IV Iberian Congress on Biotechnology

I Ibero-American Meeting on Biotechnology

BIOTEC'98

BOOK OF ABSTRACTS

Edited by

MANUEL MOTA and EUGÉNIO C. FERREIRA

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Foreword

BIOTEC'98 was the chosen name corresponding to the IV Iberian Congress on Biotechnology. Since 1992, both the Portuguese and the Spanish Societies on Biotechnology have decided to join their efforts and organise a scientific congress on biotechnology.

Ever since the first joint congress, several structuring principles were outlined. Firstly, the congress should have a significant dimension and a scientific standard as high as possible. Secondly, the congress should seek for plenary lecturers who could bring about a sound and updated overview of frontier knowledge on biotechnology. Thirdly, the congress should be youth oriented, in the sense that young researchers should be specially favoured as far as registration prices and time for exposition of their own work are concerned. Both societies share the common opinion that young researchers must be encouraged to participate, in order to discuss their work and to be exposed to the criticism of their peers.

So far, all the above mentioned rules have been fulfilled. We think that now, these rules are being accepted as terms of reference by each organising committee. In BIOTEC'98, in a total of 250 oral communications, 180 oral communications will be presented by young scientists, corresponding to 70% of the total number of oral communications.

In the present case, both societies agreed also in opening the congress to our colleagues from Northern and Latin America. This last experience revealed to be a success. More than 100 abstracts were submitted by American scientists.

The congress aims to cover the most advanced subjects investigated in each of the 10 scientific areas included in its program.

The organising committee feels that, with the close co-operation of the scientific committee, a high scientific standard was achieved.

With the publication of the book of abstracts, and a few inevitable minor logistic problems, we feel that the work of the organising committee, which lasted almost 2 years, is coming to an end.

From now on, place should be made to the congress attendants. It will be in their hands, lecturers, researchers, students, exhibitors, the conversion of the present congress in a definitely scientific event that could be remembered in the years to come.

MANUEL MOTA
July 1998

General Index

Regulation and Safety

Basics of Scientific Safety Assessment for Environmental Releases of Transgenic Plants

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***Keywords**: Transgenic plants, safety assessment, scenario method*

Public unease towards environmental biotechnology applications is due, in part, to the ever-increasing number of modified organisms whose long-term fate in the environment cannot be determined on an absolute basis. Preceding the environmental release of any genetically modified organism, extensive safety evaluations are carried out in order to gain the approval of the relevant regulatory bodies and of the general public. The purpose of the existing safety guidelines is to assure that an organism is environmentally safe, according to expertise currently available. However, safety cannot be expressed in absolute terms; it is a relative concept more adequately defined in terms of tolerability and acceptability limits, yet a rational and analytical approach towards environmental safety *is* possible and could provide the necessary foundation for constructive discussions on the real issues of interest to scientists, representatives of the regulatory community and members of the general public. A clear safety assessment methodology, with established scientific consensus on assumptions, data and methods, would also help to inspire public trust and confidence in biotechnology safety management.

The objective of the presentation is to demonstrate that there is a factual, scientific basis to environmental safety issues, by means of which the real, case-specific hazards requiring serious consideration by scientists, representatives of regulatory agencies and the public can be distiguished from conjectural hazards and naturally occurring background processes [1].

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FDA Biologics Regulations and their Impact on Manufacturing Processes

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Keywords: *FDA, Regulatory, validation, bioprocessing*

The recent changes in the FDA CBER regulations governing the manufacture of specified biologicals will provide biopharmaceutical manufacturing companies with increased flexibility for the development and implementation of their production processes. The establishment of product "comparability" between clinical manufacturing and the marketed product as well as demonstration of process and product consistency between pilot and production scales will be of key significance. A strong emphasis will be placed as well on adequate validation information in support of process changes. This presentation will provide an overview of the U.S. regulatory process and discuss the cost impact of these regulations on the development and validation of biopharmaceutical manufacturing processes.

Plenary Lectures

Madin Darby Canine Kidney (MDCK) Cells as Substrate for Influenza Vaccine Production

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Influenza vaccine production technology based on large-scale cell culture technology was developed. All recommended, egg-adapted WHO influenza A (H_1N_1) and H_3N_2 and B viruses from the last 5 years were successfully propagated on MDCK cells and subsequently used for vaccine production.

From the characterisation of the continuous cell line MDCK as well as drug safety studies we conclude that this cell line and the cell culture system are suitable to use for biological production. The Down Stream Process of the virus-containing harvest fluids guarantees sufficient inactivation of influenza viruses and adequate removal or inactivation of putative adventitious or endogenous viruses, mycoplasmas or bacteria. Clinical data reveal that the MDCK cell-derived vaccine is safe, well tolerated and meets the criteria for immunogenicity as stated in the European Community's "Harmonisation of requirements for influenza vaccines".

The tissue culture based production technology not only is feasible, but also offers a realistic alternative to the classical egg-derived vaccines to combat influenza.

From Plant Cells to Phytoremediation

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Plant biotechnology, plant cell culture, phytoremediation, organic pollutants, heavy metals

Higher plants possess a pronounced ability for the metabolism and degradation of many organic recalcitrant pollutants and can be considered as "green livers", acting as an important sink for environmentally damaging chemicals (1). On the other hand, different plant species are able to hyperaccumulate heavy metals in their tissues (2). It thus appears that plants could be developed and used for the removal of hazardous organic compounds and toxic metals from industrial wastewaters and contaminated sites. Phytoremediation can be defined as the use of green plants and their associated micro-organisms, soil amendments and agronomic techniques to remove, contain or render harmless environmental contaminants. At the same time, such biological treatments could afford new and exciting opportunities for the agricultural world, especially in European countries. However, phytoremediation is still a nascent technology that seeks to exploit the metabolic capabilities and growth habits of higher plants. In such a context, there is a significant need to pursue both fundamental and applied research to provide low-cost, low-impact, visually benign and environmentally sound green depollution strategies.

To develop such techniques, *in vitro* systems often offer advantages over the whole plants, provided that xenobiotic metabolism or metal accumulation in cultivated plant cells and tissues reflects what occurs *in vivo*. In particular, the screening of plant species to accumulate or degrade xenobiotics belonging to the same chemical family will be done much easier, quicker and independently of the climate conditions. Cell cultures are also a useful system for metabolic engineering and to obtain rapid evidence of the ecotoxicological behaviour of chemicals and metals in plants with less analytical expense. According to results obtained *in vitro*, the most promising systems have then to be tested in whole plants cultivated in controlled and well defined conditions for validation, before any application at large scale. It is therefore necessary to identify the most appropriate plant species, possessing enzymes likely to cope with the pollutants to be removed, before designing an adequate large-scale process to treat wastewaters or contaminated sites. For example, sulphonated aromatic compounds are released into the environment in large amounts: the main sources of these pollutants, rather recalcitrant to biodegradability, are the anionic detergents, the dyestuffs or their by-products. Today, about 80% of anthraquinone dyes produced in the world (30'000 tons per year) are prepared via anthraquinone sulphonic acids, giving to this family of xenobiotics a potential and actual impact on the environment. But many different derivatives of anthraquinones occur naturally in several higher plants like rhubarb. It has been shown that cells isolated from *Rheum palmatum*, and cultivated in shake flasks or in bioreactors, are able to accumulate and biotransform several mono- and di-sulphonated anthraquinones, as well as other sulphonated compounds, derived from benzene and naphthalene, also containing nitro or amino groups (3).

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New Affinity Adsorbents for the Purification of Biopharmaceuticals

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Affinity chromatography is ideally suited to the purification of high value biopharmaceutical proteins. The most commonly used adsorbents are based on natural high molecular weight ligands, but suffer from a number of problems such as difficulties in synthesis, high cost, poor chemical and biological stability and leakage. On the other hand, entirely synthetic ligands have many endearing features; notably, they are resistant to both chemical and biochemical degradation, are sterilizable *in situ* and can be produced at an affordable price. However, the availability of such synthetic ligands with suitable binding properties remains problematic, although recent advances in computer-aided molecular design and the increased access to x-ray crystallographic data now enables the rational design of affinity ligands targeted at specific sites on proteins[1-3].

More recently, an artificial protein A ligand has been designed and synthesised to mimic a key dipeptide motif on fragment B of protein A which is known to play an important role in the interaction with the Fc fragment of IgG[4]. This synthetic ligand was shown to be able to purify IgG from diluted human plasma, ascites fluid and foetal calf serum. However, it was clear from this study that the artificial protein A structure could be further refined and optimised by making small changes to its structure. Combinatorial synthesis has been used for the process of lead optimisation rather than lead discovery, since, in many situations in lead discovery, information on the target site and/or complementary partner may be available and can be used for the construction of a more focused library of near-neighbour analogues. Knowledge of the structure of the lead compound, the artificial protein A, prompted the construction of an 88-member IgG-binding ligand library synthesised by a solid phase assembly procedure on an agarose matrix using a modified "mix and split" procedure. The library was screened for binding of pure human IgG, whence selected ligands from the library were further assessed for specificity by the purification of IgG from human plasma. The potential of this strategy for the rapid identification and evaluation of chemical leads was demonstrated by the discovery of ligands with IgG binding capabilities. It was found that ligands comprising 3-aminophenol and an aminonaphthol moiety substituted on a triazine nucleus generally performed better than other ligands in the library. One immobilized ligand adsorbent, 22/8, was able to purify IgG with high yield and a purity >99% from diluted human plasma. A similar approach has now been applied to the purification of a recombinant insulin precursor (Mi3) by design and combinatorial synthesis of a intentionally biased ligand library complementary to key residues in the dimer binding interface of the protein. It is anticipated that these new approaches will generate a "tool-kit" of generic new adsorbents applied to genuine commercial targets.

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Applied Biocatalysis

Biotransformation Processes by Resting Cells

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Key words: Biotransformation, Resting Cells.

Microorganisms employ both constitutive and inducible enzymes to degrade and synthesise a great variety of chemical compounds. Reaction products that are not further degraded can usually be isolated from the fermentation medium. Such chemical reactions mediated by microorganims or their enzymes preparations are called biotransformations. The reaction types so performed are oxidations, reductions, hydrolysis, condensations, isomerizations, etc.

Purified enzymes, growing cultures or resting cells, used free or immobilised, can perform biotransformations. Enzymes purification is often tedious, time consuming and expensive, therefore it may be advantageous only in some cases. When working with growing cells both growth and biotransformation take place simultaneously in a complex medium, and under sterile conditions. Whereas, with a resting cells system, the microorganism is previously cultivated under optimum growth conditions; biomass is harvested by centrifugation or filtration. After that, cells are resuspended in a simple transformation medium, which contains water or a buffer solution with the optimised pH and the substrate. The strict separation of microbial growth and biotransformation offers the following advantages: 1) Each step can individually be optimised. 2) The cell density yielding optimum conversion rate can easily be determined. 3) The process can be conducted under non-sterile conditions. 4) Product isolation is easier since the transformation medium is less complex [1].

On the other hand, L(-)-carnitine (R(-)-3-hydroxy-4-trimethylaminobutyrate) which transports long-chain fatty acids through the inner mitochondrial membrane, has several clinical applications, consequently there is a high demand of this compound. Besides, the L(-)-carnitine production by resolution of racemic carnitine produces D(+)-carnitine as a waste product, which can be converted into L(-)-carnitine by *Escherichia coli* [2].

In this way, in the present work the biotransformation of D(+)-carnitine into L(-)-carnitine by resting cells of *E. coli* O44 K74 and of the transformed strain *E. coli* K38 pGP1-2 has been studied. Cells were cultured in a complex medium to obtain the maximum biomass. After growth, cells were harvested by centrifugation and resuspended in the biotransformation medium, which contained D(+)-carnitine in phosphate buffer pH 7.5. Incubation was maintained until the maximum conversion was achieved. Aerobic and anaerobic assays were performed. L(-)-carnitine produced was enzymatically analysed by the carnitine acetyl transferase method. Other compounds were analysed by HPLC.

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Biosint S.p.A. is also acknowledged for the kind gift of the substrate.

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Síntesis de Bloqueantes β-Adrenérgicos utilizando *Saccharomyces cerevisiae y* Lipasas

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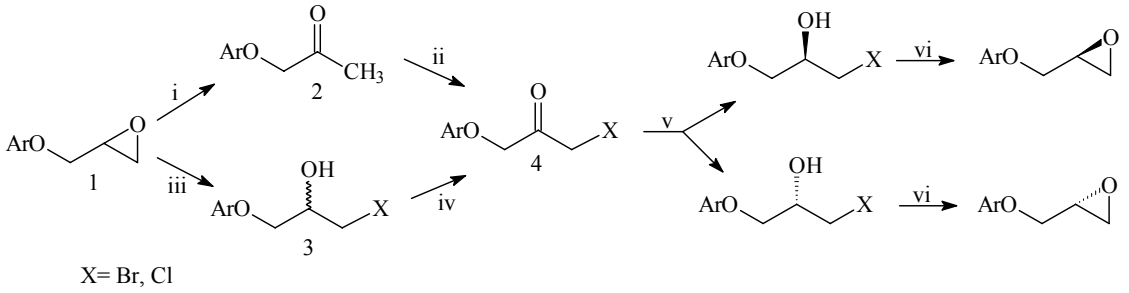
Keywords: Saccharomyces cerevisiae*, reducciones asimétricas, Pd(0)*

Los métodos biocatalíticos, han ganado interés en química orgánica como alternativa a los métodos tradicionales para la producción de compuestos homoquirales. Los métodos más utilizados son: la resolución de mezclas racémicas catalizado por lipasas y las reducciones enantioselectivas de cetonas proquirales con levaduras (Baker's yeast). En la presente comunicación se comentan los resultados obtenidos en la preparación de bloqueantes β-adrenérgicos mediante reducciones asimétricas utilizando el *Saccharomyces cerevisiae* [1], y resoluciones de mezclas racémicas usando lipasas.

Las reducciones asimétricas se realizan sobre compuestos carbonílicos α-halogenados (**4**). Estos compuestos carbonílicos pueden obtenerse vía isomerización de epóxidos (**1**) a los correspondientes compuestos carbonílicos (**2**) empleando ligandos de Pd (0) con tributil ó trifenil fosfina [2] ó bien, por oxidacion de las halohidrinas (**3**).



reactivos y condiciones: i. $Pd(AcO)_2$, Ph_3P/Bu_3P, tolueno, reflujo, 14/8 h. ii. Br_2, CH_3OH, 0 ºC, 2 h.
iii. ClH conc., $CHCl_3$, 0-5 ºC, 1 h. iv. CCP, CH_2Cl_2, t.a., 5 h. v. *S. cerevisiae*, tampón fosfato 0,1 M (ph=7), sucrosa. vi. KOH, EtOH, t.a., 30 min.

Aperturas nucleofílicas regioselectivas de estos glicidoles homoquirales conducen a la obtención de fármacos β-bloqueantes adrenérgicos tipo (*S*) Propranolol.

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Purificação da β-Galactosidase de *Kluyveromyces Fragilis* em Sistema de Duas Fases Aquosas por Bioafinidade

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Palavras chaves: purificação, afinidade, β-galactosidase, sistema de duas fases aquosas

Os sistemas de duas fases aquosas (SDFA) têm sido utilizados para recuperação e isolamento de proteínas e outros materiais biológicos, tanto em escala industrial como em pesquisas, devido a suas inúmeras vantagens, entre elas, a redução do custo operacional em relação a processos convencionais. A aplicação dos SDFA é simples, podendo ser realizada sem o uso de equipamentos sofisticados. Dos vários processos de purificação existentes na literatura, a extração líquido-líquido vem ganhando destaque por sua viabilidade industrial.
Para alcançar elevada recuperação e concentração de uma proteína específica em SDFA, é necessário maximizar o valor do coeficiente de partição, K, (concentração da proteína na fase superior/fase inferior). Seletivamente o valor de K também pode ser aumentado por ligação covalente do PEG a um ligante de afinidade. Neste trabalho foram aplicados conjuntamente dois conceitos de purificação: a utilização de bioligantes e a partição em SDFA.

Enzimas microbianas possuem vasta aplicação no setor alimentício, por serem substâncias naturais e atóxicas, por possuirem elevada especificidade catalítica, por atuarem em condições brandas de temperatura e pH e serem eficientes a baixas concentrações. A enzima β-galactosidase ainda não é produzida industrialmente no Brasil, e uma demanda crescente para produtos lácticos contendo lactose previamente hidrolizada poderá ser atendida após o desenvolvimento de processos seletivos e eficientes de obtenção desta enzima. Existem várias técnicas para acoplamento de ligantes a polímeros insolúveis (matrizes cromatográficas em geral), porém, poucos trabalhos têm sido feitos com ativação e acoplamento de ligantes à polímeros solúveis. Para a purificação da β-galactosidase de *Kluyveromyces fragilis,* este trabalho investigou o uso do ligante sintético APGP (p-aminofenil-β-D-galactopiranosídeo), um inibidor da enzima, acoplado ao polietilenoglicol (PEG) 4000. A partição da enzima β-galactosidase de *Kluyveromyces fragilis* em SDFA foi estudada em sistema constituído de 6% PEG-APGP, 8% dextrana 505.000 (p/p), pH = 6.5. A atividade enzimática foi determinada com ONPG. O coeficiente de partição (K) da enzima β-galactosidase de *Kluyveromyces fragilis* sem o ligante APGP foi de 0.4 e com o ligante foi de 1.300 tendo um aumento de 3.250 vezes seu coeficiente de partição. A metodologia completa, os resultados e discussão serão descritos posteriormente.

APOIO FINANCEIRO: FAPESP

Protein Engineering of a Lytic β-1,3-Glucanase Enzyme able to Permeabilize the Yeast Cell Wall

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A large number of recombinant proteins of clinical, scientific and industrial importance are being manufactured in yeast cells and many of these cannot be secreted. Moreover, a large fraction of the proteins that are secreted remain inside the cell and its periplasmic space. The only practical methods presently used for extracting intracellular proteins and protein particles from yeast cells are mechanical (e.g. homogenizer or bead mill) which are very inadequate as they are totally non-specific, they micronize the cell debris and release all cellular components including RNA which increases the viscosity and makes the latter purification stages of the target protein very difficult. It also can result in partial destruction of the product.

A very attractive alternative to release intracellular proteins is the use of specific yeast lytic enzymes, particularly lytic glucanases, that can selectively release specific proteins from individual cell compartments in the yeast cell. A major limitation is the low levels of expression of yeast lytic glucanases presently obtained in bacteria (e.g. *Oerskovia, Arthrobacter*). Available yeast-lytic preparations contain a mixture of glucanases and proteases, the latter causes proteolytic degradation of the protein product. We have shown that by using a pure lytic β-1,3- glucanase in the absence of any proteolytic activity it has been possible to carry out very controlled lysis which resulted in the selective release of cloned intracellular protein particles (VLPs) with very little cell lysis (wall permeabilization) and virtually no degradation products as compared to mechanical breakage or normal enzymatic lysis by crude enzymes. In order to have practical amounts of the lytic glucanase available the enzyme has been cloned in *E. coli* and subcloned in *Bacillus subtilis* protease weak strain DN1885. In our present work we are using modern techniques available to carry out the protein engineering of a lytic glucanase of high activity and improved ability to permeabilize the yeast cell wall.

We are studying the structure-function relationship of the glucanase by site-directed in vitro mutagenesis which is being used to investigate the catalytic domain using PCR for the synthesis of chimeric β-glucanases. In parallel, the use of sequential cycles of random mutagenesis and screening for constructing glucanase mutants with improved activity is being carried out. Random mutagenesis combined with selection and screening followed by recombination has proven to be a useful alternative to increase activities. The mutations are generated by PCR using conditions that increase the frequency of erroneous nucleotide incorporation by Taq DNA polymerase. Together with computer based 3-D analysis we are investigating the interaction of lytic glucanases with the yeast cell wall and thus the re-design of the β-1,3-glucanase for new and improved performance.

Improvement of Cutinase Stability in AOT Reversed Micelles: from Thermal Stability Optimization to Continuous Operation in a Membrane Bioreactor

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Keywords*: Stability, Cutinase, Micelles, AOT, Membrane Reactors*

The recombinant cutinase from *Fusarium solani pisi* has been used as a versatile biocatalyst of hydrolytic and synthetic reactions involving short chain substrates. Its globular shape and small dimensions make it suitable for microencapsulation in reversed micelles, however, anionic surfactants, like AOT, accelerate the deactivation process of the enzyme. Short chain alcohols can be used as co-surfactants in microencapsulated cutinase and have been proved to be good stabilisers[1,2]. For this reason, hexanol was used as acyl acceptor in the transesterification of butyl acetate yielding hexyl acetate, a fruity flavour ester.
Relevant parameters for cutinase activity and stability were studied using the factorial design methodology. The effects of factors and their interactions were evaluated using the response surface method. A set of conditions was selected which included low Wo (Wo=[H2O]/[AOT]) values and medium to high concentrations of hexanol in the reaction medium.
Further work included the determination of the deactivation constants (k_d) at various hexanol concentrations performing the thermostability. In the absence of the alcohol, during the incubation period, cutinase displayed an half-life of 3h at Wo=2.7. A stabilisation effect was observed with the increase of hexanol concentrations and a linear relationship exists for values above 400 mM. The non-linearity obtained at low hexanol concentrations seems to be related to diffusional limitations in the reversed micelles of AOT/isooctane.
The operational stability was tested in a continuous membrane bioreactor (MBR), with an ultrafiltration membrane with 15,000 MWCO. This type of reactor allows the the integration of the whole process by decreasing the number of downstream unit operations. The MBR was continuously operated for more than 900 hours using a molar ratio of 1:10 butyl acetate/hexanol and the conversion level was maintained at 60%. The half-life was estimated to be aproximately 2 years which represents a large improvement relatively to the thermostability results.
The high operational stability and the level of conversion obtained make this reversed micellar system promising for the biotransformation area.

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Imobilização da lipase do *Mucor miehei* em suportes inorgânicos

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Keywords: silica, lipase, imobilização, esterificação

As reacções de hidrólise da trioleína e de esterificação de vários ésteres, usando biocatalisadores com lipase do *Mucor miehei* imobilizada, têm sido objecto de estudo de vários investigadores [1, 2].

A estabilidade e a retenção de actividade dos biocatalisadores dependem fundamentalmente do método de imobilização e do microambiente que envolve as enzimas após a sua fixação. Estes temas têm sido extensivamente tratados, usando diferentes enzimas e uma larga gama de suportes poliméricos [3, 4].

Neste trabalho, e com o objectivo de promover uma reacção de esterificação num reactor em contínuo, seleccionou-se a sílica como matriz original, tendo essencialmente em consideração as suas propriedades físicas e mecânicas. Modificações químicas da superfície da sílica foram conseguidas com a realização de reacções de silanização, introduzindo diferentes grupos funcionais: $-OH$, $-NH_2$, $-COOH$ e epóxido.

As matrizes iniciais e todos os sistemas obtidos ao longo das modificações introduzidas, foram caracterizadas física, química e morfologicamente usando, entre outras, técnicas de adsorção gasosa, porosimetria de mercúrio e análise térmica gravimétrica (TGA).

A lipase do *Mucor miehei* foi imobilizada covalentemente nos vários suportes e, de entre os biocatalisadores obtidos, os que se revelaram mais activos na reacção de esterificação do caprilato de isoamilo foram os que continham grupos $-NH_2$.

Foi possível concluir que apenas quando a lipase era imobilizada em suportes com poros de diâmetros superiores a 100 Å havia expressão de actividade enzimática.

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Síntesis Enzimática de Kyotorfina en Medio Anhidro

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PALABRAS CLAVE. *α-quimotripsina, kyotorfina, sorbitol, disolventes orgánicos.*

La síntesis de péptidos tiene un gran interés tanto científico e industrial debido a las propiedades biológicas y aplicaciones que presentan. Desde un punto de vista biotecnológico, hay un gran número de ventajas potenciales en utilizar enzimas en medios no convencionales para conseguir procesos de síntesis de péptidos por proteasas. Los medios referidos con este nombre, son aquellos donde existe un estricto control de la cantidad de agua presente en el sistema, y concretamente los medios anhidros son medios no convencionales que contienen ≤ 1% v/v de agua en el sistema.
Desde un punto de vista industrial es esencial comprender los factores que controlan la actividad y estabilidad de un biocatalizador en un medio no convencional, para llevar a cabo la optimización de una biotransformación. Se han empleado diferentes estrategias para mantener un balance adecuado entre la actividad y la estabilidad de la enzima en un medio no acuoso. Así, la inmovilización del biocatalizador sobre un soporte sólido, se ha mostrado tanto como uno de los métodos más ventajosos para aumentar la termoestabilidad enzimática (Blanco *et al*., 1992), como para suprimir los problemas de agregación y autolisis de la proteína. Otra estrategia es el empleo de aditivos depresores de la actividad de agua del sistema, tales como, sales, polioles, polímeros y azúcares que se han comportado como exaltadores de la actividad y estabilidad de proteínas (Lozano *et al*., 1993; Triantafyllou *et al*., 1997).
El objeto de este trabajo ha sido optimizar la reacción de síntesis del dipéptido kyotorfina (Tyr-Arg) en un medio anhidro catalizada por α-quimotripsina inmovilizada sobre celite, etanol/hexano en cuanto a, temperatura, pH de inmovilización, parámetros cinéticos y presencia de sorbitol. Se han obtenido los mejores valores de actividad sintética de kyotorfina cuando la enzima se inmovilizaba a pH 7,8, la temperatura del medio de reacción era de 30 ºC y el medio anhidro fue hexano:etanol:aditivo, 69:30:1 v/v, a una concentración de sorbitol de 2 mmoles/g proteína. Por otro lado, el mecanismo cinético de la α-quimotripsina en la síntesis de kyotorfina se ajustó a un moddelo BiBi ordenado, pudiéndose determinar un valor de k_M para BTEE de 17,45 mM, mientras que para la AEE la k_M fue 34,61 mM y la Vm de la reacción fue 19,7 mmoles/min.

Este trabajo ha sido parcialmente subvencionado por la "Comisióm Interministerial de Ciencia y Tecnología", CICYT, nº BIO96-1016-C02-01. España.

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Enzymatic Synthesis Process for CCK-8

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***Keywords**: Proteases, organic media, enzymatic synthesis, cholecystokinin*

This work is focused on a process development, in organic media, for the enzymatic synthesis of the octapeptide CCK-8 (Asp-Tyr(SO_3)-Met-Gly-Trp-Met-Asp-Phe-NH_2), the active fragment of cholecystokinin.

On the basis of a previous systematic study of the possible synthetic routes starting from amino acids, the synthetic strategy chosen implies the obtention of the final pentapeptide CCK-5 [1-3].

A process for the synthesis of the above fragment has been developed (in stirred tank reactors of 100 mL volume), by minimising the number of steps, and using mandelyl and phenylacetyl as enzymatic labile protecting groups. All the reactions were performed in low water content media and using proteases deposited onto solid supports as biocatalyst. The scale-up of the overall synthesis reveals problems negligible at vials scale (typically 5 mL), as water activity control during the reactions and also in the upstream and downstream operations. All the reactions operated under N_2 atmosphere to avoid increase in water activity due to water transfer from the external air. Previous works showed the possibility of reducing the enzyme /substrate ratio when the reaction volume increased. Nevertheless, in the present work, as intermediate purifications are avoided, this reduction was not possible because it is associated with a reduction in the bufffer power of the reaction medium and the presence of acyl donor hydrolysis products can modify the enzyme micro-environment pH.

Moreover, mandelyl and phenylacetyl protecting groups bring different solubilities to the products obtained, which is used to separate some products between steps.

The dipeptide BocTyr-Met-OAl and the tripeptide PhAc-Asp(OBu^t)-Tyr-Met-OAl were synthetized in order to test the viability of the enzymatic coupling with the deprotected pentapeptide. Both reactions are significant and now it is necessary to optimise the tripeptide enzymatic synthesis and the octapeptide coupling.

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The Application of two Aspartic Proteinases, Cardosins A and B on Peptide Synthesis

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Keywords: *Enzymatic peptide synthesis, Biological peptides, Aspartic proteinases and Cardosins A and B.*

Since several years ago, a growing number of studies are devoted to enhancing enzymatic activity in organic solvents. In other hand, enzymatic peptide synthesis has established itself as a productive approach for the preparation of biological active peptides. It can be performed essentially in aqueous media or mixtures of water with organic solvents, being catalyzed by an enzyme dissolved in the reaction mixture, or in organic solvents that contain very low amounts of water. Concerning this, we have dedicated, in the last few years, to the use of two new aspartic proteinases, Cardosins A and B, on peptide synthesis.

Cardosins A and B are two proteinases extracted from *Cynara cardunculus* L. stigma, a cardoon traditionally used for milk clotting in the manufacturing of cheese. Cardosins are purified by a two step procedure involving extraction at low pH, gel filtration and ion-exchange chromatography. These enzymes show high preference for hydrophobic bonds.

In this work we study primary and secondary specificity towards peptide synthesis as well as stability of these enzymes on mono- and biphasic organic systems. Concluding, we pretend to evaluate the possibility of using Cardosins A and B, separately, regarding their yields on peptide synthesis, having in mind a possible industrial application.

Structural Properties of *Centaurea calcitrapa* Aspartic Proteinase

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***Keywords**:* Centaurea calcitrapa, *aspartic proteinase, structure*

The wide range of functional and structural roles of proteins in cells is essentially due to diversity of their amino acid sequences and features of their three-dimensional shapes. To understand protein function it is, therefore, necessary to understand protein three-dimensional structure.

Several aspartic proteinases 3-D structures have been analysed; early structural studies of pepsin were initiated with the objective of understanding the relationship between its structure and mechanism of action. However, commercial interests in chymosin and certain fungal aspartic proteinases such as mucorpepsin and endothiapepsin, which are used in cheese making, heightened the interest in understanding the specificity that leads to the production of peptides of desirable flavours [1]. This work has been extended to plant enzymes, some of which have also traditionally been used in cheese making. Intense pharmaceutical and academic research activity in the specificity and mechanism of aspartic proteinases followed the discovery that inhibitors of renin had antihypertensive activities. Research on retroviral aspartic proteinases, most specially on HIV proteinase, has led to the design of inhibitors which are strong candidates for antivirals [1].

Using flowers from *Centaurea calcitrapa* as source of mRNA, a full lenght clone called *cent8* was identified (EMBL Y09123) and sequenced. A three-dimensional model based on the primary structure of *cent8* gene has been constructed using COMPOSER, a rule-based modelling approach as in SYBIL software. The first step was to identify and select proteins of known three-dimensional structure that were most useful for construction of the model which was constructed excluding the 104 residues of the plant-specific region.

The 3-D structure of three plant aspartic proteinases have been modelled. The overall folding of these three enzymes is very similar. A comparison between three plant aspartic proteinases models shows that the overall folding is very similar; there is a highly conserved sequence motifs; six cysteine residues are conserved in all plant-inserts which can explain a conserved tertiary structure of this region in the three plant enzyme which models were compared.

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Covalent Structure and Proteolytic Processing of the Lectin of *Vatairea macrocarpa* Seeds

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***Keywords**: galactose-binding lectin, posttranslational processing*

Lectins are a structurally very diverse class of proteins which bind reversibly and with high specificity mono- and oligosaccharides without enzymatically modifying them. Lectins are well suited for acting in a large number of biological processes deciphering the glycocodes encoded by the tremendous variety of glycans attached to soluble and integral membrane glycoconjugates. The most thoroughly studied lectins are those isolated from leguminous plants, such as peas and beans. Although their function remains elusive, studies on plant lectins have made important contributions to our understanding of protein-carhohydrate interactions, and have been extensively used as tools for glycoconjugate purification and characterization, as well as specific reagents for biomedical research. The large majority of leguminous lectins that have been isolated and characterized belong to the *Phaseoleae*, and *Vicieae* tribes of the *Papilionoideae* subfamily *of Leguminosae*. Here we report the first determination of the covalent structure and posttranslational mechanism of a galactose-specific lectin isolated from seeds of a species of the genus *Vatairea*, *Vatairea macrocarpa* Duke, a tree growing in the northeastern of Brazil.

The primary structure of *Vatairea macrocarpa* lectin (VML) was established by Edman degradation. It is a glycoprotein composed of a mixture of 240-amino-acid doubly (28525 Da) and singly (27354 Da) glycosylated alpha chain, which is partially cleaved at the N114-K115 peptide bond yielding minor 22 kDa (beta, C-terminal) and 13 kDa (gamma, N-terminal) fragments. The primary structure of VML displays similarity with other leguminous lectins, particularly with *Erythrina*, *Robinia,* and *Sophora* lectins. Tandem mass spectrometry and methylation analysis indicated the presence of Manα1-6[(Manα1-3)(Xylβ1-2)]Manβ1-4-GlcNAcβ1-4(Fucα1-3)GlcNAc, a typical plant N-glycan, attached to N111 and N183. Equilibrium sedimentation analysis by analytical centrifugation showed that VML had a mass of 122-130 kDa, which did not change within the pH range 2.5-8.5. Mass spectrometric analysis suggested that deglycosylation of Asn111 correlates with proteolytic cleavage of the Asn114-Lys115 bond yielding glycosylated gamma (12304 Da) and nonglycosylated beta (residues 115-239, 14957 Da) chains. Some beta chain molecules are further deglycosylated and N-terminally processed yielding products of molecular masses of 13783 Da and 13670 Da. (residues 1-114) and nonglycosylated beta (residues 115-239) chains.

Our results, which are the first available for a leguminous lectin of the *Dalbergiae* tribe, indicate that proteolytic processing occured early during plant evolution.

Kinetic Parameters of a Protease Produced by *Penicillium citrinum* in Solid State Fermentation

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***Keywords**: protease, filamentous fungi, solid state fermentation,*

Protease production together with other enzymes, mainly lipases, during SSF, have been frequently reported (1,2) and could cause loss of lipolytic activity due to hydrolysis of the polypeptide chain. Nevertheless, proteolytic enzymes themselves have many important industrial applications, which fostered the attempts to isolate and characterise new sources of the enzyme. The aim of this work was the investigation of the kinetic parameters of a protease produced by a wild strain of *Penicillium citrinum* in solid state fermentation (SSF) using sunflower bran as substrate. The effect of pH, temperature and substrate concentration on proteolytic activity relative to azocasein hydrolysis was studied, using the crude enzymatic extract. There were also performed experiments to determine the stability of the enzyme related to temperature and pH. The enzyme was produced as described in previous work (3) using sunflower bran, 28 ^{0}C, 55 % humidity and phosphate buffer 0.2 M pH 8.0. Protease activity was determined in the crude extract, using azocasein as substrate according to Leighton *et al*.(4). The results showed that the optimum conditions for enzyme activity were pH 6.0 and 45 ^{0}C. Under these conditions, the crude enzymatic extract presented a Michaelis-Menten behaviour against azocasein, with $Vmax._{app}$ =102 U/mL, and $Km_{app.}$ = 2.0 mg/mL. Stability studies revealed that the protease from *P. citrinum* was quite stable against temperature: 100 % of the initial activity remained at 28 °C for 3 days, at 37 °C for 7 h, 45 °C for 5 min. At 0 ^{0}C and 4 ^{0}C the enzyme maintained 100 % of its activity for 3 weeks. pH experiments showed that the enzyme was stable in a range of pH 5 to 8.0. Even at higher pH value, enzymatic activity remained (pH 9.0, 50 % and pH 10, 27 % of the total proteolytic activity). These results show that the protease of *P. citrinum* could be used for industrial application such as detergents and support further investigation on purification and characterisation of the enzyme.

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Stereospecific Microbial Production of β-Hydroxyisobutyric Acid in Biphasic Systems

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***Keywords**: Stereoselective bioconversions, aqueous/organic biphasic systems, β-hydroxyisobutyric acid*

INTRODUCTION

Regio- and stereoselective bioconversions can be an alternative to obtain chiral compounds in an economical and fast way. The use of biphasic aqueous/organic solvents allows the integration of bioconversion and recovery in one unique step, improving product recovery and in many cases the productivity of the process. Here we describe the two-phase, stereochemical oxidation of 2-methyl-1,3-propanediol to R-(β)-hydroxyisobutyric acid (HIBA), an important chiral building block for the synthesis of pharmaceuticals.

METHODS

An acetic acid bacteria capable of oxidizing 2-methyl-1,3-propanediol isolated by Molinari *et al*. was used in a biotransformation medium consisting on citrate buffer 0.1M at the indicated pH and 28ºC. 2-Methyl-1,3-propanediol and HIBA were determined by HPLC on a Polyspher OA HY column. Mobile phase was H_2SO_4 0.005 N at a flow rate of 0.3 ml/min and an IR detector was used. Partition coefficients (K_p) were experimentally determined and calculated as the ratio between concentrations in the organic and aqueous phases.

RESULTS AND DISCUSSION

The isolated bacteria was used to carry out the bioconversion in a batch system with a yield of 100%. Dissolved oxygen and pH were the most important factors. The oxidation is a two-step reaction with aldehyde hydroxyisobutanal as the intermediate. The time course evolution of the three species was followed at different pH values and kinetic parameters were determined.

Election of the right solvent is essential in two-phase conversions. Low hidrophobicity of HIBA makes necessary the addition of adduct or ion-pair forming agents to promote its partition into organic solvents. We tested the ability of three of these agents, TOPO (Trioctyl phosphine oxide), Trioctylamine and Aliquat 336 (Tricapryl methyl ammonium chloride) dissolved in different organic solvents to extract HIBA. Partition and molecular toxicity experiments were carried out in different conditions. pH was dramatically important in the two first cases.

Since the bioconversion and the extraction have different pH optima, a compromise value should be chosen to achieve the highest productivities. Kinetic parameters and K_p values obtained at different pH values and for different agents were used for modelling the bioconversion in biphasic systems. We conclude from this model and toxicity considerations that a pH of 4 and TOPO 20% in isooctane as the extractant were the best conditions. Experimental data were in agreement with the predictions.

Efecto a Larga Distancia de los Sustituyentes sobre la Regioselectividad de la Desacilación de Diaciclonucleósidos de Tiadiazina con Lipasa PS

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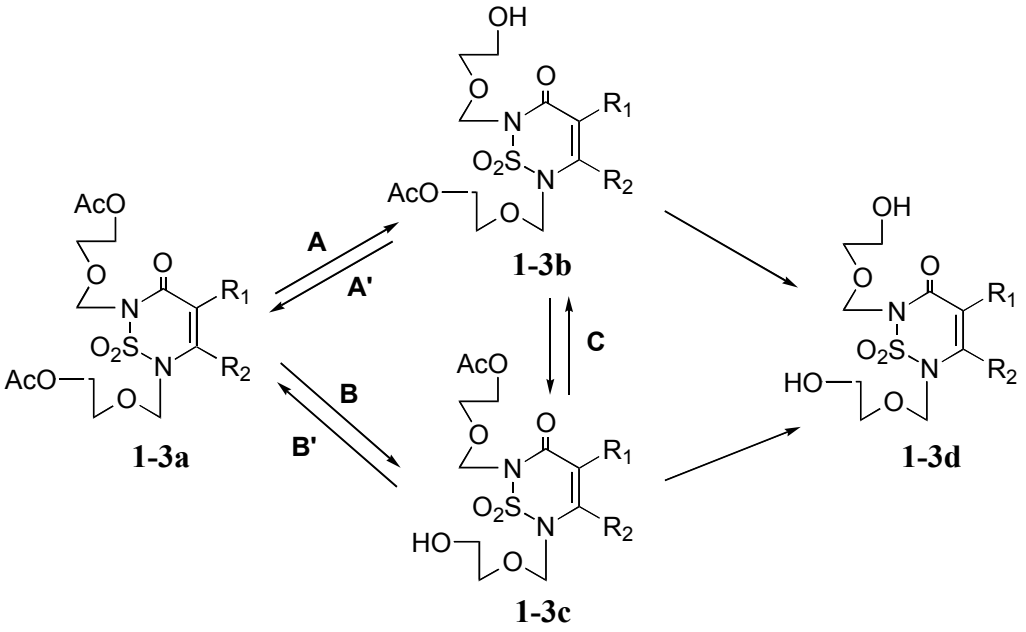
Palabras llave: Tiadiazina; Aciclonucleósido; Lipasa; Alcoholisis; Regioselectividad

Se ha estudiado la alcoholisis regioselectiva catalizada por la lipasa PS (Amano) de los grupos bloqueantes acetilo de tres diaciclonucleósidos. Pese a que la diferencia entre ellos se reduce a pequeños sustituyentes situados a larga distancia de los enlaces objetivo, el efecto que producen sobre la regioselectividad de la reacción es muy importante.



1 $R_1 = R_2 = H$. Regioselectividad nula. Rutas **A** y **B** de la misma importancia e interconversión entre los intermedios isómeros **1b** y **1c** (ruta **C**). **1b:1c** ≈1:1.

2 $R_1 = H$, $R_2 = CH_3$. Regioselectividad **2b:2c** 95:5. Utilidad sintética.

3 $R_1 = R_2 = –(CH_2)_4–$. Regioselectividad máxima. Obtención exclusiva del intermedio **2b**. No se detecta **2c**. Velocidad de reacción mas lenta que en los casos **1** y **2**.

En los tres casos se pueden obtener directamente los dihidroxilos **1-3d** por hidrólisis o alcoholisis del diacetilo **1-3a** catalizadas por la lipasa B de *Candida antarctica* (Novozym 435)

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Influencia de la relación molar agente acilante / alcohol en la resolución enantioselectiva de alcoholes quirales

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Palabras clave: lipasa, transesterificación, cinética, ping-pong bi bi, inhibición.

El estudio de la influencia de las relaciones molares en la resolución enzimática de alcoholes quirales tiene gran importancia a fin de optimizar las condiciones del proceso biocatalizado por enzimas además de estudiar las posibles inhibiciones del proceso. Los estudios cinéticos realizados sobre la lipasa de *Rhizomucor miehei,* referentes a la reacción de esterificación, responden a un tipo de cinética ping-pong bi bi, con inhibición por exceso de alcohol [1-3], de ácido [4], o bien de ambos [5], siendo incluso posible que ninguno de los sustratos produzca inhibición [6]; generalmente, estos estudios se han realizado empleando sustratos aquirales [1-6]. Al realizar el estudio cinético con un sustrato racémico habría que considerar ambos enantiómeros como sustratos independientes en el mecanismo cinético. En esta comunicación se presentan los resultados obtenidos en la transesterificación enantioselectiva de un alcohol quiral ((*R,S*)-1-feniletanol) con acetato de vinilo catalizada por la lipasa de *Rhizomucor miehei* inmovilizada por adsorción sobre una resina de intercambio de aniónico (Lipozyme® IM). La reacción se siguió mediante HPLC con columna quiral, cuantificándose la conversión, y el exceso enantiomérico del alcohol remanente a lo largo del tiempo de reacción. Esta lipasa demostró alta estereoselectividad hacia el alcohol de configuración *R*, no observándose conversión apreciable con respecto al alcohol de configuración *S,* con lo que se puede aproximar este modelo cinético al descrito en el caso de sustratos aquirales [1-6]. Respecto al estudio cinético realizado, las mejores velocidades iniciales se obtienen con una relación molar 1/1 o con un ligero exceso de agente acilante (hasta 3/1); si bien este ligero exceso de agente acilante no tiene efecto apreciable sobre la velocidad inicial, si se obtienen mejores resultados en la conversión. Los ajustes de los datos se llevaron a cabo con el paquete estadístico SIMFIT [7], obteniéndose un buen ajuste al modelo de inhibición por exceso de ambos sustratos, la cual sería competitiva y se produce por la formación de un complejo "DEAD-END", esto es, una segunda entrada del sustrato en el mecanismo catalítico que no conduce a producto final [5,6,8]. La inhibición por producto en este caso no se aprecia debido a que el análisis de los resultados se realizó con velocidades iniciales, aunque se ha descrito en algunas ocasiones [5,6,8].

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A study of media composition for the production of active *Mycobacterium sp*. cells for the selective side-chain cleavage of β-sitosterol

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Keywords: side-chain cleavage; sitosterol, Mycobacterium

Many bacterial species are capable of selectively cleaving the side-chain of sterols yielding steroid intermediates, which lead to a wide range of important steroid drugs [1]. Most studies are performed with free cells in aqueous fermentation media [2]. The low solubility of the sterol substrate limits process productivity, the use of an organic medium for microbial side chain cleavage following cell growth providing an interesting alternative [3]. However, limited published information is available on the influence of the growth medium composition [4] and growth time [5] on the yield and specific activity of sterol-degrading biomass.

In this work *Mycobacterium sp*. NRRL B-3805 cells were grown in a synthetic medium, containing fructose as carbon source, ammonium chloride as nitrogen source and, in some trials, β-sitosterol as an inducer. The effect of some operational parameters on the yield of biomass and sitosterol-cleavage activity were studied.

Low fructose (5 gL^{-1}) and ammonium chloride (0.4 gL^{-1}) concentrations proved limiting for biomass production, whereas high (10 gL^{-1}) ammonium chloride concentrations were shown to be inhibitory for both growth and specific sterol-degradation activity. Activity levels were strongly dependent both on the growth phase and on carbon to nitrogen ratio, best results being obtained at mid-exponential growth phase and with a fructose to ammonium chloride ratio of 5:1 (w/w). Sitosterol addition to the fermentation medium improved sterol-degradation activity and was observed to be more effective when done at the beginning of the fermentation runs, at a concentration of 0.5 gL^{-1}.

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An Amperometric Flow Injection Analysis Biosensor for Lactate Dehydrogenase (LDH)

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***Keywords**: Amperometric; FIA; Lactate desidrogenase; biosensor*

Anaerobic glycolysis contributes to the pathophysiology of hypoxia, anoxia or ischemia. Under these condition, there is high lactic acid production resulting in a decrease of intracellular pH, which causes a reduction of myocardial constractility and altered impulse propagation [1]. It has been reported that enzymatic level of LDH is keeping high during about four days after myocardial ischemia [2]. In the order to determinate of LDH activity in the serum is diagnostically important in clinical medicine. The reaction catalyzed by LDH is [1]:

$$\text{L-Lactate} + NAD^+ \xleftrightarrow{\text{LDH}} \text{yruvate} + NADH + H^+$$

In this work, it was carried out using an amperometric biosensor with LDH to measure pyruvate, as a previous study to provide information for a specific biosensor to determine the level of LDH in serum. Initially, LDH and NADH were immobilized in carbon paste using p-tetracyanoquinodimethane (TCNQ) as mediator [3]. The paste was packing in top of a gold pin. Pyruvate was used as substrate to calibrate the biosensor. All the assays was carried out at room temperature (28-30 ºC) and the FIA system was maintained with 100mM of Tris-HCl buffer (pH 9,0). The results of the enzymatic electrode at different pH values showed linear range 25mM up to 300mM of pyruvate and the optimum pH was 9.0. The variation coefficient of current response was 5% up to 36 injection. The long-term stability of the immobilised enzyme was maintained with 85% of initial activity after 100 assays. The amount of pyruvate of serum was evaluate and this results was compared to extinction of spectrophotometric techniques showing a significant correlation ($p < 0.01$). The current response was linear in a range 0.0050 up to 0.12 U of LDH.

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Suppoted by: UFPE, CNPq, CAPES, FACEPE, JIKA, FINEP, PADCT

Extraction of a Recombinant *S. mansoni* Antigen from an Impure *E. coli* Cellular Extract Using Aqueous Two-Phase Systems

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keywords: *recombinant protein, extraction, S. mansoni*, *aqueous two-phase system*

Aqueous two-phase systems have been used in biochemical research for the separation and purification of macromolecules such as recombinant proteins which has been exploited by pharmaceutical and food industry. The method is based on the separation of biopolymers according to their hydrophobicity, charge, size and biospecifity. This separation, i.e. partition, is influenced by phase system parameters such as molecular weight of the polymer, pH, type and concentration of phase forming components. This work describes the partition of a ***S. mansoni*** tegumental antigen produced by ***E. coli*** using an aqueous two-phase system composed by polyethylene glycol (PEG) and potassium phosphate salt. The effects of the polymer molecular weight, tie line lenght, pH for antigen partitioning were investigated. The antigen was produced in LB medium in orbital shaker (160rpm) at 28 ºC for 8 hours. Aqueous two-phase systems of total mass of 6g were prepared by weighting appropriated amounts of concentrated solutions of PEGs (550, 1000, 3350 and 8000) and by adding K_2HPO_4 and KH_2PO_4 solutions until required pH value was obtained (pH 7.0, 7.5 and 8.0). The detection of antigen in both phases were determined by dot-blotting. For aqueous two-phase system of PEG 8000 the antigen is mainly in the salt-rich phase ($K<1$), it was possible to recover nearly 18% of the antigen at pH 7.0 and 7.5 in the smaller tie line. The antigen partitioned by PEG 3350 was found mainly in the salt-rich phase for the smaller tie line at all pH values, except for the higher tie line, where it is in the PEG-rich phase in all pH values studied. For PEG 1000 and 550 antigen accumulates in PEG-rich phase ($K>1$). The system composed by PEG 550 (19.7% w/w) and potassium phosphate (17.7% w/w) yield of the antigen was 16% in the PEG-rich phase. In the all systems studied the total protein partition coefficient increased with the tie line lenght and pH in the various molecular weight of the PEG. It was observed that antigen partition in systems of the PEG 3350 is strongly affected by the tie line and pH value: by changing the pH and/or the tie line lenght of the biphasic system it is possible to manipulate the antigen partition to the phosphate-rich phase or to the PEG-rich phase and also with this separation process it was possible a the single step to remove the cell debris, that precipitate at interface of the system.

Enzymatic Acylation and Alkoxycarbonylation of A-Ring Synthons of 1α,25-Dihydroxyvitamin D_3 and 1α,25-Dihydroxy-19-*nor*-previtamin D_3 and their Stereoisomers: A Comparative Study

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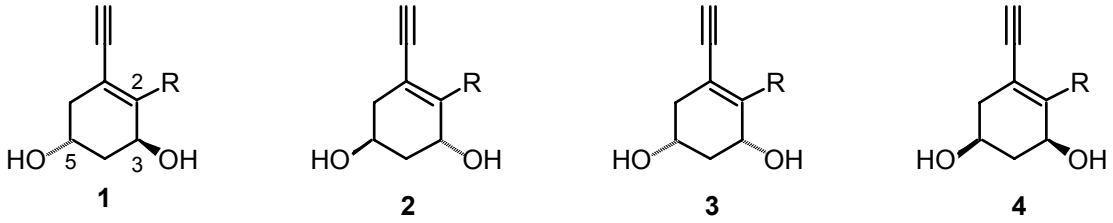
In our ongoing research related to regioselective enzymatic acylation of natural A-ring precursor **1a** of the steroid hormone 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3] and their stereoisomers **2a**-**4a**,[1] different behavior was observed. Thus, using *Chromobacterium viscosum* lipase (CVL) as catalyst and vinyl esters as acylating agents, A-ring synthons **1a**, **2a**, and **4a** are selectively acylated at the C-5 hydroxyl group, whereas isomer **3a** is acylated only at the C-3 position. Moreover, *Candida antarctica* lipase (CAL) shows a total regioselectivity through the C-5 hydroxyl group of **1a**,[2] **2a**, and **4a** in the alkoxycarbonylation reaction, meanwhile compound **3a** exhibit unexpected conduct in this process. Due to these surprising results, we try to rationalized the regioselectivity of these reactions. It would be interesting to prove the influence of the methyl group at C-2 position in the A-ring (derivatives **1b**-**4b**). Previous studies on enzymatic acylation and alkoxycarbonylation with synthon **1b** show that this methyl group is crucial to regioselectivity of the process, because in its absence (in both cases) no relevant preference was observed for one of the C-3 or C-5 hydroxyl groups. Surprisingly, the derivative **3b** continue maintaining the selectivity of its homologous **3a** in the acylation reaction.



a, R= Me; **b**, R= H

A-ring synthons of 1α,25-$(OH)_2$-D_3 **1a**-**4a** were obtained following procedures reported in the literature.[1] Preparation of 1α,25-$(OH)_2$-19-*nor*-pre-D_3 A-rings **1b**-**4b** are developing in our laboratory.[3]

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Hyperactivation of Lipase upon Adsorption onto Hydrophobic Hollow Fibers: Controlled Acidolysis of Butterfat

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*Key words:*Interesterification, immobilized enzyme, fat, membrane bioreactor, *Mucor circinelloides*

The present communication describes chemical modification of anhydrous butterfat by interesterification with oleic acid catalyzed by a commercial lipase obtained from *Mucor circinelloides*. Two reactor configurations were tested, viz. a batch stirred tank reactor containing suspended lipase and a batch stirred tank reactor in combination with a hollow-fiber membrane module containing adsorbed lipase. The goal of this research was to assess the advantage of using a (hydrophobic) porous support to immobilize lipase in attempts to engineer butterfat with increased levels of unsaturated fatty acid residues (viz. oleic acid) at the expense of medium-to-long chain saturated fatty acids (viz. myristic and palmitic acids). Reactions were carried out in the range 35 - 40 °C without solvent under controlled water activity, and were monitored by chromatographic assays for free fatty acids. The results obtained in this research effort indicate that a heterogeneous biocatalysis system encompassing the hollow-fiber membranes is a viable alternative to the traditional system encompassing pure substrate with precipitated lipase. The data also indicate that the commercial lipase from *Mucor circinelloides* is apparently hyperactivated upon adsorption relative to its suspended counterpart, with enhancement factors of the order of 100, using the same protein mass basis. A limited specificity towards short-chain fatty acid residues in butterfat triacylglycerols was also observed at 40 °C (thus allowing preservation of the delicate aroma profile of such feedstock fat), and interesterification of butterfat with oleic acid resulted mainly in exchange of myristic, palmitic and stearic acid residues of the former with the latter acid. Although hydrolysis of butterfat occurred to some extent, the enzymatic process that uses the hollow-fiber reactor was technically superior to the stirred tank system.

Specific and non-specific adsorption for affinity chromatography.

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Keywords: asparaginase, specific, non-specific, adsorption, chromatography.

Adsorption-desorption equilibrium for hydrophilic and hydrophobic bonds has been studied for Asparaginase on non-activated Sepharose-4B; also this equilibrium was studied on Sepharose-4B activated with BrCN; activated with Hexametilendiamine as spacer arm, and activated with Hexametilendiamine and L (+) Chlorosuccinamic acid as spacer arm and ligand respectively.

The experiments were performed in a batch reactor for a range of temperatures (25-29ºC) at different pH values (7.5, 8, 8.6) and ionic strengths (0-2.0)

The experimental data shown than the adsoption is highest when the ionic strength was 0.1, temperature 25ºC and for a pH of 8.6, being the adsorption equilibrium constant: K_a=77.4015 $kg.mol^{-1}$.

Equilibrium data have been correlated using the equation:

$$Log\frac{K_d}{K_a} = \frac{2A\sqrt{I}}{1+B\sqrt{I}} + K_o I \qquad \textbf{(1)}$$

based on the model developed by Morrow et al. [1] and González-Patino [2] based on Debye-Huckel theory for activity coefficients.

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Amidación Catalizada por la Lipasa de *Candida antarctica* de Esteres del Ácido Glutámico con el Grupo Amino Libre

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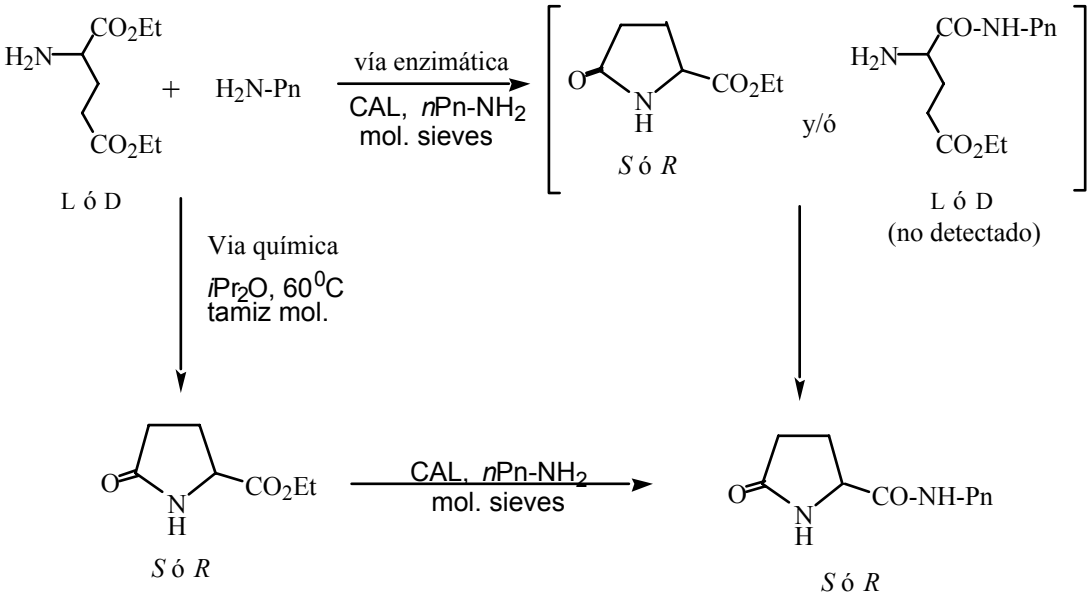
Palabras llave: Lipasa, piroglutamato, amidación

La amidación de ésteres del ácido glutámico N-protegido catalizada por la lipasa de *Candida antarctica* (CAL) muestra que la regioselectividad no se ve afectada (siempre en α) por el grupo protector. Por otro lado, la velocidad de reacción si es muy dependiente de la naturaleza del grupo protector.

En esta comunicación se presenta la reacción de amidación de ésteres del ácido glutámico no bloqueado catalizada por CAL.



Los resultados muestran que la no protección del grupo amino produce la inicial ciclación vía química del glutamato de dietilo para dar piroglutamato de etilo. A continuación, la reacción enzimática conduce al producto de amidación en posición α, tanto en el isómero L como en el isómero D, no consiguiéndose una enantioselectividad importante.

La amidación enzimática de los isómeros L y D del piroglutamato de etilo a 60°C tiene lugar inmediatamente (5 min.) sin diferencias cinéticas entre ellos. Una modesta regioselectividad aparece cuando la reacción se lleva a cabo a 45°C.

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Estudio cinético de la reacción de esterificación enantioselectiva de ácidos quirales catalizada por lipasa de *Rhizomucor miehei*

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Palabras clave: lipasa, esterificación, cinética, inhibición, enantioselectividad.

El conocimiento de la cinética de un proceso enzimático es muy importante a la hora de poder optimizar los resultados cuando se pretende llevar a cabo un proceso biocatalizado. Si se pretende disminuir los costes del proceso es necesario determinar las posibles inhibiciones existentes por exceso de los sustratos, así como las óptimas concentraciones relativas de los mismos que deben emplearse. La lipasa de *Rhizomucor miehei* ha sido ampliamente utilizada a la hora de discernir los mecanismos cinéticos de reacciones de esterificación, que responden a ecuaciones tipo ping-pong bi-bi con inhibición por exceso de alcohol [1-3],de ácido [4] o bien por ambos [5], inclusive encontrándose casos donde ninguno de los sustratos ejerce un efecto inhibitorio [6]. Generalmente, estos procesos se han llevado a cabo empleando sustratos aquirales [1-6]; no obstante, la presencia de sustratos racémicos nos permite estudiar de forma independiente la cinética de transformación de cada enantiómero.[7]. En esta comunicación presentaremos los resultados obtenidos en la esterificación enantioselectiva de un ácido quiral (ibuprofeno, ácido 2-(4-isobutilfenil)propiónico) con n-butanol, catalizada por dicha lipasa, tanto en su estado nativo (Lipozyme® 10000L), como adsorbida sobre una resina de intercambio aniónico (Lipozyme® IM). La reacción fue seguida mediante HPLC con columna quiral, determinándose la conversión, así como la disminución relativa de la concentración de ambos enantiómeros del sustrato a lo largo del tiempo. Indicaremos que, al variar las concentraciones relativas de ambos sustratos, se observa como los efectos inhibitorios del exceso de alcohol se manifiestan fundamentalmente para elevadas concentraciones del mismo en el caso del enantiómero *R*, mientras que para el caso del enantiómero *S*, sobre el que la esterificación se produce en mayor extensión, estos efectos se manifiestan ya para concentraciones menores de alcohol. Asimismo se observa como la inhibición por exceso de alcohol se manifiesta más para el derivado inmovilizado que para la enzima cruda. Por otra parte, cuando se aumenta la cantidad relativa de ácido, los efectos inhibitorios se manifiestan de forma más acusada para el isómero *R*, de forma que la enantioselección enzimática mejora de manera notable, lográndose de esta forma aumentar la eficacia catalítica de la resolución cinética llevada a cabo con ambos catalizadores.

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Secuential Kinetic Resolution of (±)-*trans*-Ciclohexane-1,2-diamine

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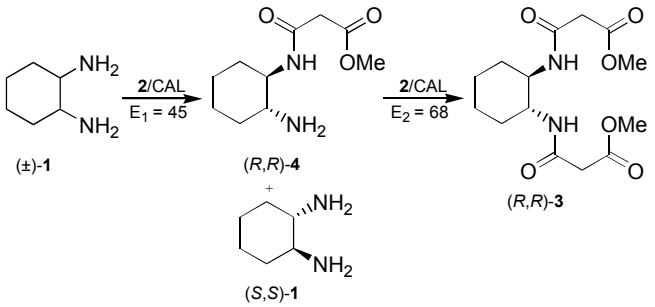
Taking into account the results obtained in our group with propane-1,2-diamine,[1] we decided to study the resolution of (±)-*trans*-ciclohexane-1,2-diamine [(±)-**1**], due to its importance in medicinal chemistry,[2] asymmetric synthesis[3] and the design of receptors of peptides.[4] We carried out the aminolysis of dimethyl malonate (**2**) with (±)-**1**, in the presence of the *Candida antarctica* lipase (CAL) and using 1,4-dioxane as solvent (see scheme). By controlling accurately the reaction conditions, is possible to obtain the substrate (*S*,*S*)-**1** or the product of the reaction (*R*,*R*)-**3**, both in enantiopure form (table, entries 1 and 2). Because the substrate has to visit the active center of the enzyme twice for the formation of (*R*,*R*)-**3**, we have determined the enantioselectivities for both processes separately (table, entries 3 and 4).[5]



Substrate	equiv. **2**	t (h)	ee-**1** (%)	ee-**4** (%)	ee-**3** (%)	c (%)	E
(±)-**1**	1	7	83	-	>99	45	-
(±)-**1**	1.2	9	>99	-	97	57	-
(±)-**1**	0.5	1	33	94	-	26	E_1=45
(±)-**4**	0.5	3	-	33	96	26	E_2=68

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Scale-up of Dipeptide AcPheLeuNH$_2$ Synthesis in a Reverse Micellar System of TTAB/Heptane/Octanol

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Key Words: scale-up, dipeptide, α-chymotrypsin, revere micelles, precipitation

Acetylphenylalanineleucinamide (AcPheLeuNH$_2$) dipeptide, an intermediate in the synthesis of some biologically active peptides, such as the opioid molecules enkephalin and dynorphin, can be synthesised by α–chymotrypsin (α-CT) in a reverse micellar system of tetradecytrimethylammonium bromide (TTAB)/heptane/octanol/carbonate buffer [1]. The synthesis of dipeptide occurs simultaneously with two side reactions, with the formation of AcPheOH and AcPheO$(CH_2)_7CH_3$ (scheme 1). The low solubility of the dipeptide in the reaction medium leads to its precipitation during synthesis [2].

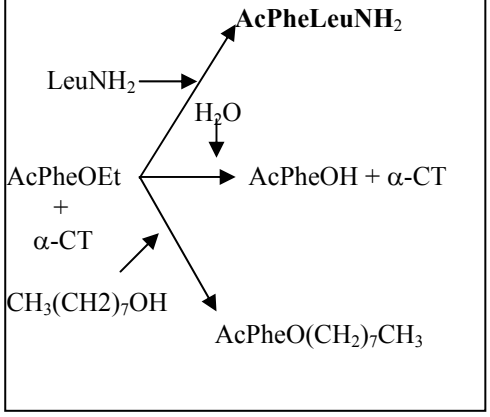
The aim of this work was to study the influence of scale up in the dipeptide synthesis. Batch reactions were performed in vessels with similar geometry, keeping D/T (impeller diameter/vessel diameter) constant and equal to 0,52 and N.D (N = stirring in rpm) constant and equal to 560 rpm.cm [3]. The volumes studied were 25, 150, 250 and 860 ml.

The results obtained are presented in table 1.

Table 1- Results from synthesis and recovery.

Reaction volume (ml)	Vi Dip (mM/min)	Yield (%)	Recovery (%)	Purity (%)
25	0,565	81	109	57
150	0,575	82	104	79
250	0,574	81	107	73
860	0,387	73	94	75

Scheme1



High reaction volumes led to a decrease in the initial rate of dipeptide synthesis and yield. However, the values obtained are very satisfactory.

With a single step filtration of the reactional media the dipeptides are recovered in the form of a white powder with a purity rounding 75% (HPLC analysis).

From a reaction volume of 860ml, 0,566g of AcPheLeuNH$_2$ were obtained with a purity of 75%.

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A Inexpensive FIA Biosensor for Ascorbic Acid (Vitamin C)

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Keywords: *ascorbic acid, FIA, biosensor, TCNQ*

It has been estimated that the food industry spends, on average 1,5 to 2,0% of value of total sales on quality control and appraisal. The food industry needs suitable analytical methods for process and quality control; that is, methods that are rapid, reliable, specific and cost-effective in their provision[1]. The ascorbic acid is important analito using as antioxidant for preservation of the industrial foods and as preventive of scorbutic disease[2,3]. The aim of this work is the development of a FIA biosensor for ascorbic acid with applications in food and pharmaceutical industries. The enzymatic electrode was prepared using ascorbate oxidase (E.C.1.10.3.3) extracted from *Cucurbita maxima* and partial purified by aqueous two-phase system (PEG/phosphate salt). This oxidoreductase enzyme was immobilised on graphite paste modified with tetracyanoquinodimethane (TCNQ), an electron-transfer mediator. The amount of enzyme for one electrode was $1{,}8x10^{-2}$ IU. At 0,2V (in 100mM of citrate-phosphate buffer (pH 6,0)), the linear concentration of ascorbic acid ranged from 10μM to 100mM, and detection limit was 10nM. The sensor was used to measure with successfully industrial samples such as orange juices and medicine presented as solvent tablets or liquids (diluted (1:1) in the same buffer). Studies of interference was made using glucose and citric acid, chemicals normally present in analysed samples, and the signal noise relation was about one. The variation coefficient was 6,27% after 100 injections of ascorbic acid and the activitity of enzyme was maintained stable about 30 days when the electrode was stored at -20 ºC.

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Experimental Design Optimisation of Penicillin acylase Purification in Aqueous Two-phase Systems

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Keywords - *penicillin acylase, aqueous two-phase systems, experimental design, purification*

Aqueous two-phase systems are formed by mixing incompatible polymers or a polymer and a salt. Due to their technical simplicity they have been widely employed on the extraction and as a first step on the purification of several enzymes. The best conditions for each enzyme are generally found by the systematic variation of several factors like polymers molecular weigh, concentration, salt concentration and pH.

In a previous work we reported the partial purification of penicillin acylase from osmotic shock extract of *Escherichia coli*. In a system at pH 6.9 containing 14% poly(ethylene glycol)(PEG) 3350, 0.42 mol/Kg sodium citrate and 1.5 mol/Kg NaCl a six fold purification was achieved with 85% yield [1]. Complex dependence of purification factor and yield was also found with the variation of tie-line length due to the variation of partition coefficients for total protein and penicillin acylase in these conditions [2].

The aim of this work was to optimise the purification of the enzyme from a crude extract. Due to the complex interdependence of the several factors involved this was performed by means of an experimental design. A central composite design centred on the previous found conditions for purification from osmotic shock extracts was used. The effect of PEG, sodium citrate and NaCl concentrations was studied. Partition coefficient of total protein and penicillin acylase, purification factor and yield were calculated for each experiment.

Purification factor data were fitted to a quadratic model with a high correlation coefficient (R^2=0.97). Yield was best fitted to an expanded quadratic model with an additional three factors interaction term (R^2=0.97). The surface defined by the first model had a maximum very close to the centre of the design:[PEG]= 12.98%, [sodium citrate]= 0.41 mol/Kg, [NaCl]= 1.52 mol/Kg. The purification predicted in this conditions was similar to the value we found before (6.2x) but the yield was lower (56%).

A compromise between purification and yield could be accomplish by superimposing the iso-response lines for this two parameters at constant NaCl concentration. At [NaCl] =1.5 mol/Kg a small region could be defined with purification factor between 5.0 and 5.5 and yield between 80% and 90%. Higher yields could even be obtained at [NaCl]=1.75 mol/Kg without great loss of purification. For the same interval as before a yield between 80% and 100% could be attained in a large area of the space.

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Chemoenzymatic Synthesis of 3'- and 5'-Carbazoylnucleosides. Preparation of 3'- and 5'-Arylidencarbazoylnucleosides.

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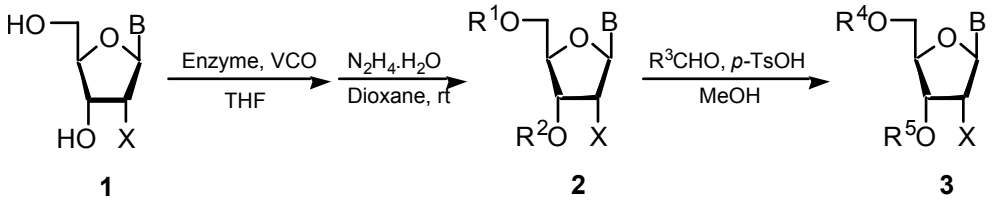
In the last few years has been enhanced interest in the development of new synthetic methods that allow access to the preparation of selectively modified nucleoside analogues owing to the discovery of several biological and biochemical properties of them.[1] The methodology of enzymes in organic solvents offers the opportunity to carry out highly regioselective transformations in mild reaction conditions avoiding time consuming protection and deprotection steps which chemical procedures require, even in some cases making possible the own synthesis.



1 **2** **3**

Enzyme= CAL, PSL

VCO= Vinyloxycarbonyl oxime

B= T, A, U; X= H, OH

R^1,R^2= H or C(O)NHNH $_2$

R^3= ^{n}Pr, Ph, *p*-PhOPh, 2-Furyl

R^4,R^5= H or C(O)NHN=CHR ^{3}

Thus, a chemoenzymatic procedure is described for the synthesis of carbazoyl nucleoside derivatives **2** and these are prepared for the first time. Following a procedure developed in our laboratory,[2] we obtain a new type of derivatives which had a carbazoyl substituent in the 3' or 5' hydroxyl group in nucleosides **1**. To prepare 5'-arylidencarbazoyl nucleoside derivatives **3**, we began with compounds **2** reacting with different aldehydes to obtain precursors with potential applications in both therapeutic and fungicide / herbicide areas.

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Extraction and Purification of Ascorbic Oxidoreductase with Aqueous Two-Phase System in Batch and in a Continuous Perforated Rotating Disc Contactor

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Keywords: *ascorbic oxidoreductase, extraction, aqueuos two-phase, contactor*

The extraction and purification of biological products is one of the areas of in biotechnological process. Among the techniques used for protein recovery and purification, liquid-liquid extraction using aqueous two-phase systems (ATPS) is one of the most attractive, due to gentle environment that provides to protein and other biological molecules (1). Aqueous two-phase systems have important potential in downstream processing as large-scale continuous operation for separations of proteins and removal of contaminants from impure extract (2). ln order to find suitable conditions for extraction of ascorbic oxidoreductase (E.C.1.10.3.3) with aqueous two-phase systems, it is necessary to known the partitioning behaviour of enzyme which depends on physico-chemical parameters of systems, namely the type and molecular mass of polymer, the tie line lenght, salt concentration and pH. The work describes the partitioning and purification of ascorbic oxidoreductase, enzyme extracted from *Cucurbita maxima* in polyethylene glycol (PEG)-phosphate salt systems in batch extraction and the best conditions obtained were using in a continuous perforated rotating disc contactor (PRDC). The study of partitioning behaviour at different tie lines was carried out varying the weight mass PEG (550, 1000, 3350 and 8000) at pH 5.0. Partition coefficient, k, was defined as ratio between ascorbic oxidoreductase activity in the top and bottom phase, respectively (3). Aqueous two-phase system was prepared total mass 6g. Total ascorbic oxidoreductase activity was assayed at 25 ºC in the both phases used 150 μM ascorbic acid in citrate-phosphate 0.1M pH 6.0 as substrate. The amount of total protein in both phases was determined. The experimental results obtained during batch extraction shown the ascorbic oxidoreductase partitions preferencially into salt-rich phase in all sytems, the best results recovery enzyme activity (about 312%) were obtained with PEG 1000 (19.7% w/w) and potassium phosphate (17.7% w/w), a purification factor of the 22 was achieved in these conditions. The total protein yield and separation efficiency in the continuous extraction after 70 minutes of operation were 37.3% and 135%, respectively. The results showed a constant increase in the capacity for extraction of enzyme from PEG-rich phase to salt-rich phase, these conditions a mass transfer coefficient (Kda/min) of the 0.05 was obtained, value higher than the one found used on a spray column.

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Adsorción de un extracto parcialmente purificado de la lipasa de *Rhizomucor miehei*. Efectos sobre la actividad y la enantioselectividad

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Palabras clave: lipasa, inmovilización, actividad, enantioselectividad

La inmovilización de enzimas por adsorción es probablemente el método más útil cuando se quiere aplicar el derivado obtenido a una Biotransformación, pues la sencillez de su procedimiento experimental no se ve empañada por problemas de desorción de la enzima, debido al medio eminentemente orgánico que va a ser posteriormente empleado [1].

Por otra parte, la obtención de extractos enzimáticos con un buen grado de pureza es de gran importancia si se pretenden racionalizar los resultados obtenidos atendiendo a la estructura del biocatalizador. No obstante, estos extractos deben ser sometidos a procesos de inmovilización para poder aumentar su estabilidad, la cual se ve comprometida al eliminar del crudo enzimático aditivos de diverso tipo que acompañan a la enzima. [2]

En esta comunicación presentamos los resultados obtenidos en la adsorción sobre una resina intercambiadora de aniones (Duolite AS 568) de diferentes preparaciones de lipasa de *Rhizomucor miehei* (cruda y parcialmente purificada por tratamiento con sulfato amónico), variando las condiciones de trabajo (naturaleza del buffer, pH del mismo, relación enzima/soporte, etc), según las diferentes metodologías descritas [3, 4]. Para cuantificar la actividad de los derivados obtenidos se han empleado como reacciones test la esterificación de un ácido quiral (ibuprofeno) y la transesterificación de un alcohol quiral (1-feniletanol), observándose unas buenas propiedades de los mismos, tanto en actividad como en estereoselectividad.

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Preparação de Membranas para Biossensores com Lipase Imobilizada

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Lipase, imobilização, membrana

A estabilidade catalítica é um dos aspectos mais críticos na construção de biossensores enzimáticos, tendo em vista a sua múltipla reutilização. O predomínio da adsorção em muitos métodos de imobilização em membranas explica, pelo menos parcialmente que, apesar da significativa expressão de actividade inicial, o decaimento nas sucessivas reutilizações seja rápido. A estabilidade operacional de um biossensor requer em geral uma ligação covalente do enzima à membrana e deve ser avaliada apenas depois de tratamentos (por exemplo, efeitos de força iónica, pH, ou tensioactivos) que favoreçam a desadsorção de enzima fracamente ligado.
No sentido de desenvolver um biossensor fotossensível para análise de gorduras, pretendeu-se desenvolver a metodologia de ligação covalente de lipase (EC 3.1.1.3) de *Candida rugosa* (tipo VII, Sigma) em diversas membranas poliméricas, preparadas por evaporação de solvente à temperatura ambiente e previamente activadas. A actividade lipolítica resultante foi avaliada pela hidrólise de trioleína em emulsão estabilizada com Triton X-100, usando o método de titulação.
Em face de conhecimentos prévios, foi numa primeira fase investigada a imobilização em membranas de acetato de celulose e de metacrilato de metilo-co-metacrilato de hidroxietilo. Membranas de acetato de celulose, preparadas em acetona ou em tetrahidrofurano, foram tratadas com periodato de sódio 0,5 M para posterior ligação covalente da lipase, por intermédio de braços extensores de hexametilenodiamina activados por glutaraldeído. Os resultados iniciais sugeriram, contudo, que a lipase era ligada apenas por adsorção física, com fraca actividade expressa na segunda e subsequentes utilizações. Decorreu daí a necessidade de melhorar a metodologia que passou por introduzir um passo prévio de hidrólise da membrana de acetato de celulose em meio alcalino (NaOH, 0,05 N) em ebulição com refluxo total. O tempo de hidrólise e a concentração de NaOH foram os parâmetros importantes a optimizar.
Nas membranas de metacrilato de metilo-co-metacrilato de hidroxietilo parcialmente hidrolizadas foram criados grupos carboxilo posteriormente activados com carbodiimida para ligação covalente da lipase. Os resultados da selecção dos melhores polímeros e métodos de activação, adequados ao fabrico de membranas para imobilização de lipase serão apresentados com vista à sua utilização num biossensor.

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Effects of Organic Solvents and Temperature upon Desorption and Decay of Lipozyme™

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Key Words: Lipase; immobilization; processing; enzymatic reaction

Industrial versatility of lipases has lead in the past few years to a renewed interest for this class of enzymes. However, two major drawbacks are in general associated with enzymatic processes: conversion is usually not high, so dilute solutions of product(s) are obtained; and intrinsically low productivities are often achieved because crude (commercial) enzyme preparations are employed rather than pure (analytical) enzymes. These drawbacks usually arise from low concentrations of active enzyme, kinetic and thermodynamic inhibition of enzyme by reactant(s) and/or product(s), and degradation of the enzyme either by heat (thermal deactivation) or by compounds present in the reaction mixture (chemical deactivation). In order to avoid the problem of low concentrations of enzyme in reaction media, immobilization has become common practice.

The present communication reports experimental work pertaining to the roles of temperature and type of solvent on (i) the desorption of enzyme from the support where it had been previously immobilized and on (ii) its loss of activity. The enzyme used was Lipozyme™, a commercial preparation of lipase immobilized onto an ion exchange resin, which was incubated in ethenol, butanol, ethyl butanoate and butyl butanoate at temperatures ranging from 30 to 70 ^{o}C. The variation of the concentration of enzyme in the bulk supernatant solution was measured spectrophotometrically at defined intervals of time. Experimental data of lipolytic activities were generated by the pH-stat method following incubation in the solvents at the aforementioned temperatures for various times.

Alcoxicarbonilación Enzimática de Aminas usando Lipasas de Diferente Origen, Libres y Inmovilizadas

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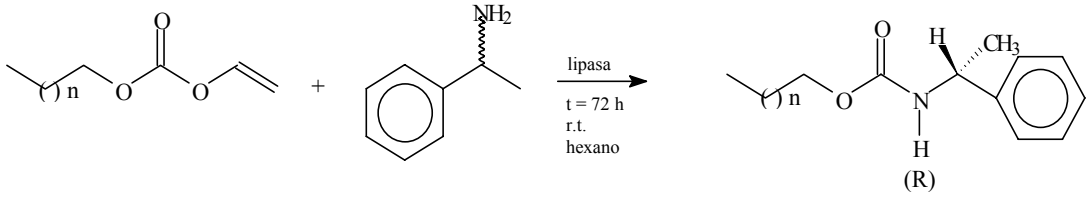
Palabras clave: lipasa, amina, carbamato

En los últimos años se ha destacado la importancia del grupo carbamato en numerosas moléculas de interés terapéutico [1]. Sin embargo, la síntesis de estas estructuras requieren reacciones específicas y en algunos casos reactivos tóxicos como el fosgeno y compuestos organometálicos. Recientemente se ha realizado la alcoxicarbonilación enzimática para obtener derivados quirales partiendo de vinilcarbonatos, siendo la lipasa de *Candida antartica* la más utilizada en este tipo de procesos [2]. Este método es interesante en la resolución de aminas racémicas, las cuales tienen un gran interés en la síntesis de pesticidas.

En el presente trabajo se ha realizado esta reacción con lipasas de diferente origen, tanto nativas como inmovilizadas, para obtener carbamatos quirales.



En este procedimiento de alcoxicarbonilación se ha realizado un estudio con el doble objetivo de analizar la influencia de la cadena carbonada del vinilcarbonato, para lo cual se han empleado el butil y el octil carbonato de vinilo.

Para la determinación del porcentaje de conversión, se empleó la cromatografía líquida de alta resolución (HPLC), y los excesos enantioméricos se determinaron por espectroscopía de RMN-H^1, utilizando el reactivo quiral tris-[3-(heptafluoropropilhidroxi-metilen)-(+)-camforato] de europio (III).

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Aproximación al Centro Activo de la Lipasa de Pancreas Porcino a través de la Resolución de 1,n-dioles por Transesterificación

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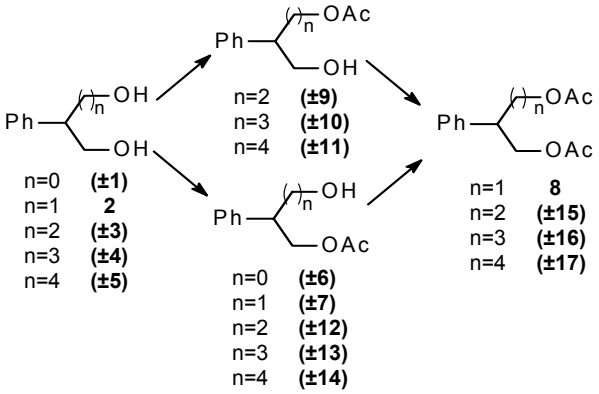
Palabras clave: PPL, diol, transesterificación, enantioselectividad, regioselectividad.

Las reglas propuestas hasta ahora para explicar el enantioreconocimiento de la PPL frente a alcoholes primarios no han resultado demasiado convincentes al resultar de ellas modelos enantioméricos[1,2] para el centro activo de la lipasa mencionada.

Con el objetivo de proponer un nuevo modelo, se ha llevado a cabo la síntesis de los dioles 2-fenil-1,4-butanodiol, 2-fenil-1,5-pentanodiol y 2-fenil-1,6-hexanodiol por métodos no descritos anteriormente. Estos compuestos, junto con los análogos 2-fenil-1,2-etanodiol y 2-fenil-1,3-propanodiol, han sido empleados como sustratos de la PPL en reacciones de transesterificación con acetato de vinilo. La conversión, regioselectividad y enantioselectividad de la lipasa frente a los sustratos mencionados ha sido analizada empleando la técnica del HPLC. A través de dicho estudio y teniendo como base la estructura cristalina de la lipasa, recientemente determinada por difracción de rayos-X[3], se propone un modelo racional del centro activo de la PPL.



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Enzymatic Peptide Synthesis using α–Chymotrypsin in Reverse Micelles. Kinetic Studies.

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Keywords: peptide synthesis, reverse micelles, α-chymotrypsin, kinetic model, central composite design.

The main objective of this study is to model the peptide synthesis in a reverse micellar system based on kinetic studies. The reaction studied was the dipeptide $AcPheLeuNH_2$ synthesis from AcPheOEt and $LeuNH_2$. The biocatalyst was α-chymotrypsin encapsulated in reverse micelles formed by the cationic surfactant tetradecyltrimethylammonium bromide in heptane/octanol. During this synthesis two other by-products, AcPheOH and AcPheOR, resulting from the ester substrate hydrolysis and transesterification are also formed, although with a much lower yield [1]. The study of a kinetic model to be applied to this synthesis reaction was done by using the experimental planning known as central composite design (CCD) [2]. By using this planning the two substrate concentrations were varied in a such a range that all the possible combinations were studied giving a complete region of enzyme responses, figure 1. From the response surface curves obtained several kinetic equations were proposed. The differential equations obtained were integrated giving the product formation at different reaction times. The kinetic equations chosen were those giving the smaller error adjustment to the experimental results, figure 2.

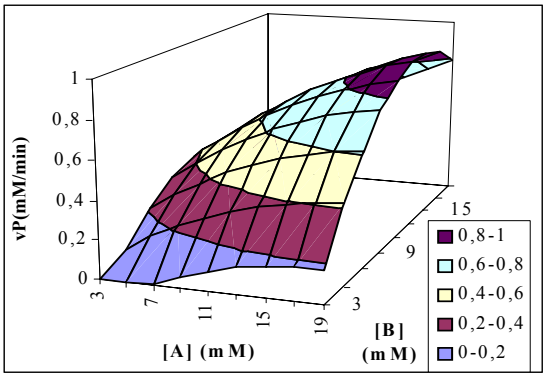

Fig. 1. Effect of substrate concentration, AcPheOEt (A) and $LeuNH_2$ (B) on the initial velocity of peptide synthesis (v_P).

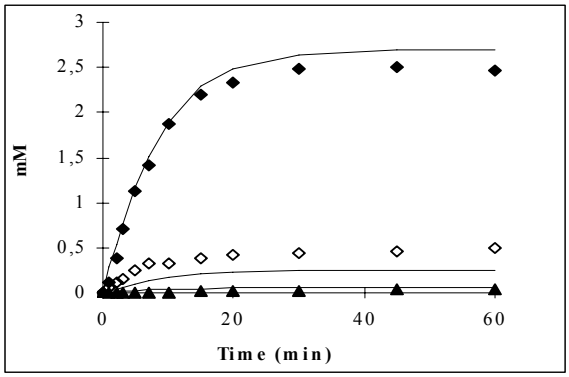

Fig. 2. Adjustment of the chosen model to the peptide synthesis(♦) and by-products production during one hour of reaction.

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Purificación de la Enzima Glutamato Racemasa durante su Inmovilización Multipuntual a Geles Glioxil-agarosa

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Palabras claves: *Inmovilización, glutamato racemasa, purificación.*

La preparación de derivados enzimáticos para su uso industrial se facilita notablemente al aumentar el grado de pureza de los extractos utilizados. Cuanto más puro sea el extracto se pueden preparar derivados mucho más activos y además se puede evitar la co-inmovilización de otras enzimas del extracto que pudiesen tener actividades catalíticas laterales indeseables. Sin embargo la utilización de extractos altamente purificados aumenta notablemente el costo de estos biocatalizadores industriales. Desde este punto de vista la asociación del proceso de inmovilización con un proceso de purificación de enzima industrial resultaría muy prometedor. La inmovilización de enzimas a soportes glioxil transcurre a través de una primera unión, al menos bi-puntual, entre cada molécula del enzima y el soporte. Por ello, proteínas con diferentes densidades superficiales de residuos lisina pueden reaccionar con los soportes activados a muy diferente velocidad. Lógicamente, las proteínas que tengan mayores densidades de residuos lisina superficiales deberían inmovilizarse más rápidamente y por ello podrían ser purificadas al mismo tiempo que se inmovilizan. Para la inmovilización de glutamato racemasa se parte de un extracto crudo procedente del sobrenadante de ruptura de fermentación de *E. coli* DH5α-pGRF [1,2]. El extracto se inmovilizó en glioxil-agarosa 10BCL (200μ moles), según la metodología de Guisán y col.[3], cargando el gel a su máxima capacidad. El proceso de inmovilización se siguió mediante electroforesis SDS-PAGE tomando muestras del sobrenadante y de la suspensión previa desorción de las proteínas. En las figuras se puede comprobar como un elevado porcentaje de glutamato racemasa presente en el extracto se inmoviliza en 30 minutos, no así el resto de proteínas, consiguiéndose un derivado activo, estable y con un porcentaje elevado de glutamato racemasa. La glutamato racemasa contiene 14 lisinas, lo que apriori le conferiría una alta velocidad de inmovilización.

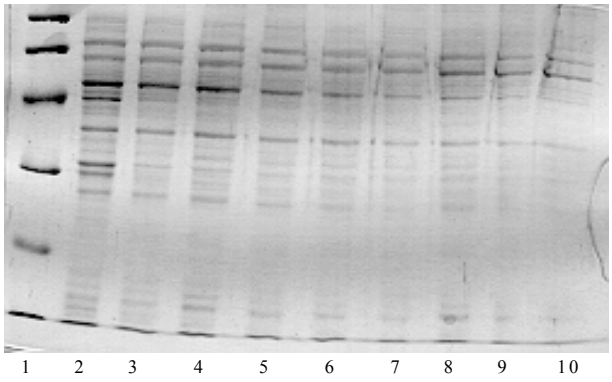
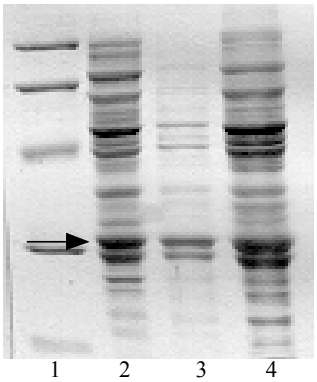


Figura 1.- *Inmovilización en gel glioxil agarosa, 1.- Patrón: ABS de bajo rango. 2-10.- Sobrenadantes de inmovilización cada 15 minutos.(2= tiempo cero)*



1.- Patrón ABS de bajo rango.

2.- Extracto con glutamato racemasa (indicada con flecha), tiempo: cero.

3.- Proteínas desorbidas del soporte, tiempo: 30 minutos.

4.- Proteínas desorbidas del soporte, tiempo: 3 horas.

Figura 2.- Purificación de la glutamato racemasa durante la inmovilización.

[1] Mansur, M. y colaboradores (1998) *Biotechnology Letters,* **20-1**, 57-61
[2] Alonso, Jorge y García, José L. (1996) *Microbiology*, **142**, 2951-2957
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Inmovilización de Glutamato racemasa - Efecto de la Densidad de Grupos Glioxil de Agarosa 10BCL

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Palabras clave: *glutamato racemasa, inmovilización, racemización, estabilidad.*

La inmovilización multipuntual de una enzima sobre un soporte pre-existente altamente activado puede provocar un interesante aumento de la estabilidad de la enzima inmovilizada frente a cualquier agente inactivante (calor, pH, disolventes, etc.)El hecho que varios aminoácidos de cada molécula enzima inmovilizada estén fuertemente unidos al soporte debe impedir cualquier alteración en sus distancias relativas y debe reducir drásticamente las posibilidades de la enzima para sufrir cambios conformacionales. La enzima glutamato racemasa cataliza la conversión del ácido glutámico a la mezcla racémica paso previo para la obtención del ácido D-glutámico, isómero no natural del aminoácido. El ácido D-glutámico es un interesante precusor de productos farmacéuticos. En el presente trabajo se plantea la inmovilización multipuntual de la enzima con objeto de mejorar su reutilización en reactores industriales y simultáneamente aumentar lo más posible su estabilidad térmica.

La enzima se obtuvo del sobrenadante de ruptura de la fermentación de *E. coli* DH5α-pGR1 [1] modificada, resultando una proteína de fusión que se hiperexpresa [2] y sigue siendo activa. El soporte utilizado fue glioxil-agarosa 10% (Hispanagar) y la preparación de los derivados inmovilizados se realizó según el protocolo de Guisán y col.[3]. Además del efecto de la densidad de grupos glioxil se ha estudiado el efecto de la temperatura, presencia sustrato, concentración de agente reductor en la actividad final y estabilidad de los diferentes derivados obtenidos. La glutamato racemasa es una enzima que posee numerosas lisinas no próximas al centro activo y ello podría posibilitar la formación de enlaces multipuntuales. En la figura se observa como efectivamente todos los derivados inmovilizados son mucho más estables que la enzima soluble y como la estabilidad aumenta a medida que aumenta la concentración de grupos activos en los geles. De hecho la enzima soluble pierde el 90% de actividad en menos de 1 hora y por el contrario el derivado más estable (preparado con los geles más activados) conserva el 70% de actividad después de 50 horas a la misma temperatura. Por otro lado el uso de un sustrato de la enzima durante el proceso de inmovilización ayuda a mantener muy altos porcentajes de actividad (más del 70%).

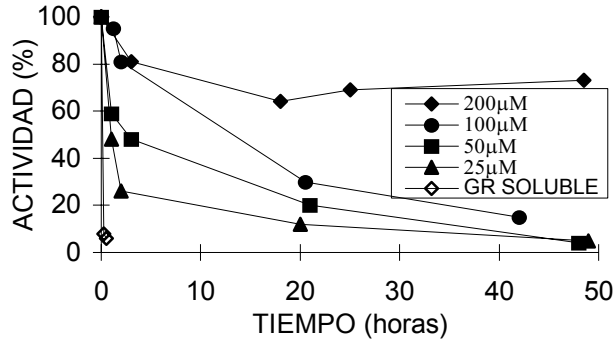

Figura 1.-*Inactivación térmica de glutamato racemasa soluble e insoluble*

1] Mansur, M. y colaboradores (1998) *Biotechnology Letters,* **20-1**, 57-61 .
[2] Alonso, Jorge y García, José L. (1996) *Microbiology*, **142**, 2951-2957..
[3] Guisán, José M. y colaboradores (1997) *Methods in Biotechnology: Immobilization of Enzymes and Cells.* Gordon S. Humana Press.

Um Gel de Poli(acrilato) como Fase Aquosa para Biocatálise em Meio Orgânico

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Keywords: Poli(acrilato) de sódio; cutinase; hidrólise; meio orgânico

Os biocatalisadores podem ser operados em meio orgânico segundo diferentes sistemas reaccionais [1]. Neste trabalho, um gel do polímero superabsorvente poli(acrilato) de sódio, com uma enzima lipolítica imobilizada, a cutinase recombinante de Fusarium solani, foi utilizado como fase aquosa num sistema bifásico com o substrato hidrófobo dissolvido na fase orgânica.
O objectivo deste trabalho foi estudar a imobilização da enzima no gel e o comportamento catalítico da enzima imobilizada em solventes orgânicos. A cutinase foi imobilizada por oclusão no gel e usada para a hidrólise de tricaprilina em iso-octano e outros solventes. As reacções decorreram com agitação orbital e a actividade enzimática foi determinada acompanhando a libertação de ácidos gordos livres pelo método de Lowry-Tinsley [2].
O processo de oclusão foi optimizado em termos de rendimento de imobilização e actividade específica da enzima imobilizada, estudando os efeitos do tempo, molaridade do tampão e pH de oclusão. A molaridade do tampão é um factor determinante para as propriedades do sistema, pois influencia as características do gel, nomeadamente, a sua porosidade.
A lipólise pela enzima imobilizada no gel é acompanhada pela emulsificação deste, o que causa um incremento da actividade aparente, em resultado de maiores velocidades de transferência de massa entre as fases. A limitação da actividade do sistema por fenómenos de transferência de massa é confirmada pela influência da intensidade da agitação do meio de reacção.
A enzima imobilizada apresenta actividade óptima a pH mais alcalino, e uma cinética do tipo de Michaelis-Menten com inibição pelo substrato a partir de 500 mM. As constantes cinéticas determinada foram Vmax = 84,7 U.I./mg e KM = 176,8 mM.
Vários solventes foram testados como fase orgânica, tendo-se observado maior actividade com o iso-octano, e menores actividades com solventes mais polares e mais apolares, usando o Log P como medida da polaridade.O método de imobilização desenvolvido neste trabalho é muito simples e rápido.
O gel de poli(acrilato) apresentou elevadas potencialidades para a imobilização de enzimas de baixo peso molecular, e demonstrou poder funcionar como fase aquosa num sistema bifásico para biocatálise em solventes orgânicos.

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An Overview of Mechanisms and Rate Expressions of Reactions Carried out by Immobilized Lipases

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Keywords*: Enzymes; chemical reaction; kinetic parameters; modelling*

The industrial versatility and the catalytic performance of lipases have been attracting increasing interest in recent years. However, in order to overcome the classical drawback of low volumetric productivities associated with use of enzymes in general, immobilization procedures have been frequently implemented, thus contributing to improve the economics of the technological processes (via reutilization for more than a single reactor pass) and to decrease the contamination of the final product (via confinement only to the reactor volume).

This work focuses on chemical reaction processes brought about by immobilized lipase reactors, and provides a brief compilation of over 75 studies on kinetics and mechanisms of reactions carried out by immobilized lipases reported in the literature in the latest decade. The mechanisms more frequently postulated are presented, as well as structural features of lipases that help in understanding their immobilization procedures, interfacial activation, catalytic performance and dependence on chemical and physical parameters.

Hidrólisis Enantio y Regioselectivas catalizadas por Lipasas Inmovilizadas en Octil-Agarosa

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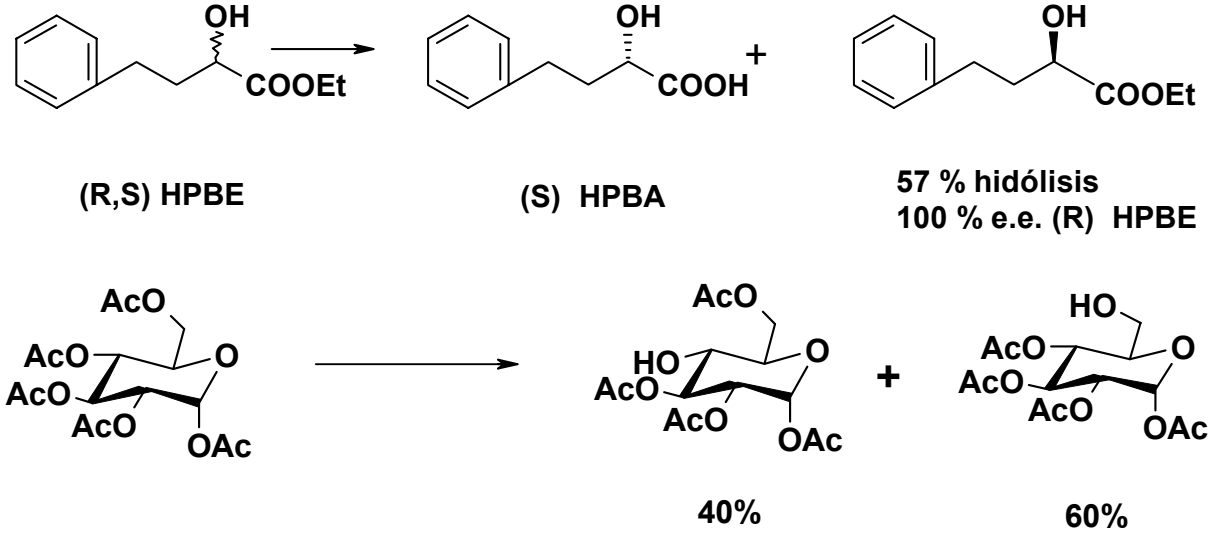
***Keywords**: adsorción interfacial de lipasas, enantiselectividad, regioselectividad*

Debido a su complejo mecanismo de acción (activacion interfacial) las lipasas son generalmente muy poco activas en medios acuosos y en ausencia de interfases. Quizás por esta razón, el enorme potencial de los procesos de química orgánica catalizados por lipasas en medios acuosos no ha sido todavía suficientemente explotado. La irreversibilidad de los procesos de hidrólisis de esteres en medio acuoso y pH neutro hace que estos procesos sean en principio muy adecuados para obtener enantioselectividades muy elevadas tanto para los isómeros de ésteres quirales no hidrolizados por la enzima como para los productos de hidrólisis.
Cuando las superficies de soportes hidrofílicos se recubren de una capa muy densa de grupos altamente hidrofóbicos (C8, C18) podemos esperar que las lipasas puedan reconocer estas superficies solidas hidrofóbicas "diseñadas a medida" de un modo similar a como estas enzimas reconocen las interfases liquidas de sus substratos naturales. De acuerdo con esta hipotesis nosotros hemos logrado diseñar un metodo de purificación, inmovilización e hiperactivación de numerosas lipasas microbianas por adsorción a geles octil y decaoctil-agarosa [1]. En esta comunicación se discuten las excelentes propiedades de estos derivados para catalizar algunos procesos de hidrólisis enantiselectiva de ésteres quirales y algunos procesos de hidrólisis controlada y regioselectiva de polioles.



• catalizados por PsF lipasa adsorbida a geles octil-agarosa

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Alcoholysis reactions with α-amylase

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Key words: alcoholysis, α-amylase, transglycosidases, alkylglycosides

Several enzymes, in particular glucosidases, have been succesfully applied in the synthesis of alkylglicosides which may have applications as surfactants in food, pharmaceutical and chemical industries. Alcoholysis reactions take place when soluble or insoluble alcohols substitute water in hydrolisis reactions, and this possibility has been particularly studied with lipases and carbohydrases. Among carbohydrases, ß-galactosidase, α-glucosidase and ß-xylosidase are particularly efficient for the transfer of galactose, glucose and xylose from lactose (or ONPG), celobiose and xylobiose respectively to alcohols, resulting in alkylglycoside. However, there are very few studies concerning the use of α-amylase in alcoholysis in contrast with its well studied transglycosidase activity. In this presentation we report the behavior of α-amylase in methanol using various substrates (starch, dextrins and maltopentaose). We compared the behavior of α-amylases from *A. niger. B. licheniformis* and *B. stearothermophilus*, observing that only the fungal α-amylase is able to transfer blucose units to methanol. Reaction conditions for optimal alcoholisis were defined and HPLC analysis combined with glucoamylase treatment was used to analyze and quantify the alcoholysis products after hydrolysis of methyl-oligosaccharides to methyl-glucoside. The specificity of the fungal amylase for the production of methyl-maltoside is demostrated as well as the stability of the enzyme in methanol. Structural differences arising from published sequences of amylases from bacterial and fungal origin are used to explain the different behavior of the enzymes in the presence of methanol, and are used to propose a mechanism for the construction of higher transferase activities in α-amylases.

Biotransformations with (*R*)-Oxynitrilase in Organic Solvents. Synthesis of Nitrogen 3-Hydroxyheterocycles

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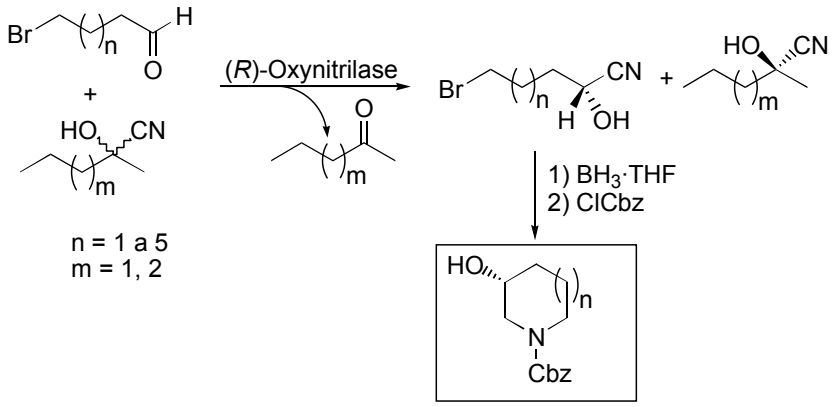
The synthesis of optically active cyanohydrins has received a considerable amount of attention during the last years; especially enzymatic catalyzed procedures.[1] (*R*)-Cyanohydrins can be easily obtained using the enzyme (*R*)-oxynitrilase as present in almond meal.[2]

We have recently studied the enzyme (*R*)-oxynitrilase-catalyzed process between different ω-bromoaldehydes and racemic ketone-cyanohydrins.[3] The ω-bromocyanohydrins obtained show a high enantioselectivity and, at the same time, we observed a high enantioselectivity in the simultaneous enzymatic decyanation of the ketone-cyanohydrin.
The optically active ω-bromocyanohydrins can be useful intermediates in the preparation of



different interesting compounds. We have used these cyanohydrins in the preparation of 2-cyanotetrahydrofuran and 2-cyanotetrehydropyran.[3] On the other hand, we have prepared (S)-pipecolic acid and other pyperidine derivatives from these compounds.[4] In this communication we use the optically active ω-bromocyanohydrins as starting material in the preparation of nitrogen heterocycles. The reduction of the nitrile function with BH_3·THF allows us to obtain, in one pot, nitrogen 3-hydroxyheterocycles from 6 to 10 in high yield and enantiomeric excess.

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Effect of Cellulase Adsorption on the Surface and Interfacial Properties of Cellulose

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In the last 10 years, the paper industries have been increasing the amount of used paper, for the manufacture of new paper products. However, use after use, undesirable changes in the pulps furnish [1] tend to occur, affecting mainly the pulps ability to release water [2]. The enzymatic treatment of wastepaper pulps [2-6] could improve the pulps drainability. Although the hydrolytic capability of the enzymes may be important in this improvement, the effect of the adsorbed protein (enzymes) on the surface properties of the fibers, should also be pondered. This work is an attempt to understand the effect of cellulase adsorption on the fibers surface and interfacial properties, and to relate these effects with the drainability improvement. Purified celluloses were used instead of lignocellulosic materials, because cellulose fibers are complex and heterogeneous materials, which would make their characterisation difficult, with the techniques applied.
The surface properties of purified cellulosic materials was characterised, before and after protein adsorption by thin-layer wicking. The celluloses interaction with water was also analysed by thermogravimetry (amount of adsorbed water) and differential scanning calorimetry (specific heat of dehydration), before and following protein adsorption. It was concluded that celluloses have a strong electron donor component, regardless of their cristallinity. The more crystalline are slightly hydrophobic. Both the heat of dehydration and the amount of adsorbed water are higher in the less crystalline celluloses. After protein adsorption, the cellulose surface become hydrophilic, and can adsorb a higher amount of water; this water is also more tightly bound to their surface [7].
The results obtained from this work were discussed on the perspective of possible mechanisms of the enzymatic drainability improvements: regardless of the preferential type of activity for this application, cellulases and xylanases have been proven to be effective in the improvement of the pulps drainage ability. However, the adsorption of the enzymes, which stabilises the fibers, probably increases the porosity of the pulps slurry, therefore increasing the velocity of water drainage.

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Biosensor for Glucose Applying Electrical and Chemical Properties of Polyaniline Film

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Keywords: Polyaniline, Biosensor, glucose oxidase

Polyaniline is a conjugated polymer composed of oxidatively coupled aniline monomers, throughout eletrochemical reaction or converted to a polymeric radical cationic salt with an organic acid. Polyaniline is a reactive polymer, and as a metal it is nobler than iron or copper. Among several system developed to study the interactions with glucose oxidase while immobilized in order to developed a biosensor for glucose measurement, we found a polyaniline potentially attractive to be used as support for biosensor development, because it has a wide range of associated properties, such as easy protonation reversibility, excellent redox recyclibility, conductivity, electrochemical and optical properties, coupled with good stability. In aim of this work is to develop a biosensor for glucose using several physical/chemical properties of polyaniline. Polyaniline film was synthetesid by eletrochemical oxidation of aniline (0,5M) prepared in nitrc acid 1M using a stainless steel electrode applying a constant current of 0,25mA (200mV) for 24h at 25^{o}C attached on metacrylate surface. The immobilization of Glucose Oxidase (GOD) was carried out as described by Parente et al. (1992), applying glutaraldehyde as bifuntional agent (leite at al., 1994).A solution of 2,5% of gluataraldehyde (phosphate buffer, 0,1M, pH 5,0) was added on polyaniline film per 30min at 25^{o}C. The first biosensor design was based on the spectrophotometric properties of the polyaniline film (polyaniline and GOD). Using 100mM of glucose in the system the polymer was reduced (650nm), which correspond to the electrons transfer to the polyaniline. The electrical properties was studied in the following system. Negative charge (-0,25mA, 200mV) was applied to the polymer, after 30, 60 and 120 min, measuring the enzyme activity by oxygen consumption.We found an increasing apparent Km values, 2,28x10^{-3}M (30 min) to 12,28x10^{-3}M (120min), respectively. This behaviour could be explained by the use of the polymer as the eletrons acepptor or the charge applied changed the protein conformation. On the other hand, when the polymer was oxidized (+0,25mV, 200mV), the initial reaction velocity increased and the apparent Km was the same. These results show the influence of redox state of the polymer on the enzyme. The following experiment was applied diferent electric charges in the polymer from -0,25 to -1,00 mA (200mV). The in this system the enzymatic reaction started after the electrical equilibrium between the polyaniline film and the substrate soltion(30seg for -0,50mA up to 6min for -1.00mA). These results could be due the charge storage in the polymer that inhibed the eletrons transfer from the substrate. These results suggesting that, using these electrical properties is possible to modulate the enzyme activity, affinity and to determine when we intend to start the enzymatic reaction.

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Processing Textile Fibres With Enzymes - An Overview

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Keywords: *Enzymes, Textiles, Fibres*

Enzyme proteins are catalysts for most reactions in living organisms and advances in biotechnology have led to their use for industrial processing of natural products. The main advantages of enzyme catalyzed reactions are: mild temperatures of processing, absence of by-products and most of the enzyme processes are claimed to be environmental friendly. In textile processing of natural fibres there are some stages where enzymes could be used. Fibre components like fats, waxes, starch, hemicelluloses, cellulose, proteins among others are ideal substrates for different classes of enzymes.

The microbial retting of bast fibres was the first biological process used in the textiles from BC times to actuality. The first use of enzymes in textile process was made in the 1857 when starch sized cloth was soaked for several hours with water liquor containing barley. In 1900 this process was slightly improved with the use of malt extract, but it was only in 1912, with the use of animal and bacterial amylases, that the process became effective and started to be applied to the industry.

In the last 10 years, new enzyme based effects and processes have been introduced such as the aged look in Denim garments and free pills cotton fabrics among others. Nowadays, some enzymes are very successful textile finishes. However, very little is known about the various processing mechanisms. This is mainly due to the lack of biochemical knowledge of textile chemists and to the lack of understanding of textile process by biochemists and biotechnologists.
In the last years, this area has suffer a rapid growth and in this paper it is intended to give an overview of the existing processes and related research.

Enzymatic Scouring of Cotton - A Clean Production Aproach

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Our objective is the development of an enzymatic process as an alternative to the conventional method of scouring, in order to decrease the environmental impact.

The conventional process for the scouring of cotton fibres is made by a chemical treatment with alkaline boiling at 100º C. It requires up to 80 Kg of caustic soda per ton of cotton. This method involves the use of large amounts of energy, and produces great volumes of wastewater. Apart from these problems, it must be taken into account that the chemical process spoils dramatically the fibre.

The scouring of cotton fibre has been studied using two characteristic enzymes of the primary wall: Pectinases and Lipases. Also, it has been analysed the influence of four parameters in the enzymatic scouring of cotton: pH, Temperature, Enzyme and Surfactant. These parameters have been optimised and a kinetic study has been carried out.
Enzymatic process takes better care of the fibre structure. This has been checked by strength tests and with the degree of polymerisation (DP) of the fibre. It has been shown that the degree of polymerisation of the fibre has been better preserved with the enzymatic process.
The absorbency tests are highly satisfactory with the enzymatic process. It has been obtained wetting times similar to the ones obtained with the chemical process.
The kinetic experiments reflect a better correlation with the enzyme concentration than with the subtract concentration (cotton). It is probably due both to the problem of mass transfer in heterogeneous medium and to the best contact enzyme/subtract in a bath with a relation cotton/water 1/20.

The waste water analysis of the enzymatic process reflects less amount of COD. It is due to the use of less quantity of surfactant in this process. It is possible too, that the more soft conditions of the enzymatic process and the better care of the fibre structure could give low levels of COD in the waste water.

Procedure		COD mgO_2/L	Weight Loss %
Chemical Treatment	Fibre	2570	5.6
	Fabric with 50 % cotton	1570	2.94
Enzymatic Treatment	Fibre	2350	3.49
	Fabric with 50 % cotton	1240	1.95

Optimization of Medium Culture for Cellulase Production by *Trichoderma reesei* Rut C-30 in Shake Flask

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Key words: Production of Cellulases, T. reesei *Rut C-30*

Cellulase is one of the most extensively investigated multicomponent enzyme system because of its ability to decompose the cellulosic biomass into glucose, which in turn can be converted to other valuable chemicals and energy. Funcionally complete cellulase enzyme systems can be produced by a large diversity of microrganisms. Among the best caracterized and most widely studied of these systems are the inducible cellulases of the fungus *Trichoderma*, particullary *T. reesei*. Cellulases produced by this filamentous fungus have a high practical potential. They are widely used in the food and feed industries and recently also in the textile and pulp and paper industries. The biosynthesis and secretion of cellulases by *T. reesei* are known to be adaptative, i.e., inducibles by the presence of cellulose, but repressed by easily metabolizable carbon sources, eg, glucose or glycerol.

The aim of this work is to delineate optimal conditions for cellulase production. In this way, results obtained with the cellulases production by *T. reesei* Rut C-30 are presented. The *T. reesei* Rut C-30 is a mutant strain which produces cellulases more efficiently than a wild type *T. reesei*, and cellulase expression is not repressed by glucose to the same extent as in some others strains [1]. *T. reesei* Rut C-30 was grown on potato dextrose agar slants at 28ºC for 5 days. Inocula were prepared by transferring the conidia to the medium TMM (*Trichoderma* minimal medium), to the medium TMM with citrate buffer and to the medium developed by Reese and Mandels [2]. The carbon source used was glucose in concentrations of 20g/l and 50g/l. The fungus was grown in flasks for 48h at 34ºC on a rotary shaker at 200 r.p.m. The 10%(v/v) inoculum concentration was used to initiate the cellulase production. The composition of production medium was the same as that for growth, except that was supplemented with specific substrates, in different concentrations. All the fermentations were carried out in 250 ml erlenmeyer flasks containing 55 ml medium for 5 days at 28ºC and 150 r.p.m.

Results obtained with different carbohydrates (cellulose derivatives, lactose, glucose, cellobiose, strips of filter paper) and at different concentrations led to filter paper activities in the range 0.5-1 FPU/ml. The maximum biomass, FP activity and specific activity,on 1% cellulose, were 16 g/l, 1 U.I./ml and 25 U.I./mg soluble protein, respectively. In this way, specific activities higher than those in the literature were obtained. We found that this strain have quantities of constitutive cellulases which are independent of carbon source present in the media and are produced upon limitation of the carbon source.

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Estabilidad de Enzimas Ligninolíticos Durante el Cultivo en Estado Sólido de Phanerochaete chrysosporium

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Palabras clave: estabilidad, ligninolítico, estado sólido, Phanerochaete chrysosporium

La lignina es uno los componentes mayoritarios de la madera, junto con la celulosa y la hemicelulosa. Se trata de un polímero aromático tridimensional y altamente irregular, lo que le confiere una gran resistencia al ataque enzimático y por lo tanto a la biodegradación. Durante los últimos años, numerosos trabajos de investigación se han centrado en el desarrollo de procesos biotecnológicos de conversión y degradación de este compuesto, con el doble objetivo de optimizar el aprovechamiento de los materiales lignocelulósicos y de minimizar el daño ecológico causado por el vertido de efluentes industriales con algo contenido en lignina (1).

El hongo de putrefacción blanca *Phanerochaete chrysosporium* presenta un especial interés, debido a su capacidad de degradar la lignina mediante la acción de enzimas secretados durante el metabolismo secundario (2, 3). La producción de complejos enzimáticos ligninolíticos por este microorganismo ha sido ampliamente estudiada, utilizando tanto cultivos líquidos (4, 5) como en estado sólido (6). Sin embargo, uno de los principales problemas que presenta la obtención de estos enzimas es su baja estabilidad, que da lugar a descensos repentinos en la actividad durante el proceso de producción (7, 8).

En el presente trabajo se ha investigado la evolución de la actividad ligninolítca obtenida por cultivo en estado sólido de *Phanerochaete chrysosporium* sobre varios tipos de soporte (espuma de nylon, carozo de maíz), utilizando los métodos descritos por Tien y Kirk (9) y por Kuwahara et al (10). Se ha realizado un seguimiento de la aparición de proteasas extracelulares en el medio de cultivo, midiendo la actividad proteásica sobre el sustrato sintético azocaseína (11) así como la producción de sustancias desactivantes (H_2O_2), y se han relacionado estos factores con las pérdidas de actividad ligninolítica observadas en determinados momentos del cultivo.

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Phanerochaete flavido-alba MnP isoenzyme pattern modification and laccase induction in decolorized olive oil mill waste waters (OMW)

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The olive oil mill waste water (OMW) is currently concentrated by evaporation in aerated lagoons which leaves a black, foul-smelling sludge which is difficult to dispose of. Polymeric phenolic compounds, a lignin-like structure, give the sludge its characteristic recalcitrant brownish-black color. An important step in the degradation of OMW is the breakdown of colored polymeric phenolics (decolorization). A significant correlation between OMW decolorization and reduction of total organic carbon (TOC) and phenolic compounds has been demonstrated.

The objective of research reported herein was to determine the pattern of ligninolytic enzymes present in the extracellular fluids of *Phanerochaete flavido-alba* decolorized OMW-containing cultures and to compare them to the enzymes produced in chemically-defined OMW-free liquid cultures. Lignin-degrading enzymes were partially purified from the supernatants of *Phanerochaete flavido-alba* decolorized OMW. The dominant enzymes, manganese peroxidases (MnPs), show different isoform patterns (analysed by FPLC) in decolorized OMW-containing cultures compared to residue-free samples. In extracellular fluids, MnP activity decreased by around eight fold in OMW-containing cultures compared to controls. Lignin peroxidase (LiP) was not dectectable in any of the samples tested.

Semipurified samples were analysed by SDS-PAGE and IEF. MnP-activity staining of IEF gels showed that MnPs from control samples had more basic pIs than those from OMW containing samples. These results strongly suggest that the MnP isoforms present in the decolorized samples were different from those present in control supernatants. Laccase induction was detected in OMW-containing, but not control cultures.The induction of laccase activity in OMW-containing cultures was demonstrated by several pieces of evidence: a protein with an apparent molecular weight of 94,000 was induced in OMW-containing cultures and laccase activity was detected as a diffuse band, with a pI similar to purified *P. flavido-alba* laccase in IEF gels of OMW-containing samples. Given the similar Mr and pI of the induced laccase reported here, it is probably that it is the same *P. flavido-alba* laccase reported previously (1).

Our results confirm that OMW influences the production of ligninolytic enzymes by *P. flavido-alba*. MnPs are the predominant enzymes in OMW decolorized by *P. flavido-alba* and laccase is not only present, but is strongly induced. These experiments may contributte to a better understanding of the enzymes implicated in OMW decolorization and suggest an important role for laccase and MnP in such biodegradation process by white rot fungi.

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Producción de la Proteinasa Extracelular de *Micrococcus* Sp. INIA 528: Modelo Cinético

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Palabras clave: Micrococcus*, proteinasa, producción, modelo cinético*

Las bacterias del género *Micrococcus* se encuentran en elevada proporción en muchos quesos, elaborados tanto con leche cruda como pasterizada, y tienen un papel relevante durante la maduración debido a su capacidad para sintetizar proteinasas y lipasas. *Micrococcus* sp. INIA 528 posee una elevada actividad proteolítica sobre las caseínas al liberar una proteinasa extracelular que ha sido purificada y caracterizada como cisteín proteinasa. La influencia de las variables y la optimización de la producción de la proteinasa se ha realizado en un fermentador discontinuo empleando como medio de cultivo basal caldo tripticasa soja (TSB) [1, 2].
En el presente trabajo se propone un modelo cinético que describe el crecimiento de *Micrococcus* sp. INIA 528 y la producción de la cisteín proteinasa. El modelo tiene en cuenta tres respuestas: biomasa, fuente nitrogenada y proteína sintetizada, conteniendo cinco parámetros: μ, velocidad específica de crecimiento del microorganismo; C_{xm}, concentración máxima de biomasa; μ_p, velocidad específica de producción de la proteinasa; Y_{xN}, rendimiento macroscópico de nitrógeno en biomasa; y Y_{pN}, rendimiento macroscópico de nitrógeno en proteinasa.

$$\frac{dC_x}{dt} = \mu \cdot C_x \cdot \left[1 - \frac{C_x}{C_{xm}} \right] \quad (1) \qquad \therefore \qquad \frac{dC_N}{dt} = -\frac{1}{Y_{pN}} \cdot \frac{dC_p}{dt} - \frac{1}{Y_{xN}} \cdot \frac{dC_x}{dt} \quad (2)$$

$$\frac{dC_p}{dt} = \mu_p \cdot C_N \cdot C_x \quad (3)$$

Las variables estudiadas fueron el pH (6,0-8,0) y la temperatura (28-40ºC). El modelo es capaz de ajustar adecuadamente todos los resultados experimentales obtenidos. La velocidad específica de crecimiento del microorganismo (μ) no es afectada por el pH del medio en el intervalo estudiado, obteniéndose valores entre 0,599 y 0,648 h^{-1}. Sin embargo, la máxima concentración de biomasa (C_{xm}) se consigue a pH 7,5 con un valor de 4,033 g/l. El rendimiento macroscópico de nitrógeno en proteinasa (Y_{pN}) alcanza su valor máximo (0,053 g_p/g_N) en el intervalo de pH 6,0-6,5, coincidiendo, en este intervalo, con la máxima velocidad de producción de la proteinasa (μ_p). En el intervalo de temperatura estudiado, la velocidad específica de crecimiento del microorganismo alcanzó un valor máximo de 0,685 h^{-1} a 37ºC. Los valores máximos de la velocidad de producción y del rendimiento de nitrógeno en proteinasa se alcanzaron a 34ºC y 31ºC, respectivamente. La evolución de la velocidad específica de crecimiento con la temperatura se ajustó a la ecuación de Ratkowski y col. (1983) obteniéndose una temperatura mínima y máxima de crecimiento del microorganismo de 9 y 47ºC, respectivamente.

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Application of Theoretical Model for Catalyst Fluid Flow Reactions with Immobilised Cell

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In this paper a theoretical model for the utilisation of immobilised whole cells in porous pellets is tried. Particular attention is given to ethanol production, enzyme based conversions, and detoxification in aqueous phase. The performance of the reactor is highlighted by determining the activity. The effects of immobilisation like decrease of activity and increased operational stability are brought forth.

Immobilised enzyme reactors are special cases of heterogeneous biocatalytic reactors. The model is henceforth applicable in this case also, as the case studies reveal.

The theoretical model values are compared with experimental values as well.

Substrate-transfer resistance in immobilised Trigonopsis *variabilis* cells

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Keywords: Ca-alginate, D-amino acid oxidase, diffusion, Trigonopsis variabilis.

D-amino acid oxidase, DAAO [D-amino acid : O_2 oxidoreductase, deaminating, (E. C. 1.4.3.3.)] is a flavoprotein using flavin-adenine dinucleotide (FAD) as cofactor used in the resolution of racemic mixtures of amino acids by selective oxidation of the dextro isomer according to the reaction

$$\text{D-amino acids} + O_2 \xrightarrow{\text{DAAO}} \alpha\text{-keto acid} + NH_3 + H_2O_2$$

An industrial application of this process requires first an abundant and cheap source of enzyme and second an immobilisation in a suitable support. In our work the yeast Trigonopsis *variabilis* was used as DAAO source[1,2]. The permeabilised yeast cells were immobilised by gel entrapment into Ca-alginate beads for the resolution of D/L-methionine mixtures.

In order to develop the design of an immobilised cell reactor, it is necessary to know the kinetic parameters of the permeabilised free cells and to study the diffusional resistance.
To achieve this, the Michaelis kinetic parameters for the free and immobilised cells were determined as well as the diffusion coefficients[3] for both substrates -D-methionine and oxygen-, for Ca-alginate beads containing different loads of permeabilised cells.

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Semisynthetic Bovine Pancreatic Ribonuclease: Activity and Stability

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The ribonucleases are a class of enzymes catalyzing the hydrolytic cleavage of ribonucleic acids, and whose activity can be demonstrated in almost all tissues both plant and animal. The preparation in the 1950´s by a company (Armour, Inc.) of 1 Kg of pure, cristalline bovine pancreatic ribonuclease was responsible for the fact that today may be considered one of the more wellknown enzymes, and as such, it has been investigated in detail as a good model enzyme and protein; indeed, since it consists of only 124 amino acids, it was the first enzyme prepared -in the late 1960´s- synthetically in the laboratory

The in vitro preparation of enzymes chemically modified with suitable moieties offers the possibility of obtaining new biocatalyts -semisynthetic enzymes- with improved properties (stability, selectivity, new activities, etc.).

The hydrophilic-lipophilic balance (HLB) of an enzyme seems to be essential in many of its properties (recognition of substrates, binding, stability, etc.).

In this context, the post-translational modification of proteins by covalent attachment of oligosaccharides is a widespread phenomenon not only in eukaryots but also has been found in proteins of eubacteria.

In the present work, the RNase has been chemically modified with several reducing saccharides (glucose, fructose, xilose, maltose, lactose, maltoheptaose). The Schiff bases formed were reduced with $NaBH_3CN$. The modification degree of the amino groups was measured with fluorescamine. Good retention of activity was observed, and in some cases, the semisynthetic RNase was much more stable at 90 and 100 ^{0}C than the native protein.

Some Physico-Chemical Characteristics of Polyurethane Foams Used for the Immobilization of Lipases

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The activity and the operational stability of the immobilized lipases depend on several parameters such as the lipase, the type of support and the immobilization method. With respect to the immobilization technique, a high operational stability of lipases is frequently observed when entrapment methods are coupled with chemical binding[1, 2].
The aim of this study was to evaluate several physico-chemical properties of two relatively hydrophilic biocompatible polyurethane foams, Hypol FHP 2002 and Hypol FHP 4300, donated by Hampshire Chemical GmbH, Germany. Hypol FHP 2002 is a toluene diisocyanate pre-polymer and Hypol FHP 4300 is composed by diphenylmethane diisocyanate groups. The immobilization of enzymes, namely lipases, is achieved by entrapment and chemical binding during polymerisation and foam formation.
Concerning Fourier Transform Infrared Spectroscopy analysis (FTIR), the following main functional groups were identified in spectra, for both foams: OH, NH, CH, C=O, CN. However, the relative amounts of the various groups seemed to be different between the samples.
With respect to the partition coefficient support/*n*-hexane[3], the estimated value for oleic acid was found to be close to zero for the FHP 4300 foam, as previously observed for the FHP 2002 matrix[5].
A higher aquaphilicity[4] value for FHP 2002 (3.7) than for FHP 4300 foams (2.8), indicates a relatively higher hidrophilicity for the first foam. The estimated porosity was 0.75, for the FHP 4300 foam, and 0.70 for the FHP 2002 counterpart.
When the FHP 2002 foams were dried under 100 mbar, at 40ºC, the equilibrium was reached after 15 minutes, corresponding to a final water activity (a_w) of about 0,25. For the other matrix tested, drying time to attain equilibrium was longer (30 minutes) and the final a_w of the foam was about 0,40. This suggests different binding forces between the matrices and the water molecules.
During vegetable oil hydrolysis in a bifasic water/*n*-hexane system, internal mass transfer limitations were observed for ratio [lipase/ pre-polymer] of about 0.55 and 0.60, for FHP 2002 and FHP 4300 foams, respectively. Whit these lipase loads the hardness and resistance to compression of the foams were about 50% of the values in the absence of enzyme.
The identification of the relationship between physico-chemical properties of the supports and the activity and operational stability of the immobilized biocatalysts, is under study.

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Application of Factorial Design to the Study of an Alcoholysis Reaction Promoted by Cutinase Immobilized on NaY Zeolite

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Key words: Zeolites, Cutinase, Immobilization, Transesterification and Factorial Design

The use of zeolites as supports for enzymatic catalysis has only recently received some attention. Promising results have already been obtained with *Fusarium solani pisi* recombinant cutinase in hydrolysis reactions with organic medium [1]. The same enzyme was also used to catalyse an alcoholysis reaction; the results obtained showed that zeolites can also be successfully used in this catalytic system [2].

The cutinase was immobilized by deposition/adsorption on NaY zeolite. The enzyme was added to the support and the preparation was vacuum dried after vortex mixing. The immobilized enzyme was used to catalyze the alcoholysis reaction of butyl acetate with hexanol in organic medium (isooctane) at 30ºC.

The influence of the water amount on the enzymatic activity was previously studied [2] and an optimum value was obtained. Although depending on the support, the optimum water amount corresponds always to the same water activity (a_w = 0.96). The enzyme preparations were equilibrated in closed vessels with salt solutions at well-defined water activity (a_w = 0.97) [3] at 30ºC, during 3 days.

Some relevant parameters for the enzyme activity such as the temperature, buffer molarity and pH of the enzyme solution, hexanol and butyl acetate concentrations were studied by means of a factorial design plan. The experimental planning was a 2^{5-1} fractional factorial design expanded further to a central composite design (CCD) according to Box [4] and Barker [5]. By knowing the system response to the experimental design, the effects of each factor refered above were calculated and its interactions were determined.

All factors influenced the initial activity. However, main effects are due to buffer molarity and butyl acetate concentration. The optimum pH was found to be between 7 and 8. The optimum buffer molarity is around 150 mM. In the studied conditions an increase of butyl acetate concentration enchances proportionally the reaction rate. An important interaction was detected between hexanol concentration and buffer molarity.

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Adsorption of Lipase on Celite using Buffer or Organic Solvent as a Dispersion Media

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The potential application of lipase for the production of several high value chemicals and the advantages associated to the use of this biocatalyst in nonaqueous medium, have been motivated our group to develop research lines relating with the production of flavor esters. Parallel to this interest, we have also carried out experimental work to establish methodologies for immobilization of pancreatic and microbial lipase on different types of supports, because of the advantages of such derivatives, which include the possibility of recovery and reuse, simplicity in operation and improved stability. The extent of stabilization depends on the enzyme structure, the immobilization method and the type of support. For immobilization of lipase, it is recommended to use a porous support material so that, suitable amount of lipase can be spread on a surface area without conformational changes. In this paper emphasis was placed on developing a procedure to obtain a lipase immobilized on celite with high activity and stability for its use in the synthesis of flavor esters. To this end, porcine pancreatic lipase was immobilized by adsorption on celite using buffer (sodium phosphate buffer, pH 7.00) or organic solvent (acetone or hexane) as a reaction media. The immobilization yield of enzyme-support complex was assessed by C, H, N mass balance determined by Elemental Analyzer (Perkin Elmer CHN 2400). The influence of the immobilization procedure, water content and enzyme loading on the derivatives activities has been studied regarding their ability to synthesize butyl butyrate. According to our results, it appears that the immobilized lipase prepared by using apolar solvent, such as hexane is highly superior to the corresponding preparation in acetone or buffer, not only in regard to the immobilized yield and to the synthesis activity but also, in relation to its stability under operational conditions. The increased stability of immobilized lipase on celite might result from the improved enzyme retention in apolar solvent. It is not yet clear whether the support can be expanded in hexane making a better distribution of the enzyme onto the surface area or if polarity of the solvent may play an important role by not stripping off the bound water from the enzyme's surface. Although both mechanisms have contributed to ensure high retention of lipase activity on the support material, it is most probably that the use of a solvent which has lower polarity (higher log P value) has allowed to maintain the configurational properties of the enzyme. This fact is not surprising since the compatibility of a solvent with enzyme activity indicated that water miscible hydrophilic solvents such as DMF, DMSO, lower alcohol and acetone (log = -0.23) are usually incompatible, whereas water immiscible hydrophobic solvents such as alkanes (hexane, log P= 3.50) retain an enzyme's high catalytic activity. This methodology seems to be attractive to enhance enzyme stability and will be subject of further studies using different kind of supports, such as controlled pore silica (CPS) and styrene-divinylbenzene copolymer (STY-DVB).

Produção e Purificação de Quitosanases em Sistemas de Duas Fases Aquosas

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Palavras chaves: purificação, quitosanase, sistema de duas fases aquosas

A partição de biomateriais em Sistema de Duas Fases Aquosas (SDFA) pode ser empregada em processos de purificação e extração de constituintes celulares de vários tamanhos, incluindo proteínas, enzimas, membranas e organelas celulares. Os SDFA são amplamente utilizados em processos contínuos, sendo os mais empregados para a produção de enzimas ou outras proteínas e formam-se a partir da adição de soluções aquosas de dois polímeros hidrófilos, como PEG (polietilenoglicol) e dextrana, ou um polímero e um sal, como PEG e fosfato de potássio. Os SDFA podem substituir as etapas iniciais da purificação e da cromatografia preparativa de proteínas e podem também ser facilmente escalonados, sem perda na eficiência do processo.
Quitosanases são enzimas que atuam sobre o biopolímero quitosana produzindo compostos de elevado valor biológico nas áreas de medicina, farmacologia, agricultura e biotecnologia.
Quitosanase de diferentes microrganismos têm sido descritas, caracterizadas e os processos de purificação envolvem diversas etapas (4 a 6) com baixos rendimentos.
Este trabalho investigou a produção da quitosanase utilizando planejamento fatorial 2^{5-1} (pH, tempo de fermentação, concentração de sulfato de amônio, concentração de quitosana e aeração), caracterização por planejamento fatorial 2^3 (temperatura, pH tempo de reação) e purificação em SDFA por planejamento 2^4 (peso molecular e concentração do PEG, concentração de fosfato e presença ou ausência de cloreto de sódio).
Níveis de até 1.5 U/ml da enzima foram obtidos em meio de cultura contendo 1% de quitosana, 4% de sulfato de amônio, pH 5 e 32 horas de fermentação. A enzima apresentou atividade ótima em pH 5.8 e temperatura de 54 ^{O}C.
Na purificação em SDFA foi observado que os sistemas formados por PEG 1500, fosfato e cloreto de sódio apresentaram um maior coeficiente de partição da enzima e melhor separação dos contaminantes proteicos. A adição de cloreto de sódio ao sistema aumentou mais de 50 vezes a partição da enzima para a fase contendo PEG.

APOIO FINANCEIRO: FAPESP

Screening and Partial Purification/Characterization of Lipases from Filamentous Fungi

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Lipases (EC 3.1.1.3) are enzymes that catalyze the breakdown of oils and fats into free fatty acids, partial glycerides and glycerol. The ability to hydrolyse long-chain-acid esters differentiates lipases from esterases (acting preferently on water-soluble esters).

Lipases are produced by animals, plants and microorganisms. In particular, extracellular lipases from microorganisms have received much attention for their potential use in biotechnology, mainly due to their availability and stability. In addition to the bacterial lipases, the lipases from fungi are at present thoroughly studied due to their regio- and stereo-specificity, high activity and stability under different conditions.

A comprehensive screening of several genera of filamentous fungi (*Fusarium, Penicillium, Aspergillus, Trichoderma)* has been carried out. The fungi were cultured wirh orbital shaking, at 25ºC during 20 days in a basal medium (Reyes & Byrde, 1973) suplemented by yeast extract, in presence and absence of olive oil as inductor for lipase activity. It was found that with 1% olive oil in the medium, the activity was higher.

In our laboratory we are also studiying the enzymatic activities present in a spent broth resulting after fermentation of a *Penicillium sp*. and in particular, the lipase present in it. Biological wastes such as this one are very good source of feed additives; in addition, they are a good source of different enzymes -proteases, lipases, carbohydrases, etc.-. This lipase has been purified and partially characterized. Since we were dealing with a protein very hydrophobic, the purification protocol consists of extraction with surfactants and a chromatographic step on Phenyl-Sepharose. In this way the protein has been purified 500-fold, with 100% activity recovered. A molecular mass of 43 kDa and an isoelectric point of 5.9 have been determined.

The hydrolitic activities of all the fungal lipases investigated have been studied using synthetic (p-nitrophenyl esters, Tween 80) and natural (tributytirin, olive oil) substrates. In some cases the synthetic activity in organic solvents (formation of ethyl oleate) was assayed. Of all the lipases screened, the one from *Penicillium sp.* was the most versatile and useful in the reaction of synthesis.

Papel del Agua y de sus Miméticos en la Activación-Estabilización de la Lipasa de *Candida rugosa*

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Palabras clave: lipasa, actividad de agua, miméticos, termogravimetría, isoterma .

La disponibilidad de agua alrededor de las moléculas activas de enzima depende de los componentes en el sistema que puedan competir por el agua, según sea su afinidad por la misma. Estos son los disolventes orgánicos, los reactivos, los aditivos y los soportes.[1,2].
Por ello es necesario disponer de métodos de medida y control del agua, para realizar la catálisis enzimática en medios orgánicos. La metodología de Karl-Ficher nos informa sobre la cantidad de agua presente en el macroentorno enzimático. Recientemente hemos comprobado que las isotermas de sorción de agua nos informan sobre el agua presente en el microentorno enzimático y sobre el valor de a_w de preequilibrado para obtener el máximo rendimiento en una reacción en medios orgánicos [3]. Esta isoterma depende del disolvente, de la enzima y del soporte.
Utilizando análisis termogravimétrico (TG) y análisis diferencial termogravimétrico (TD) hemos podido relacionar el contenido en azúcares de las isoenzimas puras A (LA) y B (LB) de lipasa de *Candida rugosa*, 8 y 3,6% respectivamente con su contenido en agua 12,8 y 6,2% en estado liofilizado. Otra alternativa para mantener la conformación cataliticamente activa en medio orgánico es el empleo de mímicos del agua.
En la figura 1 se muestra el efecto de diferentes compuestos que actúan como miméticos del agua sobre la reacción entre el (S)-ketoprofeno y el propanol.

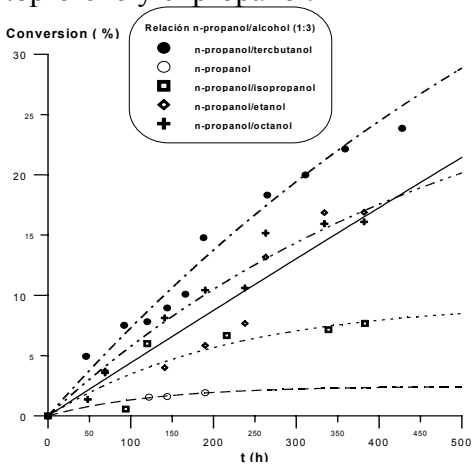

Figura 1. Efecto de miméticos del agua en la reacción de esterificación de (S)-ketoprofeno con n-propanol en isooctano catalizada con lipasa de *Candida rugosa*

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Kinetic Study of the Cellobiose Enzymatic Hydrolysis by a Commercial Extract from *Trichoderma reesei*

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Key words: Cellobiase, Kinetics, T. reesei

Cellulases of fungal origin are very effective in cellulose hydrolysis and have been increasingly used in textile, paper and food industries. Cellulases are produced as a multi-component enzyme system comprising usually three enzymatic activities: endoglucanase, cellobiohydrolase and cellobiase [1]. Cellobiase is responsible for the hydrolysis of cellobiose into glucose, but its kinetics is not fully clarified. In the present work, the substrate dependence of the initial rate of the reaction catalysed by the cellobiase of a *Trichoderma reesei* commercial extract was studied.

Hydrolysis of cellobiose into glucose was performed at 50 ºC and pH 4.8, using citrate (50 mM) buffer and an enzymic extract (Celluclast 1.5L) dilution of 1:6250. The glucose produced, at different incubation times and at different cellobiose concentrations, was measured using the glucose oxidase/peroxidase enzymatic assay [2]. Non-linear curve fitting of the cellobiose concentration-rate data, was performed using the Enzfitter (Biosoft) program.

The variation of produced glucose with the incubation time showed a good linear relationship, at least for the 30 min time interval, for all the initial cellobiose concentration studied; in this way, the initial rate of reaction for each initial cellobiose concentration was easily calculated from the slope of the straight line of glucose concentration versus time. No non-enzymatic hydrolysis of cellobiose was observed during the 30 min interval. The initial rate of reaction initially increased with the cellobiose concentration, but rapidly reaches a nearly maximal observed rate at 5 mM cellobiose and for cellobiose concentration higher than 100 mM the reaction rate might have a small reduction. The Lineweaver-Burk plot also shows a small upward curvature for higher cellobiose concentration, showing a departure from pure Michaelis-Menten kinetics, which could be compatible with substrate inhibition kinetics [3]. Also non-linear curve-fitting of the cellobiose concentration-rate data to a trial equation, was best improved when assuming partially non-competitive substrate inhibition (mean square error (MSE)=5.2×10^{-5} mM/min; reduced $\chi^2=2.2\times10^{-9}$) as compared with pure Michaelis-Menten kinetics inhibition (MSE=1.1×10^{-4} mM/min; reduced $\chi^2=9.2\times10^{-9}$). The K_m, V_{max} and K_I values, obtained assuming substrate inhibition were, respectively, 1.1 mM, 2.6×10^{-3} mM/min and 26 mM (n=8).

In conclusion, the reaction catalysed by the cellobiase from *T. reesei*, did not follow a pure Michaelis-Menten kinetics and might be consistent with a partially non-competitive excess substrate inhibition.

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Process Optimisation of Bilirubin Oxidase from *Myrothecium verrucaria* IFO 6133

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Bilirubin oxidase {E.C.1.3.3.5} from *Myrothecium verrucaria* IFO 6133 catalyzes the oxidation of Bilirubin to Biliverdin *in vitro*, followed by its further oxidation,yielding unknown substances. It is expected to be useful for the diagnosis and treatment of jaundice and Hyperbilirubinemia and it has already been employed for the enzymatic determination of Total Bilirubin in serum. Optimisation of the various process parameters influencing the production of Bilirubin oxidase, utilisation of cheap carbon sources for maximal yield and obtaining bioprocess profiles using CSTR is the objective of this study. Set of experiments was designed using Response Surface Methodology, giving adequate and reliable measurements of the response of interest. Using central composite design microbial parameters (Stab age and inoculum level), medium composition (partial substitution of molasses as carbon source for potato extract), addition of organic and inorganic nitrogen as nitrogenous source, concentration of heavy metals and environmental parameters (pH and temperature) was optimised. *Mycothecium verrucaria* IFO 6133 produces higher bilirubin oxidase in the optimised medium compared to the unoptimised medium.

Extracellular Proteases Activities from Mucor racemosus and Cunninghamela elegans

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***Keywords**: Protease; Mucor racemosus and Cunninghamela elegans*

Protease, an industrially important enzyme, accounts for nearly 60% of the total enzyme sale, with two-thirds of proteases produced commercially being microbial in origin. The major uses of free protease occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive aids and certain medical treatments of inflammation and virulent wounds. This work investigated the activites of extracellular acid and alkaline proteases from *Mucor racemosus* and *Cunninghamella elegans* by using D-glucose as main carbon source. The study was carried out using a inoculum of 10^5 spores/mL of *Mucor racemosus* and *Cunninghamella elegans* both grown in Araki & Ito [1] medium in orbital shaker (80rpm) at 28^{o}C during 96 hours. Sample were harvested each 24 hours intervals to determine cell biomass (dry weight); pH, D-glucose consumption, extracellular protein [2] and proteases [3]. The results showed activity of extracellular acid and alkaline proteases in both microrganism. *Mucor racemosus* showed the maximum acid protease activity of 447 U/L with 48 hours of cultivation with pH 3.5, while the pH increased up to 6.0 the acid proteases activity was decreased to 170 U/L. However, the maximum alkaline protease activity (480 U/L) was observed with 72 hours of growth (pH 3.5). In *Cunninghamella elegans* the pH of medium was approximaly 3.5 in the course of fermentation and maximum alkaline protease activity (367U/L) was 25% higher then acid protease activity (277U/L). The biomass yield of *Cunninghamella elegans* was twice larger than with *Mucor racemosus* obtained in the end of fermentation. These results shown that these strains of zigomycets could will be a good source of proteases.

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Preliminary studies on enzymatic deinking

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Key words: wastepaper, recycling, deinking, enzymes

Wastepaper recycling is nowadays mandatory, because of the world-wide lack of virgin fibre, and in order to reduce the cellulosic residues produced in developed societies. The incorporation of secondary fibres in paper production became an accepted reality, and research on recycling technologies, namely for effective removal of pulp contaminants (deinking) is necessary.

Traditional processes use expensive, potentially environment damaging chemicals. An alternative to these methods are biological treatments, which use enzymes to peel away cellulosic fibrils, thus removing attached ink particles which are then removed by flotation [1]. Enzymatic technology is also known as specially advantageous to deink mixed office waste (MOW), the best quality wastepaper. The reuse of MOW is usually limited by its high content of noncontact inks, which are very difficult to remove by application of current methodologies [2].

The current work aims to study the potential of enzymes in the deinking of three different wastepaper samples: (i) photocopy printed paper; (ii) MOW (a chemically treated pulp); (iii) newspaper (a mechanical pulp). The enzymatic cocktail used was a commercial preparation kindly supplied by Buckman Laboratories, which presents mainly cellulase and xylanase activities.

Enzymatic action on pulp was conveniently evaluated by determining physical, mechanical and optical properties of pulp and paper, according to the standard TAPPI procedures. Parameters as drainage rate, burst, tensile, tear and brightness were measured. Image analysis techniques were also used, allowing the study of the enzyme treatment on ink particles size distribution.

The obtained results revealed the effectiveness of the assayed enzyme preparation on ink removal trials, with the three tested pulp samples. However, enzymatic deinking requires special conditions such as pH value, pulping consistency, soaking and reaction times, mechanical shear forces and refining mixed effects (with the enzymes), which will be analysed in further work.

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Hydrolysis of β-Casein by Cardosins A and B, the Aspartic Proteinases from *Cynara cardunculus* L.

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***Keywords**: β-casein, cardosin A, cardosin B,* Cynara cardunculus *L., aspartic proteinases*

In spite of the known variability in what concerns the nature of peptide bonds cleaved by aspartic proteinases, these enzymes are characterized by cleaving preferentially bonds between hydrophobic amino acids. This preference was also shown for cardosin A and cardosin B, two aspartic proteinases from the cardoon rennet *Cynara cardunculus* L., when the oxidized insulin B chain [1] and isolated bovine αs1-casein [2] were used as substrates. Because milk caseins are the substrates of cardosins during the process of milk coagulation and cheese maturation, the aim of this work was to proceed with the study of the action of both cardosins on isolated bovine caseins and to assess the contribution of each enzyme for the overall proteolytic specifity of the cardoon rennet.
Bovine β-casein was separately digested with cardosin A and cardosin B and the casein digested peptides were separated either by SDS-PAGE or by RP-HPLC and their N-terminal amino acid sequences were subsequently determined by automated Edman degradation, thus identifying the cleavage sites.
The results showed that both cardosins have a broader specificity when compared with chymosin under similar conditions. Considering both enzymes, cardosin B revealed a higher rate of proteolysis than cardosin A. Consistently with results obtained in previous works with other substrates, both cardosins preferably cleaved bonds between residues with hydrophobic and bulky side-chains. In addition some degree of affinity for bonds involving small polar residues was also shown. The peptide bond Leu192-Tyr193 of β-casein was the most susceptible to attack by both cardosins. Nevertheless, the susceptibility of the two other identified cleavage sites (Leu127-Thr128 and Leu165-Ser166) was different for each enzyme. The order by which the peptide bonds Leu127-Thr128 and Leu165-Ser166 were cleaved by cardosin A was still affected by small changes in the pH value of the digestion buffer (6.2 or 6.8), what might occur as a result of a conformational change in the casein molecule. Based on the results obtained in the present work, a model for the action of cardosin A and cardosin B on bovine β-casein is proposed and discussed.

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Denim Bleaching with Laccases

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The fashionable light shades of Denim fabrics are just achieved with a bleaching process after stone washing. Usuall bleaching agents are sodium hipocloride and hydrogen peroxide. Recently it was introduce a enzyme product based on a laccase and an intermediate. All those products will remove indigo dye by a redox process. It is known that sodium hipocloride and hydrogen peroxide will also degrade cotton cellulose, but there are no available information about the effect of the laccase and intermediate on cotton cellulose. In this communication we intent to compare the ratios of the Indigo shades over strengh loss of Denim fabrics treated with sodium hipocloride, hydrogen peroxide and with a commercial formulated laccase product.

Production and Location of Polygalacturonase from *Aspergillus Niger* on Different Carbon Sources

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Key-words: A. niger*; induction; pectins; polygalacturonase.*

Pectinases are a group of enzymes that catalyse the breakdown of pectin-containing substances and can be produced either by sumerged or solid-state [1]. These enzymes are widely used in the industrial processing of fruits and vegetables since the decrease the viscosity of juices and facilitate extraction, maceration, liquefaction, filtration and clarification processes. Pectic enzymes are produced by many groups and species of microorganisms. The choice of *Aspergillus enzymes*, apart from the question of enzyme effectiveness, is partly dictated by the acceptability requirements of the food processing industry [2]

In this work, a process was developed for the production of *Aspergillus niger* enzyme preparations containing a high level of polygalacturonase (poly-1,4-α-D-galacturonide glycanohydrolase, EC 3.2.1.15) in liquid state surface fermentation.

In order to induce the enzyme the following substrates were used as carbon source: polygalacturonic acid, apple pectin and citrus pectin at a concentration of 0.25, 0.5 y 1% (w/v). Polygalacturonase activity was determined by the method of Rexová-Venkoba [3].

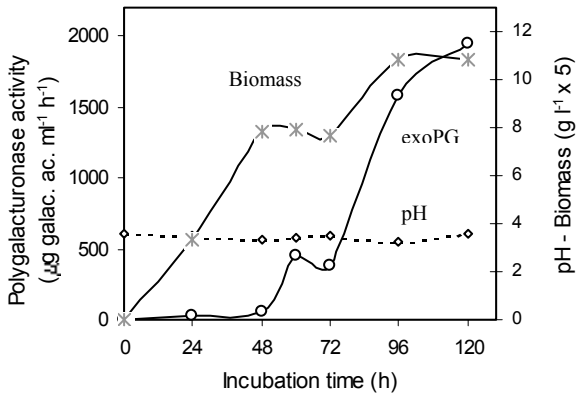

Fig. 1. Evolution of exocellular polygalacturonase activity, culture pH and biomass of *A. niger* culture supplemented with apple pectin (1%(w/v)).

A. niger was able to proliferate and induce polygalacturonase with all the carbon sources studied. Moreover, enzyme activity increased when concentrations of the carbon sources were also increased. The higher level of polygalacturonase activity was detected for the cultures supplemented with 1% apple pectin (Figure 1), descending in a 15 and 22% in the medium enriched with citrus pectin and polygalacturonic acid. The results indicated that the best inducers of polygalacturonase were esterificated substracts, while the polygalacturonic acid seemed repress the enzyme production.

On the other hand, the maximum enzyme activity was located in the extracellular fraction. Nevertheless, it was detected a certain level of intracellular and even cell-wall activities.

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Efecto del Pretratamiento con Distintos Preparados Enzimáticos sobre el Rendimiento de Extracción de Aceite de Colza por Prensado

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Keywords: *Extracción de aceites, Enzimas extractivas.*

El proceso convencional de extracción de aceite de colza se efectúa por preprensado y extracción con disolvente. Si bien el hexano es efectivo, su aplicación presenta algunas desventajas, como es emanación de compuestos volátiles orgánicos al ambiente, entonces la aplicación de un pretratamiento enzimático podría mejorar el rendimiento de extracción en el prensado, de modo tal que no sea necesario el empleo de disolventes. Además se espera reducir el contenido de fibras de la harina de colza, mejorando con ello uno de sus mayores inconvenientes en la incorporación de dietas animales.

En este trabajo se presenta el estudio de selección del preparado enzimático que entrega el mayor incremento en el rendimiento de extracción de aceite de colza por prensado.

Se estudian 15 preparados enzimáticos, principalmente comerciales, con una alta relación E/S del 10 %, de entre ellos se seleccionan 8 preparados que se analizan para una relación E/S del 5%; finalmente se seleccionan dos preparados que se estudian solos y en mezcla para menores relaciones E/S, seleccionándose la formulación que provoca mayores mejoras en el rendimiento de extracción por prensado. Estas experiencias se efectúan a un 30 % de humedad, pH natural de la semilla y temperatura recomendada por el fabricante respectivo de cada preparado enzimático.

De los 15 preparados enzimáticos estudiados, en razones de E/S del 10 y 5 % los más efectivos para mejorar el rendimiento de extracción de aceite son la pectinasa Ultrazym 100G, el preparado experimental con actividad pectinolítica Novozym SP 249, y la β-glucanasa Finizym los tres de Novo Nordisk S/A y la celulasa Maxazyme de Gist Brocades. Con estos preparados se obtienen incrementos entre el 6 y 10 % para una relación E/S del 5 % b.h. Se seleccionan las formulaciones de Finizym y Ultrazym 100G para los estudios en mezclas.

El pretratamiento enzimático con la mezcla Finizym -Ultrazym 100G no produce mayores incrementos en el rendimiento de extracción de aceite de colza por prensado que Ultrazym 100G solo, sin apreciarse efectos sincretista para la mezcla.

Se concluye que la formulación pectinolítica Ultrazym 100G es el preparado más adecuado entre los estudiados para mejorar el rendimiento de extracción de aceite de colza por prensado, presentando la mayor velocidad de incremento en el rendimiento entre 3 y 12 h de incubación. Las actividades de este preparado coinciden con la composición de la pared celular de colza.

Proteolityc Enzyme from *Penicillium citrinum*: Studies on Column Bioreactor Production

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***Keywords**: protease, filamentous fungi, solid state fermentation, biomass*

Solid state fermentation (SSF) could be in many ways advantageous over submerged fermentation system for enzyme production. Apart of offering the possibility of using agroindustrial residues as substrates, SSF uses low moisture content which could prevent bacterial contamination during the fermentation process and allows a production of metabolites in a more concentrated form, making the downstream processing less time consuming and less expensive (1). The production of a proteolityc enzyme by the filamentous fungus *Penicillium citrinum* in solid state fermentation was investigated in previous work (2), using Erlenmeyers flasks. Two different substrates were employed (soybean and sunflower bran) and the best conditions for the production of the enzyme (maximum 160 U/mL) were determined as being sunflower bran as substrate, 55 % of humidity and temperature of 28 °C, 0.2 M phosphate buffer, pH 8.0. The objective of this work was to study and to optimise the fermentation conditions of the fungus into a column bioreactor in order to establish a comparison with the growth and the enzyme production between these different fermentation systems. The experiments were carried out in a column bioreactor (1), with control of the air flow and analysis on-line of the composition of the gas produced by the fungus. Glass column reactors of 4 cm diameter and 20 cm length were filled with the inoculated and moistened solid substrate. The experiments were performed using sunflower bran, 28 ^{0}C, 0.2 M phosphate buffer, pH 8.0, and varying the humidity (45-75%) and aeration (20-20 mL / min.). Protease activity was determined in the crude extract, using azocasein as substrate according to Leighton *et al*.(3). The highest proteolytic activity (278 U/mL) was obtained at 45 % of humidity, 60 mL /min of aeration. This value is 73 % higher than those obtained for the production of the enzyme in Erlenmeyers flasks. Further studies on respirometry by measuring the produced gases during the fermentation, namely CO_2 and O_2, will be helpful to establish a correlation model between the growth of the fungus and the production of the enzyme.

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Dry Action of Cellulases on Cotton

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Today cellulases are well established in the finishing of cotton textiles. The most known applications are the ageing of denim garments, where they partly or completely substitute pumice stones, and the depilling of cotton fabrics. After the treatment, enzymes are usually inactivated and removed by washing with alkali or detergents.

If not washed with alkali, cellulases will stay adsorbed to the fabric and will remain active on the dry cotton at normal storing conditions. Three different enzyme compositions of *Trichoderma reesei* cellulases were adsorbed to a mercerized cotton fabric. Effects of storage time and enzyme concentration on physical and mechanical properties of the fabrics were analyzed and will be discussed.

Application of Mannanases Produced by *Streptomyces* UAH 58 to Biobleaching

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Actinomycetes, mannanases, biobleaching, pulp

In recent years the interest in using mannanases for the biobleaching of different types of pulps has been considered in order to reduce the chlorine bleaching chemicals consumption with the corresponding decrease in the environmental contamination, as well as to improve the quality of pulps.

The mannan-degrading enzyme complex produced by *Streptomyces* UAH 58 showed a high physiological efficiency as well as a remarkable ability to solubilize galactomannan. In addition, the absence of cellulase activity in this complex endorse its potential interest to be used for biobleaching purposes. In this work the effectiveness of this complex was assayed against pine and eucalyptus Kraft pulps.

The release of colour (A_{465}) and chromophores (A_{237}, A_{254}, A_{280}) in the supernatants of the assayed pulps was more evident when pine Kraft pulp was used at 5% pulp consistency, with an enzyme:substrate ratio of 20 U mannanase activity g^{-1} pulp and after 10 hours of enzyme treatment.

Furthermore, it was proved that the enzymatic treatment of the pulp followed by an alkaline extraction -process that is usually performed in the pulp and paper industry- improved the release of chromophoric material with the consequent positive effect on the quality parameters of the pulps, such as Kappa number, viscosity and brightness.
These results demonstrated the effectiveness of the mannanolytic complex produced by *Streptomyces* UAH 58 in the biobleaching of pine pulps.

Recycling of Cellulases During Textile Process

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Textile applications are over than 50% of the world market for cellulase enzymes. Successful applications are the ageing effects like the stone washed appearance of Denim fabrics and improved appearance on fibre and fabric surfaces by removing microfibrils and fuzz fibres. In all these applications it is intended to improve the properties of textile materials by controlled enzymatic hydrolysis. This is usually achieved with weight-loss values less than 5% over 1 hour treatment with a range of dosages 0,3-1 % protein/fabric. After the treatment much protein remains in the liquor along with oligosaccharides and a possible way to reduce the process cost is to recycle the enzyme. In this communication we intent to compare three strategies of recycling: enzyme in the liquor, enzyme adsorbed in the fabric and both of them, under normal textile processing conditions.

Indigo-Cellulase Interactions

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The redeposition of the removed Indigo on Denim fabrics during washing with cellulases seems to be a major problem. The first highlights about backstaining were suggested by the empirical observation that acid cellulases (optimum at pH 5) will yield a high redeposition than neutral cellulases (optimum at pH 7). Later, it was suggested that the use of proteases during cellulase washing will reduce Indigo backstaining with the explanation that the destruction of cellulase proteins will reduced cellulase binding to cellulose and therefore Indigo redeposition over the enzyme proteins. A discussion is made about the nature of Indigo-Cellulase-Cellulose Interactions.

Enzymatic Removal of Acid Groups from Cotton Cellulose

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Cotton cellulose during bleaching steps undergoes on chemical degradation. Degradation is a result of the opening the cellulose ring with the formation of carboxylic, acetone and aldheide groups. We used several cellulase compositions to remove these groups from the surface of cotton fibres. The method to follow the removal of carboxylic acids was the dyeing with methylene blue and verify the staining levels of the dye over cellulose. A discussion is also made the best cellulase composition to remove the acids groups from cellulose.

Production and Purification of an Alkaline Xylanase using Aqueous Two Phase Systems

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Keywords: purification, aqueous two-phase systems, bacillus, xylanase

Xylan is a major component of hemicellulose and one of the most abundant polysaccharides in nature after cellulose. Its structure is made up of β-1,4-linked β-D-xylopyranosides highly substituted. Xylanases catalyse the hydrolysis of xylan but most of the xylanases commercially available have been produced by fungi are active at neutral or acidic pH and their optimum temperature for activity is below 45^{o}C. Various applications for xylanases in bioconversion and food industries have been suggested and one of the major potential applications of xylanases involves the pulp and paper industry. This way, extremophilic enzymes which are active at alkaline conditions have great potential in bleaching process without any need for changes in pH and with the advantage in the release of polluting organic chlorine compounds**.**

The aim of this work is to optimise the xylanase production, to extract and to purify the enzyme from the crude fermentation broth without cell harvesting and to achieve higher purification factors and enzyme yields.

The composition of the culture media was optimised for the maximisation of the alkaline xylanase levels and the minimisation of celullases produced by ***Bacillus pumillus***. The produced xylanase was able to hydrolyse birchwood xylan at pH 10,0 at 45^{0} C.

The enzyme from crude fermentation broth was extracted by partitioning in aqueous two-phase systems (ATPS) composed of phosphate and polyethyleneglycol (PEG). The effect of tie-line length, PEG molecular weight and NaCl concentrations upon the purification factors and yields of xylanase were investigated by statistical design. The effect of pH and temperature on the xylanase activity were identified.

The complete methodology, the results and the discussion will be shown in a full paper.

Efecto de las Condiciones de Pretratamiento Enzimático sobre el Rendimiento de Extracción de Aceite de Colza por Prensado

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Keywords: *Extracción de aceites, Enzimas extractivas.*

Se estudia como afectan las distintas condiciones de tratamiento enzimático de semilla de colza (*Brassica sp.*) sobre su posterior extracción del aceite con prensado.

La semilla de colza molida se hace reaccionar con una pectinasa comercial Ultrazym 100 G, analizándose el efecto de la relación enzima-sustrato, humedad y temperatura sobre el tratamiento enzimático. Las distintas condiciones de reacción enzimática se evalúan para tiempos de reacción entre 3 y 12 h sobre el rendimiento de extracción de aceite en el prensado. El rendimiento se determina por medida de aceite residual en la torta prensada con disolvente. Los experimentos se realizan a nivel de laboratorio en prensa hidráulica de cilindro perforado. Todos los resultados se comparan con una muestra control.

Para tiempos de tratamiento enzimático sobre las 6 h a 45 ºC y 30 % de humedad, las relaciones Ultrazym 100G /Semilla entre el 3 y 10 % b.h. están saturadas de enzima, obteniéndose mejoras en el rendimiento de extracción de aceite del 9 % a las 6 h y del 10 % a las 9 y 12 h de incubación, en comparación con el control sin enzima. Para la relación de Ultrazym 100G/semilla del 1% b.h. el incremento en el rendimiento extracción de aceite se reduce al 6 % entre las 9 y 24 h de incubación, mejora que es igualada sólo a las 24 h de incubación cuando la concentración de la enzima se reduce a la mitad.

El efecto de la temperatura sobre la efectividad de la enzima varía con el tiempo de incubación, produciéndose un descenso después de 24 h a la temperatura de 55ºC y después de 9 h a los 60 ºC. La efectividad del tratamiento enzimático entre 3 y 24 h, sobre la extracción del aceite es similar a las temperaturas de 45 y 50 ºC.

La humedad de 10 y 20 % en el tratamiento enzimático a 45 ºC, con 1 % de relación Ultrazym/semilla, afecta apreciablemente el incremento en la extracción de aceite en comparación con el 30 y 40 %. El efecto de la humedad es similar al 30 y 40 % sobre el incremento del rendimiento de extracción de aceite. Sin embargo, con 40 % se obtienen menores rendimientos de extracción debido al efecto negativo que tiene el tratamiento con mayor contenido en agua y posterior secado, sobre la extracción del aceite por prensado.

Considerando el incremento en el rendimiento de extracción de aceite y la economía del proceso se concluye que las condiciones más adecuadas para el tratamiento enzimático de colza con Ultrazym 100G son 45ºC y 30 % de humedad con un tiempo de reacción de 9 o 12 h para una relación E/S al 1 % o 24 h de tratamiento para E/S del 0,5 % b.h..

Applied Molecular Biology

Cloning and characterization of genes of gellan gum synthesis and hydrolysis in *Sphingomonas paucimobilis*

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Keywords: Sphingomonas paucimobilis*, gellan genes, β-D-glucosidase, UDP-glucose pyrophosphorylase*

Gellan gum is a bacterial exopolysaccharide, with potential in the food, pharmaceutical and other industries. The repeating unit of this linear heteropolysaccharide is the tetrasaccharide: [→3)-β-D-Glc(1→4)-β-D-GlcA(1→4)-β-D-Glc(1→4)-α–L-Rha(1→], composed of D-glucose (Glc), D-glucuronic acid (GlcA) and L-rhamnose (Rha). Native gellan has O-acetyl and glyceryl residues in the D-glucosyl residue adjacent to glucuronyl residue. After deacylation, gellan gives rigid brittle gels similar to agar at half the concentration and with higher thermal stability. Gellan is currently produced in large scale for use as a gelling agent in foods, microbiological and plant tissue culture media, and in other non-food applications.The industrial producing strain ATCC31461 (formely *Pseudomonas elodea*) was recently classified as *Sphingomonas paucimobilis*. Bacterial strains of the new genus *Sphingomonas* are relatively ubiquitous in soil, water and sediments and have broad catabolic capabilities. Gellan biosynthetic enzymes necessary for the formation of the sugar nucleotides UDP-D-glucose, UDP-D-glucuronic acid and dTDP-L-rhamnose, that are the donors of the monomers for the biosynthesis of the repetitive tetrasaccharide unit, were detected in cell extracts of producing strains [1]. The presence of gellan lyase, β-D-glucosidase and β-D-glucuronidase activities were also reported [2]. These polysaccharide degrading enzymes are active on deacylated gellan but failed to show significant degradation of the native gellan [2]. The commercial utility of gellan has been a stimulus for studying its biosynthesis. The cloning and the functional analysis and expression profiles of genes essential for gellan synthesis or hydrolysis are indispensable for the genetic and environmental manipulation of the pathway in order to develop new polysaccharides, with distinct structure and physical properties.

In this context, we have constructed a genomic library from *S. paucimobilis* ATCC31461 in the cosmid Hypercos 1. Using degenerated primers from conserved regions of the protein sequences of UDP-glucose pyrophosphorylase (UGP) enzymes, we amplified a 600-bp DNA fragment from *S. paucimobilis* by PCR and used this fragment as a probe for screening the genomic bank; one clone was selected by colony hybridization experiments. After sub-cloning of the homology region, the 2.0kb *Sal*I fragment was sequenced and an open reading frame (ORF1) of 900 nucleotides long was identified. Genetic complementation analysis showed that a recombinant plasmid with this ORF1 inserted under the *lac* promotor restored the *galU* defect of an *Escherichia coli* mutant. This result indicates that ORF1 encodes UDP glucose pyrophosphorylase. During this study we also identified another ORF of interest in a distinct chromosomal region; homology searches indicated that the encoded protein is highly similar to several β-D-glucosidases.

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Cloning, Sequencing and Characterization of Four Genes for Type I Signal Peptidases from Streptomyces lividans

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Key words: Streptomyces, secretion, Signal Peptidase.

Both in procaryotic and eucaryotic cells those proteins to be secreted are preceded by an aminoacidic sequence at their amino end which is called signal peptide. This peptide is cleaved inmediately after or during translocation through the plasma membrane or Endoplasmic Reticulum by a specific protease, the Signal Peptidase. In certain bacteria like E.coli there is only a gene for type I Signal Peptidase, whereas in others like the Gram-positive bacterium Bacillus subtilis five different genes for signal peptidases have been described, two of them are located in plasmids.

We are interested in studying the secretion pathway of the bacterium Streptomyces lividans at molecular level. To clone the gene for type I Signal Peptidase of S.lividans two degenerated oligonucleotides were synthetized as deduced from the conserved aminoacids in the peptidases of this family (J.M. van Dijl y col., 1992). A pair of oligonucleotides corresponding to B and E boxes were used for PCR amplification of the S.lividans DNA, and a 300 bp fragment was obtained. The deduced aminoacids sequence had significative homologies with type I Signal Peptidase (Sip S) of B.subtilis. This 300 bp fragment was used for screening a BamHI E.coli library of S.lividans, and two positives clones were isolated containing an 8 kbp insert each. The complete sequence of this fragment permitted us to identify, among other proteins, four different genes for type I Signal Peptidase (named sip W, sipX, sipY and sipZ) located in a tandem array. This is the first time in which such organization is found for type I signal peptidases.

Transcription of these genes has been studied by Northern, sandwich hybridization experiments and S1 mapping using total RNA samples extracted at different growth stages. We have mapped promoters for each gene and transcription terminators for three of them. Their expression seems to be temporally controlled, being maximal at stationary phase.
To check the in vivo functionality of sip genes, a DNA fragment containing the four genes in the original array was cloned in an E.coli ts mutant (Inada, T. et al., 1989) for the signal peptidase. The strain containing the S.lividans genes for signal peptidases was abled to grow at the nonpermisive temperature, indicating that they function as signal peptidases.

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Puesta a punto de un sistema de fermentación en fase sólida para la producción de enzimas ligninolíticas por hongos basidiomicetos.

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Palabras claves: Fermentación sólida, ligninasas, maderas, basidiomicetos.

Como una alternativa al tratamiento con reactivos químicos para la producción de pulpas o para el blanqueo de las mismas se ha propuesto desde hace tiempo el tratamiento biológico con hongos basidiomicetos. Estos hongos disponen de capacidades para producir enzimas oxidantes que rompen la molécula de lignina y/o sus derivados en determinadas condiciones de cultivo. Por otro lado, actualmente la materia prima para fabricar pasta de papel esta centrada principalmente en especies del género *Eucalyptus* para gimnospermas y *Pinus* para angiospermas. En base a estos datos y a los resultados obtenidos por Giovanozzi-Sermani y colaboradores (1995) para la producción de enzimas oxidantes en fase sólida hemos diseñado una serie de cinéticas controlando los parámetros que conducen a optimizar la producción de estas enzimas en las maderas anteriormente mencionadas. Esta interesante posibilidad nos podría conducir a producir enzimas en otros sustratos de menor valor, a la vez que tendría el valor añadido de utilizar deshechos agrícolas o forestales. En nuestro laboratorio se han realizado estudios con cepas de basidiomicetos del grupo Trametoides en las que se han detectado, clonado y secuenciado varios genes que codifican para actividad lacasa y manganeso peroxidasa en cultivo sumergido. Por su parte Stewart (1996) e Iimura (1992) han demostrado con éxito que estos genes se pueden expresar de forma heteróloga en otras especies fúngicas que crecen rapidamente en sustratos sólidos y que las enzimas recombinantes obtenidas son funcionales y estables. En la fase experimental que aquí se expone se ha realizado la monitorización de la concentración de proteínas, actividad enzimática, y se han determinado la perdida de peso, cantidad de lignina, holocelulosa y pH.
Los resultados demuestran que las actividades enzimáticas son significativas si se comparan con cinéticas en cultivo sumergido aunque es necesario optimizar la metodología para recuperar las enzimas del sustrato sólido. También es interesante resaltar que las cepas de hongos se comportan de manera diferente que en el cultivo líquido resultado que se corresponde con los obtenidos con otras cepas de hongos filamentosos en especial lo referente a la actividad de agua. Como era de esperar cada hongo responde de manera diferente frente al sustrato y la producción de enzimas en parte se ve afectada por el tipo de lignina y las resinas que contiene la madera. El diseño de una cámara de cultivo en fase sólida abre las perspectivas para producir enzimas oxidantes recombinantes en cepas transformadas.

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Detection of genetic diversity in closely related landraces of *Zea mays* L. using microsatellite markers

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***Keywords**: microsatellite, germplasm, genetic analysis*

In the last few years the use of molecular markers has become widely accepted as a valuable tool for plant breeding programs as well as for evolution, conservation and genetic variation studies. Among them, simple sequence repeats (SSRs) loci also known as microsatellites have been described as powerful markers for measuring intraspecific differentiation because of their high polymorphism, codominance and abundance throughout the genome. SSRs are hypervariable DNA sequences made up of motifs of 1-6 bases of nucleotide repeats that vary in lenght. The DNA sequences flanking these microsatellite regions are generally conserved and can be used to design polymerase chain reaction (PCR) primers capable of amplifying the intervening lenghts of SSRs. In the present work, 37 maize germplasm lines belonging to the Banco Português de Germoplasma Vegetal (BPGV) and originated from the Basto region, North of Portugal, were analysed for their genetic variation using microsatellite *loci*. Following a search for maize SSRs in a public DNA database a total of 34 maize SSR-specific PCR primer pairs were selected and screened. Genomic DNA was isolated from leaf tissue from a bulk of ten different plants of each accession, with a CTAB based protocol. PCR reactions were performed in a Thermal Cycler with 40 cicles of 1 min at 94ºC, 1 min at 56ºC and 1 min and 30 sec at 72ºC. Reactions were performed in a total volume of 50µl as described by Taramino and Tingey [1] with some modifications. Produts were separated by electrophoresis in a horizontal gel system using 1X TBE on a 3% MetaPhor (FMC) agarose gel containing 0,15 µg/ml ethidium bromide. Differentiation of this germplasm measured by allele length variation at the 34 microsatellite *loci* tested was compared with estimates calculated from data at 6 isoenzyme *loci.* The microsatellite survey with the 34 primers demonstrates that microsatellite *loci* are unequivocally superior to isoenzymes for genetic variations studies, revealing a higher number of alleles per *locus*. Similarity comparisons among all SSR alleles were calculated to assess the ability of these markers to distinguish the 37 accessions. This ability depended on the SSR primer being tested. Careful selection for *loci* having high levels of polymorphism, allowed a small number of SSR markers to differentiate all the tested accessions. Since this is an rapid and relatively easy to perform technique it can be used to determine genotypes of large numbers of germplasm acessions and in the creation of core subsets from large collections which is highly valuable to breeders and other users of the collection.

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Construction of 5'→3' Exonuclease mutants of the DNA Polymerase I of *Streptococcus pneumoniae*. Development of new tools for DNA sequencing

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Key words: DNA sequencing, Streptococcus pneumoniae *DNA polymerase I, 5´→3´ exonucleases*

We are now at the forefront of the era of genome-enhanced biology, since many genomes from prokaryotic and eucaryotic organisms will be sequenced in the next few years. Thus, there is a general interest for improving manual and automatic DNA sequencing. The most utilize technique is based in the dideoxy-chain-termination. This method involve the utilization of DNA polymerases, and new versions of polymerases are been developed to improve the performance of these enzymes during DNA sequencing.

The DNA polymerase I of *Streptococcus pneumoniae* (Spn PolI) encoded by its *polA* gene is a member of the type I-like bacterial polymerases. This family of proteins is represented by the *Escherichia coli* DNA polymerase I (Eco PolI) and includes, among other members, the *Thermus aquaticus* polymerase (Taq Pol). This protein possesses 5´→3´ exonuclease and polymerase activities. The pneumococcal *polA* gene was cloned and its DNA sequence determined. Cloning of DNA encoding only the exonuclease or the polymerase activities revealed that these enzymatic domains are located, respectively, at the N-terminal and C-terminal regions of the protein. The polymerase domain of Spn PolI is able to perform DNA sequencing, yielding a very homogenous banding pattern. However, the removal of the exonucleolytic domain of the wild-type enzyme resulted in a decreased processivity during polymerization. This results suggested that a Spn PolI version carrying the 5´→3´ exonuclease domain, but lacking this activity, should retain the polymerizing properties of the wild-type enzyme, and could display a better performance than the polymerase domain of Spn PolI during DNA sequencing.

Aligment of six 5'→3' exonuclease N-terminal regions from bacterial polymerases and four related bacteriophage exonucleases indicated the possible existence of six highly conserved sequence motifs containing invariant amino acids. Ten of these residues are acidic, suggesting that this activity requires the coordination of divalent metal ions at the catalytic site. Nine out of the ten carboxylate residues appear to be important for the exonuclease reaction, since inroduction of mutations at these residues in two othre members of type I-like polymerases resulted in a decrease of the 5'→3' exonuclease activity of these enzymes.

With the aim of developing a new tool for DNA sequencing and getting more insight in the 5'→3' exonuclease activity of Spn PolI, we have introduced mutations at the invariant residues of the *polA* gene. Mutant polypeptides were purified to homogeneity and further caractherized. One of the mutant enzymes showed to be able to incorporate Dig-labeled nucleotides in manual sequencing and afficiently elongate infrared-labeled primers in automatic sequencing. These characteristics convert this polymerase into useful tool for manual and automatic sequencing.

Identification and Expected Function of Some Genes Differentially Expressed in either Heart or Heart-derived Cell Cultures in Crassostrea gigas: a new approach to better understanding bivalvia primary cell culture

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Keywords: mollusc; Crassostrea gigas; gene expression; cell cultures

The intensification of production of Japanese oyster (Crassostrea gigas), an economically important class of marine bivalve molluscs, has led to the occurrence of destructive infections diseases. The lack of an continuous cell culture for these organisms has hampered more in-depth studies of their biology and pathology and therefore the obtention of such mollusc-derived cell lines is one important goal in today's marine invertebrate research.
To date, all reports aiming at the obtention of a continuously dividing cell culture system derived from a marine mollusc have shown that it is no easy task and, to the best of our knowledge, all have failed so far. As it was done in other vertebrate and invertebrate cell systems, the possibility of introducing recombinant DNA expressing oncogenes into a primary culture derived from marine molluscs and its subsequent immortalization has been seen as a likely approach to overcome this problem, an approach which has proven to be highly successful in other cell systems.
Functional marine mollusc-specific expression vectors constructed using different promoter regions from mollusc actin genes, were unable to direct high levels of expression of these vectors when introduced into primary cultures of these organisms. This suggests that the expression of the endogenous actin genes may also be low as compared to the level of expression in the normal tissue from which the cells were obtained.
Disruption of a normal tissue structure into individual cells is known to trigger quite profound changes in gene expression, a processus associated with the appearance of altered phenotypes. As an attempt to uncover genes overexpressed under our culture conditions, we have constructed a subtracted cDNA library resulting from primary cultures derived from oyster heart as template, enriched with cDNA related to genes preferentially expressed under culture conditions. In the same way, a second subtracted library was also constructed, enriched this time with cDNA corresponding to genes which expression is higher in heart tissue.
After cloning and DNA sequencing, the identity of these cDNAÆs related genes was deduced from homology searches in the available data banks. Strong differences in the level of expression were confirmed by northern blot hybridization.
We have thus far identified several genes overexpressed in either heart tissue or cell culture and preliminary results show, in particular, that primary cell cultures express at high levels genes implicated in cytoskeleton reorganization (namely, actins, plastin). Additional sets of genes are being identified and potential implications of these data on the establishment of continuous cell lines from marine molluscs will be discussed. Some of our data has also an interest in respect to phylogenetic conservation in the related gene function.

Production Of IgG Isotype Monoclonal Antibodies Against dsRNA

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***Keywords**: Monoclonal antibodies, dsRNA, diagnostic, Immunocapture-RT-PCR.*

Monoclonal Antibodies (Mabs) specific to dsRNA can provide an attractive and reliable method for immunocapture of dsRNA virus replicative forms in plants and detection by RT-PCR technique. Previously [1,2] we have produced murine monoclonal antibodies specific for dsRNA of IgM isotype but failed to produce IgG isotype Mabs. We successfully produced IgG isotype Mabs by using Freud's incomplete adjuvant with the complex metyl-BSA- viral dsRNA in a different protocol immunization. Mabs obtained from one fusion showed binding capacities against viral dsRNA of mainly IgG isotype Mabs. The Mabs against dsRNA were useful for isolation of viral dsRNA in immunocapture-RT-PCR technique. They were also effective for detection of dsRNA infected plant tissues when ELISA technique was used. The plant viruses used in this study were: Zucchini Yellow Mosaic Virus (ZYMV), Cumcumber Mosaic Virus (CMV), Tobaco Mosaic Virus (TMV), Grapevine Leafroll Virus-3 (GLRV-3) and Grapevine Fanleaf Virus (GFLV).

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Flocculation of a *Saccharomyces cerevisiae* upon transformation with *Kluyveromyces marxianus* GAP1 gene

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Key words: flocculation, glyceraldeyde-3-phosphate-dehydrogenase, Kluyveromyces marxianus*, cell wall proteins.*

A GAPDH like protein of 37 KDa (p37) is accumulated in the cell wall of flocculent *Kluyveromyces marxianus* cells and is putatively involved in the cell interactions leading to flocculation.(1,2,3) The gene *GAP1* encoding p37 was cloned and identified as a member of the multigenic GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) family, which also includes the genes *GAP2* and *GAP3*. *GAP1* sequence revealed the absence of a signal peptide characteristic of proteins that are translocated into the endoplasmic reticulum.(4)
Aiming to elucidate the role of p37 in the flocculation process, *GAP1* was overexpressed in a non-flocculent *Saccharomyces cerevisiae*. The transformant acquired the ability to flocculate exhibiting flocs which are similar to the ones formed by *K. marxianus* .Indeed, deflocculation was only achieved using high amounts of EDTA and agitation. The presence of ions, namely Ca^{2+} and Mg^{2+}, increased flocculation rates. The different sensibility to calcofluor white indicates that the cell wall structure is modified and GADH total enzyme activity in the transformant is higher than in the wild type *S. cerevisiae*.
The presence of a N-linked carbohydrate chain in p37 indicates that the protein traffic to the cell wall is via ER. *In vitro* translation and translocation studies using a cell free system supplemented with microssomes, indicates that p37 is translocated postranslation and by a protein dependent mechanism.
This work, provides evidences that mechanisms leading to flocculation of non-conventional yeast can be used to induce flocculation of *S.cerevisae*.

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cDNA Cloning, Characterization and Tissue Distribution of the Prolactin Receptor in the Sea Bream (*Sparus aurata*)

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Keywords: Sparus aurata*, prolactin, receptor, cDNA*

Prolactin (PRL) is a polypeptide hormone secreted in the adenohypophysis of vertebrates. Its action in the target tissues is mediated by a membrane receptor which switches on a signal transduction pathway leading to a cellular response to the hormone. In fish, so far, only the Tilapia PRL-R has been cloned but not many studies have been carried out in order to further elucidate the possible involvement of PRL in other functions but osmoregulation such as reproduction and development.

In order to further elucidate the actions of PRL in teleost fish, PRL-R cDNA has been cloned from the sea bream (*Sparus aurata*) intestine and its tissue distribution has been analysed by RT-PCR. Northern blot analysis from intestine mRNA was also performed.

A sea bream PRL-R cDNA probe was obtained by RT-PCR of gills mRNA. This probe was used to screen at high stringency an intestine cDNA library constructed in Lambda ZAPII (450000 primary recombinants). A clone which spans 1114bp and encodes a 310aa polypeptide was isolated. Homology analysis showed 48% similarity to tilapia prolactin receptor. All characteristic features of prolactin receptors are present: four cysteine residues in the extracelular domain, a WSxWS box with the last serine residue substituted by a threonine, a transmembrane domain and a proline-rich-motif at the beginning of the intracellular domain.

For RT-PCR, total RNA was extracted from various embryonic and larval stages and adult tissues: pituitary, brain, liver, kidney, gills, skin, intestine, muscle, ovary and testis. The identity of the PCR products was confirmed by Southern blot. PRL-R was expressed as early as the gastrulae stage in all the tissues analysed except testis. For Northern Blot analysis mRNA from intestine was used and a major transcript of aproximately 1.4 Kb was detected.

The tissue distribution of sea bream PRL-R is similar to what has been reported in higher vertebrates supporting the idea of prolactin as also a pleiotropic hormone in fish. The precise spatial and temporal expression of the PRL-R in the sea bream is now being studied by *in situ* hybridization.

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Construction of a flocculent brewer's yeast strain producing an *Aspergillus niger* β-galactosidase

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KEY WORDS: Flocculation; Heterologous Protein; Cheese Whey; Lactose

The yeast *Saccharomyces cerevisiae* is recognised to be an attractive host for the production of heterologous protein. However, the yeast expression system has a major drawback, that is, the rather modest intrinsic secretory capacity of *S. cerevisiae*. One way of improving heterologous protein production is to use high cell density systems being the flocculating yeast production system one of the most attractive. On the other hand, lactose is available in large amount as a waste of cheese processing systems. From the possible alternatives for lactose valorization, the alcoholic fermentation is undoubtedly an attractive one. The success of this process depends on the development of a continuous fermentation system using flocculating yeast strains.

The construction of a flocculent and non-flocculent brewer's yeast strain growing on lactose and secreting β-galactosidase is presented. A plasmid (pLD1) coding for an extracellular β-galactosidase of *Aspergillus niger* having as selective marker the *CUP1* gene which confers resistance to copper was constructed. This plasmid can be employed on the transformation of wild-type yeasts of commercial use, either for the production of β-galactosidase or for lactose consumption and production of ethanol. The results clearly show that the brewer's yeast transformed with pLD1 gains copper resistance, has β-galactosidase activity, grows on lactose medium and in the flocculent host strain, its flocculence ability is maintained. Previous results have been reported describing the partial secretion by *Saccharomyces cerevisiae* of β-galactosidase from *Aspergillus niger* by expression of the gene in a multicopy plasmid under control of the *ADH* promotor [1]. However, the plasmid employed uses an auxotrophic marker and can only be transformed into *ura*3 yeast strains. In industrial application stability can become a problem as the transformants will not be under selective pressure. Because copper sulphate is cheaper and more widely available than most drugs, the approach presented in this work is potentially more feasible.

Besides this application, the main achievment with this plasmid is the possibility of studying the production of heterologous proteins in flocculent yeast using two different strains, a flocculent and a non-flocculent, having the same parental strain and secreting the same heterologous protein.

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Overexpression and Purification of the Protein Encoded by the c*mc*R Gene of *Nocardia lactamdurans*

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Keywords: Nocardia lactamdurans, cefamicina, cmcR.

The *cmc*R gene is located in the cephamycin C gene cluster in *Nocardia lactamdurans*. It is present 2.6 Kb upstream from the *pcb*AB gene and in opposite direction. The size of this gene is 645 pb and it encodes a 34 kD protein. The CmcR protein has an helix-turn-helix domain homologous to those found in the Tn10 protein family.

The *cmc*R gene was subcloned in the high copy number plasmid pVK2, giving rise to plasmid pULISR. The transformant strain *Nocardia lactamdurans* pULISR showed a 90% decrease in cephamycin production both in liquid cultures and in solid media.

Due to the unavailability of *Nocardia lactamdurans* expression vectors, the *cmc*R gene was expressed in *Escherichia coli* using the pQE32R plasmid and in *Streptomyces lividans* using the pSafR vector. Preliminary data indicates that *cmc*R gene overexpression affects the morphology of *S.lividans* in liquid cultures. The CmcR protein expressed in *E.coli* was purified to homogeneity by affinity chomatography using the QIAexpress system (QIAGEN). Adition of six consecutive histidine residues to the amino terminal end of CmcR permits the purification by interaction to Ni^{2+}groups and subsequent elution with imidazol. The CmcR protein will allow us to detect its binding capacity to specific promoters in the cephamycin cluster.

Polyclonal antibodies against the CmcR protein are being used to detect CmcR formation *in vivo.*

Respuesta al Estres Nutricional de la Expresion Genica a Partir del Promotor del Operon del Triptofano de *Corinebacterium glutamicum* Utilizando el Gen amy (α-Amilasa) como Reporter

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Palabras clave: C.glutamicum*, α-amilasa, operón triptófano.*

El desarrollo de la genética molecular de las bacterias corineformes utilizadas en la producción de aminoácidos ha conducido a la comprensión de los mecanismos de biosíntesis de estos compuestos, lo cual tiene clara proyección y aprovechamiento industrial (1).

Al final de la fase de crecimiento exponencial de las corinenbacterias se produce un cambio de metabolismo que origina una detención en la síntesis de proteínas y RNA ribosomal y se dispara la acumulación intracelular y posterior secreción de los aminoácidos. Aun no se conocen los mecanismos moleculares que disparan este cambio. Nuestro interés se centra en el estudio de ese cambio metabólico y si este se produce como respuesta a un estres nutricional o a un estres osmótico debido a la acumulación de metabolitos.Se están estudiando las señales metabólicas que controlan la expresión de los operones de RNA ribosomal y los genes para la síntesis de aminoácidos.

El principal objetivo de este estudio es observar la respuesta de *C.glutamicum* a la limitación de nutrientes mediante el uso de un gen indicador (reporter) para medir la transcripción a partir de un promotor que es diana de la regulación metabólica conocida como "stringent response". En nuestro caso, se utilizo como indicador de la transcripción el gen *amy* de *Streptomyces griseus* acoplado a la región promotora del operón del triptófano de *C.glutamicum.*

Para determinar el efecto que las diferentes condiciones nutricionales ejercen sobre la transcripción del promotor del operón del triptófano, se cuantifico la producción de α-amilasa (2) codificada por el gen *amy*.

Los resultados obtenidos indican que a medida que la limitación de nutrientes se acentúa el paso de la fase exponencial a la fase estacionaria se adelanta, así como el tiempo en que se obtiene mayor producción de α-amilasa, mientras que la cantidad total de α-amilasa producida es proporcional a la riqueza del medio.

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Fluorescence in situ hybridization (FISH) for discrimination of chromosomic domains in multigeneric hybrids

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Keywords: FISH, multigeneric hybrids, molecular cytogenetics

Fluorescence in situ hybridization (FISH) with both cloned, repetitive DNA probes and total genomic DNA probes (GISH), is a powerful molecular cytogenetic technique for the identification of the different parental origin chromosomes present in interspecific hybrids either in metaphase or in other stages of cell cycle [1, 2, 3].
The aim of the present study was to discriminate the wheat, rye and Hordeum chilense chromosomic domains in prophase and interphase nuclei of a 6x-triticale advanced line UTAD 17/85 x 6x-tritordeum advanced line HT67 F_1 hybrid by using fluorescence in situ DNA:DNA hybridization with both cloned, repetitive DNA, and total genomic DNA as probes.
The *in situ* hybridization and probe detection protocols followed [4]. The probes used were total genomic DNA from *Secale cereale* 'Petkus' labelled with digoxigenin and pHcKB6, a tandemly repeated DNA fragment isolated from *H. chilense*, labelled with biotin. *Triticum aestivum* 'Chinese Spring' DNA was used as blocking DNA to reduce cross hybridization. The slides were counterstained with DAPI.
Different multigeneric (*Triticum, Hordeum, Secale*) hybrids have been produced in *Triticeae* from crosses between triticale (x*Triticosecale* Wittmack) and tritordeum (x*Tritordeum* Ascherson et Garbner) [5]. These hybrids are of potential value because they are a source for the production of substitution or recombinant lines with improved agronomic performance. On the other hand, wide hybrids provide a useful scientific model for examining nuclear organization that may have implications for the control of gene expression and meiotic recombination or stability.
When prophase nuclei were labelled with two probes to distinguish the wheat, rye and *H. chilense*-origin chromosomes, the chromosomes originating from the three genera were not found in intermixed domains. In typical prophase nuclei, the chromosomes from *H. chilense* and rye were each clustered in one to three domains. At interphase, non-intermixed domains of H. chilense, rye and wheat chromosomes were visible and intirely consistent with the non-intermixed appearance of the prophase nuclei.
The fluorescence *in situ* hybridization (FISH) with both cloned DNA and total genomic DNA probes is a valuable molecular cytogenetic method for the discrimination of discrete genomic domains in the multigeneric hybrids analysed here.

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Bovine Freemartin Syndrome Diagnosis in a Portuguese Breed Using PCR

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Keywords: *freemartin, XX/XY chimerism, PCR, fertility, bovine.*

A freemartin has been defined as a sexually imperfect, usually sterile, female partner of a pair of heterosexual twins. In cattle about 92% of all females from heterosexual twins are freemartin and therefore infertile [1]. Chorionic fusion and vascular anastomosis between heterosexual twins in utero is the predisposing condition for induction freemartin sydrome. The establishment of vascular communication in placenta allows a reciprocal exchange of sex hormones and primordial hematopoietic tissues [2]. Thus, each twin has leucocytes formed by its own tissues as well as those formed by tissues derived from its co-twin. The sex chromosome chimaerism XX/XY observed in cultured leucocytes has widely been used as an early diagnosis method of bovine freemartins. More recently, chimerism was quick and efficiently identified by PCR [3]. In this study we have tested a fast and sensitive PCR method to diagnose bovine freemartins, including a pair of a local portuguese breed twins.

DNA was extracted by standard saline method from 5-10 ml whole blood samples collected from 5 animals: a normal bull and female of Barrosã breed, a heterosexual female twin of Holstein breed and a pair of heterosexual twins of Arouquesa breed. To test the sensivity of our PCR method, blood from a normal bull was diluted in blood from a normal cow and, based on the number of leucocytes counted, the ratio of XY/XX cells calculated. PCR was performed in 20-25 µl reactions using two sets of primers to amplify X and Y-specific fragments. PCR products were separated by electrophoresis on 2% NA agarose gels (Pharmacia). The DNA profiles were analysed with BioCapture software (Vilber Lourmat). We also determined the kariotype of the reported animals.

Results and Conclusions:

Y-specific fragments were detected in the two cases of heterosexual twins females reported in this work, whereas this sequences were absent in the normal cow. The results of karyotyping show that the intensity of the bands are proportional to the number of XY cells observed. In the artificial chimaeras, male specific signal could be detected starting with 1:50 dilution, corresponding to a ratio of 0.022 XY cells (2.2%). The sensitivity of PCR for the two pairs of primers tested was different. The ratio of XX/XY chimerism of most bovine heterosexual twins is in the 20 to 80% range [1]. Heterosexual female twins with low or whithout chimerism are very rare cases. Therefore, this method was proved to be highly useful in identifying nearly all freemartin cases.

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Análisis Genético de la Organización *argS-lysA* de *Nocardia lactamdurans*

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***Palabras clave**:* Nocardia lactamdurans*, lisina, diaminopimelato descarboxilasa.*

Nocardia lactamdurans es un actinomiceto utilizado industrialmente para la producción de cefamicina C, un antibiótico ß-lactámico de gran interés farmacéutico. Al igual que las cefalosporinas y las penicilinas, las cefamicinas derivan de 3 aminoácidos: el ácido α-aminoadípico (AAA), la L-cisteína y la L-valina. El origen del AAA en bacterias deriva de la ruta catabólica de la lisina. Este aspecto, unido a la observación de que la adición de lisina estimula la producción de cefamicina C en *N. lactamdurans*, hace que la biosíntesis de lisina en actinomicetos cobre un especial interés al influir directamente en la producción industrial de antibióticos.

La biosíntesis de lisina en bacterias tiene lugar a partir de la rama del ácido diaminopimélico (DAP), en la vía del aspartato. El aspártico en primer lugar es convertido en aspártico-β-semialdehído a través de 2 pasos enzimáticos catalizados por las enzimas aspartoquinasa (AK) y aspártico-ß-semialdehído deshidrogenasa (ASD). El "pool" de aspártico-ß-semialdehído se va a distribuir entre la rama de síntesis del DAP y la que da lugar a homoserina, treonina, isoleucina y metionina. Este flujo se encuentra controlado por la relación existente entre las actividades de la primera enzima de la rama del DAP, la dihidrodipicolinato sintasa (DAPA), y la primera enzima de la otra rama, la homoserina deshidrogenasa (HOM).

La regulación de los aminoácidos de la familia del ácido aspártico en corinebacterias, microorganismos cercanos a *N. lactamdurans,* tiene lugar básicamente sobre dos enzimas, la aspartoquinasa y la homoserina deshidrogenasa. La actividad de la homoserina deshidrogenasa es importante porque determina que proporción del precursor aspártico-β-semialdehído se deriva a la síntesis de treonina en decremento de la cantidad de precursor derivado a la síntesis de lisina.

En este trabajo describimos la complementación de un mutante de *Escherichia coli* en el último paso de biosíntesis de lisina que consiste en la descarboxilación del ácido meso-diaminopimelico mediante la acción del producto génico *lysA*. El plásmido pJ1 proveniente de una genoteca de DNA total de *N. lactamdurans*, fue capaz de complementar dicha mutación. La hibridación del plásmido pJ1 con dos sondas internas de los genes *argS* y *lysA* de *Brevibacterium lactofermentum* clonados en nuestro laboratorio, permitió identificar un fragmento *Sma*I de 4,0 kb que hibridaba con ambos genes.

La secuenciación de dicho fragmento reveló la presencia de 2 marcos de lectura abiertos completos (ORF 1 y ORF 2) y uno incompleto (ORF 3). La comparación de estas ORFs en la base de datos mostraron homología con arginil- tRNA sintetasas (*argS*) en el caso de la ORF 1, diaminopimelato descarboxilasas (*lysA*) para la ORF 2 y con el extremo amino de homoserina deshidrogenasas (*hom*) para la ORF 3. El estudio de estas proteínas deducidas mostró una alta homología con las obtenidas para micobacterias y corinebacterias.

Caracterización del Operón Ribosomal *rrn*D de *Nocardia lactamdurans*: Evidencia para su Reclasificación como *Amycolatopsis lactamdurans*

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Palabras clave: Nocardia lactamdurans, Amycolatopsis, DNAr.

Los RNAs ribosomales son moléculas muy antiguas, funcionalmente constantes, universalmente distribuidas, y moderadamente bien conservadas a lo largo de la escala filogenética. Estas razones convierten a los rRNAs en excelentes cronómetros filogenéticos, y la comparación de sus secuencias es el más poderoso instrumento para establecer relaciones filogenéticas y evolutivas entre bacterias, arqueobacterias y organismos eucariotas.

En este trabajo se ha clonado el operón ribosomal *rrn*D de *Nocardia lactamdurans*, se han secuenciado los genes para rRNA 16S y 5S, y se han comparado estas secuencias con las de otros microorganismos depositadas en las bases de datos GeneBank y RDP.

A partir de una genoteca de DNA total de *Nocardia lactamdurans* LC 411 construida en el cósmido pJAR4 [1] y utilizando como sonda de hibridación un fragmento *Hin*dIII de 2,7 kb procedente del plásmido pULRH2 [2], que contiene parte del operón *rrn*B de *Rhodococcus fascians*, se identificaron 6 clones positivos. Partiendo del cósmido pRN6 se subclonó una banda *Bam*HI de 7 kb, que contiene el operón ribosomal *rrn*D, en el vector pBluescript SK+, dando lugar al plásmido pRBN1. Desde el pRBN1 se clonó y secuenció el rDNA 16S y la secuencia obtenida se comparó con las existentes en la base de datos GeneBank. De esta comparación se han obtenido porcentajes de homología del 95,63 % con *Amycolatopsis orientalis* y 95,27 % con *Amycolatopsis sp.*, mientras que la especie del género *Nocardia* con mayor homología con el DNA secuenciado es *Nocardia crassostrae*, que aparece en el puesto 14 de esta comparación, con un 91,82 % de semejanza.

También se ha secuenciado el rDNA 5S y la comparación de su secuencia con las depositadas en la base de datos GeneBank proporcionó homologías del 93,27 % con *Saccharomonospora azurea* y del 92,50 % con *Amycolatopsis rugosa*, mientras que con miembros del género *Nocardia* las homologías eran significativamente menores, apareciendo en el puesto 21 *Nocardia asteroides* con un 88,98 % de semejanza.

A partir del programa de M. Zuker del *Whasington Universty Medical School*, se han obtenido las estructuras secundarias de los RNAs 16S y 5S de *N. lactamdurans*. La comparación de estas estructuras con las de microorganismos relacionados refuerza los resultados obtenidos con la comparación de secuencias.

De estos resultados se puede concluir que *N. lactamdurans* esta erróneamente clasificada dentro del género *Nocardia*, siendo su ubicación más probable dentro del género *Amycolatopsis*. De hecho varios microorganismos incluidos en el pasado en el género *Nocardia* han sido reclasificados como *Amycolatopsis* [3].

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Characterization of osmotic-remedial lytic mutants of *P. pastoris*

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***Keywords**:* Pichia pastoris*,* SLT2*, lytic mutants,heterologous proteins.*

The methylotrophic yeast *Pichia pastoris* has been successfully used to express high levels of many heterologous intracellular and secreted proteins [1]. This yeast expression system is similar, in many aspects of DNA manipulation, to the baker`s yeast *Saccharomyces cerevisiae* system, with the added advantage of a protein processing analogous to that of higher eukaryotes. A major bottle-neck in the processes that employ yeast cells for expression of foreign genes occurs in the release of the corresponding protein, an essential step for downstream operations leading to protein purification. The two basic alternatives for the release of heterologous proteins are secretion or cell breakage.

Secretion represents an attractive possibility because only a few proteins, in addition to the one desired, are present in a mixture of proteins secreted by yeast, but it requires substantial manipulation of the gene. However many high added value proteins produced in yeast remain intracellular instead of being secreted, so that they have to be recovered by some kind of cell breakage treatment. Cell breakage can be achieved with different chemical, physical and enzymatic methods. Physical treatment might affect the stability of polypeptides and the other methods introduce additional impurities to be removed in the corresponding downstream operations.

We have developed a new system for the release of heterologous proteins based in osmotic-remedial lytic mutants of *S. cerevisiae*, that lyse at high temperature [2]. These mutants are defective in *SLT2* gene, a MAPKinase gene responsible for cell integrity under certain conditions, and they can liberate the bulk of intracellular soluble proteins in absence of osmotic stabilisation. These proteins can be easily separated of ghosts and debris by simple spin.

The aim of our work was to obtain osmotic-remedial lytic mutants of *P. pastoris*, deficient in cell integrity, that lyse at high temperature, similar to the mutants we have characterized in *S. cerevisiae* To achieve this purpose we followed two strategies:

- Screen for autolytic thermosensitive *P. Pastoris* mutants after UV mutagenesis. We have characterized five osmotic-remedial lytic mutants, able to release their intracelular content at 37°C
- Cloning the *P. pastoris* gene homologous to the *S. cerevisiae SLT2* gene, by using PCR with degenerate oligonucleotides to amplify MAPK homologs. We isolated a 400pb fragment of *SLT2* of *P. pastoris*, and used this fragment as a probe to clone the completed gene by colony blot, using a *P. pastoris* genomic library. The *P. pastoris SLT2* gene includes an open reading frame of 1281 nucleotides corresponding to a polypeptide of 426 amino acids whose sequence shares 60.1% identity to that of *Saccharomyces cerevisiae*, 65% to the *Candida albicans* and 55.4% to the *Schizosaccharomyces pombe* protein.

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Caracterización de Factores Transcripcionales Involucrados en la Expresión del Gen *pcbAB* de *Penicillium chrysogenum*

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***Palabras clave**:* P. chrysogenum*, regulación transcripcional, proteínas de unión a DNA*

Actualmente se conoce la secuencia completa de reacciones implicadas en la síntesis de penicilina. Consta de tres pasos catalizados por las enzimas δ-(L-α-aminoadipil)-L-cisteinil-D-valina sintetasa (ACVS), isopenicilina N sintasa (IPNS) y la acil-CoA:6-APA aciltransferasa (AAT). En *Penicillium chrysogenum* estas tres enzimas están codificadas por los genes *pcbAB*, *pcbC* y *penDE*. Los genes *pcbAB* y *pcbC* se transcriben en direcciones opuestas a partir de una región promotora bidireccional de 1.1 kb.

La biosíntesis de penicilina está sometida a diferentes procesos de regulación en los que están involucrados factores de tipo nutricional y de desarollo. Fuentes de carbono que proporcionan un rápido crecimiento de microorganismo (glucosa, sacarosa, fructosa, galactosa) ejercen un importante efecto represor sobre la producción de penicilina, mientras que la lactosa se comporta de forma contraria (Martín et al., 1984, Revilla et al.,1984), por lo que se utiliza industrialmente como fuente de carbono en la producción de penicilina.

Este trabajo se centra en el efecto de las distintas fuentes de carbono sobre la transcripción génica. La región promotora intergénica entre los genes *pcbAB* y *pcbC* fue analizada para localizar secuencias reguladoras que actuen en *cis* y para detectar proteínas reguladoras. En este fragmento de DNA se localizaron secuencias de unión a proteínas (Feng et al., 1995, Chu et al., 1995). Recientemente se ha clonado el gen *pacC* de *P. chrysogenum* que codifica para una proteína de unión a DNA cuya secuencia diana se encuentra repetida varias veces en dicha región promotora. El crecimiento de *P. chrysogenum* en medios con pH alcalino, no revertió la represión ejercida por altas concentraciones de glucosa, al contrario de lo ocurrido en *Aspergillus nidulans* (S. Gutiérrez, comunicación personal; Suárez y Peñalva, 1996).

El estudio de delecciones seriadas de la región promotora *pcbAB-pcbC* acopladas al gen testigo *lacZ*, ha conducido a la cacterización de dos zonas involucradas en la expresión de estos genes. El análisis de la formación de complejos DNA-proteína de dichas secuencias mediante retraso en geles ha permitido identificar distintos complejos DNA proteína cuya presencia varia según la fuente de carbono utilizada en el cultivo. Los complejos presentes en los medios crecidos en glucosa presentan una mayor especificidad que los obtenidos de cultivos crecidos en lactosa. Ademas, este patrón en la formación de los distintos complejos, varía según el tiempo de fermentación al cual se lleva a cabo el ensayo. La especificidad de los complejos formados se ha estudiado mediante ensayos de competición directa y cruzada entre las dos zonas.

Las proteínas responsables de los complejos de unión en distintas condiciones de cultivo se unen a la misma zona y con una alta afinidad, como demuestra que la adición de extractos provenientes de otras condiciones no sean capaces de unirse al fragmento ensayado.

Las proteínas implicadas en la unión a la región intergénica *pcbAB-pcbC* se están purificando mediante cromatografía de afinidad en heparina, precipitación con sulfato amónico, cromatografía de gel filtración e intercambio iónico.

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Mecanismos de Regulación por Hierro en Dos Especies del Género *Streptomyces*: *S. coelicolor* y *S. pilosus*

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Palabras clave: *hierro, sideróforos,* Streptomyces.

El hierro es uno de los elementos químicos más frecuentes en el suelo. Sin embargo, su baja solubilidad provoca que no esté disponible para la mayor parte de los microorganismos. Al ser un elemento esencial (1) las bacterias deben presentar sistemas muy eficaces de transporte para su captación basados en moléculas de bajo peso molecular y alta afinidad por el hierro; los sideróforos (2). Estos compuestos se excretan al medio y reaccionan con el hierro férrico solubilizándolo y transportándolo al interior del organismo (3). Tanto en $Gram^+$ como en $Gram^-$ los mecanismos de captación de hierro están regulados a través de una proteína represora: **Fur** en $Gram^-$ (4) y **DtxR** en $Gram^+$ (5). Ambas proteínas están implicadas en los procesos de síntesis de sideróforos y sus receptores. El mecanismo de acción se basa en la unión de estas proteínas con secuencias de ADN específicas y actuan como un represor en presencia de hierro (6, 7).

A fin de comprobar como afecta el crecimiento en condiciones limitantes de hierro a la expresión génica de *S. coelicolor* se han realizado una serie de fermentaciones en diferentes condiciones. El medio de cultivo se suplementó con diferentes concentraciones de dos quelantes; 2,2´-dipiridil o EDDHA. Mediante SDS-PAGE se observó la aparición o sobreexpresión de diferentes proteínas de pesos moleculares mayores de 30kda y la disminución de otras. Estos resultados se observaron únicamente en los extractos de las fermentaciones suplementadas con quelantes y no así en los controles o en los que además de quelante se añadió $Cl_3Fe{\cdot}6H_2O$ para contrarrestar los efectos del quelante. El efecto observado, por tanto, es debido a los mecanismos que pone en marcha el microorganismo en ausencia o limitación del hierro. De entre todas las proteínas que aumentan su porcentaje destaca poderosamente una proteína de aproximadamente 75-80 kda. Esta proteína se está empezando a purificar a fin de secuenciarla e identificar la posible función que posee.

Con objeto de caracterizar un promotor regulado por hierro como sistema de expresión se ha realizado una fusión transcripcional del promotor del gen *des*A de *S. pilosus* (implicado en la síntesis del sideróforo desferroxiamina B) con un gen testigo (el gen *amy de S. griseus*). El promotor del gen *des*A presenta una secuencia parecida a la que reconoce el DtxR de *Corynebacterium diphtheriae* en el promotor del gen *tox*. Esta fusión transcripcional se ha clonado en un plásmido con origen de replicación de *Streptomyces* con el que se transformará *S. coelicolor* y *S. pilosus*. De esta manera se estudiará la regulación del gen *amy* en presencia o ausencia de hierro, así como la fuerza de dicho promotor.

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Streptomyces natalensis plasmid, pSNA1: Genetic organization and correlation with genetic properties

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Keywords: Streptomyces*, Plasmid, open-reading-frame, plasmid replication*

A circular cryptic plasmid, pSNA1, has been found in pimaricin-producing *Streptomyces natalensis* strain ATCC 27448. pSNA1 has been restriction mapped and its complete nucleotide sequence determined. The circular DNA molecule is 9571 nucleotides in length and has a G+C composition of 71,3 %. Its estimated copy number was 11. The nucleotide sequence of pSNA1 has been analyzed and correlated with previously published genetic data of plasmids of Gram-positive bacteria. Five open reading frames (encoding peptides larger than 90 aminoacid residues) were located on both strands of pSNA. ORF1(*korA*) encodes a polypeptide of 246 aminoacids which shares homology with products of two ORFs found in the protein database. ORF 3 codes for a potential replication protein containing 476 aminoacid residues, for which there are 2 homologous proteins encoded by other streptomycete plasmids. ORF4 (*spdA*) codes for a protein (161 aminoacids) possibly involved in intramycelial plasmid transfer. Counterparts of the products of ORF2 and ORF5, could not be found in the protein database.
Northern hybridization analysis was used to assess the number and type of RNA transcripts, further completing the characterization of pSNA1.
S. natalensis is the producer of the widely utilized polyene macrolide pimaricin. However, despite our interest in the genetic manipulation of this bacterium, no transformation has been achieved so far. The finding of pSNA1 and its genetic characterization will allow the development of pSNA1-derived vectors which may overcome genetic manipulation problems in this industrially important strain.

The Clavulanic Acid Biosynthesis Regulator in *Streptomyces clavuligerus* is a LysR-Type Protein

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Key words: Streptomyces clavuligerus, *clavulanic acid, regulation Lys-R.*

Two genes have been located and sequenced in a 2.8 kb *Eco*RI-*Bgl*II DNA fragment from the clavulanic acid-cephamycin C gene cluster of *Streptomyces clavuligerus*. *cla*R encodes a protein of 431 aminoacids with a deduced Mr of 47080, and *car* encodes a 247 aminoacid protein with a Mr 26629. The protein encoded by *cla*R, shows significant homology with transcriptional activators of the LysR family. This protein, ClaR, contains two HTH motifs located in both the amino and the carboxyl terminal end . The protein encoded by *car* posses high similarity to dehydrogenases and oxidoreductases of the SDR family. The aminoacid sequence deduced from the 5' end of *car* coincides with the N-terminal end of the purified clavulanic-9-aldehyde-reductase of *S.clavuligerus.*(1)

Transformants of *S.clavuligerus* with *cla*R in a high-copy number plasmid produce increasing amounts of the clavulanic acid and alanylclavam in defined media, while the cephamycin C production is reduced. Also the amplification of *car* originated a positive effect on clavulanic acid biosynthesis. The disruption of both genes resulted in clavulanic acid non producting strains.

Northern analysis shows that these genes are expressed as monocistronic transcripts in TSB medium what agrees with the presence of palyndromic sequences located downstream of both ORF that may act as transcriptional terminators.

A mutant disrupted in *cca*R, a gene which positively controls clavulanic acid and cephamycin C biosynthesis, lacks both *cla*R and *car* transcripts in TSB medium. This result indicates that clavulanic acid and cephamycin C biosynthesis are controlled by a cascade of regulatory proteins.

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Screening of specific genetic markers in *Quercus suber*

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Cork tree (*Quercus suber*) is a widespread culture of great Economical value in Portugal. So far there is no data available on the genetic variability of the plant among the different populations. Genetic markers are scarce or inexistent in oaks. Lately, microsatellite analysis has been considered one of the most powerful tools for variability assessment and evolutionary analysis [1,2].
This work aims the screening of VNTRs and the identification of their flanking regions for the design of primers which will be used to analyse the variability in those *loci.*

Genomic DNA was extracted from young leaves of 8 different *Q. suber* populations trees (Évora, Portalegre, Santiago do Cacém, São Brás de Alportel, Abrantes, Ponte de Sôr, Sesimbra e Mirandela) and partially digested with *Alu* I and *Hae* III. A genomic library was constructed by cloning selected restriction fragments ranging from 200 - 800 bp. A large number of recombinant clones is being screened by hybridization using $(TG)_{10}$ and $(TC)_{10}$ synthetic oligonucleotides as probes. The insert of the selected positive clones will be further sequenced in order to identify microsatellite markers.

This work is being developed within a broader project involving seven National Institutes. The microsatellite data will be crossed with those from RAPD analysis which is also being performed in our team.

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Horse Parentage Testing Using High Polymorphic Microsatellites: A Case Study

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Keywords: Paternity, Microsatellites, PCR, horse.

Introduction:

Horse interspecific hybrids (mules) are known to be sterile. However, some are subfertile and others even fertile [1]. In this study, a fertile female hybrid was analysed for the determination of the putative parents of a male descendent.
Parentage testing is being widely performed with microsatellite analysis, by studying the PCR amplification profiles of VNTRs regions. A list of specific primers is available for genetic variability analysis on horse breeds. Some of them have also been used on parentage testing. The aim of this study was to analyse several horse microsatellite markers for their application to hybrids, donkeys and horses other than the original breeds.

Methods:

Paternity tests were performed on four equines: putative mother, two putative fathers and descendant male. Unrelated animals were used as control (one she-mule, a mare and a male mule). DNA was extracted from 20 ml blood samples and PCR amplified with 5 different pairs of primers: HMS2, HMS7, [2] HTG7, HTG8, HTG10 [3]. [α ^{35}S] dATP was incorporated in the PCR reactions and amplification products were analysed by electrophoresis on a 6% polyacrylamide gel and autoradiograh detection.

Results:

All microsatellites tested were successfully amplified in the three species. However, most of them did not present enough variability in the present case that would allow correct parentage determination. More, HTG10 has produced a high level of non-specific amplification which can strongly hinder the analysis. Paternity was determined by profile analysis of the PCR amplification with HTG7. The technique has proven to be adequate for hybrids as well as donkeys and horses but this work suggests that further screening with other markers is needed before routine parental tests are to be established on a national level.

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Modelling of the cellobiohydrolase of *Coriolus versicolor*

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***Keywords**: cellobiohydrolase,* Coriolus versicolor*, homology modelling.*

In order to study *Coriolus versicolor* cellulolytic system, a cDNA library was constructed from a cellulase induced culture. The resulting cDNA library was screened using a cellobiohydrolase probe from *Agaricus bisporus*. A selected clone showed an cDNA insert of 1.4 Kbp. Sequencing data and deduced amino acid sequence identified the presence of conserved sequences from cellulose binding, and catalytic domains as well a rich Pro-Ser-Thr linker region.
From the deduced amino acid sequence, four amino acid fragments were constructed by homology modelling (SwissModel Server)[1, 2] using the structures of the PDB database with the ID: 1CBH (for binding domain), 1CB2 (for catalytic domain) and 2MCM, 7FAB, 2APR (for linker between the binding and catalytic domain) as templates. The fragments were linked using Hyperchem v 4.0 (Hypercube) and the stereochemical quality of the built model checked using Procheck v.3.4.4 [3, 4] and Verify3D server [5]. The Procheck analysis at 2 □ showed that all stereochemical main-chain and side-chain parameters were inside the expected band width as well the 3D / 1D result of the Verify3D server. The values of the RMS between model and each of the templates used for homology modelling were 0.36 □ (1CBH) and 0.54 □ (1CB2), calculated by SwissPdbViewer v 2.6 [6, 7] using the Carbon alpha fitting. The linker segment is under refinement in order to improve it.

The structural alignment between model and the catalytic domain showed identical residues in 18 of the 19 residues facing the substrate in the 4 binding sites of the active site tunnel, including Asp residues 175 and 221 involved on cellulose cleavage.

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Genotyping of Ovine αS1 Casein in Serra da Estrela Breed by PCR Analysis

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Keywords: PCR, αS1 Casein genotyping, ovine.

Introduction:

Genetic variants of milk protein in sheep were found to be related with the quantity and quality of milk yield. This was also observed for the D allele of the as1 casein [1]. More recently, Pilla *et al.* (unpublished data) have designed a method for typing the A, C and D alleles for αS1 casein.
The aim of this study was the analysis of the casein alleles within the Serra da Estrela breed, by the genotyping of αS1 casein using PCR.

Methods:

DNA was extracted by standard saline method from 15 ml whole blood samples collected from non-related animals from the breed Serra da Estrela located in different flocks dispersed within the breed distribution area.
PCR was performed in 20 µl reactions using specific primers for the amplification of allele A (A1, A2) and D (D1, D2). PCR products were digested with *Mbo* II (allele A) and *Mae* III (allele D) and analysed by electrophoresis on 12.5% acrylamide gels using PhastSystem (Pharmacia). The DNA profiles were analysed with BioCapture software (Vilber Lourmat).

Results and Conclusions:

A set of 60 animals (30 rams and 30 ewes) was sampled. The results have shown a low polymorphism within this breed. A high percentage of the individuals share the same DNA profile which indicates that they fall within the same genotype (nonA-nonA, nonD-nonD).
Although 4 phenotype variants have been detected (A, B, C and D), no sequence has been so far determined for variant B. B variant is very rare and no biological material (DNA from a phenotypic BB ewe) has been found to attempt a study at DNA level.
Therefor, the vast majority of the samples seem to belong to variant CC.
To our knowledge, no genotyping has yet been performed for national breeds at DNA level. Once that these *loci* are strongly related to milk production it is our goal to extend this analysis to other breeds within a broader project concerning the study of indigenous breeds at DNA level.

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Sequence Analysis of pRS1, a Cryptic Plasmid from *Oenococcus oeni*

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***Keywords**:* Oenococcus oeni*, plasmids, nucleotide sequence.*

Wines from Ribeira Sacra, a wine-producing region of northwestern Spain, are normally deacidified by malolactic fermentation carried out by lactic acid bacteria, mainly *Oenococcus oeni*. In order to develop cloning vectors for *O. oeni*, we have determined and analyzed the nucleotide sequence of pRS1, a cryptic plasmid from an *O. oeni* strain isolated from Ribeira Sacra wines.

Materials and Methods.
O. oeni carrying pRS1 was isolated in solid MRS suplemented with malic acid and tomato juice and incubated at 22 ºC in anaerobic jars, and identified by electron microscopy and biochemical tests. Plasmid DNA from this strain was extracted and purified by the procedure described by O'Sullivan and Klaenhammer (1993). The nucleotide sequence of pRS1 was determined in both strands by cloning restriction fragments into M13mp18/19 and using the chain termination procedure of Sanger et al. (1977).

Results and Discussion.
The sequence of pRS1 comprises 2522 bp with a G+C molar content of 35.6 %, very similar to the previously described *O. oeni* plasmids pOg32 (Brito et al., 1996) and pLo13 (Fremaux et al., 1993). Sequence analysis for both strands showed the presence of three major open reading frames (ORFs) comprising 73% of the plasmid length. ORF1 could encode a 233 amino acid peptide. This putative protein exhibits significant similarity (50 to 54% homology in overlapping regions of 137 to 238 amino acids) with ORF1-encoded protein of pOg32 and with the replication initiation protein (Rep) of a family of six closely related *Staphylococcus aureus* plasmids of which pT181 is the prototype and which replicate by the rolling circle mechanism.

Similarly, ORF2, which could encode a 255 amino acid peptide, exhibits significant homology (50.2%) with the ORF2 of pOg32, as well as with other Pre proteins (recombination-mobilization proteins) of several *Bacillus* and *Lactobacillus* strains, features also found in pLo13. Finally, ORF3, which could encode a 121 amino acid peptide, does not exhibits significant homology with the proteins present in the consulted databases. The same occurs with the third ORF of pOg32 and pLo13.

The features above mentioned suggest that pRS1 belongs to a group of small cryptic plasmids of *O. oeni* that probably replicate by the rolling circle mechanism and that have similar characteristics (i.e. three ORFs at the same strand, starting with an ATG codon, two of them coding for a Pre and a Rep proteins and the third with unknown function).

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Mechanisms of regulation of expression of the citrate transport system from *Lactococcus lactis* biovar *diacetylactis*

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Key words: Lactococcus lactis,*citrate operon, gene expresion, acid stress, processing, ribonuclease*

In the dairy industry, lactic acid bacteria (LAB) are important for their acidifying properties and their heterofermentative metabolism. The use of *Lactococcus lactis* biovar *diacetylactis* (*L. diacetylactis*) is due to its ability to synthetize some aromatic compounds, like diacetyl and acetoin, from citrate. The first step of the citrate utilisation is its transport by the cells. In *L. diacetylactis* this uptake is carried out by the citrate permease P (CitP), which is encoded by the plasmidic *citP* gene located in plasmid pCIT264. Two partially overlapping genes, *citQ* and *citR*, placed upstream of *citP* gene were previously identified. These two genes, together with *citP,* constitute the *citQRP* operon. Transcription of this operon is driven from promoter P1. Plasmid pCIT264 carries an insertion sequence (IS) of 1 kb between the lactococcal promoter P1 and *citQ* gene and provided the operon with two new promoters, P2 and P3. Promoter P2 increases expression of the *citQRP* operon in the natural host and P3 supports expression of ORF1 of unknown function. Moreover, the IS contains promoter P2´, which is only functional for expression of *citP* in *Escherichia coli*. The expression of the *citQRP* operon is independent of the presence of citrate in the growth medium. The *citQ* and *citR* genes seems to play a role in regulation of leves of CitP in the cell. The central region of *citQ* and the 5´-end of *citR* are included within a complex secondary structure, and the *cit* mRNA is processed in this structure both *L. diacetylactis* and *E. coli*. The identification of the 5´-end of the processed species showed that the endonucleolytic cleavage should interfere with translational of *citQ* and *citR*. The molecular mass of the products of *citQ* and *citR* indicates that the translation of both genes start at the first AUG of *citQ*,which is located upstream of the secondary structure.

In this work, the *citQ* and *citR* genes products were identified by the use of an *E. coli in vitro* transcription-translation system. Characterization of the *cit* mRNA processing, site directed mutagenesis of *citQ* and analysis of expression of a *citP-cat* fusion was undertaken. The results obtained revealed an interplay of mRNA processing and *citQ* translation, which influence expression of *citP* gene. Moreover, analysis of *cit* mRNA processing in several *E. coli* mutants strains reveals that a RNase E-like endoribonuclease is involved in this process.

Analysis of the physiological conditions involved in expression of the *citQRP* operon revealed that transcription of CitP is induced by acidification of the growth medium. The maximum levels of CitP were obtained from lactococcal cultures growth at pH 4.5.Thus, the maximum transport activity of CitP was observed within a pH range of 5.5-4.5. Therefore, the activation of this transport system, which is a response to acid stress in lactococci, enhances citrate utilitation by *L. diacetylactis* without any deleterious effect. Thus, the increase in expression of CitP at acidic pHs, accompanied by the enhancement of citrate uptake, could improve adaptation of *L. diacetylactis* to acidic conditions. This phenomenon could provide to *L. diacetylactis* with a selective advantage, which should enlarge its survival in fermented products.

Characterization of the *Penicillium chrysogenum* NADP-Dependent Glutamate Dehydrogenase Gene and its Use for Gene Expression

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Keywords : *beta-lactam antibiotics, Fungal promoter, DNA sequence.*

Penicillium chrysogenum and *Acremonium chrysogenum* are the microorganisms industrially used for the production of the clinically and economically important antibiotics penicillin and cephalosporin respectively [1]. The development of recombinant DNA techniques has allowed yield improvement of antibiotic producing strains. A classical strategy to enhance antibiotic production has been the introduction of additional copies of genes involved in the biosynthetic pathway or the location of the genes of interest under the control of strong promoters. In fungi, the expression of homologous genes may be negatively regulated and the heterologous gene expression is limited by the recognition of the promoter by the fungal transcription machinery.

It is known that upstream activating sequences exist in some highly expressed fungal genes [2]. Most of the genes involved in the primary metabolic pathway present high level of expression, suggesting that their promoters may contain specific enhancers or regulatory sequences that contribute to the presence of high level of transcripts in the cell. Glutamate dehydrogenase (GDH) is a key enzyme of the primary metabolism that links carbohydrate (energy) and nitrogen pathways [3]. The expression of the *gdh* gene leads to a large accumulation of both *gdh* transcript and GDH protein.

We report here the isolation and sequencing of the *gdh* gene of *P.chrysogenum* and the construction of fungal expression vectors based on its promoter. The gene was identified by hybridisation with oligonucleotide probes deduced from conserved sequences of the *gdh* gene from *Neurospora crassa*. It was included in a 7.2 kb *EcoRI* genomic fragment. The sequence of a 2816 bp fragment showed a 1600 bp coding region interrupted by two introns of 160 and 57 bp respectively with fungal consensus splice-site junctions. This ORF encodes a protein of 461 amino acids with a molecular mass of 49.83 kDa.

The performance of the *gdh* promoter was ascertained by the construction of gene fusions with the β-galactosidase gene (*lacZ*) from *Escherichia coli* and the bleomycin resistance determinant (ble^R) from *Streptoalloteichus hindustanus,* testing the constructions in *P. chrysogenum, A. chrysogenum* and *E. coli*.

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Screening of specific genetic markers in an indigenous breed of pigs

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Keywords: *Microsatellites, Bísaros, pigs, biodiversity*

A large number of autochthonous breeds of domestic animals are endangered. Strong and fast measures are needed to preserve them and assure the variability and genetic diversity among the present populations.
Pig breeds are no exception. The breed "Bísaro" is reduced to an extremely low number of animals that still conserve their phenotypic characteristics and hybrids resulting from cross matings are the most common.
This work presents a first attempt to assess the existing genetic variability among indigenous pig breeds by determining specific genetic markers that can be used as a tool for this purpose.
Microsatellites are among the most common and useful genetic markers being used [1]. However, the ones available are not known to be adequate for Portuguese breeds.
It is our goal to search for conserved sequences flanking VNTR regions and use them to design primers that can be used for PCR amplification and analysis of microsatellites.

DNA was extracted from blood samples collected from unrelated animals of the Bísaro breed.
A genomic library was constructed by cloning selected restriction fragments ranging from 200 - 800 bp. A large number of recombinant clones is being screened by hybridization using $(TG)_{10}$ and $(TC)_{10}$ synthetic oligonucleotides as probes. The insert of the selected positive clones will be further sequenced in order to identify microsatellite markers.
Similar work will also be performed in other indigenous breeds.

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Clonación, Caracterización y Análisis Transcripcional de los Genes *dmd*R y *gal*E de *Rhodococcus fascians*

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Palabras clave: Rhodococcus fascians*; represor de la toxina diftérica; galactosa epimerasa*

Rhodococcus fascians es una corinebacteria fitopatógena cuya biología molecular es aún poco conocida (1). El gen *dmd*R de *R. fascians* se ha clonado y caracterizado. Este gen es homólogo al gen *dtx*R que codifica una proteína reguladora de la expresión de la toxina diftérica dependiente de hierro en *Corynebacterium diphteriae* (2).

Utilizando una sonda procedente del gen *dtx*R de *B. lactofermentum Apa*I-*Hind*III (3), se rastreó una genoteca de DNA total de la cepa D188 de *R. fascians* construida en el cósmido pHC79. El análisis de restricción de los 5 clones positivos, mostró que uno de estos cósmidos, pRF732, contenía una banda *Eco*RI de cuatro kilobases que hibridaba con esta sonda. Este mismo patrón se observó al hibridar DNA total de la cepa D-188, digerido con la misma enzima de restricción. Se subclonó la banda *Eco*RI de cuatro kilobases en el pBluescript SK(+) en ambas orientaciones dando lugar a los plásmidos pX4 y pX5. La secuenciación de ambos insertos por el método de las deleciones unidireccionales nos reveló la presencia de dos marcos de lectura abierta: ORF1 y ORF2.

La ORF1 de 687 nucleótidos codifica para una proteína de 228 aminoácidos con un peso molecular deducido de 25 kilodaltons, que mostraba homología con las proteínas DtxR de *C. diphteriae* (53.3%), *B. lactofermentum* (57%) y *M. smegmatis* (70%) (4). Se ha detectado la presencia de esta proteína por inmunodetección utilizando anticuerpos policlonales anti-DtxR, tanto en el extracto de *R. fascians*, como en la cepa de *Escherichia coli* DH5α transformada con el plásmido que contiene el gen *dmd*R de *R. fascians*.

La ORF 2 de 978 nucleótidos se encuentra corriente abajo de la ORF 1. Codifica para una proteína de 325 aminoácidos con un peso molecular deducido de 34 kilodaltons y muestra homología con proteínas GalE (UDP galactosa 4 epimerasa) de microorganismos Gram+ como *B. lactofermentum* (68.4%) (5) y S*treptomyces lividans* (53.7%) (6).

Estos dos genes se encuentran ligados dentro del genoma de *R. fascians*. Esta organización se mantiene en *B. lactofermentum.* Experimentos preliminares nos permiten afirmar que en *R. fascians* estos dos genes forman parte de un operón que se transcribe como un RNA mensajero bicistrónico de 1.8 kilobases a partir de un promotor localizado corriente arriba del inicio del gen *dmd*R .

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The response of the *E. coli* Morphogene *bolA* to Different Forms of Stress During Exponential Growth

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Escherichia coli cells have an altered morphology under starvation or stationary-phase conditions. They are smaller and spherical in contrast to the rod-shaped morphology of growing cells. The gene *bolA* is an *E. coli* morphogene whose product causes round morphology when overexpressed [3]. This gene seems to be involved in the switching between cell elongation and septation systems during the cell division cycle [3]. In normal growth conditions the expression of *bolA* is growth-phase regulated, being specifically induced during the transition to stationary phase [2] . Such type of regulation is typical of genes containing *gearbox* promoters where the RNA is transcribed at levels inversely proportional to growth rate [2]. The gene *bolA* is governed by two promoters; the weaker P2, located further upstream from the structural gene, is σ^{70}-dependent and transcribes *bolA* constitutively. In turn, the main promoter P1 is proximal to the *bolA* structural gene and contains a -10 sequence of the type CGGCNAGTA, consensus to other gearbox-regulated genes [1]. This is the promoter responsible for late-phase induction. The modulation of P1, like other gearbox promoters, is due to the sigma factor (σ^S) encoded by *rpoS* (*KatF*) [1] .

Here we demonstrate that *bolA1p* is also induced during early logarithmic growth in response to several forms of stress and that this induction is partially independent of σ^S as seen with an insertional mutant. Northern blot was used in order to analyze the fate of *bolA1p* mRNA for a period of four hours after imposition of different stresses at an early OD of 0.3. A considerable nineteenfold increase in *bolA1p* transcript levels was seen in response to osmotic shock (0,3M NaCl), eightfold in response to sudden starvation and oxidative stress (centrifugation followed by resuspension and 50 mM H_2O_2 respectively), and lastly, a threefold increase due to both medium acidification (pH 7.0->pH 4.4), and heat shock (30^oC->44^oC). The orders of magnitude of *bolA1p* induction observed in log phase were in some cases larger than levels reached at OD 1.5. We have further correlated the timing of transcriptional response with changes in optical density and cell viabilities (in CFUs). This data, together with the morphological changes observed in the cells under the same conditions of stress, indicate that *bolA* has an important role in the general stress response triggered by the cell under adverse growth conditions. The *bolA1p* induction in response to heat shock, contradicting *gearbox* behavior, and the fourfold induction seen in a mutant strain containing a Tn-10 insertion in *rpoS,* indicate there must be alternative modes for induction. A model for such a mechanism, based on an increase in *bolA1p* mRNA stability, is proposed. The results obtained show that the *gearbox* promoter regulating *bolA* can be triggered by natural signals other than stationary-phase conditions. Such knowledge could be used in order to artificially overproduce other genes of interest in a easy and low cost fashion.

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Microsatellite DNA Variation Within and Among Portuguese Ovine Breeds: Case of *Serra da Estrela* Breed

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Keywords: *Sheep, diversity, microsatellite*

Genetic variation is the basic material of the animal breeder, wich is used to mould our domestic animal species to our needs. Loss of variation will restrict the options available to meet unpredictable future requirements. Previous, blood group and proteins studies have been used as markers to study genetic diversity and evolutionary relationships in mammals. Microsatellites markers offer great potential and various advantages for genetic comparisons within and between closely related populations.

The aim of our project is quantify the genetic variation which exists within and between portuguese ovine indigenous breeds. In this preliminary study we report the analysis of 20 microsatellites in the "Serra da Estrela" breed. All of the microsatellites contain the (dG-dT).dC-dA) dimer as the repetitive element.

Blood samples were collected from 46 unrelated sheep registered in the genealogic book.

DNA from blood samples was extracted by using a saline method (Montegomery and Sise, 1990).

The PCR analysis of microsatellites was carried out using an end-labeled primer [γ-^{33}P]ATP and T4 polynucleotide kinase. Total volume of the reaction was between 10 and 15µl. The products of amplification were electrophoresed on a 6% denaturing sequencing gels and the bands of DNA visualized by autoradiography.

All loci were found to be polymorphic in that breed. The number of alleles per locus ranged from 4 to 14 with an average of 8.5. The mean observed heterozygosity was 0.726, which ranged from 0.370 for the ETH225 microsatellite to 0.886 for the OarCP34 microsatellite.

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Single Strand Conformation Polymorphism Analysis of Exon 4 of Growth Hormone Gene and Exon 1 of α-Lactalbumin Gene in "Churra da Terra Quente" Sheep Breed

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Keywords: SSCP, sheep, variability, growth hormone, α-lactalbumin

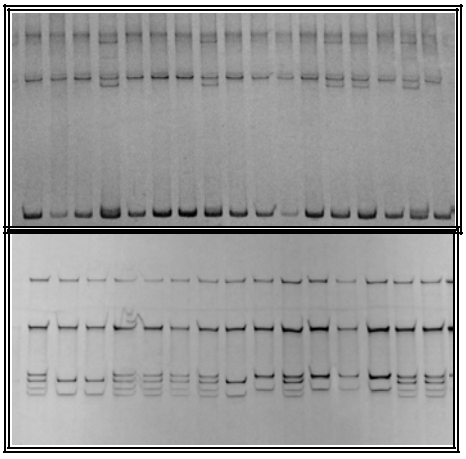
Nowadays, the loss of genetic variability is a major concern. Since 1950, the conservation of animal genetic resources has been a topic of discussion.

The ability to detect polymorphisms at the DNA level has lead to new approaches in genetic studies of livestock species. Single strand conformation polymorphism (SSCP) analysis has become one of the most common methods for the reliable detection of polymorphisms in DNA [1]. The present study is part of a research program aiming the evaluation of genetic variability of "Churra da Terra Quente" breed using SSCP analysis. The search for SSCP could also lead to the finding of genetic markers useful for improved selection of this breed, namely when applied to candidate genes associated with quantitative genetic variation in traits of economic importance. This method relies on the fact that the conformation of a single strand DNA fragment under nondenaturing conditions depends on its exact sequence. Molecules differing by as little as a single base substitution display different conformations that should result in different mobilities in a polyacrylamide gel [2].

In the present study we used a non-radioactive SSCP protocol that allowed the detection of genetic variability at the growth hormone (GH) and α-lactalbumin (α-La) genes in fourty sheep of the indigenous Portuguese "Churra da Terra Quente" breed.

Genomic DNA for polymerase chain reaction (PCR) was prepared from blood through a phenol-chloroform-proteinase K protocol. After amplification of 214 bp (exon 4 of GH gene) and 166 bp (exon 1 of α-La gene) fragments, SSCP analysis was performed by denaturing the PCR products and running the samples in a nondenaturing polyacrylamide gel electrophoresis. The DNA bands were visualized through silver staining.

Our study shows two conformation patterns at exon 4 of GH gene (top) (e.g. lane 1: frequency=72.5% and lane 4: frequency=22.5%) and three patterns at exon 1 of α-La gene (bottom) (e.g. lane 7: frequency=57.5%; lane 8: frequency=22.5% and lane 9: frequency=2%). The frequencies concern 40 animals. The DNA from two animals (5%) showed no amplification at exon 4 of GH gene, suggesting a mutation in the primer region.



The present work describes SSCP polymorphisms for the studied exons. Their association with growth and milk production parameters is being evaluated in order to find molecular markers suitable for molecular assisted selection.

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Clonagem de novos genes xilanolíticos da bactéria anaeróbia *Clostridium thermocellum*

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***Keywords**: Xilanases, genes,* Clostridium thermocellum*, celulossoma*

A degradação da celulose e hemiceluloses em anaerobiose é efectuada por um complexo multi-enzimático, constituido por celulases e hemicelulases e denominado, geralmente, por celulossoma. A dissecção molecular da estrutura do celulossoma do *Clostridium thermocellum* possibilitou a dedução da forma de organização dos agregados multi-enzimáticos secretados por organismos anaeróbios, que tem vindo a ser confirmada para o caso de outras bactéria e fungos. A manutenção da integridade do agregado de biocatalisadores depende da presença de uma proteína que, embora não participando na catálise, actua como estrutura de ancoramento das celulases e hemicelulases. Esta proteína possui, adicionalmente, domínios que permitem a ligação simultânea do complexo à parede celular vegetal e ao organismo. As xilanases são um componente importante do celulossoma, tendo sido até hoje, para o caso do *C. thermocellum*, caracterizadas 3 enzimas a partir da clonagem dos seus genes respectivos: a xilanase Z (Grépinet *et al*., 1988; J. Bacteriol. 170, 4582), a xilanase X (accession number M67438) e a xilanase Y (Fontes *et al*., 1995; Biochem. J. 307, 151). Todas elas constituem enzimas modulares, contendo um domínio catalítico unido por sequências de ligação a mais que um domínio não catalítico. Dentro dos domínios não catalíticos destas enzimas foram identificados domínios de ligação à celulose (xilanase Z e X), e domínios responsáveis pelo aumento da termoestabilidade da enzima (xilanase X e Y). Estudos anteriores desenvolvidos por Morag *et al*. (1990, J Bacteriol. 172, 6098) demonstraram, no entanto, que as enzimas xilanolíticas do *C. thermocellum* são mais numerosas. Pretendeu-se clonar outros genes xilanolíticos a partir de uma biblioteca genómica de *C. thermocellum* construída em lambda ZAPII. Identificaram-se 24 clones com a capacidade de despolimerizar o xilano. Análises de Southern e do perfil de restrição permitiram concluir que 8 dos clones contêm os genes xilanolíticos previamente caracterizados. Nos restantes clones foram identificados 3 novos genes, alargando o número total de genes codificadores de xilanases do *C. thermocellum* para 6. As possíveis razões para a existência de um número tão alargado de xilanases com actividades especificas muito semelhantes num organismo principalmente celulolítico são discutidas.

Organização molecular das celulases e xilanases da bactéria do solo *Cellvibrio mixtus*

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***Keywords**: Celulases, xilanases, genes,* Cellvibrio mixtus

A celulose e as hemiceluloses são os componentes orgânicos renováveis mais abundantes da face da terra. A sua utilização biotecnológica continua no entanto longe de ser atingida, em virtude de um desconhecimento mais ou menos extenso, dos mecanismos moleculares que estão na base da sua degradação. Com o objectivo de compreender qual a estratégia utilizada para a degradação da celulose e xilano pela bactéria do solo *Cellvibrio mixtus* encetámos um projecto que visava a clonagem dos genes codificadores de celulases e xilanases e a caracterização bioquímica das enzimas correspondentes. A partir de uma biblioteca genómica construida em lambda ZapII clonaram-se os genes codificadores de duas celulases, CelA e CelB, três xilanases, XYLA, XYLB e XYLC, e uma glucuronidase, GluA. Procedeu-se à sequenciação dos genes clonados e à análise funcional e estrutural das enzimas respectivas. À excepção da celulase A e da xilanase C, todas as enzimas apresentaram uma estrutura semelhante, contendo um domínio catalítico ligado a um, ou mais, domínios não catalíticos. Os dominios de fixação à celulose identificados nas enzimas do *Cellvibrio mixtus* apresentam elevado grau de homologia com os domínios não catalíticos de outras bactérias aeróbias. A inexistência de evidência molecular que sugira a formação de complexos multi-enzimáticos sugere que as celulases e as xilanases do *C. mixtus* actuam de forma individual durante a catálise. A existência de enzimas com estruturas moleculares muito diferenciadads indica o desenvolvimento de um elevado grau de especialização que potencialmente permite ao organismo tirar partido de um número alargado de substratos, *in vivo*. Os resultados apresentados, bem como os referentes a outras bactérias do solo, permitiram esquematizar um modelo para explicar as estratégias utilizadas para a degradação biologica da celulose e xilanas por microrganismos aeróbios..

The nitrilase of Penicillium chrysogenum

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***Keywords**: Nitrilase,* Penicillium chrysogenum*, DNA probe.*

Nitrilases are enzymes envolved in nitrile degradation converting directly the nitrile in the corresponding carboxilic acid plus ammonia without the intermediate formation of an amide. Nitrilase activity were reported in bacteria [1], fungi [2, 3] and plants [3]. Although involved in nitrile degradation, there are no phylogenic relationships between the nitrilases and the nitrile hydratase enzymes [4]. Belonging to the nitrilase / cyanide hydratase family, a C-N hydrolase superfamily including this family and between other enzymes, the aliphatic amidase of *P. aeruginosa* (NCTC 8602), was reported [5, 6] sharing several signature sequences, one of which contained an invariant cysteine residue acting as nucleophile in the catalytic mechanism [7, 8]. A fungus was isolated from wood and identified using the appropriate taxonomic keys. The colonies diameter determined after 7 days determined at different temperature in Czapek Yeast Extract Agar (CYA), at 25ºC in Malt Extract Agar (MEA) and 25% Glycerol Nitrate Agar (G25N), combined with microscopic observations and the use of glucose, saccharose, galactose, mannose, glycerol as carbon sources or the no growth in sorbose, xylose, maltose, raffinose as carbon sources, indicated a *Penicillium chrysogenum* strain. Although have been reported nitrilase activity in this fungus [3] no further studies were reported. The fungus grew in liquid medium using acetonitrile as sole or alternatively C or N sources. The nitrilase activity [9] was not detected in culture medium suggesting that the enzyme is not excreted. Based on the high conserved DNA sequence for all known fungal nitrilases around the active site Cys residue, a oligodeoxyribonucleotide probe was synthesized, labelled with [γ-^{32}P]dATP and hibridization experiments are being carried out. Attempts to purify the enzyme are in progress using ionic exchange chromatography.

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Screening of specific genetic markers on Serra da Estrela ovine breed

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Keywords: *DNA, microsatellites, polymorphism, ovine, biodiversity*

Conservation priorities concerning domestic animals should attempt the maintainance of maximum genetic diversity, including local breeds that represent irreplaceable source of genetic variation. Portugal offers numerous examples of locally adapted breeds. The study of DNA polymorphism has a wide potential application on livestock improvement as a mean of genetic profile description of different animal populations, their evolutionary relationships, detection of new polymorphic genetic markers and to find linkages of marker *loci* affecting important economic traits. Eucaryotic genome contains several types of repetitive DNA. Some of these repetitive DNAs are highly polymorphic and became excellent genetic markers. One of the most common type of repetitive DNA is stretches of simple nucleotide motifs as mono, di-, tri- or tetra-nucleotide blocks. Among the different motifs, or microsatellites, the TG repeat seems to be the most widely distributed among species and is also the most abudant dinucleotide present in mammals [1]. Microsatellite *loci* analysis is normally carried out by in vitro amplification of the repeat *locus* by polymerase chain reaction, using specific primers complementary to the repeat flanking region.

Studies are being carried out concerning the genetic variability of portuguese "Serra da Estrela" ovine breed. Blood samples were collected from non-parental animals and DNA was extracted by salt precipitation after cell lysis. Genomic DNA from several animals was pooled and completely digested with 3 restriction enzymes. In order to detect the presence of specific microsatellite *loci* we have constructed a genomic library containing the pooled restriction fragments in the range of 200-500 bp. Screening of more than 10 000 clones from the genomic library with $(TG)_{10}$ and $(TC)_{10}$ oligomers is now being performed, using hybridization technics.

DNA sequencing of selected recombinants will lead us to the characterization of the repeats flanking regions and compairison with those from other breeds. With this study, we are expecting to find specific "Serra da Estrela" microsatellite *loci*, that will enable us to precisely fingerprint the portuguese breed.

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Molecular and Sequence relationship of satellite DNA between sheep and goat chromosomes

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Keywords: Capra hircus*, fluorescence* in situ *hybridization,* Ovis aries*, repetitive DNA, satellite DNA*

Two important landraces of Portugal: Churra da Terra Quente (sheep) and Serrana (goat) were studied regarding the repetitive sequences of the higher eukaryote genome - satellite DNA. These two landraces are important genetic resources that must be studied and characterized, because they show excellent adaptabilities in rough environments and abilities for milk and / or meat production.

Several studies provide evidence that some repeat DNA sequences are generally not conserved among mammals and so they become important for evolution considerations (1). The superfamily *Bovoidea*, including sheep, cattle and goat is described as a stable Robertsonian system (2). Comparative studies of sheep and goat species suggest the three metacentric autosomes (chromosomes 1-3) have arisen from ancient centric fusion events (3). Using comparative karyotype analysis, it is suggested that this ancient fusions resulted in the formation of first sheep chromosome 1, then chromosome 3 and finally chromosome 2. Comparative karyotype measurements estimate a 5% loss of chromosomal material from these sheep chromosomes (3). Starting from these data we start our studies with satellite DNA for comparative genomic analysis between our two sheep and goat ladraces.

In this study, fragments from sheep satellite DNA were cloned and sequenced. Southern analysis was used to determine the chromosomal specificity of the cloned satellite sequences. *In situ* hybridization and G- and C-banding were used to study the chromosomal distribution of the sheep satellite DNA on sheep and goat metaphase chromosomes.

Preliminary results from is fluorescent *in situ* hybridization (FISH) with probes representing sheep alpha satellite DNA shows a different distribution of the repetitive DNA families in the two animal species-landraces: Churra da Terra Quente and Serrana. These results suggest the possibility of substantial reorganization of sheep centromeric DNA families after Robertsonian translocations.

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cDNA Cloning and Tissue Distribution of Prolactin in the Sea Bream (*Sparus aurata*)

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Keywords: Sparus aurata*, prolactin, cDNA*

Prolactin (PRL) is a polypeptide hormone secreted in the adenohypophysis of vertebrates. It has a wide spectrum of actions which can be grouped in seven broad categories: reproduction and lactation, water and salt balance, growth and morphogenesis, metabolism, behaviour, immune modulation and effects on ectoderm and skin. In fish, however, the best well documented action of PRL is water and salt balance although in a few species PRL also seems to be involved in reproduction and behaviour.

In order to further elucidate the actions of PRL among teleost fish, prolactin cDNA has been cloned from the sea bream (*Sparus aurata*) and its tissue distribution has been analysed by RT-PCR.

A sea bream PRL cDNA probe (1) was used to screen at high stringency a pituitary cDNA library constructed in UniZAP-XR ($1x10^6$ primary recombinants). A clone which spans 1358bp and encodes a 212aa polypeptide was isolated from the cDNA library. It is highly homologous to other piscine prolactin cDNAs previously isolated: 78% homologous to the European sea bass (*Dicentrarchus labrax*), 72% to Mozambique tilapia (*Oreochromis. mossambicus*) prolactin-I, 63% to Atlantic salmon (*Salmo salar*) and rainbow trout (*Salmo gairdneri*) and 55% homologous to common carp *(Cyprinus carpio*) PRL cDNA.

For RT-PCR, total RNA was extracted from several tissues, namely, pituitary, brain, liver, kidney, gills, skin, intestine, muscle, ovary and testis. The identity of the PCR products was confirmed by Southern blot. PRL was expressed in the pituitary and at extrapituitary sites, mostly the intestine and the ovary.

The tissue distribution of sea bream PRL supports possible roles of the hormone in osmoregulation and reproduction. Further studies to confirm the roles of PRL suggested by these observations are being carried out, including expression of the prolactin receptor.

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Development of a Blocking-ELISA for the detection of antibodies to Maedi-Visna virus envelope glycoprotein gp90

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Maedi-Visna; Lentivirus; Monoclonal antibodies; ELISA

Maedi-Visna virus (MVV) is responsible for a slow progressive disease of sheep. Infected animal develops a degenerative neurological disease (Visna) and a progressive pneumonia (Maedi). Arthritis and chronic indurative mastitis have been also associated to MVV infection. The virus is a member of the lentivirus subfamily of retroviruses. Like the other members of this group, including the human (HIV), primate (SIV), bovine (BIV) and feline (FIV) immunodeficiency viruses, and the caprine (CAEV) and equine (EIAV) lentivirus, MVV induces a persistent infection in the host. Identification of virus carriers in a flock is dependent on serological screening for detection of specific antibodies to MVV.

In this work, we describe the use of a monoclonal antibody (Mab16D9*env*) against viral envelope glycoprotein gp90 to develop a sensitive and specific blocking-ELISA (Blk-ELISA) for the detection of MVV antibodies in sheep. In order to assess the sensitivity of Blk-ELISA, 300 sera (Group I) were obtained from flocks known to be infected with MVV as determined by whole virus indirect ELISA. Group II, consisting on 50 serum samples originating from a MVV-free flock kept in isolation for more than 4 years, were used to check on specificity.

ELISA plates are coated overnight with a purified whole virus preparation of MVV (strain WLC-1) diluted at 1:1000 in carbonate buffer. Serum samples diluted 1:5 are added (50µl/well) and incubated 1hr at 37ºC. Without washing, MAb16D9*env* is subsequently added to each well and the plate is incubated for another hour at 37ºC. Following a four washing cycle a 1:1000 dilution of HRPO labeled rabbit anti-mouse IgG is added. After a 30 min incubation at room temperature (RT) the plate is washed and a mixture of H_2O_2/OPD in citrate buffer is added to each well. After a 20 min incubation at RT the reaction is stopped and the absorbance was read at 492 nm. Duplicate positive (PC) and negative (NC) sheep serum controls were included in each plate. The test was validated if optical density (OD) of NC was □ 0.5 and the blocking effect of PC □30%. The blocking percentage of the sample was calculated as follows: % sample = OD NC - OD sample / OD NC - OD PC x 100. Samples presenting a blocking percentage □30 were considered positive. Samples with a calculated blocking percentage □20 were negative and those presenting a blocking percentage □20% but □30% were considered doubtful. This method was compared with a standard whole virus indirect monophasic ELISA (I-ELISA). The number of positive samples detected in Group I by I-ELISA was 199 whereas 228 were positive in Blk-ELISA. The two tests disagree in 29 samples of which 22 were positive in Blk-ELISA and negative in I-ELISA. The remaining 7 samples showed the opposite result. The 29 conflicting samples were tested on immunoblot and 20 out of 22 positive in Blk-ELISA were confirmed as positive. The remaining 9 samples, 2 positive and 7 negative were not confirmed. A total of 20 samples were doubtful in I-ELISA while only 7 samples gave such result in Blk-ELISA. All sera in Group II were negative by both methods.

These results suggest that MAb16D9*env* binds an epitope on the viral envelope glycoprotein which is also recognized by the large majority of naturally and experimentally MVV infected sheep. This MAb proved to be a valuable reagent in the development of this highly specific and sensitive ELISA which is helpful tool for epidemiological surveys on MVV infections. This ELISA also allows qualitative studies on the sheep immune response to viral envelope glycoprotein gp90.

Molecular Cloning of cDNA's Involved in the Synthesis of β-Carotene in the Green Algae Dunaliella salina

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Keywords: *Dunaliella, Beta-carotene, Phytoene Desaturase (Pds), Phytoene synthase (Pys)*

Carotenoids form a family of pigments widely distributed in nature. They are essential for the protection of both photosynthetic and non-photosynthetic tissues from photooxidative damage. In addition, carotenoids serve as precursors for abscidic acid in plants and for vitamin A in animals, and they play an important role in preventing cancer in humans, especially β-carotene. They are synthesized by all photosynthetic organisms as well by several non-photosynthetic bacteria and fungi.

Although carotenoid biosynthesis has been studied in many organisms from bacteria to higher plants, little is known about carotenoid biosynthetic enzymes, or the nature and regulation of the genes encoding them. This is because these enzymes are immediately inactivated by separation from a membrane environment, thus preventing their purification and subsequent cloning of the genes encoding them.

Dunaliella salina is a green algae (Volvocidae) which is known for her halotolerance and remarkable capacity of accumulate β-carotene (up to 10% in dry weight), under specific culture conditions.

We report in this papper, the first DNA sequences from *Dunaliella salina*, related to the enzymatic pathway of β-carotene synthesis. In fact, Phytoene Desaturase (Pds) and Phytoene synthase (Pys) are the two major enzymes involved in the first steps of β-carotene metabolism in higher plants.

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Characterization of a new *E. coli* gene involved in the modulation of RNase II activity

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Key words: E.coli*, mRNA decay, exonucleases, activity modulation.*

The control of gene expression in prokaryotic systems is mainly determined by the rate of mRNA turnover. The stabilization of certain transcripts can lead to an increase of protein production. This is important for the overexpression of proteins relevant for industry. In *Escherichia coli* mRNA decay results from the concerted action of endoribonucleases and exoribonuclesases. RNase II is a processive exoribonuclease that irreversibly hydrolyses mRNA to 5'-monophosphates [1]. Its activity is sequence-independent but sensitive to RNA secondary structures [2,4].
RNase II as been pointed as the ribonuclease responsible for the majority of the exonucleolytic activity in *E.coli* extracts [3].
We have sequenced and identified a new open reading frame (orf) in *Escherichia coli*. Results obtained with a parcial deletion strain indicated that this orf seems to have an effect on the activity levels of RNase II . *In vitro* assays of the decay of a labeled transcript corresponding to *mal*E-*mal*F intergenic region [4] revealed a higher exoribonuclease activity in extracts of the orf deletion strain when compared with the wild type strain. Western analysis confirmed higher levels of RNase II in the orf deletion strain.
Sequence analysis of the orf indicates that its coding region encodes an hypothetical 67,7 kDa protein of unknown function. No significative homologies were found with sequences of known proteins.
The predicted protein size was confirmed by SDS-PAGE analysis using the pET-based expression system under the control of an inducible T7 promoter. The results concerning the RNase II activity raised the possibility of this ORF acting as a modulator of RNase II expression. Further studies to analyze the expression and purification of this protein are in progress.

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The Arginine Repressor of *Streptomyces clavuligerus* Positively Regulates Clavulanic Acid Production

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Key words: Streptomyces clavuligerus*, arginine,* arg*R, regulation, clavulanic acid*

We have reported the cloning of *arg*R a gene regulating arginine metabolism in *Streptomyces clavuligerus.*(1) Transformants containing the *arg*R gene in a high copy number plasmid showed lower activity (50%) of the biosynthetic ornithine carbamoyltransferase activity and higher levels (380%) of the catabolic ornithine aminotransferase activity than control strains.
Arginine is precursor of the β-lactamase inhibitor clavulanic acid. Arginine stimulates clavulanic acid production when added to the media and amplification of the biosynthetic *arg*G gene results in a 2.3-fold increase in the production of clavulanic acid (CA) in relation to the control strain (2). Nevertheless amplification of the repressor gene *arg*R causes higher (200%) clavulanic acid production rather than the expected decreased production. Furthermore *arg*R disrupted mutants showed lower production than the wild type strain.
ArgR was purified to homogeneity from an overexpressing strain with pT7 system. The monomer has a molecular weight of 17 kDa and the funtional protein behaves like a hexamer in a crosslinking experiment and gel-filtration cromathography.
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Estudio de la regulación de la expresión de los genes de lacasa en *Coriolopsis gallica* con vista a optimizar su producción en cultivo sumergido

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Palabras claves: lacasas, basidiomiceto, regulación de la expressión, ADNc, análisis Northern.

La lacasas pertenecen a un grupo de enzimas del tipo fenoloxidasa que contienen varios átomos de cobre en su estructura. Esta enzima ha cobrado real importancia biotecnológica por su capacidad para catalizar la oxidación de una serie de compuestos fenólicos altamente contaminantes. Los estudios bioquímicos y moleculares se han llevado a cabo principalmente en hongos basidiomicetos, clonando y secuenciando los genes que la codifican y obteniéndose los respectivos ADNc. Se ha encontrado una gran homología entre los productos de los genes que codifican para esta proteína, como son las uniones a cobre y a hidratos de carbono, no obstante los mecanismos fisiológicos que están asociados a la expresión de estos genes están aún poco claros. Se han encontrado dos genes que codifican para lacasas en *Coriolopsis gallica*, los que según estudios previos se expresarían en forma diferencial. El presente trabajo tiene como objetivo estudiar la influencia del tipo y la concentración de fuente de carbono y nitrógeno en la expresión de un gen, *lcc*1, que codifica para una lacasa mediante la técnica de Northern blot, con el fin de optimizar la producción de esta enzima en cultivo líquido

El micelio obtenido luego de crecer el hongo durante diez días en cultivo líquido fue inoculado en medios que contenían como fuente de carbono glucosa o sorbosa y como fuente de nitrógeno tartrato de amonio o nitráto de amonio y en un sistema libre de azúcares y de fuente de nitrógeno. Se determinó la actividad lacasa para cada cultivo por un periodo de cinco a siete días utilizando como sustrato ABTS (ε 29300), los azúcares fueron determinados por cromatografía de gases. El micelio obtenido de cada día fue liofilizado y su RNA extraído y cuantificado. 10 ug de RNA total correspondiente a cada día de la cinética fueron cargados en un gel de agarosa 1,2% - formaldehído y corridos a 25 V por 15 hrs para luego ser transferido a Hybon N (Nylon) e hibridado con el cDNA de *lcc*1 marcado con dCTP-32.

Las máximas actividades de lacasa fueron obtenidas cuando no se agregó ninguna fuente de carbono, ni nitrógeno, por otra parte en las cinéticas que contenian una fuente inorgánica de nitrógeno se observó una clara disminución de la actividad lacasa en los medios, siendo esta igual a cero cuando se aumentó la concentración de la sal diez veces. Con respecto a la fuente de carbono se observaron diferencias sustanciales cuando se utilizó sorbosa en vez de glucosa, siendo las actividades obtenidas por la primera muy semejantes a las de un medio que no tiene fuente de carbono. El análisis Northern muestra una clara diferencia en la transcripción del gen *lcc*1, y esta diferencia está relacionada con la fuente de carbono y con el tipo y cantidad de la fuente de nitrógeno, lo que hace suponer que la transcripción de este gen está regulada por la disponibilidad de estos compuestos en el medio extracelular.

Possible Involvement of Muramidase-1 and PBP1 in the Lysis and Killing of *Enterococcus hirae* Mutant Induced by β-Lactams Antibiotics

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The *Enterococcus hirae* ATCC 9790 and its autolytic defective EC31 mutant were tested for their susceptibility to β-lactams antibiotics. The mutant was inhibited and killed slower than the parent strain by penicillin G and ampicillin when each of these antibiotics was added at 10 times the MIC to both strains. SDS extracts of EC31 strain revealed undetectable levels of the latent form of muramidase-1 in sodium phosphate renaturation buffer. Besides, mutant cells a much reduced rate of cellular or cell wall autolysis than that of parent strain. In addition, the rate of cellular autolysis was not stimulated by the presence of trypsin but showed a substantial increase in the presence of sodium deoxycholate or Triton X-100. On the other hand, radiolabelled penicillin G used to examine penicillin-binding proteins (PBPs) from the mutant, bound to only five proteins from this strain producing marginally detectable (or undetectable) PBP1 in comparison with the six PBPs from the parent strain. This suggests that the occurrence in small amounts (or lack) of PBP1 may also contribute to the low susceptibility of this mutant to β-lactams antibiotics. To investigate whether this correlation would hold true, we analysed the PBPs of another autolytic defective mutant which also possesses a mutation in muramidase-1. This mutant displayed an identical PBP profile to the parent strain, i. e., did not lack the PBP1 and revealed a very fast kinetics of lysis and killing induced by these antibiotics. The EC31 mutant grew with a slower generation time than the parent strain and, as revealed by electron microscopy, the cell separation was severely affected with formation of chains. These results suggest that the small rate of lysis and killing by these antibiotics may be associated with the mutation of muramidase-1 and/or with the overproduction of its inhibitor. Further analysis supports the idea of a relevant involvement of PBP1 in the low susceptibility of enterococci to β-lactams antibiotics.

Gene organization and expression in oenophage fOg44 : the central genomic region encoding lytic, integrative and dispensable functions

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Oenococcus oeni *phages; lysogeny; integrase; lytic enzymes; gene deletions*

Current interest in bacteriophages of lactic acid bacteria (LAB) is centered on the exploitation of their integrative functions, for the construction of useful plasmid vectors for the genetic analysis and engeneering of their hosts, as well as on the characterization of lytic enzymes produced during the infeccious cycle, for which several biotechnological applications have recently emerged (see Gasson, 1996).
In the process of winemaking, the addition of lysozyme to the fermenting must has been proposed to avoid the growth of LAB, in those cases (such as white wines) where malolactic fermentation is detrimental to the quality and flavour of the final product. In this context, the substitution of egg-white lysozyme for endogenous lytic enzymes from relevant LAB species or their bacteriophages could be a better alternative, probably more effective, and certainly "more ecological". Eventually, the introduction of genes for such enzymes in appropriate yeast strains would allow the prevention of LAB growth, *pari passu* with alcoholic fermentation.
Recent work in our group has been concerned with the molecular study of *O.oeni* bacteriophages and the characterization of phage-host interactions [2,3]. Here we provide evidence for the localization of integrative and lytic genes in a central area of the phage fOg44 genome.
The complete nucleotide sequence of a central *Eco*RI fragment of fOg44 DNA has been determined for both DNA strands by the chain termination method of Sanger. Within this 6226 bp fragment, seven open reading frames (*orf*s) were detected, all preceded by a standard Shine-Dalgarno sequence (RBS). Two of these (*orf*217 and *orf*252, of unknown function) are missing in the deletion mutant fOg44 *del2*. The deletion in this derivative originates from a recombinatory event between direct repeats present downstream the holin gene (*orf*117) and at the putative -10 promotor region of a presumptive excisionase gene (*orf*72).
The product of the first gene in the sequenced fragment (*lys*) exhibits a high degree of similarity with bacteriophage lytic enzymes, including the presence of a C-terminal repeated stretch of 43 residues, homologous to peptidoglycan-recognizing elements of various cell-wall binding proteins. Although we observed the presence of a hydrophobic domain in its N-terminal region that could function as a secretion signal, the presence of a putative holin gene downstream of *lys*, argues for an export mechanism dependent on the formation of membrane holin pores. Northern-type experiments have indicated that *lys* and *hol* are co-transcribed, in a late stage of the infection cycle. The results are also compatible with the additional synthesis of holin-specific mRNA.
ORF72 and ORF364 are suggested to correspond to the fOg44 excisionase and integrase respectively. The genetic organization *xis-attP-int* deviates from the usual *attP-int-xis* sequence. In order to investigate the sequence requirements for integrase binding to the phage attachment region (*attP*) the *int* gene has been cloned under the control of a T7-promotor in plasmid pRSET-A (Invitrogen) and introduced in *E.coli* BL21(λDE3, pLysS). The overproduction of the expected 43kDa product, following induction with IPTG, was confirmed by electrophoresis of protein extracts on SDS-PAGE gels and immunoblotting. A similar approach to the production of phage lysin is currently beeing attempted.

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Clonaje, Caracterizacion y Análisis de la Regulación Diferencial de la Familia de Genes de Lacasa Presentes en el Basidiomiceto *Trametes sp.* CECT 20197 con Alta Actividad Ligninolitica

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La industria presenta cada vez un mayor interés por las enzimas extracelulares producidas por los hongos basidiomicetos, como es el caso de las lignina y manganeso peroxidasas y las lacasas, debido a su potencial para degradar compuestos altamente tóxicos y del tipo lignina. Estas enzimas encuantran aplicación en la industria de pulpa y papel, producción de alimento animal y protección del medio ambiente, como es la descontaminaron de distintos tipos de residuales industriales, bioremediacion, mejoramiento y recuperación de suelos. La caracterización de estas enzimas y de los genes codificantes para las mismas ayudan a comprender los mecanismos catalíticos y genéticos de este sistema oxidativo tan complejo. En el trabajo se presenta el aislamiento, clonaje y caracterización de tres secuencias de ADN genómico: *lcc*1, *lcc*2 y *lcc*3, codificantes para tres isoenzimas de lacasas, sugiriéndose de esta manera que parte de la diversidad bioquímica de las isoenzimas de lacasas detectadas en este trabajo por métodos de SDS-PAGE e isoelectroenfoque, se debe a la multiplicidad genómica de sus genes. El hongo *Trametes sp.* CECT 20197 degrada eficientemente la lignina de diversas maderas, bagazo de cana de azúcar y paja de trigo, al cultivarlo bajo diversas condiciones, además de presentar resultados significativos en relación a la remoción de color en residuales de la fermentación alcohólica, al reducir en un 67% el color inicial en 5 días de cultivo. En el trabajo se demuestra mediante análisis Northern, que la expresión de los tres genes de lacasas previamente identificados y clonados, se encuentran regulados diferencialmente. Dos de ellos son sensibles a la inducción por alcohol veratrilico: el gen *lcc*1 se induce en las etapas iniciales de cultivo, y el gen *lcc*2 solamente cuando el hongo alcanza la fase estacionaria de crecimiento. En tanto que el tercer gen, *lcc*3, aparece claramente regulado por fuente de carbono, encontrándose bajo represión catabólica al crecer el hongo en un medio con glucosa como fuente de carbono.

Study of regulation of catabolite sensitive promoter of udp gene from *Escherichia coli K-12*. Cloning of udp genes from *Salmonella typhimurium* and *Klebsiella aerogenes*. Designing and investigation of mutant promoters of udp gene.

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***Keywords**: E.coli, S.typhimurium, Kl.aerogenes, udp gene, mutant promoters*

Udp genes from *Salmonella typhimurium* and *Klebsiella aerogenes* were cloned. Nucleotide sequences of these genes were determined (EMBL: Y13360 and Y13414 respectively) and high homologous of the promoter-operator regions of the udp genes from *E.coli*, *S.typhimurium* and *Kl.aerogenes* was shown. Structure-functional organization of the promoter-operator region of the udp gene from *E.coli* was studied.

The series of plasmids with different mutant promoters of the udp gene from *E.coli* were constructed by site-direct mutagenesis. The influence of mutant promoters on the level of the udp gene expression was examined. At least two promoters of the udp gene (P1 and P2) on the DNA fragment between metE and udp genes were discovered. The start point of transcription from the promoter P2 localized at -170 concerning the start point P1. The maximum level of expression was obtained from DNA fragment bearing two udp promoters (P1 and P2). The level of expression from promoter P2 was approximately 15% from maximum. The 16bp palindrome-structure was discovered on the investigated fragment. The decreasing (10%) of expression of udp was observed when promoter region contains this palindrome.

The study of udp gene promoter P1 permits to conclude that minimal promoter activity (40% from maximum) displayed by the DNA fragment from -70 nucleotide. Our investigation showed an essential role of the region around of -60 nucleotide (similar to UP-elements of promoters *E.coli*) in the transcription activation from P1. The level of the udp expression constituted 80% from maximum (P1 and P2) if the promoter fragment included the CRP2-site (-94).

Mutant promoter-operator regions of the udp gene were used in constructing of series vectors for geterologic genes expression in *E.coli*.

The Effect of Endogenous Nucleases Present in *E. coli* Lysates on the Stability and Purification of Plasmids for Gene Therapy and Molecular Biology Applications

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***Keywords**: Gene Therapy, Plasmid DNA, Nucleases*

Gene therapy is a promising process for the prevention, treatment and cure of diseases like cancer, acquired immunodeficiency syndrome (AIDS) or cystic fibrosis. While the use of genetically modified viral systems presents some problems and raises safety concerns, formulations of genes inserted in plasmids are regarded as safer and can be used much like a conventional pharmaceutical [1]. Since the efficacy and duration of gene expression in the target tissues is presently relatively low, this mode of treatment requires repeated treatments and therefore considerable amounts of pharmaceutical-grade plasmid DNA [1].

Plasmids for gene therapy and molecular biology applications are usually produced in *E. coli* by fermentation and isolated by a variation of the alkaline lysis procedure [2]. From here on, some of the most troublesome contaminants are RNA and chromosomal DNA, usually removed by successive unit operations such as precipitation, ion-exchange [3] and gel filtration [4]. Another important issue in the downstream processing, although scarcely referred in the literature, is the stability of the plasmid in the various process streams. This work describes the action of endogenous nucleases over host nucleic acids and plasmid and demonstrates how it can affect the purification of plasmids for gene therapy and molecular biology.

Plasmid pMa5L containing the gene for a cutinase of *Fusarium solani pisi* and hosted in *E. coli* (strain WK6) was used as a model system. Cells were grown in Terrific Broth in a 4 L fermentor. Plasmid production was induced by shifting the growth temperature at mid-exponential phase from 37 ºC to 42ºC. Cells were harvested at late exponential phase (OD_{600} >30) by centrifugation and lysed by the alkaline method [1]. After the final precipitation step in the lysis procedure with potassium acetate, cleared lysates were stored at 4ºC. Aliquots of lysate were then incubated at different temperatures (22 - 90 ºC) for different periods of time. Samples were then centrifuged, precipitated with 70 % (v/v) isopropanol and analysed by electrophoresis on a 1.5 % agarose gel. Restriction analysis were also performed with *Eco* RI. The enzymatic action of endogenous nucleases over cDNA and RNA was demonstrated. Supercoiled plasmid was degraded to ´nicked´ variants under some temperature conditions and completely digested at high temperature (90 ºC).

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Single-Strand Conformation Polymorphisms Detected in Lactoprotein Genes from Algarvia Goat Breed. Association with Quantitative Traits

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Single-strand conformation polymorphisms (SSCPs) is a powerful method for identifying sequence variation in amplified DNA. SSCP analysis of DNA have been used for detection of genetic mutations in humans (Orita *et al.*, 1989), rats (Pravenec *et al.*, 1992), cattle (Kirkpatrick, 1992) and in various bacteriological (Morohoshi *et al.*, 1991) and viral (Fujita *et al.*, 1992) systems.

The search for SSCP polymorphisms could lead to the finding of genetic markers useful for improved selection of agricultural populations, namely when applied to candidate genes associated with quantitative genetic variation in traits of economic importance.

Most significant studies using the SSCP approach were accomplished on bovines in linkage analysis (Neibergs *et al.*, 1993) and to define intragenic haplotypes at the growth hormone (Lagziel *et al.*, 1996).

The present study is part of a genetic improvement programme of the indigenous Portuguese caprine breed Algarvia. Algarvia goats are reared mainly in the Algarve region where they are very well adapted to dry rural areas, and play an important role as an economic resource to the agricultural populations. This breed whose origin is unknown has as its main function milk production which is used for cheese manufacture. This work describes some SSCP polymorphisms at the αs_1-casein, β-casein, κ-casein and α-lactoalbumin genes from 77 goats belonging to the indigenous Portuguese caprine breed Algarvia and present preliminary results towards the establishment of an association of those polymorphisms with milk yield, and fat and protein content.

We have chosen the exon 1 of the α-lactoalbumin gene, the exon 7 of theβ-casein gene, the exon 4 of the κ-casein gene and the exons 9 and 10-11 of the αs_1-casein gene for the SSCPs analysis. The analysis of the amplified fragments by the PCR method using the primers is described in table 1 is shown in Fig.1 and in Fig.2. Their lengths correspond to those expected according to the position of the primers deduced from the described nucleotide sequence of the corresponding genes from *Capra hircus.* Considering the seventy-seven Algarvia goats the shown SSCPs polymorphisms were found inside each of those fragments (Figs. 3 and Table 2). The exon 4 of the κ-casein gene was monomorphic (Fig. 4) Three alleles were present in the exon 1 of the α-lactoalbumin gene. Two alleles each were present in exons 9 and 10-11 of the αs_1-casein gene and in exon 7 of the β-casein gene. The frequencies found for homozygous and for heterozygous animals for these alleles are shown in Table 2.

We have made a preliminary attempt towards the search for associations between the SSCP polymorphisms and quantitative variation in the productive traits described below. Tables 3, 4, 5 summarise the results obtained using the SAS programme applied to the analysis of linear contrasts of SSCPs relating to milk production, protein and fat content for each exon analysed.

Evidence of the existence of a nitrile hydratase in *P. aeruginosa* 8602

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***Keywords**: Nitrile Hydratase,* P. aeruginosa*, PCR, DNA sequencing.*

Nitrile Hydratase (NHase) are enzymes involved in nitrile degradation process, converting the nitrile into the corresponding amide. Usualy this amide is converted into the corresponding carboxilic acid plus ammonia through the action of an amidase. Several NHase were described [1-5, 8] being composed by two subunits (alpha and beta), containing one metal atom per alpha / beta unit. In *Pseudomonas* genus, NHase have been reported from a number of different species, e.g. *P. putida* [3], *P. chlororaphis* [4], *P.aeruginosa* SCN1 [5]. In *P. aeruginosa* strain (NCTC 8602), although it posses an aliphatic amidase [6,7], with high sequence homology with the aliphatic amidase of *Brevibacterium* R312 [8], (which posses also a NHase), no NHase activity was reported. In order to test the existence of a NHase in *P. aeruginosa* 8602, the bacteria was grown in minimal medium with trace elements and acetonitrile as C and N sources. Bacterial growth suggest that this strain could use acetonitrile as C and N sources. Based in this result, reverse and forward primers were synthesized for each subunit based in selected conserved regions among the described α and β subunits of the microbial NHases. A PCR reaction was setted up and the analysis of PCR products by agarose gel electrophoresis showed bands with the expected size. PCR products were cloned and partially sequenced. Preliminary results showed high amino acid sequence homology with *Rhodococcus* NHase, suggesting the presence of a NHase like gene. Sequencing studies are still in progress.

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Applied Physiology and Microbiology

Effect of Cu^{2+} on ethanol production in *Saccharomyces cerevisiae*

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Keywords: Saccharomyces cerevisiae*, copper, ethanol production*

Copper plays an important role in the normal growth, development and function of living organisms [1]. However, at excessive concentrations this metal exhibits toxic effects [2].
The effect of copper(II) concentration on glucose fermentation in *Saccharomyces cerevisiae* is not known and it constitutes the aim of this and further work.
When *S.cerevisiae* cells were grown in YNB, 2% glucose, in the presence of different medium copper(II) concentrations it was observed that concentrations up to 0.1 Mm did not affect the regular growth of cultures, whereas, for 0.5 and 1 mM concentrations, the cultures grew much slower, especially for 1 mM of copper(II). These results confirmed that *S. cerevisiae* cells are very tolerant towards copper(II).
A number of evidences indicate that the concentration of copper in the cell is regulated at the level of plasma membrane transport, as coppers enter the cell using a plasma proteins and then binds to a transport protein before being deliver to protein targets [3]. The total copper(II) (extracellular + intracellular) was determined during growth, using acid digestion followed by Atomic Absorption Spectrometry. As expected, the amount of copper binding is dependent on the concentration of copper in the medium and reaching the maximum after a period of 3 hours. The intracellular copper was determined as the copper not accessible to a 15% HCl solution in 1.2 M sorbitol. The quantification of the intracellular copper turned out to be very inaccurate because the levels found were very low (close to the detection limits of EA-AAS). Nevertheless, it can reliably be noticed that the intracellular copper is a minor contribution to the total copper found associate with the cell.
Fermentation experiments were performed using the mentioned above culture medium but enriched in glucose (100 g/L), and were monitored by the loss of mass of CO_2 which was checked (Ionic Exclusion Chromatography) to be a suitable indicator of ethanol production. The results showed that up to concentrations of $5x10^{-4}$ M of copper(II) the amount of ethanol produced increased with copper (II) concentration and for $1x10^{-3}$ M a slight decrease was observed. For $|Cu(II)|= 5x10^{-4}$ M the ethanol production was about the double of that obtained with no copper addition.

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Loss and Emergence of Mucoidy in *Pseudomonas aeruginosa*: Pattern of Changes in the Levels of Enzymes and Transcription of Genes of Alginate Synthesis

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Keywords: Pseudomonas aeruginosa*, cystic fibrosis, alginate, alginate genes and enzymes*

Pseudomonas aeruginosa isolates from cystic fibrosis (CF) patients often differ from environmental, or other clinical isolates, due to the mucoid phenotype of their colonies which derives from the production of high concentrations of the exopolysaccharide alginate. Mucoid strains that chronically colonize the lungs of CF patients may evolve in the CF respiratory tract from initial non-mucoid strains. Prolonged stress conditions, such as suboptimal nutrition or antibiotic stress, can also induce *in vitro* a genotypic switch to alginate production. However, the cultivation of CF mucoid isolates, under optimal conditions, gives rise, spontaneously and at a high frequency, to non-mucoid variants [1].

The low activity levels of the four GDP-mannuronic acid (GDP-Ma) forming enzymes, even in highly alginate producing *P. aeruginosa* strains, have made difficult to attribute the loss of alginate production in non-mucoid variants to a loss or decrease of a specific enzymatic activity. We have compared, using optimized conditions [2, 3], the level of GDP-Ma forming enzymes (phosphomannose isomerase, PMI; phosphomannomutase, PMM; GDP-mannose pyrophosphorylase, GMP; and GDP-mannose dehydrogenase, GMD) in three non-isogenic mucoid CF isolates [4], in the respective non-mucoid spontaneous variants and in mucoid variants which emerged during extended incubation of these non-mucoid variants under nutrient limitation. The pattern of transcription of the enzymes encoding genes, accompanying the loss/acquisition of mucoidy, were also compared. The transcriptional activation of the *algD* (encoding GMD) and *algA* (encoding the PMI-GMP activities [5]) genes (in the same operon) in mucoid variants was confirmed and no transcripts homologous to *algA* or *algD* were detected in non-mucoid strains. However, *algC* gene transcripts were detectable in both mucoid and non-mucoid cells, with higher levels in the mucoid forms. The *algC* encodes PMM and is located outside the referred alginate biosynthetic operon. Consistent with the concept that the step catalysed by GMD is a key step in the control of the alginate pathway [1], GMD was not detected or showed negligible values in non-mucoid strains. Surprisingly, PMI-GMP activities in non-mucoid strains were only slightly below the values in mucoid variants, as found for PMM. We propose that the stimulatory effect on PMI-GMP activities exerted by *algC* overexpression is one of the post-transcriptional mechanisms that might underly these intriguing relatively high values of PMI-GMP in non-mucoid cells. This observation, taken together with previous results showing the increase of PMM activity when PMI-GMP protein was overexpressed [5], reinforce the hypothesis that the two proteins may exist as an enzyme complex, thus providing the channeling of fructose 6-phosphate to GDP-mannose; this may overcome the constraints of extremely low levels of biosynthetic enzymes even in strains that produce such large quantities of alginate.

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Influence of k_La on Bioconversion af Rice Straw Hemicellulose Hydrolysate to Xylitol

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Keywords: xylitol, rice straw, hemicellulosic hydrolysate, xylose, Candida guilliermondii

Lignocellulosic materials, such as rice straw, represent abundant and inexpensive sources of carbohydrates (cellulose and hemicellulose) with potential application in diverse bioconversion processes. Xylose, the predominant sugar in the hemicellulose fraction of this biomass, can be directly converted to xylitol by biotechnological routes using yeasts. Xylitol, a five-carbon sugar alcohol as sweet as sucrose, has several industrial applications. As a sweetening agent, xylitol has been used in human food since the 1960s. It is also employed as a sweetener in the diabetic diet due to its largely insulin-independent metabolism. Moreover, the most notable property of xylitol is that it prevents the formation of dental caries, as demonstrated in repeated clinical and field studies. Thus, xylitol has become a product of high value, particularly for third-world countries where the incidence of dental caries is very high. Studies of xylitol production by fermentative process have advanced greatly in the last year, principally those concerning the use of hemicellulosic hydrolysates obtained by acid hydrolysis from agricultural and forest residues. The fermentative process for xylitol production is economically interesting, since it does not require xylose syrup purification as does the chemical process. However, the success of the fermentative process for the production of xylitol depends on the productivity of the strain and its tolerance to different chemical substances existing in the hydrolysates. In addition, a number of culture process parameters, such as pH, medium composition and aeration level, proved to have significant effects on xylitol production in hemicellulose hydrolysate media. One of the most important control variables in this bioconversion is the aeration level, which affects the biochemical pathways involved in the xylose metabolism In this work the influence of oxygen transfer rate (K_La) was evaluated in order to maximize the production of xylitol by the yeast *C. guilliermondii* FTI 20037. Rice straw hemicellulose hydrolysate obtained by acid hydrolysis was employed as a xylose-rich medium. The fermentation runs were carried-out in a 1-litre Multigen stirred-tank reactor (New Brunswick, Scientific Co., Inc. Edison, NJ, USA) containing 0.55 litre of rice straw medium. The fermenter was equipped with three six-bladed turbine impellers. The temperature was maintained at 30 C and agitation speed at 300 rpm. Air flow rate varied from 0.5 vvm to 1.7 vvm in order to obtain different initial k_La values (coefficient of oxygen transfer). Samples were taken periodically for determination of sugars, xylitol by HPLC and biomass concentrations by means of a calibration curve (dry weight x optical density at 600 nm). The results showed that this bioconversion strongly depended on the aeration rate. The maximum volumetric productivity (0.52 g litre^{-1} h^{-1}) and the highest xylitol yield (0.73 g g^{-1}) were achieved after 70 h at an oxygen transfer coefficient of 15 h^{-1}. Under these conditions 80% efficiency in relation to theoretical yield was attained.

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Analysis of D-carnitine in L-carnitine Microbial Production

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Key words: D-carnitine, D-carnitine dehydrogenase, tetrazolium salts.

L-carnitine is an important pharmaceutical compound that it is obtained by racemic resolution of D,L-carnitine with chiral salts. This method produce D-carnitine as a raw material, and then a procedure to recycle this D-carnitine into L-carnitine would be of industrial interest.
On the other hand it is well known the use of microorganisms as biocatalyst. A wide number of strains of the genera *Agrobacter*, *Escherichia* or *Proteus* were found to be useful for L-carnitine production [1]. For example, we have studied a bioreactor with immobilized *E. coli* which achieves high L-carnitine productivity values [2]. However, there is no adequate analytical procedure available for D-carnitine determination useful to follow the performance of the biotransformation of D-carnitine into L-carnitine.
The present work deals with an analytical method for D-carnitine measurements with a novel enzyme, D-carnitine dehydrogenase. This enzyme has been purified from cultures of *Agrobacterium sp.* and characterized (optimum temperature: 37 ºC; optimum pH for the oxidation: 9.0-9.5; K_M (NAD^+): 0.15 mM; K_M (D-carnitine): 5.5 mM) [3].
The analysis method has been set up by measuring the activity of D-carnitine dehydrogenase with NAD^+. These results were compared with two colorimetric assays based on the reduction of tetrazolium salts (iodonitrotetrazolium chloride, INT; 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, MTT) in presence of phenazine methosulfate (PMS). The coupling of NAD-dependent dehydrogenases reactions with the reduction of tetrazolium salts was particularly advantageous because of the relatively high absorbance of the formazan increases the sensitivity and avoid feasible interference caused for UV detection. Optimum conditions for the assays as pH buffer, wavelength, PMS, INT or MTT concentrations, or linear D-carnitine range of response were studied. The assay with MTT showed the best standard curves for D-carnitine determination. The linear relationship was reached up to about 5 mM.
This enzymatic method has been tested under two different situations: the profiles of D-carnitine consumption on *Agrobacterium sp*. growth curves, and the progress of the bioconversion of D-carnitine into L-carnitine with a transformed *E. coli* strain. In both cases, the results were in agreement with those expected.

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Mycelial Growth and Laccase Production Conditions from *Rhizoctonia solani*

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Keywords: *laccase,* Rhizoctonia solani*, bioreactor.*

The fungus *Rhizoctonia solani* is a pathogen on many plant species, including forests, fruit trees and horticultural plants (1). It is believed to cause a 20% decrease in worldwide agricultural production. As a result of this characteristic, and considering also the reported identification of laccase in strain RS22 (2), increasing interest on this fungus as ligninolytic enzyme producer and on its environmental uses has arisen.

Particularly, the laccase production has been studied because its potential to degrade both highly toxic phenolics and lignin (3). The strain S2 Albacete (identified by CNB) has been chosen, among many others from different anastomosis groups, for its higher production of laccase when grown at room temperature in Petri dishes containing a rich media. At this experimental phase, 200 activity units (A.u.) were obtained.

Subsequently, mycelial growth and enzyme production were studied on 1l baffled flasks containing a rich media with high contents of yeast and malt extracts. The results indicated that yeast extract must be part of the media while malt extract might be supressed. The effect of pH media on enzyme production was also considered; laccase production worked optimally at acidic pH -5-. Finally, among the different sources of nitrogen tested, the highest production was obtained using NO_3^- as nitrogen source.

Several batch fermentations were carried out in a lab scale bioreactor (Bioflo III, New Brunswick) at different operation conditions. Thus, at the agitation level of 400 r.p.m., air flow rate of 1vvm, and temperature of 25ºC, 1.200 A.u. were obtained 75 hours after inoculation. At the maximum production level, the enzyme was purified for later characterisation.

The ulterior objective of the present work is to reduce the colour content in wastewater effluents from textile industries using *Rhizoctonia solani* strain which produces high levels of laccase.

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Induction of Xylanolytic Activity by *Aureobasidium pullulans* Using Xerographic Paper

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Keywords: Aureobasidium pullulans*; xylanase; xerographic paper; de-inking.*

One of the possible applications of hemicellulolytic enzymes, namely xylanases, in the pulp and paper industry is at de-inking process of wastepaper, one of the critical steps in the recycling process. Enzymatic de-inking is likely to be of industrial use, since it is less costly than chemical deinking, and it is more efficient.

Aureobasidium pullulans has previously been shown to produce extracellularly a cellulase-free endo-β-1,4-xylanase of extremely high activity that might allows the use of the culture supernatant without further purification and/or concentration for the industrial enzymatic hydrolysis of xylan or xylan-containing materials. However, the commercially-available arabinoxylan (one of the best inducers of xylanase activity by *A. pullulans*) is too expensive to allow its use for any industrial enzymatic production.

Then, the aim of this work was to investigate the effectiveness of using cheaper culture media for xylanase production by the color variant-derivative strain of *A. pullulans* NRLL Y-2311-1. In this sense, we used xerographic paper as an alternative inducer, which provided an induction of xylanase production (up to 170-fold increase). These cultures were grown with glucose, sucrose or lactose as the carbon source. To minimize the cost of enzyme production, we have also used agro-industrial residues (whey, carob syrup, molasses and wine must) as alternative carbon sources. The highest level of xylanase titer (15 U/mL) was obtained when using lactose as carbon source.

Data on alternative substrates for xylanase induction, such as brewers' spent grain, wheat germ, corn cob and hardwood wood chips are also presented. The efficiency of using *A. pullulans* xylanolytic system for improving toner removal from printed wastepaper was also carried out and will be reported.

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Effect of the Carbon Source in the Regulation of Flocculation of Ale Brewer Yeast Strains

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Key words: Saccharomyces cerevisiae*, flocculation inhibition, carbon metabolism,* growth media

Yeast flocculation has been used in many fermentation processes, being traditionally exploited in the brewing industry. The ideal brewing yeast grows as discrete cells and flocculate after sugar depletion from the media, leaving clear beer[1].

Despite the commercial importance of yeast flocculation, little is known about the regulation of this characteristic. In a previous work[2], it was found that a high flocculent ale brewer strain, when transferred to fresh rich medium with glucose, was induced to growth and progressively loss its flocculation ability.

In the present work, the loss of flocculation of three ale brewer strains (NCYC 1195, NCYC 1214 and NCYC 1364), was investigated. These strains were cultured in defined medium, with the principal sugars (except maltotriose), found in the worth: glucose, fructose, sucrose and maltose. Our studies showed that all sugars tested were able to induce the loss of flocculation of the strains. In the present communication, the different hypothesis to explain the loss of flocculation in the beginning of growth of the ale yeast brewer strains, are discussed.

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Purification of Glucose 2-Oxidase from P. chrysosporium

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Keywords: Glucose 2-oxidase; lignocellulosics; Phanerochaete chrysosporium

The role of glucose oxidase in the biodegradation of lignocellulosics was earlier thought to be that of supplying the peroxidases (lignin peroxidase and Mn-dependent peroxidase) with hydrogen peroxide but there is recent evidence to suggest that this may be an oversimplification.
We have recently proposed that an additional role of this enzyme may be detoxification of quinones (1). Furthermore, the enzymatic equipment necessary for lignin biotransformation is not universal and depends on the fungus being studied. We therefore needed a simple method to purify glucose 2-oxidase that would allow for the better understanding of the role of this enzyme in different basidiomyceteous fungi.

The strategy used was based on that described by Oliveira et al (1996) in which the matrix was Sepharose 4B activated using triazine dyes and subsequently the enzyme was eluted with decreasing concentrations of ammonium sulphate. We report modifications of this protocol as our findings show that a concentration of ammonium sulphate lower than that reported is needed to avoid precipitation of glucose 2-oxidase. In order to optimise the protocol we have used cross-linked Sepharose 6B as the matrix and activated it using different triazine dyes.

The purity of the fractions collected was ascertained using silver nitrate-stained PAGE under native and denaturing conditions. Under non-denaturing conditions the enzyme could be identified using either the o-dianisidine or MBTH/DMAB assay reagents reported in the literature. We report that the recovery and specific activity of the purified enzyme depended on the particular matrix used and in certain cases it was necessary to add an additional step to obtain the enzyme in purified form but without compromising the final recovery. Purification of glucose 2-oxidase from P. chrysosporium was more time consuming than that from C. versicolor. Not only does the former produce the enzyme later but also a much larger number of contaminant proteins are present in the crude cell-free extract.

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Conversão Biotecnológica de Resíduos Lignocelulósicos em Xilitol

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Keyword: *xilitol, bagaço de cana-de-açúcar, ácido acético, fermentação*

Os processos biotecnológicos para obtenção de xilitol, adoçante não cariogênico, substituto de açúcares para diabéticos e obesos, requerem primeiramente a liberação de monossacarídeos, especificamente a D-Xilose, da fração hemicelulosica da biomassa. A hidrólise ácida destes materiais é acompanhada pela formação de compostos tóxicos, em particular o ácido acético, potente inibidor do metabolismo de xilose em microrganismos[1]. Dependendo do tipo de hidrolisado, a composição de inibidores será diferente bem como a sua influência no microrganismo e na performance da fermentação[2,3].

Com o objetivo de avaliar a bioconversão xilose/xilitol a partir do hidrolisado hemicelulósico ácido de bagaço de cana-de-açúcar, foram conduzidos experimentos empregando-se a levedura *Candida guilliermondii* ($2{,}5 \times 10^7$ cels/mL), adaptada ou não no hidrolisado. O meio de fermentação foi composto pelo hidrolisado concentrado contendo xilose, glicose, arabinose e ácido acético nas concentrações de 76,2, 11,2, 7,5 e 6,8 g/L respectivamente, tratado com CaO comercial e H_2SO_4 e suplementado com nutrientes. Os experimentos foram conduzidos em fermentador MULTIGEM (1L), pH 5,3, agitação de 300 rpm, aeração de 0,6vvm a 30^{o}C.

Foi constatado um aumento da velocidade de produção de xilitol com a adaptação do inóculo obtendo-se após 40h, aumento de 66 e 28% nos valores de produtividade (0,65 g/L.h) e rendimento (0,53 g/g) respectivamente em xilitol, em relação às fermentações com a levedura não adaptada. Tal comportamento sugere o envolvimento de mecanismo de adaptação da levedura aos compostos tóxicos presentes no hidrolisado. Observou-se também maior capacidade da levedura adaptada em assimilar o ácido acético encontrando-se um consumo de 50% deste após 40h de fermentação, não se verificando influência da adaptação na população final de células.

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Producción de Soforosa por *Candida bombicola*

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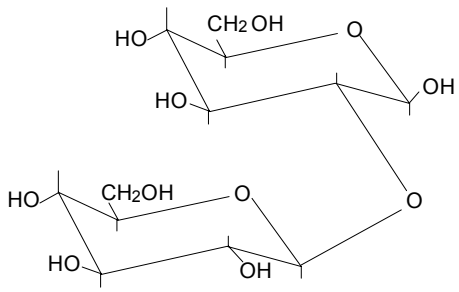
Palabras Clave: soforosa, Candida bombicola*, soforolípidos.*

La soforosa es un raro azúcar formado por dos moléculas de glucosa con enlace ß (1-2). Se aisló por primera vez a partir de glucósidos procedentes de la Acacia del Japón (*Sophora japonica*) [1]. En la actualidad se obtiene, fundamentalmente, por síntesis química [2], siendo su precio en el mercado muy elevado. La utilidad de este azúcar se basa en el poder de inducción que presenta sobre la síntesis de celulasas por el hongo *Trichoderma reesei*, estas celulasas permitirían reducir la celulosa hasta azúcares solubles [3]. No existe ningún antecedente en la literatura en el que se describa la producción de soforosa utilizando microorganismos, lo que se pone de manifiesto en este trabajo.

La levadura *Candida bombicola* es utilizada para la producción de soforolípidos [4,5], moléculas de características tensioactivas, producidas extracelularmente cuando esta levadura crece en un medio que contenga dos fuentes carbonadas de distinta naturaleza, un aceite y un azúcar. Aunque los soforolípidos están constituidos por una molécula de soforosa no se ha encontrado indicios de esta sustancia en el medio de producción. En la Figura 1 se muestra la estructura de la molécula de soforosa. Sin embargo, si en el medio de cultivo solamente está presente un azúcar, normalmente glucosa, y el resto de nutrientes se encuentra en suficiente cantidad, la levadura únicamente crece, sin producir soforolípidos, y al alcanzar la fase estacionaria de su crecimiento (unas 200 horas) empieza a generar soforosa de forma extracelular (alcanzando alrededor de 2 g/L a las 250 horas) y, también, presenta acúmulos lipídicos intracelulares considerados como los precursores de las moléculas de soforolípidos. En la Figura 2, una micrografía obtenida por Microscopía Electrónica de Transmisión, se pueden apreciar los acúmulos lipídicos intracelulares de la levadura.



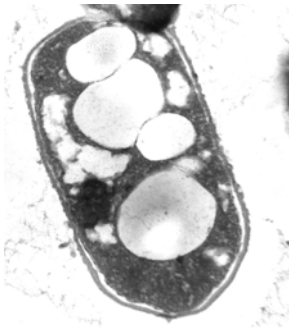


Figura 1.-Estructura de la molécula de soforosa Figura 2.- Acúmulos lipídicos en *C.bombicola*

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Structure and composition of a *Sphingomonas paucimobilis* biofilm

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***Keywords**: biofilm, exopolymeric matrix, polysaccharides*

Biofilms can be undesired structures in health and in some technological processes, but they have been successfully used in waste water treatment. To optimise the operatory condition of a waste water plant treatment, it is very important to determine the composition and structure of biofilms[1]. Biofilms are formed by microbial cells embedded in a exopolymeric matrix[2]. The polymeric matrix is mainly composed by polysaccharides, although other compounds can be found in smaller amounts such as proteins, DNA and humic substances[3]. There are several models to describe the structure of biofilms: previously biofilms were considered to have an homogenous and continuous structure[4], recently, with the advent of confocal Scanning Laser Microscopy (CSLM) a new model was developed that considers biofilms as clusters separated by water channels, the so called mushroom shape[5]. However CSLM techniques only enable observation of very thin biofilmes (200 μm) and most waste water biofilms have a thickness of 0.5 to 2.0 mm.
In this work a thick biofilm of *Sphingomonas paucimobilis* (high polysaccharide producer) was formed on glass cilinders and its structure and composition was studied by obtaining layers of biofilm detached under different rotation speeds.
The results showed that with a lower shear stress (500 rpm) 58% of the total biofilm mass was removed, 1000 rpm removed 37% and finally with a high shear stress the remaining part was detached. The inner layer of the biofilm was the most difficult to remove, suggesting that the forces of cohesion were very strong, moreover this part presented the greatest density and the smallest percentage of water. Figure 1 shows the composition in polysaccharides, proteins, DNA and cells of the removed fractions. The obtained profile points to a stratified structure: the exoplymeric substances (EPS) are more concentrated in the upper layers as well as the cells. The decrease of polysaccharide content from the top to the inner layer follows the same tendency of cells, suggesting that the rate of production is the same in each layer of the biofilm. The protein content found in the exopolymeric matrix was greater than the polysaccharide and it increased very through the biofilm layers, the same behaviour was followed by DNA. The higher ratios protein/cells and protein/polysaccharide found in the inner layer were probably due to cell lysis that occurred in a larger extent in the inner layer and these were probably responsible for the strength of the biofilm in this layer.

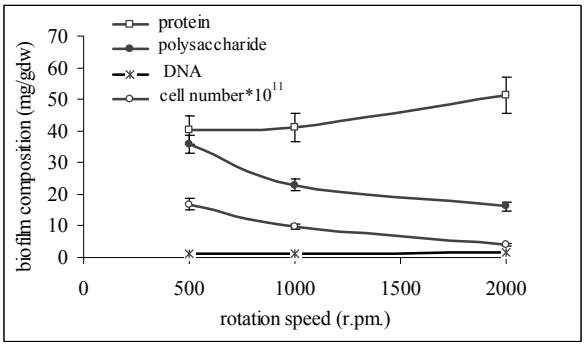

Figure 2: Sphingomonas paucimobilis biofilm composition

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Screening of Methods for Dry Weight Biomass Determinations in Cultures of Microalgae

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Key words: biomass determinations, dry weight, ash-free dry weight, microalga

Ash-free dry weight (AFDW) determinations are a common laboratory technique used to assay for the growth rate of cultures of microalgae in terms of weight of solids. Another alternative determination is dry weight (DW), a less frequently used measure of weight of solids, because it implies an extra washing step (in order to eliminate residual medium from the cell vicinity) which may promote cell lysis due to osmotic variations. Nevertheless, DW provides a wider range of information than AFDW, so efforts to use the former were undertaken.

In this experiment a saline microalga, *Pavlova lutheri*, was chosen to test several potential washing solvents for DW determinations. The solvents used were (i) an isotonic solution, (ii) a 2.25% (w/v) saline solution (i.e. with the same salinity as the culture medium), and (iii) a two-step washing procedure, with HCl 0.5 N and deionized water. The experiments were run in quadruplicate and the DW and AFDW values obtained in each treatment were compared to one another and with those of control cultures (without washing step).

Similar results were obtained for AFDW values in cultures washed with solvent (iii) and control cultures; then, a second approach was performed using this method for cultures harvested at different growth phases and using different sample volumes, all of which yielded comparable results with those of the control. Hence, the two-step washing process described is a suitable and reproducible method for biomass dry weight determinations.

The Effect of Nitrogen Source on Biosurfactant Production by *Bacillus subtilis*

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Keywords: biosurfactant, Bacillus subtilis

In recent years, the interest in microbial surface-active agents has been increasing due to their properties as emulsification, dispersing, foaming, gelling, detergency, metal sequestration, floculation action, etc. These properties render biosurfactants capable of being utilized in many applications in food-processing and cosmetic industries, agriculture and crude oil recovery. The microbial surfactants, synthetic compared to the ones, presents advantages as biodegradability, low toxicity and specificity. Also, considering the production of biosurfactant through fermentation, it makes possible the use of industrial residues, rendering the process economically advantageous.

A wide variety of microorganisms can synthesize different types of surface-active agents. *Bacillus subtilis* produces one of the most effective biosurfactants known, a lipopeptide called surfactin. This product lowers the water surface tension to 27 dyne/cm, an expressive low value.

The aim of this study was to evaluate the effect of nitrogen source and concentration for biosurfactant production by *Bacillus subtilis* ATCC 6633.

Batch fermentations were carried out in 500 ml Erlenmeyers flasks with 100 ml of mineral salt medium containing sucrose, KCl, KH_2PO_4, K_2HPO_4, $CaCl_2$, a trace metals solution and EDTA (pH 7). The nitrogen sources $NaNO_3$, $(NH_4)NO_3$, $(NH_4)_2SO_4$ and urea were added into the medium to establish the initial concentration of nitrogen of 20 g/L. After incubation at 30ºC for 48 hours with an agitation speed of 200 rpm, culture samples of whole broth were centrifuged to remove cells and the supernatant fluid was monitored for surface tension and emulsion index. Once selected the best nitrogen source, surfactant formation by *B. subtilis* strain was determined by varying sodium nitrate concentrations (0.5; 1.0 and 4.0 g/L).

Biosurfactant formation was affected by the nature of the nitrogen source, the best results for surface tension being obtained for sodium nitrate (33.2 dyne/cm) and urea (32.6 dyne/cm). However, the uptake of sodium nitrate by *B. subtilis* strain resulted in the formation of a product with the greatest emulsion index on kerosene of 44.8%. Maximum decrease of surface tension was attained at 0.5 g/L of sodium nitrate, with no significant variations being observed for higher amounts of this salt. Nevertheless, the emulsion index value directly increased with the increment of the nitrogen source concentration. Based on the experimental results it can be concluded that the addition of 4.0 g/L sodium nitrate to the medium as the nitrogen source favoured biosurfactant formation on basis of the surface tension an emulsion index of the culture supernatant.

Free Energy of Adhesion of Nitrifying Bacteria to Limestone and Basalt

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Keywords: *adhesion, surface properties, thin-layer wicking, contact angle*

Any surface in contact with a biological fluid is a potential target for microbial cell adhesion. In this process, the surface properties of both interacting bodies, like surface charge and surface free energy play an important role. Surface energy considerations enable the computation of the free energy of adhesion between two surfaces - the thermodynamic model. According to this model, bacterial adhesion will be favoured if the process itself causes the free energy to decrease, $\Delta G^{IF} = \Delta G^{LW} + \Delta G^{AB} < 0$, where LW means Lifshitz-van der Waals interactions (apolar) and AB stands for Lewis acid-base interactions (polar).
To determine the surface free energy components of a solid, contact angles of three different liquids (for which apolar and polar components are known) need to be measured. However, when the solid material is in a particulate form an alternative technique "Thin-Layer Wicking" has to be used [1]. In the present study the two methods were compared by determining the free energy of interaction between nitrifying bacteria and supporting materials (basalt and limestone) when immersed in liquid medium (Table 1).

Although the absolute values of ΔG^{IF}_{132} obtained by both techniques were different, the tendency observed was the same. From the data presented in Table 1 it is clear that although adhesion is not thermodynamically favourable ($\Delta G^{IF}_{132} > 0$), adhesion of both bacteria to limestone would be more auspicious than adhesion to basalt.

Table 1 Interfacial free energy of adhesion (ΔG^{IF}_{132}) between nitrifying bacteria (1) and support materials (2), immersed in water (3), in mJ/m^2, at 20 ºC

Interaction	Contact angle			Thin-layer wicking		
	ΔG^{LW}	ΔG^{AB}	ΔG^{IF}	ΔG^{LW}	ΔG^{AB}	ΔG^{IF}
Nitrosomonas/W/L	-0.93	17.67	16.73	-1.20	9.39	8.19
Nitrosomonas/W/B	-0.61	27.21	26.60	-1.20	12.94	11.75
Nitrobacter/W/L	-0.93	24.22	23.29	-1.37	14.13	12.76
Nitrobacter/W/B	-0.61	36.73	36.12	-1.36	19.43	18.07

The higher hydrophobicity of limestone and the establishment of Ca^{2+} bridging can be possible explanations for this fact. The differences observed on the absolute values can be related to the porosity of the material plates in the thin-layer wicking method. Although the plates are prepared with very small particles and a high concentration of solids, there are some pores remaining among the particles, which are responsible for an increase in liquid spreading. Furthermore, limestone and basalt used in this technique were submitted to a previous dehydration by heating and the same procedure was not followed in contact angle measurements, which imply a different degree of surface hydration.

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Characterisation of a peptide antibiotic produced by a new thermotolerant *Bacillus* strain

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Key words: Bacillus licheniformis*; Peptide antibiotic; RP-HPLC*

An antibiotic producing bacterial strain was isolated from an hot spring environment in S. Miguel (Açores). The code name I89 was given. The strain was identified by phenotypic methods as *Bacillus licheniformis*. Due to the high intra-specific diversity degree of *B. licheniformis* a more fine characterisation of the I89 strain was required. As reference strain it was used the *B. licheniformis* ATCC 10716, producer of bacitracin.
The mol percent of guanine plus citosine of the DNA of the I89 and ATCC 10716 strains (47,13 % and 47,86 %, respectively) and the high degree of homology of their DNA (78,4 %) confirmed that the two strains belong to the same genospecies. Nevertheless, some phenotypic and genomic differences revealed that the two strains are distinct. The I89 strain showed sensitivity to β-lactam antibiotics, not observed for the ATCC 10716 strain. Analyses by PFGE and by RFLPs showed that the two strains differ on their genomic organisation. Also, for the I89 strain it was detected the presence of a stable plasmid in low copy number, not present in the reference strain.
The I89 strain produces a peptide antibiotic with activity against Gram positive bacteria. The peptide antibiotic was recovered from culture media by partition in 1-butanol, concentrated by freeze-drying and submitted to RP-HPLC. At this moment we are interested in determining the sequence of the peptide antibiotic.
Results obtained in the RP- HPLC analysis showed that the peptide produced by the I89 strain is a peptide with different characteristics from those of the bacitracin produced by the ATCC 10716 strain.

Bioemulsifier Production by *Penicillium citrinum*

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Keywords: Penicillium citrinum, *bioemulsifier, lipase*

In this work was studied the fermentation profile of a lipase-producing strain of *Penicillium citrinum* growing in a 5L fermentor, using a non-expensive medium containing olive oil as carbon source. In our conditions, we observed that during fermentation *P. citrinum* produced an bioemulsifier, wich inhibited the lipase activity. Therefore, this bioemulsifier was measured and analysed by TLC and HPLC. Pre-inocula consisted of 100 ml MA medium inoculated with 10^5 spores per mL and cultivated for 5 days at 30°C in a rotatory shaker. This was used to inoculate 2.9 L MA medium [1] in a BIOFLO 2000 Fermentor (New Brunswick Scientific Co.). Fermentations were carried out at 30° C, 400 rpm and 2.0 L . min $^{-1}$ aeration rate. Samples were harvested at 12 hours intervals. Dissolved oxygen was not limited and remained at more than 80% during whole fermentation. Total extracellular carbohydrate quantification was performed by phenol-sulfuric acid assay. Bioemulsifier production was measured at 202 nm. Bioemulsifier activity was carried out as described by Cirigliano and Carman [2]. HPLC analysis of culture supernatant samples were performed in a LC 6 A Liquid Cromatograph (Shimadzu Co.) using ultrahydrogel 500 Å column. Samples were further submitted to acid hydrolysis during 15 hours at 140° C before TLC in Silica gel G plates (Analtech Inc.). D-glucose, D-frutose, D-galactose and sucrose were used as standards (Sigma Co.). The results showed a low production of the bioemulsifier in the first 48 hours of fermentation and an increasing to the maximum production at 60 hours. Bioemulsifier activity increased to a peak at 60 hours (1,6 $U.mL^{-1}$), remaining in this level thereafter. Total extracellular carbohydrate reached a plateau of 0,1 $mg.mL^{-1}$ at 60 hours of fermentation. HPLC analysis showed an unique peak with the same retention time during whole fermentation, indicating the presence of a polyssacaride with molecular weight around 10 kDa. TLC analysis of hydrolysed samples showed D-glucose-like bands.

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Estudio de la inducción de lipasa extracelular en una cepa de *Pseudomonas cepacia*

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Palabras clave: lipasa, inducción, Pseudomonas cepacia

Las lipasas de origen microbiano son enzimas extracelulares que se producen como respuesta a la presencia de una molécula inductora en el medio de cultivo. Los inductores más utilizados para la producción de lipasas microbianas son el aceite de oliva y el ácido oleico [1]. También se ha descrito la inducción de la producción de lipasas mediante el empleo de diferentes aceites vegetales, grasas animales, ácidos grasos, monoésteres de sorbitano e, incluso, alcanos [2]. Hasta el momento no se ha llevado a cabo ningún estudio sistemático acerca de los factores estructurales que afectan a la producción de lipasa. Por ello y para intentar conocer un poco mejor el mecanismo de la inducción, decidimos estudiar la influencia que diferentes compuestos, con diverso grado de similitud estructural con el ácido oleico, presentaban sobre la inducción de una lipasa microbiana, e intentar así encontrar una serie de motivos estructurales que configurasen un inductor tipo. El estudio se realizó con una cepa de *P. cepacia* que presenta una buena actividad lipásica cuando se utilizan tanto aceite de oliva como ácido oleico como inductores. Los cultivos se llevaron a cabo en medio líquido BYPO [3] al cual se añadieron los potenciales inductores de lipasa a una concentración del 1%. La producción de lipasa se determinó a distintos tiempos de cultivo, empleando el sustrato cromogénico glutarato de 2,3((±)di-O-lauril)gliceril resorufinilo.
Para este estudio se emplearon cinco grupos generales de compuestos: aceites y grasas; ácidos grasos de distinto tamaño de cadena, saturados e insaturados; triglicéridos; estructuras químicamente relacionadas con el ácido oleico y detergentes (Brij, Tween y Span). De los distintos aceites utilizados, los mejores inductores fueron el aceite de pescado y el de almendra. Los ácidos grasos saturados apenas produjeron inducción de la producción de lipasa, independientemente del tamaño de su cadena carbonada, mientras que los correspondientes insaturados con un tamaño de cadena entre 14 y 22 carbonos sí produjeron inducción. Los triglicéridos empleados se comportaron de manera análoga a los correspondientes ácidos grasos de los que estaban formados. Los análogos de ácido oleico utilizados mostraron la necesidad de tener un extremo polar y una insaturación próxima al carbono en posición 9 para inducir la producción de lipasa. También se probaron detergentes que incluyen en su estructura la presencia de ácidos grasos, siendo los mejores inductores los que contenían la estructura del ácido oleico. El mejor inductor de todos los probados fue el ácido cis $\Delta^{9,10}$ epoxiesteárico.

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The Specific Growth Rate of *Xanthomonas campestris* Increases After Adaptation to FCCP

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Keywords: Xanthomonas campestris*, FCCP, weak acids, growth stimulation*

Introduction and objective: Sublethal concentrations of weak acids have been identified as stimulators of the metabolism of *Xanthomonas campestris* [1]**.** This may be due simply to their utilization as carbon sources or to their action as metabolic uncouplers. In order to clarify this point, the effects of different concentrations of a non-metabolisable protonophore (FCCP) in batch cultures of *X. campestris* were investigated and the optimal concentration for stimulation of the cell metabolism determined.

Methods: *X. campestris* was grown in shake flasks in a well defined medium [2]. FCCP was added as a pulse at the end of the exponential phase to give concentrations of 0.2, 0.4, 0.8, 1.9 and 3.9μM. The initial specific growth rate following this addition was estimated by using the logistic equation. The concentrations of biomass and xanthan were determined by gravimetry and glucose concentration was determined enzymaticly.

Results and conclusions: The addition of 0.2μM FCCP did not alter the growth profile of *X. campestris* but higher concentrations stopped culture growth immediately after pulsing. However, cultures were able to adapt to the presence of 0.4, 0.8 and 1.9μM FCCP (but not to 3.9μM FCCP) and a second exponential growth phase, characterized by higher initial specific growth rates when compared to growth before FCCP addition, was observed (table 1).

Table 1 Effects of FCCP concentration on *X. campestris* cultures (μ_1 and μ_2 : initial specific growth rates before and after pulses, respectively, Yx : biomass final yield, Yp : product final yield, Yp/x : product/biomass final yield).

Tests	FCCP pulses (μM)	Adaptation time (h)	Growth			Product	Final yields		
			μ_1 (h^{-1})	μ_2 (h^{-1})	X_{final} (g/kg)	P_{final} (g/kg)	Yx (gX/gS)	Yp (gP/gS)	Yp/X (gP/gX)
F1	-	-	0.065	-	2.60	9.83	0.132	0.514	3.89
F2	0.2	-	0.065	0.065	2.65	9.47	0.141	0.516	3.66
F3	0.4	4	0.064	0.078	2.59	9.49	0.138	0.519	3.76
F4	0.8	6	0.064	0.121	2.47	9.79	0.131	0.538	4.11
F5	1.9	33	0.064	0.206	1.81	8.92	0.115	0.603	5.24
F6	3.9	>52	0.066	-	1.01	7.35	0.065	0.552	8.49

These results closely resemble those obtained with acetic acid [unpublished data] and suggest that weak acids stimulate the microbial metabolism due to metabolic uncoupling. It was concluded that 0.8μM FCCP is the optimal concentration for further investigation of metabolic stimulation in *X. campestris* cultures.

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Efeito do Farelo de Arroz, $(NH_4)_2SO_4$ e Concentração Inicial de Açúcar na Fermentação do Hidrolisado Hemicelulósico de Eucalipto a Xilitol

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Keyword: *hidrolisado de eucalipto, xilitol,* Candida guilliermordii

O xilitol ($C_5H_{12}O_5$), um poliálcool natural de carbono, de grande valor sócio-econômico devido as suas diversas propriedades, tais como, poder adoçante semelhante ao da sacarose, anticariogenicidade, substituto de açúcares na dieta de diabéticos por não requerer insulina para seu metabolismo. Este poliálcool é comercialmente produzido por via química a partir da redução catalítica da xilose obtida de materiais lignocelulósicos. Vários estudos vem sendo conduzidos para o desenvolvimento de um processo biotecnológico para a obtenção deste adoçante a partir de resíduos agroindustriais e florestais [1,2].

Neste trabalho foram avaliados a bioconversão xilose/xilitol por *Candida guilliermondii* FTI 20037 cultivada nos hidrolisados hemicelulósicos de eucalipto contendo 21 e 54 g/L de xilose. O hidrolisado foi tratado pela elevação de seu pH inicial 1,7 para 7,0 com CaO comercial, seguido do abaixamento para 5,5 com H_3PO_4 e submetido à adsorção por carvão ativo (10%) sob agitação de 200 rpm, 30 ^oC por 1h. Em seguida foi autoclavado e suplementado com nutrientes. Os fatores e os níveis avaliados foram farelo de arroz (fator A) nas concentrações 5,0 e 20,0 g/L, $(NH_4)_2SO_4$ (fator B) 1,0 e 3,0 g/L e concentração de xilose no hidrolisado (fator C) 21 e 54 g/L utilizando um planejamento fatorial 2^3. Os experimentos foram realizados em frascos Erlenmeyer de 125 mL, a 200 rpm e 30 ^oC por 72h. O inóculo utilizado foi de 3 g/L de células ($1{,}85.10^8$ células/mL).

Verificou-se através da análise estatística que a variação da concentração de farelo de arroz não apresentou efeito significativo ($p<0{,}05$). O projeto experimental foi então reduzido a um planejamento fatorial 2^2 com ponto central. Pela estimativa dos efeitos, a variação de 1,0 a 3,0 g/L na concentração do $(NH_4)_2SO_4$ (fator B) mostrou uma diminuição de 2,66 g/L de xilitol, enquanto que a variação da concentração de açúcar no hidrolisado (fator C) de 21 a 54 g/L favoreceu um aumento de 15,14 g/L de xilitol e a interação destes dois fatores (BC) resultou uma diminuição de 1,50 g/L. Isto indica que o nível de um fator interfere no nível do outro. Observa-se ainda, que a variação da concentração do açúcar de 21 para 54 g/L aumentou a produção de xilitol em 16,63 g/L quando a concentração de $(NH_4)_2SO_4$ foi de 1g/L Esta mesma variação proporcionou um aumento de 13,63 g/L quando a concentração de $(NH_4)_2SO_4$ foi de 3 g/L. A análise de variância do fatorial 2^2 é mostrada na TABELA 1. Observa-se que os fatores apresentam um bom nível de significância e um coeficiente de determinação de 0,996.

TABELA 1 - Análise de variância do planejamento fatorial 2^2 para os fatores $(NH_4)_2SO_4$ e concentração de açúcares.

Efeitos	SQ	GL	MQ	F	p
B	14,178	1	14,178	27,17	0,0065
C	458,288	1	458,288	878,39	0,0000
BC	4,515	1	4,515	8,65	0,0423
Erro total	1,910	4	0,522		
Total	479,067	7			

$R^2 = 0{,}996$; GL = graus de liberdade ; SQ = soma quadrática ; MQ = média quadrática.

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Hydrophobins and Biodeterioration of Paint by Fungi

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Key words: *biodeterioration; attachment, paint surfaces; hydrophobins; fungi.*

Paint coatings are susceptible to biodeterioration by fungi often affecting the appearance and physical/chemical properties of the paint.
Investigations into adhesion of fungal spores to paint films is therefore of fundamental importance.
Hydrophobins are small, cystein rich, hydrophobic proteins with the ability to assemble into an amphipathic protein film when confronted with a hydrophilic-hydrophobic interface, such as between water and air [1]. This is of special importance for the emergent growth of aerial mycelium and differentiation.
Hydrophobins play an important role in the attachment of conidia to plant surfaces prior to infection, and they enhance adhesion of *Schizophyllum commune* mycelia to solid hydrophobic surfaces.

We report here on a simplified extraction procedure for hydrophobins from conidia and mycelia in order to investigate the possible role of hydrophobins on the adhesion to paint films of a wide range of fungi.

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Purification and Characterization of the First Benzaldehyde Dehydrogenase from an Halophilic Bacterium

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***keywords**: Benzaldehyde dehydrogenase, Halophilic microorganisms, stereospecific synthesis*

In the last few years, the use of enzymes for industrial purpose has revealed a rapid growth owing to the advantages they confer to conventional chemical methods [1]. For example biocatalysts are highly specific and efficient and are able to produce chiral compounds [2]. Enzymes obtained from microorganisms already have numerous industrial applications, although till now their application fields are restricted by peculiar reaction conditions. In fact, these technological processes frequently involve exposure to extremes of temperature, pressure, ionic strenght, pH and organic solvents, and hence there is a continuing need to isolate microorganisms/enzymes which are able to function and catalyse specific reactions under these imposed conditions [3]. In particular organisms able to growth in environments containing high salts concentration (halophiles), are good candidates for isolate enzymes able to resist at extreme conditions.

In this work we describe the purification and characterization of a benzaldehyde dehydrogenase activity from an halophile strain named "CA" (an halotolerant microorganism isolated from saline environments and not yet taxonomically characterized).

The enzyme was purified by ammonium sulphate fractionation, hydrophobic chromatography on Sepharose 4B and sulphate-mediated ion-exchange chromatography on DEAE-cellulose. Preliminary studies indicated that the enzyme is very thermostable with an optimum of temperature at about 80°C. It showed also a broad spectrum of activity in the pH range from 7 – 11 with the maximum at pH 9.5.

At our knowledge this is the first benzaldehyde dehydrogenase enzyme detected in halophilic microorganisms. Studies are in progress to well characterize the enzyme in view of its potential application for stereospecific organic synthesis.

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Pectinase Production by *Aspergillus niger* C28B25 in Solid State Fermentation Using Polyurethane Foam as an Inert Support

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Keys Words: Pectinases, Solid-State Fermentation, Polyurethane foam, Improvement.

Introduction. Pectinases have been used widely in food industry and have been obtained by Solid State Fermentation (SSF) at higher levels to those observed in Submerged Fermentation (SmF) [1]. However, biodegradable supports contaminate crude enzymatic extracts (ECE) with a series of byproducts [1-3]. In this work, a study was carried out using polyurethane foam (PUF) as an inert support [4-5].

Materials and methods. SSF was performed according to [4] using a basal mineral medium (30 mL/g PUF) with pectin (15 g/L), sucrose (32 g/L), salts and a mixture of oligoelements (OLI = $MnCl_2$, $CuSO_4$, $ZnSO_4$; 1 mg/L) at pH 4.5, a_w = 0.995, which was inoculated with 10^8 spores/(g of carbon source). Temperature was kept at 35 ºC. Incubation time was t = 24 h (optimal with basal medium). The following factorial experiment was designed (2x2x2):

Treatment (T_i)	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**	**T_6**	**T_7**	**T_8**
OLI (ml/L)	0.7	0.7	0.7	0.7	1.3	1.3	1.3	1.3
Sacarose (g/L)	22.3	22.3	41.7	41.7	22.3	22.3	41.7	41.7
Inocula (N_O/g C)	$7x10^7$	$1.3x10^8$	$7x10^7$	$1.3x10^8$	$7x10^7$	$1.3x10^8$	$7x10^7$	$1.3x10^8$

Determinations were made by triplicate for: 1) Excreted protein concentration (PT), 2) enzymatic activity (PE) in ECE and 3) biomass (X).

Results: The table below shows that treatment T4 was found to be the best, since: 1) X increased by 40%, 2) PE in ECE was 3-fold higher, and 3) specific activity (PE/PT) was also 6-fold higher. Similar results were obtained with repetition by triplicate of T4.

Experiment	**X** (gX/g PUF)	**PT** (mg/mL ECE)	**PE** (U/mL ECE)	**PE/PT** (U/mg PT.)
Basal medium	0.53 ± 0.03	0.07 ± 0.001	2.6 ± 0.18	44.6 ± 3.37
T_4	0.74 ± 0.02	0.03 ± 0.002	7.6 ± 0.67	254 ± 3.35

Discussion and Conclusions: Pectinase production by *Aspergillus niger* in SSF using PUF as a support resulted in 10 times higher specific activities, obtained in a time 3-fold shorter to those obtained previously [2] using sugar bagasse cane as a support. Additionally, resistance to catabolic repression on pectinase production by SSF was confirmed as described earlier [1-2].

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Microbial Hydrolysis of 2-Isoxasoles

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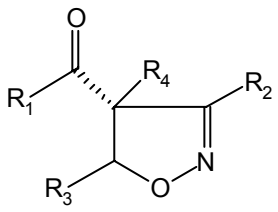
***Key words**: microbial hydrolysis, 2-isoxasoles*

The aim of our work was to hydrolyse ester groups in the substituted 2-isoxasoles. The classic, chemical hydrolysis esters groups in these compouds is impossible to carry out, since due to heating, both in an acidic and basic media the heterocyclic ring undergoes opening. Our aim was effected using the fungal strain *Aspergillus niger*. Diffrent derivatives of 2-isoxasoles having the general formula:



where $R_1 = OCH_3, OC_2H_5, OC_6H_5$
$R_2 = CH_3, C_6H_5$
$R_3 = C_6H_5$, p-$OCH_3C_6H_5$, p-$NO_2C_6H_5$
and $R_4 = H, C_6H_5$
Some of these 2-isoxasoles were mixtures of regioisomers A and B.

Reactions using *A. niger* were performed in aqueous medium (phosphate buffer, pH 7,0), at 30°C, 200 rpm.

For all tested compounds the hydrolysis was achieved. The regioselective hydrolysis was observed for 2-isoxasoles (mixtures of regioisomers), in which:
1) $R_1 = OCH_3$, $R_2 = CH_3$, $R_3 = C_6H_5$
2) $R_1 = OC_2H_5$, $R_2 = CH_3$, $R_3 = C_6H_5$
3) $R_1 = OCH_3$, $R_2 = C_6H_5$, $R_3 = C_6H_5$
The enantioselective hydrolysis was observed for 2-isoxasoles (one regioisomer A or B), in which:
1) $R_1 = OC_2H_5$, $R_2 = CH_3$, $R_3 = C_6H_5$, $R_4 = C_6H_5$; $[\alpha]_D^{20}$ for obtained acid = +133°
2) $R_1 = C_6H_5$, $R_2 = CH_3$, R_3 = p-CH_3-OC_6H_5; $[\alpha]_D^{20}$ for obtained acid = -103°
3) $R_1 = OCH_3$, $R_2 = CH_3$, R_3 = p-NO_2-C_6H_5; $[\alpha]_D^{20}$ for obtained acid = +23°
4) $R_1 = OC_2H_5$, $R_2 = CH_3$, $R_4 = C_6H_5$; $[\alpha]_D^{20}$ for obtained acid = +13,7°
5) $R_1 = OC_2H_5$, $R_2 = C_6H_5$, $R_4 = C_6H_5$; $[\alpha]_D^{20}$ for obtained acid = +13,6°

Potential Biotechnological Applications of Thermophilic Rhodanese

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Keywords : *Rhodanese, Cyanide detoxification, Extremophiles,* B. acidocaldarius.

The use of cyanide compounds in industrial processes is very extensive and continuously increasing; it is estimated that more than 3 million tons of cyanide per year are used worldwide in the production of chemical intermediates, synthetic fibers and rubber, pharmaceuticals, as well as in coal processing and metal plating [1]. Since a notable quantity of cyanides is still present in the process waste waters, the problem of effluents detoxification must be taken in great consideration. The standard way of cyanide treatment in industrial waste waters is chemical oxidation by alkaline chlorination or by using potent oxidants like hydrogen peroxide or ozone. Such conventional techniques are characterised by their universal applicability because of their insensitivity toward the nature of the waste material and by their relatively low costs. However, their main handicaps are the need of hazardous reagents like chlorine and their potential for creating toxic residues requiring a post treatment.
An alternative to chemical methods could derive from biological treatments; it has been shown that certain types of microorganisms can decompose the toxic substrate cyanide [2]. Recently we have examined several strains of extremophilic microorganisms for the presence of rhodanese, an enzyme protein which catalyses the conversion of cyanide ion into the less toxic thiocyanate. Apart from the theoretical interest, the rhodanese enzyme isolated from thermophilic microorganisms could significantly improve its potential biotecnological applications in wastewater detoxification and CN^- monitoring in consideration of its enhanced thermostability, long shelf life, resistance to denaturing agents and general robustness.
The highest level of activity, tested both on whole cells and on sonified homogenates, was exhibited by the thermoacidophilic eubacterium *Bacillus acidocaldarius*. The enzyme was isolated and purified to homogeneity by a procedure including pH precipitation and the sequential use of cation-exchange, hydrophobic interaction FPLC chromatography, and Superdex gel filtration.
B. acidocaldarius whole cells previously subjected to one cycle of freeze-thawing, were also immobilised by entrapment in various polysaccharide matrices and in an insolubilized gelatin gel [3]. On the basis of the results obtained with the different immobilizates in terms of activity yield, possibility of regeneration and operative stability, the gelatin immobilised whole-cell rhodanese was employed in a continuously fed membrane reactor suitable conceived and working as a plug flow reactor (PFR).

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Yeast Cell Wall Dynamics: p54 - a Protein Accumulated at Late Growh in *Saccharomyces cerevisiae*

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***Keywords**: yeast cell wall proteins, late growth*

A protein with an apparent molecular mass of 54 kDa (p54) was present in higher amounts in the wall of flocculent, post-diauxic *S.cerevisiae* NCYC 1195, when compared to non-flocculent, exponential cells. The protein was isolated and 6 peptides were obtained after endoproteinase LysC hydrolysis, one of which highly identical to a sequence found in yeast elongation factor EF-1a. Synthesis was monitored *in vivo* using ^{14}C labeled amino acids, reveling that p54 wall incorporation took place throughout growth, and also that it was the only SDS-extractable protein targeted to the cell wall at significant levels by stationary cells. The surface localization of p54 was further investigated using laser confocal fluorescence microscopy. Flocculation was not correlated with p54 accumulation as it became clear after western analysis. Furthermore, its accumulation did not take place when the same cells were grown at 10°C, a condition that prevented the flocculation onset. The presence of p54 in the cell wall during growth and its accumulation at late growth phases provide new evidences regarding the structure dynamics of yeast´s outer organelle.

Influence of Physico-Chemical Surface Characteristics on the Adhesion of *Alcaligenes denitrificans* to Polymeric Supports

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Keywords: *initial adhesion, polymeric supports, surface properties*

One way to avoid the washout limitation in continuous biological systems and to increase productivity is to retain the cells inside the reactor via immobilisation onto a supporting material. From a physical-chemical point of view, adhesion of cells to solid surfaces is determined by the interplay of electrostatic and hydrophobic interactions. Thus, the nature of the surface of the microorganisms and supports are deciding factors. The two characteristics of relevance are electrokinetic potential and hydrophobicity. Studies were performed to determine which type of material promotes a stronger adhesion of *Alcaligenes denitrificans* and the development of the most stable biofilm, to be used as biomass carrier in denitrifying inverse fluidised bed reactors. The supports are: high-density polyethylene (HDPE), polypropylene (PP), polyvinylchloride (PVC) and polymethyl-methacrylate (PMMA). This study also aims at determining if in the initial adhesion process prevails the electrostatic interaction or hydrophobicity. The zeta potential of the cells and supports was calculated by electrophoretic mobility measurements and the hydrophobicity was determined by contact angle measurements, using the concept that a material is hydrophobic when the interfacial free energy of interaction of its particles immersed in water is negative, and is hydrophilic when that energy is positive [1]. Initial adhesion tests were performed with four slides of each type of support horizontally placed in a sterile small container and 100 ml of bacterial suspension were added. After 2 hours of incubation at 27ºC and 90 r.p.m., the slides were rinsed with sterilised water. They were then covered with a 0.1% acridine orange solution and observed under an epifluorescence microscope. The images were acquired by microscope photography and then digitised. The number of bacteria per square mm was enumerated by image analysis.

In the pH range 6-9 all the above mentioned materials to be used as carriers were found to be negatively charged as well as the bacterial cells, causing a potential energy barrier which difficults the adhesion process. All the supports studied are hydrophobic. PP is the most hydrophobic material, PMMA is the less hydrophobic whereas HDPE and PVC presents an intermediate behaviour. As bacteria cells are hydrophilic, adhesion is favoured to polypropylene. This is confirmed with the results of adhesion tests (Figure 1). In this way, adhesion seems to be dominated by hydrophobic interactions.

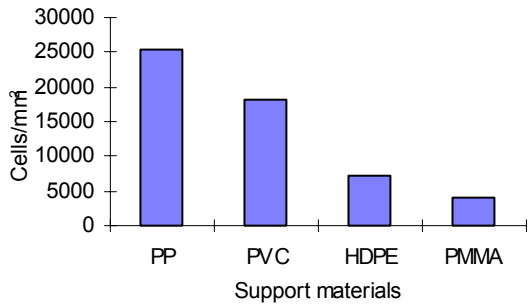

Fig.1 - Results of adhesion tests performed, expressed by the number of bacteria per square mm of the polymer surface.

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Diferences Between *Aspergillus niger* Saccharases Produced by Solid and Liquid Fermentation

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Key Words: Saccharase, Solid-State Fermentation, Enzyme excretion, Aspergillus.

Introduction: Saccharase synthesis (β-D-fructo furanósidase fructo hydrolase EC. 3.2.1.26) is a good model to study the regulation of enzyme production and excretion by *Aspergillus niger*. This enzyme is codified by *suc 1* gene, and it has been cloned and sequenced previously [1-2]. At this work, we demonstrate the existence of differential synthesis and excretion of saccharases by *A. niger* on solid (SSF) and liquid state fermentation (SmF) [3].

Methodology. To conduct SSF polyurethane foam (PUF) was used as innert support [4], it was embedded (25 mL/g PUF) with saline basic medium [5] and sucrose as carbon source (100 g/L). Incubation was conducted at 30º C by 60 h, taking samples every 12 h. Crude extract was obtained pressing softly and washing with distilled water. For SmF 125 mL Erlenmeyr flask were used, 25 mL of basic media plus sucrose (100 g/L) as carbon source were added and incubated at 30º C with agitation at 200 rpm by 60 hours. Same numbers of samples were obtained for both systems. Biomase was obtained by dry weight. Saccharase activity was determined by reducer sugars(E) and excreted protein (PRT) was measured by Coomasie blue method. PAGE were realized to detect saccharase molecular weight and zymografies were used to detect sacchrase activity [6].

Results: *A. niger* grows much faster by SSF ($\mu = 0.2/h$) than by SmF ($\mu = 0.1/h$). Maximal activity of the excreted sacharase (E = 5.6 U/mL at 36 h) was obtained earlier in SSF than in SmF (E = 4,9 U/mL at 60 h). A very similar behavior was observed for production patterns of intracellular invertase, because in SSF saccharase appears earlier (E = 1.8 U/mL a las 24 h) than in SmF (E = 3.1 U/mL a las 60 h). Specific activity in SSF was 94 U/mg PRT and for SmF it was 355 U/mg/PRT. Electrophoretic and y zymographic study shows excreted saccharase by SSF has a molecular weightr (PM = 80 kDa) minor to the SmF (100 kDa < PM < 160 kDa) molecular weight and this shows a very long activity dot on the zimography.

Discussion and Conclusions. Kinetic differences between SSF and SmF production patterns could be related more evenly to μ differences. Zymographic differences suggest: In SmF a more glycosilated and heterogeneous saccharase is produced, as compared with a homogeneous and lighter form produced by SSF. These results confirm our hypothesis about the fermentation system has a strong effect over the synthesis and excretion of hydrolases.

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Purification of High Molecular Weight Xylanases from *Bacillus* sp.

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Keywords: Bacillus *sp., thermophilic bacteria, xylanases, pulp bleaching.*

One of the major industrial applications of hemicellulolytic enzymes, mainly endo-β-xylanases, involves its use for prebleaching kraft pulps allowing to reduce the consumption of chlorinated chemicals commonly used in the conventional process. Due to the high temperature and alkalinity of the pulp in industry, screening for novel cellulase-free xylanases displaying activity under these extreme conditions is an important feature for pulp applications, avoiding the need for cooling and pH shifts.

The thermophilic *Bacillus* sp. 3M isolated in our laboratory from Azores hot springs has previously been shown to produce extracellularly cellulase-free, alkaline and thermotolerant, endo-β-1,4-xylanase activity, with optimum at 60ºC and pH 7, showing high stability at 55ºC (retaining 100% of its original activity after a 3-days preincubation at this temperature) [1]. Of the maximal xylanase activity produced by *Bacillus* sp. 3M, 47% was still present at 80ºC and more than 85% at pH 9.0 (conditions generally used in the kraft pulp process [2]).

Most of the xylan-degrading microorganisms studied sofar produce a range of β-xylanase enzymes, but in general, these enzymes are monomeric proteins with low molecular mass (between 11 and 80 kDa) [3]. On filtrates of *Bacillus* sp. 3M grown on oat spelts xylan, five xylanolytic enzymes, corresponding to proteins of apparent molecular masses ranging from 90 to 220 kDa, were detected on Congo Red-stained PAGE zymograms using arabinoxylan incorporated into the gel.

The purification of these xylanases was carried out using ammonium sulphate fractionation, hydrophobic interaction chromatography, anion exchange chromatography and affinity chromatography techniques. The purity of each protein was checked by PAGE.

The xylanases purified to electrophoretic homogeneity were characterized in terms of their optimal pH and temperature, as well as their thermostability, in order to predict the efficiency of their isolated use for improving the bleachability of pulps.

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The Effect of Hexoses as Co-Substrates on Xylitol Production by *Debaryomyces hansenii* CCMI 941

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***Keywords**: Xylose, Hexose, Xylitol,* Debaryomyces hansenii

Xylitol is a compound with high industrial potential, used as an anticariogenic sweetener in food and related products. Any economical feasible xylitol bioproduction can only be achieved by utilizing an inexpensive source of xylose. Hemicellulosic hydrolysates are generally considered as suitable xylose-containing syrups for yeast fermentation [1]. Depending on the nature of the hydrolysate, the relative amount of xylose can be variable, but it is usually the most abundant sugar. However, the presence of some readily metabolised hexoses (glucose, mannose and galactose) can result in a poor xylose conversion to xylitol.

In this work the influence of glucose and galactose as co-substrates on xylose metabolism was studied under oxygen-limiting chemostat cultivation by the xylitol over-producing *Debaryomyces hansenii* [1].

Steady-state metabolic data were collected from *D. hansenii* cultures using either xylose as the sole C-source or mixtures of glucose and galactose (at different ratios) at a constant dilution rate of 0.06 h^{-1}. The steady-states obtained were characterised in terms of metabolic rates and biomass macromolecular composition.

Xylitol was the major product detected among all the tested conditions. The maximal values for xylitol yield and volumetric productivity (0.56 gg^{-1}xylose and 0.21 $gL^{-1}h^{-1}$ respectively) were obtained in the presence of glucose (2 gL^{-1}) as co-substrate, showing that the addition of small amounts of glucose, but not galactose, potentiates the xylitol yield, leading to an increase of 30% comparing to the single xylose-containing feed medium.

Ethanol and glycerol were also detected in small amounts on cultures grown on xylose and glucose mixtures but only when the hexose concentration was upper or equal to 6 gL^{-1}. This by-product accumulation was correlated with glucose metabolism, since a direct relationship between the increase of ethanol (and glycerol) concentration and the increase in the glucose concentration in the feed medium was found.

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The Influence of Nitrogen Source for Chitin Production by *Mucor javanicus*

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Keywords *Chitin, Nitrogen Source and* Mucor javanicus.

Laboratory studies on chitin production were carried out with purpose of knowing the role of nitrogen medium in growing and developing of *Mucor javanicus* culture. Taking into acount the facility of obtaing chitin by using microrganisms and the large applicability of this biopolymer (health care, food and beverages, agriculture, waste and water treatment and others), this preliminary report plays important and additional constraints about the effets of nitrogen on the evolutionary and productive aspects *Mucor javanicus* culture. In this context we have investigated the influence of different concentrations of nitrogen in the content of chitin. Experiments were carried out by using an inoculum of 10^7 spores/mL described in [2] medium containing different thiamine and asparagine concentrations (media "A"; "B"; "C"). Erlenmeyer flasks have been incubated with orbital agitation at 22ºC during 96 hours. On the basis in an alkali-acid treatment the mycelia of chitin was extracted, having been identified by infrared spectrum of the insoluble fraction, as described in [1]. The total nitrogen content have been determined by assuming the Kjeldhal Method.
The resulting analysis of three mycelial growing are listed below (Table).

MEDIUM	ASPARAGINE (g/L)	THIAMINE (mg/L)	BIOMASS (g/L)	CHITIN (mg/g)	TOTAL N (%)
A	2.0	0.05	6.8	47.0	0.06
B	1.0	0.05	1.0	10.8	0.02
C	2.0	0.10	13.3	45.0	0.10

In a first aproach, the analytical data demonstraded that when the asparagine and thiamine concentrations have been decreased at 50% and remained the same respectively, the chitin content diminished at circa of four times. On the other hand, concerning the medium "C", where the asparagine and thiamine concentrations were basically inverted in relation to "B", the chitin performance was similar to the original medium in terms of content. Summarizing, the thiamine as source of nitrogen doesn't favored relative elevated chitin content. Furthermore, the chitin perfomance content is influenced directely by asparagine concentration when is used as source of nitrogen.

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Yeast Response to Acid Stress: Physiological Role of the Plasma Membrane H^+-ATPase

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Saccharomyces cerevisiae*, weak acid stress, plasma membrane H^+-ATPase*

The antimicrobial effect of weak acids used as food preservatives at low pH relies not only on the high concentration of protons around the cell but also on the intracellular dissociation of their acid form which leads to additional acidification of the cytosol. Moreover, the undissociated forms of many weak acids (like octanoic acid) are highly lipossoluble and stimulate the passive influx of H^+ into the cell by increasing the non-specific permeability of the plasma membrane [1]. The ability of yeast cells to grow and maintain viability in the presence of weak acids at low pH reflects their capacity to maintain control over intracellular pH (pH_i) by excluding H^+. Their pH_i depends on both the activity of the H^+-ATPase in the plasma membrane and plasma membrane passive permeability to H^+. Our laboratory proved that, at optimal concentrations, weak acids (octanoic, succinic and acetic acids) induce the activation of *S. cerevisiae* plasma membrane ATPase *in vivo,* either in growing cells [2,3] or in cells exposed to acid stress for a few minutes [4]. This response, that counteracts the negative effects of weak acids, was found to be vital in the restoration of pH homeostasis during recovery from acid stress [4,5]. However, when strong acids (HCl or H_2SO_4) were used as the acidulants, stressing low pH did not induce plasma membrane ATPase activation; on contrary, a reduction of this enzyme activity was observed either in growing cells at low inhibitory pH [6] or in cells exposed to acid stress for a few minutes [4]. *S. cerevisiae* plasma membrane H^+-ATPase activity was also found to be stimulated during octanoic acid-induced latency [5], as observed before during exponential growth in the presence of inhibitory concentrations of this acid [2]. The time dependent-pattern of ATPase activation during latency correlated with the decrease of the intracellular pH of individual cells, suggesting that this activation was triggered by intracellular acidification [5], as proposed for acetic acid-induced activation [4]. Very recent results concerning the acquisition of thermotolerance by yeast cells previously exposed for 10 minutes to a mild acid stress with HCl at pH 3.5 (leading to a slightly lower pH_i, compared with unstressed cells) but not by cells exposed to 50 mM acetic acid at pH 3.5 (leading to pH_i around 5.4) will also be presented. Interestingly, these cells pre-incubated with acetic acid exhibited a more active plasma membrane ATPase although this cell response was not enough to the adequate control of pH_i and acquisition of cross-stress tolerance.

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Ligninolytic Enzymes from Corncob and Barley Straw Cultures of *Phanerochaete chrysosporium* in Semi Solid State Conditions

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Key words: P. chrysosporium*, corncob, barley straw, MnP, LiP.*

In this work, the production of ligninolytic enzymes by *Phanerochaete chrysosporium* in stationary cultures, operating with barley straw or corncob as carriers, was studied. These carriers are utilized by the fungus both as an attachment place and as a source of nutrients.
Veratryl alcohol has been shown to activate the ligninolytic system as well as to increase the level of ligninolytic enzymes in *P. chrysosporium* by protecting ligninases against inactivation by H_2O_2 (Tonon and Odier, 1988) or against proteolytic decay (Faison *et a*l., 1986). However, the mechanism of stimulation by veratryl alcohol has not been determined, yet.
By supplementing the cultures with veratryl alcohol (final concentration 2 mM) it was enhanced two and five fold Manganese-dependent peroxidase (MnP) activities in barley straw and in corncob cultures, respectively. However, the addition of this alcohol had only effect on Lignin peroxidase (LiP) activities from corncob cultures. Moreover, it was found high laccase activities in corncob cultures, which showed that *P. chrysosporium* is able to produce laccase in spite of always held belief that laccase is absent in this microorganism (Table 1). This is agreement with a recent report by Dittmer *et al*. (1997) and with a previous work (Rodríguez Couto *et al*., 1997).
Some selected samples were studied by SDS-PAGE. Major protein bands appeared for molecular weights about 43 kDa and around 40 kDa, which agreed with previous results reported for MnP (de Jong, 1993) and Lip enzymes (Linko, 1991), respectively.

Table 1. Maximum MnP, LiP and laccase activities obtained

	MnP max.	LiP max.	Laccase max.
Straw	302	300	
Straw + veratryl	520	304	
Corncob	384	155	
Corncob + veratryl	1780	235	295

MnP activity was determined spectroscopically according to Kuwahara *et al*.(1984), LiP activity was measured by Archibald (1992) and laccase activity by Niku-Paavola e*t al*. (1990).

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Xylose Metabolism in *Candida guilliermondii:* Effect of pH on Xylitol Production and on Xylose Reductase Levels

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Keyword: *xylitol, xylose reductase, sugar cane bagasse*

The effect of pH on xylitol production and on xylose reductase activity in the yeast *Candida guilliermondii* FTI 20037 grown in sugar cane bagasse hydrolysate was investigated.

Bagasse hydrolysate, obtained by acid hydrolysis, was concentrated under vaccum and after clarification, initial pH was adjusted to 3.5, 5.0 and 7.0. Fermentations were carried out in ERLENMEYER flasks containing 50 mL of medium at 200 min^{-1}, 30 ^{0}C. For enzymatic analysis, cells were disintegrated by sonic disruption technique. Xylose reductase (E.C.1.1.1.21) and xylitol dehydrogenase activities (E.C 1.1.1.9) in the cell extracts were determined spectrophotometrically at 340 nm as described by ALEXANDER, 1985, with some adjustments in the method. One International Unit (IU) was defined as the amount of enzyme that catalyzes the oxidation/reduction of 1 μmol NAD(P)H/NAD^+/min under the assay conditions. Protein was determined by the method of BRADFORD, 1976. Sugars were analyzed by HPLC and cell concentration was measured by turbidimetry. The results of the present work revealed that the initial pH affected both sugar consumption and xylitol production. At pH 3.5, we observed a slow consumption of sugars and xylitol accumulation after 48h was very low (3.7 g/L). Although no significant differences in xylitol yields were found at pH 5.0 and 7.0 (0.71 $g.g^{-1}$ and 0.69 $g.g^{-1}$ respectively) after 48h, the initial pH of the medium markedly influenced the maximum volumetric productivity (0.39 $g.l^{-1}.h^{-1}$ and 0.56 $g.l^{-1}.h^{-1}$ for pH 5.0 and 7.0 respectively).

Regarding the key enzymes of xylose metabolism, xylose reductase especific activities at pH 7.0 were, in general, higher than the activities at pH 5.0 which were also higher than the especific activities at pH 3.5, along the fermentations. The high xylose reductase especific activity attained at pH 7.0 (0,15 $UI.mg^{-1}$) after 24h could explain the high volumetric productivity (0.42 $g.l^{-1}.h^{-1}$) found at the same pH value, which was 60% higher than that found at pH 5.0. The decrease in the productivity and XR activities when initial pH was <7.0 could probably be related to the inhibitory effect of the undissociated acetic acid molecules during xylose metabolism by the yeast, which occurs at acid pH values.

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The Use of Lignocellulosic Wastes for Xylanase Production by Mesophilic and Thermophilic Strains

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Keywords: *xylanase, xylosidase, celulase,* Aspergillus*,* Humicola

Faced with market, environmental and legislative pressures, the pulp and paper industry is modifying its pulping, bleaching and effluent treatment technologies to reduce the environmental impact of mill effluents. Enzymatic bleaching using xylanases is one of such technologies which in short term, offers the help to reduce substantially chlorine usage. The effectiveness of xylanase filamentous fungi production is largely influenced by the carbon source and in general, the carbon source has been estimated as the major cost factor in enzyme production.

The effect of medium composition on endoxylanase, β-xylosidase and total cellulase activity was studied. Sugar cane bagasse and corn cobs have been used for xylanase production by the mesophilic species *Aspergillus niger*, *Aspergillus awamori* and *Humicola fuscoatra* and by the thermophilic strain *Humicola* 14-1.

Semi-solid fermentation with all of them resulted about 2000 U/L of endoxylanase for genus *Aspergillus* over sugar cane bagasse and about 100000 U/L for *Humicola* over corn cobs. The highest endoxylanase production by *Humicola* was reached at 45^{o}C after 96-120 hours of semi-solid cultivation. In the optimized condition the production by the fungus reached 148 U/ml of thermophilic endoxylanase. On the other hand *Aspergillus niger* and *Aspergillus awamori* produced also high amounts of β-xylosidase reaching over 4500 U/L and 13000 U/L, respectively.

Cellulotytic activities (C1 and Cx) of *Aspergillus* grown on sugar cane bagasse are negligible while no celulase activity was detected on the supernatant of *Humicola* after growth on corn cobs, whether the cultivation was carried out on liquid or semi-solid fermentation.

The results have shown the feasibility of using semi-solid fermentation with a waste from agroindustry for celulase-free xylanase by filamentous fungi strains and support further investigation on purification and characterization of the enzymes.

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Biotransformation of Olive Mill Waste Water. Growth and Antimicrobial Activity of Olive Lactic Acid Bacteria

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Keywords: Olive mill waste water, Bacteriocins, Phenolic compounds

The lactic acid fermentation of green olive table is an economically important industry in Portugal. This fermentation is mainly carried out by *Lactobacillus plantarum*. The bacterial growth is often affected, limited or inhibited by the composition of brines, i.e, high levels of NaCl, phenolic compounds [4] and low pH and temperature of fermentation. On the other hand, olive mill waste waters (OMWW) are a toxic residue from olive oil production. The polyphenols of OMWW can inhibit the growth of some bacteria and Hep2 human cells [2]. The utilization of this product as part of a culture medium for the growth of *L. plantarum*, may be of interest to several points of view. Firstly, OMWW is a direct source of nutritive and toxic substances present in the pulp of olives. Consequently its utilization permits to study their effects on the growth and the metabolism of *L. plantarum*. A recent study indicates the possibility of using OMWW as part of a culture medium for the growth of *L. plantarum* has been tested [3]. A growth medium constituted by OMWW and some adjuvants such as tryptone and agar were used to the growth of *L. plantarum*. A liquid/solid system was employed. This biphasic system allows to harvest easily the diffusate medium to study the effect of OMWW on the bacterial growth, the metabolism and the production of an antimicrobial compound, and wich phenolic compounds this bacteria is able to use in its metabolism.The phenolic content was analized by HPLC until 72h [1]. It was verified that this acidophile/halophile bacteria can grow on OMWW but at a lower rate than on MRS broth. On the other hand, the production of antimicrobial compound, detected by a simultaneous method proposed by Bart ten Brink, is higher on OMWW than on MRS broth, comparing with biomass production. The combination of a solid and liquid culture medium, and the possibility to do some variations in the concentrations of the constitutive medium substances, in a way that resembles as close as possible the natural environmental of brines (4-8% NaCl, low pH and high levels of phenols) seems an important advantage to understand the real problems of table olive fermentations and its control. In summary, this bacteria can grow under stress condition (NaCl, pH, low temperature, high concentration of phenols), can degradate some phenolic compounds and always keeping its capacity of production an antimicrobial substance. Therefore it is possible that this bacteria can have a significative role on the biotransformation of OMWW.

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Biodegradation of Textile *Azo* Dyes by *Phanerochaete chrysosporium*

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Key-words: Azo *dyes,* P. chrysosporium*, biodegradation, decolorization, effluent treatment.*

Azo dyes are used extensively in the textile and dyestuff industries and effluents from these industrial processes are usually resistant to biological treatment.

Textile *azo* dyes with bioaccessible groups such as guaiacol and 2,6-dimethoxyphenol, for lignin-degrading fungus as *P. chrysosporium* were synthesised (Fig. 1).

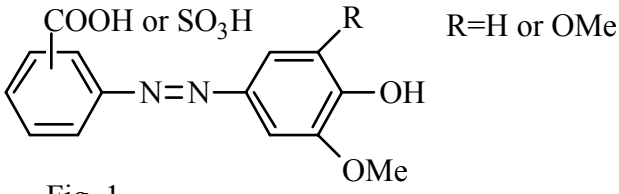
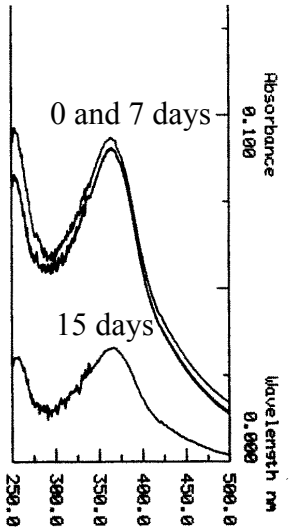


Fig. 1

Some of these dyes were studied in order to compare the biodegradation ability related to their chemical structure, when cultured with *P. chrysosporium* .

The degradation of dyes was followed by UV-Visible spectrophotometric assays studying the degree of decolorization by the decrease of the absorbance in the maximum wavelenght of the dye (Fig. 2) or by the possible formation of new products and by biomass production.



Degradation of

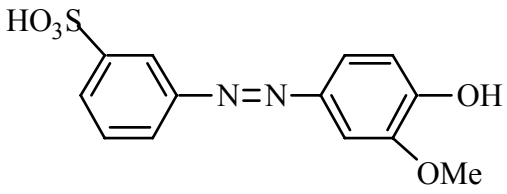


Fig. 2

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Comparative Study in the Production of Chitosan from *Mucor racemosus* and *Cunninghamela elegans* (Mucorales, Zygomycets)

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Keywords*: Chitosan; Zygomycetes;* Mucor racemosus *and* Cunninghamela elegans*.*

Chitosan is a natural polymer derived from chitin, a polysaccharide found in the exoskeleton of shellfish like shrimp or crabs. Technically speaking chitosan is a naturally occurring substance that is chemically similar to cellulose which is a plant fibre. It is a polysaccharide formed primarily of repeating units of beta (1-4) 2-amino-2-deoxy-D-glucose (or D-glucosamine). Chitosan has a great potential in the agriculture and biotechnology, biomedical and pharmaceutical industry. Commercially available chitosan is usually produced from shrimp and crabshell chitin by deacetylation using strong alkali. Such conversion appears to have to have limited potential for industrial acceptance because of seasonal and limited supply, high processing cost, processing difficulties, and inconsistent physiochemical characteristics of chitosan. However, chitosan also could be obtained in the vast majority of fungi (Zygomycets) as the principal fibrillar polymer of the cell wall. In this work the comparative study on chitosan contents between the *Mucor racemosus* and *Cunninghamela elegans* was made. The study was carried out using a inoculum of 10^5 spores/mL of *Mucor racemosus* and *Cunninghamela elegans* growning in medium described by Araki & Ito [1] in orbital shaker (80 rpm) at 28^{o}C during 96h. The chitosan extraction was carried out each 24h of fermentation as described by White et. al. [2] and identified by infrared spectrum. Later on to the process of physical identification the samples were hidrolysed chemically and the content of D-glucosamine was determined as described by Blix, G. [3]. The results showed that among the two samples of fungi studied the *Mucor racemosus* showed better yield of isolated chitosan with 35,1mg chitosan/g cell biomass, while *Cunninghamela elegans* showed 20,5mg chitosan/g cell biomass in the first hour of cultivation. However, these yield decreased in the later hours of cultivation in both. The infrared spectrum of the insoluble fraction showed similar peaks those showed by the standard chitosan (sigma). From, these results, we conclude that chitosan production by fungi is a good alternative compared with deacetylation of chitin. Furthermore, the *Mucor racemosus* showed to be a better producer of chitosan than *Cunninghamela elegans*.

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Biotechnological Application of Thermophilic Actinomycetes in the Pulp and Paper Industry

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Actinomycetes, hemicellulases, oxidative enzyme, biobleaching, pulp

Nowadays the screening for new hemicellulolytic and oxidative enzymes to be applied in biopulping and biobleaching processes is one of the main objetives in biotechnological research. The use of these enzymes in pulp and paper industry involves savings in energy and chemicals with a concomitant decrease in the environmental pollution.

In our laboratory, several thermophilic strains of actinomycetes has been isolated from volcanic soils from Nicaragua. For this study four of these strains have been selected for their ability to decolourise polymeric dyes to show up lignin degradation (Poly R-478 and Remazol Brilliant Blue). Moreover, in order to establish the potential application of these strains in pulp and paper industry, a complete screening of hemicellulolytic and oxidative enzymes produced by these strains was performed. The production of these enzymes were carried out under different culture conditions: submerged cultures in shaking flasks and solid-state fermentation in agar plates using in both cases carboxymethylcellulose and wheat straw as inducers.

In all strains the highest levels of xylanase activity were obtained in submerged culture and the maximum level achieved for this activity was 20 U mL^{-1}. By contrast, oxidative enzymes (L-DOPA peroxidase and ABTS phenoloxidase) achieved maximum values in solid state conditions (maximum value for L-DOPA peroxidase: 0.6 U mL^{-1} and maximum value for ABTS phenoloxidase: 3 U mL^{-1}). In all cases, the presence of wheat straw was necessary to induce both hemicellulolytic and oxidative enzymes. In solid-state conditions water activity (a_w) resulted to be an important parameter for the production of the oxidative enzymes.

Zimograms of L-DOPA peroxidase and ABTS phenoloxidase activities produced in solid-state fermentation by the selected strains revealed different patterns of bands depending of the culture conditions.

In order to know the potential application of the strain which produced the highest levels of phenoloxidase activity in pulp and paper industry, a biobleaching assay was performed. The treatment of eucalyptus unbleached pulp with a crude enzyme supernatant (2 U mL^{-1} of phenoloxidase per gram of pulp) of this strain and further alkaline extraction resulted in a noticeable release of colour (A_{465}) and chromophores (A_{237}, A_{254}, A_{280}) from the substrate.

The high optimum temperature (70°C) of the ABTS phenoloxidase produced by the assayed strain and the possitive effect obtained on the unbleached eucalyptus pulp demonstrated the suitability of this strain for biobleaching purposes.

Isolation, characterization and expression of genes encoding enzymes with lipase activity in *Yarrowia lipolytica*

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***Keywords**: yeast, lipases*

Yarrowia lipolytica is a dimophic yeast with very high secretory capacities and able to grow in alkanes and fatty acids as the sole carbon source. Using PCR, we have isolated two DNA fragments that after sequencing show homology with fungal lipases. By genomic DNA subcloning, we have isolated a gene nominated *YlLIP1* located on *Y. lipolytica* chromosome IV. The gene presents an ORF of 1458 bp which encodes a putative protein of 486 amino acids with an apparent molecular weight of 55.4 kDa. The nucleotide sequence of the 5' and 3' ends shows the consensus signals for translation initiation and transcription termination motifs in the yeast.
A disruption of *YlLIP1* has been performed. The deleted *ΔYllip1* strain shows
lower lipase activity in comparison to the wild type.

The DNA encoding YlLip1p was expressed in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Both yeast species are able to synthesize a functional enzyme under the control of *GAL1* promoter.

We are currently isolating the second gene, both in the wild type and in the *ΔYllip1* strain.

Relação entre a Actividade da Lacase Produzida por *Lentinus edodes* e a Formação de Carpóforos

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***PALAVRAS CHAVE**: Biodegradação,* Lentinus edodes*, lacase, actividade enzimática*

Os resíduos lenhocelulósicos são constituídos maioritariamente por celulose, lenhina e hemiceluloses, que são os polímeros naturais mais abundantes na biosfera, sendo contudo a sua estrutura molecular uma barreira à sua biodegradação. A biodegradação da lenhina é de extrema importância para as transformações biotecnológicas dos resíduos lenhocelulósicos, dado ser ela o maior obstáculo para uma utilização eficiente desses mesmos resíduos, numa vasta gama de processos industriais.

A capacidade de degradação da lenhina pelos basidiomicetas tem sido associada à produção de complexos enzimáticos formados, principalmente, por enzimas do tipo das fenoloxidases. Estudos feitos sobre estas enzimas revelam que elas podem, teoricamente, levar a cabo a oxidação e degradação da lenhina, tendo, também, como principal efeito, a polimerização desta molécula. A síntese do complexo enzimático e a actividade das enzimas, que o compõem, dependem da natureza e composição do substrato. Elementos como o carbono, oxigénio, hidrogénio, azoto, fósforo, sódio e enxofre, são necessários em grandes quantidades e, embora em menores concentrações, existem determinados elementos minerais também essenciais, absorvidos normalmente sob a forma iónica, que são igualmente importantes.

Neste trabalho estuda-se a influência da concentração de alguns catiões (Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, Na^{+}, Fe^{3+} e Zn^{2+}), na actividade enzimática da lacase produzida por uma estirpe de *Lentinus edodes* (Berk.) Sing. (*L.e.* 1-3), bem como na capacidade de formação de primórdios e a sua diferenciação em carpóforos.

Aplicando a técnica de análise estatística denominada "Cluster Analysis", é possível determinar quais os catiões/concentrações com efeito semelhante no traçado da curva representativa da evolução da actividade da lacase.

Os catiões estudados demonstraram ter influência na actividade enzimática da lacase, produzida pela *L.e.* 1-3, bem como na formação de primórdios. As frutificações produzidas, na cultura em meio líquido pela estirpe de *L. edodes*, embora de tamanho reduzido e com uma tonalidade bastante clara (raramente com a cutícula diferenciada nas zonas tão características da espécie) apresentaram de uma maneira geral, morfologia semelhante às frutificações produzidas em substrato sólido. Os catiões que tiveram efeito mais semelhante, entre si, relativamente ao traçado da curva representativa da evolução da lacase foram o sódio (0,08 ppm) e o zinco (0,0766 ppm).

Comparison of *Aspergillus niger* Pectinase Overproducing Mutants in Different Water Activities of the Culture Medium

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***Key words**: water activity,* Aspergillus, *mutants, pectinases.*

The wild type strain *Aspergillus niger* C28B25, previously characterized as pectinase producer[2], has been used to isolated a series of mutants using a novel approach[1] . Two criteria to select the mutants have been used: resistance to 2-deoxyglucose (2DG) and the ability to grow and sporulate normally in two different water activities (a_w = 0.96 and 0.99), thus two classes of mutants were isolated, series AW96 and AW99, respectively. Growth and pectinase overproduction patterns in such mutants were accordingly to their adaptation to either a_w value. Specific growth rate (μ) was estimated in Petri dishes using an image processing technique[3] to analyze micelial growth. Pectinase production was determined both in solid stated fermentation (SSF, a_w=0.96), using coffee pulp as a substrate and in submerged fermentation (SmF, a_w=0.99), using pectin as a carbon source. After induction of arginine auxotrophy in one representative strain from each series, a diploid was isolated by means of a parasexual cross between such strains and it was selected for further analysis.
This diploid showed the wild type phenotype of 2DG sensitivity, suggesting that there are at least two different *loci* involved in 2DG resistance in *A. niger*. However, pectinase production profiles by this diploid either in SSF and SmF were not as those previously determined for the wild type strain, since diploid strain retained overproduction pattern in SmF. Thus 2DG resistance phenotype seem to be independent from pectinase overproduction.
Finally, comparison of μ and relative enzymatic activities (q_s and q_x) showed a reciprocal relation, depending on whether the strains were grown on a_w = 0.96 or a_w = 0.99. This supports the hypothesis of reciprocal regulatory relations between μ and q_s or q_x. Thus, this also suggests a compromise between secretion vesicles and those involved in cell wall formation, both acting in the hyphae tips. These results can be helpful in designing new techniques to isolated overproducing strains in media with different a_w values.

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Microflora Variation in Biofilms Formed on AISI-1020 Carbon Steel and Brass Coupons

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Keywords: biofilm, carbon steel, brass, cooling system.

Industrial cooling systems use seawater or freshwater, depending on the location of the industry and on the availability of either one. Either seawater or freshwater have microorganisms that are able to adhere to surfaces forming biofilms. In cooling systems, biofilms constitutes a serious problem. It may cause reduction in the efficiency of heat transfer, resistance to fluid flow, as well as microbially induced corrosion (MIC).

The experiments were conducted in an industrial cooling system that uses freshwater on its heat exchangers. AISI-1020 carbon steel and brass (70% Cu, 30% Zn) coupons were placed in bypasses installed in the system at a point that allowed the circulation of the cooling water. The water flow inside the duct had an average speed of 0.3 m^3/seg. During the experimental period the temperature and the pH varied from 21 to 27^oC and from 6.9 to 7.6 respectively.The coupons were withdrawn with 30 and 60 days of exposure for quantification of aerobic and anaerobic bacteria. The bacterial adherence was confirmed by Scanning Electron Microscopy (SEM).

After 30 days of coupons exposure, the value of aerobic bacteria corresponded to 98% and to 30% of the total bacteria quantified in biofilms formed on carbon steel and brass coupons respectively. After 60 days, the percentage value recorded for aerobic bacteria in biofilms formed on carbon steel coupons was 95%. This result showed that there was only a slight variation of percentage in comparison to the value found within 30 days.However, after 60 days of brass coupons exposure, there was an expressive decrease on the percentage of the aerobic bacteria quantified. At that time, the aerobic bacteria in biofilms of brass coupons corresponded to only 3% of the total bacteria quantified.

Considering anaerobic bacteria, in biofilms of carbon steel coupons, the number of these microorganisms corresponded to only 2% and 5% of the total bacteria quantified, after 30 and 60 days of exposure, respectively. Differently, there was an increase in the number of these bacteria in biofilms formed on brass coupons according to time of exposure; 70% after 30 days and 97% after 60 days.

The results showed formation of a predominantly aerobic biofilm on carbon steel coupons. In brass coupons, however, there was predominance of an anaerobic biofilm. A possible explanation to this fact is the higher resistance these bacteria have to the toxic effect of ion Cu(II) present in brass.
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Fumaric Acid Production from Cassava Bagasse by *Rhizopus formosa*

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***Keywords**:* Rhizopus*, cassava residues, fumaric acid*

The southern part of Brazil (mainly the Parana State) is a great producer of cassava roots, amounting 3.2 millions of tons in 1997 [1]. The cassava root processing for producing starch gives daily a residue called "cassava bagasse", which still contains 50-70% of starch (dry weight). Other residues are generated in the process, namely the "água vegetal" and the external peels. The starch content of these residues is atractive to look up in technologies in order to obtain more valuable products. Environmental problems can also not be ignored, which emphasizes the search of new alternatives for these residues. One possibility of re-utilization of this starchy residues is its conversion to reducing sugars, which are easily consumed by microorganisms. The hydrolysis process performed by enzymes make the process more suitable and specific than the acid process; it requires simple equipments, low temperature and neutral pH. Besides, the enzymatic hydrolysis doesn't affect the lipidic or proteic components.
Many authors [2,3,4] use glucose as substrate and the fungi *Rhizopus arrhizus* and *Rhizopus oryzae* for fumaric acid production. However, the production of this metabolite using *Rhizopus formosa* seems to be new. This work aims to study the fumaric acid production by *Rhizopus formosa*, using the enzymatic hydrolysate of cassava wastes (mainly the cassava bagasse). The hydrolysis conditions of the process, such as incubation time, added enzyme amount and the granulometric size of the raw material were tested using a statistical experimental design. The best conditions for hydrolysis were the size of cassava bagasse less then 0.84 mm, 100 μL of Thermamyl (1 h, 90 °C) and 471 μL of AMG (Novo Nordisk) (24 h, 60 °C) added per 145 g bagasse. Afterwords, the conditions of fumaric acid production were studied. The results showed that *Rhizopus formosa* produced fumaric acid (21.3 g/L) using batch conditions, C/N ratio of 168, and 120 h of fermentation. This value could be increased using a longer time of fermentation. Nevertheless, at 168 h of fermentation the fumaric acid production decreased, probably due to the consumption of metabolic products and to the lack of glucose in the culture medium.

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The Effect of Cytochrome P-450 Inducers in Monolayer Cultured Hepatocytes

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***Keywords**: cell culture, immortalised hepatocytes, cytochome P-450, inducers*

The effect of cytochrome P-450 inducers was evaluated in enzymatic activities of phase I and phase II reactions in three different clones of hepatocytes. The studied hepatocytes, two adult rat cell lines (P9 and LQC3) were isolated by electroporation (LQC3) and by calcium phosphate precipitation method (P9) and a human cell line (HepG2) obtained from ECACC, UK. They were cultured in monolayer and exposed to three well-known inducers of cytochome P-450. These have been choosen according to their capacity to induce different subfamilies of cytochome P-450. Namely, 3-methilcholanthrene inducer of 1A subfamily, phenobarbital inducer of 2B subfamily and dexamethasone as inducer of 3A subfamily. Phase I activities were analysed by quantification of testosterone hidroxylates by HPLC and measurement of 7-ethoxyresorufin O-deethylase and 7-ethoxycoumarin O-deethylase activities by spectrophotometric assays. Phase II reactions were evaluated by fluorescence measurements of glutathione and glutathione, and activities of S-transferase and UDP- glucuronyltransferase. The cellular viability was followed by the study of catalytic activity of lactate dehydrogenase and all values are expressed by μg of cellular protein, quantified by Pierce`s method.

Production of the Antibiotic Undecylprodigiosin in *Streptomyces coelicolor* A3(2)

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Keywords: *Undecylprodigiosin (Red),* Streptomyces coelicolor *A3(2), antibiotic.*

Undecylprodigiosin is a tri-pyrolle pigmented antibiotic made by *Streptomyces coelicolor* A3(2). It is made from mono- and bipyrrole precursors by a convergent branched pathway (1, 2, 3) and is the major component (ca. 60%) of a mixture of four red pigments made by the strain, of which butylcycloheptylprodigiosin (ca. 34%) is the next most abundant compound.

Recent nucleotide sequence analysis of the right end of the *red* cluster has identified three open reading frames (*redX, redW* and *redY*) located between the regulatory genes *redD* and *redZ* that are likely to be involved in production of the monopyrrole moiety (4).

A deletion of *redX*, *redY* and *redW* has been created using a non-replicating vector (pSET151) carrying flanking regions of the *red* cluster and a selectable marker. To facilitate screening for, and analysis of the required mutants, the hosts for these experiments wereM511 and M52, *act*II-ORF4 deletion mutants of *S. coelicolor* A3(2) strains M145 and M600 respectively, which are deficient in the production of the other pigmented (blue) antibiotic made by these strains. The *redXWY* mutants (M591 and M592) do not produce the red pigment, indicating that these genes are required for the production of undecylprodigiosin. M591 and M592 are being characterised by cross-feeding and co-synthesis with previously isolated *red* mutants, by mutasynthesis using monopyrrole analogues, and by analysis of the metabolites produced by TLC, HPLC (and possibly mass spectrometry) to define more precisely the role these genes play in undecylprodigiosin production.

To the right of *redD* in the *S. coelicolor* A3(2) chromosome lies the gene "*trkA*", which is believed to encode a membrane-associated protein involved in potassium uptake and potentially osmoregulation (5). *In vitro* transcription studies indicate that the *redD* promoter and three of the four "*trkA*" promoters are probably recognized by *S. coelicolor* ^{hrdD}, one of a family of four closely related factors that includes ^{hrdB}, the major and essential factor of *S. coelicolor* (6). This, and the possibility that undecylprodigiosin production may be osmotically regulated, implies that both genes might be coordinately regulated. Consequently, "*trkA*" is being disrupted and its role in Red production under conditions of osmotic stress assessed.

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Microbial, Selective Hydrolysis of Nucleotides

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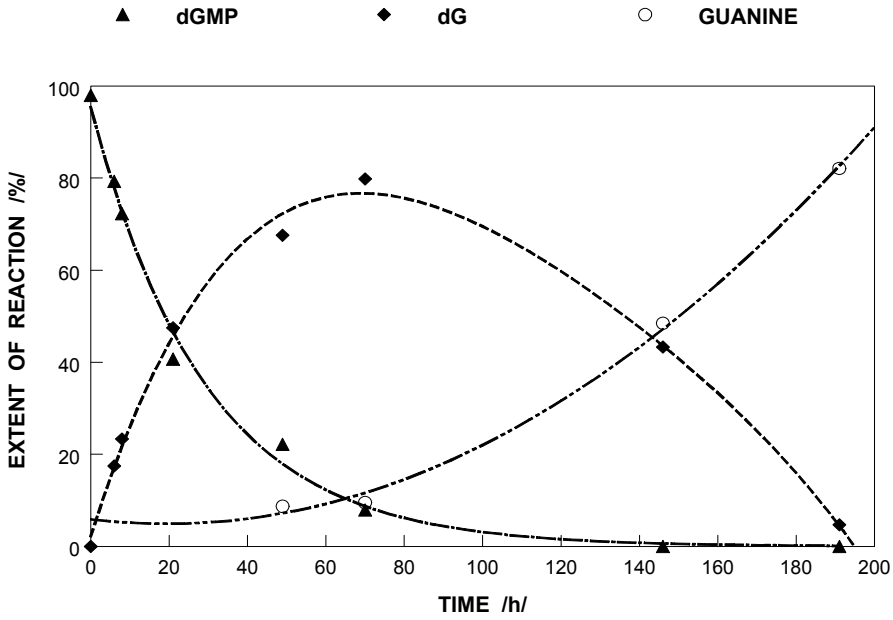
Keywords: *nucleotides, nucleosides, microbial hydrolysis*

Xanthomonas maltophilia[1] and *Pseudomonas putida* (G(-) bacteria) were used to hydrolyse the following nucleotides: 2'-deoxyadenosine-5'-monophosphoric acid (dAMP), 2'-deoxycytidine-5'-monophosphoric acid (dCMP), 2'-deoxy-guanosine-5'-monophosphoric acid (dGMP), tymidine-5'-monophosphoric acid (dTMP). Reaction was performed in aqueous buffor, pH=7 with 0,5% glucose, concentration of nucleotides was 1mg/ml. The following products were obtained: 2'-deoxyadenosine (dA), 2'-deoxycytidine (dC), 2'-deoxyguanosine (dG) and tymidine (dT) or respective free amines.

Nucleotide conversion - time dependence, using *Xanthomonas maltofilia* is shown on the figure below.



2-Deoxyguanosine was obtained with 80% yield and then selectively hydrolysed yielding guanine. Yields of other nucleosides were as follows: dA - 86.4%, dC - 96.8%, dT - 81%. We found that density of bacteria influenced yield of reaction. The reaction was controlled by HPLC (spectrophotometric detector UV/VIS, Supelcosil LC-18-DB column, mobile phase: aqueous buffor, pH=4-NaH_2PO_4, flow rate 1ml/min, injection volume 20µl, detection at λ=254nm).

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Evidence for the Functional Importance of Tryptophan Residues in D-Amino Acid Oxidase from *Rhodotorula gracilis*

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D-amino acid oxidase, Rhodotorula gracilis*, catalytic residues.*

D-amino acid oxidase catalyzes the oxidation of D-amino acids to the corresponding imino acids which are further hydrolysed non-enzymatically to α-keto acids and ammonia. The native enzyme is a dimer of two identically subunits and contains one molecule of tightly non-covalently bounded FAD [1]. The elevated turnover [2], the tight binding to the coenzyme FAD, the stable dimeric aggregation state and its possibility to oxidize cephalosporin C [3] are relevant properties to the explotation of this flavoenzyme in biotechnological and industrial applications.

Chemical modification studies have been carried out to identify relevant residues involved in catalysis. To data, arginine [4], lysine [5] and histidine [6] residues have been described essential for the enzymatic activity.

In order to look for other important residues for enzyme activity, we have performed chemical inactivation studies by using pure enzyme preparations[6]. Both apo- and holoenzyme have been treated with increasing concentrations of NBS (N-bromosuccinimide), specific reagent for tryptophan residues [7], and TNM (tetranitromethane) and N-acetylimidazole specific reagents for tyrosine residues [7]. Complete inactivation was observed in both apo-and holoenzyme when treated with 0.1 mM of NBS; by contrast, neither TNM nor N-acetylimidazole inactivated them. To characterize the extension of modification the difference UV absorption spectra of both apo- and holoenzyme were recorded. Also, the fluorescence emission spectra of unmodified and treated enzymes were recorded. Our results have shown that D-amino acid oxidase from *Rhodotorula gracilis* has two essential tryptophan residues for enzyme activity, however, no essential tyrosines could be detected in *Rhodotorula gracilis* enzyme by contrast with other D-amino acid oxidases from other sources [8].

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The Effect of Different Salinities on the Aqueous Flow in the Formation of Biofilms

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Keywords : Biofilm, Biocorrosion, SRB

The coasts of the oceans provide suitable conditions for the installation of petroleum refineries and petrochemical industries due to transportation facilities and availability of salt water that can be used for various applications, such as the cooling of equipments and products, and injection waters for the secondary recovery ofpetroleum. The seawater presents several microorganisms that deposit on metalic surfaces forming biofilms. Biofilms can acelerate corrosion process, many times due to the salinity of the water. Beyond this, microorganisms can be sensitive to NaCl levels, consequently affecting, both the formation of biofilms as well as the corrosion process.

Experiments were done using carbon steel AISI 1020 coupons internally located in PVC dutes, in the direction of the aqueous flow, aiming at the formation of the biofilms, analogously to what would happen in the internal walls of industrial tubes. Seawater has been used with different NaCl concentrations (from 12,8 to 80 g/L), flow-rate of 8 LPM (0,48 m^3/h), temperature of 30 $\pm 1^0$C and pH 8,0.

After 30 days of contact of the coupons with the aqueous flow, biofilms were analised in relation to the number of aerobic bacteria, anaerobic bacteria, sulfate-reducing bacteria (SRB), ferrobacterias, fungi and total sulfides. Coupons containing biofilms and after the removal of the biofilms were evaluated by Scanning Electron Microscopy (SEM).

For the 12,8 g/L salinity (expressed in NaCl) aerobic bacteria predominated, corresponding to 36% of the total number of bacteria present in the biofilms, 26% ferrobacteria, 16% of anaerobic bacteria, 13% SRB and 9% fungi. Higher salinities, such as 35, 50, and 80 g/L promoted higher adherence of ferrobacteria, reaching 35-55% of the total microorganisms.

Specific quantifications of the various microorganisms indicated that aerobic, anaerobic and SRB bacteria were sensitive to increasing salinity, the opposite behaviour observed for ferrobacteria and fungi. No correlation was observed between the levels of sulfide formed and the amount of SRB cells present in the biofilms. The extent of resistence of the carbon steel to biocorrosion depended on the salinity levels of the water.

Formulation of Fermentation Media from Agricultural Wastes for Xylitol Production

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Keywords: *acid hydrolysis, autohydrolysis, barley, corn, hemicelluloses, xylose*

INTRODUCTION

Solubilization and depolymerization of hemicelluloses from lignocellulosics can be performed by means of either a mild acid hydrolysis or a technology free of chemicals consisting of hydrothermal treatment. Liquors from both kinds of treatments contain extractives and solubilized hemicelluloses (mostly xylose and xylose oligomers). Xylose can be bioconverted into xylitol, a sweetener with dietary and clinical properties, but the feasibility of using lignocellulosics for fermentation is affected by the concentration of both xylose and inhibitory compounds [1].

This work deals with the solubilization of hemicelluloses by either acid prehydrolysis or hydrothermal treatment of agricultural wastes and further detoxification by solvent extraction [2] to remove fermentation inhibitors such as acetic acid and lignin-derived compounds.

MATERIALS AND METHODS

Agricultural residues. Barley, corn cobs and leaves locally collected were dried, ground to 1 mm and stored until use. Their composition was determined using standard methods.

Hydrothermal treatment. The ground materials were subjected to autohydrolysis in a Parr reactor at 160-200 °C during 1-3 h with 8 g water/g dry solid.

Acid hydrolysis. The ground materials were hydrolysed with 1-3 % H_2SO_4 at 130 °C during 1-5 h using a liquid:solid ratio=8 g water/g dry solid.

Solvent extraction. Operational conditions for the solvent extraction with ethyl acetate were optimized for maximing the removal of lignin-derived compounds.

Microorganisms. *Debaryomyces hansenii* NRRL Y-7426 was used.

Fermentation. Culture media made from raw and from detoxified liquors were fermented in an orbital shaker at 30 °C, pH=6.5 and 200 rpm under microaerobiosis.

Analytical methods. Xylose, glucose, xylitol, acetic acid and furfural were analysed by HPLC, using a Interaction Ion column, mobile phase 0.005 M H_2SO_4, flow rate 0.4 mL/min and IR and UV detection. Water-soluble lignin was semiquatitatively estimated by spectrophotometry.

RESULTS

Operational conditions for both acid hydrolysis and autohydrolysis were selected as those leading to high xylose concentrations with and minimum furfural contents. Xylose concentrations in the range 10-40 g/L were attained in liquors from autohydrolysed barley and from acid-hydrolysed corn cobs. Solvent extraction of acid-hydrolysed liquors with ethyl acetate removed acetic acid almost quantitatively and more than 70 % of the solubilized lignin. The sugar solutions obtained after detoxification show good potential as fermentation media.

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Aspectos Cinéticos de la Transformación del Ácido Oleico en el Ácido 10, Hidroxi-8E-octadecenoico por *Pseudomonas sp* 42A2 NCIB 40044

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Keywords: Pseudomonas*, ácido oleico, biotransformación, ácidos grasos hidroxilados, cinética.*

Las modificaciones de los ácidos grasos insaturados llevadas a cabo mediante microorganismos, ofrecen la posibilidad de obtener nuevos compuestos en condiciones más suaves y con mayor especificidad que mediante procesos químicos.

La introducción de grupos funcionales aumenta la reactividad de la molécula, los ácidos grasos hidroxilados tienen propiedades de emulsionantes, tensioactivos, lubricantes, plastificantes y se aplican, entre otras, en la industria de detergentes, pinturas, de síntesis de polímeros y resinas.

Los ácidos grasos hidroxilados ocupan un lugar importante entre los productos naturales debido a sus propiedades biológicas. Estos productos, denominados recientemente oxilipinas, forman parte del sistema defensivo de las plantas[1]. Entre otros, se ha aislado el ácido 10, hidroxi-8E-octadecenoico aislado en plantas de *Oryzae thyphina* por su actividad fungitóxica frente a hongos fitotóxicos como *Cladosporium plhei* i *Epichloe thyphina* [2].

En el estudio del uso alternativo de substratos renovables, se ha estudiado la utilización del ácido oleico de calidad industrial como substrato para la producción de nuevos derivados mediante biotransformación.

En el transcurso de la producción del ácido 7,10 dihidroxi-8E-octadecenoico [3], se aisló y caracterizó un nuevo intermediario, el ácido 10,hidroxi-8E-octadecenoico [4]. El parámetro cultural que más afecta a la transformación es el oxígeno [3]. En el trabajo que se presenta, se estudia el efecto del oxígeno en el crecimiento y la producción del ácido 10,hidroxi-8E-octadecenoico a partir del ácido oleico por *Pseudomonas* 42A2 .

Del estudio cinético llevado a cabo a diferentes K_La (15-200), estudiadas, se desprende que la producción aumenta desde 0,65 g/l a 7,4 g/l; que la producción específica (q_p)aumenta exponencialmente con la velocidad de crecimiento y que la producción volumétrica aumenta linealmente con la K_La.

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Biotechnological Applications of Acidophilic Fungi

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***Keywords**: metal resistance, metal sequestering, bioremediation, pharmacology, enzymes.*

We have explored different biotechnological applications of our collection of acidophilic fungi (700 isolates) obtained from the Tinto River, an extreme environment of low pH (pH 2-2.5) and high concentration of heavy metals. Due to the unexpected level of biodiversity found in the Tinto River we postulate that the microorganisms found here were well adapted to perform at the extreme conditions of the habitat. Different screening programs were developed to evaluate metal resistance profiles, metal sequestering properties, production of antibiotics with different specificity, and production of extracellular enzymes of technological and biomedical interest by representatives of the main groups of fungi isolated from the Tinto River. The results showed that some of the isolates are resistant to high concentrations of toxic metals. Also, some of the resistant strains are capable to specifically sequester metals with high efficiency and at concentrations relevant to industrial interests (metallurgy). Of course, these fungi can also be used to develop bioremediation technologies for metal-polluted waters in which recovery and elimination can be performed at the same time. In addition, some of the filamentous fungi isolated from the Tinto Rivershow interesting antibacterial and antitumoral activities, together with enzymatic activities that exhibit activities at acidic pH. The current status of the biotechnological applications studies of the Rio Tinto fungi will be presented.

Influencia de las Condiciones de Fermentación en la Actividad Sintetasa de las Lipasas de *Candida rugosa* Y *Rhizomucor miehei*

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Palabras clave: lipasa, fermentación, inductor, actividad sintetasa

Numerosos estudios revelan la actividad sintetasa y la enantioselectividad de las lipasas de *Candida Rugosa* (CRL) y *Rhizomucor miehei* (RML). En principio se pensaba que eran enzimas inducibles sólo en presencia de triglicéridos. No es así, pues la lipasa es inducida también por diferentes sustratos que no son susceptibles, a priori, de ser degradados por dicho biocatalizador.

Se ha procedido al cultivo de *Rhizomucor miehei*, y de *Candida rugosa* en distintos medios y con diferentes inductores. Para la levadura de *Candida rugosa*, se emplearon dos medios, uno con fuente de carbono adicional [1], y otro con el inductor como única fuente carbonada [2]. En ambos medios se han empleado distintos inductores, demostrándose hasta el momento que el ácido oleico y el dodecanol resultan ser los que mejores resultados presentan a la hora de producción de lipasas. Por su parte, el *Rhizomucor miehei* ha sido cultivado en un medio rico en extracto de malta [3], en un medio con extracto de levadura [4] y en un medio sin fuente adicional de carbono [2], empleándose hasta el momento aceite de oliva, aceite de girasol, y ácido oleico como inductores, siendo el medio basado en extracto de malta con aceite de oliva como inductor el que mejores resultados ha proporcionado hasta hoy. Las lipasas crudas presentan diferente actividad lipásica y esterásica, según el inductor empleado y su porcentaje.

Las enzimas crudas obtenidas se han usado en una serie de reacciones standard para evaluar los cambios en la enantioselectividad y en la actividad sintetasa de las lipasas producidas. Estas reacciones son: esterificación del R y del S-Ketoprofeno, esterificación del 1-feniletanol, resolución de aminas quirales por vía alcoxicarbonilación, y por vía de formación de amidas.

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Comparison of Tannase Production (Tannin-Acyl Hidrolase) by *Aspergillus niger* on Liquid and Solid State Fermentation on Polyurethane Foam

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Key Words: Tannase, Solid-State Fermentation, Polyurethane foam, Production, Stability

Introduction. Tannase (tannase acyl hydrolase E.C: 3.1.20) breaks down tannic acid, producing gallic acid and glucose, [1] and it can be used to reduce tannins concentration on foods, beverages and effluents from industry. As the level of tannase production by *Aspergillus niger* on liquid culture (SmF) is very low, solid state fermentation (SSF) had been suggested as an alternative method for its production, since obtained levels of tannase are higher on solid sustrates. A much easier tannase secretion on SSF by this organism has also been found [2]. In this work, we compare tannase production by *Aspergillus niger* in SmF and SSF, we also discuss the differences of production in the fermentation systems. Foam polyurethane (PUF) was used as an inert support on SSF[3], in this way, we can measure biomass (X=g/L) and reduce contaminants on medium culture produce by support degradation.

Methodology. Strains Aa-20, V9-A35 and C13-B35 of *Aspergillus niger* from ORSTOM-UAM collection were used. Strains were grown on basal mineral medium [2] containing 2,5 % glucose and 1% tannic acid. Cultures on SSF were carried out on washed and dried polyurethane foam cubes (PUF) (ρ=17g/L), impregnated with liquid medium (30 mL/g PUF) which was inoculated with $2x10^7$ spores/g of carbon source. SmF cultures were conducted in 125 mL Erlermeyer flask containing 30 mL of liquid medium, inoculated with $2x10^7$ spores/mL, and then agitated at 200 rpm (T=30°C). We analyse biomass (X=g/L) by gravimetry; tannase activity by HPLC technique [4] and proteolytic activity with azocoll [5]. Protein total excreted (PTE=mg/L) was evaluated by Bradford [6].

Results. When we compare PE for the three strains tested (from t=24 h, to t=120h) we found for SSF, that PE showed a proportional increase with that observed with biomass (PE $\approx -\beta\Delta X$), in contrast for SmF, PE diminished as biomass increased. Reduction of PE in SmF was associated with a high proteolytic activity in the medium, which was not detected on SSF. Specific activity (PE/PTE) was approximately 14,000 U/(g PTE) and 650 U/(g PTE) for SSF and SmF, respectively. Thus, enzymatic titres for SSF and SmF were PE (380 U/L) and PE (480 u/L), respectively.

Conclusion. Although, tannase production by the three strain of *A. niger* were very similar for solid and liquid techniques, the tannase produced by SSF was more stable and total protein were lower; moreover, we do not observe undesirable proteolytic enzymes production on SSF fermentation. As it has been shown by these results, SSF has advantages for tannase production in terms of an increment in purity and stability when compared to SmF techniques.

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Bioengineering/Bioprocess Engineering

Cuantificación de Componentes Intracelulares Mediante Citometría de Flujo en la Levadura *Candida Bombicola*

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Palabras Clave: Citometría, fluorimetría, cuantificación, lípidos intracelulares

La necesidad de una mejor descripción de las transformaciones con microorganismos ha llevado a plantear modelos cinéticos cada vez más complejos[1,2]. Dentro de los diferentes tipos de modelos, los que proporcionan una mejor descripción de la evolución de los procesos con microorganismos son los Modelos Metabólicos, que tratan el sistema como una red compleja de reacciones. Dentro de estos modelos, hay que distinguir los que consideran la evolución de especies intracelulares, normalmente denominados Modelos Químicamente Estructurados (MQE), o también Modelos de Célula. Los MQE son capaces de describir la evolución de diferentes componentes durante el crecimiento o la producción teniendo en cuenta tanto el metabolismo de la fuente carbonada como el de la nitrogenada. El desarrollo de MQE plantea la necesidad de analizar y cuantificar componentes intracelulares, debido a que su concentración (g/L) se encuentra en las ecuaciones cinéticas implicadas en el esquema de reacción planteado para cada modelo de este tipo.

En este trabajo se aborda la obtención de datos experimentales, de la evolución de componentes intracelulares, en la producción de un soforolípido producido por la levadura *Candida bombicola* utilizando Citometría de Flujo (CMF). Esta técnica se revela como una herramienta útil para estos estudios, siendo empleada para el análisis de células individuales en sistemas microbianos, proporcionando una información que, a menudo, no es posible obtener mediante otros tipos de técnicas. Sin embargo, los datos obtenidos mediante CMF no pueden ser empleados directamente en la formulación de modelos, ni en la determinación de sus parámetros, ya que los datos proporcionados por la técnica son en unidades relativas. Para transformar las unidades relativas en valores absolutos- esto es, de concentración (g/L)- se ha realizado un calibrado para diversos componentes intracelulares de *Candida bombicola* con el apoyo de otras técnicas de análisis que permiten la cuantificación: Espectrofluorimetría (ESF) para DNA, RNA y proteínas y Espectrofotometría de Absorción (EFA) para lípidos intracelulares. Los datos obtenidos por ambas técnicas se pueden expresar en concentración (g/L) por extrapolación del valor medido de la intensidad de fluorescencia o absorbancia a 500 nm, en las respectivas curvas patrón obtenidas para cada componente. La misma muestra, además, fue analizada mediante CMF, correlacionándose con los resultados obtenidos con las técnicas cuantitativas empleadas en cada caso. De esta manera se ha determinado la evolución de las especies antes citadas a lo largo del crecimiento de la levadura *Candida bombicola*, así como, en experimentos aparte, en la producción de soforolípidos, cuando a la levadura se le suministran dos fuentes carbonadas, una hidrófila –glucosa- y otra lipófila –aceite de girasol-. Estos datos, antes de ser aplicados en la formulación de Modelos Cinéticos Estructurados, son de gran ayuda para proponer rutas metabólicas que puedan constituir un metabolismo simplificado del crecimiento, del catabolismo de nutrientes y del metabolismo de la síntesis de los productos, que sirva como base para la formulación de Modelos Metabólicos.

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Faster Development of Industrial Fermentation Processes. New Engineering Tools for Better Processes Faster

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***Keywords**: monitoring, control, optimisation, intelligent systems, industrial processes*

Development of pharmaceutical fermentation processes is carried out mostly by trial and error procedures (i.e., very expensive and time consuming). The biochemical complexity of these processes and the unawareness of advanced monitoring techniques and of powerful methods for process diagnosis and supervision, are the main factors responsible for the prevailing empiricism.

Faster Development of Fermentation Processes for the Pharmaceutical Industries is a capacity building project for an industrial pharmaceutical pilot-plant [FASTERPROD,1997]. The project objective is to achieve shorter development cycles of products of strategic value to CIPAN (Companhia Industrial Produtora de Antibióticos).

The main project paradigms are:

knowledge generation (R)
a quantitative knowledge basis of the processes is systematically being built by:
1. rigorously planned and controlled experiments, at several scales, with high sampling frequencies and monitoring of essential variables unavailable until now
2. computation of physiological variables and a macroscopic analysis of the process with recent biochemical evidence on metabolic pathways and fluxes.

effective use of knowledge (D)
with modern tools for **early process diagnosis** and **intelligent systems supervision**:
multivariate statistical methods and intelligent systems such as hybrid neural-networks
which can take into account the complex interaction between process and operating variables with process productivity as well as the heuristic knowledge about the process.

This communication integrates and summarises the authors posters, which deal with: 1) process development in a modern pharmaceutical pilot-plant, 2) monitoring industrial fermentation processes, 3) multivariate statistical process diagnostics of industrial antibiotic production processes and 4) artificial neural networks - alternative approaches in modelling industrial fermentation processes.

A description is thus made of the methods of FASTERPROD [1997] demonstrating their general value to other biochemical and chemical process industries.

FASTERPROD,1997, http://alfa.ist.utl.pt/~qmenezes/fasterprod/

Application of Image Analysis Techniques in Biotechnology, Wastewater Treatment and Food Technology

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Keywords: Image Analysis, Wastewater Treatment

Image analysis (IA) is commonly used nowadays in a wide range of applications due to the development of faster computers, advanced frame grabbers, and sophisticated software. IA allows the enhancement of pictures as well as automatic identification and isolation of particles so that they can be properly studied. It also provides an extremely fast means of getting morphologic information, thus saving tremendous effort and time.
Although the availability of commercial sophisticated software some efforts have been made at DEB in software development using MATLAB (The Mathworks, Inc.) programming environment. This programming approach permits to tailor the software to our specific needs. In-house software currently in use and development include:
automatic differentiation of flocs and granules through fractal dimension [1];
monitoring methanogenic auto-fluorescence [2]. IA was used to quantify the blue-green light intensity (co-factor F_{420}) developed during the start-up of a CSTR with a VFA based synthetic substrate and during the steady state operation of an anaerobic filter fed with a synthetic dairy waste;
determination of the reduction in mobility of ciliates exposed to toxics [3]. Ciliated protozoa play an essential role in the purification of wastewaters by removing, through predation, the major part of the dispersed bacteria, in the aeration tank;
automatic quantification of filamentous bacteria (to characterize bulking phenomena in activated sludge processes);
automatic counting of viable/non-viable yeasts [4] by epifluorescence microscopy with acridine orange as dying agent;
simultaneous monitoring of lactic acid bacteria and yeast during *Vinho Verde* fermentation using phase contrast microscopy coupled to image analysis [5].

Other developments cover automatic determination of the number of yeast flocs and their size distribution [6], dynamics of bacterial adhesion [7], estimation of the tortuosity of porous media [8], and automatic detection and counting of ink spots in recycled paper [9].

A list of image analysis resources (hardware, software) may be browsed throughout the Laboratory of Image Analysis web page at **http://www.deb.uminho.pt/lab_imagem**.

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New Stationary Phases for Hydrophobic Interaction Chromatography

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Key words: Cellulases, Hydrophobic Interaction Chromatography, Lipase, Stationary Phases

Purification procedures for enzymes were usually based on multistep series of non-specific techniques such as ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. Hydrophobic interaction chromatography has become a popular technique for fractionation of proteins. The separation takes place by differential interaction with hydrophobic substituents on supports and the strength of the binding depends not only on the type of ligand and matrix but also on the type and concentration of salt, pH, temperature and additives. However, negative side-effects have also been noted. The binding is, in many cases, too strong to be useful in a chromatographic process and may even be practically irreversible. One example deals with the hydrophobic interaction between the lipase of *Chromobacterium viscosum* and a Phenyl-Superose column, where elution is only obtained with a gradient 0 to 65%(v/v) ethyleneglycol. In this respect, ligands with intermediate hydrophobic character are of great interest, as they provide an adequate binding strength without the drawbacks mentioned above. This approach has been applied to the purification of enzymes, lipases and cellulases, under study in our laboratory.
This presentation describes the chromatographic purification of *C. viscosum* lipase using three new ligands covalently bond on Sepharose CL-6B: 1,4-butanediol diglycidyl ether [1], polyethylene glycol [2] and polypropylene glycol [3]. The crude commercial preparation of *Trichoderma reesei* cellulases was also purified by using a HIC step on Sepharose CL-6B modified by covalent immobilization of 1,4-butanediol diglycidyl ether. The effectiveness of some salting-out salts (in different concentrations) at various pH values in increasing enzyme-adsorbent interactions is described. It was found that the extent of retention of lipase and cellulases is affected by the salt used and increases with increasing ionic strength in the eluent buffer. Using 20%(w/v) ammonium sulphate in the eluent a total retention of enzyme on the columns was obtained and most of the bound enzyme was effectively desorbed by simply washing the support with 10 mM phosphate buffer.
In conclusion, the properties of the gels prepared seem to provide an adequate approach to enzyme fractionation based on their hydrophobic properties.

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An Expert System for Optimal Selection of Multistep Protein Purification Processes

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A hybrid Expert System (Prot_Ex), that combines expert rules and mathematical correlations to manipulate physicochemical databases for selecting the optimum sequence of operations for purification of proteins, has been built. This paper describes how the physicochemical data of the protein product and the other proteins present in the culture (contaminants) was used to select a sequence of operations with a minimum number of steps to achieve a defined level of purity. An algorithm was developed to model the amount of each protein eliminated after each step. The system was tested in a practical example and it was found that the process selected was sufficiently sensitive to important changes in the physicochemical parameters of the product and protein contaminants and rather robust to small variations or errors in the measurements of such parameters (10 %). Two criteria were implemented to select the optimum sequence of purification. One of these uses the Separation Selection Coefficient (SSC) and the other uses the final level of Purity. The sequences suggested by the expert system have been investigated experimentally using both criteria and it has been shown that both are valid for practical application, but the sequences suggested by the Purity criteria have fewer steps than those suggested by the SSC criteria.

To calculate the SSC the expert system will read the database containing the information on the properties of the main contaminant proteins present in the expression system used. Such a database has been obtained for *E. coli* and CHO cell culture. This database contains data on pI, concentration, m.w., hydrophobicity and charge as a *f*(pH) for the 13 predominant proteins present in a commercial strain of *E. coli* and is then used to select the first high resolution purification step. After each high resolution step, the concentration of contaminant proteins decreases and the number of steps has to be sufficient to eliminate contaminants until the product reaches the desired level of purity. In order to find the new concentrations of all proteins after each separation step we developed a simple algorithm based on the behaviour of a chromatographic separation that will give an approximate value of the concentration of each of the contaminants after each separation step.

A consultation was carried out using the Expert System to find all the steps necessary to achieve the desired level of purity (e.g. 98 %) for the purification of the protein somatotropin, produced in *E. coli*. Once a process was found the original databases were randomly varied at the levels of 10 % and 20 % to see the effect on the sequence proposed by the system. The suggested sequence will be compared to a "good" industrial process that has been published.

Considering that the most important parameter when choosing a separation is the final purity level, and that we have developed an algorithm to calculate the purity after a separation, we implemented this criteria. In this case the final overall Purity of the protein is compared when a particular chromatographic technique has been applied. After determining which chromatographic technique gives the highest purity the system chooses this as the technique to use for this step.

In this presentation we will show an experimental comparison of the use of both selection criteria that have been tested. Consultations were carried out using both criteria (SSC criteria and Purity criteria) implemented in our Expert System (Prot_Ex) for the purification of a protein mixture.

Both criteria suggest valid purification sequences that have been experimentally validated, however the sequences suggested by the Purity criteria have fewer steps than those suggested by the SSC criteria. On a large scale (and sometimes in the lab) it is desirable to obtain the highest purity and the highest possible yield minimizing the resources used. One way to obtain this is by minimizing the number of purification steps. The steps suggested by the Purity criteria are more optimal, particularly when there are a large number of contaminants present in similar concentrations.

In conclusion, the sequences suggested by the expert system developed (Prot_Ex) have been investigated experimentally using both criteria and it will be shown that both are valid. These criteria are recommended as a starting point for the experimental purification process instead of the commonly used trial and error method.

RNA Separation by Boronate Affinity Chromatography

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The isolation of RNA from biological samples is of increasing importance in genetic diagnostics, large-scale sequencing, and in the preparation of plasmid-based therapeutics and vaccines. One of the few methods of selective adsorption of RNA in the presence of DNA employs immobilized boronic acid derivatives, which above their pK_a's form a cyclic complex with vicinal diols. The deoxyribose sugar backbone of DNA lacks the 2',3' vicinal diol of RNA, and is poorly adsorbed to boronate matrices.

In this study, the adsorption of total yeast RNA on phenylboronic acid agarose (Amicon) was examined by batch equilibrium adsorption methods. Adsorption affinity and capacity were found to increase markedly at lower temperatures, suggestive of an enthalpically-favored interaction process. Previous work in the field has employed Mg^{+2} to enhance binding, putatively through screening of electrostatic repulsion between the negatively-charged phenylboronic acid ligand and the phosphate backbone of the RNA. We found that the affinity and capacity of RNA adsorption are greatly enhanced by use of other divalent cations, notably barium. Several lines of evidence suggest that the contribution of divalent cations to adsorption is not solely mediated by screening of electrostatic repulsion.

On-line estimation of biomass through pH control analysis in aerobic yeast fermentation systems

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Key words: On-line control, pH control, growth monitoring, proton titration, yeast

Biomass determination is a basic parameter in fermentation processes. Therefore, simple and reliable on-line estimation procedures are highly desirable, particularly in fermentation processes using *Saccharomyces cerevisiae* [1,2], which are widely used in industry.
Experimental evidence of the existence of a direct relationship between proton production and growth has been presented in the past [3,4]. The determination of the equivalents of acid or base consumed by the culture per unit time has been a widely used parameter for on-line control processes [5,6]. Nevertheless, the majority of studies have been restricted to the establishment of empirical relations, while the physiological basis of the models proposed remained unclear.
Many studies on changes in medium pH associated with growth processes pointed to metabolic activity as the principal cause of medium proton exchange [3,7]. This fact was confirmed in a recent study in which the nitrogen assimilation pathway was certified as the main contributing pathway under aerobic conditions and respiratory metabolism [8].
The aim of this work is to verify the applicability of the model described above to those aerobic conditions in which respiro-fermentative metabolism is involved with production and consumption of ethanol. Experiments have been performed with dilution rates ranging from 0.11 to 0.38 h^{-1}, using urea as the nitrogen source, in order to evaluate the contribution of the fermentative pathway to the total specific rate of proton production or consumption by the culture (qH^+, $mmol^{-1}$ h^{-1} $gbiom^{-1}$), determined as in Castrillo and Ugalde [8].
The experiments have shown that production or consumption of ethanol does not contribute significantly to the specific rate of proton production (qH^+), thus extending the previously obtained relations [8] for all aerobic conditions in which other major acid/base contributions are not involved. This constitutes the fundamental metabolic basis by which many fermentation processes can be monitored through the accurate titration of the pH control reagent. Those relations may be usefully applied in fermentation control processes as formal rules, extensive for many different microorganisms displaying similar physiological patterns. Tests in batch and chemostat culture confirm the validity of qH^+ as a formal control parameter in fermentations.

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Modelo Cinetico del Consumo de Glucosa y Fructosa por la Levadura Fructofilica *Zygosaccharomyces bailii* Creciendo en Mezclas de ambos Azúcares

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Zygosaccharomyces bailii es una levadura que provoca el deterioro de alimentos y bebidas. Al contrario que la mayoría de los microorganismos prefiere la fructosa a la glucosa, es decir, consume más rápidamente la primera hexosa que la segunda. Esta propiedad, llamada fructofilia está basada en dos mecanismos que han sido identificados previamente (1): Existe un sistema altamente específico de transporte para la fructosa, con muy baja afinidad (Km 70 mM) y otro general para hexosas con afinidades semejantes para glucosa y fructosa (Km 6 mM) que se inhiben competitivamente. Este sistema es inactivado por la fructosa.

En el presente trabajo hemos intentado construir un modelo que describa el consumo de glucosa y fructosa cuando el microorganismo esta creciendo sobre ambos azúcares. El modelo está basado en las constantes cinéticas micaelianas del transporte previamente citadas, así como en el proceso de inactivación que tiene lugar cuando la fructosa está presente.

Tres tipos de experimentos han sido utilizados: Cultivos *batch* con un solo azúcar, cultivos continuos con mezcla de hexosas, a varias diluciones y experimentos de pulso de fructosa en cultivos continuos limitados por glucosa.

Los cultivos *batch* fueron empleados para calcular las constantes cinéticas del consumo de cada azúcar. La tasa especifica de consumo de azúcar fue calculada como $q_s = ds/dt * 1/x$, en la cual ds/dt fue calculada por diferenciación numérica a partir de los datos de desaparición de azúcar. Las constantes cinéticas encontradas fueron muy semejantes a las previamente publicadas para los sistemas de transporte inicial.

La ecuación para describir la inactivación fue obtenida empíricamente a partir de los datos de pérdida de actividad con el tiempo y a diferentes concentraciones de fructosa. La inactivación tiene una cinética exponencial decreciente con el tiempo que puede ser descrita por la ecuación $q_{max} = q_{min} + A\exp(-t/B)$, en la cual q_{min} es el valor mínimo de consumo al que tiende asintóticamente el proceso de inactivación a cada concentración de fructosa. La variación de q_{min} con la concentración de fructosa sigue una ecuación similar. Introduciendo la inactivación que afecta a q_{max} en los modelos cinéticos micaelianos y la inhibición competitiva entre ambos azúcares, hemos llegado a un modelo cinético que predice valores de consumo bien correlacionados con los experimentales.

Los experimentos de pulso solo pueden ser modelizados por las ecuaciones anteriores, una vez transcurrido un tiempo que oscila entre 20 minutos para la glucosa y 2-3 horas para la fructosa. Obviamente el pulso desencadena reacciones celulares en las que el transporte deja de ser la reacción con mayor coeficiente de sensibilidad, según la teoría del control de flujo metabólico.

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Predicción del Coeficiente de Transporte de Oxigeno en Tanques Agitados Utilizando Redes Neuronales

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Palabras Clave: Coeficiente transporte oxígeno, redes neuronales, tanques agitados

Las redes neuronales son técnicas informáticas, englobadas dentro del concepto de minería de datos, que se basan en la extracción de conocimiento (patrones y relaciones no triviales) a partir del análisis de gran cantidad de datos. Son, por tanto, de utilidad en aquellos campos donde no se cuenta con un modelo adecuado que describa el proceso, como el control de fermentaciones, la modelización de sistemas biológicos o, en general, en todos aquellos casos donde se deban realizar complejas traducciones de datos y, sin embargo, no se disponga de una función de relación (la visión artificial, el reconocimiento del habla y de manuscritos), como puede ser el caso de la modelización de ciertos sistemas.

El coeficiente volumétrico de transporte de materia, k_La, uno de los parámetros más importante para el diseño y cambio de escala en biorreactores, es función de muchas variables, tales como las propiedades físicas del líquido, los parámetros geométricos del sistema y las variables de operación. En la bibliografía se han propuesto un elevado número de ecuaciones, de tipo empírico, para su predicción en tanques agitados. Estas ecuaciones relacionan un número determinado de variables, aunque todavía no se dispone de una única ecuación, especialmente en los casos más complejos, cuando cambia la reología del sistema, por ejemplo. Además, estrictamente, sólo tienen aplicación en el intervalo experimental de las variables estudiado. Unido a esto se encuentran las discrepancias entre valores obtenidos por los diversos autores en el mismo intervalo de condiciones de operación, probablemente como consecuencia de los distintos métodos de medida, y de la influencia de variables no consideradas en dichos trabajos.

La complejidad del problema hace muy interesante disponer de una red neuronal que, a partir de los datos experimentales disponibles en la bibliografía, sea capaz de predecir el valor del k_La en un amplio intervalo de condiciones de operación, desde la escala de laboratorio a la industrial.

En este trabajo se ha construido una red neuronal, para la predicción de k_La en tanques agitados, que consta de 17 entradas (que tienen en cuenta tanto los parámetros geométricos, como las propiedades físicas y las condiciones de operación) y ha sido entrenada con datos experimentales obtenidos en tanques de 2, 4 y 25 litros, en un amplio intervalo de condiciones de operación, tanto con fluidos newtonianos como no newtonianos. Con la aplicación de la red a este sistema, se es capaz de predecir satisfactoriamente los valores de este parámetro, con un error medio inferior al 12%. Así mismo, la red ha permitido estudiar, por separado, la influencia de cada una de las variables o condiciones de operación en el valor del coeficiente de transporte.

En una segunda etapa se pretende entrenar la red con datos de otros autores, aprovechando una de sus principales características: su robustez frente al ruido. Este hecho puede permitir, una vez entrenada, la discriminación entre las fuentes con ruido y las precisas, introduciendo las condiciones en que se han obtenido los datos y comparando la salida de la red con el valor experimental.

Linear and Non-Linear Programming for Growth Media Optimization

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Keywords: *Solid State Fermentation, Single Cell Protein, Animal Feed.*

Growth media optimization is one of the most important steps towards bioprocess development. It influences not only fermentation step if not also most upstream and downstream operations. It has already been pointed out **[1]** that growth media have to be designed in a global context of bioprocess development, however most of the time only technical criteria related to cell growth or product formation are actually employed **[2]**. The aim of this work is to present a methodology that overcome these limitations.

The procedure combines the needs of the three main components of any biotechnology: the microorganisms, the costumes and the process. A general linear model relates raw material proportions with raw material unit cost and some types of constrains related to each formed referred components. That is:

$$\text{MIN:} C_{rm} = \sum p_i m_i \qquad (1)$$

Constraints:

$$\sum_{i=1}^{n} \sum_{j=1}^{m} a_{ij} m_i = A_j \qquad (2)$$

Where A_j are certain types of components requirements. In the case of microorganisms the A_j have to be experimentally optimized using a general non-linear program with partial production unit cost as objective function.

$$\text{MIN:} C_p = f\left(A_j\right) \qquad (3)$$

The methodology has been applied to develop a growth medium for protein enrichment of some sugar cane by-products using yeast ***Candida utilis***. The problem was then to determine the optimal proportion of bagasse, cachaza(mud), molasses, inoculum, water, nitrogen and phosphate sources.

For microorganisms, nutritional and ambient requirements have been used as constraints. This means total sugars and initial water content and also the mutual proportion of two sorts of inorganic nitrogen sources. The costumes in this case are ruminant animals so nutritional restrictions for ash and crude fiber content of the final product have been introduced. The process incorporated constrains which limits the maximal water content of medium so that no percolation takes place and a minimal cost is needed for product drying.

For the optimization of microorganisms requirements a central rotational design has been employed which permitted to find that optimal conditions for growth are: Total Sugar Content 37,2 % DM, Initial Water Content 68 % and Mutual Nitrogen Source Proportion: urea 75,8 % and ammonium sulfate 24,2 %. These data were then used to solve the linear problem whose solution finally gave the optimal proportion for each raw material: bagasse 5%, cachaza 8,82%, molasses 20,4 %, inoculum 9,39 %, mixed inorganic nitrogen source 0,88 %, phosphate 0,17 % and water 53 %. True protein (Berstein, **[3])** of optimal medium was around 9 % DM, crude fiber 12 % DM and ash 8% DM, after 24 hours of fermentation.

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Immobilised Particles in Gel Matrix-Type Porous Media. Homogeneous porous media model

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Keywords: *gel, immobilised cells, diffusion, tortuosity, porosity, and simulation.*

Diffusion in pure gels and gels with immobilised cells were analysed. A model of diffusion in immobilised cell gel assuming of homogeneous cells distribution in gel was improved by introducing a tortuosity value. By theoretical analysis and numerical modelling it was shown that tortuosity of a gel with immobilised cells is the result of two factors: 1) a tortuosity generated by cells, T_c, and 2) a tortuosity of the gel matrix, T_g. Both variables are function of cell volume fraction, ϕ_c. Hence, the total path length in the gel with immobilised cell is $L_\Sigma = T_c T_g L$ and total tortuosity will be $T_\Sigma = L_\Sigma / L = T_c \cdot T_g$.

Based on this approach it is possible to analyse the data for a gel + cells system, where in general tortuosity seems too large. It is shown in this work that the diffusivity $\eta = D_e / D_0$ for this system is a complex function of: 1) diffusivity in gel, η_g and 2) diffusivity in immobilised cells, η_c. Hence $\eta_c = D_e / D_g$, or $D_e = \eta_c D_g$, and $D_e / D_0 = \eta_c \dfrac{D_g}{D_0} = \eta_c \eta_g$, where D_g is the diffusion coefficient in pure gel matrix, and D_0 is the diffusion coefficient in bulk liquid. Diffusion in a pure gel without immobilised cells is represented by the equation $\eta_g = D_g / D_0 = \varepsilon_g / T_g$, where ε_g is the gel porosity, and T_g is the pure gel tortuosity (average ratio of molecule path to porous media thickness).

The total diffusivity, with tortuosity T_c and T_g depending on volume fraction of immobilised cells $(1-\phi_c) = \varepsilon_c$, is $\eta = \eta_g \eta_c = \dfrac{\varepsilon_g}{T_g} \cdot \dfrac{\varepsilon_c}{T_c(\varepsilon_c) \cdot T_g(\varepsilon_c)}$.

For the general case, the tortuosity created in the matrix by cells presence is $T_c(\varepsilon_c) = 1/\varepsilon_c^\beta = 1/(1-\phi_c)^\beta$ and the tortuosity of gel matrix filled with cells is $T_g(\varepsilon) = 1/\varepsilon_c^\gamma = 1/(1-\phi_c)^\gamma$. Thus: $D_e / D_0 = \eta_g \cdot (1-\phi_c) \cdot (1-\phi_c)^\beta (1-\phi_c)^\gamma = \eta_g (1-\phi_c)^\alpha$, and $\alpha = 1 + \beta + \gamma$, where β and γ are order values in the range from 0 to 1, and η_g is the ratio D_g/D_0 for pure gels. In the particular case when $T_g(\varepsilon_c) = T_c(\varepsilon_c) = T$, and $T \sim 1/\sqrt{\varepsilon_c}$ the equation becomes $\eta = \dfrac{D_e}{D_0} = \dfrac{\varepsilon_g}{T_g} \cdot \dfrac{\varepsilon_c}{T^2} = \eta_g \varepsilon_c^2$.

The developed model gave the possibility to describe the dependence of D_e / D_0 on ϕ_c. Comparison with numerous published experimental data showed a good approach. Not all experimental data could be fitted with this homogeneous model. These deviations case might be explained by non-homogeneous cell distributions inside the gel matrix.

Mixing as a Determinant of the Type and Amount of Metabolites Produced by Fermentation: Examples on Plant Cell Culture and Polysaccharide-producing Bacteria

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Key words: Mixing, hydrodynamic stress, alginates, plant cell culture, rheology.

Cells cultured in bioreactors are exposed to different types of stresses. Given a specific genotype, the production level of certain metabolite is determined mainly by the environmental conditions prevailing in the fermentor. These conditions are determined, in turn, by the rheology of the broth, the mixing and the oxygen transfer in the fermentor. The present work shows two examples regarding the effect of mixing conditions on a plant cell culture (*Beta vulgaris*) and on a polysaccharide-producing bacterial culture (*Azotobacter vinelandii*).

The scale-up of *Beta vulgaris* cells from shake flasks (considered a system with low hydrodynamic stress) to a stirred tank operated at an impeller tip speed of 0.17 m s^{-1} (high hydrodynamic stress) was analyzed in terms of cell growth, production of betalains and rheology. Kinetic profiles of biomass and betalains were similar in both systems. The broths (with cells) obtained in either shake flasks and stirred tank were viscous and pseudoplastic, whereas the cell-free medium was Newtonian in the cultures developed in shake flasks and pseudoplastic for those performed in a stirred tank. The rheology of the cell-free medium was associated with a high accumulation of extracellular compounds (protein and carbohydrates) which viscosify the broth. We postulate that by increasing viscosity, this extracellular material plays an important role in the reduction of turbulence and its accumulation could be the response of the cells to the hydrodynamic stress.

On the other hand, the effect of the agitation speed on alginate production and its composition by *Azotobacter vinelandii* was evaluated. To separate the hydrodynamic effect of the agitator speed from the influence of dissolved oxygen tension (DOT), this was controlled at a constant value of 3% by gas blending. The higher the agitation speed (between 300-700 rpm) the higher the growth rate and the alginate production. The formation of cell flocks or microaggregates (at least one order of magnitude larger than the individual cells) at lower agitation speed (300 rpm), could be causing dissolved oxygen gradients along the aggregates and, therefore, could explain the lower growth rate and alginate production. Therefore, decreasing the oxygen diffusion barrier would facilitate mass transfer and would improve the alginate yield. Nevertheless, when the agitation speed was increased, the quality of the alginate (in terms of its molecular weight), decreased one order of magnitude. This phenomenon is probably due to the production of an alginase under high DOT.

Using two different biological models, we have shown that mixing conditions could be responsible of one order of magnitude difference in either the production of extracellular compounds (in plant cultures) and in the molecular weigh of alginate (in cultures of *A. vinelandii*).

The Role of Activity of Water in Solid State Fermentation - Choice of a Method of Determination in Laboratorial Scale Process

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The solid state fermentation (SSF) is a type of fermentative process related to the microorganisms growth and consequent metabolites production in a humidified solid substrate. This technique is used in the East from the Antiquity for production of drinks, fermented foods and enzymes. Even so, after the 2nd World War it was forgotten in the western countries due to the great development of the process in liquid media for production of solvents and pharmaceutical products.
In the last years, that type of process had a renewed interest, mainly in development countries, because it has some advantages over the submerged fermentation, such as: smaller reactor volume, smaller amount of effluents, larger biomass concentration and products due to the little amount of water in the culture media, what also takes, to a smaller expense of energy.
The microorganisms used in this process can be fungi, yeast or bacteria, which require a minimum amount of water for the development of the physiologic activities and activation of the enzymatic reactions that occur in the process. The culture media, in SSF, is a solid substrate with a certain moisture content, turning the water a limiting factor of the process. The level of available water in the substrate for the microbial growth, is better expressed in terms of water activity (Aw), that indicates the state of water in the materials. Several types of substrates with same moisture content can present different water activities, due to the different hygroscopic characteristic of the materials. Then, the previous study of this property of the solid substrate is needed.

Several authors studied the influence of the Aw in SSF and they showed the importance of this parameter in the process. Aw is defined as the relationship between the pressure of vapor of water of the substrate (p) and the pressure of vapor of pure water (p_0) in the same temperature. In equilibrium with the environment is equal with its relative humidity (%RH). Therefore, it is equal the equilibrium relative humidity of (%ERH) as in the equation: Aw = p/p0 = %URE/100. There are several methods and equipments for determination of Aw. When one have to choice of one of them, it should be taken into account the following attributes: accuracy/precision, reproducibility, speed, low cost, and durability of the equipment and easy handling. Among these, the graphic interpolation method, is shown quite interesting, once it doesn't need sophisticated equipments and it is relatively fast. For the determination of Aw by this technique, only dessecators containing saturated salt solutions are used and the time spent in each determination is about 1 hour. In this work, it was studied the viability of the use of this method for determination of Aw of the substrates used in SSF.

Based on the results obtained, it can be concluded that this method could be considered efficient, because of the three different samples analyzed, the largest variation coefficient (% V.C.) was 1.79. In this work, in substitution to the dessecators, pots tightly sealed, generally used of foods preserves were used. It decreased the cost of the method in more than 90%. Taking into account the attributes required in the choice of a method, the graphic interpolation one demonstrated a good performance. In laboratorial scale it can be used in the previous study of the hygroscopic characteristic of the culture media, as well as in the process monitoring. The equipments for determination of Aw are generally expensive; the acquisition of one of them can be considered a high and unnecessary investment, because when the process reaches pilot plant or industrial scale, the ideal it is the monitorament "on-line", what can be made controlling the %RH of the atmosphere in the reactor. Being considered those observations, it can be concluded that the option for the use of the one of these equipments, in this case, takes to a high relationship cost/benefit emphasizing, once again, the advantages of the method used in this work.

Lipase Production by *Candida rugosa* : Structured Modelling and Sensibility Analysis

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Key words: Lipase; structured modelling; Candida rugosa*; fermentation; sensibility analysis*

Lipases from different sources have found numerous applications in bioorganic synthesis, hydrolysis and modifications of fats and oils, and transesterifications of triacylglycerols. The use of enzymes for the chemical synthesis of some interesting pharmaceutical compounds is becoming economically attractive, especially when synthesis involves an intermediate with a chiral centre. Knowing the mechanisms of lipase production is important in order to implement control strategies and to obtain a reproducible biocatalyst.

It has been developed a structured mathematical model for lipase production by *Candida rugosa* in batch, fed-batch and continuous fermentation [1] [2] [3]. In this model lipase production is induced by extracellular oleic acid present in the medium. The acid is transported into the cell where it is consumed, transformed and stored. Lipase is excreted to the medium where it is distributed between the available oil-water interphase and aqueous phase. A lipase adsorption law describes the total lipolytic activity in the system. Cell growth is modulated by the intracellular substrate concentration.

Model parameters have been determined separately for the different fermentation strategies in order to achieve a better performance of the variables evolution. Some parameters have been determined experimentally and the others have been found by optimisation of an error function between experimental and simulation data. The whole model has been validated against experiments not used in the determination of the above model parameters.

A Sensibility Analysis of some of the model parameters has been realised in order to determine those parameters that must be known with a better accuracy.

The software used has been programmed in Fortran 90 under a Windows environment that allows graphical output for a qualitative assessment of the model results.

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On-line Control of Xylitol Production by *Debaryomyces hansenii* CCMI 941: Evaluation of a Strategy

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***Keywords**: Fed-batch cultivation,* Debaryomyces hansenii*, Xylose, Xylitol, KBCS*

Xylitol can be over-produced by the yeast *Debaryomyces hansenii* CCMI 941 when grown on xylose, but its production is extremely dependent on the environmental conditions, e.g. oxygen availability [1,2]. In this work we present a preliminary study for the development of an automated control strategy for the xylitol bioprocess based on the knowledge-based control system (KBCS) approach. This approach can be divided into two parts: a basic level and a supervisory level. The later is able to perform classification and decision-making tasks in real time as well as diagnostics of physiological phenomena as long as there is an availability of critical on-line information [3]. For this bioprocess, biomass, xylose, dissolved oxygen and xylitol concentrations are the most important ones for control purposes.

Since reliable biomass, xylose and xylitol on-line biosensors are not yet available in the literature, other common on-line measurements, e.g., DOT, total volume of alkaline added and respiratory rates were screened for the most accurate estimation procedure.

It was used a 6-L stirred tank reactor operated under fed-batch cultivation with a 2.5-L initial volume. The chemically defined medium (with 50 gL^{-1} xylose), temperature, pH and aeration rate were previously described [4]. During batch operation, the stirring speed was used to maintain fully aerobic conditions. Fed-batch operation started when xylose concentration reached 30 gL^{-1}. The operational variables, stirring speed and feed rate, were time-changed in order to achieve both semi-aerobic conditions and a residual xylose concentration always above 30 gL^{-1}. The physiological phases, fermentation phases and decision criteria/actions relevant to the KBCS are shown and discussed.

The total volume of alkaline added was chosen as the most suitable "on-line" monitoring parameter since it enables to distinguish the Lag and Log growth phases as well as to predict with accuracy the biomass, xylose and xylitol profiles during the whole fermentation. For the fed-batch phase a 0.72 gg^{-1} xylitol yield was obtained with a 0.68 $gL^{-1}h^{-1}$ overall xylitol productivity. Further fed-batch cycles are predicted to enhance the overall bioprocess productivity.

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Produção de Nemátodos Entomopatogénicos *Steinernema spp.* em Fermentador Airlift Não Convencional: Avaliação da Eficácia.

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Palavras–chave: Fermentador airlift não convencional, produção em massa, nemátodo entomopatogénico.

Desde a década de 70 que os nemátodos entomopatogénicos do género *Steinernema* começaram a ser usados em larga escala no controlo biológico de insectos praga. Razões importantes para o sucesso destes entomopatógenos são as vantagens ambientais do uso de um biopesticida em substituição dos pesticidas orgânicos, e os avanços conseguidos na sua produção em massa e formulação [1]. Um dos avanços observados foi a produção segundo o método de Bedding [2] em suporte sólido muito aplicado na produção à escala comercial em países como a Alemanha, Austrália, China, França, Holanda Suiça e Suécia. No entanto, a quantidade produzida, segundo esta metodologia, é limitada e o recurso à mão de obra é significativo. Assim sendo, a tecnologia de fermentação em meio líquido passou a ser o principal desafio na produção à escala comercial de *Steinernema*, de tal modo que, hoje em dia, estes nemátodos são produzidos correntemente em fermentador [3]. Apesar disso, e devido a aspectos característicos da biologia de *Steinernema*, nomeadamente, o dimorfismo sexual, a diferença de densidade entre os sexos e as necessidades diferenciadas de oxigénio ao longo do processo de desenvolvimento, são vários os problemas que se põem no que respeita ao "design" e à operação dos fermentadores. Neste trabalho, desenvolveu-se um fermentador do tipo "airlift" com um "design" não convencional que possibilitou o aumento da taxa de acasalamento e a satisfação das necessidades de oxigénio e de que resultou o aumento significativo do número de estados infectantes produzidos.

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Effects of Air Pressure on Batch Cultures of *Kluyveromyces marxianus* With Different Lactose Concentrations

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Keywords: Kluyveromyces marxianus*, pressure, yield*

Oxygen is an indispensable nutrient and can be the limiting factor for growth or product formation [1]. The traditional way of improving oxygen transfer rates to bioreactors, by increasing stirring rate, have several limitations like power consumption and cell sensitivity to high shear stress. Several *Kluyveromyces* strains have been reported to exhibit a "Kluyver effect" for lactose: even under oxygen limited growth conditions, certain disaccharides that support aerobic, respiratory growth, are not fermented [2]. Therefore the aim of this work is to improve biomass yield of these types of yeasts increasing the oxygen in the culture with the increase in air pressure.

Batch fermentations, in a pressure reactor, were carried out using different air pressures and different lactose concentrations. Non-aerated fermentations were also made as control. Under non-aerated conditions the strain, *Kluyveromyces marxianus* ATCC10022*,* grew poorly, and ethanol production was high for all lactose concentrations. A small increase in air pressure, 2 bar, led to a 3 fold increase in biomass yield, for all lactose concentrations. Alcoholic fermentation occurred till lactose was exhausted and the ethanol produced was completely consumed. Maximal ethanol yield decreased with the increase of air pressure. The growth of this yeast in 100g lactose/L confirmed its lactose fermenting capacity however, with lower lactose concentrations it was possible to observe the total consumption of lactose. The presence of alcoholic fermentation in the culture leads to the conclusion that this *Kluyveromyces* strain is not a "Kluyver-positive" yeast.

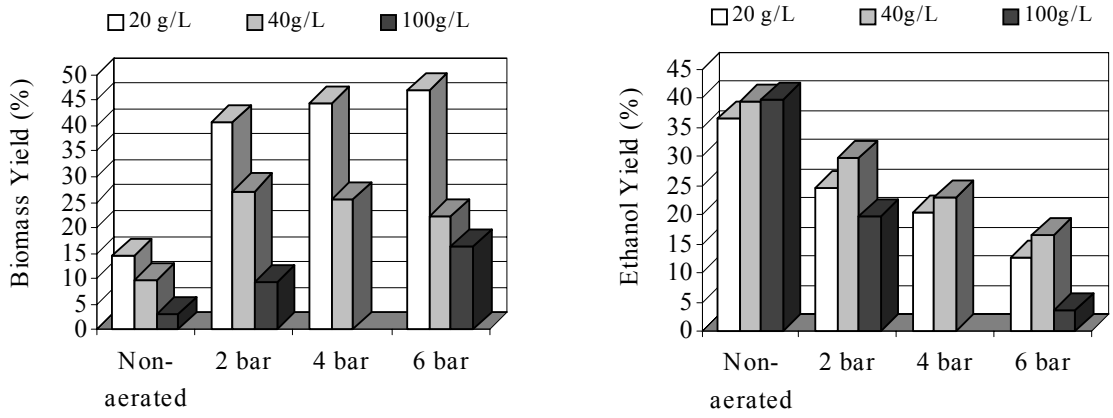

Figure 1. Biomass and ethanol yield with air pressure and without aeration for different lactose concentrations.

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A Novel Method for the Determination of the Growth Kinetics of Micro-organisms on Hydrophobic Volatile Organic Compounds

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Keywords: growth kinetics; VOC; fed-batch.

A novel method for the determination of microbial growth kinetics on hydrophobic volatile organic compounds (VOC) has been developed. Generally, these compounds are poorly water-soluble. Therefore, when investigating the growth kinetics in a batch bioreactor the VOC concentration decreases significantly with time as a result of the microbial growth. Due to this significant decrease the specific growth rate is difficult to determine. Alternatively, the use of a continuous system allows a constant biomedium VOC concentration to be achieved, however, wall growth and other operational complexities make this strategy unappealing. The method presented here provides a constant VOC concentration in the biomedium, and allows a faster kinetic characterisation when compared with commonly used continuous systems. A stirred tank bioreactor was operated as a fed-batch system where the VOC was continuously fed to the biomedium via the gas phase. A constant concentration of the VOC was assured due to a high K_La of the VOC. The concentration of the VOC could be changed by altering the concentration of the VOC in the gas entering the bioreactor. This flow of air was saturated with the VOC and then mixed with a flow of pure air to obtain a predetermined VOC concentration in the biomedium. Both flows were controlled via mass flow controllers.

The growth kinetics of *Pseudomonas* JS150 [1] on chlorobenzene (MCB) was assessed in this system. MCB concentrations in the biomedium were measured by GC analysis, biodegradation product evolution was followed by chloride determination, and biomass evolution by increases in absorbance of the biomedium. A constant concentration of MCB was successfully maintained in the biomedium at concentrations as low as 1ppm. The results obtained were fitted with the model proposed by Luong [2], where μ_{max}=0.24 h^{-1}, K_S= 4.7 ppm, and 248 ppm was the maximum concentration where growth was possible. This last value is in accordance with published values. A simple modification of our system enabled the study of oxygen concentration on the growth kinetics of *Pseudomonas* JS150. The affinity constant for oxygen, K_{O2}, was obtained using the Monod model and was 0.37 ppm, a high value when compared with other studies.

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Influence of System Composition on Glucoamylase Extraction in Aqueous Two-Phase Systems

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***Keywords**: ATPS, glucoamylase*

The concentration of system components is one of the factors that influence the partitioning of proteins in aqueous two-phase systems (ATPS).
In this work, the influence of system composition on glucoamylase extraction was studied. Initially, experiments with several molecular weights of polyethylene glycol (PEG) and pH values were conducted to select a system in the basis of the partition coefficient value (K). Two systems (PEG 300/phosphate at pH 7.0 and PEG 4000/phosphate at pH 9.0) were chosen for this study. Clarified broth of *Aspergillus awamori* containing glucoamylase was used in the experiments. The variation of system components composition (tie-line length) was expressed by the stability ratio [1].
The experiments showed that the partition coefficient of glucoamylase (K_{GLU}) increase dramatically, from 50 to 4,121, with the increase of the stability ratio in PEG 300/phosphate systems at pH 7.0. However, in PEG 4000/phosphate systems at pH 9.0, K_{GLU} did not change in all assayed compositions.
The different responses for K_{GLU} in PEG 300 and PEG4000/phosphate systems could be attributed to the salting-out effect and molecular weight of the enzyme. For PEG 300 systems, the increase of the salting-out effect by means of the increase of the stability ratio, results the extraction of the enzyme to the top phase. However, for PEG 4000/phosphate systems, the influence of the molecular weight of the enzyme was higher than the influence of hydrophobicity, avoiding the glucoamylase extraction to the top phase, owing the difficulty of interaction between the enzyme and PEG molecules.
The influence of protein solubility and molecular weight on K values depends of the system composition regarding the molecular weight of the polymer and the concentration of the components.

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Heterologous Protein Production in *Escherichia coli* Under Different Process Conditions

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Keywords: *heterologous protein,* E.coli*, lactose, IPTG*

In this work, process variables that affect the concentration of a heterologous protein in *E. coli* have been investigated. *E.coli* BL21(DE3)pLysS cells have been transformed with plasmid pET bearing the troponin C (TnC) gene, which is transcribed by T7 RNA polymerase. The system under control of the *lac* UV5 promoter is chemically inducible by IPTG or lactose. Induction experiments have been performed in 2 L bioreactors at 37^{0}C and pH 7.0. A growth phase on semi-synthetic medium preceded each induction experiment, which was then started by the addition of IPTG (0.1 g / g DCW) and glucose (inducer and carbon source, respectively) or lactose (inducer and carbon source, simultaneously). TnC content in the cells was assessed through SDS-PAGE, transmission densitometry, determination of total cell protein, and dry cell weight (DCW) analysis. Hence, parameters used for analyzing process performance were: percentage of TnC with respect to cell protein (TnC-Pi), percentage of cell protein with respect to DCW (Pi), intracellular TnC content (TnC-X) and TnC concentration in the reactor (TnC-V).

In the experiments employing IPTG, different ways of transferring the carbon source have been used, allowing for **mean glucose concentrations** (g/L) of 6.0, 3.2 and almost zero in the reactor. Although TnC-Pi values were similar in all cases (around 25%), it was found that Pi values decreased upon the instant of maximum TnC-Pi, and later also. However, this decrease was sharper in the cases of non-limitant glucose concentrations (from 55% to 35%) when compared to the cases of limitant glucose concentrations (from 55% to 45%), indicating that there is a probable degradation of cell protein by proteases in the former.

Experiments employing **lactose** were characterized by the addition of the inducer through pulses. High Pi values (45-50%) were observed during the whole induction period and later also, in contrast to the case of induction by IPTG, which is probably due to the natural limitation of carbon source availability imposed by the cleavage of lactose. However, TnC-Pi values (around 18%) were lower when compared to the cases of induction by IPTG.

Experiments with IPTG under different **initial specific growth rates (μ_o)** (0.2 and 0.4 h^{-1}) resulted in similar TnC-Pi values (20%) and different Pi values (45% and 37%, respectively) with a simultaneous increase in extracellular protein concentration (cell lysis) in the latter, indicating that μ_o also has an influence on TnC production.

In experiments with IPTG, as well as in those with lactose, TnC-X reached maximum values around 100 mg/g. Thus, considering the peculiarities of the system used (strong promoter, rapid transcription, low toxicity of the target protein, and a probable lack of rare codons that could inhibit translation of the mRNA), lactose a non-toxic and less expensive molecule than IPTG is probably the most appropriate inducer, since Pi values remained constant hours after induction ceased, an interesting feature for downstream purposes.

L(-)-Carnitine Production with *Escherichia coli* 044 K74 in Continuous Bioprocesses

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Key words: L(-)-carnitine, Escherichia coli*, packed-bed reactors, cell recycle reactors.*

L(-)-carnitine is a fine chemical with pharmaceutical, nutritional and animal food applications which functions in the transport of activated fatty acids over the mitochondrial inner membrane in eukariotes [1]. Its chemical synthetic procedure renders a racemic mixture of D,L-carnitine and expensive selective precipitation with chiral salts is necessary for the L-isomer isolation. Biological processes are being studied as an alternative to chemical methods. Enterobacteria, especially *Escherichia coli, Agrobacterium* and *Proteus* are capable of forming L(-)-carnitine from cheap achiral substrates as crotonobetaine [2]. Nowadays, continuous bioprocesses with immobilized cells in packed bed reactors could have industrial interest to get efficiently high levels of L(-)-carnitine.

In this way, the purpose of our research has been devoted to compare the behaviour of *Escherichia coli* 044 K74 cells immobilized in different continuous reactors, for the trans-crotonobetaine to L(-)-carnitine biotransformation under growing and resting cell condition.

E. coli 044 K74 was grown anaerobically at 37ºC in a complex medium with pH adjusted to 7.5. Resting cell were handled in a 50 mM phosphate buffer pH 7.0 with different crotonobetaine concentrations. The analysis of biomass was performed by following the optical density at 600 nm. L(-)-carnitine concentration was measured with the carnitine acetyl transferase enzymatic method, while that of crotonobetaine was measured by HPLC [3].

Continuous L(-)-carnitine production from crotonobetaine was studied with *E. coli* cells passively immobilized in continuous packed-bed reactors with two different supports: glass beads or polyurethane foams. Thus, packed-bed reactors had a similar behaviour, producing carnitine yields of 26%, working with an optimum dilution rate of 1 h^{-1} and getting productivities close to 2 g L^{-1} h^{-1}.

Biotransformation rates with immobilized resting cells took place to the same extent as that with growing cells, but enhanced the carnitine yield: however after a week of continuous L(-)-carnitine production the biotransformation capacities diminished due to a progressive cell washout. The reactors with immobilized resting cells completely recovered the L(-)-carnitine production rate after feeding the system with complex medium for 12 h. Continuous reactors with immobilized resting cells look promising for bioconversions when run under continuous/regeneration cycles.

This work has been partly founded by the "Acciones Integradas Hispano-Alemanas nº 106B and 68B" and by the CICYT project BIO96-1016-C02-01.
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Modification of the Glycosylation Percentage in the Lipases Produced by the Yeast *Candida rugosa*

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Key words *: glycosylation, isoenzymes, lipase, tunycamicine*

The yeast *Candida rugosa* produces and secretes extracellular lipases in fermentation processes when it grows on insoluble carbon sources [1]. Five different lipase gene sequences have been isolated from *Candida rugosa*, namely *Lip1, Lip2, Lip3, Lip4* and *Lip5*[2]. These isoenzymes are highly similar in amino acid sequence but they do differ in their biochemical properties like the isoelectric point, hydrophobicity, substrate specificity, N-terminal sequence and carbohydrate composition [3]. The carbohydrate moiety of these enzymes has been suggested to play a role in their catalytic activity. In this work, *C. rugosa* lipases with different degree of glycosylation have been produced using the antibiotic tunicamycin. This drug inhibits the glycosylation pathway in yeast by blocking the Dol-PP-GlcNac synthesis in the endoplasmatic reticulum. Different antibiotic concentrations (10μg/ml, 20μg/ml and 30μg/ml) were tested. Partial characterisation of the produced enzymes has been performed, and tunicamycin effects on cell viability, protein production and lipase activity have been investigated.

Production of *Candida rugosa* lipases has been carried out in batch cultures using 4g/L of oleic acid as carbon source in 1L bioreactors. The different lipase isoforms produced under these conditions have been analysed by SDS-PAGE in order to visualise changes in the migration profile of the less glycosylated forms. Total desglycosylation was not achieved in any of the experiments carried out, and therefore, a mixture of enzymes with different glycosylation percentage was obtained. Protein and activity profiles of the lipases produced under different tunicamycin concentrations suggest that higher antibiotic concentrations are related to a lower lipase activity and a higher intracellular accumulation of the enzyme.

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Manganese Peroxidase Production by *Bjerkandera* sp. BOS55 in Stirred Tank Reactors

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Keywords: Manganese peroxidase, Bjerkandera *sp. BOS55, stirred tank reactors.*

Lignin biodegradation is initiated by several extracellular oxidative enzymes secreted by white-rot fungi, including lignin peroxidase (LIP), manganese-dependent peroxidase (MnP), manganese-independent peroxidase (MIP), laccase and H_2O_2-generating oxidases. Since the synthesis of ligninolytic enzymes occurs during secondary metabolism in response to nitrogen or carbon limitation, the productivity of the process is quite low, which implies one of the major obstacles to implement new biological treatments and to carry out other environmentally clean processes such as biopulping and biobleaching of pulps by using oxidative enzymes at large scale [1].

In contrast to the well known model organism *Phanerochaete chrysosporium*, *Bjerkandera* sp. strain BOS55 is N-unregulated. Sufficient or excess N-nutrients stimulate high MnP titers in parallel with the high biomass production [2, 3]. This characteristic of the fungus makes it an outstanding candidate for large scale fermentation in order to produce MnP in stirred tank reactors. This type of reactor can be scaled up beyond the laboratory size and cultures parameters such as pH, dissolved oxygen and shear can be measured and controlled.

In this work, we evaluated MnP production in stirred tank fermenters of 2, 10 and 50 L. The highest activity over 1,000 U/L was obtained in the 10L fermenter, which can be explained by the bioreactor set-up found under these conditions. Good oxygen and nutrient transfer rate were maintained in this operating volume. Though a bit lower, a still very high activity was found in the 50L fermenter, where a maximum around 400 U/L was achieved. Similar values were obtained with the 2L fermenter. These results makes viable the use of the extracellular fluid of the fungus for application studies.

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Protease Fermentation Process by a Shipworm Bacterium

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Keywords: *optimization, enzymology, metabolism, bioprocessing*

A symbiotic bacterium, *Teredinobacter turnirae*, isolated from the gland of Deshayes of the marine shipworm is capable of producing a complex mixture of extracellular enzymes. Among these, we have been particularly interested in a protease enzyme which displays activity in a wide range of pH, high salt concentration and temperature. Namely, this enzyme is active over a pH range of 3.5 to 11.9, has tolerance to saturated NaCl concentrations and is thermally stable to 40˚C.

In this work, a summary will be presented of the fermentation optimization studies at the shake flask level involving media composition effects on cell growth and enzyme production. The first results in controlled bioreactor experiments, including fed-batch fermentation, will be reported as well. Through such physiology and metabolic studies, an increase of over 10 fold in enzyme production with a decrease of 5 fold in the fermentation time relative to the early data in this project was obtained.

Effects of Polysaccharides on *Yarrowia lipolytica* Lipase

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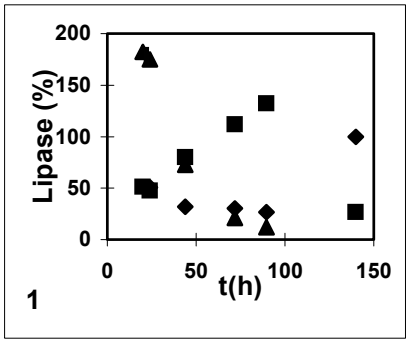
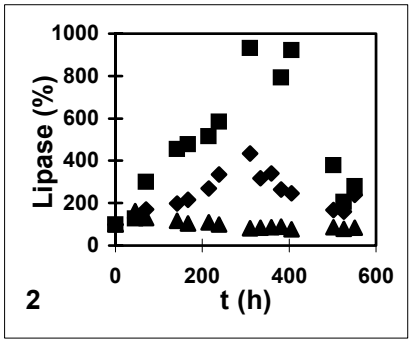
Lipase (E.C.3.1.1.3.) modulation ability and specificity can be altered on some natural polymers presence. These features may be exploited for biotechnological purposes.

Enhancement of lipolytic activity, on polysaccharide presence, *in vivo,* has been investigated for bacterial cells since the seventies, but studies on yeast cells are scarce. Moreover, the interaction mechanisms between the enzyme and each polysaccharide seems to be different, as those microorganisms present distinct cell-wall composition.

Unfortunately, despite of the technical relevance of enzyme activity enhancement mediated by polysaccharides, the literature does not present enough information in this field. Thus, the main objective of this work is to study some polyssaccharides-lipase interactions on different *Y.lipolytica* growth phase (*in vivo*) and the singular effect of each polysaccharide on the crude lipase preparation (*in vitro*). For this purpose, a wide type strain from *Y.lipolytica* (IMUFRJ50682), previously selected in the vicinity of Rio de Janeiro, was used.

The results of this work points out that, some non-metabolized polysaccharides, like glycogen and gum arabic, can be used to improve lipase properties, increasing the enzyme activity by a factor of 2 and 1,5 respectively, over the control experiment. On the other hand, molecules, like pectin and polygalacturonic acid may be suitable to immobilize the enzyme, promoting an stabilization or a specificy change that result on the enzyme modulation loss. Typical profiles are presented in Figure 1. Moreover, all of them were able to antecipate lipase liberation to the culture media, for at least 50h, as shown in Figure 2. This different behaviour can be exploited in practical terms to modulate lipase activity and/or antecipate lipase liberation to the culture media, decreasing fermentation time and consequently increasing productivity. (◆) represents control experiment, (■) glycogen and (▲) polygalacturonic acid.





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Inmovilización de Microorganismos en Matrices Orgánicas Poliméricas. Aplicación a la Obtención de Etanol.

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***Palabras clave**: inmovilización, fermentación,* Saccharomyces cerevisiae*, etanol.*

Las fermentaciones llevadas a cabo con microorganismos inmovilizados presentan una serie de ventajas frente a los sistemas tradicionales de células libres (2,3):

- Fácil separación entre células y productos formados.
- Alta densidad celular (mayor velocidad de reacción y mayor rendimiento).
- Operación en continuo con altas velocidades de flujo.
- Eliminación de sistemas de recirculación de biomasa.
- Fácil control.

El objetivo del presente trabajo es el estudio de la inmovilización de células de *Saccharomyces cerevisiae* en matrices orgánicas poliméricas; utilizando posteriormente las biopartículas formadas en la obtención de etanol (1). Se han utilizado dos resinas orgánicas de bajo coste: látex PE-1098 y PB-1302H. El trabajo se ha llevado a cabo en varias etapas diferenciadas, evaluando la toxicidad del material, caracterizando las partículas/biopartículas formadas y finalizando con experiencias de fermentación.

Evaluación de la toxicidad del látex sobre las levaduras.
Partiendo de suspensiones acuosas de látex más levaduras, y operando tanto en forma aerobia como anaerobia se analizó el crecimiento y/o producción de etanol.

Formación de partículas/biopartículas.
La formación de partículas/biopartículas se realizó insolubilizando el látex o la mezcla látex/levaduras con iones Ca^{+2} y Al^{+3} en proporciones adecuadas para conseguir buena resistencia mecánica y actividad adecuada (partículas de tamaño medio 1.5 mm). Las concentraciones (mol) utilizadas células/Ca^{+2} /Al^{+3} fueron (ml^{-1} látex): 10^7 /4.8 10^{-3} /7.4 10^{-4} .
Previamente se llevaron a cabo ensayos con solo partículas de látex para determinar la permeabilidad de los sustratos y productos en el soporte.

Ensayos de fermentación.
Se han realizado experiencias de fermentación alcohólica, en reactor de mezcla perfecta, operando tanto en discontinuo como en continuo, con soluciones de sacarosa de 100 g/l, pH 3.9 y temperatura 30 ºC.

Resultados representativos de un proceso discontinuo corresponden a una producción de 30 g/l etanol, baja proliferación de células en el medio, y alta viabilidad celular (80 %)..

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Expression of Exo-ß-glucanase in Batch Cultures of Recombinant *S. cerevisiae* Immobilized in Ca-Alginate Beads

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Keywords: Recombinant, exo-β-glucanase, immobilization.

S.cerevisiae secretes into the culture medium exo-β-glucanases with capacity for hydrolysing β-glucan which is a structural component of the cell wall. For this reason, these enzymes give to cells an autolytic property, and also play an important role in morphogenetic processes. However yeast exo-β-glucanases present basically a trophic fuction[1].

The use of recombinant microorganisms for industrial production of enzymes and proteins is now widespread. However, plasmid instability should be avoided because lead to a less recombinant metabolic activity and therefore a lower level of enzymatic expression. Immobilisation has been proposed as an useful technique for improving the stability of recombinant microorganisms. Physical restrictions imposed by cell entrapment allows to improve retention of plasmid bearing cells and thus delay overgrowth of plasmid free cells.

In this work, a number of immobilised cultures of genetically modified *S. cerevisiae* pRN5 containing the *EXG1* gene which codes the exo-ß-glucanase (EXG) production and of *S. cerevisiae* MAX18-9B, a defective in EXG activity strain, were carried out. Erlenmeyer flasks of 250 mL containing 100 mL of medium were used for batch experiments. The temperature (30ºC), and agitation rate (150 rpm) were ensured by an orbital shaker. Yeast was entrapped in Ca-alginate beads [2]. Plasmid stability and enzymatic expression level were analyzed for both strains growing in selective medium (leu) and non-selective medium (leu-ura). The assay for determination of enzymatic activity is based on the release of glucose from laminarin which is the specific substrate for the exo-ß-glucanases[3].

The fraction of plasmid bearing cells was 10% higher when operating with selective medium. In this case, a higher level of enzymatic activity was also observed. A redox balance from the production of the different metabolites was evaluated for determining the cofactor availability. Protein synthesis requires of reduced cofactors (NAD(P)H) availability, and so recombinant cells presented a reduced cofactor pool a 66% higher when compared to Exg^- strain. In the case of the Exg^- strain, the requirement of NAD(P)H cofactor is inferior, because this strain lacks in *EXG1* gene which codes exo-β-glucanase so only a residual glucanase activity could be expressed.

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Xylitol Production Using a Chemostat with Total Biomass Retention by *Debaryomyces hansenii* CCMI 941

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***Keywords**: High-Cell Density,* Debaryomyces hansenii*, Xylose, Xylitol, Chemostat.*

Xylitol is a polyol used as an alternative sweetener by food industries, nowadays manufactured by means of an unspecific sugar chemical reduction. The development of a bioprocess is extremely desirable but has been delayed mainly due to the low xylitol volumetric productivity usually attained. A possible way to overcome this problem is to increase the biocatalyst (biomass) concentration to attain high-cell densities and develop a xylitol bioprocess based in a two-stage fermentative process. The yeast *Debaryomyces hansenii* has been reported as one of the best xylitol producing yeasts [1,2] and therefore, it was used in this work.

A preliminary study for the development of an effective *D. hansenii* biomass production process from xylose as sole carbon source, and the evaluation of the xylitol production rates for different conditions were carried out.

To obtain a *D. hansenii* high-cell cultivation, it was used a continuous stirred tank reactor with a working volume of 1 800 mL coupled to a micro-filtration membrane unit (0.2 µm, 45 cm^2) to allow total biomass retention. The dilution rate was controlled by the permeate flow rate and varied between 0.017 - 0.10 h^{-1}. The maximal biomass concentration obtained was 70 g/L which compared favorably with the biomass concentration usually attained under classical batch cultivation, 10 g/L for this strain [1]. The former biomass value was obtained under fully xylose oxidative metabolism without xylitol production.

In a second stage of the fermentative process, xylitol production was induced by decreasing the oxygen availability. The variables, stirring rate (100-300 rpm) and aeration rate (0.10 - 1.70 L/min), were studied to optimize the xylitol yield and volumetric production rate.

Throughout the conditions tested, xylitol was the main metabolic product found, accompained only by residual amounts of glycerol, acetate and ethanol (always less than 1 gL^{-1}). In spite of xylitol yield reached values close to the theoretical maximum (0.91 g/g) its volumetric production rate was unexpectadly rather low, due to a low xylose uptake rate.

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Dextran and Fructose production using *Leuconostoc mesenteroides* NRRL.B512(F) with sucrose as substrate

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Keywords:dextran, fructose, dextransucrase, fermentation

Dextran and fructose have many industrial applications. Dextran is widely used as a blood volume expander, in food industry and as a chromatographic media. Fructose is a low caloric sugar.

The strain *Leuconostoc mesenteroides* NRRL.B512(F) grows in sucrose rich media which induces the production of an extracelular enzyme: dextransucrase. This enzyme also uses sucrose as a substrate to produce dextran and fructose as follows:

$$n\ C_{12}H_{22}O_{11} \xrightarrow{\text{enzyme}} (C_6H_{10}O_5)n + n\ C_6H_{12}O_6$$

Sucrose Dextran Fructose

In this work a 2 l bioreactor (Setric Genie SET02, Incheltec, France) with control units for temperature, pH and agitation was used. Strain growth and products concentration for various operating conditions was studied.

Batch fermentations were carried out for sucrose concentration in the range 10 to 120g/l, temperatures from 20 to 40ºC, pH of 6.9 (optimum pH for strain growth) and 5.5 (for mimizing loss of enzyme activity) and aeration rate of 0.05 vvm. Fed-batch fermenations were carried out with different start-up times and feed flowrates.

Cell growth was significantly higher for T=35ºC and fermentation took 4 hours less than at 20ºC. Experiences at 25ºC and controlled pH at 6.7 resulted in higher cell growth than at pH 5.5. At lower pH values (5.5) dextransucrase production was faster in the earlier hours of fermentation and the decrease in acctivity was slower. Cell growth and product formation were not affected by aeration. Results with fed-batch operation were similar to those obtained with batch operation.

Studies of metabolic engineering of the strain were made. The evolution of the Carbon/Nitrogen ratio throughout the fermentation was studied. A fermentation was carried out correcting the nitrogen level around the 4th and 7th hour. Results in enzyme productivity were improved.

No growth of the bacteria was observed in the presence of other carbon sources than sucrose, such as maltose, lactose and galactose.

The enzyme production in the presence of these sugars together with sucrose was analysed. None of the three sugars was consumed by the bacteria and the cellular growth and enzyme activity were smaller comparing with the results of a fermentation using only sucrose.

The kinetics of enzymatic conversion of sucrose in the presence of dextransucrase follow Michaelis-Menten equation. When maltose is used together with sucrose reaction rate decreased.

The Use of a Bioreactor as a Temporary Immersion System in the Micropropagation of European Chestnuts

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Keywords: Bioreactor, Temporary Immersion System, Media

It is well documented that shoot growth is better in an agitated liquid medium than in solid medium, resulting in higher of organogenesis shoots from axillary buds. [1][2] A different type of bubble jar bioreactor is a temporary immersion system that offers all the advantages of liquid medium without visible sign of limited gas exchange or symptoms of hyperhydricity [3].
All these advantages appear to be result of a physical conditions created in the culture vessel: renewed direct contact with the medium during each flooding means a more efficient supply of nutrient elements compared with the solid medium; immersion times are short and for the majority of the time the tissues are merely covered with a capillary film. So there is no desiccation, resistance to gas diffusion is low and there is minimum disruption of gas exchange between the plant and the atmosphere; the atmosphere inside the vessel is almost completely renewed at regular intervals. [3]
Woody plants, in general, are more difficult to propagate asexually than herbaceous species, and methods for large-scale regeneration of true-to-type clones are limited.
Thus, an efficient micropropagation method for european chestnuts (*Castanea sativa* Miller) resistant to an importante disease caused by the fungi *Phytophthora cinnamomi* Rands and *P. cambivora* (Petri, Buis) [4], is required. We tested a Temporary Immersion System for micropropagation of this woody specie.
The chestnuts were collected in the winter and grown on solid medium until the experimentes were performed. The bioreactor was used as a Temporary Immersion System, in which plants were submerged for 15 min. each 2h in G.D. [5] liquid medium. To be able to compare results, experiments using plants grown in solid medium and in glass tubes, were performed.
After fourth week, the results clearly showed a difference between plants developed in the bioreactor and the plants grown in solid medium. The average number of axilary shoots per plant at fourth week, from the liquid medium and the solid medium was repectively: 12.27±8.85 and 1.30±0.30.
The fresh weight average was respectively: 1.056±0.606 g. and 0.109±0.090 g. Identical results were obtained for the others parameters .

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Influence of Salt Content, Degree of Proteolysis and Aeration on the Production of a Polymer via Fermentation of Whey-related Media by *Rahnella aquatilis*

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Key words: *whey, gum, exopolysaccharide, bacteria, lactan*

Utilization of whey as fermentation feedstock has been attempted widely by the dairy industry. Production of lactan, a polysaccharide composed of mannose, galactose and galacturonic acid (at the molar ratio 5:3:2), starting from a semi-defined medium containing lactose via fermentation under aerobic conditions with *Rahnella aquatilis* was described previously. In this communication, the effect of salt, previous hydrolysis and aeration were studied during the polysaccharide production from whey in alternative fermentation media: hydrolyzed whey (under (i) aerobic and (ii) anaerobic conditions), hydrolyzed whey with 2.0% NaCl (w/v) (iii) and 0.5% NaCl (w/v) (iv),.and plain whey (v).

The growth of biomass and the variation in concentration of organic acids, lactose, peptides and free amino acids were monitored. The polysaccharide production and the variation of viscosity of were also followed throughout 48 h of fermentation. Under the different conditions tested, *Rahnella aquatillis* showed a maximum specific growth rate of 0.61 h^{-1}, 0.60 h^{-1}, 0.61 h^{-1}, 0.64 h^{-1}, and 0.46 h^{-1} for hydrolyzed whey under aerobiosis and under anaerobiosis, hydrolyzed whey with 2.0% NaCl (w/v) and 0.5% NaCl, and plain whey, respectively; the final yields of the various organic acids were: 0.07, 0.18, 0.07, 0.04 and 0.05 ($g/g_{lactose}$) for acetic acid; 0.06, 0.07, 0.00, 0.04 and 0.02 ($g/g_{lactose}$) for lactic acid; 0.08, 0.09, 0.03, 0.04 and 0.04 ($g/g_{lactose}$) for formic acid; 0.01, 0.04, 0.01, 0.01 and 0.02 ($g/g_{lactose}$) for succinic acid; and 0.11, 0.09, 0.14, 0.19 and 0.00 ($g/g_{lactose}$) for acetoine. Lactose was almost completely depleted during the 48 h of fermentation for hydrolyzed whey; however, lactose was only partly consumed in plain whey (final yield of 0.48 $g/g_{lactose}$).Small peptides (< 2,000 Da) and most free amino acids were consumed by 24 h in hydrolyzed whey fermented under anaerobiosis and plain whey, but these peptides were present until the end of fermentation in the remaining media. *R. aquatilis* showed similar behavior in free amino acid consumption in hydrolyzed whey with NaCl and hydrolyzed whey fermented under aerobiosis. Plain whey yielded very low concentrations of free amino acids throughout the whole fermentation. The yield of polysaccharide was 0.56, 0.26, 0.39, 0.40 and 0.44 $g/g_{lactose}$ for hydrolyzed whey fermented under aerobiosis and under anaerobiosis, hydrolyzed whey with 2.0% NaCl (w/v) and 0.5% NaCl, and plain whey, respectively.

Produção de Xilanases e Carboidratos por Fermentação a Partir do Resíduo da Extração do Amido de Mandioca

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Palavras-chave: *Xilanase, resíduo lignocelulósico, carboidrato*

O aproveitamento de resíduos sólidos orgânicos tem sido alvo de muitos trabalhos na área biotecnológica, nos últimos anos. Este material, derivado principalmente das indústrias de exploração florestal, de alimentos e agroindústrias, constitui-se em um grave problema para estes segmentos uma vez que seus processos são cíclicos e o acúmulo de rejeitos alcança volumes insuportáveis. Por outro lado, o aproveitamento destes resíduos nos processos biotecnológicos torna-se altamente atrativo devido a sua constituição bioquímica e o custo irrelevante desta matéria-prima. Constituídos basicamente por celulose, hemicelulose e lignina os resíduos, uma vez fracionados por pré-tratamentos químicos, físicos ou enzimáticos, disponibilizam açúcares fermentescíveis que podem ser convertidos a insumos de grande interesse industrial, como álcoois, ácidos orgânicos, biomassa proteica e enzimas. Neste trabalho avaliou-se a potencialidade do bagaço de mandioca como substrato principal para produção de xilanases, enzimas cuja principal aplicação está nos processos de biopolpação e biobranqueamento das indústrias de celulose e papel, e de carboidratos avaliados na forma de glicose e xilose. O bagaço de mandioca foi submetido, inicialmente, a pré-tratamentos para liberação de frações hemicelulósicoas. Utilizou-se processos de hidrólise química (H_2SO_4) e física (autoclave a 121 ^{0}C/15 min). A fração líquida foi recuperada e foram preparados meios de cultivo suplementados com 0,2% de extrato de levedura sendo o pH acertado para 5,0 com NaOH. Utilizou-se como inóculo 5 x 10^8 cel/mL de *Penicillium janthinellum* que fora cultivado em ágar-malte a 25 ^{0}C/72 horas. As fermentações foram conduzidas em incubadora com movimento rotatório a 60 e 100 rpm a 25 ^{0}C por 72 horas. As amostras para quantificação da atividade enzimática e do teor de carboidratos foram retiradas a cada 12 horas. A atividade das xilanases foi avaliada pela liberação de xilose, medida pela técnica do DNS, após reação com xilana "birchwood" e a liberação de monossacarídeos foi também avaliada pela técnica do ácido 3,5 dinitrossalicílico. Os resultados encontrados mostraram que, nos ensaios realizados para produção de xilanases, o pré-tratamento utilizado é essencial para viabilização do processo, uma vez que tratando-se de uma enzima induzida, a xilanase só é produzida em presença de xilooligômeros e ausência de altas concentrações de monossacarídeos. Com relação a liberação de monossacarídeos avaliados na forma de glicose e xilose, observou-se que a maior liberação ocorre sempre entre 48 e 72 horas de cultivo, fato que ocorre, provavelmente, pelo fungo passar a metabolizar os monossacarídeos liberados após este tempo, uma vez que os meios utilizados, não apresentam complementação com fonte de carbono. Conclui-se, portanto, que o bagaço de mandioca é um resíduo viável para utilização nos processos biotecnológicos aqui estudados.

Producción de Ácido Oxálico con *Aspergillus niger* en Fermentador de Tanque Agitado y Air-Lift

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Palabras clave: Aspergillus niger*, ácido oxálico, tanque agitado, air-lift.*

El hongo filamentoso *Aspergillus niger* es capaz de producir elevadas concentraciones de ácido oxálico cuando se cultiva a pH 6 sin limitación de las fuentes de nitrógeno y fósforo (1,2). El lactosuero, subproducto de las industrias lácteas, ha resultado ser un substrato adecuado para la producción de este ácido. Debido a que es un producto natural, dispone de la cantidad suficiente de nutrientes de modo que no es necesario suplementar el medio con fuentes de nitrógeno y fósforo. En cultivos en matraces agitados se ha alcanzado una concentración de 37 g/L con un rendimiento de 0,62 g de carbono de ácido oxálico / g de lactosa consumida, cuando se emplea la concentración óptima de lactosuero: 100 g/L (3).

Con el objeto de producir ácido oxálico a gran escala se ha empleado un fermentador BIOSTAT B de B. BRAUN altamente instrumentalizado. El fermentador permite el control de pH, temperatura, agitación y oxígeno disuelto, así como el funcionamiento en continuo mediante el control del volumen de operación y las bombas de entrada y salida. El fermentador dispone de dos cubas: tanque agitado y air-lift de 5 litros de volumen operativo que en procesos biotecnológicos puede considerarse escala piloto.

Se ha empleado una cepa de *Aspergillus niger* 1120, que se creció en matraces Erlemmeyer de 250 mL con 50 mL de 50 g/L de lactosuero para formar una suspensión de pellets con la que se inoculó el biorreactor. El medio de cultivo se preparó con 100 g/L de lactosuero. Las condiciones de fermentación fueron: 30ºC, pH 6 y oxígeno disuelto por encima de 50% de saturación. La velocidad de agitación se varió para determinar el óptimo que se encuentra entre 200 y300 rpm.

En trabajos previos (2) se demostró la existencia de inhibición por producto por lo que es necesario trabajar a concentraciones bajas de ácido oxálico, lo cual implica conversiones y rendimientos bajos. Para incrementar la conversión del substrato y mejorar el rendimiento, se separó el ácido oxálico por precipitación y el medio sobrenadante se recircula al reactor.
Del estudio realizado puede concluirse que tanto el valor de la velocidad de producción como el del rendimiento en producto alcanzados en los reactores de tanque agitado y air-lift, son similares a los valores alcanzados en experimentos llevados a cabo con anterioridad en matraces agitados. Se han obtenido valores de 0,5 g de carbono de ácido oxálico / g de carbono de substrato, con una velocidad de producción de 6 g/L·d.

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Immobilization on Alginate of Isolated Acetic Bacteria from Vinegar Fermentation for Continuous Production: Comparison Between Free and Immobilized Cells

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***Keywords**: acetic bacteria, alginate, immobilization, continuous production.*

The use of acetic bacteria immobilized on various supports for vinegar production has been the object of many studies. The intention has been to improve production, with continuous or semicontinuous systems, those which need the cells fixed on a matrix.
The entrapment on alginate is the most frequently used technique for immobilization. Alginate is a polymer of mannuronic and guluronic acid extracted from marine alga. The advantages of this alginate matrix are: low price, its acceptance as an additive in the food industry to thicken solutions, physical and chemical stability, high porosity, high retention of biological material, facility to form beads of different sizes.
The isolated cells from industrial fermenters showed a high efficiency in the transformation of ethanol into acetic acid. Practically all the ethanol is consumed and transformed into acetic acid after a few days. The isolated bacteria were able to resist ethanol at the concentration of 6.5% (v/v) in shaken cultures, showing a substantial productivity at 5% concentration.
A clear difference in growth capacity at high concentrations of acetic acid is observed between shaken pure cultures and industrial fermenters. In pure cultures with isolated bacteria, the maximum acetic concentration is reached at about 9% (w/v); meanwhile, production in the industrial fermenters reached the maximum acetic acid concentration at about 12% (w/v).
In the shaken culture, with a medium containing yeast extract, glucose, ethanol and acetic acid, the isolated strain only grew in the acidic range between pH 2.5 and 4.5, with a optimal growth at pH 3.5 . Acetic acid was tolerated up to 9 % (w/v) in this medium.
Acetic production by free and immobilized cells in batch culture was analyzed. A clear difference in the production was observed between free and immobilized cells in Ca-alginate(3%) when they were cultivated in batch. The production rate was about double in the case of immobilized cells, the maximum concentration of acetic acid (5.5%, w/v) in the first case was reached in 5 days, meanwhile the free cells showed the same amount in 10 days.
3%(w/v) Ca-alginate was the optimal concentration for immobilization. At this concentration no leakage of cells occurred and it provided high porosity of the matrix, and subsequently a good product diffusion. Observations using the scanning electron microscopy (SEM) showed how the entrapped cells were distributed in the pores of the matrix. The entrapped cells did not lose their capacity to be divided and to form colonies of great cellular density on the edges of the matrix (bead of alginate). On the other hand, the matrix showed abundant pores that thoroughly crossed the bead, guaranteeing gas and reagent diffusion
The continuous production of acetic acid by the immobilized cells was investigated under optimal conditions for the batch system. The bubble column reactor used for the continuous production was provided with reservoirs for influent and effluent media and a pump to supply the influent. The flow rate was 10 ml per day with a concentration in the effluent of 5.5% of acetic acid. Thus, all the ethanol introduced into the bioreactor was transformed into acetic acid. In these experimental conditions, a continuous productivity for several weeks was observed.
Currently, we are studying the production of cells fixed in alginate using a continuous system(bubble column reactor), in which the principal parameters that can affect production are automatically controlled.

Efeitos da Imobilização na Estabilidade e Viabilidade de uma Levedura Recombinante

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Palavras chave: Saccharomyces cerevisiae*, levedura recombinante, estabilidade, viabilidade, imobilização*

A estabilidade e a viabilidade da levedura recombinante imobilizada *Saccharomyces cerevisiae* L36 contendo o plasmídeoYIpG com gene de expressão da amiloglicosidase, foram determinadas em meio contendo amido dextrinizado, durante fermentação contínua e em bateladas repetidas. Os resultados foram comparados com aqueles obtidos em cultivo com as células não imobilizadas, em suspensão. Esferas de gel de pectina foram usadas como suporte de imobilização e introduzidas em um reator convencional de mistura, contendo uma cesta de tela de aço inoxidável, de maneira a constituir um reator de leito fixo.

A estabilidade do plasmídeo foi superior nas células imobilizadas, apresentando valores aproximadamente constantes, ao longo do tempo, da ordem de 80% tanto nos cultivos em contínuo como em bateladas repetidas. Nos cultivos com células imobilizadas a proteína secretada apresentou valores constantes ao longo das bateladas sucessivas. Para as células em suspensão, a perda da estabilidade mostrou-se crescente ao longo das bateladas apresentando um valor final de cerca de 70%. Verificou-se também menor produtividade da proteína heteróloga, uma vez que o etanol produzido afetou o crescimento das células em suspensão. Quanto ao número de células não viáveis, observou-se um aumento gradativo para ambas as células, com valores finais inferiores a 10%.

Os resultados obtidos indicam, portanto, que a imobilização da levedura proporcionou uma maior estabilidade do plasmídeo, quando comparada às células em suspensão.

Selection of Media Composition and Fermentation Conditions for the Enhance Production of *Fusarium solari pisi* Cutinase cloned and expressed in a Recombinant *Saccharomyces cerevisiae*.

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***Keywords**: Cutinase, Culture conditions, recombinant,* Saccharomyces cerevisiae

Advances in molecular biology and the exploitation of recombinant DNA procedures for the expression of heterologous genes in microbial hosts offer an excellent opportunity to develop fermentation processes. The combination of manipulating both the genetic design of recombinants and the conditions of culturing the organisms may be used to achieve stable host-vector associations during culture of recombinant organisms in bioreactors.
Saccharomyces cerevisiae strains have been sucessfully used in the large-scale production of recombinant proteins (ex. vaccine of hepatitis B) with the advantage of these to be excreted to the extracellular media, leading to a high proportion of specific product. When a foreign protein is expressed in a recombinant yeast, the expression rate is determined by the expression vector, host cell type, plasmid copy number and culture conditions (pH, temperature, pO_2, media composition, etc).
The aim of this work is to evaluate the direct influence of the media composition and fermentation conditions on the host-vector stability and that enhance simultaneously the production of *Fusarium solari pisi* cutinase cloned and expressed in the recombinant *Saccharomyces cerevisiae* SU50-pURY320. This specific objective is very important for a future utilization of economic carbon and nitrogen nutrients and development of a sucessfully integrated bioprocess design for scale production.
Batch fermentations were performed in shake flasks and a 5 L fermenter. In the first case the expression behaviour of the cutinase was examined at different experimental conditions levels of inoculum (media composition and optical density 600nm of inoculation) and production cultures (culture/flask volume ratio, media sterilization method, media composition and carbon and nitrogen nutrients sources).
The scale-up of the experimental conditions obtained in shake flasks was performed in the 5 L fermenter by controling the dissolved oxygen level in the liquid media. The pH control and on-line measurements of the O_2, CO_2 and N_2 in the air composition for the inlet and exhaust gases were analysed.
The results obtained until now have been promising as it was found experimental conditions for a good host-vector stability and high cutinase productivity that can get the final goal, the large-scale production and extraction process integration of this recombinant protein.

Simultaneous Saccharification and Fermentation Process of Pretreated Municipal Organic Wastes

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Segregation of the organic fraction of MSW seems to be one realistic approach in the future strategy for the treatment of these residues. This fraction contains cellulose and hemicellulose which can be used for the production of liquid fuels and chemicals by fermentation. Simultaneous saccharification and fermentation (SSF) process is a viable option for direct bioconversion of cellulosic materials into fuels and chemicals. The SSF process uses cellulase enzyme to saccharify cellulose to glucose, which is simultaneously converted to final product by microorganisms in the same reactor.

In this work the performance of acetone-butanol-ethanol (ABE) and ethanol production by SSF Process, using the organic fraction of domestic wastes (DOW) as substrate, has been investigated.
Microorganisms utilized were *Clostridium butylicum* NRRL B-592 (obtained from TU Wien) in SSF-ABE process and *Kluyveromyces marxianus* EMS-26 (a thermotolerant yeast obtained in our laboratory) in SSF-Ethanol process. Enzymes used in the SSF work were Celluclast 1.5L and Novozyme 188 (Novo Nordisk) with enzyme loading of !5 FPU/g substrate plus 12.6 IU β-glucosidase/g substrate. The SSF were carried out at 37ºC in SSF-ABE and 42ºC in SSF-Ethanol, with 10% (v/v) inoculum addition.

Sustrate was pretreated in a Steam Explosion pilot plant at a range temperature of 180-220ºC and 2-6 minutes residence time.

Glucose concentration was measured by HPLC using a AMINEX HPX-87P carbohydrate analysis column. Acids and solvents were analyzed using a Hewlett-Packard 5890 gas chromatograph with a flame ionization detector.

In a SSF-Ethanol process using 10% DOW as substrate pretreated at 200ºC and 6 minutes residence time, a final ethanol concentration of 10.9 g/L can be obtained

Influência da Aeração sobre a Associação entre Formação de Butanodiol e Crescimento de *Klebsiella pneumoniae*

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Keyword: *butanodiol, levedura, microrganismo, hidrolisado de eucalipto*

Os processos fermentativos que empregam matérias-primas impuras como os hidrolisados hemicelulósicos são dificultados pela presença de inibidores formados durante o processo de hidrólise. Para contornar este problema pode-se empregar o sistema de fermentação descontínua alimentada, em que o substrato é adicionado durante o processo, sendo diluído quando entra em contato com o meio. Desta forma, os inibidores também são diluídos, o que resulta na redução dos seus efeitos tóxicos. A fim de se definir o melhor tipo de aeração para o processo fermentativo de produção de 2,3-butanodiol a partir de hidrolisado hemicelulósico de eucalipto empregando-se este sistema, o processo foi estudado em três condições: não aerado, aerado com vazão de ar constante, e aerado com vazão de ar variável mantendo-se constante a vazão específica de aeração. Em um fermentador de 500 ml de volume útil (NEW BRUNSWICK Sc. Co.) foram introduzidos 210 ml de uma cultura de *K. pneumoniae* previamente adaptada em meio sintético(1) contendo 20% de hidrolisado não tratado. O reator foi alimentado com 300 ml do hidrolisado contendo os mesmos nutrientes do meio sintético(1), a uma vazão de aproximadamente 10 ml/h. As concentrações de células, de substrato e de produto foram determinadas por densidade ótica (600nm), método colorimétrico (Carboidratos Redutores Totais) e cromatografia em fase gasosa, respectivamente.

A maior concentração final de produto foi atingida na condição de aeração constante (6,0 g/l de butanodiol em 24 h de fermentação), com um valor de concentração celular intermediário ao dos outros dois tipos de aeração. Avaliando-se a relação entre as velocidades específicas de formação de butanodiol e de crescimento da bactéria constatou-se a ocorrência de dois tipos de associação entre formação de produto e crescimento. No ensaio não aerado a formação de produto foi associada ao crescimento, enquanto que com aeração constante foi parcialmente associada ao crescimento. Os resultados indicam que este tipo de classificação dos processos fermentativos não é sempre definido apenas em função da espécie do microrganismo e do produto formado, mas também das condições operacionais.

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Comparative Studies of Alcoholic Fermentations using Flocculent and Non-Flocculent Strains of *Saccharomyces cerevisiae*

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Keywords: *Alcoholic fermentation; Extractive fermentation;* Saccharomyces cerevisiae*;* Rhizomucor miehei *lipase*

Alcoholic fermentation is one of the oldest applications of biotechnology. However, inhibition problems, by substrate and/or product, arise when the purpose is to carry out fermentations of high glucose concentration. The use of yeasts with high ethanol tolerance and/or the integration of fermentation with the *in situ* recovery of product can be an approach to overcome some of those problems [1].

In this work, the performance of alcoholic fermentations with 200, 300 and 400 g/l of glucose using a flocculent *Saccharomyces cerevisiae saké* strain and a non-flocculent *Saccharomyces cerevisiae* DER24 strain were compared. The fermentation processes were carried out at controlled pH, an agitation rate of 150 rpm and a temperature of 30ºC. In the extractive fermentation systems oleic acid was used as organic solvent (extractant).

The obtained results for batch fermentations (without extraction) showed that: i) the ethanol yield for the flocculent strain was higher than the one observed for the non-flocculent strain; ii) the specific uptake rate of glucose was lower for the *S. cerevisiae saké* yeast.

When oleic acid was introduced in the fermentation systems, in a 5:1 organic phase / fermentation medium ratio, an improvement on fermentation performances was observed, corresponding to a fermentation time reduction and to a glucose consumption increase. In those systems, higher values of both ethanol yield and specific uptake rate of glucose were obtained with *S. cerevisiae* DER24 strain.

For both *S. cerevisiae* yeast strains, extractive fermentations of 300 g/l of glucose (fermentation medium / organic solvent ratio of 1) coupled with enzymatic esterification of ethanol by a free lipase from *Rhizomucor miehei* (10.2 mg/ml) led a high glucose consumption (residual glucose ≈ 4 g/l). In this case, an increase of organic extractant / fermentation medium ratio was not necessary, as in the physical extractive fermentations [2].

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Modelling Glycolytic Kinetics in *Lactococcus lactis* using *in vivo* NMR Data

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Keywords: *Lactococcus lactis,* in vivo *NMR, Glycolysis, Metabolic Engineering*

To improve the performance of *Lactococcus lactis* as a cell factory, a global comprehensive model of the central pathways, such as glycolysis, is mandatory. Moreover, a rational approach to manipulate organisms for maximal production of desired metabolites can be achieved only if a detailed description of the cell metabolism is available. In this presentation, we will report studies aiming at the characterization of the glycolytic flux in nongrowing cells of *Lactococcus lactis* MG5267 and a derivative strain harbouring the plasmid pNZ2500 (overproducing α-acetolactate synthase, *als*) by *in vivo* NMR. The on-line NMR system, recently developed in our group in which a cell suspension was maintained at controlled conditions of temperature and pH, was used to quantify intracellular metabolites as well as extracellular substrates and products during glucose fermentation under anaerobic and aerobic conditions.
Lactate was the major end-product from the metabolism of 20 mM [1-^{13}C]glucose in both strains; only trace amounts of 2,3-butanediol and acetate were detected. Two resonances due to carbon atoms 1 and 6 of intracellular fructose 1,6-bisphosphate (FBP) were clearly detected after addition of glucose (maximal concentration, 40 mM). The distribution of the label at the two sites of FBP shows the occurrence of scrambling of the label at the level of trioses phosphate and backflux through aldolase, and allowed a detailed characterization of this triangle. The intensity of the resonances from FBP started to decline at the onset of glucose depletion. At the same time, the resonances due to 3-phosphoglycerate (3-PGA) and phosphoenolpyruvate (PEP) ppm started to increase steadily and reached a plateau (30 and 13 mM, respectively) only when the levels of FBP were undetectable.
Moreover, ^{31}P-NMR spectra obtained using the same experimental setup, allowed measurements of NTP concentration as well as of glycolytic intermediates and NDP-hexoses. 3-PGA was the predominant phosphorylated metabolite in starved cells; following glucose addition, resonances due to G6P and FBP were detected as well as the NTP resonances. The results are in good agreement with those derived from carbon-13 experiments.
The data obtained *in vivo* for FBP, 3-PGA, and PEP were complemented by enzymatic assays in extracts where G6P, DHAP, FBP, F6P, 3-PGA, 2-PGA, PEP and pyruvate were measured. All the information available was used as input for the construction of a mechanistic model of glycolysis. The dynamics of intermediate metabolites accumulation confirm that the hydrolysis of FBP and the inter-conversions in the aldolase triangle represent an important control step in glycolysis. Upstream intermediates (G6P, FBP, DHAP) accumulate immediately after glucose addition, in opposition to downstream intermediates (3-PGA, 2-PGA, PEP). The other key features in glycolysis regulation were the accumulation of NAD(P)H, 3-PGA and PEP influencing the backflux through the aldolase triangle and glucose intake due to PTS activity. The mechanistic model was adjusted directly to the transient intermediate metabolite concentrations (instead of using the calculated metabolic rates) in order to have an unbiased error distribution for the estimates of kinetic coefficients. This mechanistic model was then used to calculate metabolic control coefficients for the different metabolic steps in order to identify candidates for engineering.

Proposta de uma Ferramenta para Cálculo de Velocidades em Processos Fermentativos

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Palavras-chave: Método Spline, velocidade específica, fermentação.

A velocidade específica gerada a partir de valores experimentais é parâmetro de relevante importância para análise da cinética de processos fermentativos. Para tanto, é necessário a adoção de um modelo matemático que melhor represente o comportamento das variáveis envolvidas.

Utilizando-se o Método Spline, foi construído um programa de computador para tratamento de dados experimentais em processos fermentativos. O programa, a partir dos dados experimentais de concentração celular, consumo de substrato e formação de produto, determina as respectivas velocidades de processo. Estes dados podem ser informados diretamente ao programa ou serem importados de outro aplicativo, no formato texto.

O usuário visualiza graficamente a curva gerada, e pode realizar alterações nos pontos de entrada com o objetivo de melhorar o modelo matemático obtido. A partir do modelo gerado, são calculadas as velocidades específicas de processo em intervalos determinados pelo usuário.

O Método Spline foi utilizado em decorrência de seu melhor comportamento, conforme descrito na literatura corrente [1]. O programa é operado em microcomputadores tipo Pentium e foi desenvolvido para a plataforma Microsoft Windows95, utilizando Borland Delphi 2.0.

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Specific Oxygen Uptake Rate for the Xylitol Production by *Candida guilliermondii* IM/UFRJ 50088

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Keywords: Xylitol, optimization, oxygen uptake, Candida guilliermondii

Xylitol is a naturally occurring power sweetener, anticariogenic, whose human metabolization independs of insulin, indicating its use for diabetics. Industrially, it is produced by catalitic reduction of xylose from xylan-rich hemicellulosic materials hydrolysis, having the disadvantage of low-specific catalisis, and resulting in the production of other polyalcohols besides xylitol.

Alternatively, the biotechnology process has shown atractive due to the high specificity of enzymes, involved in xylose catabolism, leading to high conversion yields. The principal variables which dictate the optimum conditions for the bioconversion of xylose to xylitol are: dissolved oxygen, initial substrate and cell mass concentrations, which also display interdependence.

In this context, experiments were carried out in shaken flasks and in a bench bioreactor with a dissolved oxygen concentration sensor, aiming to investigate the influence of these three variables.

The oxygen supply was standardized by the gas-liquid mass transfer coefficient (K_La) measurements for each fermentation. It was obtained the ratio: oxygen transfer rate/cell concentration that maximized the xylitol production. This value was called Specific Oxygen Uptake Rate for the xylitol production, since the bioreactor was operated in a condition of oxygen supply equal to cell demand.

Enzimatic Complex Mixture Purification by Ethanol Precipitation

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***Keywords**: ethanol, precipitation, glucoamylase, xylanase, fractionation*

Ethanol precipitation is a technique that has been applied to several types of proteins on industrial scale and can, in some cases, fractionate enzymes. Two target enzymes, glucoamylase and xylanase, were recovered from clarified culture broths of *Aspergillus awamori* and *Penicilium janthinellum* respectively, by ethanol precipitation. All experiments were carried out with absolute ethanol, at 5°C and pH 4.5

The **glucoamylase complex** presents, at least, three isoenzymes, with very close values of molecular weight, from 72 to 80KDa. It was verified that below 40%v/v of ethanol, none of the medium proteins were precipitated and from 40% to 60%v/v, all enzymes of the glucoamylase complex has a great solubility decrease making this system unable to the fractionation of the isoenzymes. Between 60%v/v to 80%v/v ethanol all glucoamylase was precipitated and almost 35% of the initial total protein content, representing low molecular weight contaminants (6KDa), remains soluble. With a single stage ethanol precipitation with 60%v/v ethanol, 100% of glucoamylase activity was recovered in the precipitated phase with an increase of the specific activity of 1.7.

A three step fractionation was performed under 20%, 60% and 80%v/v of ethanol for the recovery of the **xylanolitic complex**. In the first step, around 20% of the total protein and 10% of the beta-xylosidases were precipitated. In the second step, 75% of the remaining beta-xylosidases (molecular weights of 107 and 115kDa) was recovered and an increase of the specific activity of 4.3 was obtained. In the third step, 82% of the remaining xylanolitic complex (three endoxylanases with molecular weights of 20-30kDa) was precipitated. The enzymes of the xylanolitic complex have a wide range of molecular weight, from 20 to 115 kDa, allowing fractionation of the isoenzymes while that of the glucoamylse complex, showing a narrow molecular weight range, are not allowable of fractionation through this method.

The above results confirm that the solvent concentration necessary for the precipitation depends strongly on the molecular weight of the protein, beeing the higher the molecular weight of the protein the lower the solvent concentration necessary. Accordingly to this behavior, almost total glucoamylase precipitation was verified between 60 and 80% v/v ethanol, while for β-xylosidase, 10% of the activity was recovered with only 20% v/v of ethanol, beeing the remaining of the enzyme recovered with 60%v/v of ethanol. Although other protein features related to solubility, like the glycosilation level, can drive this process, this does not seem to be taking place in the present study since glucoamylase has 5 to 20% (w/w) [1] and β-xylosidase has 30% (w/w) [2] of carbohydrate content.

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Effect of Cell Purge on the Stability of Microfiltration Cell Recycle Fermentation Systems

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High cell concentration fermentations are operated on reactors with cell recycle on ultra/microfiltration membranes to produce different products. These type of processes generally lead to a significant improvement in productivity, unless there is a large drop in the specific production rates. In fact, the objective is not just to obtain a high cell concentration culture but a high concentration of active cells[1]. Therefore, to operate successfully, both physiological and engineering parameters must be optimized[2].

The rheological behavior of the fermentation broth during operation is quite important since it determines mixing and mass transfer rates within the fermenter. Only a few of the large number of publications (for ex. 3) related to cell recycle studies consider the effect of broth rheology, which is critical for operational stability.

In this work the fermentation process was operated with complete cell retention at three different dilution rates and broth rheology evaluated. It was found that the cell concentration increase is responsible for the viscosity rise observed, leading to hydrodynamic changes on the broth behavior and consequently to operational instability. Note that for implementation of industrial fermentations, process operation has to be trouble free[4]. To avoid these rheological problems and therefore to operate long term fermentations with a good stability, a strategy to control cell concentration has been proposed: the introduction of a cell purge to the cell recycle system so that partial cell retention fermentations are operated instead of complete cell retention. To obtain long term stable fermentations, the quantification of the cell bleed rate and the precise moment to start bleeding are of paramount importance and have been tested and discussed.

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Influence of Aeration on the Stability of Recombinant *S. cerevisiae* During Discontinuous Operation

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Key words: recombinant S. cerevisiae*, exo-β-glucanase, bioreactor, stability.*

The use of recombinant microorganisms in industrial processes usually has to face the problem of plasmid instability. The proper selection of operational variables in the bioreactor such as agitation rate, aeration level or dilution rate are some of the strategies proposed to overcome this problem. The stability of recombinant cells can be enhanced by means of an adequate control of the oxygen supply.(1).

In this work, the influence of the aeration rate on the stability of a recombinant *S. cerevisiae* strain expressing the *EXG1* gene was analyzed. A number of batch experiments were carried out in a BIOSTAT® B bioreactor with a working volumen of 2 L. Two fermentation processes with a non-selective medium were carried out operating either with anaerobic and aeration. Temperature, pH and agitation rate were automatically controlled and set to 30°C, 5 and 200 rpm, respectively.

As it can be seen in Table 1, higher decrease in recombinant cells (24.2%) was observed under anaerobic condition. However, when aeration was used, the fraction of recombinant cells decreased only at 4.3%. Redox balance were estimated from metabolites and substrate concentrations. A higher enzymatic activity and availability of reduced cofactors (NAD(P)H) were observed under aerobic conditions.

	$\%\Delta P^+$	cofactors mM	EXG $activity_{max}$,U	μ_{max}, h^{-1}
Anaerobic	24.2	11.1	178.7	0.186
Aerobic	4.3	22.1	194.4	0.224

Table 1. Decrease of recombinant cell fraction %ΔP+, exo-β-glucanase (EXG) activity, maximum growth rate, and redox balance (in mM cofactors).

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Design of a Neural Network to Predict the Behaviour of a Recombinant System In Fed-Batch Fermentations

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Modelling the behaviour of recombinant system cultures is a difficult task since there are two different cell populations growing at different rates (cells with plasmid and cells without). Also the rate at which the recombinant enzyme is synthesized depends on the cellular condition that often is characterized by the growth rate. Different approaches have been presented to mathematically describe the profiles of the state variables (substrate, biomass, protein, enzyme and concentration of intracellular components) as a function of time in fermentations of recombinant systems. These mathematical models involve a great number of parameters that have to be estimated from experimental data assuming constant values but these will often depend on the state of the system. The complex interrelationships between the variables cannot be described through common expressions. These nonlinear characteristics decrease the prediction capability of the model. Neural networks reduce the effort to model complex relationships and can give a similar or higher prediction degree. In the present work we determined the structure of a neural network (number of hidden layers, number of nodes in each layer, and the activation functions) able to simulate fed-batch fermentations of the recombinant system *Bacillus subtilis* DN1885(pCH7). Experimental data obtained for different feed rate strategies were used to estimate the parameters in the net. A comparison between the prediction given by a mathematical model previously derived for the system and those given by the neural network will be presented.

Formation of Biofilms on Suspended Particles in an Airlift Bioreactor

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Key words: Biofilm Airlift Reactor, exopolymers, Pseudomonas fluorescens

The Biofilm Airlift Reactor is one of the new technologies applied to waste water treatment.This is a promising system for aerobic treatment since it is possible to achieve a high biomass concentration in the reactor that leads to a high substrate consumption.
In this research, a biofilm of *Pseudomonas fluorescens* was studied using suspended basalt particles in an airlift bioreactor. In order to investigate the effect of substrate loading, biofilm formation was followed under different glucose influent concentrations (25, 53, 78, 150, 191 mg/L). During these experiments, the observed reaction rate (r_{fa}), the specific removal rate (q_{obs}), the respiratory activity, biofilm thickness and its composition in proteins and polysaccharides (total and extracellular) were measured. Biofilm detachment was followed by measuring the Total Suspended Solids (SST).
Results - Higher substrate loadings resulted in thicker hairy biofilms much more susceptible to detachment, leading to lower Biofilm/SST ratios (Figure 1).
Substrate uptake rates and the specific removal rates clearly showed that, despite increasing organic load and consequently increasing biofilm thickness, there is no decrease in the biological activity (Figure 2). This fact seemed to be confirmed by the respiration rates.
An increase of exopolymer content occurred at the beginning of each test, followed by a decrease and finally attaining a stable value. These experimental data confirm the key role of exopolymers during the early phase of microbial adhesion. Results also showed that polysaccharides were the mainly component of the exopolymer matrix, although it was possible to detect some proteins.
The **total** proteins and polysaccharides amounts were also determined in all those biofilms. In such samples, a preponderance of proteins was observed.
Assuming that cell mass can be indirectly quantified by measuring the total proteins content, as some authors propose, it seems credible that a substantial part of these biofilms were bacterial cells. This is in agreement with the activity results indicated above.

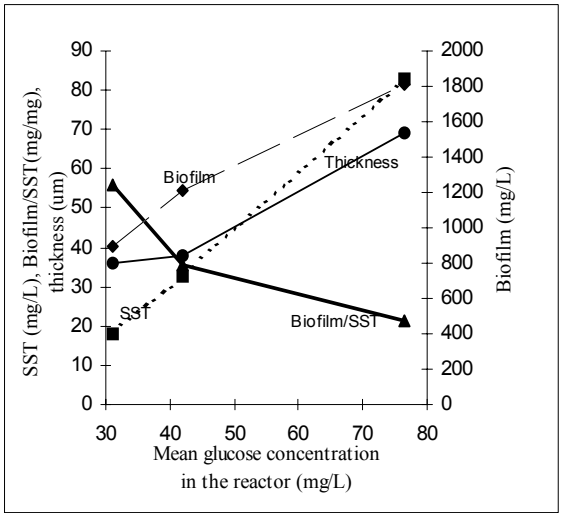

Figure 1 - Steady-state values of biofilm concentration, SST, Biofilm/SST ratio and Biofilm thickness

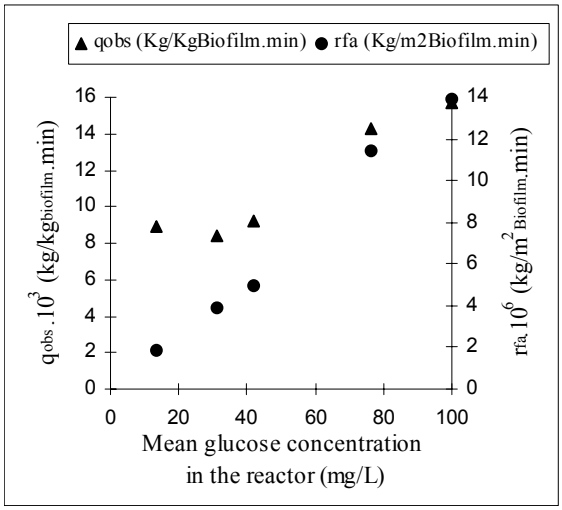

Figure 2 - Steady-state values of r_{fa} and q_{obs} obtained under different substrate conditions

Production of Lactan Using Plain Whey, Whey Permeate and Synthetic Medium as Feedstock

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Key words: *dairy, products, exopolysaccharide,* Rahnella aquatilis, *fermentation*

Whey (or whey permeate), a by-product of cheese manufacture, has created a worldwide problem of waste disposal owing to its high biological oxygen demand. Production of lactan has been previously described based on a semidefined medium rich in lactose using *Rahnella aquatilis*. This research was aimed at obtaining lactan directly from whole whey without additional nutrients, as well as and whey permeate obtained after ultrafiltration, using a similar type of strain, and the fermentation process was compared with that using the synthetic medium previously tested.

The growth of biomass growth rate, the polysaccharide production rate and the viscosity of the broth were monitored. Organic acids, lactose, peptides and free amino acids were also determined. The growth curves were similar for the three media, showing a maximum specific growth rate of 0.61 h^{-1}, 0.65 h^{-1} and 0.63 h^{-1} for whey, whey permeate and synthetic medium, respectively. The major increase in polysaccharide production was observed between 12 h (beginning of stationary phase) and 24 h for whey and the synthetic medium; however, the increase in the case of whey permeate is less pronounced and occurs essentially after 24 h. The yield of polysaccharide was 0.59 $g/g_{lactose}$, 0.56 $g/g_{lactose}$ and 0.37 $g/g_{lactose}$ for synthetic medium, plain whey and whey permeate, respectively. The larger amount of citrate present in whey was used by *Rahnella aquatilis* with significant formation of acetic acid in the first 12 h and acetoine thereafter; whey permeate and synthetic media did not lead to acetoine formation. The final yields of the various organic acids for the synthetic medium, whey and whey permeate, respectively, were: 0.08, 0.07 and 0.03 ($g/g_{lactose}$) for acetic acid; 0.02, 0.06 and 0,00 ($g/g_{lactose}$) for lactic acid; 0.08, 0.08 and 0.02 ($g/g_{lactose}$) for formic acid; 0.04, 0.01 and 0.00 ($g/g_{lactose}$) for succinic acid; and 0.00, 0.11 and 0.00 ($g/g_{lactose}$) for acetoine. Lactose was almost completely depleted by 48 h of fermentation in the case of whey and synthetic medium, but only part of lactose was consumed in the whey permeate (final yield of 0.43 $g/g_{lactose}$). Small peptides (< 4,000 Da) and most free amino acids were consumed by 24 h in whey and synthetic medium. The whey permeate possessed low amounts of peptides (virtually consumed by 12 h) and very low concentrations of free amino acids, which increased slightly between 12 and 24 h.

Faster Development of Fermentation Processes. III. Intelligent Systems for Peniciflin Fermentation Process Modeding

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***Keywords**: modelling, industrial processes, artificial neural networks*

Artificial Neural Networks (ANN) are emerging as important modelling tools in bioprocesses due to their inherent non-linear behaviour. ANN are non-mechanistic models that require large quantities of process data for training - they learn by example. There many issues that must be considered in building robust ANN models of real bioprocesses for which the data available is both noisy and limited to a narrow range of operating conditions.

In this work ANN were used to model penicillin fed-batch fermentation in an industrial pilot-plant [FASTERPROD,1997]. The fermentation data set used in modelling was chosen through multivariate statistical techniques to ensure that the range operating conditions was adequately covered.

Culture age, feed rates and broth volume were used as model input variables and predictions of biomass, substrate and penicillin concentration were obtained after optimisation of network parameters. Results from feedforward, global recurrent and hybrid models were compared against real process data. Model validation was carried out testing its predictive capacity in fermentation sets not used in model development.

Neural networks proved to be an extremely attractive alternative to mechanistic models as process control models, when model development time is important, but their extrapolation capacity is limited to the range of operating conditions used in training the network and must be considered in process optimisation.

FASTERPROD,1997, http://alfa.ist.utl.pt/~qmenezes/fasterprod/

Faster Development of Fermentation Processes. II. Derived Variables an Invaluable Tool in Quantitative Physiology

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***Keywords**: biomass estimation, CER, OUR, industrial processes*

Biomass concentration is the single most important and difficult to monitor variable in an industrial fermentation process. The use of complex media with a high content of insoluble metabolisable substrates interferes with any direct monitoring method. Without the knowledge of the amount of viable biomass in the fermenter it is not possible to quantify the metabolic parameters needed to develop any rational optimisation strategy for the process.

The use of indirect methods such as the evaluation of cell mass through off-gas analysis is one way to overcome the above difficulties. In fact, this has proved to be a valuable method of gaining insight into the performance of many aerobic processes [HEINZLE E DUNN,1991]. The transfer rates of oxygen and carbon dioxide to and from the broth can be calculated by a mass balance over the fermenter to yield a usually good estimation of oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER). The respiratory quotient – RQ (the ratio of CER to OUR) - is also a known indicator of metabolic shifts from growth to product formation or switches between primary substrates [HEINZLE E DUNN,1991].

In this report we present the first results obtained with O_2 and CO_2 gas analysers in a 10-tank industrial fermentation pilot-plant facility, recently upgraded with respect to basic instrumentation, dedicated advanced analytical equipment and automatic data acquisition. Assuming constant stoichiometric coefficients as suggested by a constant unitary RQ in the production phase, OUR and CER values can be used to estimate on-line cell mass and also product concentrations. These possibilities have been investigated and the results will be discussed in terms of their potential as a more rational and faster approach to fermentation process development [FASTERPROD,1997].

In addition, the local area computer-network and all software components developed for remote supervision of this pilot-plant, based on the industry-strength package LABTECH CONTROL®, will be fully described [FASTERPROD,1997].

HEINZLE, E., DUNN, I.J., 1991, "Methods and Instruments in Fermentation Gas Analysis.", *in* "Biotechnology.", *in* "Measuring, Modelling and Control.", vol. 4, pg. 27-74, Ed. K. Schügerl, 2nd ed., *in* "Biotechnology", Eds. H.J. Rehm, G. Reed, VCH, Germany.

FASTERPROD,1997, http://alfa.ist.utl.pt/~qmenezes/fasterprod/

Faster Development of Fermentation Processes. I. Monitoring Real Processes in an Industrial Pilot-Plant

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***Keywords**: monitoring, industrial processes, FIA, biosensors*

Monitoring of industrial fermentation processes with complex media is still mostly based in off-line analysis of broth samples. The use of in-situ or on-line sensors is limited to pH, temperature, dissolved and off-gas analysis. The lack of sterilisable sensors or of reliable sampling devices that can work unattended for long times under these conditions, are the two hindering factors [SCHÜGERL,1991].

Off-line monitoring of real fermentation processes can be very effective in providing the information needed for process development and control. Many fast, high-specificity and high-selectivity analytical techniques, such as biosensors, FIA or HPLC, now universally available can be used quite successfully in the characterisation of industrial media. Unfortunately, some of these techniques are still unknown to many bio-industries.

Here we report on applications of such techniques and their combinations, in monitoring substrates (C&N-sources), metabolic products (antibiotics and by-products), and inorganic salts (e.g., phosphates) in fermentation processes in an industrial fermentation pilot-plant.

Three cases will be described. Sugar analysis with several methods (wet-chemistry analysis, biosensors, HPLC) to determine media composition and understand carbohydrate uptake. Precursor analysis by a FIA-biosensor set-up and comparison with results from a commercial enzymatic kit. HPLC analysis of a secondary metabolite and comparison with chemical analysis results to determine process selectivity and product decomposition.

It will be shown that combining different techniques important process information can be obtained on media composition, product decomposition, by-product formation or selective substrate uptake, thus enabling a faster development of fermentation processes [FASTERPROD,1997].

SCHÜGERL, K., 1991, "On-Line Analysis of Broth.", *in* "Measuring, Modelling and Control.", vol. 4, pg. 149-178, Ed. K. Schügerl, 2nd ed., *in* "Biotechnology", Eds. H.J. Rehm, G. Reed, VCH, Germany.

FASTERPROD,1997, http://alfa.ist.utl.pt/~qmenezes/fasterprod/

Continuous Ethanol Fermentation from D-xylose with Cell Recycle using the yeast *Pichia stipitis*

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Keywords: *xylose, ethanol,* Pichia*, continuous fermentation, cell recycle*

The hemicellulose fraction of some lignocellulosic materials can represent up to 30% of the total biomass content, with D-xylose as the principal sugar component. This work encompassed the continuous alcohol fermentation of this particular sugar.
In the brazilian context, despite the fact that a large amount of sugar cane bagasse is annually consumed, there has been a surplus of ca. 9 x 10^6 tonnes, being these raw material considered approprieted for bioconvertions, since there are no production costs attached to it.
The system was composed of a mechanical stirred bio-reactor, continuously fed with medium containing xylose in a concentration of 50 g/L. The outlet was sent to a conical settler, where the yeast, bearing flocculation characterists, was separated from the fermented broth and recycled to the bio-reactor, aiming to operate it with a higher cell density, resulting in increased volumetric productivity.
The best results obtained for the continuous system described above were: Yp/s = 0.45 g/g (ca. 90% of fermentation efficiency), and Qp = 2.7 g/L.h, for a dilution rate of 0.27 h^{-1}, which corresponds to a residence time of 4 hours.
The strain showed to be sufficient stable to withstand variations in the substrate feed rate, and had its flocculation characterists enhanced with the elapsed time.

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Cassava Wastes Hydrolysate: an Alternative Carbon Source for Citric Acid Production by *Candida lipolytica*

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***Keywords**: citric acid, cassava hydrolysate, yeasts*

Citric acid is a commercially important product obtained mostly by fungal submerged fermentation. Its global production reached now 736 thousands of tones per year. From the total production, 70% is consumed in food and beverage industry, 12% in pharmaceutical industry and 18% in a variety of applications. Despite this important amount of produced citric acid, its consumption raises between 3.5% and 5% each year [1]. New economical alternatives for its production are then required.
This work shows the ability of solid and liquid wastes of cassava processing to be used as substrates to produce citric acid by yeasts. Cassava bagasse, brown peels and "água vegetal" are starch rich residues normally discarded in the State of Paraná, Brazil. These residues can be pretreated with amylolitic enzymes and use as carbon source in different fermenting processes. *Candida lipolytica* NRRL Y-1095 was chosen for its excellent potential for industrial production of citric acid from a wide variety of carbon sources, including glucose, hexadecanes, n-alkanes, edible oils and different hydrolysates. A two step hydrolysis was conducted in order to form fermentable sugars, mainly glucose, easily consumed by microorganisms. In these steps, the medium was added of α-amylase and amyloglucosidase. Hydrolysis rate reached a maximum of 100–120 g/L of reducing sugars, after 25 h of incubation using both liquid and solid residues. Batch fermentations were performed in two stages, a carbon-limited growth phase and a nitrogen-limited phase. Conditions were fixed in 28° C, pH 5.5 and 150 rpm (200 rpm for the second stage). The composition of both growth and production media were presented before [2]. After inoculation (40 to 48 hours) the cells were separated from the medium by centrifugation. The production medium was then added to the cells, being obtained 10 g/L of citric acid and a smaller amount (2.5g/L) of isocitric acid after 96 h of culture, showing that cassava wastes are suitable for citric acid production by *Candida lipolytica* NRRL Y-1095. Optimization assays are being developed to increase the amount of produced acid.

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Fermentação Alcoólica de Matéria-Prima Amilácea em Biorreator com uma Levedura Recombinante Imobilizada em Gel de Pectina

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Palavras chave: Saccharomyces cerevisiae*, levedura recombinante, imobilização, etanol, glicoamilase.*

Este trabalho tem por objetivo a avaliação do desempenho da levedura amilolítica recombinante *Saccharomyces cerevisiae* L36, portadora da codificação genética de glicoamilase, durante a fermentação alcoólica de matéria-prima amilácea. O processo foi conduzido com as células imobilizadas em gel de pectina, em sistema contínuo e de bateladas repetidas, em um reator de mistura convencional, adaptando-se uma cesta de tela de aço inoxidável no seu interior (reator tipo cesta).

No sistema contínuo, embora não tenha se verificado um consumo total das dextrinas presentes no meio, para os valores de vazão específica de alimentação (D) empregados, foram obtidos valores de produtividade em etanol de cerca de 1,0g/L.h, similares aos obtidos no sistema em bateladas repetidas, com uma concentração de etanol próxima a 70 g/L, significando um rendimento da ordem de 85% em relação ao rendimento estequiométrico. A produtividade em glicoamilase foi de cerca de 1,0U/L.h em ambos os processos, com valores de atividade enzimática no meio de 57,7U/L e 54,8U/L, respectivamente, para o sistema em bateladas repetidas e sistema contínuo.

A levedura recombinante imobilizada mostrou, portanto, desempenho satisfatório em ambos os tipos de processos estudados, indicando que a imobilização das células foi eficiente na manutenção da estabilidade do plasmídeo.

Studies on Hydrophobic Interaction Chromatography of *Trichoderma reesei* Cellulase Complex

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Key words: Cellulases, Hydrophobic Interaction Chromatography, T. reesei

Interest in the application of enzymes present in the cellulase complex produced by the fungus *Trichoderma reesei* has considerably increased in consequence of their potential applications in textile, paper and food industries. It is established that *T. reesei* produces different types of cellulases: two cellobiohydrolases (CBH I and II: EC 3.2.1.91), four endoglucanases (EG I, II, III and IV: EC 3.2.1.4) and one ß-glucosidase (EC 3.2.1.21). The enzymes appear in multiple isoforms in culture filtrates of *T. reesei* and act in synergism during hydrolysis of cellulose [1].

Complex cellulase systems present considerable purification problems. Ion-exchange chromatographic methods have been used most often, but affinity chromatography has been attempted too. This work reports new studies for cellulase complex purification by hydrophobic interaction chromatography (HIC). In HIC, the proteins are separated based on differences in their content of hydrophobic amino acid side chains located on their surface. The addition of salting-out salts to equilibration buffer and sample solution reduces the availability of water molecules in solution, increases the surface tension and improves the ligand-protein interactions. Decreasing salt concentration of eluent, the elution and fractionation are done according to differences in surface hydrophobicity. The type of ligand and matrix, the salt and its concentration, pH, temperature and additives are the most important factors to consider for separation processes using HIC.

The crude commercial preparation of *T. reesei* cellulases (Celluclast 1.5L) was purified by using a HIC step on Sepharose CL-6B modified by covalent immobilization of 1,4-butanediol diglycidyl ether [2]. The first step on the purification procedure consists in a gel filtration on Sephadex G-25M. The cellulase active fractions obtained were then pooled and concentrated using a Millipore Ultrafree 10,000 filter. The enzyme solution was put on the hydrophobic column described above and the influence of mobile phase composition (type of salt and concentration, pH) on the chromatographic behaviour of cellulase complex was described. All the experimental results obtained with the HIC step described above seems to provide an interesting approach for cellulases fractionation.

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Producción de Escleroglucano con *Sclerotium rolfsii*: un Modelo Cinético No Estructurado-No Segregado

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Palabras Clave: escleroglucano, producción, modelo cinético, determinación parámetros

El escleroglucano es una goma natural producida por hongos del género *Sclerotium.* Este polisacárido microbiano presenta características reológicas interesantes, siendo empleado en diferentes tipos de industrias, como la textil, la papelera, en alimentación e incluso, en la extracción del petróleo [1].

En este trabajo se ha empleado el microorganismo *Sclerotium rolfsii* PROIMI F-6656 para la producción de escleroglucano. El estudio experimental se ha llevado a cabo en incubadora orbital empleando una agitación de 300 r.p.m., una temperatura de 30ºC y un medio de producción sintético (compuesto de sales minerales y sacarosa).

Se han analizado la biomasa y el producto obtenido por peso seco, previa separación del polisacárido y el micelio. Después de estudiar la influencia de la fuente nitrogenada empleada y su concentración no solo en el rendimiento de la producción sino también en el comportamiento reológico del biopolímero sintetizado por el hongo, se ha propuesto un modelo cinético. Este modelo incluye el crecimiento del microorganismo y la producción del polisacárido, siendo del tipo no estructurado-no segregado [2].

El modelo cinético propuesto considera dos respuestas, de acuerdo a las siguientes ecuaciones diferenciales:

$$\frac{dC_X}{dt} = \mu \cdot C_x \cdot \left(1 - \frac{C_X}{C_{X_0} + Y_{XN} \cdot C_{N_0}}\right) \quad (1)$$

$$\frac{dC_P}{dt} = m \cdot C_X + n \cdot \frac{dC_X}{dt} \quad (2)$$

El tipo de ajuste realizado ha sido no lineal, mediante el algoritmo de Marquardt [3] en simple-respuesta, aplicando el método integral mediante un algoritmo de Runge-Kutta de cuarto orden acoplado al anterior. Los valores de los parámetros cinéticos de crecimiento (μ e Y_{XN}) y de producción (m y n), obtenidos por ajuste de los datos experimentales, resultaron estadísticamente significativos. La reproducción obtenida de los resultados experimentales es buena y sin tendencia en el error.

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Modelo Cinético de la Hidrólisis de Lactosa con β-Galactosidasa de *E. coli*

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Palabras clave: modelo cinético, lactosa, β-galactosidasa, Escherichia coli

La hidrólisis de lactosa es una reacción de interés en la industria alimentaria. Se puede llevar a cabo utilizando tanto ácidos como enzimas. La hidrólisis enzimática de la lactosa no provoca en el alimento los problemas asociados a la hidrólisis ácida y lo hace apropiado para el consumo de muchas personas con bajos niveles de actividad β-galactosidasa intestinal. Además revaloriza, junto con otros procesos enzimáticos, los sueros [1]. La β-galactosidasa de *E. coli* es una enzima de la que se han realizado numerosos estudios referentes a su estructura, a su inmovilización y se ha determinado su actividad en a la hidrólisis de lactosa [2, 3]. Sin embargo, no se puede decir que exista un modelo cinético de esta reacción con esta enzima, aunque los hay propuestos para β-galactosidasas de otros microorganismos, considerados más seguros para su uso en la industria alimentaria, como *A. niger* y *K. fragilis* [4, 5].

En el presente trabajo se ha estudiado el efecto de la temperatura (4-40ºC) y las concentraciones de enzima (2-10 mg/l), lactosa (25-75 g/l) y monosacáridos (0-15 g/l) sobre la velocidad de hidrólisis de lactosa. Los datos se han ajustado a diversos modelos cinéticos mediante regresión no lineal acoplada a una integración numérica de las ecuaciones cinéticas, por el uso de datos integrales. Primero se han analizado los datos a temperatura constante, y posteriormente a diversas temperaturas. Los criterios de discriminación utilizados han sido estadísticos (F, t) y físicos (signos de los parámetros, variación de su valor con la temperatura de reacción, etc.). Se ha elegido un modelo cinético tipo Michaelis-Menten con inhibición acompetitiva por glucosa, cuya ecuación cinética es la siguiente:

$$r = \frac{\exp\cdot(13{,}72 \pm 0{,}6 - \frac{7239 \pm 186}{T}) \cdot C_E \cdot C_{lac}}{\exp\cdot(11{,}32 \pm 6{,}1 - \frac{4894 \pm 1910}{T}) + C_{lac} \cdot (1 + \frac{C_{glu}}{\exp\cdot(1{,}363 \pm 1{,}9 - \frac{1007 \pm 480}{T})})}$$

Es de destacar la inhibición por glucosa que presenta esta enzima, en lugar de la habitual inhibición por galactosa de la mayoría de las β-galactosidasas.

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Effect of Liquid-Phase Surface Tension on Hydrodynamics of a Three-phase Airlift Reactor with an Enlarged Degassing Zone

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Key Words: *Airlift, surface tension, gas holdup, solids holdup, hydrodynamics*

Airlift reactors are especially useful for operations requiring solid-suspension without high shear force (e.g. fermentation and cell culture). Fermentation broths are complex mixture of cells, sugars, electrolytes, proteins, etc. and exhibit high viscosity, low surface tension and non-Newtonian characteristics [1]. However, the majority of hydrodynamic data for airlift reactors has been obtained with air/water systems, with properties different from the real conditions of operation. It is, nevertheless, known that both liquid viscosity and surface tension affect gas holdup. The small bubbles formed in liquids with reduced surface tension may enhance gas holdup.

It is considered that dilute aqueous alcohol solutions simulate reasonably well the liquid-phase behaviour in bioreactors [2], being the surface tension the only physical property which differs significantly from water. Aqueous solutions of ethanol represent a coalescence inhibiting system, which will affect bubble size and the overall hydrodynamics.

The aim of this study is to compare the behaviour of a three-phase airlift reactor (60 l), of the concentric draught-tube type, with an enlarged degassing zone when water and an aqueous solution of ethanol are used as liquid-phase. The concentration of ethanol used (10 g/l) is higher than the limiting concentration (0.11 mol/l) reported by Zahradník *et al.* [3], in order to observe the maximum deviation possible. Ca-alginate beads were used as solid-phase. Gas and solids holdup in the riser and in the downcomer, circulation and mixing times and riser and downcomer interstitial liquid velocity were measured for both liquid-phases, changing solids loading (0% to 30% (v/v) and airflow rate (from 1.9 to 90.2 l/min).

The reduction in surface tension with the addition of ethanol has a great influence on the hydrodynamics of the airlift reactor being observed, as primary consequence, the increase of riser gas holdup and the augment of the entrance of gas into the downcomer. Deriving from the increase of the riser and downcomer gas holdup a decrease of solids holdup in these sections is observed. However, the difference between gas and solids holdup in the riser and in the downcomer remains practically constant when ethanol is added. Consequently, due to the maintenance of the driving force for the circulation, the circulation time for the ethanol solution is similar to the one observed for water. The lower riser and downcomer solids holdup observed for ethanol causes a decrease of the riser and downcomer interstitial liquid velocity. With the exception of the high solids loading and the low airflow rates, the presence of ethanol in the liquid-phase increases the mixing time in the three-phase system.

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Influence of Temperature and Water content on Solubilities of *Guevina avellana* and *Rosa moschata.* Oils in Ethanol-Water mixtures near azeotrope.

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Keywords: Guevina avellana, Rosa moschata, oil, ethanol

OBJECTIVE

The objective of this work was to determine solubilities of *Guevina avellana* and *Rosa moschata* oils in near-azeotrope ethanol-water mixtures at different temperatures with variable water content. These parameters need to be known to develop a semicontinuous extraction process for enzymatically-treated seeds in optimal conditions, as the water greatly influences the solubility of oils in ethanol (Abraham *et al*., 1988, 1993).

MATERIALS AND METHODS

Experimental conditions. Oil from the two different seeds was incubated at different temperatures between 20 °C and 60 °C. Water content was varied between 92 % and 99%, which is the range within which ethanol concentration is expected to be, because of water absorption or loss in contact with seeds.

Solubility of oil in water-ethanol mixtures was determined from saturated oil-solvent miscellas by weighing the oil in an analytical balance AND HR-120.

Miscellas were obtained by placing an excess of oil in contact with the solvent into screw-capped tubes with a magnetic stirrer bar inside. Saturation is achieved after 24 hours stirring. A water thermostate Techne TE-8J was employed to control the temperature.

Materials. *Guevina avellana* and *Rosa moschata* seeds were collected during 1997 in Chile producer areas and they were kindly supplied by Forestal Casino Ltda.,Santiago de Chile

RESULTS AND DISCUSSION

Solubilities about 0.015 mg oil/mL solvent were obtained at room temperature by employing 96% ethanol, increasing.those values up to 0.04 mg/mL when 99% ethanol was the solvent. Solubilities were notably enhanced at higher temperatures. The presence of water limits the oil solubility. The moisture of seeds must be adjusted so the ethanol employed does not reach a water content that can cause a very low solubility of oil (Rohn, 1993).
Temperature affects the solubility, this showing a linear behaviour in the studied range. Below 40 °C the values of solubility are very low.
Water content affects the solubility more than temperature does. Although lower water content and technically optimum, a compromise solution is needed to make the process available. An optimum drying of seeds, allowing the azeotropic ethanol to loss water and increasing the ethanol content can lead to the best conditions for this extraction process.

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Fermentation of Low Grade Glycerol by *Clostridium butyricum* and *Clostridum acetobutylicum* Strains

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***Keywords**: glycerol, 1,3-propanediol, butanol,* Cl. butyricum, Cl. acetobutylicum

In recent years there has been an increasing interest in using Biodiesel as a less pollutant, alternative fuel [1]. The Biodiesel is produced by the transesterification of plant seed oils and yields glycerol as the main by-product. A subsequent utilisation of this "low grade" glycerol could help to decrease the Biodiesel production costs and facilitate the implementation of Biodiesel as an alternative fuel.

It has been shown that some clostridial species can ferment glycerol. *Clostridium butyricum* can use glycerol as the sole carbon source with a high conversion to 1,3-propanediol, which can be used as a monomer on biodegradable polymers synthesis. *Clostridum acetobutylicum* can use glycerol as a co-substrate of glucose to produce solvents such as butanol.

In this work a low grade glycerol, obtained through the transesterification process using rape seed oil, was used without prior purification.

Batch fermentations regulated at pH 6.5 were performed with *Cl. butyricum* VPI 3266 and VPI 1718. A concentration of glycerol as high as 40 g/l could be fermented with a maximum conversion of 0.60 g 1,3-propanediol/ g glycerol consumed and a 100% consumption of this glycerol.

Continuous cultures of *Cl. acetobutylicum* ATCC 4259 were performed at different pH values. A maximum concentration of butanol of 8.4 g/l was achieved.

Attempts to optimize culture conditions in order to improve the efficiency of these processes are still under investigation.

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Study of the Effective Power on Yeast and Filamentous Fungi in Aerated and Non Aerated Systems

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Key-words - bioreactor, bioprocess, aeration/agitation

A great number of industrial processes that use microorganism are carried out in aerobic conditions, and the combination of agitation and aeration are usually employed. The aeration, not only supply the biocatalist's metabolic necessities but also promotes a reduction of the impeller power consumption. The agitation leads to oxygen diffusion through the bioreactor, attaining a more homogeneous aerated system, and allows to reach an adequate microbial population development and the best oxygen system supply. Firstly, it was studied the influence of the aeration on power consumption in water, with different classes of impellers. The main objective was to obtain experimental data for a further comparison to those determined with microbial suspensions prepared with *Aspergillus niger* and *Saccharomyces cerevisiae* cells. In order to compare the influence of the aeration on power consumption, tests were made in aerated and agitated systems, and the results compared with those obtained without aeration. Correlations were established between the ratio of the required impeller power with or without aeration (P_G/P_O) and the aeration number ($N_A = Q/N.D^3$). In this mathematic model (P_G) stands for gassed power input and (P_O) stands for ungassed power input. The correlations were obtained for different types of impellers, being specific for each kind of fluid, either water or microbial suspensions. One of the most important characteristics of these correlations is the possibility to calculate the energy which would be saved with the introduction of air into the system. The volumetric air rate (Q) must supply the microbial demand and also reduce the energy consumption by the mechanical agitation system. Exponential decay was found to predict the performance of the parameters studied, $Y = Y_0 + A.e^{-(X/B)}$, and typical results achieved in the present work are displayed in the following tables.

Table 1 - Constants for different diameters and geometric impellers to *Saccharomyces cerevisiae* suspension.

D (cm)	Y_0	A	B
8,43 (1)	0,416	0,540	0,260
7,70 (2)	0,381	0,658	0,822
5,16 (3)	0,432	0,552	0,965
6,80 (4)	0,472	0,536	0,629

Table 2 - Constants for different diameters and geometric impellers to *Aspergillus niger* suspension.

D (cm)	Y_0	A	B
8,43 (1)	0,292	0,540	0,309
7,70 (2)	0,258	0,641	0,308
5,16 (3)	0,098	0,839	2,707
6,80 (4)	0,210	0,688	2,039

(1)flat blade turbine (3 blades); (2)flat blade turbine disc (18 blades);
(3)pitched blade turbine (4 blades); (4)marine propeller;

Water constants were $Y_0 = 0,299$, $A = 0,734$ and $B = 0,736$ for a pitched blade turbine with 4 blades and 5,16 cm diameter (D) impeller.

Automatic Determination of Yeast Cells Viability by Image Analysis

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***Keywords**: Image Analysis, Epifluorescence, Viability, Yeast*

There are numerous methods to determine cell viability. Such methods usually measure the loss of replication ability, cell damage or the reduction in metabolic activity [1]. Since conventional methods are time absorbing in detection and enumeration, have poor sensitivity and specificity and the reproducibility is relatively low, rapid methods are needed. The direct epifluorescence technique can be used to give rapid estimates and is often used for monitoring the microbiological quality of milk and other food-stuffs [2]. An advantage of this method over other rapid methods is that morphological characteristics of microorganisms can be ascertained [3]. However, large number of cells are necessary to obtain statiscally significant data, which makes this task laborious and time consuming. Image analysis and other technological improvements have extended the applications of epifluorescent microscopy, hence avoiding operator fatigue.

Epifluorescence microscopy with acridine orange as dying agent, is used for determination of viable and non-viable yeast cells. This fluorescent dye is a hydrophobic weak base which is known to bind to negatively charged, particularly phosphate groups. It attaches to adjacent phosphate groups in single-stranded nucleic acids (RNA) and fluoresces red/orange due to dye-dye interactions. Molecules of acridine orange will also intercale singly into double-stranded nucleic acids (DNA), and fluoresce green [4]. The cells are viewed thereafter in an ultra violet microscope and the images can be rapidly photographed. Subsequently these photos are digitised and the images can be enhanced by the Corel Photopaint™ 6.0 program (Corel Corp.) with the possibility of multiplying, adding and subtracting of the channel (RGB) images which can increase the contrast between viable (red/orange) and non-viable (green) cells. Image analysis can be performed in the Global Lab Image 3.21 (Data Translation, Inc.) where many parameters can be calculated, yet only area calculation and particles (cells) numbers are useful to this work.

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Modeling of Xylitol Production from Xylose in a Continuous Membrane Bioreactor

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Keywords: *xylitol, bioreactor, membrane, modeling.*

This study presents a mathematical model which describes the behavior of the principal variables involved in the continuous bioconversion of xylose to xylitol by *Candida guilliermondii,* carried out in a membrane bioreactor with total cell recycle. The bioproduction of xylitol is a promising of competitive process, since it is possible to obtain an increase in volumetric produtivity by operating the system with high cell densities.

The fermentation system model may be considered semistrutured and the following dependent variables were evaluated: cell mass, xylose, xylitol and dissolved oxygen concentrations, as well as xylose reductase and xylitol dehydrogenase enzymatic activities.

The inhibitory actions due to high xylose concentrations on microbial growth, and the influence of dissolved oxygen level were evaluated. The xylose uptake together with xylitol production and consumption were evaluated considering the inhibitory influence of the respecting substrates, and also over enzymes activities.

The model parameters were estimated by using in experimental data available in the literature. The results of the mathematical model were compared to experimental data to confirm its acuracy. Simulation studies were carried out for several dilution rates and the results were evaluated.

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Study of Clavulanic Acid Production by *Streptomyces clavuligerus*

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***Keywords**: Clavulanic acid;* Streptomyces clavuligerus*; β-lactam; antibiotic.*

Clavulanic acid is a potent inhibitor of ß-lactamases, which, when used with traditional ß-lactam antibiotic sensitive to ß-lactamase, protects them against hydrolysis by the enzyme and so minimise the bacterial resistance. It is produced by *Streptomyces clavuligerus* simultaneously with others ß-lactam antibiotics such as cephamycin C and penicillin N. As for the most secondary metabolites, the production of **clavulanic acid** occurs in two phases: trophophase (growth) and idiophase (production).

In this work, the best medium for growth of *Streptomyces clavuligerus* and the best one for production of **clavulanic acid** were elected, amongst several media described in literature, with the aim of using these media for further studies on the effect of aeration on growth and clavulanic acid production.

Biomass concentration was determined by dry weight of a known volume of sample until constant weight at 100ºC (dry cell weight). Carbon sources were quantified by high-performance liquid chromatography, except maltose, which was determined by the traditional method described by Nélson (1944). Clavulanic acid was determined by bioassay and spectrophotometric methods.

The microorganism showed significant growth (**22 g/L**) in the medium containing glycerol, malt extract, and bacteriological peptone. The results allowed to estimate the cell productivity (**Q_p = 3,67 g/L.d**) and the yield factor on biomass (**$Y_{X/S}$ = 1,94 g cell dry wt/ g glycerol consumed**). For this medium, different concentrations of nutrients were tested. From the results obtained in this set of experiments it could be observed that the most suitable composition for growth was: glycerol, 10 $g.L^{-1}$; malt extract, 4 $g.L^{-1}$ and bacteriological peptone, 6 $g.L^{-1}$.

Concerning the production, amongst the evaluated media, the highest level of **clavulanic acid** (**3,00 mg/L**) was obtained after four days, when maltose was used as the main carbon source. The necessity of mineral salts and iron for **clavulanic acid** production was evaluated. At last, batch cultivation in 2-L bioreactor was carried out with the production medium elected in this study. Further experiments have been developed in order to enhance the production of **clavulanic acid**.

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Environmental Biotechnology

Biofiltration in Latin America: State of the Art and Development of a Specific Technology

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***Keywords**: Gas, treatment, carriers, agricultural, sub-products.*

Facing the increasing concern about environment, depollution activities take a major place in our industrial society. Following the example of water and soil treatment, biological gas cleaning has been developed since the 1920's [1]. However, it is only a few decades ago that biological gas cleaning became a competitive alternative to the physico-chemical technologies. Nowadays, it is commonly used for the cleaning of a wide variety of gaseous pollutants. Biofiltration is the most widely used biological gas treatment technology. It consists of a filter-bed, generally, composed of organic matter (peat, compost, sawdust...) serving both as carrier for the active biomass and as nutrient supply, through which the polluted gas passes. Several examples of successful applications have been published [2,3,4,5] and nowadays some industrial plants treat up to 200,000 m^3 per hour. Nevertheless, biofiltration is not developed in Latin-America. Apart from our work and that of Revah's group [6,7,8,9] the literature does not mention other work done in Latin America. The specific technology is lacking and the carriers commonly used are expensive and/or unavailable in Latin America. Therefore, different agricultural byproducts, locally available in large quantities were investigated using a self-developed biofilter.

Five different organic substrates; peanut's shell, rice shell, and fibers of coconuts, maize and sugarcane were selected and physico-chemically analysed. Peanut's shell was selected and a long-term biofiltration experiment was performed using a 20 liters lab-scale biofilter treating air polluted artificially with methanol. The methanol loading rate was progressively increased from 3 to 45 kg methanol m^{-3} day^{-1} during a 10 months experiment. The removal efficiency decreased from 95 to 75 % with increased loading rate with a maximum removal rate of 33 kg methanol m^{-3} day^{-1}. At the end of the experiment, a nitrogen and phosphorous depletion was observed in the carrier material but without a significant effect on the removal rate. The present work confirms the potential of a low-cost agricultural bye-product as biofilter material.

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Bacterial Desulfurization of Dibenzothiophene

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***Keywords**: Dibenzothiophene, Microbial Dessulfurization, Sulfur Metabolism.*

Dibenzothiophene (DBT), a aromatic sulfur heterocycle, represents a model compound of the organic sulfur integrated in the coal matrix and petroleum [1]. This sulfur contributes to the corrosion of refining equipment, and burning these high-sulfur products provokes the emission of sulfur diooxide (SO_2) to the atmosphere [2], the major contributor of acid rain.

To avoid SO_2 emission it is necessary to remove the sulfur from these fuels. Though inorganic sulfur can be reduced by physical or chemical means, none of them can be applied to removing organic sulfur. Therefore, microbial processes able to desulfurize the above raw materials have recently received much attention. While the vast majority of microorganisms capable of cometabolizing DBT use a carbon-destructive pathway, only few microrganisms are known to use an alternative 4S-pathway that selectively removes the organic sulfur (under the form of sulphate) without decreasing the caloric value of the substrates [3]. The aim of this work was screening novel bacteria able to remove the sulfur from DBT using the 4S-pathway.

Fourteen methylotrophic bacteria previously isolated were used for this screening. Parallely, it was isolated novel bacteria able of using DBT as the only sulfur source available from soil samples in the Expo 98 area in Lisbon. The methylotrophic strain #7 was the most promising in the ability of degradation of this compound. On the other hand, the isolated strain #1B grew both on n-hexadecane and glucose as carbon source whereas DBT was only used as sulfur source. When DBT was tested as single carbon source, it was not detected bacterial growth.

The strain #1B was then selected for further degradation studies on glucose due to its ability to degrade faster DBT compared to the other strains. A 90% degradation of the DBT available in the medium was obtained after three days of culture as monitored by gas chromatography. The effect on the bacterial growth of other carbon sources (hydrocarbons, sugars) was studied and the results are shown.

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Evolución de poblaciones en un biofiltro sumergido de doble etapa

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Palabras clave: *biofiltro sumergido, biopelícula, competencia poblaciones, nitrificación.*

La tecnología de biofiltro sumergido aplicada al tratamiento de aguas urbanas tiene un notable potencial. El sistema tiene características propias de interés, como:

- Existencia simultánea de microorganismos adheridos (biopelícula) y en suspensión en los poros del lecho.
- Modelo hidrodinámico de flujo tipo pistón que permite la estratificación de la biomasa.
- Competencia de poblaciones autótrofas y heterótrofas.

Un biofiltro de doble etapa de 2 m de altura y 35 l de volumen ha operado en continuo durante 155 días la primera etapa y 79 días, la segunda con agua residual urbana pretratada. Los rendimientos promedio para un THR de 4.5 horas, son: Eliminación DQO = 88% Nitrificación = 100% Eliminación SST = 84%. A lo largo de los biofiltros, además de grifos para la toma de muestras líquidas, que permiten seguir la evolución de las especies en disolución, se dispusieron entradas que facilitan la toma de muestras de soporte colonizado y de biomasa suspendida.

Aplicando técnicas de respirometría cerrada con tres sustratos: acetato, amonio y nitrito, a las diferentes muestras sólidas, se han obtenido interesantes resultados con evidentes connotaciones de tipo tecnológico:

- La actividad máxima de los microorganismos heterótrofos calculada a partir de las respirometrías es menor que la actividad calculada mediante balances de materia al reactor.
- La mayor parte de la materia orgánica carbonosa y de amonio es oxidada por microorganismos suspendidos y no por la biopelícula.
- Los microorganismos oxidantes de nitrito se encuentran principalmente adheridos al soporte.
- La actividad endógena es elevada a lo largo de ambas etapas del reactor.
- En la primera etapa (en la que se elimina la mayor parte de la materia carbonosa del agua) se observa presencia de bacterias nitrificantes adheridas al soporte y no en suspensión.
- En la segunda etapa (nitrificante) se observa la existencia de microorganismos heterótrofos tanto adheridos como suspendidos.

Se presenta cuantificación de todas las conclusiones.

Purification of the Effluent from Olive Oil Extraction Industry in Constructed Wetlands (*Phragmites australis*)

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Key words: olive-oil mill wastewater's, purification, constructed wetlands, expanded clay (CECA)

The olive extraction industry generates one of the most pollutant agro-industrial effluents, giving rise to several ecological alterations in the receiving rivers, due to high concentrations of COD (36-100 Kg/m^3), polyphenols (1-8 Kg/ m^3), colour and smell. (1)
The *Phragmites* beds are constructed wetlands in which the roots support a wide microbial population. These biological filters with plants are often used for effluent treatment, due to low operation and maintenance costs, simplicity of the system and high resistance to high resistance to high organic loads and toxicity shocks.
The working mechanism is characterised by a complex integration of biological, chemical and physical processes and a concerted action between plant rhyzomes, microorganisms and the support matrix (2).
The system under study was installed at the Cooperativa Agrícola of Ervedal e Figueira e Barros, in Alentejo and is made of eight pilot beds of vertical sub-superficial flux with an area of 0,6m^2 each. The support matrix is made of layers of gravel (15-25mm) and expanded clay (LECA) (3-8 mm and 8-16mm), six of them being cultivated with *Phragmites*.
The experiments allowed the evaluation of the influence of different factors which condition the working of the beds, and that can be grouped in external factors, such as effluent quality (COD, TSS, polyphenols, pH, colour), temperature and evapotranspiration, and design factors, such as retention time, stratification, microbial population and vegetative and radicular development of the plants, operation factors such as hydrautic load and addition of nutrient supplements.
The assays were batch and removals of COD in the order of 50-90%, polyphenols 50-80%, suspended solids 30-95% were obtained. The pH of the effluent was in the order of 7-8, depending on the retention time, quality of effluent and temperature.

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Description of the Degrading Process of Toluene in a Vapor Phase Biological Reactor (VPBR)

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Groundwater contamination by volatile organic compounds and other xenobiotic agents is a persistent water quality issue. Vapor phase bioreactors (VPBRs) offer a low-cost and low maintenance method to eliminate these volatile contaminants under aerobic conditions. Although VPBRs are being installed in the field, an understanding of the microscale process within the bioreactor is very limited. In a VPBR, the microscale process includes mass transport of the contaminant and oxygen from the bulk gas phase through a liquid film into the biofilm, where biodegradation occurs. In order to model or design a VPBR, it is critical to determine the mass transfer, kinetic and stoichiometric coefficients. A flat plate VPBR was operated to degrade toluene using *P. putida* 54G, an environmental isolate.

Oxygen microelectrodes were used to measure oxygen concentration profiles through the gas, liquid, and biofilm phases. The linear shape of the dissolved oxygen concentration profile in the outer biofilm suggested an absence of reaction in this layer. Oxygen consumption in the basal biofilm followed zero order kinetics.

Enumerations of cells in the biofilm were made using culture techniques (selective and non-selective for toluene) and microscopic techniques (total and respiring cells), and an analysis of the progression of the state of the culture was made by examination of various fractions of the populations. Long-term exposure to higher levels of toluene produced lower fractions of total respiring cells and toluene-culturable cells, but higher fractions of respiring cells that could not grow on toluene. Also the fraction of the total respiration that was not associated with toluene uptake increased with higher toluene exposure. A combination of cryosectioning and respiration rate data were used to demonstrate that more respiring cells and a higher respiration rate both occurred at the base of the film, suggesting a deterioration in physiological state with continued exposure to toluene. The accumulation of dead cells on the top of the biofilm contributed a resistance to the transport of substrates to deeper layers of the biofilm suggesting a protective role of the outer layer against the harmful effect of the toxic. These results highlight a new conceptual biofilm model in which both microbial growth and inactivation are controlled by substrate transport, leading to a structure that itself controls substrate availability.

Keywords: Oxygen microsensors, Vapor Phase Biological Reactor (VPBR), Volatile Organic Compound (VOC), Biofilms, Bioremediation.

Selective Removal of Cu^{2+}, Cd^{2+} and Pb^{2+} by Non-viable Biomass

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Key words: *Selective removal, biosorption, cadmium, copper, lead*

The use of microorganisms for metal ion removal (biosorption) may represent an interesting alternative to the existing technologies, especially when it provides a more efficient and economic process [1, 2]. Since industrial effluents usually contain more than one single metal, the use of non-viable biomass has received increasing attention because of both its low cost and high selectivity [1].
The goal of this work was to establish both the pH profile for the Cu^{2+}, Cd^{2+} and Pb^{2+} (1 mM) removal by a yeast strain, from a portuguese brewery industry-Centralcer- and the effect, on its biosorption capacity, of adjusting the pH during the process. The effect of the presence of multiple metal ions on the biomass selective removal capacity was also studied.
The results obtained showed that the optimum initial pH for the three cations removal was in the range 4.5-5.5. Furthermore, a gradual pH increase was observed during the removal process, until a final value of 7-8 for each metal. However, the pH adjustment led to lower uptake and removal yields, showing that the pH gradient may influence positively the biosorption capacity of the cells [1].
Regarding the selective removal of these three cations, it was verified that Cd^{2+} and Pb^{2+} were significantly affected by the presence of the other metals in solution, being observed a decrease in the cells removal capacity with the increase of the interferent cation concentration. Although Cu^{2+} was the cation with the lowest uptake in the absence of other metals, it was not affected by the presence of Cd^{2+} and Pb^{2+} in solution. In fact, these initial experiments seem to indicate that despite the fact that Cu^{2+} binds to a lower number of anionic groups on the cell surface, it is preferentially removed by the biomass used in this work. On the other hand, Cd^{2+} uptake showed the highest decrease when in the presence of increasing concentrations of Cu^{2+} and Pb^{2+}.
It is evident from the results above that for the successful application of biosorption, it will be necessary to understand the influence on metal removal of the solution composition and the physical and chemical characteristics of the ions present [1].

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Trivalent Chromium Removal using Flocculating Yeasts – effect of pre-Treatments on Removal Efficiency

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Key words: Heavy metals, biomass

The use of heavy metals in industrial applications with the production of contaminated wastewater is a serious environmental problem. The importance of developing efficient and inexpensive treatments to these wastewaters is clear, taking in consideration that heavy metals are a threat to public health due to their accumulation through the food chain and that its reuse is possible.
An alternative to traditional treatments, often inadequate when applied to large volumes of diluted solutions, is the use of waste biomass, since many micro-organisms are able to accumulate and concentrate heavy metals from solutions.
Trivalent chromium uptake by the flocculating yeast *Saccharomyces cerevisiae* NRRLY265 was studied, being characterised the effects of permeabilization with ethanol and heat pre-treatments on equilibrium.
In diluted chromium solutions (0-10 p.p.m.), the higher removal yield was observed for cells without pre-treatment, followed by cells permeabilized with ethanol and pre-treated with a heat shock. In concentrated solutions (40-150 p.pm.), cells permeabilized with ethanol removed chromium more efficiently, while cells without pre-treatment exhibited the lowest removal yield.
Sorption equilibrium experimental data was well described by Langmuir model, confirming that chromium uptake capacity was higher for ethanol permeabilized cells. The higher biomass affinity to the metal was obtained for intact cells.
These results indicate that living cells with no pre-treatment have a higher affinity to chromium in diluted solutions. Pre-treatments increase the cell uptake capacity in concentrated solutions. This may be explained by cell denaturation, in heat treated cells and by an increase in permeability caused by ethanol exposure, resulting in new binding sites for chromium.
The presented results demonstrate the possibility of using industrial residual biomass as a means to remove heavy metals from aqueous solutions.

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Biosorption of Chromium

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Key words: Biosorption, biofilm, chromium, heavy metals

For small scale industries as local electroplating units and tanneries, operating at relative tight budgets, instalation and maintenance of conventional pollution abatement technology may be very expensive. Besides, discharges of these units may be unacceptable at local and cumulative scale. Therefore, at the moment there is still a world-wide market for robust and low cost heavy metal removal systems.

A research effort is being made to develop and optimize biosorption systems for heavy metals by growing biofilms over granular activated carbon, GAC. Metals in waste streams too dilute to recover usefully, may be economically removed by enhancing activated carbon surfaces for futher applications. Polluting organic compounds may also be remediated usefully.

After a selection procedure, biosorption studies were carried out with *Arthrobacter viscosus* supported on GAC, in mini-columns with an expanded bed. Concentration values of Cr (VI), measured by Atomic Absorption Spectroscopy, were considered with a maximum of 100 mg/l and the achieved values of uptake were determined for the some volume of metallic solution passed through the columns.

The effect of the following parameters on the performance of the biosorption system, metabollically active, was considered: pH, presence of lactose, acetic acid and EDTA. Best performances were achieved at pH 2.6, with an uptake of 32 mg Cr/g_{GAC}, and the presence of other compounds does not affect the metal removal process.

Particular attention was payed to the effect of a competing metal, cadmium, on the biosorption of hexavalent chromium and, in fact, this was severely affected by the existance of the other metal in solution as the uptake was reduced to half of the original value, for identical initial metal concentration.

The survival of the biofilm demands subtoxic levels of metal concentration in the original solution and inactivated supported biomass was also considered as a biosorption matrix. Lower uptakes were reached, 20 mg Cr/g_{GAC}, although the system mantained a higher fixation capacity over more cycles of biofilm formation-biosorption-metal fixation by heat treatment. Again the presence of a competing biosorbate, Cd, reduces the uptake of Cr (VI).

Similar studies with Cr (III) indicated that this ion does not biosorb, probably as it precipitates at the original pH of the solution, rapidly covering the fixation sites of the biofilm.

Treatment of Contaminated Marine Sediments in a Stirred Tank Reactor

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Keywords: *sediment, stirred tank, hydrodynamics, mass transfer*

For many years, treatment of sediments and solids containing organic contaminants was limited to thermal treatments. However, incineration is an expensive treatment technology [1]. This leads to the development of new technologies, which offer alternatives that are technically and economically attractive, such as biodegradation.

Hydrodynamics and mass transfer characteristics of a three-phase stirred tank reactor were studied in a 8 L reactor. The optimum solid loading was also analyzed. The gas holdup (ε) and the overall volumetric mass transfer coefficient (K_La) were examined as a function of the gas flow rate and the stirring speed over the ranges of 0.5-3 vvm and 0-100 rpm, respectively.

Three-phase systems were consisted of air, seawater and uncontaminated marine sediment because the purpose of this study was to examine reactor performance, and not biodegradation However, since the majority of contamination in soils is associated with the finer fraction [1], the solid phase used for some assays was the finer fraction obtained after sieving the sediment (cut point=125μm). The sediment was characterized by the moisture and solids content.

The solids loading was ranged from 5 to 20% p/v, both bulk sediment and the finer fraction. The slurry mixing was not good for 15 and 20% solids loading and the sediment was settled. Hence, the study was developed for slurries with 5 and 10% sediment. Results showed the following common characteristics in all the studied systems: 1) K_La and ε increased with increasing the stirring speed over a range of gas flow rates (Fig.1). 2) K_La and ε increased with the gas flow rate up to 1.5 vvm. A little effect of the gas flow rate on K_La and ε was seen for higher Q_g which could be due to these air flow rates were too high for the agitation conditions (Fig.2)

Results indicated that the stirred tank reactor allowed better hydro dynamic and mass transfer conditions for the systems including the finer fraction of sediment. Therefore, it seems advisable to carry out a pretreat-ment of the solid for reducing the amount of polluted material to be treated and costs.

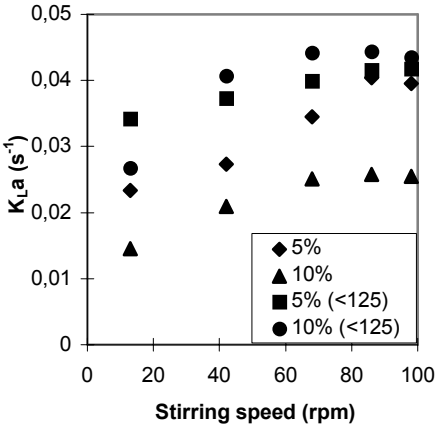

Fig. 1 K_La as a function of the stirring speed. Q_g = 12.000 mL/min

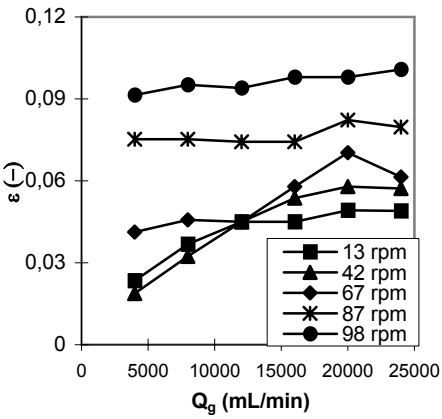

Fig. 2 ε as a function of Q_g (5% finer fraction of sediment)

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A New Method for Reducing Biofouling in Paper Pulp Production Processes

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Key words: pulp and paper industry, biofouling, biocides, Pseudomonas fluorescens

Biofilm growth on paper and pulp mills decreases the operational efficiency of the process, since it contributes to the release of microbes into the process water, affecting product quality (holes, specks and spots in the paper), paper machine breaks, corrosion, bad odours and health problems.
The use of strong biocides in this industry has been decreasing since they are highly toxic. They were replaced by less toxic, but less effective compounds. As a result, biological growth is still a common problem in the paper industry. Only a few studies were published on biofilms in the paper machine environment, in spite of paper and pulp production being one of the largest industries in the world.
This work presents a novel approach based on the retention of bacterial cells on the cellulose fibres in order to reduce biofilm formation. The retention agent, in this case, was a non-oxidizing biocide (mixture of two carbamate solutions). Tests were performed to determine the combined effects of carbamate and pH on: i) cell (*Pseudomonas fluorescens*) adhesion to stainless steel coupons; ii) zeta potential of the cells; iii) retention of the cells by the pulp fibres (measured by total protein).
Addition of the carbamate biocide changes the pH of the paper pulp suspension and can also shift the bacterial surface charges from negative to neutral or positive values, depending on the pH value and the biocide concentration (Table 1). Therefore, cell adhesion to the fibres is promoted, since the latter are negatively charged : in fact, a cell retention of 45% to 75% was obtained within less than 5 minutes of contact between the carbamate and the pulp suspension (Figure 1). This effect increased with the biocide concentration (from 100 to 300 mg/L). In papermaking, the time span between the biocide application point and the head box of the papermachine is very important. The results suggest that the best time interval would be 30 minutes, which is compatible with the residence time in the process.

Table 1. Zeta potential of *Pseudomonas fluorescens* (the standard deviation in brackets) without biocide and treated with different concentrations of biocide at several pH.

System	pH	Zeta Potential (mV)
	5.5	-27.400 (±7156)
P. fluorescens	6.7	-20.988 (±6.906)
	8.3	-26.235 (±7.906)
P. fluorescens	5.9	0
+	6.8	0
100 mg/L of biocide	8.3	-25.633 (±5.948)
P. fluorescens	5.5	18.681 (±3.103)
+	6.7	0
200 mg/L of biocide	8.3	-29.547 (±5.076)
+		
P. fluorescens	5.9	17.905 (±2.953)
+	6.7	12.241 (±6.093)
300 mg/L of biocide	8.3	-28.463 (±6.423)

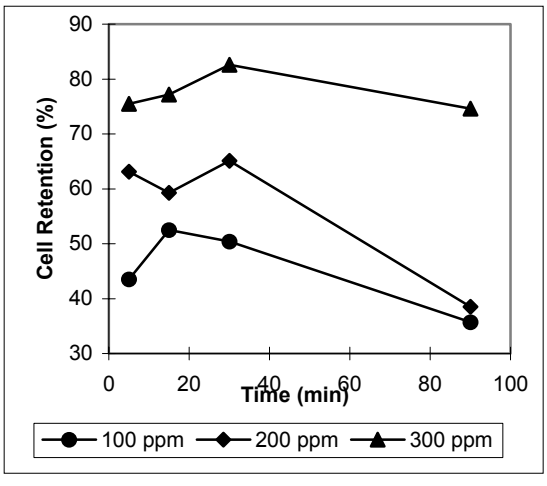

Figure 1. Cell Retention(%) as function of time, obtained in the assays with pulp suspensions in presence of several concentrations of biocide

Microscopic Analyses of Sludge from an Activated Sludge Pilot Plant

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Keywords : *wastewater treatment, microorganisms, protozoa, filamentous*

The biomass in activated sludge plays two roles. Firstly, it metabolises organic material contained in the waste water, and secondly it build up structured aggregate to be able to separate the sludge from the treated water by settling. Initially, the microfauna analyses and the biotic index aid to determine the optimum operation of an aerated reactor. On the other hand, the settling capacity of the sludge frequently presents difficulties, which can be caused by different "pathologies". Foaming and bulking are the most common. Ocurrence of these problems need to analyse the filamentous microorganism presence in the secondary settler.

To search for the most efficient operation of the wastewater treatment, a procedure to analyse activated sludge microscopically has been set up. These analyses are important complement to the analysis of the physic and chemical parameters, commonly followed in an activated sludge plant. The present results has been obtained from a pilot plant designed to remove organic material, nitrogen and phosphorous, which is continuously feeding with a synthetic urban wastewater.

With the aim of following the evolution of the pilot plant, a date sheet has been developed, filled with the next information : Operational parameters, captured pictures from the samples and their description, flocs and filament characterisation (size, presence of filaments and their effects in the floc structure, floc morphology, etc...), filaments identification and qualitative determination of their abundance, and finally microorganism identification and biotic index determination.

During the period of study (28/05/97 up to 10/12/97) a biotic index between 8 and 9 was obtainded. This belongs to quality class I [1]. Predominant presence of free-swimming ciliates and stalked ciliates was observed. *Aspidisca cicada* and *Epistylis*, respectively were dominant in each case. These results indicated the presence of properly colonised sludge with an optimum biological activity and a high depurative efficiency which was corroborated with the physical and chemical analyses.

Nostocoida limicola II was the predominant filamentous microorganisms. Their presence in excess of these filaments caused bulking, periodically. These filaments are the main indicative, in addition with the presence of Metazoa, of a high sludge age. The same filaments with sporadic presence of *Opercularias* may indicate low level of dissolved oxygen.

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Monitoring of *Bacteroides* Species in Environmental Samples by 16S rRNA Amplification

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Keywords: Bacteroides*, PCR, rRNA 16S.*

Some species of *Bacteroides* have been proposed as "indicator organisms" for the evaluation of faecal pollution, alternatively to coliformes. Comparatively, the genus displays several advantages; while outnumbering coliformes by about one hundred times, different *Bacteroides* species colonise human and animal digestive tracts, therefore behaving as "indicators" of the type and origin of the contamination, in addition to its intensity. Although they tolerate O_2 exposition, being strict anaerobes makes them unable to grow in aerobic environments, so "overgrowth" is prevented.
Specific primers have been designed for PCR detection of most *Bacteroides* spp., based on a large number of sequences available in Genbank, corresponding to the rRNA 16S of the genus, and on published results [1], that point this gene as strongly conserved.
We assessed the sensitivity of PCR for *Bacteroides* spp. detection in water samples by evaluating the smallest number of cells required for amplification. After recovery by filtration or centrifugation, cells were boiled to extract DNA. In addition, the QIAmp Tissue Kit (QIAGEN) was used for faecal DNA extraction.
Our results suggest this methodology as suitable for the enumeration of *Bacteroides* spp. in water and faeces. Thus, its suitability for the monitoring of the sanitary quality of waters, wastewaters and treated effluents may be expected.

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Process Costing for Bioengineered Clean Technologies

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Biological resources are many. For example, whey, molasses, liquid waste urea, liquid waste benzo(a) pyrene, nitrogenous waste from a fertiliser unit are good agro resources for exploitation. The bioprocessing of the above materials to get alcohol, ammonia, methane, water and biomass as fertilizer are established technologies and vary in keeping with the amount of materials to be handled. Ours is a tropical country with good temperature throughout the year which enables the optimum activity of bacteria and microbial enzymes and growth of bacteria as well.

The concept of clean technologies assumes significance when we are handling huge quantities of agro wastes of fertiliser waste waters.

For example whey is a waste from cheese cremery and will result in enviro nuisance if left to itself; it will also, be a breeding ground for mosquitoes and flies. Molasses is a sugar industry waste of 35% to 50% sugar. But after conversion to alcohol, at the bottom of the distillation column, the stillage having minimal sugar can be inoculated with methanogenic culture and converted to CH_4 and CO_2. Thus the COD gets reduced. Then it can be diluted and fish can be raised and then the waters can be sent to the estuary or river. The urea wastes are treated in a packed immobilised bed of urease with urea water percolation and conversion of urea to ammonia. The water can be used after neutralisation for Indl. cooling and raising of paper grass. The following table is indicative of the brief outcomes of the waste processes.

No	Waste	Product	Remarks
1	Whey	Alcohol	EcoCleansing + VAP
2	Whey	Biogas	-d0-
3	Molasses	Alcohol	EcoPreservation + Fish + Biogas
4	Urea Waste	Water + NH_3	Water for cooling + EcoPreservation
5	Pyrene	Water + Pyrenol	Water for cooling + arable purpose
6	Fertilise Wastes	Cyanobacteria	Biofertiliser + water

In this paper the process costing in each case is given using the common sense based approach taking into account the current prices of RM and utilities. The costing methodologies are elaborated in detail as well.

Protozoan Population as an Indicator of the Aerobic or Anoxic State of the SBR Operating Cycle

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Key words: SBR, protozoa, nitrification, denitrification, biological indicator.

The use of the microfauna analysis to estimate both the quality of the effluent and the performance of the treatment plant is becoming more and more common. The importance of protozoan population in biological wastewater treatment systems has been widely studied for a number of treatment options, such as the activated sludge system, trickling filter systems and rotating biological contactors. However, only a few studies were published on protozoan population in sequencing batch reactors (SBR).
The aim of this research was to investigate the changes in microfauna during a typical operating cycle and the potential of protozoa to act as indicators of the operational phase.
The SBR was operated over a 10 months experimental period with the purpose of achieving nitrification/denitrification. The inoculum was mainly composed by a sample of mixed liquor from the aerated tank of a municipal wastewater treatment plant. The feed was a brewery wastewater after anaerobic pre-treatment. During the last 10 days, microscopic analysis of the reactor contents was performed throughout each cycle and protozoa were identified. At this time, the operating cycle consisted of a long aerated period followed by an anoxic phase. The dissolved oxygen (DO) was regulated by on-off control of the air flow.
During the aerated period (DO=3-4 mg/L) crawling and attached ciliates were the predominant protozoa in the system. With the decrease of the DO concentration throughout the unaerated phase, these species showed a reduction of 40% and the appearance of free swimming ciliates and flagellated protozoa could be observed (Figure 1), indicating a poorly aerated sludge. Crawling and attached ciliates always re-established themselves as the aerobic conditions took place again, indicating that the cyclic operations had no detrimental effect on protozoa. The presence of *Vaginicola* sp., a common specie in nitrifying activated sludge systems, was often observed.

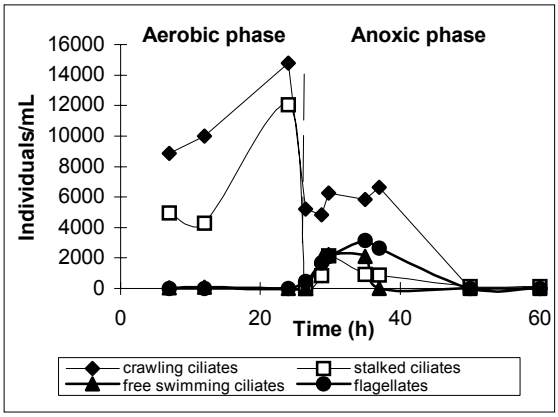

Figure 1. Changes in microfauna during a typical operating cycle of the SBR .

Table 1. Protozoa observed during a typical operating cycle.

Functional group	Functional sub-group	Observed species
Ciliated protozoa	Stalked	*Vorticella* sp.
		Epistylis sp.
	Free swimming	*Vaginicola* sp.
		Uronema sp.
		Colpidium sp.
	Crawling	*Aspidisca* sp.
		Chilodonella sp.
Flagellated protozoa	-	*Peranema* sp.

The results showed that the dominance of species of a certain functional sub-group of protozoa (Table 1) in the mixed liquor is related with the operational phase of the SBR.

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Physiological Responses of the Ciliated Protozoa *Tetrahymena pyriformis* to Toxic Compounds

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Keywords: *Protozoa;* Tetrahymena pyriformis*;* In vitro *toxicity; Biological indicators.*

The essential role of protozoan community in aerobic wastewater treatment is well-known and documented [1,2]. On the other hand, it is recognized that changes in this community may affect the whole food web of these systems, thus affecting the biological performance of the plants. The sensitiveness of protozoa to environmental changes have, therefore, enhanced their potentiality as biological indicators of water quality [3].
The aim of the present study is to emphasize the indicator value of ciliated protozoa, by assessing physiological responses of *Tetrahymena pyriformis* after exposition to four toxics: copper, zinc, the antibiotic ciclohexamide and the neutral surfactant Triton X-100.

Tetrahymena pyriformis can be cultured in axenic conditions, *i.e.*, free from bacteria or other organisms, in a defined medium. Physiological responses were assessed *in vitro*, in a series of miniaturized assays in 24 and 96 well culture dishes, at 20±1ºC. Physiological condition of *Tetrahymena pyriformis* was assessed in assays of MTT (metabolic activity), ATP (energetic demand) and cytoskeleton morphology (structural changes). These miniaturized assays are low cost and low time consuming, which may be relevant in monitoring programs.

Diferential responses to the toxic compounds encourage further research in order to find wether a certain pattern of physiological responses will allow, in the future, to identify the toxic or group of toxics involved.

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Puesta en Marcha de un Reactor Aerobio para la Depuración de Efluentes de Industrias Conserveras

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Palabras Clave: lodos activos, industrias conserveras, atún, depuración aerobia.

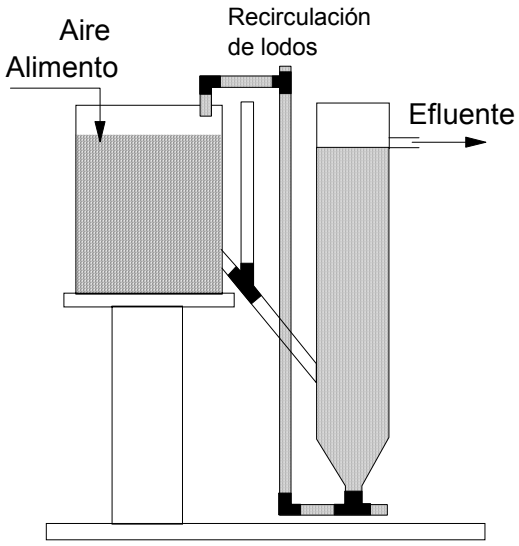
El proceso de lodos activos es uno de los tipos de tratamiento biológico aerobio con el que se consigue eliminar sustancias orgánicas biodegradables (coloidales o disueltas) así como nitrógeno y fósforo. El principio del proceso de lodos activos se fundamenta en que si a una comunidad de microorganismos se le suministra constantemente materia orgánica y oxígeno, los microorganismos consumen la materia orgánica y la transforman en nueva biomasa microbiana y en productos finales oxidados.

En este trabajo se pretende determinar las condiciones de operación adecuadas para depurar un agua residual procedente de una balsina de cocción de atún (cocción de atún sumergido en agua a una temperatura de 95-98ºC). Esta agua presenta una DQO_T=26.073 mg/L; sólidos totales:19,8 g/L; sólidos volátiles:7,3 g/L; NO_3^- =2,3 g/L; PO_4^{3-} =1 g/L.

El equipo empleado en la depuración consta de un reactor de 4,5 L aireado y perfectamente mezclado debido a la turbulencia provocada por la aireación. En esta unidad tiene lugar la depuración. El efluente depurado pasa a un decantador donde se separa el agua de la biomasa que es recirculada al reactor. El diseño del equipo se ha mejorado respecto a unidades previas, ya que la conexión entre el reactor y el decantador no es horizontal sino inclinada, lo cual evita la decantación de biomasa en este conducto.
Para la puesta en marcha del equipo se empleó un lodo procedente de un digestor aerobio. En los primeros días se alimentó el reactor con agua sintética (Tabla 1) con el objeto de obtener una alta densidad celular. Una vez alcanzada una concentración de lodo de 1,5 g/L se procedió a sustituir el agua sintética por agua residual de forma gradual para que la flora bacteriana se adaptase a las nuevas condiciones. De este modo se alcanzó una velocidad de carga orgánica de 1,5 kg DQO/kg SSV·d con un grado de depuración del 90%.

Glucosa	1000 mg/L
NH_4Cl	95,5
Urea	56,3
KH_2PO_4	22,6
$FeSO_4 \cdot 7H_2O$	12,6
$NaHCO_3$	390
Extracto de levadura	35

Tabla 1. Composición del agua sintética



Seguimiento de la Actividad y Cinética de Microorganismos en el Proceso de Fangos Activos Mediante Técnicas Respirométricas

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***Keywords**: fangos activos, respirometría, actividad, cinética*

En un sistema de fangos activos con nitrificación tienen lugar muchos procesos biológicos, principalmente la oxidación de materia orgánica carbonosa y la oxidación de nitrógeno amoniacal, en donde intervienen numerosos tipos de microorganismos. La cuantificación de la actividad de los microorganismos frente a los diferentes sustratos constituye una buena herramienta para el seguimiento del proceso.

Las técnicas más adecuadas para determinar la actividad, por su facilidad y corta duración, son aquellas basadas en la medida de la velocidad de consumo de un determinado sustrato o la producción metabólica. Las técnicas respirométricas se basan en la medida de la concentración y consumo de oxígeno provocada por la adición de un determinado sustrato.

Se han aplicado las técnicas respirométricas para el seguimiento de la actividad y la determinación de parámetros cinéticos de la biomasa de un reactor de fangos activos. El reactor se utiliza para el post-tratamiento del efluente de un digestor anaerobio que trata aguas residuales de matadero.

Para la realización de la respirometría una pequeña cantidad de fango (en torno a 50 ml), procedente del reactor, se introduce en un matraz de 0,5 l junto con agua de dilución tamponada y saturada de oxigeno. El matraz se cierra para evitar el contacto con el aire y la concentración de oxígeno se mide con un oxímetro conectado a un ordenador. La inyección de un sustrato provoca un aumento en la actividad de los microorganismos correspondientes, y en consecuencia una mayor velocidad de consumo de oxígeno proporcional al consumo de sustrato. Para la actividad de los microorganismos heterótrofos se inyectó acetato como materia carbonosa, la actividad autótrofa nitrificante se determinó mediante la adición de amonio y nitrito. La inyección de sucesivas cantidades de sustrato, en una misma respirometría, permite obtener valores de velocidad a distintas concentraciones. Estos valores se utilizaron para la obtención de los parámetros cinéticos del modelo de Monod (μ_{max} y K_s)

Se obtuvo relación entre la actividad en el reactor y la determinada mediante respirometrías tanto para la oxidacción de materia carbonosa como para la nitrificación. Las actividades realizadas sobre la oxidación de nitrito se correlacionan linealmente con el grado de inhibición de *Nitrobacter* en el reactor.

Para la nitrificación se obtuvieron valores de μ_{max} entre 7,3 y 15,3 mgN-NH_4^+/gSSV h; y valores de K_s entre 0,3 y 6,2 mgN-NH_4^+/l. La velocidad obtenida utilizando estos valores cincidió con la con la calculada mediante balances en el reactor. Para la oxidación de nitrito se obtuvieron valores de μ_{max} entre 6,8 y 4,1 mgN-NO_2^-/gSSV h y valores de K_s entre 10,1 y 1,1 mgN-NO_2^-/l; sin embargo en este caso la correspondencia con los valores obtenidos en el reactor fue peor. Para el caso de las cinéticas realizadas con acetato no se obtuvieron valores representativos al trabajar en la respirometría con valores de concentraciones por debajo de K_s.

En la determinación de actividades mediante respirometría es fundamental minimizar la concentración de sustratos que acompañan a la muestra de fango antes de iniciar la respirometría. En este sentido la técnica más adecuada es la decantación y el lavado del fango.

Optimized Feed Strategy for Nitrogen Biological Removal using a Sequence Batch Reactor

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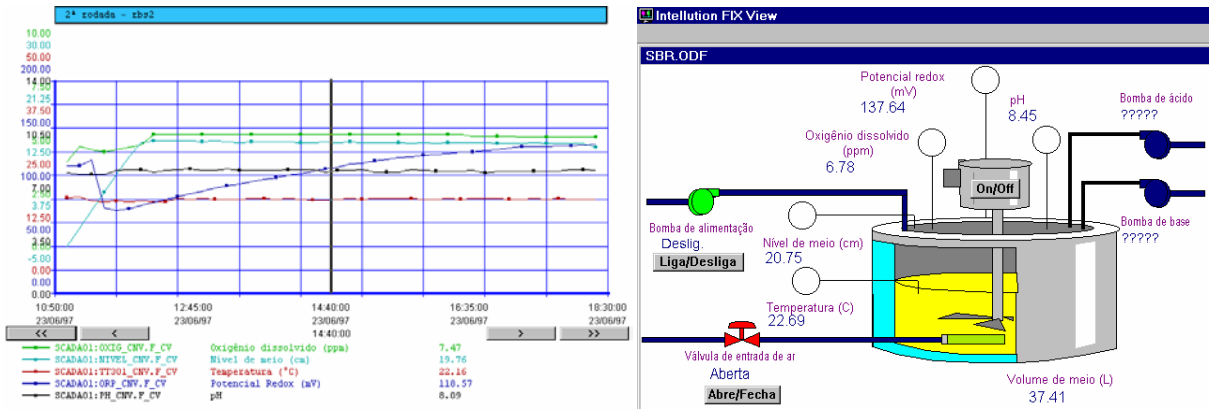
Keywords: Sequence Batch Reactor, Wastewater Biological Treatment, Optimization,
Dymanic Simulation

Nitrogen accelerates eutrophication process naturally ocurring in lakes, rivers and other aqueous systems. Nitrogen biological removal usually consists of nitrification, a two-step process carried-out by autotrophic microrganism, under aerobic conditions, which converts ammonia to nitrate, and denitrification, when nitrate is converted to molecular nitrogen in presence of heterotrophic bacteria under anoxic conditions.

A bench-scale instrumented sequence batch reactor was implemented (on-line measurements for pH, dissolved oxygen, level, redox potencial and temperature; off-line measurements for ammonia and nitrate), connected to a process PC computer running FIX-DMACS supervisory control software (Intellution Inc.), which mantains a real time experimental database.

Experimental database obtained allowed parameter estimation[1] of a mathematical model (6 ODE's) process optimization and monitoring. Optimization of squared errors was carried out under MATLAB / Optimization Toolbox (The Mathworks Inc.). Results pointed to a discrete feed strategy[2,3].



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[3] Souza, S.G.M., Coelho, M.A.Z., Araújo,O.Q.F. e Russo,C., *12º Congresso Brasileiro de Engenharia Química*, set/98, Porto Alegre-RS, Brasil.

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Evaluation of Biosorption of Heavy Metal Mixtures by Waste Biomass from Typical Brazilian Beverage (Aguardente) Distillery using a 2^4 Factorial Design.

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Biosorption of heavy metals on non-viable cells can be used as potential technology for removal of toxic metals from industrial waste streams, in terms of efficiency and operational suitability. The advantages of using non-viable cells are numerous. Inactive biomass has the advantage of being independent of supply of nutrients for cell growth and maintenance, and it does not involve any time loss to culture propagation or contamination. Besides having rapid and efficient metal uptake, biomass behaves as ion exchanger and metals can be desorbed readily and recovered. Moreover, if biomass employed is a waste material (fermentation by-products, for instance) then biosorption represents a cheap alternative to conventional treatments, due to the use of a low cost sorbent material. Our previous work has shown that waste biomass from typical Brazilian's beverage distillery (sugar cane spirit - Aguardente) was effective in the removal of heavy metals such as chromiun, iron and nickel from both single metal solutions (synthetic media) and multimetal ion system (metal processing industry -ACESITA). Since many parameters were found to affect the capacity of biomass to bind more than one metal simultaneously a complete factorial statical design 2^4 with center point was used to evaluate the influence of the following experimental factors: pH (2.0 and 4.0), biomass concentration (2 and 4 g/L), presence or absence of bleaching earth (celite) and contact time (3 and 6 hours). Biosorption runs were carried out in 250 mL Erlenmeyer flasks containing 50 mL of industrial waste solution with the following composition: 602 mg/mL of iron (III), 2.7 mg/mL of nickel and 7.8 mg/mL of chromium (VI). Before mixing with the biomass and/or celite, the pH was adjusted to various values from 2.0- 4.0. The flasks were agitated on a shaker incubator (150 min^{-1}) at constant temperature (30^0C) for a maximum period of time of 6 hours. The response of the process under investigation was the metal specific uptake (q in mg/g). The experimental data were analyzed by response surface regression procedure using Statgraphics Statical Software (version 2.7). The pH values (positive effect) and the biomass concentrations (negative effect) had statical significance as principal effects (at the 5% level). The presence of celite and contact time had no effect on the biomass capacity metal removal. The maximum metal uptake was obtained, working at the highest level of pH (4.0), minimum level of biomass (2.0 g/L), absence of celite and with a contact time of 3 hours. Under such conditions the following metal specific uptake values were found: 155 mg/g for iron, 38 mg/g for chromium and 0.4 mg/g for nickel, this represents reductions in the concentration levels of about: 51% for Fe, 30% for Ni and 94% for Cr. The results also shows that there are several interactions among factors which make the system optimization complex. The factors effects and interactions are different for each metal involved in this biosorption.

Kinetics of Nitrification with Different Volatile Organic Compounds at Varying Concentrations on Nitrifying Sludge

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***Keywords**: nitrification, ethanol, acetate, propionate, butyrate*

Effect of increasing concentrations of volatile organic matter (VOM) such as acetate, propionate, butyrate and ethanol on a nitrifying sludge were evaluated. Sludge was taken from nitrifying reactor in steady state regime. The concentrations of each compound ranged from 0 to 2 g/l in batch culture when ammonia nitrogen (NH_4^+-N) was 0.250 g/l. Experiments were also conducted in a continuos reactor where the ammonium loading rate was 0.15 g/ld (0.5 NH_4^+-N/l in the input). The VOM fed (during 14 h) was made up a mixture of acetate, propionate and butyrate at 4:1:1 ratio with a loading rate ranging from 0 to 0.78 g/ld (0 to 3 g VOM/l in the input). Nitrate formation was measured by capillary electrophoresis and microbial protein by Lowry´s method [1].
Nitrate formation profiles for both acetate and ethanol were similar. Figure 1 shows nitrate formation for only acetate. Figure 2 shows nitrate formation at different concentrations of butyrate. Respiration profiles with propionate were similar to those for butyrate.

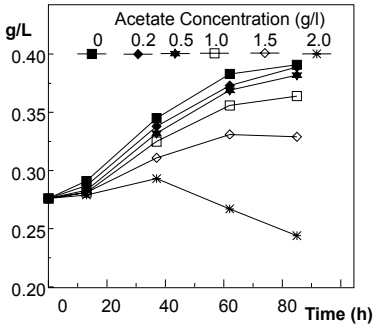

Figure 1. Nitrate formation at different acetate concentrations.

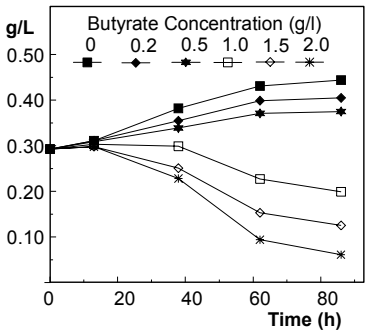

Figure 2. Nitrate formation at different butyrate concentrations.

With acetate and ethanol the respiration were almost like a mixotrophic while with butyrate and propionate, the inhibition of nitrification was quite evident after 0.5 g/l. Calculation obtained form nitrification rates suggested that for similar concentrations existed a different negative effect of each one of the compounds evaluated. For instance, the specific nitrate formation rates showed that the inhibition of nitrification at 0.5 mg/l of ethanol, acetate, propionate or butyrate was 57, 53, 73 and 64%, respectively. On the other hand, when the mixture of VOM, in the range of 0 to 0.78 g/ld, was fed into nitrifying steady state reactor the nitrification efficiency (g NO_3^-N/g NH_4^+-N) was not affected showing a steady 87%. Microbial loading growth rate was slightly increased while the VOM increased.
The results suggest that the nitrification conditions must be defined in terms of the type of VOM, since each volatile compound affected the kinetics of nitrification in different way.

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Nitrification in a Circulating Bed Reactor: pH and Dissolved Oxygen Effects

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Key words: Nitrification; Circulating Bed Reactor; pH; Dissolved Oxygen; Biofilm Activity

This study is aimed at investigating the influence of dissolved oxygen and pH on the nitrifying activity of a biofilm reactor, the Circulating Bed Reactor(CBR).
The effect of the dissolved oxygen concentration (DO) on reactor nitrification activity was studied in short term experiments (10 –12 hours), by manipulating the oxygen partial pressure in the incoming gas and thereby the dissolved oxygen in the reactor. A Biological Oxygen Monitor (BOM) was used for the measurement of the respiration rate of biofilm particles taken from the reactor, at two different operating condition, without and with pH control. The respirometric method used has been described elsewhere [1]. The biofilm mass was estimated by means of Total Protein (TP) measurements (Lowry method).
Fig. 1 presents the influence of bulk oxygen to ammonia ratio on reactor ammonia removal efficiency. The transition from ammonia limitation to oxygen limitation was found to occur when the bulk oxygen to ammonia ratio was about 1.5 – 2 g O_2/gN–NH_4^+. This value is lower than the ones reported in literature, 2.5 – 4 g O_2/gN–NH_4^+ [2].
The specific ammonia oxidation rate (SAOR) as determined from CBR operation and the one obtained in the BOM are compared in Fig. 2. For the case of no pH control, the oxidation rate values obtained by both procedures are similar, once the ammonia concentration was not limitin (15 mg N–NH_4^+/L for CBR and 10 mg N–NH_4^+/L for the BOM). Due to pH control implementation, the oxidation rate in the reactor increased and the ammonia concentration became limiting (<5 mg N–NH_4^+/L). This explains the large difference between the values obtained in the BOM (where ammonia is not limiting) and in the CBR.

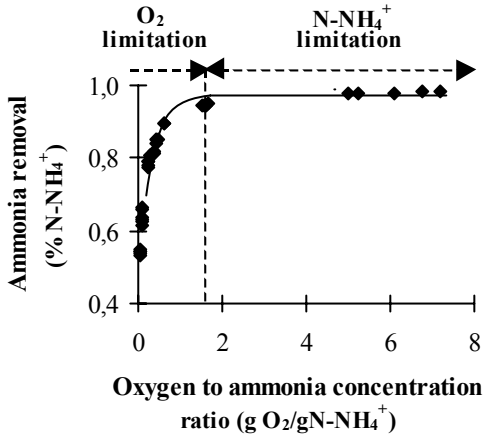

Fig. 1 Ammonia removal as a function of bulk O_2/N–NH_4^+ effluent concentration.

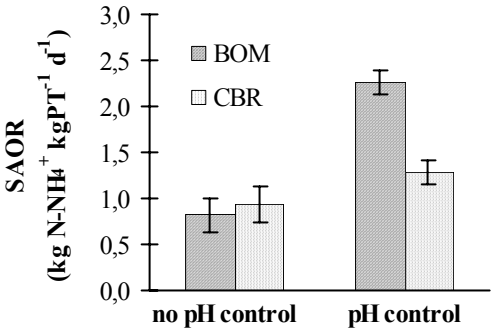

Fig. 2 SAOR obtained in CBR and determined in the BOM

The main conclusions to be drawn from the present work are:
– the lower transition value from ammonia limitation to oxygen limitation, appears to be due to the high turbulence in the CBR which results in reduced resistances to oxygen transport;
– the respiration rate is a valuable tool for the characterisation of biofilm activity, and could be useful for controlling and identifying disturbances (in particular those due to a pH effect) that affect nitrification reactor performance.
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Análisis Químico y Electroforético de un Lodo Nitrificante Asociado al IVL

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Palabras clave: Lodo nitrificante; exopolímeros; IVL

Los lodos que se utilizan para el tratamiento de aguas residuales pueden sufrir el fenómeno de esponjamiento (bulking). Se han estudiado los exopolímeros (EP) como una posible causa, pero no se sabe bien cual de sus componentes es el agente causal (exopolisacáridos y proteínas) [1]. En el presente trabajo se analizan cada uno de ellos y se relacionan con el índice volumétrico del lodo (IVL)

Se empleó un lodo nitrificante, no filamentoso, en régimen estacionario, con una eficiencia nitrificante de 98%. Se midieron la biomasa microbiana y proteínas exopoliméricas (PEP) por Lowry y los exopolisacáridos (EPS) por el método del fenol sulfúrico. Los flóculos del lodo no filamentoso se disgregaron por la adición de EDTA 0.1N, seguidos de una diálisis (membrana de 10 KD). Para determinar el tipo de PEP se realizó electroforesis en gel de poliacrilamida. Los sólidos en suspensión se midieron por seco. Al lodo se le midió el IVL

Los resultados obtenidos indicaron que los EPS del lodo presentaron una media de 30±3 mg/l. Sin embargo, las PEP variaron significativamente (de 6 a 50 mg/L), siguiendo un patrón oscilatorio. El valor del IVL fue de 11.2±3 mg/g. En al Figura 1 se presentan los perfiles de PEP y EPS con respecto al tiempo, del reactor nitrificante en régimen estacionario.

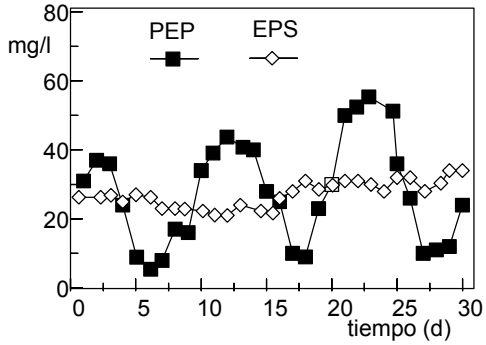

Figura 1. Perfiles de los exopolímeros del lodo nitrificante

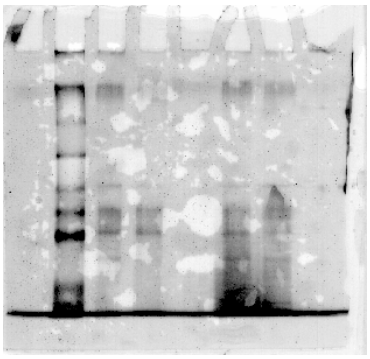

Figura 2. Electroforegrama de las PEP del lodo nitrificante

Se hizo el análisis de las PEP, del reactor nitrificante, por electroforesis en placa (Figura 2), donde se muestran 7 columnas (correspondientes a diferentes días consecutivos de muestreo). Se encontró que el tipo de proteínas varía con el tiempo. Se observaron desde 17 tipos distintos, hasta 6, donde estas últimas permanecieron siempre constantes. Este resultado coincide con las oscilaciones de PEP, mostrados en al Figura 1.

Los análisis químico de las EP y electroforético, y del IVL, con respecto al tiempo, sugieren que son las PEP las que dan gran variabilidad al lodo nitrificante, aun estando el reactor en régimen estacionario.

Urbain V., Block J. C. and Manem J. *Wat. Res*. 27, 829-838, 1993.

High Hydraulic Load Trickling Filter Performance in the Wastewater Treatment Plant of Toluca (Mexico)

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KEYWORDS: Trickling filter, domestic wastewater, distribution system.

The performance of three high hydraulic load trickling filters, operating in parallel in the treatment plant "Toluca Norte" was evaluated. Biofilters are 6.1 m high and 40 m diameter, wastewater is distributed by four rotatory arms, the influent concentration is 125 mg BOD_5/l, the organic loading is 0.44 kg BOD_5 m^{-3} d^{-1} and the hydraulic loading is 21.6 m^3 m^2 d^{-1}. Support is cross-flow modules with a specific area of 12.8 m^2 m^{-3}.

This study was based on measures of disolved oxigen, flow distributuion over the filter surface and water velocity through the filter, as well as everyday control analysis of the plant.

Removal efficiencies in soluble organic matter were 45 % in soluble BOD_5 and 30 % in soluble COD, considerably lower than the designed of 65 % in soluble BOD_5. This insufficient performance is caused mainly by two factors. Firstly, the organic matter concentration in the influent is considerably lower than the design one of 301 mg BOD_5/l. Secondly, and due to the low rotatory speed of the distribution arms (0.042 rpm), the water velocity trough the filter is too high, giving a short time of permanence inside the filter, around 30 s. Operation with a higher rotatory speed in similar climate conditions (Sept. 1996) showed better performance, although an adequate washing protocol was needed.

The performance of the trickling filters between July and September 1997 showed a temperature effect, decreasing performance as autumn arrived.

Under these operation conditions, the high shear forces allow a constant and homogenous detachment of biofilm, avoiding pounding and plugging processes. The presence of nematodes and high dissolved oxygen levels in the effluent indicate a satisfactory aeration of the biofilm.

Flow measures along the distribution arms showed that the hydraulic superficial load is higher near the centre than on the periphery, making substract- biomass contact worse near the axis of the filter.

The sole control over organic and hydraulic loading rates does not guarantee the development of a stable biofilm and an adequate substract-biomass contact. It is also necessary a careful control over the radial distribution of the wastewater and the rotatory speed of the distribution system.

Characterization of Nitrite-Oxidising Bacteria from a Municipal WWTP Sludge

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Keywords: *Gibbs energy, nitrification, nitrite-oxidisers, Nitrobacter*

Nitrite and ammonia are non desirable compounds in water streams. They are toxic for aquatic life and stimulate the growth of organisms and plants, which consume dissolved oxygen. The most utilised method to remove these compounds is the biological nitrification-denitrification process. Nitrification is carried out in two sequential steps: 1) Ammonia oxidation to nitrite and 2) Nitrite oxidation to nitrate. Last step is generally attributed to genus *Nitrobacter* since other nitrite-oxidisers are associated to marine environments. Recent studies carried out with in situ identification techniques have discovered that *Nitrobacter* is not present in samples coming from several municipal WWTP [1]. The possibility to control the oxidation to nitrate would allow to get energy and volume saving in the nitrification unit, being the kinetic knowledgement of this process very important.

The aim of this work is to characterise nitrite-oxidising genus contained in a municipal WWTP.

Microorganism was seeded in chemiostat. Operation conditions were kept similar to a conventional municipal WWTP, it means, hydraulic retention time (HRT) of 5 days and low nitrite concentrations. These conditions favoured slow-growth bacteria selection but with high substrate affinity. An identification of microorganisms present in the medium was carried out by FISH method (Fluorescent In Situ Hybridation) after several HRT. *Nitrobacter* was found to be the predominant genus. It can be concluded that chemiostat conditions are not favourable for the growth of another nitrite-oxidants genus.

A kinetic study of these microorganisms was developed using a BOM (Biological Oxygen Monitor). Maximum activity (V_m), Monod constant (k_S), substrate and product inhibition constant (k_I and k_P) were obtained at different pH values. Product effect (nitrate) fits into a non-competitive kinetic, showing a lineal relationship between reaction rate and Gibbs free energy [2].

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Biological Treatment of Wastewater Using Immobilized Biomass in an Airlift Reactor

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Keywords *: Immobilized biomass, airlift, wastewater, Pirimiphos-methyl.*

Systems with immobilized biomass for wastewater treatment provide high biomass concentrations, and hence, in high volumetric removal rates, short liquid residence times and good operating stability. Also, high biomass concentrations enable high sludge ages and therewith minimal sludge production [1].

For this research an internal airlift loop reactor was used. A schematic representation of this reactor is given in Figure 1. The reactor had a total volume of 4.5 l . Particles of biolite with a diameter of 0.25-0.315 mm were used for cell immobilization. The initial carrier concentration for all experiments was 10% on volume. The reactor was inoculated with sludge from a municipal wastewater treatment plant of Alcalá.

The composition of synthetic water was variable: sodium acetate, glucose, sacarose, and pirimiphos-methyl with addition of saccharose were used as the C-source. A mixture of nutrients (NH_4Cl, KH_2PO_4, K_2HPO_4 and $MgSO_4$) was present in all experiments.

Parameters used for the systems characterisation were COD, BOD, organic substrate concentration, immolized biomass, suspended biomass, superficial air velocity, dissolved oxygen, temperature and off -gas composition.

In all experiments, the C-source conversion was higher than 90% even at maximum loading substrate rates assayed.

Substrate	Loading rate
Sodium Acetate	12.10 Kg./d.m^3
Glucose	10.84 Kg./d.m^3
Saccharose	2.84 Kg./d.m^3
Saccharose + 4ppm pirimiphos-methyl	0.59 Kg./d.m^3

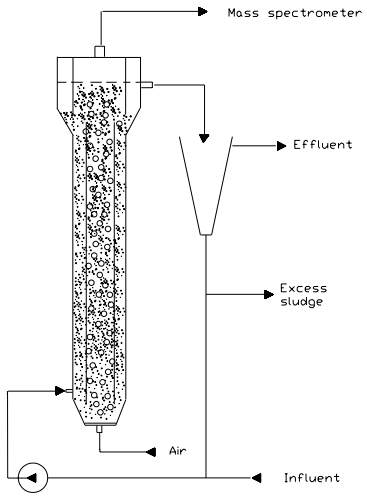

Figure 1. Schematic representation of the airlift reactor.

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An Innovative Process for Nitrification of Wastewaters: The Circulating Floating Bed Reactor

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***Keywords**: Nitrification; Biofilm; Airlift reactor*

The Circulating Fluidised Bed Reactor (CFBR) is a biofilm airlift system developed in France by Lyonnaise des Eaux, for secondary and tertiary treatment of wastewaters. This system is characterised by the separation of the reactor in two sections: an up-f low aerated section and a down-flow non-aerated section. A floating support of is used as support. The reactor design is simple without any complex technical devices [1, 2].
1 CFBR of 5 L was operated with 20% v/v support. The support was the same that those used in the industrial scale units. Size distribution of particles was between 1 and 4 mm. Ammonia concentration in the influent was around 50 mg-N-$NH_4^+ l^{-1}$and temperature between 14 and 27 ºC. Till operating day 65, ammonia loading rate (ALR) was up to 0.6 kg N $m^{-3}d^{-1}$. During this period, the produced biomass was detached and biomass concentration reached a pseudo-stationary state (200 mg-protein/L). After day 65 ALR was increased till 1.2 kg-N·m^{-3}·d^{-1} and ammonia removal was 90%. This value is lower than the 2 kg N $m^{-3}d^{-1}$ obtained in tertiary nitrification in an industrial scale reactor [2]. Between days 65 and 150, up to 20% of the newly produced biomass was retained in the reactor, being the rest washed out from the system. Biomass concentration increased till 1200 mg- protein/L at the end of the operation.

Between days 145 and 155 the influence of temperature both on ammonia and nitrite specific oxidation rate was determined by using a respirometer. Ammonia specific conversion was 1.9 and 0.57 g-N/g-protein·d at 30 and 10 ºC, respectively. Nitrite specific conversion was 4.5 and 1.2 g-N/g-protein·d at 30 and 10 º C, respectively. By applying a diffusion-reaction model [3] it was estimated the average specific surface and deep of the biofilm in 220 m^2/m^3 and 0.17 mm, respectively.

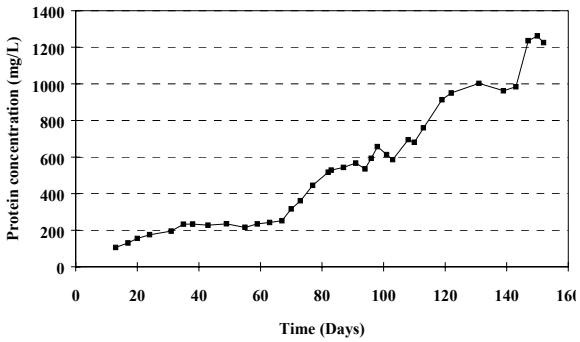

Figure 1. Protein concentration in the reactor.

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Effect of Clay Particles on the Activity of Autotrophic Nitrifying Bacteria

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***Keywords**: nitrification, kinetics, kaolin particles*

Searching for new technologies to improve amonia removal in water and waster water is an up-to-date subject. The improvement of the process may be attained by the design of new reactors or new operating methologies, including the addition of inorganic particles that may play a significant role on the oxidation process of amonia and nitrite.
The aim of this work was to evaluate whether the presence of different concentrations of kaolin particles interferes with the kinetic process of nitrification. The nitrification experiments were performed with a mixed culture of nitrifying bacteria, consisting of *Nitrosomonas* sp. and *Nitrobacter* sp. The following experiments were carried out:

a) Study of some physico-chemical properties of kaolin, namely the effect of the particles on the pH of solutions with different initial pHs, adsorption and desorption of amonia, nitrite and nitrate by kaolin particles at different pH values.
b) Study of the effect of the concentration of kaolin particles on the specific oxidation of amonia and nitrite to nitrate in batch reactors with and without aeration, with and without pH control. In all the experiments the initial amonia concentration was high (in the range of 480 mg/L to 650 mg N-NH4/L.
c) Respirometric assays to evaluate the effect of the concentration of the particles and the time of contact on the endogeneous respiration rate and the substrate (amonia and nitrite) oxidation rate.

The physico-chemical studies of the the kaolin particles showed that, for clay concentrations below 0.5 g/L, the final pH values were near neutrality. However, for higher concentrations (1 and 2,5 g/L) the final pH values of the solutions were more basic, whatever the initial pH values. In addition, for solutions with initial values lower or equal to 7, the final values increased, conversely to the case of initial values higher that 7. With respect to the adsorption of amonia, nitrite and nitrate, negligible adsorption of those substances was observed.
The nitrification studies carried out showed that in the presence of 1 g/L and 2,5 g/L of particles, the nitrification was more efficient in the reactors without pH control (with and without aeration). For lower kaolin concentrations the nitrification kinetics seemed to be more efficient in reactors with pH control and reactors with aeration.
The respirometric experiments showed that the presence of 0.5 and 1 g/L of kaolin particles produced a stimulation in the specific endogeneous respiration rate of the bacteria, and a higher exogeneous substrate specific consumption rate. These results were obtained in two different situations: in one case the tests were carried out immediately after the additionof kaolin particles and in the other case there was a 7 days pre-contact of kaolin with the bacteria consortium before the test.

Characteristics of a Denitrifying Biofilms in a Fluidized bed Reactor

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Key Words: *denitrifying biofilm; fluidized bed reactor; biofilm thickness; proteins/polysaccharides ratio; C/N/P ratio*

The aim of this work was to study and optimise the operating performance (stability) of the fluidized bed reactor (FBR) by monitoring the variation of the bioparticle density (support+biofilm) and the thickness of the biofilm that coated the support over the time, under various operating conditions. Basalt was used as the support medium for biological growth and the denitrifying microorganism seeded was a heterotrophic culture of *Alcaligenes denitrificans* (ATCC 15173). Different C/N/P conditions were used in the FBR (hydraulic retention time, superficial velocity, recycle ratio, temperature and pH were kept constants) and denitrifying biofilm thickness, density and composition (in terms of total proteins and totals polysaccharides) were monitored along the time. The results obtained showed that the growth of the biofilm is not strongly affected by the C/N/P variable conditions, even in low nitrate removal efficiency. The biofilm that has achieved the highest denitrification activity has also presented a total amount of proteins far above the amount of polysaccharides (see Table 1). Nevertheless, for the conditions studied, the proteins/polysaccharides ratios are not significantly higher in the more active biofilm, in contrast with the results founded by Lazarova *et al.*[1]. Therefore the proteins/polysaccharides ratio does not indicate a good performance of denitrifying activity.

Table 1 - Relationship between the operating conditions, the denitrifying activity and the content of proteins and polysaccharides

C/N ratio ($mgC/mgN\text{-}NO_X^-$)	[P] (mgP/L)	Denitrifying Activity (%)	Thickness (μm)	mgTotal Prot/mgSV	mgTotal Polysac./mgSV	Ratio Prot/Poly
1.78	1.14	45	0.395	0.390	0.163	2.5
3.6	1.14	93	0.402	0.307	0.107	2.9
3.6	0.49	49	0.344	0.227	0.107	2.1
1.81	0.16	15	0.384	0.167	0.181	0.9
1.43	0.16	25	0.385	0.268	0.244	1.1

During all the experiments was observed that when the attached support biofilm was grown, the biofilm and bioparticles density decreased resulting in the expansion of the fluidized bed at a given liquid recycle flow rate (similar results were founded by Livingston *et al.*[2]). After approximately a period of 20 to 25 days' operation, the mean bioparticle density decreased reaching values of decrements between 50 to 55%. At this point, manual operations of removal of biomass were required to keep the reactor within good operating conditions. Therefore, the current reactor does not produce the required shear stress that would allow the control of biomass growth on the support particles. This has caused a considerable reduction of the bioparticle density, previously reported, and hence various operating difficulties, namely excessive biomass growth, bioparticle washout and bioparticle clogging. The maximum thickness achieved by the biofilm, before clogging occurring, ranged between 350 and 400 μm.

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Influence of Increasing Organic Load Rate of Kraft Mill Effluent on Activated Sludge System Stability

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Keywords: *Activated sludge,* Pinus radiata *Kraft mill, Organic load rate, Stability.*

Activated sludge processes are widely employed to treat wastewater with varying physical-chemical and biological characteristics. The system stability depends on several parameters, as Organic Load Rate (OLR), Hydraulic Retention Times (HRTs), toxic compounds, sludge characteristics, etc. On the other hand, environmental impact of Kraft mill wastewater is mainly due to the release of biodegradable organic matter and inhibitory constituents, which are toxic to aquatic organisms. Besides, the presence of large amounts of solubilized ligninic materials explains their characteristic dark colour.

The goal of this study was to evaluate the stability of the activated sludge system as *Pinus radiata* Kraft mill effluent OLR increase.

An activated sludge system (reactor (4L), settler (4.5L)) was operated during 200 days. The reactor was fed with *Pinus radiata* Kraft mill wastewater with two different bleaching processes: ECF (Elementary Chlorine Free) ($D_oE_{op}D_1D_2$) and traditional ($C_{50\%}/DE_oD_1D_2$) sequences. The OLR ranged between 0.7 - 6.0 gCOD/L·d, while the relationship between F(Food)/M(microorganisms) was 0.4 h^{-1} in average. The sludge was periodically recycled and the excedent was purged in order to obtain 30 days aged sludge and controlled SVI (Sludge Volume Index); furthermore oxygen concentration was kept between 2 and 4 mgO_2/L. Finally, pH, COD, BOD_5 were also monitored. The results show that while the HRT decreased from 48 to 6 h, the activated sludge system removed efficiently BOD_5 (85-90%), COD (40-60%) and phenolic compounds (35-86%). Due to the system start-up and operation, it was not destabilised between 48 and 10 h of HRT. However sludge characteristics changed and bulking was produced at 6 h of HRT.

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Commercial Microbial Inocula as Reference Organisms in a Biodegradability Test (BOD_5)

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Key Words *: BOD_5, assays methods, commercial inocula*

Biodegradability appears as an useful criterion for evaluating the fate of a chemical and its environmental impact. Biodegradation studies, as well as the complexity of the phenomena which are involved during biodegradation reactions has been object of study, generally using the Biochemical Oxygen Demand (BOD) determination. The principle of this test is the assessment of the amount of oxygen used for the respiratory activities of microorganisms which utilize organic matter as substrate for growth.

In any biodegradability assay it´s necessary a microbial inoculum which is responsible by the oxidation of the carbonaceous material, soluble, present in the sample. Generally, it is used a mixed microbial inoculum from an activated sludge treatment plant, treating domestic sewage. However, this fact leads to problems as: inoculum sampling, maintenance of its viability, impossibility to control their quantitatives and qualitatives characteristics and presence of pathogenics microorganisms.

In this context, the aim of this work consisted in the study of the potencial of two commercial microbial inocula (BI–CHEM® and BIOLEN M112) as reference organisms of the 5-day BOD test, using the dilution method (APHA, 1992) and the manometric method (OxiTop®, Instruction Manual, 1997). In the assays performed was tested the standard glucose-glutamic acid solution, proposed in the methods standards, and the value of BOD_5 of this solution for the microorganisms of the two inocula was determined. Simultaneously was effectuated the microbiological characterization of these inocula with the API identification system. The mean values of biochemical oxygen demand obtained were BOD_5 = 199.4 mg/L O_2 (CV= 4.52%) and BOD_5 = 176.8 mg/L O_2 (CV = 4.66%) for the BI–CHEM® inoculum and BOD_5 = 209.8 mg/L O_2 (CV = 7.35%) and BOD_5 = 228.6 mg/L O_2 (CV = 9.37%) for the BIOLEN M112 inoculum, with the dilution and manometric methods, respectively. These results are included in the range referred for the activated sludge in the standards of the methods cited, which seems demonstrate that these commercial inocula are fitted to be used in BOD_5 test as an alternative to the activated sludge inoculum. With this preliminary study is pretended to proceed with the validation of these methods performing the BOD_5 test with the two commercial inocula and with an activated sludge inoculum using samples as industrial effluents.

The authors acknowledge to DGA, Programme Specific for the Environment, for the financial support.

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Selection of Bacteria And Fungi Petroleum Hydrocarbons Degraders*

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Keywords: Bioremediation, Biodegradation, Petroleum, Light Arabian oil

In Brazil, imported products containing microorganisms have been used in the attempt of solving the effects caused by petroleum spills and some of its derivatives through bioremediation. This process of biological treatments of residues consists of the action of microorganisms in order to mineralize or detoxify the pollutants. Some of the factors that determine the persistence of some polluted compounds in the environment are the microbial flora and the absence of nutrients. Microorganisms adapted to the contaminated site are recommended to increase the low rate of degradation under field conditions. In this context, it is justified the necessity of selection of cultures capable of degrading contaminants under biological and physical-chemical characteristics of the polluted ecosystem in order to develop and implement processes that eliminate or minimize the impacts caused by the introduction of pollutants in the biosfera.

The aim of this study is to isolated oil-degradading microorganisms from Brazilian soil contaminated by petroleum and to study them in laboratory test.

First, some mixed cultures collected from soil contaminated by petroleum have been obtained and they were collected in the TEBIG/PETROBRÁS (Angra dos Reis-RJ), and others were gotten from different points of landfarming (REDUC/PETROBRÁS, Rio de Janeiro-RJ). In this process, 32 cultures (bacteria 17, fungi 13 and mixed cultures 2) and 24 culture collection (bacteria 7, fungi 10 and mixed cultures 7) were tested considering the growth capacity in a medium containing light Arabian oil as the single source of carbon. These experiments were made in 500 mL Erlenmeyer flask containing 100 mL of mineral medium with 1% of light Arabian oil and 10 mL of inoculum at 150 rpm and at 30°C during a process of less than 30 days. A test control without microorganisms was carried out simultaneously.

The 15 cultures grown in this medium were tested to verify their ability to degrade the hydrocarbons present in light Arabian oil. The 15 cultures were able to metabolize these compounds. Two mixed cultures labeled Nd and Nz degraded 22,0% and 20,9% respectively, after 7 days of incubation in 100 mL of mineral medium containing light Arabian oil. The extraction of hydrocarbons from the culture medium was accomplished by partition techniques using water/carbon tetrachloride and their quantification in a organic phase was perfomed by infrared spectroscopic and gas chromatographic measurements.
Among all microorganismis tested, the Nd and Nz cultures were selected as promising agents able to degrade hydrocarbons of light Arabian oil.

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Phenols Degradation by an Olive Oil Wastewater Isolated Yeast

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***Keywords**: Olive oil mill wastewaters, yeast, phenols degradation*

Olive oil production is a large-scale agricultural activity of the European southern regions with a very strong economical significance. In Portugal the olive oil extraction, from about 200 000 to 500 000ton of olives per year, is one of the most important agro-industries and the amount of wastewaters, produced during only 3 to 4 months per year, is estimated to be about 225 000m^3 [1].

The wastewaters from the olive milling process (OMW) are highly pollutant due to a high organic carbon and suspended solids content and to the presence of recalcitrant components, mainly aromatics like phenols and polyphenols, reported to be hardly degraded by microorganisms. In the literature, caffeic, gallic, p-coumaric and vanillic acids have been reported to be among the phenolic compounds found in OMW [2].

In INETI, it was firstly performed an yeast isolation from several OMW [1] and the yeast designated as DER 101 was chosen to proceed for the degradation studies. We have started by testing the yeast resistance, at 30ºC and 150rpm, when inoculated in YNB synthetic media containing one of the phenolic products, at several different concentration levels (350- 12000 mg/l). The yeast was previously grown in three different inocula media, two of them containing 350g/l of cathecol.

Unexpectedly, DER 101 has shown to be able to grow and to completely degrade amounts of 12g/l of caffeic acid, 3g/l of vanillic acid, 12g/l of p-coumaric acid, 8g/l of gallic acid and 1.5g/l of cathecol, in about 24-144h. The results obtained allowed a characterisation, in terms of kinetic constants, of the yeast DER 101 ability to degrade each of these phenols.

However, when this yeast was inoculated in several different OMW media, the maximum degradation obtained, achieved in 48h, was 34% of the total phenols content in the OMW, probably due to the presence of other inhibition compounds.

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Biochemistry of Hydrocarbon Biodegradation in Indigenous Fungi Isolated from Contaminated Soils

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Keywords: biodegradation, hydrocarbons, fungi, oxidoreductases

Hydrocarbon compounds are major environmental pollutants as a result of improper disposal processes or spills of petroleum and petroleum-derived products. Diverse microbial populations can metabolize the hydrocarbons found in petroleum; this property of microorganisms is of biotechnological importance in the context of bioremediation. The compounds metabolized include simple aliphatic hydrocarbons (methane, n-alkanes, isoalkanes,olefins, cycloalkanes, etc.) as well as simple and complex aromatic compounds (benzene, toluene, naphthalene, anthracene, phenanthrene, etc.). Although it is known that bacteria, fungi and in less extent algae can oxidize hydrocarbons, most of the detailed biochemical and genetic studies concerning the metabolic pathways for hydrocarbon degradation have been made in bacteria. The metabolic versatility and ease of handling of fungi and the few species studied so far stresses the importance for broadening the scope for the study of many more fungi, with emphasis on the biochemical basis of hydrocarbon degradation and their potential for bioremediation processes.

In this work we describe the isolation and the initial biochemical characterization of the oxidoreductases alcohol dehydrogenase (ADH) and alcohol oxidase (AO) of an indigenous filamentous fungus (strain YR-1), isolated from an oil refinery in Salamanca, Gto., at central México. The primary and secondary selection of the microorganism were achieved using minimal medium supplemented with methanol and hexadecane, respectively. The enzyme activities were detected in crude extracts and in the high-speed (40,000 rpm) supernatant, which suggested that the enzymes are cytosolic. Zymogram analysis revealed the presence of one main band of ADH and of AO activity; both types of enzymes exhibited different electrophoretic migration. It was observed that in cells grown in minimal medium the production of AO varied with the carbon source: the levels of the enzyme activity were stimulated by the presence of the hydrocarbons decane and hexadecane and repressed by the presence of glucose. The AO enzyme employed different substrates, such as methanol, ethanol and decanol. Cell-free extracts of the fungus YR-1 possesse proteins that are immunodetected with antibodies directed against the alcohol oxidase of the methylotrophic yeast Hansenula polymorpha; these observations suggested the possibility of the existence of common structural features and antigenic determinants in both oxidases.

Ascomycete Yeasts in The Aerobic Decoulorization of Azo Dyes

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Key words: azo dyes, biodegradation

Azo dyes are among the most widely used coloured materials in textile industries and its biodegradability is, therefore, an important issue in the biological treatment of waste waters containing dyes. However, these treatments are usually ineffective in reducing colour of textile dye waste-water since these compounds are tipically resistant to oxidative degradation. Most biodegradation studies on azo dyes involve bacterial species, and anaerobic or microaerophylic conditions are usually refered to as being favourable to a reduction step producing colorless amines. Oxidative biotransformation of dyes by the white-rot fungus Phanerochaete chrysosporium has also been reported.

The yeasts are highly versatile microorganisms and our group has succeeded in isolating a number of yeast species which revealed an interesting potential in the colour removal of azo dyes in aerated culture media. This work describes the time-course decoulorizaton of four sulphonic monoazo dyes under aerobic conditions by the ascomycete yeasts Issatchenkia occidentalis and Candida zeylanoides, and the effect of medium composition and pH on the process. Preliminary experiments were conducted in order to establish the range of dye concentrations within which total decoulorization is observed, and the inhibitory effect of the dye on the microrganisms growth.

The extent of decoulorization of the dyes by I. occidentalis in shake flasks, in a nutrient medium containing glucose as carbon and energy source and dye concentrations up to 200 mM, approaches 100% in less than 25 hours. In the course of the process a decrease in the pH from ca. 4.8 to 2.8 is observed. In biostat experiments, performed at pH 5, decoulorization was negligible and strong adsorption of the dye to the cell wall was observed. However, if pH was allowed to decrease, total decoulorization occured in 14 hours. The yeast C. zeylanoides appears to be less efficient, since decoulorization times, in shake flasks, extended to 50-60 hours. Dye concentrations up to 200 mM did not negatively affect the biomass yields or the specific growth rates of either microorganism.

Enhanced Naphtalene Degradation by a Co-Culture in a Biphasic System

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Keywords: *bioavailability, naphtalene, bioremediation, apolar-phase.*

Biodegradation of polycyclic aromatic hydrocarbons (PAH) is very slow due to their weak solubility and low rates of solubilization in water, which limit the bioavailability of this widespread type of toxic contaminants. Therefore, surfactant and apolar-compound amendments have been suggested as strategies to facilitate the transfer of PAH from the solid to the aqueous phase, hence, to accelerate bioremediation processes.

We comparatively studied the effect of the addition of anionic detergents, or of a non-biodegradable organic phase (20%, v/v) on naphtalene degradation rates by a bacterial co-culture, that we had isolated from a site with heavy and old PAH contamination.

Growth on naphtalene, added as a single particle or dispersed by sonication, in solution in dimethyilpolysiloxane, and in the presence of Tween 80 or Triton X-100, was exponential while naphtalene was detectable in the culture medium. Thereafter, linear growth occurred. While observed exponential growth rates ($0.20\pm0.03\ h^{-1}$) were similar for all tested systems, the length of logarithmic growth was longer on dispersed naphtalene and was significantly extended with dimethyilpolysiloxane. In addition, subsequent zero-order growth was significantly faster in the biphasic system. Unexpectedly, surfactants had no any comparable effect. Apparently, after depletion of the starting aqueous-naphtalene concentration, PAH degradation was controlled by its rate of dissolution. Moreover, it is likely that the transfer of naphtalene to water was faster from the organic than from the solid phase, provided that an adequate concentration-gradient between the organic and aqueous phases was maintained. So, the presence of an organic phase may facilitate PAHs bioavailability. As many apolar compounds easily and rapidly separate from the bulk medium, the use of biphasic-systems with organic-phase recycling may help to overcome present rate limitations in the utilisation of microorganisms to treat water contaminated with PAHs, like in Pump and Treat Processes.

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Phenol Biodegradation with *Pseudomonas putida* DSM 548 Immobilized in a Large-Pore Support

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Phenol biodegradation with *Pseudomonas putida* DSM 548 was studied in batch reactor with suspended biomass, continuous rotating-disc biofilm reactor, CSTR with fixed biomass in PORAVER particles and fluidized bed biological reactor with fixed biomass in PORAVER particles. The feed contained phenol, potassium phosphates, ammonia sulphate, magnesium sulphate, sodium, calcium and iron chlorides.

The kinetics of phenol biodegradation and biomass growth in a batch reactor at 26 ºC, pH~6.8 for phenol concentrations up to 100 mg ℓ^{-1} is well described by Haldane equation $\mu = \mu_m S/(K_s + S + S^2/K_i)$ with μ_m=0.436 h^{-1}, K_s=6.19 mg ℓ^{-1}, K_i=54.1 mg ℓ^{-1}.

In the rotating-disc biofilm reactor the rate of removal of phenol is 0.05 - 0.15 mg h^{-1} cm^{-2} of support. The maximum conversion was 93% corresponding to the rate of removal of phenol of 0.02 g h^{-1} ℓ^{-1} reactor. The maximum specific growth rate is μ_0= 0.04 - 0.08 h^{-1}. When biomass is fixed in PORAVER particles, the maximum specific growth rate is μ_0= 0.0147 h^{-1}.

In the fluidized bed biological reactor with biofilm thickness up to 350 mm and space time of 28 - 73 s, the maximum specific growth rate is μ_0= 0.02 - 0.1 h^{-1}. The rate of phenol removal is 0.01 - 0.085 mg h^{-1} cm^{-2} of support.

Poly R-478 dye Decolorization by *Phanerochaete chrysosporium* in Packed bed Bioreactor

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Keywords: *Dye decolorization,* Phanerochaete chrysosporium*, packed bed bioreactor.*

Textile industries consume substantial volumes of water and chemicals for wet processing of textiles. Colour is the first contaminant to be recognized in wastewater and has to be removed before discharging into waterbodies or on land. The presence of very small amounts of dyes in water is most of the time highly visible and affect water transparency and gas solubility in rivers and lakes. Nowadays the removal of synthetic pollutants dyes is gaining great importance .During the past two decades several physico-chemical decolorization techniques have been reported, few, however, have been accepted by the textile industry.

Several studies have shown that microbial treatment can be a very promessing choise for synthetic dyes degradation. Ligninolytic peroxidases secreted by white-rot fungi are capable to degrade polyaromatics hydrocarbons (PAH). These synthetics organic compounds have a resemblance with the lignin. For that reason this biological treatment may be use as an alternative in effluent decolorization.

In this study, Poly-R 478 is selected to develop an optimun degradation process. To achieve this a continuous process is designed using *Phanerochaete chrysosporium* immobilized on a polyurethane foam in a pulsed packed bed bioreactor. All over the process some environmental conditions were followed such as Mn^{2+} concentration, oxygen and air effects on the decolorization, hydrogen peroxide level, and also adequate hydraulic residence time was evaluated. During the process several parameter like Manganase Peroxidase production, redox potential, glucose comsuption, total organic carbon and nitrogen level, oxalic acid production, dissolved oxygen on the outlet effluent and Poly-R 478 decolorization percentage were followed daily. The operation is carried out over 120 days giving an decolorization average of a 75%. This study has given operational parameters to achieve the higher decolorization of the Poly-R dye by *Phanerochaete chrysosporium.* This continuous treatment is a very interesting process and it can be use for further research on the treatment of others pollutant compounds

Biosorção de metais pesados de efluentes industriais com biomassa de *Chlorella vulgaris*

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Palavras-chave: *Microalgas, biosorção, metais pesados, efluentes industriais.*

A utilização de biomassa microalgal para a remoção de metais pesados tem tido, na última década, grande desenvolvimento.

As paredes celulares das microalgas biológicamente activas (vivas) ou inactivas (mortas), contendo macromoléculas (proteínas, polissacáridos) a que está ligada grande variedade de grupos funcionais (hidroxilo, carboxilo, aminas, imidazole, sulfatos, fosfatos,...) podem ligar-se reversivelmente a quantidades significativas de iões metálicos contidos em soluções aquosas e funcionar da mesma forma que resinas de troca iónica [1].

As propriedades mais importantes das células são as de serem efectivas para níveis de concentração muito baixos e apresentarem uma especifidade muito forte para determinadas espécies químicas, características que os métodos químicos normalmente não apresentam.

Este trabalho visa a remoção de Zn^{2+}, Cd^{2+}, Pb^{2+} e Cu^{2+} do efluente obtido na lavagem dos fumos dos fornos de calcinação durante o fabrico do óxido de zinco. A lavagem é feita com água em contra-corrente e com recirculação. O efluente sai com elevada carga de metais pesados, nomeadamente de Zn, e com um pH de 3-4.

O estudo da remoção dos metais com biomassa de *Chlorella vulgaris* foi feito a partir de soluções sintéticas contendo dois ou mais metais nas concentrações encontradas no efluente referido, utilizando o pH, o tempo de contacto e a temperatura anteriormente definidos [2].
As melhores condições de biosorção determinadas foram depois aplicadas ao efluente real.

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Results from a Experimental Pilot-Scale Low-Cost Wastewater Treatment Plant

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Keywords: *wetlands, biofilm, HRAP, nutrients, fecal indicators.*

Results from a experimental plant constructed in Leon on low-cost depuration processes are shown. Three different systems have been used for research: conventional stabilization pond, high rate algal pond (HRAP) and constructed wetlands, all of them treating low loaded domestic wastewater from a rural village at Northwest of Spain, in a continental extreme climate.

Experiments were conducted since 1990 up to date, in order to evaluate microbial population dynamics (free and attached bacteria, phytoplancton, and zooplancton), and to estimate optimal design parameters for organic matter, nutrients, parasites, and fecal microorganisms removal.

Results have lead to the design of several full-scale constructed wetlands for low population rural areas.

Several remarkable conclusions may be pointed out:

- Constructed wetlands show a great buffer capacity against flow, nutrient, and organic load, leading to a well stabilized effluent (accomplishing all European standards). However, great differences in resistance against pollutants concentrations were observed for the tested plant species.
- Use of ligneous plants as a final step in a constructed wetland system gives excellent removal efficiency, specially for fecal indicators.
- Constructed wetlands designed for high root-rhizome development are suitable for effluent nitrification, solids retention, and pathogen microorganisms removal.
- Use of a mineral substrate with high specific surface lead to optimal phosphorus removal, while removal efficiency for ammonia, organic matter and solids decreases respect reactors with high root development.
- In cosnstructed wetlands, biofilm associated to the substrate and the rhizosphere plays a main role in nutrient removal compared to the plant uptake effect. However macrophytes seem to create a microenvironment which enhance bacterial processes.
- HRAP systems show lower area requirements and higher stability against weather changing conditions than conventional ponds, for the same pollutant removal efficiency.
- Besides HRAP effluent has better settle properties than that from the stabilization pond, due to the presence of microalgae aggregates. Algae diversity in HRAP is lower than in stabilization ponds, while abundance is higher.

Decoloración de Poly R-478 por *Phanerochaete chrysosporium* en Cultivos Discontinuos

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Palabras clave: Phanerochaete chrysosporium*, Poly R-478, decoloración.*

Gran parte de los colorantes sintéticos empleados hoy en día son vertidos al medio ambiente, ya que su tratamiento es sumamente complejo [1]. Varios autores recomiendan la utilización de métodos biológicos como alternativa al tratamiento de diferentes contaminantes recalcitrantes [3]. Se ha estudiado la capacidad del *P. chrysosporium* BKM-F-1767, hongo conocido por su notable actividad ligninolítica, de degradar un colorante sintético polimérico. El objetivo de este estudio es obtener la relación entre la actividad enzimática y la decoloración del Poly R-478. Es usual encontrar en la bibliografía afirmaciones de que esta relación existe [2,3], pero no se aportan datos concretos.

La decoloración se ha realizado in vivo, en estado semisólido, empleando erlenmeyers de 250 ml, sin agitación y carozo de maíz como soporte-sustrato (7 mm de longitud y 4 g por matraz).

El medio de cultivo es el Kirk Medium suplementado con Tween 80, alcohol veratrílico y MnO_2 para mejorar la actividad ligninolítica. La temperatura se controla a 30 ºC el pH a 4,5 y el cultivo se mantiene en completa oscuridad. La aireación de los matraces se produce de forma pasiva durante todo el proceso, ya que se emplean tapones de celulosa comprimida. El colorante se añade en forma de disolución acuosa hasta una concentración final del 0,08 %.

La decoloración se sigue mediante estudio fotométrico a la longitud de onda de máxima absorvancia del colorante. Para eliminar los efectos de coloración de la muestra debido a la presencia de carozo de maíz, y los posibles cambios en el comportamiento óptico del colorante, se han de preparar dos tipos de controles, sin colorante y sin inóculo.

La decoloración es efectiva empleando las condiciones comentadas, lo que reafirma la capacidad del complejo enzimático ligninolítico del *P. chrysosporium* para el tratamiento de compuestos xenobióticos normalmente vertidos al medio ambiente.

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Beneficios de la Implementación de la Eliminación Simultánea de Nitrógeno y Fósforo en Plantas de Lodos Activos

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Keywords : *nutrient removal, phosphorus, domestic wastewater, pilot plant.*

El tratamiento convencional de depuración de aguas residuales urbanas más implementado es el proceso de lodos activos. El objetivo principal de las plantas depuradoras construidas hasta el momento es la eliminación de la carga orgánica del agua residual, pero el aumento de los problemas ambientales ha hecho que la actual legislación exija, además, la eliminación de nitrógeno y fósforo.

Existen varias razones que aconsejan ampliar la depuración biológica a la eliminación de nutrientes. Estas razones son medioambientales y económicas. Desde el punto de vista medioambiental el mayor beneficio que se obtiene es el control de la eutrofización. Además, en plantas de lodos activos con eliminación biológica de nutrientes se obtienen una serie de beneficios económicos : ahorro en el consumo de energía de aireación [1], reducción, o incluso eliminación, de la adición de productos químicos necesarios en la depuración físico-química, menor producción de lodos y la posible revalorización de los lodos producidos. [2,3]

El objetivo de este trabajo ha sido cuantificar algunas de las ventajas que se consiguen con la eliminación biológica simultánea de nitrógeno y fósforo. El estudio se ha realizado en una planta piloto de lodos activos de 150 l con configuración A_2O (anaerobio-anóxico-óxico). Se ha tratado un agua residual sintética de composición similar al agua residual urbana con fuente de carbono compleja (400 mg/l de DQO, 40 mg/l N, 8 mg/l de P). Los parámetros analizados a lo largo del estudio han sido : DQO, $P\text{-}PO_4^{3-}$, $N\text{-}NH_4^{+}$, $N\text{-}NO_3^{-}$, $N\text{-}NO_2^{-}$ y SSV.

A partir de los resultados obtenidos se ha cuantificado : la capacidad de eliminación de nutrientes (95% DQO, 93% nitrógeno, 90% fósforo), la producción de lodos (Y_{obs} = 0.20 - 0.25 g SSV/g DQO) y la acumulación de fósforo en los lodos (0.07-0.10 g P/g SSV).

Estos resultados reflejan que operando una planta de lodos activos con configuración A_2O se puede cumplir la legislación sobre vertido de nutrientes, se producen menos lodos en exceso y se obtienen lodos potencialmente utilizables como fertilizantes por su alto contenido en fósforo.

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Paper Mill Effluent Decolorization by *Phanerochaete chrysosporium* and *Bjerkandera* sp. BOS55

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Keywords: *Paper mill effluent, Phanerochaete chrysosporium, Bjerkandera sp. BOS55.*

Conventional biological treatment of highly coloured wastewaters, though effective in organic matter removal, finds its major drawback on the lack of efficiency on colour removal and even an increase in final colour may be expected. Additionally, complex compounds present in some wastewaters are hardly biodegradable by these technologies or in the best case, their degradation requires prolonged adaptation periods. White-rot fungi are unique microorganisms since they are able to cause extensive biodegradation of lignin and this ability faculties these microorganisms for the degradation of many other complex compounds, even those with toxic properties [1,2].
In this work soda cook liquor coming from paper mill effluents of wheat straw pulping (pH 8.5) was treated with two white-rot fungi, *Phanerochaete chrysosporium* y *Bjerkandera BOS55*. The following table resumes results obtained for the treated effluent with *Phanerochaete crysosporium* and *Bjerkandera BOS 55*.

Fungi	Inocula of day [a]	%Decolorization	%COD reduction
P. chrysosporium.	0	90.6	84.4
	6	60.6	53.0
Bjerkandera sp. BOS55	0	84.3	86.7
	6	61.4	91.0

[a], effluent.

In order to analyze the effect of the addition of the effluent on the process efficiency, cook liquour was added at days 0 and 6 (this corresponding to the onset of secondary metabolism). In both cases a lower colour removal was observed when the effluent was added at day 6. The adsorption of colour by the biomass was similar at both times for *P. chysosporium*, while *Bjerkandera* presented a four-fold absorption when the addition was performed at day 0. Both microorganisms are able to reduce organic content, showing another point of interest to be taken into account.

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Micoteca da Universidade do Minho (MUM)

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Key words: *Culture of fungi; Deposit facilities; Contract research; Training courses*

The need to obtain fungal strains with specific degrading capabilities as well as the continuing experimental activity in mycology, in connection to environment preservation and biodiversity issues, consolidated the idea that in Portugal one feels the need to organise a fungi culture collection with the double purpose of keeping and supplying strains and act as a centre for research, information and training.

The collection kept in the Department of Biological Engineering at the University of Minho includes over 160 identified species and more than 50 mutant and transformant strains.

Identification and preservation services are basically the result from research activities and specialized services for the Industry with emphasis on biodeterioration and biodegradation. More specifically a project on the deterioration of paint films is being carried out in cooperation with the International Mycological Institute, Surrey, UK.

The available specialized books include around 300 titles of which 100 are monographys.

A manual on food-borne fungi was prepared to support the advanced course on the same subject, which took place in Dec. 97.

A database on the collection is also being built which will allow the publication of the corresponding catalogue.

Acknowledgements

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Estudio de la Bioadsorción de la Fracción Rápidamente Biodegradable de Agua Residual Urbana Mediante Respirometría

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Keywords : *Respirometría, Fracción, Biodegradable, SBR.*

Los procesos de tratamiento biológico de aguas residuales están fuertemente influenciados por las diferentes cinéticas de biodegradación de los componentes de la materia orgánica. En este trabajo se ha usado la respirometría para la determinación de la fracción rápidamente biodegradable usando el método propuesto por [1].

El método se basa en que la velocidad de consumo de oxígeno (oxigen uptake rate, OUR) permanece constante con un valor relativamente alto mientras se degrada la fracción rápidamente biodegradable y después disminuye rápidamente a un nivel inferior. El alto valor inicial de OUR es una consecuencia de la utilización de la materia rápidamente biodegradable del agua residual y de la obtenida por hidrólisis de la materia particulada biodegradable. Una vez que la fracción rápidamente biodegradable se ha consumido, la OUR cae al segundo nivel, que representa la velocidad de utilización de la materia rápidamente biodegradable derivada de la hidrólisis de la materia particulada biodegradable. En un respirograma, a partir del área entre estos dos niveles de OUR, puede calcularse la fracción orgánica rápidamente biodegradable.

Para la determinación experimental se ha usado un respirómetro discontinuo cerrado, conectado a un ordenador, que registra el valor de la concentración de OD existente en el interior de la cámara respirométrica.

Se ha aplicado esta técnica a un sistema de dos reactores SBR. El primer reactor tiene como objetivo adsorber sobre la biomasa la materia orgánica presente en el influente. Se ha determinado la fracción rápidamente biodegradable del agua residual urbana usada como influente y de la corriente de salida del reactor una vez que ha tenido lugar la etapa de bioadsorción.

Como promedio de diferentes experiencias la fracción rápidamente biodegradable del influente es el 48 %, siendo la DQO total 338 mg O_2 / L, y la de la corriente de salida del primer reactor tras la bioadsorción es el 60 %, con una DQO total de 147 mg O_2 / L, con lo que podemos establecer que el 46 % de la materia rápidamente biodegradable presente en el influente es adsorbida en el primer reactor durante el llenado. De la materia no rápidamente biodegradable presente en el influente el 45 % es retenido en el primer reactor.

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The Use of the Reed *Phragmitis communis* and Associated Microbial Community for the Development of a Bioremediation Process

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Keywords: Phragmitis communis*, bioremediation, microbial consortia, haloorganics, GAC*

The aim of this study was to evaluate the role of microorganisms-host plant relationships in bioremediation processes and to enhance the efficacy of such processes. Rhizomes of *Phragmitis communis* inhabiting a heavily contaminated environment were collected, and cultivated in laboratory soil systems. Haloorganic compounds (chloro-, nitro- phenols) are supplied to the soil-plant systems and biodegradation is monitored. Plant roots were analysed for the adsorption and degradation of the target compounds, when deprived of microbial activity. Disappearance of the substituted phenols was quantified by HPLC and chloride release monitored.

In parallel, chloro- and nitro- aromatic degrading consortia were isolated from the rhizosphere of *Phragmitis communis* collected from the same site. Batch enrichments were established using the haloaromatic compounds. Isolated consortia were able to degrade mixtures of such pollutants. A granular activated carbon (GAC) biofilm column for the degradation of a model compound, 4-chlorophenol, was established using an isolated consortium. To improve this process, a second natural biofilm is being established on a GAC column using microbial communities extracted from rhizosphere samples by differential and dispersion centrifugation techniques.

Delignification and Bleaching of Oxygen-Delignified Kraft Pulp by White-rot Fungi

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Due to increasing environmental concern, the pulp and paper industry has made substantial changes to conventional pulping and bleaching technologies. The proposals towards cleaner processes have been focused on the bleaching with chlorine-free sequences to reduce emission of organic halogen compounds. The aim of developing bleaching sequences based on biotechnological processes has led research to different strategies, referred as a pretreatment with xylanases or as an alternative bleaching process by using white-rot fungi and their oxidative enzymes [1].

White-rot fungi produce extracellular oxidative enzymes which initiate the catalytic oxidation of lignin. Whole cultures of various white rot fungi have been found to cause extensive brightness gains and delignification of kraft pulps [2]. *Bjerkandera* sp. BOS55 was proved to achieve the greatest brightness gains (up to 14 ISO units) and delignification (up to 50% reduction) compared with 24 other white-rot fungal strains [2]. During the course of biobleaching, manganese peroxidase (MnP) was the major oxidative enzyme activity detected in the extracellular fluid. Previous observations with other MnP-producing white-rot fungi indicate that MnP utilizing Mn(II) as a cofactor, known to mediate oxidation reaction between the enzyme and lignin, is largely responsible for biobleaching of hardwood kraft pulps. As expected, manganese nutrient additions greatly increase the MnP production; however, the biobleaching was not affected by the Mn nutrient regime, ranging from 0.004 μM Mn corresponding to a vitually Mn-free trace element solution with EDTA-extracted pulp to 1,000 μM added Mn. The lowest Mn concentration tested was at least several orders of magnitude lower than the Km known for MnP. Consequently, it was concluded that Mn is not required for biobleaching in *Bjerkandera* sp. BOS55 [3]. Exogenous additions of physiological organic acids at 1 to 5 mM greatly stimulated brightness gains and pulp delignification under the manganese-free conditions 2- to 3-fold compared to control cultures not receiving acids. The stimulation was attributed to the role of organic acids on enhancing the production of MnP as well as the physiological concentrations of low molecular weight compounds [4].

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Toxicidad y Biodegradabilidad de Compuestos Clorofenolicos

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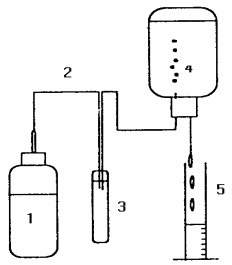
Keywords: *Biodegradabilidad clorofenoles, toxicidad.*

Los clorofenoles pueden ser vertidos como efluentes industriales, también se forman en la cloración de las aguas superficiales y residuales, aparecen en lodos de tratamiento de pulpa de papel y son intermediarios del metabolismo de biocidas, insecticidas, PCBs, etc. Debido a su toxicidad, el tratamiento biológico de aguas industriales que contienen altos niveles de clorofenoles puede ser dificultoso. Este trabajo estudia la biodegradabilidad y toxicidad de algunos clorofenoles en ensayos discontinuos anaerobios.

Para llevar a cabo los test de biodegradabilidad y toxicidad se utiliza el esquema de la fígura 1.



Fígura 1. Montaje experimental.
1. Digestor.
2. Circuito de biogás.
3. Cámara de seguridad.
4. Frasco de Mariotte.
5. Probeta calibrada

Los digestores utilizados tienen un volumen de 125 ml. Estos digestores están agitados a una temperatura de 35º C y conectados a un frasco de Mariotte relleno de una disolución de NaOH. El biogás es recogido en el frasco de Mariotte. El CO_2 del biogás es absorbido en el medio básico y solo el metano alcanza el espacio libre del recipiente, desplazando un volumen equivalente de líquido. Se añaden diferentes concentraciones de sustancia tóxica desde el nivel 0 (blanco) hasta superar el umbral de toxicidad. Una vez que el experimento haya comenzado se sigue la producción de metano. Los ensayos de biodegradabilidad y toxicidad determinan el porcentaje de actividad metanogénica que pierde un lodo en presencia de un compuesto inhibitorio (en nuestro caso PCP, 2,4,6-TCP, 2,4-DCP, 4-MCP y fenol).

En la siguiente tabla se muestran los resultados obtenidos para un compuesto, el PCP:

Concent. (ppm) PCP	% Inhib.			Veloc. de elimin. de tóxico (mgtóxico/gSSVdía)									Veloc. de elimin. de sustrato (mgAcH/SSVdia)											
	Anaerobias con sustrato						Anaerobias sin sustrato						Anóxicas con sustrato						Anóxicas con sustrato					
	1ºAlim			2ªAlim			1ºAlim			2ªAlim			1ºAlim			2ªAlim			1ºAlim			2ªAlim		
2.5	20	0.5	1.1										16	0.5	0.9									
5	35	0.8	0.9	31	1.0	1.0							41	0.7	0.9	40	0.9	1.0						
7.5	75	1.0	0.3	83	1.2	0.5							53	0.9	0.8	46	1.0	0.9						
10.5	90	1.5	0.1	85	1.7	0.2		0.3					67	1.3	0.7	72	1.6	0.9		0.8			0.8	
12.5	93	1.7	0.1	90	2.2	0.1							79	1.4	0.7	79	1.2	0.6						
15				96	2.3	0.1										86	2.0	0.5						

La toxicidad se determina a partir de la reducción de la actividad del fango al ir aumentando la concentración de tóxico. Por otro lado, también se evalúa en que medida el compuesto tóxico es biodegradado por los microorganismos.

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Biodegradação de Compostos Aromáticos em Leitos Construídos de Macrófitas (*Phragmites australis*)

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***Palavras chave**: Efluentes industriais, Leitos Construídos de Macrófitas, Remoção de Compostos Aromáticos, Nitrobenzeno, Dinitrofenol, Trinitrofenol*

O recurso a tecnologias cada vez mais limpas na produção de compostos nitroaromáticos gera efluentes com menor indíce de contaminação tornando pouco efectiva a depuração por via físico-química mas, ainda restritiva para os processos biológicos atendendo à sua elevada toxicidade. A utilização de leitos construídos de macrófitas na depuração destes efluentes surge assim como uma opção, de baixo investimento e manutenção, na charneira entre o processo físico-químico e o biológico .

Tomando como base para este estudo um efluente líquido com diferentes teores de nitrobenzeno(MNB), dinitrofenol(DNF) e trinitrofenol(TNF), i.e., os principais contaminantes numa unidade de produção de MNB e utilizando um leito piloto de macrófitas (1,5x0,5x1 m) com uma área superficial de 0,75 m^2 plantado com *Phragmites australis,* suportadas num solo argilo arenoso, procedeu-se ao estudo da influência da concentração de MNB, DNF e TNF na eficiência de remoção dos mesmos, para as seguintes condições operatórias: fluxo subsuperficial misto, taxa de inundação de 70%, tempo de retenção hidráulico médio 5dias, carga hidraulica média 6,4 cm/dia .

Procedeu-se a uma alimentação contínua do leito com teores de MNB, DNP e TNF de 5 -10, 5 - 15 e 10 -70 ppm respectivamente, a um pH inicial de 9, durante 120 dias.
O conteúdo em nitrofenóis nos influente e efluente foi determinado por HPLC.

Obtiveram-se taxas de remoção máximas de 0,72, 0,41 e 1,4 g C/m^2.dia para o MNB, DNP e TNP respectivamente, oscilando o pH de saída entre 6 e 8 diminuindo com o aumento do teor total de aromáticos na alimentação . Não se detectaram durante o período de teste compostos aromáticos no efluente do leito.

Com o intuito de esclarecer preliminarmente eficiências da ordem dos 100% comparou-se a qualidade do influente com o teor de ácidos húmicos à saída do leito . Os resultados obtidos sugerem que entre os diversos mecanismos de remoção poderá estar envolvida a humificação de parte dos compostos fenólicos através de reacções de polimerização catalizadas por fenoloxidases.

Não obstante, os resultados apresentados serem referentes a um estudo à escala piloto é de salientar que o processo se encontra já implementado à escala industrial.

AGRADECIMENTO: Este estudo foi realizado em total cooperação e com o suporte económico da Anilina de Portugal .

Biodegradation of Hydrocarbon Slurries Retained in Oil Separators of Vehicle Washing Facilities

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Keywords: Biodegradation, Oil Separator, Hydrocarbons, Bioaugmentation, SBR

Wastewater discharges from vehicle washing facilities represent a major source of hydrocarbon (HC) contamination of water, soils and municipal wastewater treatment plants. For that reason, oil separators are mandatory at such facilities. Periodically, separators are cleaned and contaminated HC sludges are disposed off. The final disposal is somehow problematic because oily sludges are classified as hazardous wastes. An approach to solve this problem could be the use of bioaugmentation techniques and Sequencing Batch Reactors (SBR). Thus, the present study concerns a basic characterisation of HC contaminated sludges and a preliminary assessment of their biodegradation potential using batch processes. The assessment was performed using a non-adapted biomass inoculum and an enriched one, registered under the reference Bioactiv HGS.
Grab samples of HC slurries were collected in oil separators operating at two vehicle washing facilities located at the city of Braga. COD, BOD, solids and pH were analysed according to Standard Methods. Total hydrocarbons were analysed by infrared spectrophotometer according to ASTM, D3921-80. The samples had a pH of 5.5 (sample A) and 10.7 (sample B). COD values were very high, 29371 ±1929 mg/L and 66376 ± 838 mg/L. Average total solids were 406.6 g/L and 311.0 g/L, respectively. BOD_5 determination, using non-acclimatised biomass, reached values 35% and 9% lower than COD, indicating the recalcitrant nature of oily sludges. The respirometric assays reinforced such results: oxygen consumption of undiluted samples was very low, but respiration rate increased with dilution. Furthermore, an acclimatisation potential was observed, the second feeding provided a higher degradation rate when compared with the first one. Experiments performed without any nutrient (N,P) supplementation displayed very low respiration rates. Following the indications provided by respirometric tests, batch treatability experiments were performed with a sample dilution of 1:10, being air and nutrients added. The values of COD and solids decreased along experimental time. After 90 days of operation, total and volatile solids reached values 50% lower than initial ones, approximately 15 g/l and 2 g/l. Regarding HC determination, it should be mentioned that the method was rather difficult to perform. Indeed, the paper filtration step during extraction procedure was affected by the high solids content, meaning all HC measurements could be underestimated. Nevertheless, the assays performed with enriched biomass were the first ones where no HC was detected. Reactors inoculated with unacclimatized microbial populations also remove HC compounds but at longer retention times. In the blank assay (reactor not inoculated, only aerated) a significant decrease in HC content was also observed. Such result indicated that overall hydrocarbon reduction was also due to a volatilisation phenomenon enhanced by air stripping. Therefore, despite some indications on the profitability of enriched biomass to remove HC, the results can not be considered sufficient to state a clear advantage of such procedure. The present study indicates that a further optimisation of process parameters should be performed before the set-up of a full scale SBR for HC degradation.

Degradation of Phenolic Compounds by a *Rhodococcus* Strain

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***Keywords**: phenol, degradation, kinetics, bioreactor, Rhodococcus*

The effluents of crude oil refineries, petrochemical industry, pesticides, insecticides, explosives, resins and dye-manufacturing companies are sources of phenol pollution in water. The high toxicity of these compounds may be overcome not only by standard physicochemical processes but also through mineralization and/or biotransformation by certain bacterial strains. Several heterotrophic microorganisms capable of growth in phenol as the sole source of carbon and energy have been isolated from the Gernika area (Bizkaia, Basque Country). They may also be grown in the presence of a second substrate such as glucose or phenols mixtures. One of these strains, belonging to the *Rhodococcus* generum, displays a high phenol-degrading ability, among other hydrocarbons. Phenol degradation kinetics have been studied and the corresponding parameters and constants have been calculated. Growth and degradation conditions have been optimized in batch cultures as well as in discontinuous, semicontinuous and continuous reactors. This bacterium has also been both entrapped in chitosan and immobilized in membranes. Finally, efforts have been made to study the catabolic pathway through which these pollutants are degraded and to test phenol degradation in synthetic wastewaters.

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Biofiltration of Alkylbenzene Contaminated Waste Gases

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Keywords: *flue gas, VOC, contaminated air, gas phase bioreactor, biodegradation*

Biological processes have been developed and accepted over the past decades as alternative technologies for the treatment of waste gases. In this study, a gas phase biofilter was set-up for the removal of alkylbenzene contaminated air. Optimal conditions were determinated. Kinetics of contaminant removal was evaluated batchwise and compared to data generated during biofiltration studies.
Reactor configuration and methods used for waste gas analysis as well as other relevant methods were described elsewhere (1).
Several experiments were performed, feeding the biofilter with different substrates, consisting in (i) a mixture of identical concentrations of toluene, ethylbenzene and *o*-xylene, (ii) toluene, (iii) ethylbenzene, (iv) *o*-xylene. The first experiment was performed with the mixture. Inlet concentration was increased stepwise from 300 $mg.m^{-3}$ to 2000 $mg.m^{-3}$, the latter concentration being often considered as the upper limit for biofilters treating recalcitrant aromatic contaminants. More than 95% alkylbenzene removal was reached up to inlet concentrations of 1200 $mg.m^{-3}$. No diffusion limitation was observed. The reaction followed zero order biodegradation kinetics with a rate constant around 0.022 $g.m^{-3}.s^{-1}$. The maximum elimination capacity reached with this system was 70 $g.m^{-3}.h^{-1}$, which is highly satisfactory, compared to other results reported in the literature (2). Elimination capacities with single compounds were of a same order of magnitude.
Under steady-state conditions, representative biomass samples were removed from the biofilter in order to perform kinetic batch assays. The expected theoretical biofilter elimination capacity, evaluated according to batch results, gave a much higher elimination capacity than the experimental one. This might be explained by the different conditions characterizing either batch or continuous reactor studies or the presence of some inactive biofilm portions attached on the support material (3).

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Application of a diffusion-reaction model to biofouling in heat exchangers

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***Keywords**: Biofilm, kinetics, diffusion-reaction model, heat exchangers.*

Modelling the processes that take place inside biofilms under known operating conditions is crucial, since the results obtained may be used to predict biofilm behaviour in other circumstances. The processes related with substrate consumption that take place inside a biofilm can be adequately described by the diffusion-reaction model developed for the heterogeneous catalysis. In fact, the overall "observable" or apparent reaction rate in a biofilm system comprises the transport of the reactants outside and inside the biological matrix and the biochemical reaction involving the immobilised microorganisms. Assuming that the reaction inside the biofilm is well described by the Monod kinetics, the mass balance for the substrate is:

$$D_A \frac{d^2 C_A}{dz^2} - \frac{k_1 C_A}{k_{s1} + C_A} = 0 ,$$

with the following boundary conditions: z= L $C_A = C_{As}$ and for z= 0 $dC_A/dz = 0$,
where L is the biofilm thickness, C_A the substrate concentration in the biofilm, C_{As} the substrate concentration at the biofilm surface, and k_1 and k_{s1} are the Monod kinetic constants.
The diffusion coefficient inside the biofilm (D_A), can be regarded as an average "effective diffusivity", and replaced by a mass transfer coefficient that takes into account all the mechanisms of mass transport in the matrix.

The aim of this communication is to present a methodology to solve the diffusion-reaction model without using any simplifications to solve it analytically. Therefore, there will be no restrictions on the reaction order (it may vary from zero to one, according to Monod). Also, the Monod kinetic constants in the biofilms are not considered to be equal to the ones in suspension. Finally, instead of theoretical "effective diffusivities" experimental values of mass transfer coefficients are introduced in the model equations.
The methodology, using experimental data of substrate consumption, biofilm thickness, mass transfer coefficients inside the biofilm, bulk substrate concentrations, for, at least, more than two different situations, involves modelling for parameter estimation (including least square parameter estimation and numerical integration). The solution of the model will be the concentration profiles inside the biofilms and the Monod kinetic constants. The proposed model was applied to biofilms formed under turbulent flow and low organic substrate concentrations in heat exchanger surfaces.

Biofilm Kinetics in an Airlift Reactor

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Key words: Biofilm Airlift Reactor, kinetic parameters, diffusion-reaction model

A mathematical model based on the porous-catalyst model has been frequently used to describe the diffusion-reaction phenomena occurring in a biofilm. The main goal of the present work was to apply this model to biofilms developed in an airlift reactor operating at different substrate concentrations, in order to obtain the concentration profiles and the values of the Monod kinetic parameters (μ_{max} and K_S) within the microbial films. An additional purpose was to verify whether the kinetic behaviour of microorganisms in biofilms differed fundamentally from that in a homogeneous suspension.
The porous catalyst model had to be adjusted to incorporate the experimental values of the mass transfer coefficients measured in a special flow cell. It was assumed that the biofilm activity followed a Monod-type kinetics and appropriate numerical methods (1) were developed to solve the differential equations thus obtained. The input data to this model were: substrate concentrations, substrate uptake rates (r_f), biofilm thickness values (L_f) and mass transfer coefficients within biofilms.
In this work, thin biofilms of *Pseudomonas fluorescens* were grown in a concentric-tube airlift reactor with suspended basalt particles. Biofilm characteristics were determined in several assays with different glucose concentrations. During these experiments, r_f and L_f were also measured. The experimental data were then introduced in the model and the results obtained clearly demonstrate that all biofilms were completely penetrated by the substrate, as expected in thin and highly active microbial layers (Figure 1). This suggests that the **B**iofilm **A**irlift **S**uspension **R**eactor (BAS Reactor) is a promising technology for aerobic treatment, since it can maintain highly active biomass, achieved by growing thin biofilm on small carriers. The constant K_s (0.73×10^{-3} kg/m^3) obtained from the model was significantly lower than the one measured in a suspended culture (6.21 kg/m^3), while μ_{max} (0.24 h^{-1}) was similar to the suspended value (0.31 h^{-1}). Taking into account the K_s value and the concentration profiles, it may be concluded that the reaction inside the biofilm is of zero order. The model fitting was good, as confirmed by the similarity between the experimental and calculated values of the substrate uptake rates (Table 1).

Table 1. Substrate uptake rates and biofilm thickness values at different glucose concentrations

Glucose (mg/L)	L_f (μm)	$r_{f,experimental}$ (kg/m$^2_{Biofilm}$ s)	$r_{f,model}$ (kg/m$^2_{Biofilm}$s)
13.5	7	3.155×10^{-8}	1.513×10^{-8}
31.0	36	6.450×10^{-8}	8.206×10^{-8}
42.0	38	8.335×10^{-8}	8.754×10^{-8}
76.5	69	19.000×10^{-8}	16.05×10^{-8}
100	105	23.25×10^{-8}	24.54×10^{-8}

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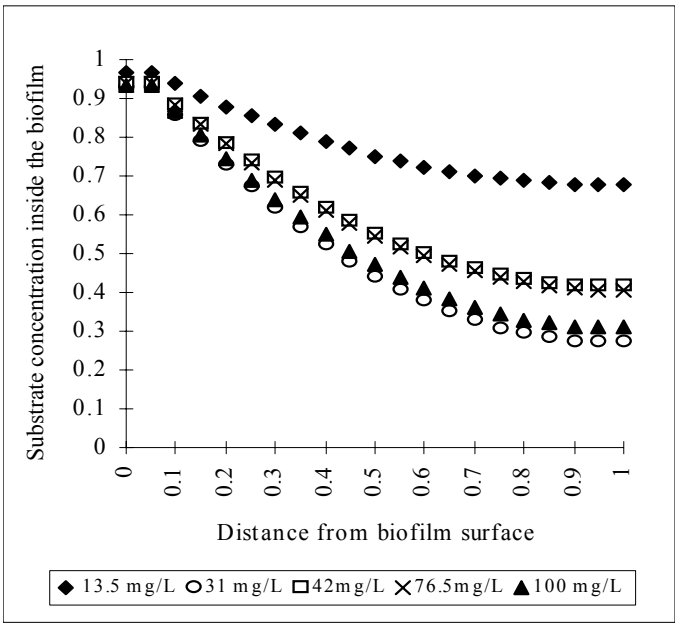

Figure 1 - Concentration Profiles

Treatment of Volatile Organic Compounds in a Bioreactor Using PVC Tubes as Carrier

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Key words: Gas treatment, biofilm, methanol, mist bioreactor

The biological treatment of waste gases is nowadays considered as an interesting alternative to the more classical physico-chemical processes. This is especially true for gases presenting a low concentration of pollutants. Usually most of the biological processes for waste gases treatment are included within two categories; biofilters and bioscrubbers [1]. In this work a new reactor concept intermediary between biofilter and bioscrubber is presented and experimented.

The reactor concept presented in this paper is based on the "Mist-Foam" concept previously described [2]. The "Mist-Foam" concept is characterised by the creation of a mist formed by contacting the waste gas and a liquid nutrient solution inside an atomising nozzle. After being created, the mist passes (down-flow) trough a synthetic carrier serving as support for the microorganisms. Due to the characteristics of the mist created (mainly small droplet size), the liquid nutrient supply settles on the carrier and form a homogeneous and thin liquid layer. Since this system allows to operate at very low water content, it is expected to enhance mass transfer by reducing the thickness of the liquid layer separating the gas phase and the biofilm growing on the carrier.

In the present work, the carrier selected was formed by 19 PVC tubes of 1 m height and 20 mm external diameter. This carrier was placed in a glass lab-scale reactor (diameter 0.10 m, height 1.7 m, working volume of 7.8 litres) and tested during a gas treatment experiment. Methanol was chosen as model pollutant in order to characterise the performance of the system developed. During the 70 days of the experiment, air was injected in the reactor with a specific gas flow rate of 110 $m^3/m^3.h$ containing from 1 to 4 g/m^3 of methanol. These concentrations correspond to a volumetric loading rate from 2.6 to 10.5 $kg/m^3.d$. A clear biofilm growth was observed since the beginning of the experiment but preferentially on the reactor's wall, over and under the PVC tubes and not into them. A clear degradation appeared progressively but never overpassed 50% of the load applied. The maximum degradation rate obtained during the experiment was 3.5 $kg/m^3.d$. At the end of the experiment the reactor was opened and the carrier was dismantled for analysis. A clear clogging of 18 tubes was observed making them useless. Additionally, batch methanol degradation assays were realised using different biofilm samples in order to evaluate their capacity to degrade methanol. The biofilm having been developed on the wall of the reactor presented a specific degradation rate of 1.08 $g/g_{VSS}.h$ without any lag phase. By comparison, the biofilm developed into the carrier's tubing presented a specific degradation rate of 0.6 $g/g_{VSS}.h$ with a lag phase of 30 hours. These results suggest that 0.02 m PVC tubing is not a suitable biofilter carrier.

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Real-Time Expert Control of a Pilot WWTP with Nitrogen and Phosphorus Removal

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***Keywords**: real-time, expert system, distributed control, nitrification/denitrification, wastewater*

In the present work, the design and implementation of a multilevel distributed control system is shown. This control system is applied to a pilot WWTP with biological removal of organic matter, nitrogen and phosphorus. The operation scheme is based on an A2/O system (Anaerobic, anoxic, oxic) and includes organic matter removal, nitrification/denitrification, and Enhanced Biological Phosphorous Removal (EBPR).

The pilot plant is formed by four independent reactors and a settler. It has a total volume of 150 L and treats about 500 L per day. The pilot plant is fully automated and configurable from the control system. The control and monitoring system is based on two different computers. One is in charge of three automatic analysers of phosphate, ammonium, and nitrate and nitrite [1], in addition to an automatic sampling system. The other computer controls the plant, monitoring three probe controllers and supervising a PLC programmed for the actuation over the mechanical elements of the plant.

The expert system (ES) developed in G2 [2], which runs in a Sun workstation, is on the top of the system architecture. The ES is fed with in-line data (pH, T, DO, ORP, aeration and flows) and on-line data (NO_3^-, NO_2^-, NH_4^+ and PO_4^{3-}) generated by the two computers. These data is sent to the ES using a TCP/IP connection through an Ethernet network. Qualitative data (odours, colours, microbiological observations data) and discrete data from off-line analyses (COD, TSS, VSS, TKN, and SVI) are sent to the ES using a form page in WWW.

The ES systematises the knowledge about the process being based on the existing scientific knowledge and the practice acquired in our particular system. All this knowledge is structured through a whole of rules and procedures for each subsystem of the pilot plant, co-ordinated with a supervisory agent. The knowledge base has been validated on the pilot plant, showing an excellent performance to manage the pilot WWTP. The system developed detects and controls all the wrong and special operations, as for example: pump failure, feeding problems, probes malfunctioning, analysers control and maintenance, etc.

The main achievement of this prototype is a versatile framework able to deal with different plant configurations, based on object-oriented paradigm and rule-based reasoning. The on-line feature is an important innovation of this system, particularly for data monitoring and supervisor control. In our system, different control strategies can be implemented for activated sludge control of carbon, nitrogen, and phosphorus removal with different plant configurations. In addition, the developed KBES can be adapted to a new plant in a short time because of object-oriented design.

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Motility Assessment of the Ciliated *Tetrahymena pyriformis* after Exposition to Toxic Compounds using Image Analysis

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Keywords: *Protozoa;* Tetrahymena pyriformis*; motility; image analysis.*

Ciliated protozoa play an essential role in the purification of wastewaters by removing, through predation, the major part of the dispersed bacteria, in the aeration tank. A complex food web exists and a change in the protozoan community will affect this whole ecosystem and, therefore, the biological performance of the plant. Being very sensitive to environmental changes, protozoa are an important tool as biological indicators of water quality[1].

Image analysis is commonly used nowadays in a wide range of applications within the biological sciences. It allows the enhancement of pictures as well as automatic identification and isolation of particles so that they can be properly studied. It also provides an extremely fast means of getting morphologic information, thus saving tremendous effort and time.

Few studies exist on the subject of motility measured by image analysis [2,3], since only recently processing and analising images by computer in reasonable time is possible.

The aim of the present work is to study the relation between toxicity and the motility of *Tetrahymena pyriformis* in aquatic environment. This is a parameter connected with its viability, because energy is needed for cilia movements and, therefore, to *T. pyriformis* motility.

Four toxics were used: copper, zinc, the antibiotic ciclohexamide (wich inhibites protein syntesis) and the neutral surfactant Triton X-100 (wich directly disrupts cell membrane).

Main problems lie on the frequent non-linear movements of *T. pyriformis* and on the impossibity of calculating the vertical component of its movement, since one single plan can be digitalized to the computer. These aspects had to be accounted for in the recollection of the images to be processed. Brownian movements of dead cells are important in the discussion of results.

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Estudio Fenomenológico de Reactores Granulares para el Tratamiento de Aguas Residuales

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Palabras claves: *Reactores granulares UASB y EGSB, estudio fenomenológico, gránulos, comportamiento hidrodinámico, velocidad superficial.*

La presente investigación tiene como objetivos el diseño y construcción de reactores granulares anaerobios UASB y EGSB a nivel laboratorio para estudios de comporta-miento hidrodiná mico y del proceso anaerobio asociado al sistema de flujo y suspensión de gránulos; proponer y analizar el modelo de fluidización, crecimiento y estabilización del lodo granular, en funció n de la velocidad superficial y tipo de aguas residuales a tra-tar. El objetivo final consiste en estudiar las distintas variables que influyen en la depura-ción de aguas residuales en reactores granulares en busca de entregar una metodologí a para la optimización en el diseño de dichos reactores.

En los estudios realizados para ambos reactores se establecieron las diferencias entre ambos, entregando, de esta manera, los lineamientos para el diseño óptimo de los mismos.

En el reactor UASB se estudió su puesta en marcha y operación. La puesta en marcha se realizó con una solución de agua sintética. Después de reemplazar esta solución por aguas de conservera y aumento gradual en la VCO, se llegó a condiciones de operación con valores de VCO de 8 Kg/m ^{3}·d. El tiempo de operación fue de 68 días, lográndose una remoción entre 60 y 70% bajo condiciones de operación y producción especí fica promedio de metano de 0,3 L CH_4/g $DQO_{eliminado}$ para valores de VCO = 4 Kg/m^3·d.

El reactor EGSB operó durante 82 días, de los cuales 50 consistieron en la puesta en marcha, hasta alcanzar una VCO de 12 Kg/m^3 ·d. A lo largo de la experiencia la remoción de carga orgánica estuvo entre 70 y 90 % y se obtuvo una producción específica de gas correspondiente al 85 % del valor teórico. Se observó una fuerte dependencia de la hidrodinámica en el buen desempeño del sistema. Estudios hidrodinámicos reflejaron que el sis tema se comporta como reactor de flujo pistón en su parte inferior y de mezclado perfecto en la parte superior, además se observó la existencia de *by-pass*, con una fracción de 45, 36 y 2 % para velocidades superficiales de 4.5, 6.4 y 7.8 m/h, respectivamente. El aumento en la velocidad superficial de 4.5 a 6.4 m/h produce un incremento de 40% en el diámetro granular; por otro lado, se produjo una disminución en el diámetro al au mentar la velocidad a 7.8 m/h. Mediante análisis, se determinó que el arrastre del material celular se produciría a una velocidad superficial de 12.5 m/h. El aumento en el tamaño de grá nulo se produce al aumentar la velocidad superficial y/o concentración de alimentación. Al tratar aguas de conservera diluidas, se observó una disminución tanto en tamaño granular como en producción de metano.

Se concluye que los sistemas UASB no son recomendables para tratamiento de aguas diluidas o bajo condiciones desfavorables de temperatura. El uso de relaciones altura/diámetro mayores en sistemas EGSB hace que é stos presenten mayor flexibilidad para tratamiento de aguas diluidas o concentradas en un amplio rango de temperatura.

Role of Manganese Peroxidases and Lignin Peroxidases of *Phanerochaete chrysosporium* in the degradation of the colourants present in a sugar refinery effluent

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***Keywords**:* Phanerochaete chrysosporium*, LIP, MNP, sugar colourants, degradation*

Anion-exchange resins are used in sugar refinery to decolourise sugar liquor. The pre-regeneration of these resins is made with 50 g/l NaCl, giving rise to an effluent containing different types of colourants, the most important being: (1) phenolic compounds, coming from the cane plant, (2) caramels, which are produced by thermal degradation and condensation reactions of sugars, (3) melanoidins, formed from sugar-amino acid reactions via the Maillard reaction and (4) hexoses's alkaline degradation products (HADPs).

Previous studies made in our laboratory demonstrated that *Phanerochaete chrysosporium* was able to degrade all these colourants.

Phanerochaete chrysosporium, when cultured under nitrogen-limited conditions, is known to produce two families of extracellular glycosylated heme proteins, designated lignin peroxidases (LIPs) and manganese peroxidases (MNPs), along with an H_2O_2-generating system as the major components of its lignin-degrading system.

In this paper, the involvement of these peroxidases in the degradation of the four types of colourants, present in the sugar refinery effluent, is discussed.

Utilization of Residual Biomass from Lactic Fermentation Industry for Cadmium Sorption

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Keywords: biosorption, heavy-metals, waste water, cadmium

Several heavy-metals, especially zinc and cadmium, are present in effluents from different industrial processes. The deleterious effect to the environment provided several studies in biosorption because it is a promising technology which involves low operational cost.

The present work has the objective of using a lactic fermentation residue in fixed bed column for cadmium removal from aqueous solution.

Several biomass treatments were tested, involving imobilization with PEI (polyethyleneimine) and glutaraldehyde and caustic treatment with NaOH. The experimental sorption isotherms were calculated according to Langmuir model, determining the maximum uptake value (qmax) for all conditions tested.

The best value was reached using residual biomass imobilized and caustic treated (qmax=139,0 mg/g of biomass) being selected as biosorbent for treatments in packed columns.

A glass column of 1.5 cm ID and 62 cm length was used containing the selected biomass. A solution of 10 mg/L $CdSO_4$. 7 H20 was fed to the column from the top and pumped at a flowrate of 10 ml/min in a continuous flow. The breakthrough point was reached in 62 h and the total volume treated was 37 L.

The column efficiency was superior to 98%, being considered satisfactory to industrial application.

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Oily Sludge Treatment in a Liquid-Solid (LSC) Bioreactor

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Keywords: LSC bioreactor, aeration, recycle, oily sludge, microbial consortia

Large amounts of oily sludge are generated as residues by the refineries, representing a real environmental problem. Those residues are formed during production, transportation and refining operations and are basically composed of oil, water and solids. Some of the main characteristics, as varied composition, impair their reutilization, and confer on them a high recalcitrance.

Thus, this work dealt with the study of the technical viability of treating oily sludge biologically in an agitated and aerated liquid-solid contact reactor (LSC type, 14L capacity). It was employed, in this study, an oily sludge generated by the Duque de Caxias Refinery (REDUC), at Rio de Janeiro, Brazil, which was submited to microbial action of native consortia, previously estimulated. The process was carried out in a batch wise, due to high operation times.

Microorganisms were able to grow in a medium containing 5% (v/v) of oily sludge as the only carbon and energy source. High microbial populations were established after 4 days of process (10^8-10^9cfu/mL). The aqueous effluent was recycled, increasing the process efficiency. Such efficiency was measured by the level of *n*-paraffins, pristane and phytane (GC), polycyclic aromatic hydrocarbons (UV-FIA) and oil and grease (IV absortion) uptake, after 42 days: 97.4, 92.6 and 87.3%, respectively.

A procedure for identification showed the predominance of bacteria, though yeast and filamentous fungi species were also isolated. The main genus identified were: *Pseudomonas, Flavobacterium, Xanthomonas, Ochrobactrum, Rhodotorula* and *Candida*. The importance of aeration became evident, since all bacteria species isolated are strictly aerobic microorganisms. Such conclusion allowed the adoption of strategies in the process optimization. Besides, a significant consumption of the oily sludge confirmed the potential of the biotreatment system.

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Biodegradation of Recalcitrant Compounds to Anaerobic Digestion by *Trametes versicolor* Pre-treatment

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Keywords: *Anaerobic treatment, Recalcitrant compounds,* Trametes versicolor*, Biodegradation,* Pinus radiata *Kraft mill.*

A large amount of organic matter, toxic and intensely coloured waste effluents are released into the environment annually by the pulp and paper industry. The primary contribution to the colour and toxicity of these streams is the pulp bleaching plant effluent which contains high molecular weight compounds, modified and chlorinated lignin and its degradation products. Conventional bacterial waste treatment as anaerobic treatment is an improving technology for cleaning-up forest industry wastewater. One advantage of this process is due to the high retention of active bacterial aggregates inside the high-rate reactors such as granular upflow anaerobic sludge bed (UASB) reactors. However, these processes are relatively inefficient at removing coloured pollutant.

On the other hand, the potential advantages of enzymatic treatment as compared with conventional treatment, include: application to biorefractory compounds, operation at high and low contaminant concentrations, reduction in sludge volume (no biomass generated) and the ease and simplicity of process controlling. Several enzymes are known to act on recalcitrant compounds contained in Kraft mill effluent (like lignin), especially oxidative enzymes such as laccase, lignin peroxidase, and manganese peroxidase.

The goal of this study was to evaluate the biodegradation by anaerobic treatment of the recalcitrant compounds pre-treatment with *Trametes versicolor*. Batch system cultures containing 200 mL medium and *Pinus radiata* Kraft mill wastewater were inoculated with homogenised mycelium of *T. versicolor*. Cultures were performed at 30 °C and 150 r.p.m. in an orbital shaker.

Anaerobic Sludge Blanket (200 mL) (UASB) was inoculated with 20 gVSS/L of anaerobic flocculent sludge (0.9 gCOD/gVSS·d). Flow rate, pH, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD_5), Total Solid (TS), colour, tannin and lignin, total phenol compounds (UV_{215}), phenol compound, Total Alkalinity (TA) and Alkalinity ratio were measured during the operation in both reactors. Methane gas production was also monitored. In order to compare the increase in biodegradability of recalcitrant compounds, an UASB reactor working with Kraft mill effluent was used at a first stage without pre-treatment and then with fungi pre-treatment.

Anaerobic results without fungi pre-treatment show a removal of COD and BOD_5 of around 50% and 70% respectively, 50% of tannin and lignin and no colour reduction. However, anaerobic treatment with *T. versicolor* pre-treatment improved colour, COD and tannin and lignin removal.

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Cinética de degradación de AGV

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Palabras claves: *Degradación anaerobia, AGV, cinética, simulación.*

La cinética proporciona una base racional para el análisis, control y diseño de los sistemas de tratamiento anaerobio. Existe una importante variación en los valores de las constantes cinéticas reportadas por distintos autores. Esto se debe a la variabilidad del modo de operación, medio utilizado y condiciones de trabajo [1].

El objetivo del trabajo es el estudio cinético de la degradación anaerobia de AGV y formación de HAc en tanque agitado con volumen útil de 2.5 L. El inóculo utilizado ha sido purines de cerdo adaptados sobre glucosa a 37ºC. Como fuentes de carbono se han utilizado ácidos puros (HAc, HPr y HBut) así como mezclas de los mismos en proporción 2:1:1 en DQO, además de nutrientes para mantener una relación C:N:P 100:7:1.

En la experimentación se ha variado la concentración total de AGV (1.2-3.5 g/L) manteniendo la temperatura a 37ºC, el pH entre 6.8-7.2 y la concentración de sólidos suspendidos volátiles entre 3.7-4.6 g/L. Las variables medidas son la concentración de AGV (GC-FID), la composición de los gases (GC-TCD) y el caudal, así como los SSV iniciales y finales.

Los parámetros cinéticos se han determinado a partir de los datos de los ensayos con ácidos puros, suponiendo una cinética de Monod mediante linealización de Lineweaver-Burk. Dichas constantes se han utilizado para simular en mezclas de AGV los datos experimentales de consumo de HAc, HPr, HBut, así como formación de HAc, mediante un programa de simulación desarrollado en lenguaje ISIM [2].

Los parámetros cinéticos obtenidos son : Ks (mM) 2.118; 0.209; 1.973, y Vm (mM/h) 0.641; 0.053; 0.452, para HAc, HPr y HBut respectivamente.

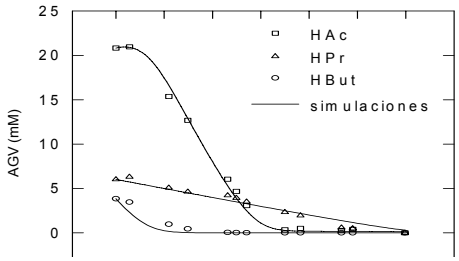

Fig. 1 Simulación mezcla AGV 2.0 g/L

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Solvent Resistant Bacteria for Effective Bioremediation. Case study: Biodegradation of Aromatic Hydrocarbon Mixtures in Heavily Contaminated Groundwaters and Soils by Immobilized *Pseudomonas putida* DOT-T1

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Keywords: Bioremediation, BTEX, Solvent-tolerance, Pseudomonas putida *DOT-T1*

Effective decontamination of polluted sites as well as the biodegradation of industrial wastes is often hindered by susceptibility of the intervenient microorganisms to the pollutants themselves. This is particularly true for many organic solvents (such as aromatic hydrocarbons) which tend to dissolve within the cell membrane, disrupting its integrity and leading ultimately to cell death.
Hence, microorganisms resistant to high concentrations of organic solvents offer an enormous potential in the bioremediation of heavily contaminated groundwaters and soils. Unfortunately, these microorganisms are rare, or at least, they have been seldom reported in literature. Recently, we have isolated a *Pseudomonas putida* (DOT-T1) able of tolerate and metabolize toluene and related hydrocarbons at concentrations up to 90% (v/v) [1]. The molecular and physiological mechanisms responsible for solvent tolerance have been intensively studied and are now partially elucidated [2,3].
Aims - Having obtained, characterized and expanded the metabolic capabilities of this solvent-resistant bacterium, the next logical step is to actually use it in bioremediation. Hence, main goals of the research presented here are:
- to develop, characterize and evaluate a compact, effective system for *in-situ* biodegradation of heavily contaminated groundwater using both free and immobilized *Pseudomonas putida* (DOT-T1);
- to assess the feasibility of using *Pseudomonas putida* (DOT-T1) for soil clean-up;
Methods - *Pseudomonas putida* (DOT-T1) was cultivated in suspension, in soil reactors and in gel beads. Degradation of mixtures of alkylsubstituted benzenes at high concentrations was evaluated both under steady and dynamic conditions. The intrinsic kinetic parameters were measured for all the substrates involved, both individually and in mixtures. The effect of the metabolism of each hydrocarbon on the metabolism of the others was determined to assess the feasibility of these cells to biodegrade complex streams. Biodegradative capacities as well as survival and colonizing capacity of *P. putida* introduced both in soil microcosms and soil reactors were measured.

Main conclusions - Due to its great tolerance to organic solvents, *Pseudomonas putida* (DOT-T1) was able to degrade both single aromatics and aromatic mixtures (BTEX) at rates up to 10-12 times higher than those so far reported in literature for comparable systems. *P. putida* (DOT-T1) was also able to colonize and attain high cell numbers in soils that had been subjected to discharges of toluene of 10% (v/v) [4]. Besides, it was rather effective in eliminating toluene from these soils. These results as a whole clearly demonstrate the potential of using solvent-tolerant cells for bioremediation purposes.

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Application of Highly Aerobic Reactors in the Wineries Effluents Treatment. Development of microbial inocula.

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Keywords: *winery, wastewaters, biodegradation*

The wine making, like most industries, produces polluting liquid effluents that show tremendous negative impact on the environment. Concerning effluents from wine cellars, several alternative solutions have been tried, for example, classical treatment techniques, land dispersion, evaporation or collective mixed treatment. We have built a new type of aerobic bioreactor to minimize energy consumption on aeration.

The importance of development of apropriate microbial inocula was evaluated by adaptation of several biomass obtained from different origins.

The winery effluents were obtained from the EVN (Dois Portos, Portugal) white wine vinification (ca. COD 6,300 mg/L, pH 4.5) and from Lungarotti and Coop Vitivinicola of Orvieto, CVO (Italy) wineries (COD 3,000 - 4,000 mg/L).

Different microbial inocula were developed and were obtained from different origins: the activated sludge of a winery cellar treatment plant, the activated sludge of a paper industry treatment plant, two activated sludges of Lungarotti winery and two activated sludges of the CVO winery. Other inocula were obtained from comercial freeze-dried biomass and a liquid starter.

Viable counts of different microbial groups showed that bacteria and moulds were the main microorganisms present in the active sludges. Yeasts were also found but in small number. The predominant species belonged to the genus Pseudomonas; however, Bacillus, Aeromonas and Flavobacterium were also present. After about one month of continuous operation, strong increase of dispersed bacteria forms and disappearance of protozoa and bacterial floculant forms were observed.

Our results show that pH increases with incubation time as an indication of acid degradation. The increase of aeration rate resulted in a more rapid COD removal. The effect of dilution rate (waste feeding rate) was studied and we verified that COD removal rate increased to a maximum (retention time less than 2 days) with the feeding rate. After an adaptation, biomass obtained from Pegões and from freeze-dried inoculum showed COD conversion levels higher than 90%. Reactors have been working in stable conditions for more than three months.

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Biological Treatment of Saline Wastewater from Cultures of *Dunaliella salina*, by Halophilic Bacteria

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Keywords: halophilic bacteria, saline wastewater, Dunaliella salina

The halophilic bacteria are generally found in natural environments containing NaCl concentrations of over 20%, and they confer a reddish hue to the salt pans and to the salt lakes such as the Dead Sea. These bacteria belong to the family of the Halobacteriaceæ, chemoheterotrophic organisms which grow in presence of simple carbon sources such as amino acids, sugars, glycerol and related compounds [1, 2].
This feature confers one potential application for them in the handling of wastewaters with high NaCl concentrations. The case in study is one wastewater resulting from mass production of *Dunaliella salina* used as a βcarotene yielding crop. This medium has a NaCl concentration of between 16 and 24% and an organic load of 1,78g/l AFDW, glycerol being the main organic component present in a concentration of *ca.* 660 mg/l.
Batch biological treatment of this wastewater was tried out at laboratory level using bacteria isolated from the wastewater itself. The growth of these bacteria in wastewater was favoured by the addition of the following salts: NH_4Cl, K_2HPO_4, KCl, $MgSO_4.7H_20$, $CaCl_2.2H_20$. In a first round of experiments, each salt was added separately and in a second one they were added in combination, using response surface methodology (RSM)[3] to find optimum conditions of biomass production and glycerol removal from wastewater.
In the first phase, highest glycerol removal observed was 70-84% in 8 days, as opposed to 54% in the control, and this was obtained with the addition of 125 and 500 ppm of NH_4Cl and with the addition of 24 ppm of K_2HPO_4.
Optimum salt combinations for glycerol removal from wastewater, reaching a maximum value of 99% in 18 days were the following: [NaCl]=195g/l, $[Mg^{2+}]$=1,47 mM, $[K^+]$=2,72 mM with a constant $[NH_4^+]$=2,36 mM; and a removal of 98% in only 8 days was obtained for the following conditions: [NaCl]=209g/l, $[PO_4^{3-}]$=0,334 mM, $[NH_4^+]$=5,26 mM. The optimum conditions for glycerol removal are not those yielding maximal biomass production. For this purpose the conditions are [NaCl]=199g/l, $[PO_4^{3-}]$=0,327 mM, $[NH_4^+]$=7,15 mM, measured as yielding 0,362 units of optical density at 520 nm. Meaningful dry weight determinations are a difficult problem at lab scale and the authors are still trying to deal with it.
These data indicate that the biological treatment of saline wastewater in continuous production will have to be made more efficient by addition of these salts.

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Anaerobic biodegradability and toxicity of formaldehyde

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Keywords: *Formaldehyde, Anaerobic degradation, Toxicity, Biodegradability, Methanol.*

The objective of this work was the study of the anaerobic biodegradation of formaldehyde (FA) in batch assays, even in the presence and absence of a co-substrate composed by volatile fatty acids (VFA). Two different VFA mixtures were used (containing acetate, propionate and butyrate), in order to determine its influence on FA degradation; as well as to determine the toxic effect exerted by FA on VFA removal. A kinetic model based Monod's equation was used to describe FA degradation using the initial rates method. Abiotic assays carried out in the absence of biomass as well as with previously autoclaved biomass samples in order to determine the extent at which other processes such as volatilisation, adsorption or chemical reactions could occur.

Biomass used in this work was not previously aclimated to FA degradation, being its maximum methanogenic activity around 0.6 g COD/g VSS·d. Besides, a significant sulfate reducing activity was evidenced in the assays carried out with sulphate, being around 12% of the organic substrate used by sulphate reducing bacteria.

During the experiments, both in the absence and in the presence of a co-substrate, methanol was detected as the key intermediate. FA degradation was strongly enhanced in the presence of this co-substrate, being this effect mainly related to acetate since propionate and butyrate were poorly degraded. Besides, FA exerts a clear toxic effect on VFA removal, being acetate completely degraded only when FA concentrations became lower than 100 mg/l. At higher FA concentrations methanol was also accumulated in the medium. Abiotic assays confirmed that FA was mainly biologically degraded, the maximum fraction removed by other processes amounting up to 11%.

The results obtained after modelisation according to the initial rates method indicate the suitability of a model based on Monod's equation, being obtained initial maximum rates of 50.8 mg FA/g SSV·d y 91.9 mg FA/g SSV·d, in the absence and presence of a co-substrate, respectively.

According with these results and the previously reported by other authors [1,2] a degradation pathway for anaerobic degradation of formaldehyde is proposed, in which hydrogen accumulation in the medium, its reaction with FA to produce methanol and the final methanol degradation to methane or through the formation of acetate are the main steps considered.

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Treatment of Domestic Sewage from Small Communities in an Anaerobic Filter

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The special distribution of the population in Galicia, dominated by many small rural communities, between 500 and 5000 people, together with the high pluviometric characteristics of the region and the future wastewater European Directives (EC urban wastewater treatment directive from 1994), makes the research on wastewater treatments on this field an interesting subject for further study.
The treatment of domestic wastewater from rural places of Galicia, characterised by a low organic load, involve the use of systems that could work with low Hydraulic Retention Time (HRT) together with a low maintenance cost and an easy management. The anaerobic digestion is an alternative technology to the traditional treatments, as it can be found in several studies (Ruiz *et al*, 1998), for this type of effluents.
The experimental set-up consisted of an anaerobic upflow filter (AF) with a 1.5 L volume operated with recirculation. Regular porous cubes of poliurethane foam were used as support material for the biofilm (30% v/v). The poliurethane foam was seeded with a flocculent sludge from an anaerobic digestor treating tuna wastewater. The reactor temperature operation was controlled at 20 ºC.
Previous batch assays were done to determine the methanogenic potential and the organic matter reduction of the rural domestic wastewater with anaerobic bacteria immobilised on this type of support. Successive batch experiments were done with the same biomass to determine the toxicity degree of the wastewater. Methanization percentage was higher in the first batch, being near 50% in the next ones. The organic matter reduction was of 85%.
The reactor started up was with a synthetic effluent containing 740 mg COD/L, and it was operated until a 6 hours HRT was attained. The reactor methanogenic activity was of 0,5 g COD/VSS·d. After that, the reactor was fed continuously with domestic sewage, containing an organic load between 800 and 170 mg COD/L. It started with an HRT of 36 hours, which was continuously decreased to an HRT of 7 hours. For the different organic loading rates and HRT used the COD reduction percentages was near 80% and a higher reduction was obtained for suspended solids.
These results show the feasibility of the Anaerobic Filter for the treatment of a low-strength wastewater (domestic sewage) and its use in rural communities could be recommended.

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Treatment of the Waste Water of a Laboratory for Milk Analysis in an Anaerobic Filter

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Keywords: *Anaerobic digestion, anaerobic filter, milk*

Effluents from a laboratory for milk analysis, located in the north of Spain, are characterised by their relatively high concentration of organic matter (10 kg-DQO/m^3) and low flowing rate (10 m^3/d), the composition of such wastewater being similar to the produced in some dairy factories. In order to fulfil the legal requirements, it is studied the treatment of these waste waters by using an anaerobic filter (for lowering the COD content) and a Sequencing Batch Reactor (removal of N and P). In the present communication it will be presented results of the anaerobic treatment of the waste waters in a Laboratory scale anaerobic filter.

An anaerobic filter of 2L volume was operated. Temperature was maintained at 37 ªC in a thermostatic chamber. It was employed a support of corrugated plastic, with an internal diameter of 18.6 mm, porosity of 93% and apparent density of 65 kg/m^3. The system was inoculated with a sludge from an anaerobic UASB plant treating the waste water from a fish-canning factory. Influent was obtained by diluting unskimmed milk in water, this being similar to the origin of waste water in the laboratory (dilution of the analysed milk in water). COD concentration was increased from 2 (first operating days) to 10 kg-COD/m^3 and the organic loading rate from 2 to 10 kg-COD/m^3·d. Hydraulic Retention Time was between 0.3 and 6 days.

For determining if the unskimmed milk or an intermediate could be toxic or inhibitory for the innoculum, it was determined the activity of the sludge and the toxicity of milk to the anaerobic sludge by using the method of Soto [1]. No toxic effect of the milk was found, being this result similar to the referred by other authors [2]. Biomass activity of the inoculum was 0.5 kg-COD/kg-VSS·d.

Removal of organic matter was similar to the obtained by Strydom [3]. Up to 95% of removal of the soluble COD and 85% of the total COD, was obtained. This is due to the presence of the relatively high percentage of solids in the effluent. The presence of volatile fatty acids was low, around 150 mg/L of acetic acid, the concentration of propionic acid being between 25 and 100 mg/L . Methane yield was 350 L/g-COD.

Nitrogen concentration in the effluent was around 180 mg/L, a large fraction of it being ammonia. For obtaining the removal of this nitrogen, around 800-1000 mg-COD/L will be required in the water fed to the SBR. The COD concentration of the effluent from the anaerobic filter was higher than 1500 mg/L, this indicating that denitrification in the SBR might be not limited by the COD content in the water.

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Characterisation and Laboratory Studies of Pre-Treatement of Winery Wastewaters

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The characterisation of the wastewaters produced by a modern Portuguese wine co-operative was carried out using an automatic sampling system during the vintage and non-vintage periods. In each period average daily and hourly samples were collected and analysed. Continuous flow rate, sewage temperature, air temperature and pH measurements were recorded by a data-logger system.

In the winery studied the major part of the pressed solids and husks are sold to distilleries which significantly reduces the organic content of the wastewater and provides an income.Even so the wastewater concentration is still higher than the limits defined by the municipality for domestic sewage discharge. Laboratory experiments were performed using an anaerobic filter reactor and an UASB reactor to evaluate the viability and applicability of such pre-treatment to this kind of wastewater, and to compare the different reactors.

7 months of experiments revealed good COD reduction (50-90%) operating at reduced hydraulic retention times (1-3days). Thus this approach is a simple and favourable solution for the pre-treatment of this kind of wastewater.

Diseño Avanzado de Vertederos Sanitarios con Reciclo de Lixiviados Tratados Anaerobicamente

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Key Words: *vertedero, lixiviado, digestión anaerobia, filtro anaerobio, asentamiento*

Existen experiencias que indican que es posible disminuir el tiempo de estabilización de la materia orgánica en vertederos sanitarios en un orden de magnitud, operando con recirculación de sus lixiviados tratados anaeróbicamente, con lo cual se logra una mayor productividad de biogas y un uso más eficiente del terreno ocupado por el vertedero.

De esta manera, se construyó un vertedero piloto con capacidad para aproximadamente 1.500 toneladas de basura compactada el que se ha operado con recirculación de lixiviado tratado en un filtro anaerobio de 6 m^3 relleno con Flocor R.

La construcción del vertedero se realizó considerando las técnicas utilizadas en el diseño de vertederos controlados. En él se pretende simular dos celdas de un vertedero con el fin de analizar su comportamiento físico (asentamiento, composición y aspectos geotécnicos) y químico (producción de lixiviado y biogas).

Para evaluar el comportamiento del sistema se ha medido la variación de la concentración de DQO del lixiviado generado en el vertedero y tratado biológicamente, la producción de biogas y el grado de asentamiento de los residuos sólidos urbanos (RSU).

A la fecha se ha conseguido un asentamiento del 3% en el vertedero piloto, para una operación sin recirculación, el cual se ha incrementado sustancialmente al comenzar la operación con recirculación de lixiviado tratado. La producción de biogas del vertedero ha alcanzado los 12.000 m^3, en 6 meses de operación, llegando a un 40% de metano.

Los resultados obtenidos con el filtro anaerobio arrojan degradaciones del 85% de DQO, con producciones importantes de biogas.

Physico-Chemical Properties of Porous Microcarriers in Relation with the Adhesion of an Anaerobic Consortium

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***Keywords**: microcarriers, surface properties, methanogenic activity.*

In anaerobic digestion the use of reactors with high cellular density has revealed great advantages, enabling the operation with low hydraulic retention times and high cellular retention times. The attached microorganisms are less affected by unfavourable environmental conditions. The early stages of bacterial adhesion can be described by van der Waals forces of attraction and electrostatic forces of repulsion as formulated in DLVO-theory and by a thermodynamic approach [1]. The energy of the electrostatic interactions is determined by the zeta potencial and the van der Waals forces can be understood in terms of hydrophobic attraction. The thermodynamic approach postulates that adhesion is favoured when the interfacial free energy of interaction between bacteria and the adhesion surface is negative. Apart from the above mentioned surface properties, other characteristics are also important in adhesion, such as surface roughness, porosity, and chemical composition. In the present work the biomass colonisation capacity of four porous mineral microcarriers was assessed and related with their physico-chemical properties. The surface tension of bacteria was determined by contact angle measurements considering van Oss approach [2] and the surface tension of mineral supports was obtained by the Thin-Layer Wicking Technique [3]. Table 1 represents the amount of adhered biomass expressed per liter of fixed bed volume.

As far as electrostatic interactions are concerned both pozzolana and clay behaved similarly showing an oscillatory pattern of zeta potential with pH: negative for pH between 6.9 and 7.5 and positive outside this pH range. The interaction energy between the support, the biomass and the medium was in the same range for all the supports. Visual inspection by scanning electron microscopy (SEM) and EDS analysis of the cleaned surfaces showed that sepiolite have the roughest surface and a higher level of magnesium while foam glass was the less rough material. However, the specific methanogenic activity of the adhered biomass showed an inhibition of 50% on sepiolite relative to the non adhered biomass and the less inhibited adhered biomass was observed in pozzolana with only 2.3% of reduction relative to the non adhered biomass. This reduction in activity was more related to diffusion limitations than to the release of toxic components from the supports to the medium, because chemical composition did not reveal the presence of toxic elements, although clay showed a higher level of aluminium.

Table 1 - Attached biomass ± 95% confidence interval

Support material	Attached volatile solids ($gVS/L_{fixed\ bed}$)
Clay	7.2±0.2
Foam Glass,	9.4±0.7
Pozzolana	6.7±0.2
Sepiolite	15.0±0.9

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Methods to Extract the Extracellular Matrix from Anaerobic Granules

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Keywords: anaerobic granules, extraction, exopolymeric matrix

The successful operation of a UASB reactor is dependent on the formation of highly flocculated compact sludge aggregates, called granules. The structure and composition of the anaerobic granules are determinant for its organic carbon removal ability as well as for its mechanical characteristics. The granules are composed by bacteria, embedded in a matrix composed by exopolymeric substances (EPS) (poysaccharides, proteins, etc.)[2]. The EPS content is about 0.6% to 20% of the volatile suspended solids (VSS) of granules, however this value is dependent on the extraction method used. Several methods have been reported for the extraction of exopolymers from bioaggregates, including high-speed centrifugation, steaming, ultra-sonication, treatment with NaOH or EDTA, treatment with Tris/HCL buffer treatment with phosphate buffer and heat, with a cation exchange resin[3] and treatment with formaldehyde or glutaraldehyde. Many of these methods have a low efficiency in terms of selectivity of exopolymer extraction and promote cellular lysis or intracellular material loss[4].

In this work some of the above mentioned extraction methods were assayed in order to determine their ability to extract the polymeric matrix from anaerobic granules. The extraction efficiency was determined by the yield in exopolymers and by the effect on cell lysis or intracellular material loss. The extent of cellular lysis could be evaluated by the amount of DNA extracted. Cell permeability could be inferred from a high protein content, due to the release of periplasmic proteins.

From the results it is apparent that the composition of the polymeric matrix of granules are dependent on the extraction method selected (Figure 1): Sonication extracted the greatest amount of protein and the smallest amount of polysaccharide, suggesting that some of the protein obtained was probably intracellular. The greatest amount of polysaccharide was obtained using Dowex resin and glutaraldehyde. Dowex resin is a cation exchanger resin that removes the divalent ions responsible for sludge flocculation[4], however this method requires high speed of agitation that can promote some cellular lysis affecting especially the outer cells of granules. The smallest ratio protein/polysaccharide was obtained with glutaraldehyde. Glutaraldehyde has been used as a fixation agent to avoid the release of intracellular enzymes[5]. So, it is expected to have a minimum effect on cell lysis and cell's permeability.

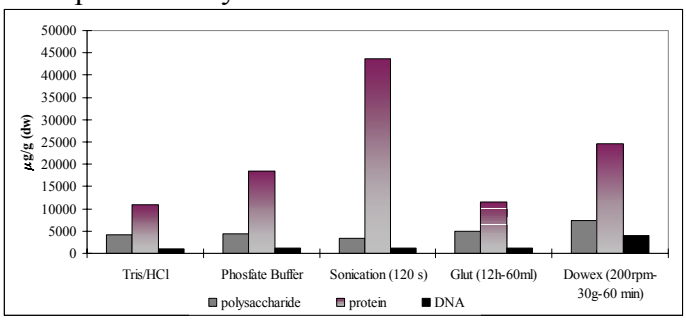

Figure 1: results of the biofilm extraction assays

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Combined Anaerobic Treatment of Olive Mill and Piggery Effluents

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Keywords: Olive mill wastewater, piggery effluent, anaerobic co-treatment, anaerobic filter

The strong pollutant nature of olive mill wastewater (OMW) is an important factor for water contamination in all countries where production of olive oil is a significant economic activity. The environmental impact of OMW is due to its high organic content of 50 to 157 Kg COD m^{-3} and to a consortium of aromatic compounds associated with complex lipids which inhibit biological degradation processes. Among the OMW purification solutions which have been proposed, anaerobic treatment is a method which allows construction and maintenance expenses to be offset by utilisation of the biogas produced. Nevertheless, the anaerobic process has been difficult to apply due to the presence of inhibitory compounds and to the unfavourable C/N ratio and pH. In order to get stable biotreatment, high dilution of OMW and addition of alkalis are needed. In addition, physico-chemical and biological pre-treatments have been suggested as a means to reduce OMW concentration and toxicity.

The purpose of this study was to find an integrated and economical process for the Alentejo region where both pig rearing and olive processing are significant activities and notorious sources of pollution. Anaerobic digestion of OMW was performed to evaluate the operational conditions required to develop a feasible method for the untreated OMW, avoiding water dilution and chemical correction. Piggery effluent (PE) contains significant organic carbon and nitrogen concentrations and was chosen to be mixed with OMW to satisfy these requirements economically.

The effect of influent concentration and composition on the performance of an up-flow anaerobic filter treating a mixture of these wastes was investigated by gradually increasing the proportion of OMW in the feed from 8% to 83% by volume. Organic loads ranged from 3 to 10 kg COD m^{-3} d^{-1}, with substrate organic load ratio (OMW COD/PE COD) varying from approximately 6 to around 30. Hydraulic retention times of 6-7 days were employed throughout. Increases in organic load and feed OMW content resulted in reduced biogas methane contents but higher overall methane production rates. Total COD removal efficiencies of 70-77% were achieved consistently, while the methane yield of 0.341-0.349 m^{-3} kg^{-1} total COD removed indicated stable operation even at the highest feed OMW content.

The anaerobic filter used proved to be stable throughout the study, and recovered rapidly following temperature shock, interrupted feeding and air ingress. This suggests that a full-scale system would be economical with respect to control and maintenance requirements. The digester was also effective in removing nitrogen from the mixed feed, suggesting that co-treatment could be used in reducing discharges of nitrogen originating from PE. The results of this study indicate that, in regions where both OMW and PE are readily available, combined anaerobic treatment may be an economically attractive means of OMW disposal.

Assessment of the Anaerobic Biotreatability of Some Textile Effluent Components

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Key words: textile effluent, anaerobic treatment

The textile industry produces high quantities of effluent which are difficult to treat in conventional activated sludge systems. The major contributers to the organic load of the effluents of a cotton manufacturing process are the sizing agents (commonly modified starch compounds), surfactants and dyestuffs. Bulking sludge and foam formation occur frequently in activated sludge systems if sizing agents and surfactants are found in the effluents[1], while dyes are very resistant to degradation under aerobic conditions. Decolourisation of dyes with azo-based chromophores can be achieved under the reducing conditions prevailing in anaerobic bioreactors[2]. An additional problem related to textile effluents are toxic shocks. In the present work the feasibility of an anaerobic pre-treatment step for textile effluents was preliminary assessed as it would allow colour removal, the reduction of the organic load to the aerobic step and the protection of the main activated sludge system from toxic shocks.

In a first series of batch tests the biodegradability of representatives of the major textile effluent component types was determined by incubation of the test compounds under anaerobic conditions with anaerobic sludge obtained from a lab-scale digester. Performance was monitored by following gas production, VFA accumulation and COD removal and compared to a glucose-fed control. It was found that only the starch based sizing agent was readily biodegraded indicating that the presence of this easily biodegradable carbon source can be essential to maintain reactor operation. Two commercial reactive azo dyes and a commercial non-ionic surfactant mixture could not maintain reactor operation. However, high colour removal levels (>95%) were obtained for dye concentrations up to 1500 mg COD/L after 7 days of incubation. In a second series of tests the inhibitory effects of the test compounds towards the VFA-consuming bacteria in the anaerobic inoculum was assessed by introducing a VFA spike into the test vials. Performance was compared to a control receiving no test compound. Inhibition with two azo dyes started from 500 mg COD/L on, while complete inhibition ocurred at 1500 mg COD/L. Inhibition for the commercial non-ionic surfactant mixture ocurred at all tested concentrations (lowest tested concentration 200 mg COD/L). The application of an anaerobic pre-treatment step could thus be impaired due to inhibitory effects. However it was found that the application of a coagulation step using ferrous sulphate and lime from which only the sludge was fed to the anaerobic inoculum apparently reduced inhibitory effects. This scheme could introduce the possibility of segregation of the raw textile effluent components in addition to a controlled release of the inhibitory compounds.

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Dye Elimination using a Pulsant Fluidized Reactor with *Trametes versicolor* Pellets

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Keywords : *white-rot fungi, waste water, dye, pulsant reactor, peroxidases*

Textile wastewater is characterised by its high production and its great variability of composition. Biologic treatment of textile effluents is difficult due the presence of dyes, non-biodegradable and toxic.

Some authors have studied the degradation of toxic compounds dyes like using inespecific ligninolytic enzymes (LiP, MnP and laccase) produced by white-rot fungi [1,2,3,4].

The objective of this work is the evaluation of *Trametes versicolor* capacity in dye elimination using a pulsant fluidised bed, where the air is introduced by pulses. This kind of aeration avoids fungus adhesion and the operational problems derived from this, and improves the material transfer [5].

We achieved to operate the reactor in discontinuous during two months approximately. The enzymatic activity was maintained. In order to find the relation between the dye elimination, the enzymatic production, the enzyme stability and micelial adsorption some different dye addition strategies were used. It was obtained a 0.37g of dye elimination per g of biomass with a maximum laccase activity of 1400 AU.

In order to evaluate the process during the treatment the followed parameters were : pH, au, glucose, nitrogen, laccase enzymatic activity, colour and toxicity. Microscopic fungus observation during the process gave an idea about morphologic fungus changes and the dye situation inside the fungus.

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Estudio de la Toxicidad y Biodegradabilidad del NTA en el Tratamiento de Aguas Residuales

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Keywords:*NTA, Toxicidad, Biodegradabilidad, Tratamiento biológico.*

El NTA por sus características de tensoactivo se ha utilizado en la industria de los detergentes como sustitutivo de los fosfatos o en combinación con estos para disminuir su concentración en agua residual. Por ello el NTA aparece como un componente mas del agua residual urbana e industrial. Una característica a destacar del NTA es su capacidad para actuar como fuerte complejante de metales formando complejos muy estables [1], aumentando la concentración de estos en el agua residual.

En el presente estudio se analiza la toxicidad y biodegradabilidad del NTA en tratamientos anaerobios y aerobios, así como la adaptación del fango a este compuesto.

En procesos anaerobios, los test de toxicidad metanogénica[2] se llegó a la conclusión que el NTA era tóxico a concentraciones muy altas, a partir de 1500 ppm NTA; pero la presencia de este compuesto hacía disminuir la producción de metano total en todos los ensayos realizados, manteniéndose una DQO recalcitrante importante al final de los test. Por otro lado la biodegradabilidad del NTA aumentaba con la adaptación del fango al compuesto. En un reactor anaerobio de lecho fluidizado (V=3 L, THR = 3 d), se llegó a una eliminación del 50 % para una concentración inicial de 100 ppm NTA. La eliminación de la DQO en el reactor no disminuyó a lo largo del periodo de operación del 85 %. A THR menores (0.75 d), la eliminación del NTA no superó el 40 %, mientras que la eliminación de la DQO no varía.

En procesos aerobios la toxicidad se analizó por técnicas respirométricas[3] obteniéndose que el NTA no afectaba a la degradación del sustrato fácilmente biodegradable y era necesario la presencia de éste para facilitar la degradación del NTA. Mediante ensayos de DBO_5, se corrobora el hecho de que el NTA no es biodegradable; pero no inhibe la biodegradación de otros sustrato. En dos reactores SBR (ciclo de 4 h) analizó la adaptación del fango aerobio al NTA obteniéndose una eliminación del NTA de hasta el 95 %.

Puede concluirse que el NTA no afecta a los tratamientos de aguas residuales y se elimina con mayor facilidad en procesos aerobios que anaerobios.

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Influência da Velocidade Ascensional Num Reactor Anaeróbio Alimentado com Águas Residuais Urbanas

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A aplicaçao da digestao anaeróbia a efluentes de baixa carga, como os esgotos domésticos, está a ser estudada nos últimos anos, polo interese que supoe dispôr de uma tecnologia eficaz e de baixo custo.

No presente trabalho estuda-se a influência da velocidade ascensional, na eficácia de um reactor UASB ao que se alimentam águas residuais urbanas da cidade de A Corunha. O reactor UASB, construido em metacrilato, tem um volume útil de 1l, e está provisto de tomas de mostra laterais. Inicialmente inocula-se o reactor com 200 ml de lodo granular, com uma concentraçao de sólidos en supensao voláteis (SSV) de 18,4 g/l e uma actividade metanogénica de 0,26 g DQO_{CH_4}/g SSV.d.

A posta em marcha realiza-se alimentando o reactor com uma mescla de sacarosa, AGV (ácido acético, propiónico e butírico) e nutrientes durante 52 días, com o fim de comprobar o funcionamento da equipa. A demanda química de oxigênio (DQO) desta alimentaçao é de 750 mg/l. A temperatura de operaçao mantem-se no intervalo de 20-25°C. Fíja-se a velocidade ascensional nesta posta em marcha em 1,2 m/h regulando o caudal de recirculaçao. A eliminaçao de DQO acadada é do 92%. Porém, produce-se um lavado de lodos finos que supoe uma concentraçao média no efluente de 80 mg SSV/l. Ao final deste período de posta em marcha a actividade média dos lodos mantem-se no valor inicial.

A continuaçao alimentou-se o reactor com águas urbanas. As características das águas expresadas em valores meios son: 587 mg DQO_t/l; 248 mg DQO_S/l 192 mg SS/l. Durante um tempo de operaçao de 285 dias provam-se velocidades ascensionais e tempos de retençao hidráulica (TRH) diversos. Aliás, foi necessário acrescentar ao esquema un pequeno decantador, e recirculaçao de biogás para favorecer a homogeneizaçao. Os resultados obtidos resumem-se na Tabela 1, conxuntamente com os referidos a um estudo prévio.

Tabela 1. Eficiência de depuraçao frente á velocidde e o TRH.

Período	v. ascen. (m/h)	TRH (h)	VCO_a (g DQO/l.d)	DQO_t	DQO_S (% Eliminaçao)	SS
III	1,7	9,0	1,6	64	59	64
IV	0,5	8,0	1,8	75	70	75
V	0,25	8,0	1,7	78	74	82
VI	0,25	5,6	2,7	60	54	70
Estudo prévio*	0,07	9,0	2,1	73	63	80
"	0,07	5,0	3,2	53	39	63

*Estudo realizado num reactor UASB sem recirculaçao

Inicialmente previa-se que um aumento na velocidade ascensional (en relaçao ao estudo previamente realizado) melhoraria o contacto entre as águas e o lodo e isto conduciria a maiores porcentagens de depuraçao. Os resultados obtidos indicam, porém, o contrário: a depuraçao melhora ao diminuir a velocidade ascensional, dentro do rango de operaçao do presente trabalho. Porém, a aplicaçao de uma certa recirculaçao si se apresenta como benefactora do processo, ja que a velocidades de 0,25 m/h a depuraçao é maior que a velocidades de 0,07 m/h.

Anaerobic treatment of wastewaters containing formaldehyde and urea in UASB reactors

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Keywords: *Formaldehyde, Urea, UASB, Industrial adhesives manufacturing.*

Formaldehyde (FA) is a raw material used in different industrial processes such as fibreboard adhesives manufacturing, being therefore usually present in the effluents generated by these factories, with concentrations up to 1 g/l. Besides, these wastewaters usually contain urea as well as a variable amount of other organic substrates.
The objective of this work was the study of the continuous anaerobic treatment of these effluents in a UASB reactor. Two synthetic influents were prepared, one of them only with glucose and formaldehyde, in order to study the fate of formaldehyde under anaerobic conditions; and the other one contained FA, urea and glucose in concentrations according to the composition of the wastewaters generated by this industrial sector. Both influents were treated in 0.1 l anaerobic UASB reactors operating at 37ºC (reactors R1 and R2, respectively).

The biomass used as inoculum in both reactors was obtained from an industrial digester treating sea food processing wastewaters. This biomass was characterised by means of maximum methanogenic activity tests (0.89 g COD/g VSS·d) and toxicity assays (100 mg FA/l as the IC_{50}).

Both reactors were initially operated only with glucose, being the influent characteristics gradually changed to the ones previously described. Reactor R1 was operated under stable conditions with FA levels up to 2 g/l, at organic loading rates of 6.0 g COD/l·d and 0.6 g FA/g VSS·d, with COD removal efficiencies up to 93%. These results indicate the importance of the technology used as well as its flow pattern, which allowed the operation at high VSS and low FA concentrations simultaneously, being possible in this way the treatment of effluents with very high FA content [1].

Reactor R2, operating with similar influent characteristics as those generated by industries (0.95 g FA/l and 0.35 g urea/l), achieved OLR values of 3.45 g COD/l·d and Nitrogen Loading Rates of 0.58 g N/l·d. Urea present in the influent was completely hidrolised [2], which caused an increase of the total ammonium content in the reactor, although did not affected the COD removal efficiency (around 90-95%). However, the maintenance of the pH level below 8 is a key factor in order to avoid increases free ammonia concentration of in the medium, which could deteriorate the performance of the system due to its inhibitory effects on the biomass.

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Influence of the C/N/P ratio on nitrate removal in a denitrifying biofilm fluidized bed reactor

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***Key Words**: denitrification; biofilm; fluidized bed reactor; C/N stoichiometric ratio; C/N/P ratio*

Reactors of attached biomass are highly effective, and hence are considered as a preferred technology in removing nitrogen from wastewater. With the aim of further improving the quality of waters, the research herein discussed presents a study of the process of biological removal of nitrates of a synthetic effluent with a low degree of nitrates (40-95 mgN-NO_3^-/L) using a fluidized bed reactor. *Alcaligenes denitrificans*, a heterotrophic microorganism, was an initial inoculum to seed the basalt used as the support of adherence. With the purpose of the study and the denitrification process optimisation, several C/N/P ratios were applied and the effects of variations in the removal of nitrates, organic carbon and residual nitrate accumulation were analysed. The findings from this experimentation showed that the phosphorus and the organic carbon were limiting factors to the removal efficiency of nitrates (Figure 1b). In the absence of the required concentrations of the phosphorus (Figure 1a) the denitrification efficiency was very low for any value of the applied ratio C/N, but the quantity of organic carbon removal recorded notable values. Figure 1b, *Zone A_3*, indicates that the amount of organic carbon supplied was insufficient for completing the denitrification process, but in *Zone B_3* when the organic carbon and the amount of phosphorus were sufficient the nitrate removal efficiency increased from 45 to 95%. The growth of the biofilm, expressed as biofilm thickness (see Table 1), was neither strongly affected by the different quantities of phosphorus employed nor by the applied ratios C/N. The ratio C/N experimentally achieved for no limiting organic carbon and phosphorus concentrations was of 3.4 mgC/mgN-NO_3^-, being approximately twice the value of the stoichiometric requisites (1.43 mgC/mgN-NO_3^-). The values recorded for the specific denitrification rate were comprised within the ranged between 0.001 and 0.002 mgN-NO_3^-/mgSV h.

Table 1- The effect of C/N/P conditions in the denitrifying activity

C/N ratio applied (mgC/mgN-NO_X^-)	[P] (mgP/L)	Mean Thickness (µm)	Experimental C/N ratio (mgC/mgN-NO_X^-)	Denitrifying Activity (%)
1.78	**[7×P]** = 1.14	0.395	0.08	45
3.60	**[7×P]** = 1.14	0.402	3.40	**93**
3.60	**[3×P]** = 0.49	0.344	3.40	49
1.81	**[P]** = 0.16	0.384	0.25	15
1.43	**[P]** = 0.16	0.385	0.25	25

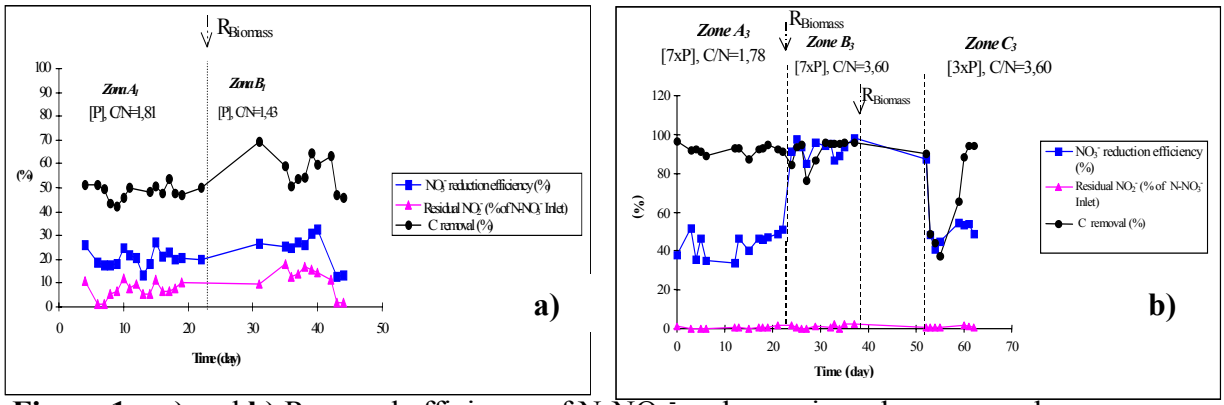

Figure 1 – a) and **b)** Removal efficiency of N-NO_x^- and organic carbon removal.

Optimizacion del Proceso de Nitrificacion-Denitrificacion Mediante la Utilización de Antioxidantes

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Palabras claves: Desnitrificación, metanización, antioxidantes, radicales libres

Tradicionalmente los sistemas de tratamiento de aguas residuales han estado enfocados en la eliminación de materia orgánica, sin embargo, las nuevas tendencias han llevado al desarrollo y utilización de nuevas tecnologías para la eliminación de nutrientes; dentro de los cuales el nitrógeno ha recibido gran atención. Una de las tecnologías más viables de remoción de nitrógeno es la Nitrificación-Desnitrificación. En el cual el amoniaco es transformado en nitrito y nitrato en una etapa aerobia. En una segunda etapa anóxica el nitrato es transformado en nitrógeno molecular. Este último proceso lo llevan a cabo bacterias facultativas que coexisten junto a bacterias anaerobias metanogénicas, que en ausencia de oxigeno utilizan el nitrito y el nitrato como aceptor final de electrones. Usualmente se alimenta el reactor de desnitrificación con nitratos, ya que con nitritos la actividad desnitrificante disminuye.

En este trabajo se estudia la utilización de agentes antioxidantes, para disminuir la posible acción de radicales libres que pueden producirse en la metabolización del nitrato, de este modo se espera aumentar la actividad desnitrificante cuando el reactor es alimentado con nitritos. Los resultados preliminares indican que es posible aumentar la actividad desnitrificante en un 80%. También se espera lograr eliminar el efecto inhibitorio entre los procesos de desnitrificación y metanización, causada posiblemente por la presencia de radicales libres originados a partir de nitrito.

Las ventajas del uso de antioxidantes in la desnitrificación son: 1) Se podría metanizar y desnitrificar en un mismo reactor, 2) alimentar el reactor de desnitrificación con nitritos, manteniendo una alta actividad desnitrificante, 3) Disminución en la materia orgánica necesaria para la desnitrificación al alimentar con nitritos, 4) Ahorro en el consumo de oxígeno en la nitrificación ya que no se necesita oxidar el nitrito a nitrato.

Influence of Lipid Acclimatization on the Oleic Acid Toxicity towards Methanogenic Acetoclastic Bacteria

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***Keywords**: anaerobic filters, LCFA toxicity, lipids, acetoclastic bacteria.*

The acetoclastic bacteria are probably the weakest link in the overall anaerobic digestion chain[1]. Long chain fatty acids (LCFA) are one of the most important inhibitors of the anaerobic consortium and toxic effect is dependent on the temperature, sludge structure (granular or dispersed), presence of calcium or magnesium saltz. Sludge origin is considered to be unrelevant[2]. It is suggested that LCFA exert a bactericidal effect on methanogenic bacteria[3]. From the existing literature acetoclastic bacteria do not adapt to LCFA neither upon repeated exposure to toxic concentrations, nor after prolonged exposure to non-toxic concentrations. However, most of the results were obtained in batch tests which does not take into account the real operating times in anaerobic digestion. In this work, the resistance to oleic acid toxicity and the its biodegradation capacity was followed during a long term operation in a fixed bed reactor. Two bioreactors (RI and RII) were running in parallel for 426 days. In a first period RI received a lipidic substrate (whole milk based) and RII received a non fat substrate (skim milk based). Following this period both digesters received the same substrate which was initially composed f skim milk and oleic acid (Period II) and after by oleic acid as the sole carbon source (period III). The effect of feeding increasing lipid concentrations on biomass resistance after changing the feed to oleic acid was evaluated. It was observed that biomass from RII developed a higher resistance during the period I, but, after changing the feeding to be composed of skim milk and oleic acid, the biomass from RI which had been fed with lipids, exibited an higher tolerance to oleic acid toxicity. This suggests that the acclimatization with lipids is beneficial to treat LCFA based wastewaters. However, when oleate was the sole carbon source fed (Period III), both sludges lost their potential resistence. In this final period the biomass was found to be encapsulated with oleate, which was evidenced by the extremely high values of the background methane production in batch assays without any added substrate and after successive washings with an anaerobic buffer. Furthermore the degradation rates of this background substrate (probably adsorbed oleate) was higher than the maximum achieved oleate degradation rate measured by biodegradability tests (105.4±0.9 versus 86.3±4.6 mg COD-CH_4/gVS.day), indicating that adsorbed oleate can be more easily degraded than added oleate. It was also observed that added oleate retarded the degradation of adsorbed oleate.

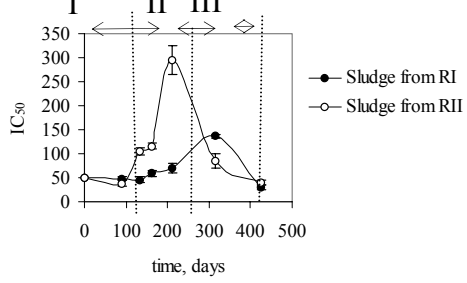

Figure 1 - Toxicity limit along the operation period

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Calibration of a Simplified Model Describing an Urban Waste Water Treatment Pilot Plant based on N/D and BEPR Criteria

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Keywords: *Modelling, monitoring, N/D, BEPR*

A simplified model of the IAWQ Activated Sludge Model No. 2 (ASM2) [1] has been developed and calibrated. In order to use models for applications in real-time parameter identification or retrofit of existing municipal waste water facilities modifications to the ASM2 are necessary to minimise the complexity of the model and the number of parameters involved.

The model has been applied to a municipal waste water treatment pilot plant with an A^2/O multistage process treating 500 liters per day of a complex synthetic waste water. The A^2/O process allows for biological organic matter, nitrogen and phosphorus removal. This fully automated pilot plant is equipped with in-line sensors (pH, T, DO and ORP) and on-line analyzers for ammonium, nitrite, nitrate and phosphate allowing process monitoring. Ammonium is analyzed through a commercially available continuous flow analyzer (CFA). A FIA analyzer is used in order to determinate phosphate, and another for simultaneous nitrate and nitrite analysis [2]. All the elements of the plant (pumps, valves, stirrers,...) and analyzers are computer controlled and a data acquisition system has been implemented.

Data obtained from batch and continuous operation experiments and simulations with MATLAB have been used for calibrate the modified model. The main differences between this model and the ASM2 are the state variables chosen and the processes considered, reducing significantly the number of parameters to be identified. With reference to this, just two COD fractions (slowly and readily soluble biodegradable organic matter) have been considered instead of the five different COD fractions considered in the ASM2. Phosphorous accumulating organisms (PAO) are not considered in the modified model because they are heterotrophs, so heterotrophic and autotrophic biomass are sufficient to describe the processes involved.

This model still has to be validated but good results have been obtained in comparison with simulations done using the ASM2.

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Influence of C/N Ratio on the Start-Up of Up-Flow Anaerobic Filter Reactors

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Keywords: *Biomass adhesion, C/N ratio, start-up, Upflow Anaerobic Filter.*

The reduction of start-up time is one of the key parameters to increase the competitiveness of high-rate anaerobic reactors [1], being the long start-up duration one of the main disadvantages associated with anaerobic digestion. One of the facts which could improve the start-up efficiency and rapidity could be better understanding- and the consequent exploitation-of the role of the C/N ratio in the feed on biomass aggregation and adhesion. Several researchers [2], [3] agree that nutrients excess or limitation influence the production of extra-cellular substances which are suspected to be responsible for biomass attachment. The present work aims to investigate the mentioned issue by experimentally implementing fed-batch and continuous operation anaerobic systems characterised by different C/N ratios and analysing the resulting biomass adhesion.
Batch reactors of 250 ml were used and PVC Raschig rings (11mm diameter) were the tested support. The reactors were maintained the temperature constant at 35ºC and provided constant shaking at 70 rpm during 4 weeks. Two identical anaerobic filters were set up in order to confirm under continuous operation the results obtained from the fed-batch study. PVC Raschig rings (20mm diameter) were the tested support and lactose ($C_{12}H_{22}O_{11}\cdot H_2O$) was used as substrate. By adding NH_4Cl and KH_2PO_4 to lactose, two different C/N/P ratios theoretically corresponding to 100:1:1 and 100:7:1 were prepared. The necessary micro and macronutrients were added to the former solution.
The followed parameters were influent flow rate, gas flow rate and composition (CH_4 and CO_2), Volatile Fatty Acids (VFA), Total Organic Carbon (TOC), Chemical Oxygen Demand (COD), Total Kjeldahl Nitrogen (TKN) and alkalinity. At the end of experiments Total and Volatile Suspended Solids (TSS, VSS) were determined in the biomass, as well as the specific methanogenic activity and Extra-cellular Polymeric Substances (EPS).
The results obtained from batch experiments indicated that a nitrogen limitation in the very first phase of reactor start-up seems to majors enhance biomass adhesion. Hence a new alimentation strategy was formulated: that consisted of initial low nitrogen –for two weeks- and later nitrogen excess and a further experiment in continual operating systems was then promoted in order to verify the results. The new strategy leads to higher attachment when nitrogen is limited during the two first weeks with an improvement of 36% in VSS, and higher content in PS for adhered and suspended biomass. This is in accord with what suspected by [2]. The hypothesis on biomass pelletization formulated by [3], which considers nitrogen excess a necessary condition for EPS releasing, seemed not to be valid for bacterial adhesion on fixed supports.

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Nitrate and Organic Matter Removal Using a Combined System of Slugde Blanket-Support with Biofilm

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In domestic wastewater effluents with medium-low load on organic matter, nutrient elements like nitrogen and phosphorus, must also be eliminated.The application of combined systems, where anaerobic digestion is followed by a post-treatment with denitrification-nitrification processes shown efficient treatment of those effluents. However, anaerobic and denitrification processes can be carried out in one step, reaching high efficiencies. (Hendriksen *et al*., 1996 and Hanaki *et al*., 1989). With a previous sludge adaptation step, anaerobic and denitrificant bacteria can coexist, and the organic matter in excess which is not removed in the denitrification process, is used by the methanogenic bacteria. The nitrate needed in the methanogenic-denitrification reactor is recirculated from the nitrification unit.

The main objective of this work was to study the capacity of a denitrification-methanogenic rector fed with synthetic effluent with a composition similar to the one of a domestic wastewater.
It was used a 1L reactor, fed continuously in an upflow mode. The operation temperature was in the range of 17-24 °C. As seed was used a mixture of sludge from an anaerobic digestor and a denitrification reactor, previously adapted in a batch mode. The reactor had two parts, at the bottom part was located a sludge blanket and at the top, poliurethane foam with immobilised biofilm was placed. Methanol was used as carbon source, with a concentration of 450 mg COD/L and 50 mg N-NO_3^-/L were added in order to obtain a COD/N-NO_3^- of 9. Also 50 mg N-NH_3/L and 250 mg/L of bicarbonate were presented to simulate the domestic effluent.

After 60 days, the reactor operated with a HRT of 4 hours, with an organic and nitrogen loading rates of 2,7 g COD/L.d and 0,3 g N-NO_3^-/L.d, respectively. During the operation period the organic matter and nitrate removals were always between 90 and 98%. The biogas contained 60% of nitrogen and 40% of methane. The biomass at the bottom granulated and formed a compact sedimentated bed. It was observed the colonisation of the support foam placed at the top part that remained stable with the flow increment.

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Treatment of Wastewaters from an Industry Producing Synthetic Resins: Characterization and Full-Scale Plant Operation

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Keywords: *industrial wastewater, wood industry, formaldehyde, phenol, nitrogen removal*

The most common resins produced in Galicia are copolymers composed of formaldehyde, urea, melamine and/or phenol. Wastewaters generated during the process are thus expected to contain high concentrations of nitrogen as well as moderatly recalcitrant compounds as formaldehyde and phenol. In the present work, wastewaters generated during the production of synthetic resins were characterized. Data of operation of a full-scale treatment plant are presented.
Characterization of the effluents were carried out by measuring different parameters according to Standard Methods (1): pH, alkalinity, conductivity, TSS, VSS, COD, TNK, N-NH_4, and phosphates. Formaldehyde was analyzed colorimetrically according to the Hantzsch reaction (2).
Wastewaters were characterized by taking samples at eight different locations in the plant. Daily, monthly and seasonal variations were evaluated. The most important measured parameters varied in the following range: COD, 146-4300 mg/l; Total Nitrogen, up to 6373 mg/l; N-NH_4, up to 1044 mg/l; formaldehyde, 4-3543 mg/l. Phenol concentrations were ususally low since most resins were composed of urea-formaldehyde and/or melamine. According to samples analyzed so far, pH, alkalinity, conductivity, phosphates, TSS and VSS were highly variable, depending on the sampling point.
The effluents were treated in an anoxic-aerobic full-scale reactor. More than 85 % COD removal and 60 up to80 % nitrogen removal were reached. Formaldehyde removal reached 99 %.

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Nitrification in a Fluidized Bed Reactor

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***Keywords**: nitrification, fluidized bed, free ammonium inhibition*

Biological nitrification of different types of wastewaters is usually required in order to produce good effluent quality. The fluidized bed technology can offer many advantages related to the use of small particles of support material for the accumulation of active biomass, and the recirculation required to obtain sufficient upflow velocity which improves mixing conditions and mass transfer [1].

Results of nitrogen oxidation of synthetic wastewater and anaerobically treated poultry wastewater are compared in this work. Attention was focussed on the determination of the influence of hydraulic retention time and loading rates on the nitrification process, and the inhibitory effect of free ammonia (FA). The inhibitory concentrations of FA were compared with the values obtained by Anthoninsen [2] for the activated sludge process. Experimental work was done in a laboratory scale aerobic fluidized bed reactor (FB), 4.2 l working volume, with sepiolite as carrier material. The bed expansion was kept close to 30%, dissolved oxygen was within the range 3.0-4.0 $mg \cdot l^{-1}$, and temperature was maintained at 35 ± 1ºC.

With the synthetic feeding the reactor was operated at 8.0 ± 0.3 pH, with nitrogen loading rates (B_{VN}) in the range 0.20-1.26 kg NH_x-$N \cdot m^{-3} \cdot d^{-1}$ and hydraulic retention time from 1.8 to 8.0 h. The nitrification efficiency was found to be higher than 95% when nitrogen loading rates were below 0.84 kg NH_x-$N \cdot m^{-3} \cdot d^{-1}$, and lower than 70% when nitrogen loading rates were above 0.84 kg NH_x-$N \cdot m^{-3} \cdot d^{-1}$.

With the anaerobic effluent the FB was operated at two pH ranges 6.0 ± 0.3 and 8 ± 0.3, and various loading rates, up to a maximum B_{VC} of 1.2 kg $COD \cdot m^{-3}$ d^{-1} and B_{VN} of 2.5 kg $TKN \cdot m^{-3} \cdot d^{-1}$. Efficiencies were higher than 80% for nitrification and higher than 50% for COD removal. The results obtained with the anaerobic effluent showed that the FB is competitive with conventional biological treatment systems for the ammonia oxidation and organic matter stabilization.

Operating with pH 8, for NH_x-N concentration greater than 10 $mg \cdot l^{-1}$ (FA>1 $mg \cdot l^{-1}$) nitrite accumulation was observed which implies nitrobacters inhibition. When NH_x-N concentration was higher than 60 (FA>10 $mg \cdot l^{-1}$) nitrosomonads inhibition was also observed an nitrification stopped. However, at pH 6, nitrification efficiency was not affected by a NH_x-N concentration of 60 $mg \cdot l^{-1}$, because FA was bellow the inhibitory level (FA < 1.0 mg/l). Nitrification efficiency was independent of free nitrous acid (FNA) in the range studied (FNA<0.002 $mg \cdot l^{-1}$). By contolling reactor pH and avoiding nitrification inhibition conditions, the system accomplish complete nitrification untill a maximum nitrogen loading rate B_{VN} of 2.5 kg $TKN \cdot m^{-3}$ d^{-1}.

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Tratamiento de aguas residuales de matadero en un reactor UASB floculento. Determinación de parámetros cinéticos

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***Keywords**: aguas residuales, matadero, UASB, cinéticas*

Las aguas residuales de matadero presentan un alto contenido en materia orgánica de la que aproximadamente la mitad corresponde a la fracción soluble. La fracción no soluble corresponde a materia coloidal y suspendida no degradable o lentamente biodegradable.

Los digestores UASB han sido utilizados con éxito en el tratamiento de estas aguas, si bien se han detectado problemas de operación relacionados con la adsorción y atrapamiento de materia particulada en el fango.

Con el fin de cuantificar la importancia de este fenómeno y sus posibles efectos sobre el comportamiento del sistema se ha procedido a determinar experimentalmente, en un reactor UASB de lodos floculentos, la eliminación de materia orgánica y la producción de metano y, a partir de los datos obtenidos, la acumulación. Ello ha permitido obtener las constantes cinéticas para la eliminación y la producción de metano, así como la producción de fango observada Y_{obs}= 0,4355 g SSV/g $DQO_{consumida}$.

Por otra parte un balance al nitrógeno nos ha llevado a determinar el rendimiento celular $Y_{X/S}$= 0,185 g SSV/g $DQO_{consumida}$ y la constante de descomposición endógena k_d= 0,0067 d^{-1}.

Por diferencia entre la producción observada Y_{obs} y el rendimiento celular $Y_{X/S}$ se ha obtenido el factor de acumulación Y_{acum}= 0,2505 g SSV/g $DQO_{consumida}$, lo que confirma la importancia del fenómeno.

Asimismo se ha medido la actividad metanogénica específica observándose que se mantiene sustancialmente constante después de 170 días de operación, si bien el valor obtenido 0,058 gCH_4-DQO/g SSV resulta pequeño en comparación con la actividad máxima determinada en un ensayo utilizando acético, propiónico, butírico (2:1:1) que fue de 0,22 g CH_4-DQO/g SSV.

Por tanto se puede concluir que la elevada acumulación de materia observada no afecta negativamente a la actividad metanogénica específica del fango en las condiciones en que se realizo el experimento, B_v entre 1 y 6 g DQO/ l día (B_x entre 0,05 y 0,4 g DQO/ g SSV día) y THR superiores a 15 horas. Sin embargo, la acumulación de materia en suspensión, principalmente grasas, provocó la flotación brusca de fango.

El arrastre de sólidos en el efluente, expresado como diferencia entre DQO total y soluble, tampoco ha sido significativo manteniéndose en todo momento por debajo del valor de la DQO correspondiente a la fracción no soluble de la alimentación.

Reactor anaerobio de lecho fluidizado: puesta en marcha y cinética de la metanogénesis

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Palabras claves *:lecho fluidizado, anaerobio, puesta en marcha, cinética, inhibición*

Reactores anaerobios de biopelícula fija tales como filtro anaerobios, lechos fluidizados o expandidos y UASB han sido desarrollados para el tratamiento de residuales líquidos de alta carga orgánica [1]. De forma especial, debido a sus variados usos y ventajas, los lechos fluidizados han demostrado su eficiente utilización en el tratamiento anaerobio [2]. Un tema de elevado interés en los mismos lo constituye la puesta en marcha y la cinética de los distintos procesos y especialmente de la metanogénesis, por ser la etapa limitante del proceso.

El trabajo presenta la puesta en marcha de un Reactor Anaerobio de Lecho Fluidizado (AFBR) de 1.8 L de volumen total utilizando como soporte piedra pomez blanca (Biolita) con 200-300 μm de diámetro y HAc como única fuente de carbono. Se mantuvo una relación C:N:P de 100:7:1 garantizando de esta forma la presencia de macro y micronutrientes. En un período de 75 días se alcanzó una velocidad de carga de 14,8 $kgm^{-3}d^{-1}$ con una eliminación de acético superior al 80 %, Fig. 1.

El estudio cinético se llevo a cabo en continuo, manteniendo una concentración de HAc de entrada de $2.5gL^{-1}$ y variando la velocidad de dilución. Se puede comprobar a partir de la Fig.2 una inhibición, por que se ha utilizado la expresión de Haldane. Los parámetros cinéticos así obtenidos son : Vmax = 0.74 $gL^{-1}h^{-1}$; Ks = 0.185 gL^{-1} ; Ki = 1.0 gL^{-1}

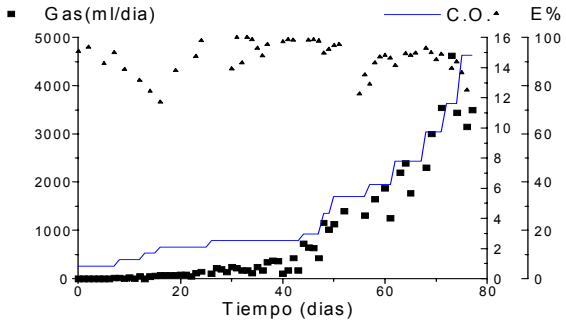

Fig.1 Puesta en marcha.

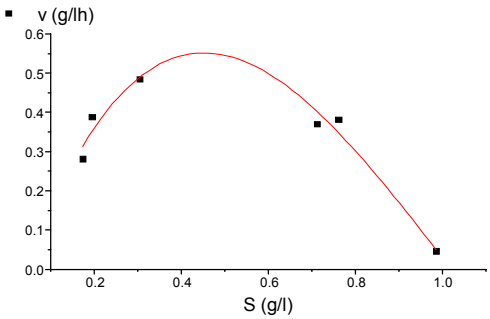

Fig.2 Velocidad de consumo de HAc.

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Aplicación de Ensayos de Actividad al Seguimiento de un Reactor UASB de Dos Etapas

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Palabras claves : *Tratamiento anaerobio, Ensayos de actividad.*

La degradación anaerobia del agua residual es posible cuando tenemos un fango con propiedades capaces de llevar adelante las diferentes etapas de la degradación, por vía anaerobia, de la materia orgánica. Su determinación tiene que ver con la actividad del fango, mediante el desarrollo de ensayos anaerobios. Se han realizado ensayos de actividad acidogénica y acetogénica a fangos de un reactor UASB de doble etapa, a nivel de planta piloto, con el objetivo de caracterizar mejor las propiedades biológicas del lodo y en consecuencia estudiar la adaptación del mismo al agua residual que se va a tratar, en este caso agua residual urbana. Para ello se tuvieron en cuenta dos periodos de operación, que difieren en el tipo de fango inoculado en la segunda etapa del reactor. Los resultados obtenidos fueron los siguientes:

Periodo-I.

Etapa	**Nivel**	**Actividad metanogénica (g DQO/g SSV d)**	**Actividad acidogénica (g DQO/g SSV d)**
1ra (R-1)	1 (0.3 m)	0.08	0.31
	2 (1.3 m)	0.06	0.20
2da (R-2)	1 (0.3 m)	0.03	
	2 (1.3 m)	0.13	
	3 (2.0 m)	0.12	
	4 (2.7 m)	N.D*	

*N.D no se detecta.

Periodo-II

Etapa	**Nivel**	**Actividad metanogénica (g DQO/g SSV d)**
R-1	1 (0.3 m)	0.06
R-2	1 (0.3 m)	0.12

En el periodo 1, dado los valores de actividad obtenidos, podemos indicar que la primera etapa funciona como un buen reactor de acidificación en cambio en la segunda etapa (inoculada con lodo granular), se observa que en el primer nivel la calidad del fango es mala, el grado de mineralización del mismo es elevado, que impiden la existencia de una actividad metanogénica buena. Luego en el periodo 2, con la inoculación de un fango floculento en R-2, menos pesado, que permite mantenerlo expandido y en buen contacto con la materia orgánica, se observa una actividad metanogénica superior, de vital importancia para que la segunda etapa opere como reactor metanogénico.
Se realizaron también, ensayos de biodegradabilidad (a 20 y 35 ºC) al efluente procedente de R-1 usando fango de R-2 como inóculo y se observó que a ambas temperaturas el agua residual sufre una hidrólisis.

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Tratamiento Anaerobio de Aguas Residuales Urbanas en Sistemas de Doble Etapa (Hidrolítico-Metanogénico)

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La aplicación de la digestión anaerobia a efluentes de baja carga, como son las aguas residuales urbanas y numerosos efluentes mixtos de polígonos industriales (Soto, M. et al., 1995) requiere la operación eficaz y estable a muy bajos tiempos de retención hidráulica (TRH) y a temperatura ambiente. Además en procesos con una considerable cantidad de partículas orgánicas, como son las aguas residuales urbanas, es conveniente la separación de la digestión anaerobia en fases. En una primera etapa, la hidrolítica, las partículas de materia orgánica son atrapadas y convertidas en compuestos solubles, incrementando la biodegradabilidad global del efluente, al tiempo que se sustituye al decantador primario. La depuración completa del agua residual se alcanzará en una segunda etapa, que tanto puede ser aerobia como anaerobia (metanogénica).

El objectivo del presente trabajo es un estudio del tratamiento anaerobio de efluentes domésticos en un sistema bi-etapa. La primera etapa consiste en el tratamiento en un reactor hidrolítico de flujo ascendente (RHFA) tipo lecho de lodos y una segunda etapa se utiliza dos digestores alternativos: un filtro anaerobio relleno con espumas de poliuretano, y un digestor metanogénico de flujo ascendente (RMFA).

El digestor hidrolítico tiene un volumen activo de 2 litros, operándose inicialmente con un TRH de 4,4 horas. En fases posteriores el TRH se redujo progresivamente hasta 2,3 horas, manteniendo constante el caudal alimentado, y reduciendo el volumen activo. Los digestores utilizados para la etapa metanogénica tienen un volumen de 0,5 litros. El TRH varió en un rango de 24-2 horas para el RMFA y 30-6 horas para el FA.

El reactor hidrolítico operó un total de 610 días. A partir del día 50, el reactor muestra una eficacia media en la eliminación de sólidos entre un 50-60%. Esto se refleja en una eliminación mayor de la DQOtotal que de la DQOsoluble. Durante esta etapa la concentración de AGV en el efluente se situa en el entorno de 50-60 mg/L. En el día 225 se baja el TRH en el reactor hasta 3,5 horas . Como consecuencia de esto, se observa un sensible aumento en la eficacia de eliminación de las partículas orgánicas en suspensión, llegando a valores de entre un 65-75%. La eliminación de la DQOsoluble en este período muestra un pequeño descenso debido al aumento de los AGV procedentes de la solubilización de los sólidos, que pasó a situarse en un rango de 100-120 mg/L. Finalmente, a partir del día 540, el TRH se bajó a 2,3 horas consiguiéndose, de nuevo, una mejora en la acidificación y en la eliminación de sólidos en suspensión

El filtro anaerobio permitió alcanzar una depuración adicional del efluente prehidrolizado, con rendimientos en la DQO eliminada en un rango de 40-60% y en los sólidos del 25-60% en esta segunda etapa. Así, la combinación RHFA-FA permite conseguir depuraciones globales del 72% en DQO, 83% en SS y 93% en DBO_5 para un TRH global de 8,3 (6+2,3 horas).

El trabajo realizado con el reactor metanogénico de lecho de lodos tuvo como objectivo el estudio de la granulación. Para ello, se alimentó con sacarosa (300 mg/L) como co-sustrato añadido al agua residual prehidrolizada. Se alcanzó un TRH de 2 horas detectándose unicamente inicios de granulación.

A New Device to Select Microcarriers for Biomass Immobilization: Application to an Anaerobic Consortium

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***Keywords**: microcarriers, biomass immobilization, methanogenic activity.*

Sand is the most widely used microcarrier in fluidised bed reactors[1]. However, the choice of sand is found to be far from the optimum, as far as biomass retention capacity is concerned. Other materials have been evaluated, such as granular activated carbon[2] foam glass[3] sepiolite, pozzolana[5] diatomaceous earth[4] or pumice stone. The use of porous microcarriers reduced the start-up time by more than 50% as compared to sand[3], allowed the application of higher organic loading rates and favoured the biofilm growth[2]. So far the comparative studies of different microcarriers for biomass colonisation have been made either in continuous mode, operating one reactor with each support[2], [4] or in batch tests run simultaneously[6]. In this work a new device to compare biomass retention capacity of different microcarriers was designed. The microcarriers are randomly distributed in parallel mini-bioreactors under selected and identical flow conditions. Four porous microcarriers (sepiolite, pozzolana, clay and foam glass (Poraver™)) were compared in terms of their ability to retain an anaerobic consortium developed in a synthetic dairy waste. Sepiolite was found to have the highest biomass retention capacity and the better internal porous volume for biomass immobilisation (Table 1). The average specific methanogenic activity (SMA) of the immobilised biomass in the different support materials was found to be inversely correlated to the amount of attached biomass (Figure 1). The individual acetate propionate and butyrate consumption rates revealed that acetoclastic bacteria were the most inhibited by the immobilization whereas syntrophic activity was enhanced for all the materials. Internal diffusion limitations, potential stimulation/inhibition of released components from the different materials and the more suitable hydrogen environment in the biofilm can be put forward to explain these observations.

Table 1 - Attached biomass concentration, expressed per internal porous volume (±95% confidence interval).

Material	Attached biomass g VS/L (internal porous volume)
sepiolite	38.4±2.4
clay	35.1±1.0
pozzolana	29.3±1.3
foam glass	19.3±1.4

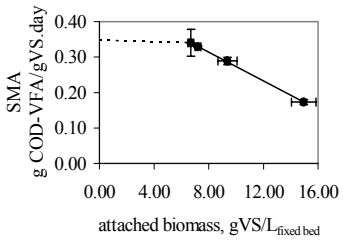

Figure 1. SMA of the attached biomass. Extrapolated value represents non attached biomass activity.

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Effect of Recycling Ratio on the Performance of a Coupled Nitrification-Denitrification System

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Keywords: *nitrification-denitrification, nitrate, ammonia, DUSB, activated sludge.*

There are several technologies based in the biological nitrification-denitrification process used to remove nitrogen from wastewater in practice, as recycling systems, alternated tanks, or carrousel systems, being recycling systems very used for the treatment of industrial wastewaters due its simplicity and versatility [1]. In this work a nitrification-denitrification recycling system was employed, the denitrifying unit being a Denitrifying Upflow Sludge Blanket reactor (DUSB) [2]of 1.6 L. This reactor was selected due to the good settling characteristics of developed biomass which allow to get a high content of suspend solids into the reactor [3], and therefore to denitrify high loads of nitrate. The nitrification unit consisted of an activated sludge reactor of 2 L with an external settler of 1 L. The objectives of this work were to study the effect of Hydraulic Retention Time (HRT) and recycling ratio (R/F) on nitrification-denitrification efficiency of the system.
Before to be coupled, the nitrifying unit was feed with 3 L/d of an autotrophic influent (1.0 g N-NH_4^+/L and 1.9 g C-$NaHCO_3$/L), its nitrifying efficiency being practically 100%. The DUSB was also previously feed with 3 L/d of a heterotrophic influent (1.0 gN-NO_3^-/L and 1.3 g C-NaCOOH/L), its denitrifying efficiency being 90-80%. Once stationary state conditions were obtained, both reactors were connected as follows: The influent to the DUSB consisting of an acetate and ammonia solution (synthetic medium, with the same concentrations as before) plus a recycling flow coming from the nitrifying reactor. The system was operated at three R/F ratios (1, 5 and 3). Initially, it was operated during 25 days with the first ratio (1), the individual efficiency from reactors being between 90-100%. When the recycling ratio was increased to 5, a drastic efficiency decrease to 20% was observed, while the denitrifying reactor had a similar behaviour, decreasing its efficiency to 40%. The further reduction to the R/F ratio to an intermediate value of 3, however does not allow to recover the original activity of nitrifying reactor, while the denitrifying unit efficiency was quickly recovered to 90%. The reason of the problems was related to the decrease of pH to 6.5 observed during the increase of R/F to 5 due to the inhibition of nitrifying bacteria. A systematic addition of bicarbonate to the nitrifying reactor allowed to recover a suitable pH between 7 and 9, with an important increase on the overall efficiency of the system.

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A New Method to Study Interactions between Biomass and Packing Material in Anaerobic Filters

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***Keywords**: anaerobic filters, biomass-support interactions, methanogenic activity.*

In anaerobic filters the biomass immobilization is achieved by entrapment in the void space and by adhesion to the surface of the packing material**.** In the upflow mode, the adhered biomass is usually considered to play a minor role due to its low proportion as compared with the total accumulated biomass[1]. However, depending on support properties such as bed and carrier porosity, surface area, and type of material, a thick biofilm can be formed increasing its importance on the overall performance[2]. If hydraulic or toxic shocks are applied the adhesion of biomass provides a more stable immobilization than the entrapment. On the other hand, the study of anaerobic filters is limited by the difficulty of determining biomass quantity and quality as well as its evolution with time and operating conditions. It is believed that a general behaviour can not be predicted and it is proposed that biomass-support interactions should be characterized for each particular application. In the present work a methodology based on the placement of several parallel mini-bioreactors in the same bioreactor was tested with two different applications: (I) Comparison of three materials (glass, Plexiglas and polyvinyl chloride (PVC)) and three carrier sizes in terms of biomass distribution between the adhered and entrapped fractions. (II) Evolution of biomass characteristics (distribution in the support and specific methanogenic activity) by regular withdrawal of some accumulated biomass. Figure 1 represents the layout of the support section for both applications. Entrapped biomass was more concentrated for the smaller pore sizes of support material and the glass, the most smoth and hydrophilic surface accumulated a thinner biofilm essencially composed of *Methanospirillum*-like bacteria. In the experiment II, a continous growth of the adhered biomass was observed, achieving a maximum of 40% of the total biomass (Figure 2). The evolution in biomass quality was assessed by measuring the potential specific methanogenic activity against direct (acetate, H_2/CO_2) and indirect (propionate, butyrate and ethanol) substrates. The acetoclastic activity achieved a maximum of 621.4 ml CH_4@STP/gVS.day on the 162nd day whereas the other measured specific activities remained stable after the start-up with the exception of propionate activity, which increased continuously during all the trial period.

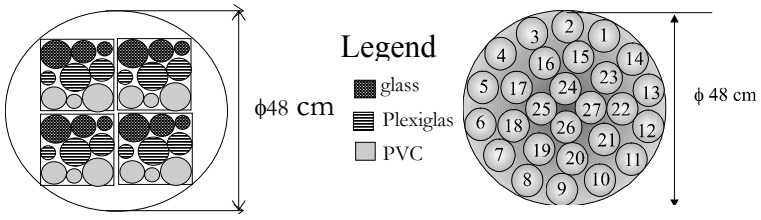

Figure 1 Layout of the support section in the experiment I (a) and II (b)

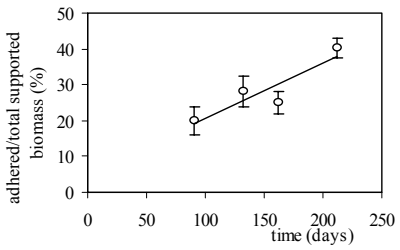

Figure 2. Time course of the ratio adhered/total supported biomass

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Influence of Lipid Acclimatization on the Support Matrix Colonisation in Anaerobic Filters treating Oleic Acid

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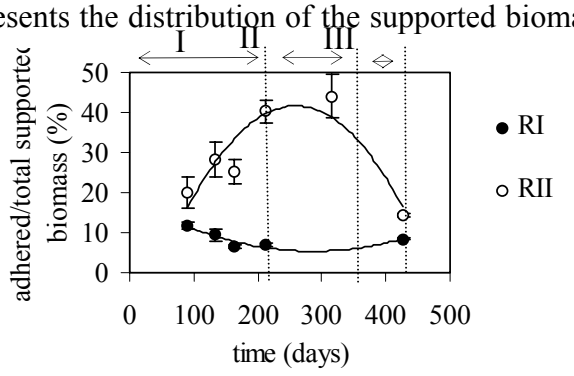
***Keywords**: anaerobic filters, biomass-support interactions, LCFA, lipids.*

Two main problems are associated with the treatment of lipid containing wastewaters: the adsorption of a light lipid layer around biomass particles resulting in biomass flotation and washout and the acute toxicity of LCFA against both methanogens and acetogens, the two main trophic groups involved in LCFA degradation[1]. Notwithstanding the possibility of decreasing the toxic effect by addition of calcium or magnesium salts, flotation problems, which are particularlly important for UASB operation, can not be reverted by calcium addition and, in general, arise for LCFA concentrations bellow the toxicity threshold[2]. Oleic acid, one of the most abundant LCFA present in effluents has been tentatively degraded in UASB and EGSB reactors, without success due to granule disaggregation and washout[1],[2]. In this work the biomass developed in anaerobic filters was studied in terms of distribution in the support (PVC Raschig rings) and by periodically measuring the specific methanogenic activities against specific substrates (acetate, H_2/CO_2, propionate, butyrate and ethanol), according to the methodology already described[3]. Two bioreactors (RI and RII) were running in parallel for 426 days. In a first period RI received a lipidic substrate (whole milk based) and RII received a non fat substrate (skim milk based). Following this period, both digesters received the same substrate which was initially composed f skim milk and oleic acid (Period II) and after by oleic acid as the sole carbon source (period III). Figure 1 represents the distribution of the supported biomass. In the reactor initially fed with lipids (RI), the adhered biomass was always very low as compared with total biomass. However in RII the biofilm was continuously built-up achieving a maximum of 40%, but it was very sensitive to the substrate change from skim milk to oleic acid. The biofilm formed in the presence of lipids was thinner, but more resistant to oleic acid than the one formed in the absence of lipids.The specific acetoclastic and hydrogenophilic activities were very close for RI and RII along all the trial period (Figure 2 - for acetoclastic activity), but the metanogenic activity against butyrate was clearly enhanced in the reactor RI during the period I.



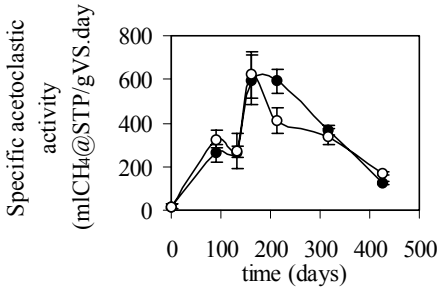


Figure 2 - Specific acetoclastic activity along the trial period

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Behaviour of an UASB Reactor Acidifying Saccharose at Room Temperature (20 - 25ºC)

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Keywords: UASB reactor, acid phase, two-phase digestion.

In this paper,the behaviour of an UASB reactor acidifying a solution of saccharose with 11000 mg COD/L, at room temperature (20-25ºC) is studied, measuring the influence of operational pH in the rate of acidified organic matter.

Introduction:

Cheese whey has, due to lactose fermentation, a very important acidification potential, which causes very low pH values while it is kept in store and the inhibition of VFA production. For this reason, one-phase anaerobic digestion is a problematic procedure in this cases, and makes it advisable to operate in a two-phase system. That is why in this paper it was studied the influence of pH in the acidification process, using saccharose instead of lactose.

Materials and methods:

A 1 litre UASB reactor was used, fed with a synthetic solution (11g. COD/L), to which macronutrients were added. Tests of the following parameters were carried out to check the reactor´s behaviour: COD, alkalinity, pH and VFA.

Results:

Table 1. Operational parameters at HRT=0,33 days. Values in mg/L.

INFFLUENT		EFFLUENT						
$NaHCO_3$	COD	pH	COD	acid COD	AcH	PrH	BuH	iBuH
2300	11071	4,0	10488	5142	3231	760	100	199
4900	11244	4,5	10800	7954	4618	1410	374	120
6250	11133	5,0	10400	7232	3535	1166	913	0
7250	11157	5,5	9959	8791	3058	1147	1807	0
7500	11040	6,0	9626	8404	2669	1215	1836	0

Conclusions:

At a constant HRT of 0,33 days, with the addition of different $NaHCO_3$ concentrations, it was possible to get operational pH between 4 and 6. The highest VFA rate was achieved at pH 5,5, and the lowest at pH 4. For every pH value the acetic acid was always the most important. When pH was underneath 5, propionic acid occupied the second place, and butiric acid the third. For pH over 5, the concentrations of butiric and propionic acid were second and third, respectively.

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Slaughterhouse Wastewater Treatment using Anaerobic Fixed Film Reactors

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KEYWORDS: Anaerobic, fixed film, poultry, slaughterhouse.

Slaughterhouse wastewater contains high concentrations of biodegradable matter, which can be removed by anaerobic treatment. Various anaerobic reactors have been tried achieving different but mostly successful results. In suspended biomass reactors, after long operation periods, sludge flotation due to accumulation of colloidal greases is observed [1]

The aim of this work is to study the convenience of anaerobic fixed film reactors for slaughterhouse wastewater treatment, and the influence of operation conditions, such as organic loading rate, influent concentration, direction of flux, recirculation and intermittent operation over the efficiency of the process.

Experimental work was done in two lab-scale reactors, one upflow and another downflow, with a working volume of 2 l each, using PVC corrugated tubes as vertical support, and kept at 35 C. The wastewater came from a poultry slaughterhouse and chicken blood was used to increase its organic concentration when needed. COD, TKN-N, NHx-N, TSS, VSS and alkalinity were determined following Standard Method's procedures.

For organic loading rates (Bv) of 10 kg COD m^{-3} d^{-1}, efficiencies between 80 and 90 % in COD removal were achieved. This performance was found not to be significantly influenced by the direction of flux, the influent COD concentration or recirculation ratios between 0 and 12. A small increase in volatile solids in the effluent was detected with a recirculation ratio of 12. Accumulation of non-attached biomass was observed in the downflow reactor.

Satisfactory stability was determined under intermittent operation, with a 60 hour-stop per week, very common in slaughterhouses habitual operation. During the stop, a slight mineralization was observed in solids, as well as in nitrogen producing nearly the absence of organic nitrogen in the effluent at the end of the stop. The reactor reaches its normal performance within the first 24 hours. Besides, an increase on COD removal efficiency with increasing effluent carbonaceous matter concentrations in the range of 150 to 700 mg/l was detected.

Bv up to 20 kg COD m^{-3} d^{-1} produced a slight decrease in the performance efficiency of COD removal, but pH was always above 7.5, far from acidification conditions. The ammonification rate was sharply reduced from 60 to 10 % in organic nitrogen removal.

From all these results we can conclude that the anaerobic fixed film reactors studied are efficient, stable, with a firmly attached biomass and appropriate hydraulic characteristics for slaughterhouse wastewater treatment.

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Effect of the Carbon Source on the Performance of a Denitrifying Unit Treating Wastewaters from a Fish Canning Industry

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Keywords *- denitrification, carbon source, nitrous oxide.*

Denitrification is a biological process allowing the reduction of nitrate from wastewaters to molecular nitrogen. To carry out this process it is necessary the presence of a carbon source acting as an electron donor. Previous works have been developed using different kinds of carbon source: glucose, acetic acid or glycerol [1] and also wastewaters coming from different sources [2]. Acetate has been proved to be an adequate carbon source allowing the obtention of very high denitrification activities.

Employed reactor was an anoxic filter full of PVC corrugated rings. In a first adaptation step reactor was fed with a synthetic solution containing sodium acetate and sodium nitrate (1 gNO_3Na-N and 2 gCH_3COONa-C) and in a second step wastewater coming from the cookers of a fish canning industry anaerobically pre-treated was used. Biomas concentration into the system was 8.34 gVSS/L. The system was kept thermostatized at 37±1ºC and without pH control.

C source	HRT (d)	C/N	removed nitrogen gN/Ld
EADC*	0.27	2.0	2.5
Acetate	0.33	1.2	3

*EADC- Effluent from an Anaerobic Digestor treating wastewater coming from fish Cookers.

The obtained results (see table) shown that the use of an effluent from a fish canning industry allows to obtain similar nitrogen removal rates by denitrification as those obtained when acetate is employed. A 20% of efficiency decrease was observed when initial feed of acetate with a C/N = 2 was changed for a wastewater feed with a C/N = 1.2. When the change of carbon source was carried out, N_2O in the biogas was produced. The presence of nitrous oxide in the gas phase is related with instabilities of denitrification processes [3]. This phenomena has still been previously observed when a failure in the feeding system was produced and also because of nutritional deficiencies. pH value was kept during the whole system operation period between 9 and 10 and N_2 content in biogas was always between 90-100%.

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Metano como Reductor y Fuente de Materia Organica en Procesos de Desnitrificacion Biologica

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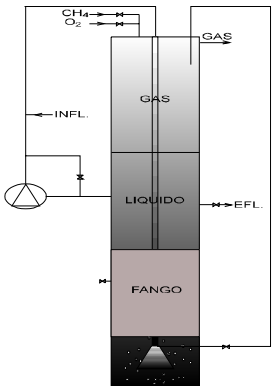
***Keywords**: desnitrificación, fuente de carbono, metano.*

Para eliminar el nitrógeno de las aguas residuales, se utiliza, normalmente, una etapa de nitrificación seguida de un etapa de desnitrificación. Debido a que la desnitrificación es, por lo general, la última fase del tratamiento de aguas residuales (cuando la mayor parte de la DQO ha sido oxidada) y a que las bacterias desnitrificantes son heterótrofas, es frecuentemente necesario añadir una fuente adicional de carbono. Con este propósito han sido utilizados compuestos como metanol, etanol, acetato y azúcares [1]. El uso de éstas fuentes externas de carbono representa un significativo coste y se tiende a utilizar fuentes de carbono que sean baratas y a la vez efectivas. De acuerdo con distintos autores, el metano se presenta como una posible y única fuente de carbono en procesos de desnitrificación ya que, además de ser barata, se encuentra disponible en grandes cantidades en numerosas plantas de tratamiento. Según la bibliografía, parece posible usar metano como fuente de carbono para la desnitrificación de agua residual tanto en presencia de oxígeno como en condiciones anóxicas [2]. Para bajas concentraciones de oxígeno, las dos hipótesis que se manejan en la bibliografía son la existencia de bacterias desnitrificantes consumidoras de metano capaces de usar nitrato como aceptor de electrones incluso en presencia de oxígeno, o bien la existencia de una asociación de bacterias metanotróficas que producen un compuesto intermedio que es utilizado por las bacterias desnitrificantes, aerobias o anaerobias, como fuente de carbono.

El proceso se ha llevado a cabo en un biorreactor continuo con biomasa adherida provisto de una recirculación de líquido, recirculación de gas y sistema de refrigeración. La agitación del reactor se consigue haciendo pasar el líquido recirculado por un eyector tipo Venturi. La sobrepresión, con el fin de evitar la entrada de aire, se mantiene gracias a una columna de agua que tiene que atravesar el gas de salida. El reactor se ha alimentado primero en discontinuo y después en continuo (flujo=2L/d) con una concentración conocida de nitrato y se ha seguido su evolución y la composición de la fase gaseosa en el reactor. La composición del gas de entrada es de 10% O_2 y 90% CH_4.



Los análisis de la fase líquida indican que, trabajando en discontinuo, la concentración de NO_3^- disminuye de 100 ppm a 0 ppm en 7 días mientras que, operando es continuo, se pasa de 170 ppm a 130 ppm, es decir, no se logra eliminar completamente el NO_3^- lo que puede deberse a que el tiempo de residencia no es suficiente. En cuanto a la fase gaseosa se ha observado una reducción de la concentración de CH_4 y se ha detectado CO_2.

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Monitoring Methanogenic Fluorescence by Image Analysis

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***Keywords**: Methanogenic bacteria, Image analysis, co-factor F_{420}.*

The presence of the co-factor F_{420} gives to the methanogenic bacteria the specific ability of auto-fluorescence when excited at a wavelenght of 420 nm. The F_{420} is found in other bacteria such as *Streptomyces*, but at levels far below those found in methanogenic bacteria [1]. Therefore, in pratice, it is possible to use the blue-green autofluorescence to differentiate between methanogenic and non-methanogenic bacteria [2]. However, the analysis of the intracellular F_{420} is not a suitable routine method due to its complexity and the lack of commercially available F_{420} molecule. Furthermore, the usefulness of this property in the identification of different genera of methanogenic bacteria is not evident and the great variability of F_{420} levels in the different genera of methanogenic bacteria, along with the sensitivity to environmental conditions, makes difficult the interpretation of experimental data. Due to the above mentioned problems, the very high initial expectations in the use of intracellular F_{420} analysis as a tool to measure the methanogenic activity were partly frustrated. However, the intensity of the blue-green emitted light after excitaton at 420 nm is potentially useful to predict the overal methanogenic activity. In general, hidrogenotrophic bacteria present higher levels of F_{420} than acetoclastic bacteria. For instance, *Methanosaeta* (ex *Methanothrix*) possess only residual levels of F_{420} [3].

Image analysis was used in this work to quantify the blue-green light intensity developed during the start-up of a CSTR fed with a VFA based synthetic substrate and during the steady state operation of an anaerobic filter fed with a synthetic dairy waste. The possibility of release of F_{420} to the medium keeping its blue green fluorescence was considered by discounting the backgroug blue green intensity in order to eliminate unrelevant information (originated probably from dead cells). The images were acquired by microphotography and subsequently digitised to a 24 bit (true color) JPEG format by a HP Deskscan. A program was written in MATLAB 5.1 (The Mathworks, Inc.), to calculate the number of bacterial cells and its fluorescence intensity. The image was split in Red (R), Green (G) and Blue (B) components. After concluding that the B channel originated a greater distinction between the dark background and the fluorescent cells, all the subsequent images were processed in the B channel only. The following operation consisted on achieving a mask of the objects brigther than a pre-determined threshold (the value of 0.88 was found to be adequate for choosing only the brighter cells). The background was then computed and subtracted from the image which compensated for illumination differences in the image. The backgroung was calculeted by extracting the darker pixel in 4x4 squares into a 128x128 image and retransforming it with the bicubic extrapolation method. A new mask was obtained from this image with the pre-set threshold 0.85. Cells in a bright background were poorly recognised by this mask, but the first mask was very useful in recognising those cells. Therefore the cells could be recognised using both masks by performing a logical *or* operation and a cell, to be considered as one, has to appear in at least, one of the two masks.

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Influence of Light and pH in the Activity of Nitrifying Bacteria

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Keywords: Nitrifying bacteria, wastewater treatment, nitrification, microbial activity

Nitrifying bacteria are aerobic microorganisms able to consume ammonia and nitrite producing nitrato. These bacteria involved in this process belong to the family *Nitrobacteriaceae*. The main genera involved in this process are *Nitrosomonas* and *Nitrobacter*, that consume ammonia and nitrite, respectively. Use in industrial scale are connected to the treatment of industrial effluents, in plants of terciary treatment. The change load and modification of the effluent composition have influenced negativily in the nitification process, due to sensible of these bacteria and their low time generation. Therefore, several factors can influence the microbial activity and the growth of this microorganisms. This work was aimed to isolate nitrifying bacteria present in the biological sludge of a waste treatment plant and to evaluate the light and pH influence in the activity these bacteria.

The cells count of the biological load were realized by *pour plate* method [1], and two oxidize bacteria of ammonia and oxidize nitrite were isolated. The experiments with this bacteria was carried out in shaker, in erlenmeyers of 500mL at 30^0C. The sinthetic medium utilized had the following composition: $MgSO_4.7H_2O$ (0,10g/L); $CaCl_2.2H_2O$ (0,18g/L); KH_2PO_4 (0,70g/L); $FeCl_3.6\ H_2O$ (0,014g/L); $NaHCO_3$ (0,50g/L); $MnSO_4.\ H_2O$ (0,135g/L); Na_2HPO_4 (13,5g/L); $ZnSO_4.\ 7H_2O$ (0,14g/L). Amonium cloride was used as ammonia source to the bacteria oxidize ammonia in the concentration of 0,05g/L and sodium nitrite (0,05g/L) was used to the bacteria oxidize of nitrite. To observe the influence of the light, it was used laminated paper in the erlenmeyers that couldn't take light. The system was iluminated with fluorescence lamps (15000lux), distant of 20cm of the frasks.

The process was evaluated by quantification of ammoniacal-N, nitrite-N, nitrate-N and protein attained concentrations, at the beginning of the tests and after 24 hours. The influence of the pH was evaluated to the values 7.0 and 8.5, and these experiments were carried out in absence of light.

All bacteria presented a best activity in absence of light with increase of ammoniacal-N removal efficiences (24%). Analogously, the increase of the medium pH (7.0 to 8.5) increased the activity these species up to 50%.

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Education on Biotechnology

GMP Training Programs at the University of Maryland, Baltimore County

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Keywords: *GMP, Regulatory Engineering, biotechnology, education*

Compliance with Good Manufacturing Practices (GMPs) requires that employers institute a comprehensive training program focusing on both regulatory and technical issues. To assist companies in meeting these requirements, UMBC has developed a four-course certificate program which concentrates on Regulatory issues, GMP requirements, quality control aspects and facility design issues associated with for biopharmaceutical manufacturing. Additionally, UMBC has developed an interactive CD-ROM based training program which companies can utilize for in-house self-paced training. This presentation will highlight UMBC's current GMP training programs and present plans for future offerings.

Novas Exigências do Mercado de Trabalho: Estudo de Caso Regional da Engenharia Enfocando a Biotecnologia

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Keywords: *formação profissional, mercado de trabalho, ensino de biotecnologia*

Buscou-se analisar alguns aspectos das novas exigências do mercado de trabalho que vêm causando um profundo impacto sobre os formandos nos cursos profissionalizantes. Estas novas exigências são decorrentes da mudança radical ocorrida nos processos produtivos e de gestão de negócios que passaram a ser implantados em vista do processo de globalização da economia mundial.

Partiu-se, neste trabalho, do pressuposto de que o sistema universitário tem objetivos pluralistas no processo de formação, buscando atender à diversidade de demandas formativas. Entretanto enfoca-se especificamente a formação de mão-de-obra para o segmento produtivo, função bastante significativa, principalmente nos cursos de engenharia.

Utilizou-se a técnica de entrevistas pessoais para avaliar a demanda do setor produtivo, sendo que estas conduziram à conclusão de que em princípio a qualificação técnica dos cursos de engenharia atendem razoavelmente as necessidades das empresas. Observou-se que o mercado busca nos recém-formados o conhecimento básico das engenharias. As empresas entendem que a adequação da formação profissional a seus processos produtivos é tarefa que lhes cabe. Deve-se considerar que os processos produtivos variam de empresa para empresa e são objeto de constantes melhorias e mudanças em vista dos avanços tecnológicos.

Os entrevistados, apresentaram como sendo a maior deficiência dos cursos de engenharia a falta de um enfoque de relações humanas, com acento maior para as técnicas de liderança e comunicações. Isto parece ser decorrente da mudança no ambiente profissional em que os engenheiros passaram a atuar, no qual devem prevalecer características que fortaleçam a habilidade do trabalho em equipe, com forte enfoque nas relações humanas. Em síntese, buscou-se uma avaliação preliminar dos impactos que o novo modelo produtivo está causando no perfil profissional do engenheiro e os reflexos no recrutamento de recém-formados.

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Brazilian Education In Bioprocess Engineering

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Brazilian studies in Biochemical Engineering are done mainly in Food Engineering and Chemical Engineering careers. Pharmaceutical and Agricultural careers, are developing more the Fermentation Technology, without the engineering concern, or with a small dedication to this subject in terms of Unit Operations.
Food Engineering become to be a professional career in 1967, at UNICAMP - the State University of Campinas, in the Food Engineering School. First group of professionals finished the course in 1971, but a complementation group finished in 1970.
Education Ministry of Brazil proposed in 1976, a minimun curriculum to be followed by the six Engineering Courses, and now, in 1996 a new legislation that try to flexibilize the Engineering and others careers, offering to the Universities, the possibility of indicate 50% of the necessary contents to prepare the professional. This flexibility is intended as a good manner to prepare the right professional to the right region, that is, the region where he lives and will exert his influence. Brazil is a big and diversified country, and regional differences in engineering formation are noted, when one analize the technical knowledge of the Engineers formed in the south and southeast and that of the north/northeast.
The centerwestern engineer is a medium term between both. Climate, soil, industrial development, are the responsible by the main differences encountered. Big Industries in Bioprocess Engineering are mostly in the south/southest region, but natural resources to instal biochemical industries, and be used as carbon and nitrogen sources at less cost are encountered in the north/northeast, as well as the manpower to be employed.
This paper will present and discuss the curricular regional differences and the implications on the day-by-day of the professional Food Engineer and the Chemical Engineer, mainly with respect to the Biochemical Engineering undergraduate formation. Some graduate courses will be also presented and discussed

O Ensino de Processos Biotecnológicos na Formação de Engenheiro Químico

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Palavras-chaves: *Engenharia Química, Biotecnologia, Ensino*

Pode-se definir Biotecnologia como o estudo e usos de agentes e materiais biotecnológicos, objetivando a produção de bens e serviços para usufruto do homem. Dentre os vários setores da Biotecnologia, há alguns específicos onde o Engenheiro Químico pode e deve atuar. Entretanto, no Brasil ainda é reduzido o número de profissionais da Engenharia Química atuando na Indústria de Biotecnologia.

O objetivo deste trabalho é a discussão dos fatores que levam a este quadro, destacando-se a distorção curricular na grande maioria dos cursos de Engenharia Química, onde não são compulsórias as disciplinas da área biotecnológica.

Na pesquisa realizada entre as Instituições de ensino Superior brasileiras que formam Engenheiros Químicos, constatou-se que grande parte dos cursos oferece, no máximo, uma disciplina na área biotecnológica, o que denota, ainda, a falta de preparo de Engenheiro Químico para atuar nesta área.

Virtual Cell: A New Approach for Undergraduate Biochemistry Practical Lessons

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Keywords: tutorial, biochemistry, internet, WWW, education

The Virtual Cell project has the goal of offering undergraduate students an support tool to help them improving their basic knowledge on biochemistry. This is being done through the development of softwares to be used in the practical lessons of this subject. The project is divided in four modules – *Enzymes and Metabolic Integration, Methods of Glucose determination, Liver metabolism and Acid Basic Balance* – each one developed as interactive tutorials. The first allows users to assay with the glycolytic enzymes focusing on basic enzymatic kinetics. The second module has nowadays glucose determination methods explained in their general principles and applications. *Liver metabolism* describes the major hepatic reactions with focus on their role in the global metabolism and homeostasis. The last one explains the systems involved in maintenance of acid basic balance, mainly on their biochemical aspects.

The modules are being developed in hypertext format with pictures and links to related sites in the World Wide Web (WWW). They are organized in sections that comprise a *revision* – historical aspects, anatomy, physiology – on useful topics, the *main subject*, updated *online resources* as support reference and *online tests* for learning evaluation. Students can also send their doubts through email. The modules are available via the internet to the users. Using this way to spread information allows professors and Bioinformatics team updating contents easily. Tutorials are being developed in Hypertext Markup Language - HTML, in LIKA intranet (Windows 95), with Pentium 133MHz - RAM 32Mb personal computers direct connected to the internet via our data processing center (NPD).

Bioinformatics Sector team and Biochemistry Department professors are evaluating the modules during biochemistry practical lessons for the medical course. Initial results are encouraging, mainly because the excellent feedback. Three aspects of the model can be mencioned: interactive interface, easy access and easy update. The modules will be incorporated to the biochemistry courses after conclusion of the evaluation period.

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Food and Plant Biotechnology

Synthetic and Natural Citrus Juices Debittering by *Rhodococcus fascians* Cells

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Keywords: Limonin, Rhodococcus fascians*, continuous reactor, immobilized cells, Navelina orange*

Bitterness due to limonin in citrus juices has become a serious economic problem. The naturally occurring form and precursor of limonin is limonoate-A-ring lactone. This limonoid, found contained in the citrus fruit, moves into the liquid phase after being extracted, where it is converted to limonin in a process accelerated by the acidic pH values of the juice and also by heat [1]. The limonin and its precursors species debittering with *R. fascians* cells in batch system at pH 4.0 and under presence and absence of aeration has been studied [2]. It reached a high celular retention on polyurethane foam pads in a continuous reactor rising the debittering capability with regard to free cells [3]. Besides, it studied the shifts in metabolism and morphology of *R. fascians* cells at debittering conditions of citrus juices [4].

In the present work the behavior of *R fascians* cells in immobilized continuous systems on synthetic citrus juices at the characteristic conditions of citrus juices (pH 4.0, 25 ºC and absence of aeration of the citrus juice) has been studied. The solid support used was polyurethane foam, due the fact that it has high structural stability and biomass retention capacity as well as being harmless to cells. A column reactor was packed with polyurethane foam cylinders and inoculated with *R. fascians* cells previously grown on synthetic juices. The continuous experiment started with the addition of the synthetic juice containing limonin (45.7 mg/L) at a dilution rate of 0.2 h^{-1}. Once steady-state conditions were reached, the dilution rate was changed gradually until it reached a value of 0.6 h^{-1}. It was select the highest limonin consumption conditions. Furthermore, two aspects of the operational stability of the reactor containing immobilized *R. fascians* cells wereere studied: continuous and discontinuous (after a storage period) operation.

On the other hand, the biotransformation by *R. fascians* cells from natural citrus juices was optimized in batch systems. Therefore, variables as cellular growth, pH and aireation of the culture were cheked in order to establish the higher limonin consumption conditions within a natural citrus juice

Finally, within every experiments, it was determinated not only limonin levels but sugars (sacarose, glucose and fructose) and citric acid consumption as well, as they could be taken up by *R. fascians* cells.

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Effect of *Ante-Mortem* Administration of Irreversible Serine and Threonine Proteases Inhibitor 3,4-DCI on Rabbit Meat Ageing

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***Keywords**: rabbit meat, ageing, proteolysis, proteases, 3,4-DCI.*

It is generally accepted that endogenous proteolytic enzymes or proteases (EC 3.4.-.-) have a major role on meat texture and flavour improvement during ageing (*post-rigor mortis* phase) [1]. However, their relative contribution to meat ageing process is still unknown, since the regulation of *in situ* activities depends on many factors [2]. Several serine proteases, with exopeptidasic activity (EC 3.4.14,16.-) as well as endopeptidasic activity (EC 3.4.21.-), were identified in mammalian skeletal muscles [3]. Threonine-dependent activity is associated with both forms (ATP-dependent or 26 S and ATP-independent or 20 S) of proteasome (EC 3.4.99.46). In this work it was investigated the possible role of serine and threonine proteases on mechanical, structural and biochemical changes occurred in rabbit meat during ageing.

Rabbits (*Oryctolagus cuniculus* L.) were administrated (iv., 5 μmol/kg b.w.) with recommended [4] serine and threonine proteases irreversible inhibitor 3,4-dichloroisocoumarin (3,4-DCI), or with inhibitor solvent (control), before slaughter. The development of meat ageing was assessed for different types of muscle (I, IIB and IID) by objective tenderness (*Warner-Bratzler* shear force), *in situ* exopeptidasic and endopeptidasic activities (variation of free amino acids and TCA-soluble peptides contents, respectively), protein solubility (at 300 mOsm and pH=6.25), myofibrillar proteins degradation (5%T and 10%T SDS-PAGE profiles) and structural myofibrillar weakening (phase-contrast microscopy), over a 9 day period of refrigeration (+4°C) after slaughter.

The results showed no significant differences (H_0: $p>0.05$) between 3,4-DCI treated and control animals for any of the parameters quantified throughout the storage period. The SDS-PAGE profiles were also similar between the two animal groups. These results are in agreement with our previous reports [3] on the absence of detectable serilendopeptidasic activity (upon hemoglobine at pH=5.50 and casein at pH=7.00) in rabbit skeletal muscles.

Our study suggests that endogenous serine proteases and threonine-dependent activities of proteasome have no role either on structural or biochemical changes occurred during rabbit meat ageing. A similar experimental design has been carried out in our lab in order to investigate the possible role of other protease types (cysteine, aspartic and metalloproteases), which results will be published in the future.

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Proteolytic Specificity of *Cynara cardunculus* Rennet vs. Animal Rennet

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Key words*: ovine cheese, enzyme, thistle, clotting, ripening*

The enzymatic action of proteinases in crude aqueous extracts of flowers of *Cynara cardunculus* were compared with that of proteinases in commercial animal rennet using ovine raw milk cheeses as substrate and urea-PAGE and RP-HPLC as analytical tecniques. Urea-PAGE of the water-insoluble extract (WISE) has shown that ovine β-casein is less susceptible to proteolysis than ovine α_S-caseins, and that the animal rennet act more intensely, in quantitative terms, than the plant rennet on ovine β- and α_S-caseins. The water-soluble extract (WSE) of cheese produced via the plant rennet exhibited electrophoretic bands which, upon transfer by blotting and N-terminal sequencing, indicated that they were accounted for by fragments of ovine β- and α_{S2}-casein. The peptide profiles of the WSE of cheeses were characterized by different patterns throughout ripening for the two rennets utilized, thus conveying important qualitative information for fundamental differentiation of proteolysis effected by either rennet. The peptides β-(f128-*), β-(f166-*) and β-(f191-*) were produced only by plant rennet, whereas the peptides β-(f164-*) and β-(f191-*) were produced only by animal rennet; the peptide β-(f1-190) was the primary product of ovine β-casein breakdown in the WISE of cheese using both rennets. The complementary peptides α_{S1}-(f1-23) and α_{S1}-(f24-191) were produced from ovine α_{S1}-casein by both rennets; although the bond Phe23-Val24 was cleaved right upon contact, in cheese manufactured with *C. cardunculus,* evidence of such clevage was apparent only after 28 d in the case of animal rennet. The peptide α_{S1}-(f24-165) was only produced when plant rennet was employed, whereas the peptide α_{S1}-(f120-199) was only produced with animal rennet. The peptides produced from ovine α_{S2}-casein in cheese could not be identified as products of proteinases contributed by the rennet, and were probably a result of action of proteinases or peptidases produced following microbial contamination.

Response of Sphaeroplasts and Permeabilized Cells of *Salmonella choleraesuis* LT2 to the Action of the Bacteriocin AS-48

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***Keywords**: AS-48 bacteriocin, biological activity,* Salmonella*, biopreservative,*

The features of the antimicrobial peptide AS-48 produced by *Enterococcus faecalis* subsp. *liquefaciens* (broad activity spectrum, heat-resistance and stability under conditions of storage) [1] make it an attractive candidate as a natural food preservative, similarly to other cationic peptides such as nisin and certain pediocins.
In spite of its broad spectrum, AS-48 is not fully effective against many Gram-negative strains involved in food spoilage or even pathogenic processes, which limits its usefulness in this field. To exert its effect, AS-48 needs to cross the cell wall barrier and to reach the cytoplasmic membrane. Resistance of Gram-negative bacteria is mediated by their peculiar cell-wall structure. An intact outer membrane (OM) prevents the diffusion of hydrophobic solutes, because its outer leaflet contains highly ordered quasicrystalline LPS, being this effect particularly remarkable in *Salmonella choleraesuis* cells [2].
The role of the outer membrane of *Salmonella* in protection against AS-48 has been demonstrated by the high resistance of intact cells and the high sensitivity of sphaeroplasts. We have attempted to bypass the OM barrier by using cells of this bacteria subinjured by heat treatment (60 ^{o}C for 3 min) and chelating agents as permeabilizers. According to the results obtained, each of the agents tested separately facilitates the activity of AS-48 on *Salmonella* in a time- and concentration-dependent fashion. The combination of two or more treatments decreased the amount of AS-48 required to reduce the surviving population of injured salmonellae. The activity of AS-48 is also significatively increased when tested at pH 9, even on intact cells.

Methods

Sphaeroplasts of *S. choleraesuis* LT2 were prepared by the method described by Rassoulzadegan *et al* [3]. Aliquots (1 ml) of the sphaeroplast suspension were treated with peptide AS-48 dissolved in deionized water. Lysis was monitored by decrease in $O.D._{620}$
AS-48 assays on *Salmonella choleraesuis* LT2. An overnight culture in Luria broth medium was centrifuged, washed and resuspended in 10 mM sodium phosphate buffer (1ml) pH 7.2 ($O.D_{620}$ = 0.155, c.a. 10^8 CFU/ml).
For AS-48 assay, the cells were added of increased concentrations before being treated with the different physico-chemical agents. Cell viability was determined right after addition of AS-48 (T_o) or after 6h incubation (T_6) at 37°C. The number of colony forming units was determined by serial dilution in sterile saline solution and plating the adequate dilutions on trypticase soja agar plates.

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Instability of the Karyotype in *S. cerevisiae* Wine Strains during Fermentations

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Key words: Instability, karyotype, S. cerevisiae.

Two wild strains and one commercial strain of *S.cerevisiae* were respectively used to start three fermentations in volumes of 500L of Albariño grape-must. In each case, 40 clones of *S.cerevisiae* species were isolated at four successive stages of vinification. Their chromosomal profiles (PFGE, TAFE system) were analysed in a gel-doc system and phenotypical properties were tested by microvinifications and other assays. In one of the three fermentations, 38 clones had a karyotype similar to the starter, but two other showed differences affecting the number of bands on the chromosome VI. Nevertheless, assimilation of carbon compounds and enological properties (ethanol, SH_2, acetic acid and glycerol production, flocculation, Killer, and ethanol and SO_2 tolerance) were similar in all the 40 strains. The instability of karyotype during fermentations and its enological implications are discussed.

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Pesticide Products Affecting the Stability of the Chromosomal Profil in *S. cerevisiae* Wine Yeast Starters

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Key words: pesticide, chromosomal polymorphism.

S.cerevisiae wine strains belonging to fermenting musts (D.O. Rías Baixas) were cultured (200rpm/28ºC/24h) in presence of a pesticide product used on the vineyard, in order to know the stability of the chromosomal profile during vegetative growth. Volumes of 10 ml of grape must were supplemented with 50mg/L of the pesticide and used for successives cultures every nineteen hours. Every 50 and 100, 150 and 200 generations, the karyotype of 10 clones was analysed. The analysis of the karyotypes was carried out in 10 clones isolated after. The clones with a modified karyotype were tested for several enological properties and compared with the parental strain. The pesticide wastes reaching the musts from the grape could be responsible, among the other factors, for the chromosomal polymorphism on *S.cerevisiae* wine populations. We comment their effect on the fermentative behaviour of strains.

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Efeito dos Processos de Conservação Sobre o *Lactobacillus Plantarum.* Indução de Mecanismos de Resistência.

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Palavras-chave: Conservação, Congelação

A fermentação da Azeitona de Mesa é um processo espontâneo basicamente conduzido por Bactérias Lácticas (BL), principalmente o género *Lactobacillus (L. plantarum e L. paracasei)*. O controlo dos processos fermentativos poderá passar pelo uso sistemático de inóculos de BL, cuja utilização a nível industrial exige estudos detalhados para a optimização, não só da sua produção como também, e particularmente, da sua conservação.

A conservação de inóculos visa garantir a estabilidade das bactérias ao longo do tempo e é igualmente uma forma de preservar todo um património microbiológico através da criação de uma colecção de estirpes de interesse cientifico e tecnológico. A eficácia dos processos de conservação traduz-se pela viabilidade das estirpes e também pela manutenção das suas caracteristicas fisiológicas, metabólicas, tecnológicas e de genoma.

É por isso fundamental recorrer a condições de conservação óptimas que garantam a sobrevivência e a estabilidade das estirpes bacterianas ao longo do tempo. A congelação e a liofilização são os dois procedimentos de conservação mais usuais.

Neste trabalho estudou-se o efeito de diversos factores intervenientes na preparação de pré-inóculos no comportamento de *L. plantarum* (LB 32A), nomeadamente pH e composição do meio de cultura, considerando o crescimento e viabilidade das células, acidificação do meio de cultura e identificação dos ácidos gordos da parede celular. Estes mesmos aspectos foram avaliados também após sujeição da massa celular formada, a diferentes processos de conservação: congelação a -80ºC e em azoto líquido e liofilização. A suplementação do meio de cultura com cálcio parece induzir uma melhoria da sua resistência à congelação, melhoria essa correlacionada com uma transição morfológica das células.

O estudo das modificações sofridas pelas estruturas das membranas celulares, principalmente, no decurso dos tratamentos de conservação possibilitará a indução de mecanismos de resistência aos mesmos, por adaptação das células durante a sua cultura em condições adequadas.

Survival of Pathogens in a Portuguese Bottled Mineral Water

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Several studies have investigated how long enteric pathogens persist when deliberately inoculated in bottled waters. Some contradictory results have been found that may be due to the different techniques used between labs, different strains used and different waters. Concern about the use of Escherichia coli as an efficient indicator have been expressed since it was found that pathogenic bacteria such Salmonella typhimurium persisted at least 70 days in still mineral water.

Survival of Escherichia coli, Salmonella typhimurium, Staphylococcus aureus and Yersinia enterocolitica inoculated into a Portuguese bottled mineral water was investigated under light and dark conditions. It was observed that survival was longer under dark conditions for all the organisms investigated. For both conditions Salmonella typhimurium and Escherichia coli showed the highest survival followed by Staphylococcus aureus and Yersinia enterocolitica.

The authocthonous flora increased during storage, apparently not being affected by light or dark conditions.

Characterization of Bacterial Isolates from Serra da Estrela Cheese via Analysis of their Electrophoretic Protein Profiles

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Keywords: Cheese ripening, Lactic acid bacteria, SDS-PAGE, taxonomical identification

Improvement of traditional food processing procedures requires in-depth knowledge of prevailing adventitious microflora. In recent years, serious efforts in that direction have been taking place via certification towards standardization of Serra da Estrela cheese; this cheese, manufactured from raw ewe's milk and coagulated enzymatically using a wild variety of the thistle flower, is a widely appreciated dairy product with unique organoleptic characteristics.

One-hundred and thirty two cheese samples from six different dairies at eleven times within the ripening period were analysed microbiologically using selective media for enumeration of the various microbial groups. Colonies were isolated, purified and kept in slants for posterior identification. Bacterial protein extracts from the lactic acid bacteria isolates were prepared and identified by computer-assisted comparison of sodium dodecyl sulphate electrophoretic patterns of whole-cell proteins (SDS-PAGE). Profiles were compared with those of reference strains comprising the following species: *Lactobacillus plantarum*, *L. paracasei* subsp. *paracasei*, *L. pentosus*, *L. brevis*, *L. fermentum, L. curvatus*, *Lactococcus lactis* subsp. *cremoris*, *Lc plantarum*, *L. lactis* subsp. *lactis*, *Enterococcus faecium*, *E. durans*, *E. faecalis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *L. mesenteroides* subsp. *dextranicum*, and *L. mesenteroides* subsp. *mesenteroides*. The relative abundance of identified isolates were correlated with ripening time, geographical location, herd size and total milk and cheese production of the corresponding dairy.

Biotic Interaction Between Traditional Portuguese Cheese's Ripening Microflora and *Listeria monocytogenes* Scott A

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Key Words: Listeria monocytogenes*; Traditional Portuguese's Cheeses; Organic Acids; Hidrogen Peroxide; Listeriocins.*

Listeria monocytogenes in cheese has been responsible for several outbreaks and sporadic cases of listeriosis in North America and Europe. This pathogen behaves differently in cheeses depending on the type and processing technology. Last years studies concludes that soft and semi-soft cheeses provides best survival and developing conditions to this pathogen [1].
Recently, Lactic acid bacteria, as well as *Enterococcus spp.* have been studied for their potencial application on the control of *Listeria monocytogenes* on foods, as they produce substances that inhibit this organism, such as organic acids, hidrogen peroxide and bacteriocins.

Our study concerns about the role of the ripening microflora of some portuguese traditional hard and semi-hard cheeses on *Listeria* colonization.

From ripening microflora 531 isolates were screened for *Listeria* antagonistic activity on a primary selective assay using PCA (Difco 0479-01-1) and All Purpose Tween (Difco 0654-17-0) plates previously inoculated with 10^6 CFU/ml of the indicator strain (*L.monocytogenes* Scott A). Two hundred and eight (39,2%) of them exhibited inhibitory capacity towards the indicator strain.

The inhibitory mechanism identification was accessed by a deferred antagonistic test using active cell-free supernatants, acording to the Pilet *et al.* (1995) [2] technique.
Sixty-two of those isolates (29,8%) produced filtrable substances, which were able to inhibit the pathogen. Twenty-five inhibitions were attributed to the organic acids production, 37 to the hidrogen peroxide production and 14 to the simultaneous production of these two products. No listeriocin production was observed. The agents that expressed a capacity anti-*Listeria*, belonged to the *Enterococcus*; *Lactobacillus*; *Lactococcus*; *Leuconostoc* and *Staphylococcus* Genera.
These results represent a relevant beginning to the control of *Listeria spp* in traditional cheeses from Portugal.

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Single-Strand Conformation Polymorphism (SSCP) Analysis in the Serrana Transmontana Breed of Goats

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Keywords: SSCP, Goat, Variability, Polymorphism

Denominations of origin (D.O.) have been attributed, in 31/01/94, to the following products of Serrana breed of goats from Trás-os-Montes region (Portugal): cheese "Serrano Transmontano" (licence issued 20/94); meat "Cabrito Serrano Transmontano " (licence issued 21/94) [1]. These represent an important factor for the livelihood of farmers and shepherds because they guarantee products' quality.

The Serrana breed of goats, whose origin is lost in time, probably had its origin in the Serra da Estrela from the wild goat *Capra pyrenaica*, a ascendant of the goat breeds of the Iberian Peninsula, is most representative of the Portuguese goat breeds, that can be found throughout the Country both north of the river Tejo and in the peninsula of Setubal to the south.

This goat breed is considered a breed with great productive potentialities and with considerable possibilities for expansion, due to its high level of adaptability to varied conditions, productive and reproductive indices and its products of high quality: milk (cheese) and meat [1].

The aim at the research was to study molecular markers capable of helping in a molecular characterization of the Serrana breed of goats, attributing their products with a denomination of origin, and to support molecular-assisted selection (MAS); consequently, we proceeded to DNA analysis through single strand conformation polymorphism (SSCP).

The genomic DNA was acquired from blood taken from animals of flocks owned by members of the Associação Nacional de Caprinicultores da Raça Serrana in Trás-os-Montes. The DNA extraction was carried out by the high salt method [2]. After PCR amplification for a exon of the receptor of the growth hormone (GHR), and denaturation of this product, we proceeded to SSCP analysis in polyacrylamide gel. Some polymorphism has been found.

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Grape Processing Wastes as a Nutrients Source for Yeast Cultures

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Key words: nutritive medium, wastes, pectinases.

The winery-wastes belonging to the Albariño grape variety (N.W.Spain) has been used to elaborate a nutritive medium. The medium has been obtained from grape-skin *(Vitis vinifera* var. Albariño) by aqueous extraction at the higher temperatures. It was tested to growth five commercial and wild strains of *Sacharomyces cerevisiae, Aureobasidium pullulans and Sporobolomyces roseus* species. Yield of biomass and pectinases production were quantified and compared with the yield obtained with the other synthetic and natural media. The results show the interest of the winery-wastes recycling for industrial purposes.

Action on Bovine k-casein of Cardosins A and B, Aspartic Proteinases from the Flowers of the Cardoon *Cynara cardunculus* L.

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***Keywords**: k-casein, cardosin A, cardosin B,* Cynara cardunculus *L., aspartic proteinases*

Animal rennets are probably the first milk-clotting enzymes used in cheese manufacture, however in several mediterranean countries – namely Portugal – flowers of cardoon are traditionally used as rennets in ewe cheeses since the Roman Era. On the contrary to cardosin A and cardosin B, two milk-clotting enzymes from the cardoon *Cynara cardunculus* L., the other vegetable rennets studied [1] [2] [3] [4] are inadequate to be used as rennet substitutes since they all have a low ratio of milk clotting to nonspecific proteolytic activity. Because of the importance of k-casein in the process of milk-clotting and in cheese manufacturing, the aim of the present work was to study the action of cardosin A and cardosin B on isolated bovine k-casein and at the same time to assess the contribution of each cardosin for the clotting process.

In this way, bovine k-casein was separately digested with cardosin A and cardosin B and the casein digested peptides were separated either by SDS-PAGE or by RP-HPLC and their N-terminal amino acid sequences were subsequently determined by automated Edman degradation, thus identifying the cleavage sites.

The cleavage of k-casein by both cardosins was not affected by small changes in the pH value of digestion buffer (6.2 and 6.8), both enzymes showed a broader specificity when compared with chymosin under similar conditions and cardosin B revealed a higher hydrolysis rate. Consistently with results obtained with other caseins [5] [6] cardosins preferably cleaved bonds between hydrophobic and bulky side chains but also showed some degree of affinity for bonds involving small polar aminoacids. The peptide bond Phe105-Met106 was the most susceptible to cleavage by cardosin A and cardosin B. In spite of the preferential cleavage of this bond, minor cleavage sites in para-k-casein region were also identified when k-casein was digested with cardosin B. Two of these para-k-casein fragments have in their sequences peptides with opioid antagonistic activities. The caseinomacropeptide fragment was not cleaved neither by cardosin A nor by cardosin B. Based on these results, a model for the action of cardosin A and cardosin B on bovine k-casein is proposed and discussed.

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Efeito de Agentes Químicos, Permeabilizadores da Membrana Celular, na Actividade Proteolítica de Células em Suspensão de *Cynara cardunculus* L.

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Palavras-chave: Cynara cardunculus *L., actividade proteolítica, permeabilização, agentes químicos, sonicação.*

A cultura de células em suspensão de *Cynara cardunculus* L. tem vindo a ser estudada como potencial fonte de enzimas coagulantes e proteolíticas. Técnicas como a imobilização ou a permeabilização das membranas podem ser encaradas como alternativa para o melhoramento da actividade proteolítica da suspensão celular.

Com este trabalho pretendeu-se optimizar a actividade proteolítica de células, previamente filtradas a partir da cultura de suspensão celular de *C. cardunculus*, para diferentes volumes e valores de pH relativos ao tampão de extracção e diferentes tempos de sonicação. Procurou-se ainda testar a permeabilização da membrana celular, mediante a utilização de agentes químicos, através da actividade proteolítica.

Os estudos de optimização da actividade proteolítica, em suspensão celular com sete dias de subcultura, em *erlenmeyer* (500 ml), foram efectuados pelo método de Twining (1984) utilizando a caseína derivatizada com isotiacianato de fluoresceína (FITC) a 1%, sendo a actividade proteolítica definida como o aumento da fluorescência relativa. No tratamento com os agentes químicos éter e tolueno, estes foram adicionados directamente nas suspensões celulares (1:1), para o caso do dimetilo sulfóxido -DMSO- e citocromo-c, as células, previamente filtradas, ficaram em contacto com soluções a 10% e 0,01% respectivamente.

O método de extracção utilizado na determinação da actividade proteolítica foi optimizado, tendo-se obtido um protocolo experimental visando a sua aplicação em células em suspensão de *C. cardunculus*. A sonicação revelou-se mais eficaz do que os agentes químicos ensaiados (éter, tolueno, DMSO e citocromo-c). Destes agentes foi o DMSO que produziu melhores resultados.

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Produção de Concentrados Protéicos dos Rejeitos da Indústria de Laticínios Através do Processo Adsortivo

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Palavras-chave: *concentrados protéicos, lactosoro, processo adsortivo*

Indústrias como as processadoras de alimentos produzem uma grande quantidade de rejeitos ricos em proteínas, aminoácidos e outras substâncias que podem ser utilizadoas para a alimentação animal.

Os experimentos conduzidos em escala laboratorial à temperatura de 25 $\pm$ 1°C; vazão de ar 66,66 l/h e em um volume de 100 ml, pelo período de 30 minutos mostraram que o processo adsortivo com bolhas por flotação em coluna é indicado para a recuperação do material protéico, presente em rejeitos das indústrias de laticínios, com atividade superficial de soluções diluídas. Este processo apresenta baixo custo operacional e de instalação.

No lactosoro permaneceu vitaminas hidrossoluveis, minerais, lactose e proteínas de alta qualidade tornando-o um produto muito nutritivo, por possuir proteínas constituídas de aminoácidos essenciais à nutrição animal.

A eficiência do processo está associada às características de equilíbrio entre a proteína no líquido e a operação e projeto da coluna de flotação, dependendo também da natureza das substâncias, do pH e do tamanho das bolhas.

Obtivemos ao final do processo, um recuperação de 73% das proteínas inicialmente existentes no lactosoro usando-se o carboximetilcelulose como agente auxiliar na precipitação de proteínas.

Atualmente, os concentrados protéicos de soro de leite estão sendo utilizados na fabricação de Hamburgueres, Sorvetes Dietéticos e Refrigerantes.

Apoio: FINEP-FUNCAMP.

Aroma Production by *Kluyveromyces marxianus* in Solid State Fermentation Using "Giant Palm" Bran as Substrate

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***Keywords**: Aroma,* Kluyveromyces marxianus*, solid state fermentation, giant palm bran*

Flavor and fragrance compound synthesis by biotechnological process nowadays plays an increasing role in the food, feed, cosmetic, chemical and pharmaceutical industries, due to an increasing preference of the consumer for natural food additives and others compounds of biological origin. Many microorganisms are able to produce flavors by fermentation of simple nutrients such as sugars and amino acids [1].
In this work the production of fruity aromas by the yeast *Kluyveromyces marxianus* in solid state fermentation, using "giant palm" bran (*Opuntia ficus indica*) as substrate was studied. In order to test the influence of the process parameters on the *K. marxianus* culture to produce volatile metabolites, a 2^5 statistical experimental design was performed. The parameters studied were pH, carbon-nitrogen ratio, cultivation temperature, initial water content and inoculum size. The volatile metabolites were measured by the headspace analysis on a Hewlett Packard 5890 Gas Chromatograph equipped with a 0,53 mm, 30 m polar capillary column and a FID detector at temperature of 250 °C. Experiments were performed in 250 mL erlenmeyer flasks filled with 20 g of palm bran and covered with six layers of gauze. A sensorial evaluation was employed to characterize the aroma of the cultures. At the end of 96 hours of incubation, total and reducing sugars were quantified by Somogyi-Nelson method, final pH and water content were determined.
The initial pH of the medium and the carbon-nitrogen ratio were statistically significant at 5 % level on the production of volatile compounds and on the residual sugar content. Aroma production increases in a slightly acidic pH (6.5) and in the highest level (21) of carbon-nitrogen ratio studied . These results show the feasibility of using "giant palm" bran as a substrate to produce fruity aroma by *Kluyveromyces marxianus*.

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Phase Contrast Microscopy Coupled to Image Analysis as a Rapid Method to Monitor Wine Flora

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Keywords. Image Analysis, Wine, Phase Contrast Microscopy

In food industry, there is a need for the development of rapid methods to monitor microflora at any time during processing. Conventional methods are time consuming and have poor sensitivity and specificity. The use of routine microscopic techniques coupled to image analysis is a powerful method that has already been applied successfully to several industries.

Vinho Verde wines, together with Port wines, provide Portugal's main wine exports. *Vinho Verde* wines are characterised by having a relatively low alcohol content and quite a high malic acid content. High levels of acidity are sometimes undesirable. To control the acidity of the wines, deacidification process can be done using lactic acid bacteria that convert malic acid to lactic acid – the malolactic fermentation.
The presented results describe the use of phase contrast microscopy coupled to image analysis to simultaneously monitor bacteria and yeast during *Vinho Verde* fermentation.

Several factors had to be considered during software development – different size and motility of bacteria and yeast as well as different brightness and the presence of impurities. To solve this problem an algorithm for each of the species under consideration was prepared. For the counting of bacteria, a method based on the difference between two consecutive images caused by bacteria movement was developed. For yeasts, a two-stage process was prepared. Firstly, all unwanted pixels were eliminated and then individual objects were individualised and counted.

This method has proved to be suitable for simultaneous counting of lactic acid bacteria and yeasts.

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AFLP Analysis of Genetic Diversity in Collections of Grapevine (*Vitis*) and Orange Trees (*Citrus*). Applications in Cultivar and Clone Identification

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Keywords: *AFLPs, Cultivar identification, Clone identification,* Vitis*,* Citrus

Genetic improvement of many cultivated woody species relies on the identification of sports or somaclonal variants and their vegetative propagation. This method known as clonal selection has been highly effective in the development of cultivars for wine grapes and oranges with improved agronomic and organoleptic features. Unfortunately, cultivars produced by clonal selection are genetically and morphologically very similar and can only be distinguished on the basis of their agronomic behaviour. This genetic similarity hampers their genetic identification and prevents the protection of newly developed cultivars by the breeders.
In order to develop molecular methods to distinguish specific clonal selections, we have used AFLPs (Amplified Fragment Length Polymorphisms) (1) and SAMPLs (Selective Amplification of Microsatellite Polymorphic Loci) (2) molecular markers to analyze the levels of genetic diversity in several collections of grapevines and orange trees. When different *Citrus* species or different non-clonal grape varieties were considered, both AFLPs and SAMPLs allowed the characterization of ca. 100 loci per reaction, with 50 to 80% of them being polymorphic for the presence or absence of the amplified fragment. With these genetic materials a single AFLP or SAMPL reaction is enough to distinguish all the different accessions tested in *Vitis vinifera* (3) and in *Citrus* providing a fast alternative to the identification of species or cultivars of non-clonal origin.

AFLPs and SAMPLs were also useful in the identification of genetic differences among grapevine clones belonging to the same cultivar and among different orange cultivars originated from somaclonal variants. Using four primer combinations, we were able to analyze the ocurrence of polymorphism for more than 200 and 500 loci, respectively in *Vitis* and *Citrus*. The results of this analysis were enough to distinguish most of the clones and cultivars tested. Thus AFLPs and SAMPLs provide a rapid screening tool for the identification of nucleotide sequence variation in anonymous regions of a given genome. Their application for cultivar and clone identification, shown here for *Vitis* and *Citrus*, can have important implications in the protection and management of clonal selections.

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RAPD analysis of genetic variability in *Quercus suber*

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Keywords: *RAPD,* Quercus suber, *biodiversity, DNA polymorphism*

The production of cork plays an important role in the economies of Mediterranean countries. However, genetic improvement of the cork tree (*Quercus suber*) has lagged behind. In the foreseeable future one can also envisage potential new uses being given to cork by innovative materials technologies. Different individuals can be valuable sources of useful genes. A thorough understanding of the genetics and evolution of this species, is thus of pressing importance.
A major goal in the present study is to assess the genetic variability available in the almost wild Portuguese populations of this species. We have evaluated the feasibility of using random 10-mer primers for PCR amplification of DNA, as a source of polymorphic markers to characterise the individuals and their populations.

Total DNA was extracted from leaves of 90 cork trees originated from the populations of Azaruja (Évora), Santiago do Cacém, S. Brás de Alportel, and Besteiros. These samples represent four of the nine populations to be covered by this study, geographically spanning the country. As a control, total DNA was extracted from cork tree plantlets presumably being parasite-free as they had been regenerated by meristem culture. Total DNA was isolated as well from three specimens of *Quercus ilex* also cohabiting in the population of Azaruja.
Fifteen primers were found to generate polymorphic band profiles, and can be used in the RAPD analysis of variability. Individual trees are discriminated by many of the primers tested, even though a general species-specific commonly shared band pattern is observed. Some of the primers are also capable of differentiating the different populations. The polymorphism thus obtained will be used to generate reliable phenograms.

The RAPD data will be crossed with those from microsatellite analysis which is also being performed in our laboratory. This work is being developed within a broader project involving seven National Institutes and is supported by the PRAXIS XXI project 3/3.2/FLOR/2100/95.

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Identification of Genes involved in Lignin Biosynthesis in Maize

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***Keywords**: COMT, CAD, CCR, lignification, maize*

Lignin is an essential component of vascular plants being required for structural support and in pathogen defence. In spite of its importance, lignin represents an undesirable component of agronomically and industrially important plants. In forage crops, such as maize, high lignin content and/or lignin composition were linked to a reduced digestibility. The characterisation of the pathway that leads to lignin biosynthesis in such an important crop species represents the first step toward future biotechnological applications.

Previously, the caffeic acid O-methyltransferase (COMT) of maize was cloned and characterised by northern blot analysis, by *in situ* hybridization and by studying its expression in transgenic tobacco and maize plants, using its promoter fused to the GUS reporter gene [1, 2]. We also identify this gene with the *bm3* mutants in maize [3]. All these analyses showed that in maize the COMT mRNA was mostly detected and expressed in the elongation region of the root, in the tracheary elements and in the metaxylematic vascular cells. In the meristematic region of the root, corresponding to the root tip, no COMT mRNA and no GUS expression were detected. These results clearly indicate that in maize the lignification process takes place mainly in the elongation region of the root.

To clone other genes related to lignin biosynthesis, a cDNA library of the non-meristematic part of the root was constructed. Using poly(A)+ RNA from the root meristem (not lignifying), several clones were isolated by differential screening and characterised. Among the clones analysed, were identified some peroxidases, two SAM-synthetases, two new O-methyltransferases (which might be related to lignification). Also using cDNA clones from other plants as heterologous probes, we can cloned a cDNA for cinnamyl alcohol dehydrogenase (CAD), a cDNA for cinnamoyl CoA reductase (CCR) [4], and other genes of the biosynthetic pathway of the lignin. Here we describe its sequences and its patterns of expression.

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Expression of *rbc*S and *rbc*L Genes during Senescence and Regreening of *Zantedeschia aethiopica* Floral Spathe

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***Keywords**: Rubisco, rbcS, rbcL, regreening,* Zantedeschia aethiopica

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco) is a key enzyme in both Calvin-Benson and oxidative glicolate cycles, catalising both the carboxylation and oxygenation of ribulose-1,5-bisphosphate. In algae and plants, Rubisco is a soluble stromal protein, showing an Mr of 550 kDa. In its native form, Rubisco has two different types of subunits, namely eight large (rbcL) and eight small (rbcS) subunits. *rbc*S genes, present in nuclear DNA as a multigene family, are translated in cytoplasmic ribosomes and transported across envelope membranes, while *rbc*L genes, present has a single copy in chloroplast DNA (cpDNA), are translated in plastid ribossomes. Due to these features, the establishment of the quaternary struture of Rubisco occurs in stroma chloroplast, as a consequence of a coordinate action of both nuclear genome and cpDNA. The expression of *rbc*S and *rbc*L genes are under control of several distinct factors, such as light and cytokinins.

The floral spathe of *Zantedeschia aethiopica* (L.) Spreng. undergous a senescence-regreening process during its development [1], namely evident changes in photosynthetic capacity, in particular in what concerns Rubisco activity and content, as shown previously [2, 3]. In this work we report the identification and cloning of a *rbc*S and *rbc*L genes from *Z. aethiopica*. *rbc*L gene was identified by screening a cpDNA library using an heterologous probe, while *rbc*S gene was obtained by random sequencing of cDNA clones. Expression analysis during spathe development were performed by Northern blotting followed by hybridisation with homologous ^{32}P-labelled probes. The results will be discussed considering the effect of endogenous cytokinins as senescence inhibitors and regreening inducers.

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Making Molecular Markers a Routine Tool in Agriculture

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Keywords: Molecular markers, Isozymes, RAPDs, AFLPs.

The modernization of Portuguese agriculture will require extensive utilization of new technologies, including molecular biology techniques. Analysis using molecular markers has therefore been carried out in the laboratory of Genetics and Plant Breeding, University of Algarve, during the past few years. Isozyme and DNA markers (RAPDs and AFLPs) are currently used for several purposes including genetic fingerprinting of commercial and traditional cultivars of fruit species such as *Citrus*, fig, almond and strawberry Assessment of genetic variability in traditional germplasm of *Citrus*, evaluation of apomixis level in rootstock varieties and identification of hybrid plants and their progenitors are other applications. Molecular markers were used to characterize entries in already established germplasm field collections allowing the detection of several errors on them. Molecular characterization of plant materials is now being used simulytaneously with traditional methods in establishing new germplasm collections. Furthermore, discrimination between zygotic and nucellar (somatic) rootstock seedlings, variety purity assessment and cultivar fingerprinting are three main domains where molecular markers have been used in collaboration with private plant growers. Molecular markers linked to resistance genes have also been investigated in pineapple and *Brassica* sp. in collaboration with other research teams. The discrimination limitations of isozymes are usually overcome by the complementary utilization of the more numerous, but dominant, RAPD markers. Additionally, AFLP markers are used when a more fine genomic analysis is required and RAPD markers are unable to perform it.

Bioprocess Engineering to Get Agrobiological Products - the Case of *Bacillus thuringiensis*

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Brazilian studies on *Bacillus thuringiensis* production, a bacterial insecticide, using submerged fermentation are in development since 1971 and Master and PhD thesis were proposed to determine the best way to producce Bt in small regions. A Brazilian Patent of the productive process was deposited in 1976.

Brazil is a big country and several insect pests cause serious problems in the field (raw material) or during the storage (raw and processed goods) being as far as 40% the total losses. Initial projects tried to determine the convenient culture media composition and the operational conditions in terms of temperature, agitation and aeration rates, time for geting total sporulation in batch culture. Two thesis Master and PhD were developed, before 1976. In a second phase, the studies tried to find agricultural residues and wastewaters, to get a culture medium with the necessary nutrients, but a low cost. Several by products were studied to design culture medium composition, related to the Brazilian region/country. The number and diversity of residues and wastewaters is very big. Studies in continuous fermentation were tried, and a Master thesis was developed.

In this aspect, media containing sugar cane molasses, corn steep liquor, cassava meal, "manipueira" (a wastewater from cassava meal production), coconut wastewater, sugar cane bagasse, milk and cheese wastewaters, cashewnut molasses, and so an, were studied, and the culture medium was established.

The study of solid state fermentation began at 1978, just to adapt the production technology to regions were manpower were defficient. This type of research was subject of a PhD thesis. Studies both in the endo and exo toxins of Bt, were developed and a second patent of a thermostable exotoxin production was deposited in Brazil. The S. Paulo Governor Prize was done to this patent as the best invention of 1985.

Following this work it was verified the importance of water activity determination, to conduct the process in a best way, and a Master Science thesis was developed. The process of recuperation of the product by drying method get another Master Science thesis, comparing the conventional drying to the spraydrying method.

By now the Master Science thesis that is being developed treat of the use of chicken blood as a Nitrogen source to get *Bacillus thuringiensis* doing the comparation with ox blood. Brazil has a lot of small and big slaughterhouses, and this type of wastewater is lost, causing pollution problems to the ecosystem, so, its use is very interesting.

This paper will present the results of those mencioned Master and PhD thesis and the possibility of small and localized production of *Bacillus thuringiensis* in distant regions of the country, using no specialized manpower and residues and wastewaters producced in the region, as well as a better use of the Carbon and Nitrogen sources, now lost or object of environmental pollution.

Determination of *Subtilisin* Inhibitor in Legumes by pH-state Method

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Keywords: Subtilisin*, Protease inhibitor, Enzymatic hydrolysis.*

Legume seeds contain a wide range of components with adverse effect on enzyme activity, digestibility, nutrition and health. Inhibitors of digestive enzymes are common components of grain legumes and reduce protein digestibility, depress growth and cause pancreatic hypertrophy. Among these inhibitors, trypsin and chymotrypsin inhibitor presence in legumes has been extensively studied. However, information on the inhibitory activity of bacterial proteases, like *Subtilisin*, is limited.

Usually, methods for determination of inhibitory activity consists of a prior extraction of inhibitor from seeds and a subsequent analysis through enzymatic hydrolysis: a given protein substrate (casein, BAPA, TAME, …) is hydrolyzed by the enzyme which inhibitor is analyzed in presence of some amount of extracted inhibitor [1] [2] [3].

The aim of this work has been the evaluation of the inhibitory activity of a bacterial protease, *Subtilisin Carlsberg*, in a legume, chickpea (*Cicer arietinum*), by using the pH-state method.
This method, usually used for following proteolytic reactions [4], allows a direct analysis of *Subtilisin* inhibitory activity from chickpea: previous inhibitor extraction is not necessary and hydrolysis substrate is the same legume protein.

In this method ground legume suspensions are hydrolyzed and hydrolysis rate is measured for several enzyme and substrate concentrations.

From results obtained a ratio between reaction rate, enzyme amount and legume amount has been found. From this ratio and for a zero hydrolysis rate, the activity of *Subtilisin* inhibitor present in chickpea (amount of inhibited enzyme per weight of sample) can be determined.

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Substantial Reduction in Time for Production of Mature Transgenic Woody Fruit Plants

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Keywords: sweet orange; Citrus*; woody; transformation; mature*

Transformation systems from mature plant material of woody fruit species have to be achieved as a necessary requirement for the introduction of useful genes into specific cultivars and the rapid evaluation of resulting horticultural traits. Maturation and aging seem to be responsible for the explant regenerative potential decline found in plant tissue culture of most woody species [1], which difficults the application of genetic transformation to mature tissues of commercial varieties. Citrus is the most extensively grown fruit crop worldwide and sweet orange (*Citrus sinensis* L. Osbeck) accounts for approximately 70% of the citrus total production. Up to 20 years may be needed for sweet orange plants to lose juvenile characters and be useful for commercial cultivation. Transformation of juvenile tissues of this genotype, although in a low frequency, has been already achieved [2]. We report here the development of a transformation procedure from mature tissues of this genotype that greatly reduces the time required for plants to flower and bear fruits.
Several factors were optimized for attempting the trasformation of mature tissues of sweet orange plants. Improved regeneration frequency from mature explants was obtained by invigoration of the plant material through grafting of mature buds on juvenile seedlings. Cocultivation of the explants in feederplates after inoculation with *A. tumefaciens* resulted in enhanced transformation frequencies. Furthermore, *in vitro* shoot-tip grafting of the regenerated mature shoots on seedling rootstocks provided a rapid and efficient system for plant production. This procedure led to the obtention of mature transgenic sweet orange plants, flowering and bearing fruits in 14 months after transferring to the greenhouse, that had stably integrated and expressed the transgenes. A field trial is being carried out, in order to analyze the inheritance and stability of the transgenes and the phenological characteristics of the transgenic trees.
We are already using this transformation and regeneration system to introduce genes capable to confer agronomically important characters to citrus cultivars. Furthermore, we are trying to adapt the developed system to other woody fruit species.

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Peroxidase Activity Related to Lignin Biosynthesis in Cell Suspension Cultures of *Ficus carica*

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Key words: lignin, biosynthesis, suspension culture, peroxidase

Lignin biosynthesis in plants is a complex enzymatic and non-enzymatic process. However, lignin is polymerized from only three molecules: p-hydroxyphenyl, guaiacyl and syringyl residues. The process of regulation of these biosynthetic pathways is therefore of great importance. We have been using fig tree (*Ficus carica* L.) cell suspension cultures to establish the protocols for these studies. In order to characterize the growth of fig tree cells, suspension cultures of *F. carica* were obtained from *calli* from the hypocotyl. These suspension cultures were maintained on B5 medium [1], supplemented with 20 $g.L^{-1}$ sucrose, 1 $mg.L^{-1}$ 2,4-D and 0.1 $mg.L^{-1}$ BAP. They were kept in the dark, at 28 ± 1 ºC, and subcultured every 8 days. The growth of the cultures was characterized by a dissimilation method [2] which allowed to distinguish a lag phase of 9 days from a long exponential phase of 21 days, and a stationary phase from the 30th day of culture. The growth rate of the suspension culture was 0,07 day^{-1} and its doubling time (td) was 9.9 days.
The presence of lignin was histochemically tested in the cells using the floroglucinol test, which was positive, indicating that there were coniferaldehyde terminal groups in guaiacyl lignins, and the Maüle test, which was negative, indicating the absence of syringyl lignins [3]. Peroxidases are of primordial importance for lignin biosynthesis. Two peroxidases were studied: guaiacol peroxidase (GPX) and ascorbate peroxidase (APX). The activities of GPX and APX were detected only in the culture medium, after filtration and ultrafiltration (with a 10 kDa membrane) of a sample from a suspension culture in the stationary phase. The highest GPX activity was 94,1 nmol guaiacol $(mg\ protein)^{-1}\ min^{-1}$. The APX activity was higher [211,9 nmol ascorbate $(mg\ protein)^{-1}\ min^{-1}$].
These results confirm that there is synthesis of guaiacyl lignins in the cells of *Ficus carica* in suspension cultures. The use of reverse phase-HPLC in order to detect aromatic compounds in the cells indicates they probably produce ferulate and cinnamate, that are both intermediate compounds of the biosynthetic way of guaiacyl lignins.

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Achillea millefolium Hairy Roots: Growth, Essential Oil Composition and Antibacterial Activity in Comparison with Parent Plant Roots

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Two hairy root clones of *Achillea millefolium* were obtained by transformation using *Agrobacterium rhizogenes* strains LBA 9402 and A4 70GUS. The growth of the hairy root cultures, measured by the dissimilation method, showed similar rates up to 40 days after subculture. A4 clone reached the stationary phase by that time, whereas the LBA clone showed a decrease in dissimilation only 60 days after subculture.

The two clones of hairy roots, and roots from the parent plant were subjected to hydrodistillation to estimate the oil yields. The essential oil isolated from the parent plant roots was obtained in 0.1% (v/w) yield, whereas that from the hairy roots attained 0.05% (v/w). The oils isolated by distillation-extraction, both from the hairy root clones and from the parent plant roots, were analysed by GC and GC-MS. Oxygen-containing sesquiterpenes constituted the major fraction (24.3%) of the oil isolated from parent plant roots, *epi*-cubenol (16.6%) being the main constituent. The essential oil composition was quite similar for both hairy root clones, showing only some differences in the relative amounts of the individual components. The fraction of oxygen-containing monoterpenes was dominant in both cases (28.9% for A4 and 42.0% for LBA), neryl isovalerate being the major constituent of these oils (24.1% and 36.5% for the A4 and LBA clones, respectively).

The antibacterial activity was determined by the agar diffusion method. The essential oil from the parent plant roots showed activity against 3 out of the 10 bacterial strains tested. Growth inhibition zones were obtained against *Bacillus subtilis* (8.4mm), *Staphylococcus aureus* (9.7mm) and *Micrococcus luteus* (12.0mm). The essential oil from the hairy roots showed activity against 2 out of the 5 bacterial strains tested, namely *B. subtilis* and *Staph. aureus*, with 9.4mm and 6.6mm growth inhibition zones respectively.

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Induction of Proteinase Inhibitors in Callus of *Lycopersicon sculentum*

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***Keywords**: Callus, proteinase inhibitors, tomato,*

The proteinase inhibitors (IPs) are molecules whose involvement in plant response to mechanical stress conditions have been widely studied [1]. IPs induction have been described upon the attack of fungi, insects and mechanical wounding [2]. The accumulation of IPs occurs in the damaged tissue as well in places located distal to the wound site. However, the factors involved in the IPs activity in low differentiated tissues of tomato or potato are unknown. In this work we studied some factors that regulate IPs activity in callus of *L sculentum* cvs. Packmor and Roma.

Callus were induced frown shoots of tomato seedlings using MS culture medium. Callus showed an inhibitory activity against tripsin (T) and chemiotrypsin (QT) higher than those found in tomato leaves. Packmor callus have a higher IPs activity against QT than T, while Roma callus showed a high IPs activity against T. In addition, water stress induced by PEG-8000 did not affect IPs activity while salt stress or low temperature (4ºC) affected differentially the activity of IPs in both tomato cultivars.

By the other hand, abscisic and jasmonic acid affected IPs activity depending of the cultivar. This may reflect differences in the sensibility of the cells to the hormones, differences in local concentration of hormones or differences in the rate of uptake of both hormones.

Thus, tomato callus represent a suitable system for study at molecular level factors involved in the regulation of IPs activity.

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Experimental Formulations of *Bacillus subtilis* for the Control of Root Rot (*Fusarium solani* f. sp. *phaseoli* and *Rhizoctonia solani*) of Bean (*Phaseolus vulgaris*)

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Keywords: Bacillus subtilis, formulation, Fusarium solani, Rhizoctonia solani.

The aim of this work has been to obtain a biological fungicide able to control root rot of bean.
Six experimental formulations of *Bacillus subtilis* were developed for the control of *Fusarium solani* f.sp. *phaseoli* and *Rhizoctonia solani* .
Bean seeds of two cultivars (IAPAR 14 and Pérola) were inoculated with cells and metabolites of *B. subtilis* using the following substracts: a) peat, b) pectin, c) sodium alginate, d) an aqueous suspension of *Bacillus subtilis,* and e) two wettable powder products (WP 179 and WP 80) formulated with an inert material, a wetting agent, and a dispersant . The difference between WP 179 and WP 80 was the temperature used to dry the powders. Besides, a conventional treatment using a recommended fungicide (Benomyl) was tested as a control.
All of these products were tested in a greenhouse at Embrapa (Brazilian Company of Research in Agriculture, in Jaguariuna City, São Paulo State, Brazil), but among them, only peat, pectin and the fungicide formulation were tested at field conditions (at Santa Helena and Mateiro Farms of Guaira, São Paulo State, Brazil).
The following variables were evaluated: a) seedling emergence, b) root nodulation, c) dry weight of roots, d) incidence and severity of the disease and e) viability of the bacteria in each substrate.
In field conditions, the results of seedling emergence have shown that peat was the best substrate for the formulation of *Bacillus subtilis*, while the natural polymer, pectin, is shown to interfere negatively in the emergence of plants . In the greenhouse, the results have shown that the best treatment of bean seeds was the aqueous suspension containing cells and metabolites of *Bacillus*, followed by the peat substrate which is seen to be as good as the fungicide recommended to control the pathogens.

Effect of Spray-Drying and Storage on Carotenoids Content of *Haematococcus pluvialis* Biomass

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Key words*: Microalgae,* Haematococcus, *carotenoid, astaxanthin, spray-drying*

Microalgae biomass of *Haematococcus pluvialis* was spray-dried at different temperatures to obtain a powder rich in carotenoids suitable to incorporate in fish fed. Microalgae are the basis of essentially all marine and freshwater aquatic food chains. Carotenoids have been added to trout and salmon diets to colour their flesh as the wild species. For convenience of use, the dried powder is the most adequate form to incorporate *Haematococcus pluvialis* (known to accumulate large quantities of carotenoids specially astaxanthin) in fish feed.

Under the conditions studied, the drying temperatures that yielded the highest content of astaxanthin and ß-carotene were 220°C at the inlet and 120°C at the outlet of the drying chamber. The powders obtained were stored under different conditions in order to study the effect of temperature and presence of oxygen in the degradation of both astaxanthin and ß-carotene. Results showed that both pigments suffered the same level of degradation during storage.
According to the results achieved by the pigments stability study during storage, astaxanthin and ß-carotene exhibited fast decomposition with high moistured powders, reaching a degradation of 40% after 6 weeks storage. A powder with a moisture content of 87 g/kg, obtained with drying temperatures of 180°C (inlet) and 110°C (outlet), was found to be the less sensitive to degradation.

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The Effect of Red Pepper Seeds on the Stability of the Carotenoid Pigments in Paprika (*Capsicum annuum* L.) Powder

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***Key words**: carotenoid pigments, HPLC, multivariate mathematical-statistical methods, paprika, pepper seeds*

The spice paprika prepared from dried ground fruits of *Capsicum annuum* or *Capsicum frutescens* is used as colour and flavor additive to foods. Its deep red colour, upon which rests much of its commercial value is due to a complex mixture of carotenoids in which capsanthin and capsorubin are present in the greatest amounts. Other carotenoids include hydrocarbons such as β-carotene and its variously oxygenated derivates including cryptoxanthin, zeaxanthin and violaxanthin. The major pigments capsanthin and capsorubin, together with cryptoxanthin and zeaxanthin have been shown to be present as fatty acid esters with capsanthin occurring as the dilaurate ester. The long conjugated unsaturation of carotenoids renders them prone to oxidative degradation caused by the air and light.

As an attempt to control colour degradation of paprika powders during storage, some Hungarian and other world enterprises add ground seeds into paprika powders. In fact, paprika seeds contain an effective antioxidant (γ-tocopherol), which inhibits oxidation of the carotenoids. Some authors refer that after grinding, oil from seeds diffuses and surrounds most of the particulate of the paprika powder protecting the pigments from surrounding oxidising agents and improving the visual appearance of the products.

The objectives of our work were the separation and quantitative determination of colour pigment of paprika powders, and the elucidation of the influence of various environmental conditions (time of storage, concentration of seeds added to the powder, presence or absence of light) on the stability of the pigments using multivariate mathematical statistical methods such as SPM, NML and stepwise regression analysis. For separation of carotenoid pigments by HPLC we used a Lichrocart Merck endcapped reversed-phase C_{18} column with a pre-column RP-18. The quantification was realised using an external standardisation. The length of storage significantly influenced both the overall velocities of decomposition rate and its selectivity. The concentration of the seeds in the samples has also a significant impact on the selectivity of the decomposition of the pigments.

Efeito da Fonte de Carbono e da Regulação Hormonal no Crescimento das Culturas de Células em Suspensão de *Dittrichia viscosa* (L.) W. Greuter

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Key words: *culturas em suspensão,* D. viscosa

Dittrichia viscosa (L.) W. Greuter [sin. *Inula viscosa* (L.) Aiton] é uma planta perene da família das *Compositae* que cresce na região oriental da Península Ibérica. A esta espécie têm sido atribuídas propriedades antipirética e antiflogística, sendo ainda usada no tratamento de feridas e de doenças gastroduodenais [1-5].

No presente trabalho foi estudado o efeito da fonte de carbono e da regulação hormonal no crescimento das culturas de células em suspensão de *D. viscosa*, previamente estabelecidas há pelo menos 6 meses, com intervalos de repicagem de 12 dias. Estas culturas foram mantidas em meio MS [6], suplementado com: a) 30 g sacarose, 1 mg/l NAA e 0,1 mg/l Kin; b) 30 g sacarose, 1 mg/l 2,4-D e 0,1 mg/l Kin; c) 30 g/l glucose, 1 mg/l NAA e 0,1 mg/l Kin; d) 30 g/l glucose, 1 mg/l 2,4-D e 0,1 mg/l Kin. Estas culturas foram mantidas às escuras, sob agitação orbital a 160 rpm, a 24 ºC±1 ºC.

As culturas de células em suspensão de *D. viscosa* suplementadas com glucose, NAA e Kin foram as que registaram melhor crescimento com a maior produção de biomassa, quer em termos de peso fresco quer em termos de peso seco. Partindo de um inóculo inicial de 1,5 g (p. f.) ao fim de 336 h, tinha-se 13,5 g de biomassa (p. f.) a que correspondeu 0,88 g em peso seco. A este tempo correspondeu também ao início da fase estacionária. Nas culturas suplementadas com sacarose, NAA e Kin registaram-se os valores mais baixos de produção de biomassa. A mobilização da glucose, do amónio e do nitrato no meio, nas culturas suplementadas com glucose, NAA e Kin foi quase total, ou seja ao fim de 330 horas, início da fase estacionária, praticamente não existia qualquer um dos nutrientes atrás referidos. Foi também a partir desta altura que se verificaram os valores mais baixos de condutividade do meio de cultura. O mesmo não se verificou para as restantes culturas.

A produção de biomassa nem sempre corresponde a uma produção de metabolitos secundários. Por esta razão, neste momento averigua-se se as culturas produzem flavonóides com interesse medicinal e que já foram identificadas em plantas a crescer no campo.

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Cultivo da Alga Marinha *Ulvaria oxysperma* sobre Redes no Ambiente Natural, Ilha do Barbado, Guaraqueçaba, Paraná, Brasil

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keywords: Ulvaria oxysperma, *Brazil, alga*

O ambiente marinho pode ser uma fonte inesgotável de alimentos. O uso de produtos marinhos é comum em países orientais e em alguns países americanos, como Venezuela, Chile e Peru. O seu consumo é pequeno no Brasil, no entanto, a alteração de hábitos alimentares, com intuito de utilizar uma alimentação balanceada, tem despertado o uso destes alimentos em nosso país. As algas são a principal fonte de alimentos de peixes e outros organismos aquáticos. Elas são seres autotróficos, podendo ser unicelulares ou formar agregados de células com aspecto talóide ou folheáceo. A maioria se reproduz sexualmente. Os anterozóides fundem-se com as oosferas formando o zigoto. Este fixa-se a um substrato e desenvolve-se formando uma nova alga. As algas são freqüentemente divididas de acordo com sua pigmentação em: Chlorophyta (verdes), Phaeophyta (pardas) e Rhodophyta (vermelhas). As algas podem ser usadas como diversos tipos de alimentos, sendo que, no Brasil, o consumo de "folhas de algas tostadas" tem aumentado intensamente. No litoral do estado do Paraná, três algas verdes são abundantes: *Ulva lasctuca*, *Ulva fasciata* e *Ulvaria oxysperma*, sendo que a última é colhida artesanalmente para a produção de "folhas de algas tostadas".

A criação do Parque Nacional do Superagüi (13.638 ha) foi importante para a manutenção deste biossistema, no entanto, ela inviabilizou a permanência da população nativa com mínimas condições de subsistência. O cultivo de algas, associado a pesca artesanal, pode ser a alternativa que torne viável a sua manutenção, evitando o êxodo. Após autorização do IBAMA, a Ilha do Barbado foi escolhida para a implantação do cultivo da *Ulvaria oxysperma*. Os objetivos deste trabalho são de avaliar: a) a implantação natural dos zoósporos na rede (algodão-nylon, malha 18-20 e 0,7 por 3 a 5 m), b) a competição da *Ulvaria* com outras algas, c) a presença de predadores, d) o comportamento frente a maré e e) as características físicas (das algas úmidas e secas) e químicas (umidade, cinzas, proteínas, lipídeos, carboidratos e teor calórico) da alga produzida.

A rede foi um bom substrato para fixação de *Ulvaria oxysperma*. A presença de *Enteromorpha* sp foi verificada em proporções pequenas nas extremidades das redes. A densidade de algas na rede foi extremamente elevada, quando comparada com o substrato natural (manguesal). A presença significativa de predadores não foi observada. A alternância de imersão ou não das algas pela ação da maré é importante. Por outro lado, movimentos bruscos provocam a fragmentação e o desprendimento de algas. As algas cultivadas apresentam coloração verde-grama, mais intensa que as nativas, e sua textura é mais espessa. A alga seca à temperatura ambiente e sob sombra apresentou coloração verde. A composição química da alga preparada é adequada como alimento (umidade residual = 15,4-17,8%, cinzas = 12,4-18,2%, proteínas = 4,8-9,9%, lipídeos = 0,4-0,9%, carboidratos = 55,1-59,9%, teor calórico = 251-276 kcal.100^{-1}g).

(ECOPLAN, DQ/UFPR, CNPq)

Avaliação de Diversidade Genética em Clones *E. globulus*

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Keywords: Diversidade genética, marcadores de RAPD, E. globulus

A utilização de tecnologias de marcação molecular num programa de melhoramento genético florestal (para avaliação do grau de relacionamento genético entre clones selecionados) constitui uma ferramenta valiosa para actividades de gestão de populações. A identificação de linhas e populações, permite perspectivar o delineamento de intervenções visando a preservação de um máximo de variabilidade genética, procurando obter uma larga plasticidade adaptacional a diferentes condições edafo-climáticas. Podem igualmente complementar um conjunto de informações fenotípicas relativas a populações melhoradas, auxiliando o delineamento de cruzamentos que possam proporcionar a selecção sustentada de novas combinações genéticas, algumas delas traduzindo-se num ganho real em características desejáveis.

Utilizou-se um conjunto aleatório de marcadores de RAPD ("Random Amplified Polymorphic DNA") para estimar o nível de homologia genética de uma amostragem constituída por dois grupos distintos de plantas *E. globulus* : um grupo, composto por individuos selecionados, de origem clonal (resultantes de diversas etapas de selecção) e o outro de proveniência seminal (originário de lotes de semente de origem diversa).

Resultados preliminares, baseados num reduzido número de primers, permitiram vislumbrar uma base de variabilidade genética similar entre os dois grupos. Este facto parece indicar que o esforço de melhoramento genético sustentado, não tem aparentemente acarretado substanciais perdas na variabilidade genética representada na amostragem analisada.

Estes resultados integram-se no esforço de caracterização, gestão e mobilização do material vegetal, resultante do programa de melhoramento genético convencional do RAIZ, numa perspectiva de futura implantação de estratégias de selecção e melhoramento genético assistido por tecnologias de marcação molecular.

Continuous somatic embryogenesis derived from mature zygotic embryos of *Olea europaea* (cv. Galega Vulgar and Cordovil de Serpa

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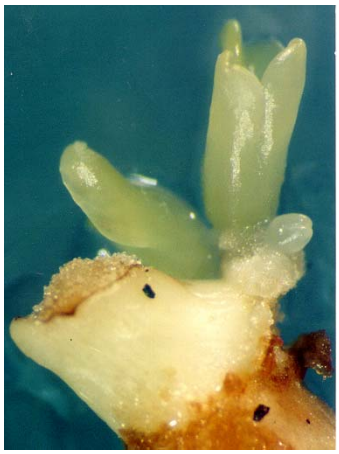
Olive is a crop of great economical importance in Portugal. With the aim of supplying trees of selected productivity, for the renewal of old orchards, techniques for *in vitro* clonal propagation of cv. *Galega* and cv. *Cordovil* were applied. Somatic embryogenesis was induced directly on mature zygotic embryos on half-strength MS medium supplemented with 0.44µM BA or 0.22µM BA+0.49 µM IBA or in the absence of growth regulators

On cv. *Galega*, the percentage of explants originating primary somatic embryos range from 3.6% to 12.5%. The best response was obtained at darkness, in the absence of growth regulators (10.3%) or in medium supplemented with 0.22µM BA+0.49 µM IBA (12.5%) both solidified with gelrite. Somatic embryos obtained were isolated at the globular, heart, torpedo and cotyledonary stages. Continuous cycles of secondary normal and abnormal embryos were formed at the basis of the hypocotyl of primary embryos near the surface of the culture medium. Two clones were chosen from these initial somatic embryo sources: embryos originated in the absence of growth regulators (clone Gv81Ff) and embryos coming from medium supplemented with 0.44µM BA (clone Gv74Ba). Conversion of somatic embryos from both clones was studied changing sucrose concentration and the type of solidifying agent. Plantlets obtained from somatic embryos were aseptically transferred to vermiculite with Hoagland's solution. Acclimatisation to soil was done two months after.

Somatic embryogenesis was also obtained with cv. *Cordovil* at darkness, in the absence of growth regulators in medium solidified with gelrite. Primary somatic embryos obtained produced continuous cycles of secondary embryos at the basis of the hypocotyl of primary embryos, near the surface of the culture medium



1

2

Fig.1. Formation of primary somatic embryos directly from the hypocotyl of a mature zygotic embryo
F**ig.2** Formation of secondary embryos in the basis of the hypocotyl of a primary embryo

Distinguishing Traditional Portuguese and Brazilian Citrus Varieties by Isozyme, RAPD and AFLP Markers

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***Keywords**: Citrus, isozymes, RAPDs, AFLPs.*

"Cravo" is a traditional mandarin variety cultivated in Brazil where is of relative economic importance, particularly in the state of São Paulo. "Carvalhais" is a traditional mandarin cultivated in Portugal where supposedly it was originated. Considering the phenotypic similarities between both mandarins, Hogdson (1967) and Amaral (1982) hypothesize that they should constitute one single clone. To test this hypothesis, we analysed six leaf isozyme systems: PGI, PGM, IDH, MDH, GOT and LAP, by starch gel electrophoresis of leaf extracts from both *Citrus* varieties. Almost all loci were shown to be identical in both mandarin cultivars. An exception was the locus PGM-1, which displayed in mandarin "Cravo" an additional enzyme band. However, a similar band whose expression seems to be environmentally dependent, can be observed in other *Citrus* biotypes.

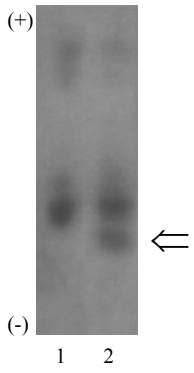

Fig.1.PGM zymograms of mandarins "Carvalhais" (1) and "Cravo" (2). The arrow indicates the polimorfic band in the gel zone of enzyme activity PGM-1.

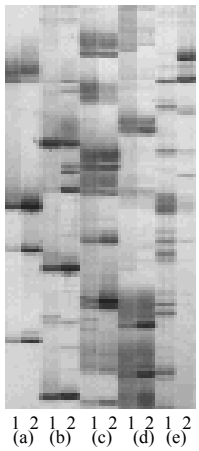

Fig.3. AFLP markers of mandarin varieties "Carvalhais" (1) and "Cravo" (2) generated by the following primer combinations: (a) E-ACG/M-CAT; (b)E-ACG/M-CTG; (c) E-ACG/M-CAA; (d) E-ACG/M-CTT; (e) E-ACG/M-CAC.

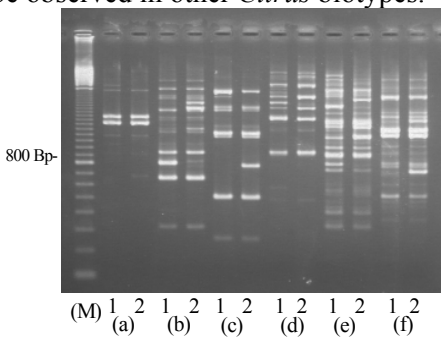

Fig. 2. RAPD markers amplified in mandarins "Cravo" (1) and "Carvalhais" (2) by primers: (a) OPM 9; (b) OPM 15: (c) OPM 18; (d) OPN 20; (e) OPK 4; (f) OPA 4. Lane (M) molecular marker.

Seeking better genetic discrimination, DNA of both mandarins was submitted to RAPD analysis. Forty nine 10-mer primers were assayed. Twenty five primers were found to be polymorphic, and the estimates for genetic similarity between both varieties, using the coefficients DICE and SM, were 0,901 and 0,890 respectively. Additionally, a more fine analysis of both genomes was carried out using the AFLP technique. One hundred and five AFLP markers were identified by the eight primers with three random nucleotides used in this study. Fourteen AFLP markers were polymorphic and the genetic similarities values estimated using coefficient DICE and SM, were respectively 0,929 and 0,867. In view of the above results, we concluded that although "Carvalhais" and "Cravo" are genetically very close, as expected from two mandarin cultivars, they are genetically different enough not to be considered the same clone. We assume that growing both mandarin cultivars under identical environmental conditions could allow their genetic differences to be expressed into two differentiated phenotypes.

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Discrimination among Fig (*Ficus carica* L.) Clones by Isozyme and RAPDs

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***Keywords**: Fig,* Ficus*, Isozymes, RAPDs, AFLPs.*

Isozymes and RAPDs have been previously reported [1] to constitute suitable molecular markers for discrimination among fig traditional varieties and for molecular characterization of fig germplasm collections. In this work we tested the suitability of those markers to discriminate among eleven different clones of the same Turkish fig variety "Calimyrna", selected in the Ege University, Turkey. The Turkish fig variety "Sarizeybek" was assayed as a control for the intervariety discrimination ability of the molecular markers. Five isozyme systems: PGI, PGM, IDH, MDH and GOT, were analysed after starch gel electrophoresis of leaf extracts. As expected, the low number of isozyme loci available for study (6), was insufficient to discriminate among the clone selections of the variety "Calimyrna". However, the variety "Sarizeybeck" could be distinguished by its different GOT heterozygous pattern (Fig.1). In a second step of our study, a more detailed genomic analysis of fig clones was attempted using the RAPD technique. One hundred primers (10-mer, Operon Technologies Inc.) were tested for their ability to generate clearly defined and reproducible RAPD amplification products in a bulk of genomic DNA isolated from different fig varieties.

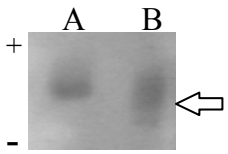

Fig.1. GOT patterns of clone 11 of "Calimyrna" fig variety (A) and "Sarizeybek" variety (B).

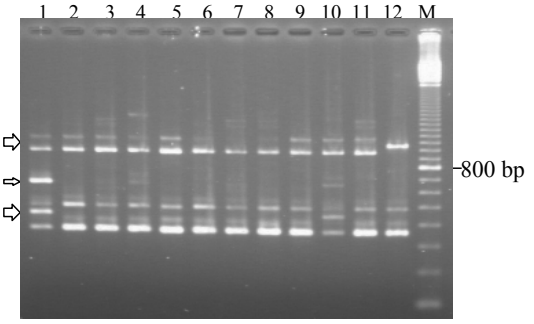

Fig.2. RAPD patterns generated by primer OPN 10. Arrows indicate the polymorphic markers found among the clones of the variety "Calimyrna"(1-11) and a polymorphic marker characteristic to the variety "Sarizeybek"(12). Lane (M) one hundred base pair molecular marker.

Twenty primers were selected. Twelve RAPD markers, representing approximately 10 per cent of the obtained 135 markers, differentiated between "Calimyrna" and "Sarizeibek" fig varieties. However, only the primer OPN 10 revealed two markers, well characterized and confirmed, that were polymorphic among the eleven clones of "Calimyrna" (Fig.2). These results, confirm the suitability of isozyme and RAPD markers to discriminate between fig varieties, but also point out the relatively poor ability of these markers to distinguish between clones of the same variety. The AFLP technique that generates a higher number of DNA markers per reaction is being tested for such purpose.

[1] Elisiário, P. J., Neto, M. C., Pica M. C., Leitão, J. M., 1st International Symposium on Fig, Izmir, Turkey, 7, 1997.

Sanitation of Virus Diseases of Almond and Construction of Vectors to Introduce Virus Resistance

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Keywords: Almond, coat protein strategy, genetic transformation, PDV, virus diseases

In Algarve region, several viruses were found to affect almond orchards. Prune Dwarf Virus (PDV), Prunus Necrotic Ringspot Virus (PNRSV) and Apple Mosaic Virus (AMV) could be detected in samples collected from different regions of Algarve.

To obtain virus-free material, a sanitation program was initiated 2 years ago by testing in separate or in combination, different treatments applied to in vitro cultures, such as thermotherapy, chemotherapy and culture of isolated meristems, followed by regular testing for the presence of PDV. The evaluation of the treated material for PDV eradication proved to be difficult, when using ELISA technique (enzyme linked immunosorbent assay). The results were very similar to those obtained for control samples because the virus title in *in vitro* shoots could be too low to be detected by ELISA. However, the presence of the virus was confirmed by immuncapture/reverse transcriptional-polymerase chain reaction (IC/RT-PCR. Meristem culture alone revealed in fact, very often insufficient to eliminate PDV from other *Prunus* sp. (like sour cherry). In parallel, we started to root the micropropagated shoots for subsequent transfer to soil and further screening for the virus elimination, using ELISA. Almond rooting is highly genotype-dependent and several rooting protocols were compared for their efficiency.

Because PDV can be spread in the orchards just by pollen, a long-term maintenance of good sanitary conditions is better ensured by introduction of resistance to this virus. For this goal we have followed the coat protein strategy by isolating the coat protein gene from infected almond leaves using IC/RT-PCR, inserting the cDNA fragment in a cloning vector, and later, in a transformation vector, pBI121 (carrying the kanamycin resistance gene *npt* II, and the β-glucuronidase, *uid*A gene), by replacing the *uid*A coding region for cpPDV (pBI121cpPDV). This construct was tested in the transformation of *Nicotiana tabacum* and transgenic plants are being tested for virus resistance, while the same construct is being transferred to almond. For the other viruses detected in almond (PNRSV and AMV), IC/RT-PCR protocols are being developed for diagnosis of in vitro material and for isolation of cDNA fragments encoding viral coat proteins, for further cloning in transformation vectors.

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Effects of Hemicellulase and Salicylic acid on Tropane Alkaloid Production in Hairy Root Cultures of *Brugmansia candida*

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Keywords: Brugmansia candida*, hairy roots, tropane alkaloids, hemicellulase, salicylic acid.*

The tropane alkaloids scopolamine and hyoscyamine are employed in medicine as anticholinergic agents. Since their chemical synthesis is difficult and expensive, these compounds are still extracted from plants that belong to several species of the Solanaceae. Hairy roots of *Brugmansia candida* (Solanaceae) have been obtained in our laboratory through transformation with *Agrobacterium rhizogenes* and were cultured according to Pitta-Alvarez and Giulietti (1). In an attempt to increase the productivity of the cultures, treatments with different elicitor preparations have been tested. In this research, we have investigated the influence of two different elicitors: salycilic acid and hemicellulase. In every experiment, the corresponding elicitor was added to 18-day old cultures, and these were exposed for periods of 24, 48 and 72 hours. The effects on growth, alkaloid accumulation and release into the medium were evaluated. Salicylic acid, a compound involved in plant defense, was tested at three concentrations: 0.01, 0.1 and 1.00 mM. At concentrations of 0.01 and 1 mM, salicylic acid increased scopolamine and hyoscyamine accumulation (ranging from 30 to 170%), particularly after 24 and 48 hours. There was also an increase in the release of both alkaloids into the medium. Hemicellulase, in the two concentrations tested (60 µg/ml and 120 µg/ml), had a positive effect on scopolamine (100%) and, especially, hyoscyamine accumulation (300%) in the roots after 24 hours. This effect was also seen, although to a lesser extent, after 48 hours. Release of alkaloids into the medium was enhanced at 48 hours, but only in the case of scopolamine.

[1] Pitta-Alvarez, S. I.; Giulietti, A. M.. In Vitro Cell Dev. Biol. Plant. 31: 215-220; 1995.

Effect of Sodium Chloride Concentration and Storage Temperature on the Viability of *Bifidobacterium lactis* and *Lactobacillus acidophilus* in milk

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Key words: Bifidobacterium lactis, Lactobacillus acidophilus, processing, salt, survival

The growth and viability of *Bifidobacterium lactis* and *Lactobacillus acidophilus* were studied in reconstituted skim milk so as to assess their dependence upon temperature, NaCl concentration, and storage time; such dependence was modelled in mathematical terms from first principles. Storage temperatures of 5, 10 and 15°C were combined with NaCl concentrations of 0, 3 and 6% (w/w) for each strain *per se*, and for both strains as a coculture, using a factorial experimental design; the milk media were stored for up to 8 weeks and sampled on a weekly basis. Samples were assayed for number of viable bacteria and level of acid production.

Cultures of *B. lactis* exhibited no significant loss of viability either with increasing NaCl concentration or increasing storage temperature within the experimental ranges tested. On the other hand, viability of *L. acidophilus* decreased with increasing temperature and increasing NaCl concentration within the aforementioned ranges; a decrease of three to six log cycles during 8 wks of storage was observed. When cocultured with *L. acidophilus*, *B. lactis* was significantly less tolerant to higher NaCl levels and higher temperatures than when in pure culture (viable bacterial numbers decreased up to three log cycles), although final numbers were still above the threshold required for commercial application. The mixed culture with *B. lactis* had no overall detrimental effect on the viability of *L. acidophilus*; an increased sensitivity of *L. acidophilus* to NaCl concentration was, however, apparent in a coculture environment. Surprisingly, addition of 3% NaCl to the pure culture of *L. acidophilus* inhibited the decline in viable numbers during subsequent refrigerated storage, whereas similar effects were not observed in the presence of *B. lactis*.

A mechanistic model, which postulates that the behaviour of pure and mixed microbial populations is described by specific death rates that vary with temperature (following Arrhenius relationships) and NaCl levels (following simple inhibition kinetics) in the milk medium, was successfully fitted to the experimental data; thereby the model considered met the requirements of simplicity and physicochemical meaning. Activation energies (E_{act}) for the death rates of *B. lactis* and *L. acidophilus* were 14 and 9-15 kcal mol^{-1}, respectively. The higher E_{act} observed for pure cultures of *L. acidophilus* than for cocultures of the same strain indicates that coculturing reduces the heat lability of *L. acidophilus*.

The work developed is useful in attempts to predict the effects on survival of environmental conditions that are easily manipulated, for both pure and mixed cultures, which may be a useful tool for optimization of the cheese environment where such species are to be eventually incorporated as the sole starter.

Quality Control of Infant Formulae in Terms of Major Constituents and Nucleotides

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Key-Words : Infant formulae, nucleotides, moisture, ash, protein, fat.

The infant formulae industry tries to obtain adapted formulae with a composition similar to that of the human milk. The first important modifications include reduction of the protein and electrolyte contents, modification of the casein/whey protein ratio, addition of lactose and supplementation with unsaturated lipids, vitamins, trace elements and others.
The nutritional value of 12 samples of different infant formulae was determined via their characterisation, using Portuguese Norms NP, in terms of major constituents viz., moisture, ash, protein and fat. Carbohydrate and organic acid composition was determined by HPLC-RI/UV, with an NH_2 chromatographic column. The results obtained agreed with National [1] and European legislation [2].
Owing to the recent recommendation of infant formulae supplementation with nucleotides by several organisations, namely the Committee for food of the European Community [3], our study included the monitorization of cytidine 5-monophosphate, uridine 5-monophosphate, guanosine 5-monophosphate and adenosine 5-monophosphate. Increased attention is being paid to the role of dietary nucleotides in infant nutrition. The dietary nucleotides are important to maintaining normal growth and development in infants. These compounds have several functions, such as, modulate lipoprotein metabolism, modify the composition of the intestinal microflora, improve gastrointestinal tract repair after damage and participate in the immunitary response mediated by T cells. Moreover, rapidly growing tissues such as the intestinal epithelium and lymphoid cells lack significant capacity for *de novo* synthesis of nucleotides and require exogenous sources of these compounds.
An HPLC method with diode array detection, was optimised for that purpose. Sample preparation was simple and involved protein removal and filtration operations prior to injection onto a reversed-phase column C_{18} ($S_{10}ODS_2$). Gradient elution was carried out using a mixture of two solvents. Solvent A consisted of 5 mM tetrabutyamonium hydrogensulphate (TBAHS) and 20 mM potassium dihydrogenphosphate (KH_2PO_4) and solvent B of 5 mM TBAHS, 100 mM dipotassium hydrogenphosphate (K_2HPO_4) and 10% (v/v) acetonitrile. The pH of both solutions was adjusted to 5.2.
Only one of the 12 analysed infant formulae presented the four nucleotides, the levels of which were within the ranges recommended by the Scientific Committee for food of the EC. Six other samples showed traces of cytidine. No nucleotides were detected one the other infant formulae.

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Comparison of Enhanced Hemolytic Agar, Oxford agar and Palcam Agar for Isolation of *Listeria monocytogenes* from Production Lines of Fresh to Cold-smoked Fish

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Key words: Listeria*;* L. monocytogenes*; Enhanced Haemolytic Agar*

Only the hemolytic species of *Listeria* - *L. monocytogenes*, *L. ivanovii*, *L. seeligeri* - are associated with human pathogenicity. *L. ivanovii* has been reported to be involved in human pathology only rarely, and *L. seeligeri* has been reported only once to be the cause of meningitis in a non immunno-compromised adult(1). *L. monocytogenes* is the only species among the genus which has been consistently involved in known foodborne outbreaks.Therefore, the introduction of an isolation medium on which the pathogen can be differentiated from the non-pathogenic *Listeria*, was achieved using the EHA (Enhanced Haemolysis Agar)(2). In EHA, *L. monocytogenes* and *L. seeligeri* can lyse ovine erythrocytes in the presence of the enzyme sphingomyelinase. *L. ivanovii* does not show a noticeable hemolysis in the EHA. In this study, EHA was compared with the two conventional *Listeria* isolation agars Oxford and Palcam (Merck) for their ability to isolate *L. monocytogenes* from fish and cold-smoked fish products as well as from environmental sites along the cold-smoking processing chain and from the trout farms. 132 fish samples and 41 environmental samples were analysed. The EHA was shown to be both discriminatory and effective but, unless it is produced as a commercial medium, the preparation is time and labour-consuming. Apart from the most important feature of EHA-the elective ability of distinguishing *L. monocytogenes* from the other *Listeria* spp.- no differences were found in the recovery of the different *Listeria* species by the three plating media. However, the abilities of the three methods for isolating *L. monocytogenes* varied depending upon whether isolation was performed from the Fraser primary enrichment or the Fraser secondary enrichments after 24 and 48 hours. EHA was shown to be less effective when isolating from Fraser primary enrichment. The typical *L. monocytogenes* colonies were masked when the growth of competitive flora was high. However, when the number of competitors were low, the *L. monocytogenes* colonies were easily distinguished from the *L. seeligeri* and *L. innocua* colonies.

References:

(1) Lovett, J. and Twedt, R., *Listeria*. Food Technology, April, 188-191, 1988.
(2) Beumer, R.R.; te Giffel, MC. and Cox, L.J. Optimization of Enhanced haemolysis agar (EHA), a selective medium for the isolation of *Listeria monocytogenes*. In: *Listeria monocytogenes* detection and behaviour in food and in the environment. Ed. R. Beumer. Thesis Landbouwuniversiteit Wageningen. Koninklijke Bibliotheek, Den Haag. Netherlands, 1997.

Purificação da Bacteriocina de *Lactobacillus plantarum* e sua Detecção em Substratos Naturais

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Palavras-Chave: Bacteriocinas, Purificação, Bioconservantes.

O processo de produção de azeitona de mesa, economicamente relevante nos países mediterrâneos, envolve a fermentação da salmoura por bactérias ácido lácticas (BAL), a qual se deve principalmente ao crescimento de *Lactobacillus plantarum*. Do seu metabolismo resultam compostos importantes, do ponto de vista organoléptico, bem como substâncias inibidoras do crescimento bacteriano. De entre os vários compostos inibidores sintetizados pelas BAL, as bacteriocinas têm sido alvo de grande interesse nos últimos anos, por serem substâncias proteicas, termoestáveis, efectivas contra bactérias patogénicas e degradativas. O crescente interesse pelo estudo destas bacteriocinas deve-se à importância histórica das BAL nos processos fermentativos de alimentos e à emergente resistência dos consumidores a conservantes químicos. A utilização da nisina como conservante natural, apesar das limitações impostas pelas suas propriedades, está já regulamentada em alguns alimentos. Muitas bacteriocinas, apesar de efectivas em meios sintéticos, não mantêm a sua actividade antibacteriana, quando aplicadas em alimentos. No entanto, estudos preliminares de bacteriocinas de BAL, adicionadas ou produzidas *in situ*, encorajam o seu uso como sistemas de biopreservação em alimentos fermentados. O estudo das propriedades químicas e biológicas, bem como a avaliação da sua eficácia em alimentos torna necessária a obtenção de quantidades significativas destes péptidos purificados. O objectivo deste trabalho foi a purificação e estudo comparativo de uma bacteriocina produzida por *L. plantarum*, isolado de salmoura de azeitonas, em MRS e em salmoura das *cvs*. "Negrinha" e "Santulhana". A pesquisa de bacteriocinas foi efectuada pelo método simultâneo, segundo adaptação da técnica proposta por Bart ten Brink (com. pessoal). Na quantificação da sua actividade antimicrobiana utilizou-se o método semiquantitativo das diluições seriadas. A bacteriocina foi concentrada no sobrenadante da cultura em MRS, por "salting-out", tendo sido testadas várias concentrações de sulfato de amónio e diferentes valores de pH. Como controlos, utilizou-se caldo MRS não inoculado, o tampão em que se ressuspendeu o precipitado e as soluções de cloreto de sódio, nas condições utilizadas. O mesmo método de concentração foi aplicado em salmouras inoculadas com esta estirpe produtora em várias fases da fermentação. Como controlo, utilizou-se salmoura isenta de estirpes bac+ (BAL produtoras de bacteriocina) e não foi detectada nenhuma actividade inibitória. O tamanho da bacteriocina foi estimado por ultrafiltração, prevendo-se que o seu peso molecular se situe entre 10- e 3-kDa. Estudos preliminares, com ureia, sugerem que a bacteriocina no estado bruto se encontra associada em complexos proteicos. A dissociação destes complexos origina o aumento da actividade. Avanços na purificação das bacteriocinas, incluem separação de fracções por cromatografia líquida de troca iónica e SDS-PAGE. A informação recolhida na análise das várias fracções e sua comparação com os dados obtidos a partir da salmoura, permitirá explorar as suas potencialidades, como agentes de biocontrolo, para prolongar o tempo de vida dos alimentos e impedir o risco microbiológico.

Survival of Probiotic Microbial Strains in a Cheese Matrix during Ripening: Simulation of Rates of Salt Diffusion and Microorganism Survival

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Key words: Bifidobacterium lactis, Lactobacillus acidophilus, dairy, ripening

The growth and survival of *Bifidobacterium lactis* and *Lactobacillus acidophilus* strain Ki in a semi-hard Gouda cheese at various axial locations during 9 weeks of ripening at 13 °C were assessed using non-linear regression analyis. The final average salt levels ranged in 2-4 % (w/w). Enumeration of viable *B. lactis* and *L. acidophilus* was done using the spread plate technique on selective MRS-agar and TGV agar, respectively. During the whole storage period studied, the average numbers of *L. acidophilus* decreased by two log cycles to 0.2-5 x 10^7 cfu/g, whereas those of *B. lactis* decreased by less than one cycle to 6-18 x 10^8 cfu/g. Viable numbers of both probiotic strains underwent a slow decline during the first 3 weeks, followed by a sharper decrease towards the end of ripening; such decrease was more significant for the outer than for the inner cheese portions.

Salt transport was successfully described by Fick's second law of diffusion considering that the cheese behaved as a finite slab for modelling purposes. Salt diffusivity remained virtually constant with time and was estimated to be 0.2 $cm^2 d^{-1}$. Theoretical salt concentration profiles were in good agreement with experimental data. The mathematical models postulated and fitted to the microbial viability data encompassed either a linear relationship between specific death rate and salt concentration or a constant death rate; the fitting procedure followed a methodology of increasing model complexity. Decision on the better model was taken based on a *F*-test of the ratio of incremental sum of squares of residuals to sum of squares of residuals of the more complex model; it was thereby concluded that viability of the probiotic strains was better described by a first order process irrespective of local salt concentration. The values for the specific death rate constant α_0 (viz. 0.9-1.0 d^{-1}) were within the range usually found in the literature; interestingly, this parameter increased with the increase of the average salt concentration within the cheese matrix, and the linear negative effect was slightly more important for *L. acidophilus* (r=0.993) than for *B. lactis* (r=0.975). Sodium chloride was apparently responsible for a masked inhibitory effect upon survival of both *B. lactis* and *L. acidophilus*.

Prediction of the profiles of viable numbers of *B. lactis* and *L. acidophilus* in cheese with respect to both ripening time and axial location for several salt concentrations, which evolves as a logical output of this research effort, is important in attempts to predict potential viability by the time of consumption.

Effect of Antioxidants in the Survival of Spray Dried *Lactobacillus bulgaricus* During Storage

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Lactobacillus bulgaricus is important for a number of industrial applications and it is frequently used as starter culture for dairy fermentation. The development of concentrated cultures for inoculating the production vat directly has eliminated many of the problems customarily involved in preparing and maintaining starter cultures in the dairy plant.

Although frozen concentrates of starter cultures are widely used in the production of fermented foods, there is a market for dried starter cultures. Commercial production of spray dried starters has not been widely successful because cells of many bacterial strains are killed by spray drying and subsequent storage. Although the mechanisms of cell death during storage in the dried state are not completely understood there are evidences that membrane lipids oxidation and survival during storage may be related.

The effect of antioxidants in the survival of spray dried Lactobacillus bulgaricus during storage at 4C and 20C was investigated. Higher survival was observed during storage at 4C and for both storage temperatures viability increased in the presence of these compounds.

Estudo do Perfil em Ácidos Gordos da Fracção Lípidica de Patés de Fígado do Mercado Nacional

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Palavras-chave: patés de fígado, ácidos gordos, isómeros trans

A presença de isómeros *trans* nos lípidos alimentares e os seus efeitos nutricionais constituem, actualmente, uma preocupação nos países industrializados. Foi demonstrado que os ácidos gordos *trans* afectam de modo adverso o perfil lipídico do soro, elevam os níveis de LDL e diminuem os níveis de HDL, não estando ainda completamente esclarecidos os seus efeitos sobre outras lipoproteínas. Assim a ingestão de isómeros *trans* dos ácidos gordos insaturados poderá ser considerada como um factor de risco na etiologia da doença coronária. A determinação do teor de isómeros *trans* num alimento poderá ainda assumir particular importância como meio de garantir a genuinidade de um produto.

Este trabalho teve como objectivos: *i*) determinar o diagrama dos ácidos gordos dos patés para avaliar a sua qualidade, (do ponto de vista nutricional e de segurança alimentar); *ii*) avaliar a genuinidade dos patés de fígado por comparação da composição em AG dos patés com os das matérias primas.

Analisaram-se 15 amostras de marcas diferentes 3 lotes por marca com diferentes períodos de validade, num total de 45 amostras. Estas foram adquiridas aleatoriamente no mercado nacional.

Para determinar a possibilidade de utilização do perfil de ácidos gordos na detecção de eventuais fraudes, procedeu-se à análise da gordura de fígados de animais referidos na rotulagem (porco, vitela, pato, perú, frango e galinha) e outras matérias-primas presentes na grande maioria das amostras, como toucinho e banha.

Para a análise do perfil de ácidos gordos e seus isómeros *trans*, quer dos patés quer das matérias primas, usou-se a cromatografia em fase gasosa, de coluna capilar, dos ésteres metílicos obtidos por transesterificação.

Os resultados obtidos evidenciaram que os ácidos gordos mais abundantes eram, respectivamente, o ácido oleico com cerca de 40%, o ácido palmítico com cerca de 20%, o ácido esteárico e o ácido linoleico com teores da ordem de 12%.

Relativamente à relação ácidos gordos saturados/insaturados verificou-se que os teores de saturados rondavam os 35%, sendo os restantes 65% referentes a ácidos gordos insaturados. Dentro destes cerca de 40% são ácidos gordos monoinsaturados nomeadamente ácido oleico.
No que se refere aos ácidos gordos *trans*-insaturados, verificou-se que os patés fornecem teores médios da ordem de 0,7%.
Quanto á genuinidade verificamos que só foi possível identificar as diferentes matérias primas quando estremes. Nos patés a confirmação da informação do rótulo não foi possível.

Proteolysis of Goat Caseins: Comparative Study of the Action of Cardosins A and B From *Cynara cardunculus*

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Key words: Enzyme, caprine cheese, urea-PAGE, HPLC

Animal rennet extracted from the abomasa of milk-fed calves has been the traditional coagulant in cheesemaking. However, increasing worldwide cheese production has led to a shortage in the supply of such traditional rennet, and this fact has stimulated search for alternative milk coagulating enzymes. Aqueous extracts of the thistle (*Cynara cardunculus)* have acquired the status of successful rennet substitute, and has accordingly been used for decades in the manufacture of goat and ewe's milk cheese in several rural areas of Portugal and Spain. The clotting activity of those extracts is attributed to the presence of two aspartic proteinases, cardosins A and B, which are believed to be similar (in terms of specificity and kinetic behaviour) to chymosin and pepsin, respectively. Studies of breakdown of independent caseins provide information that can be used to better understand the fundamental processes involved in cheese ripening. Although such studies are available for bovine and ovine caseins, information is scarce concerning goat caseins. In this work, proteolysis of goat Na-caseinate at 30°C in phosphate buffer at pH 6.5 as effected by cardosins A and B was followed by urea polacrylamide gel electrophoresis (urea-PAGE) and by reversed-phase high performance liquid chromatography (RP-HPLC). Hydrolysis of β-casein occurred to a much greater extent by the action of cardosin A than by cardosin B, and it was also faster. However, the α_s-casein region was degraded following similar patterns by both proteinases, with cardosin B acting slowlier than cardosin A. The RP-HPLC peptide profiles of the Na-caseinate hydrolysates indicated that both cardosins A and B yield several different peptides by 10 h of hydrolysis, most of them eluted in the hydrophobic region (50-60 min).

Purificação e Caracterização de uma Proteinase Aspártica Isolada a Partir de Células em Suspensão de *Centaurea calcitrapa*

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Palavras-chave: proteinase aspártica, purificação, suspensões celulares, Centaurea calcitrapa.

A coagulação do leite é uma das operações mais antigas em tecnologia alimentar e reside, em certos casos, na utilização de enzimas proteolíticas de origem vegetal.

Muitas Compostas da vegetação expontânea de Portugal produzem enzimas com actividade coagulante do leite. Entre elas, encontra-se o "cardo" *Centaurea calcitrapa*.

Neste trabalho, foram isoladas proteinases aspárticas com actividade coagulante, a partir de células em suspensão de *Centaurea calcitrapa*.

O extracto enzimático, obtido por sonicação, foi precipitado com 70% de sulfato de amónio, seguido de três passos cromatográficos, dois de troca iónica e um de interacção hidrofóbica. Deste processo, resultou o isolamento de uma banda com o peso molecular aparente de 66 kDa.

A temperatura óptima para a actividade proteolítica da proteinase isolada foi de 52ºC e o pH óptimo foi de 5,1. É glicosilada e fortemente inibida pela pepstatina, pertencendo à classe das proteinases ácido aspárticas.

A realização de ensaios de coagulação demonstrou possuir actividade coagulante.

On the Microbiological Profile of Traditional Portuguese Maize Bread

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Key Words: Panification; Corn; Rye; Microorganisms

The earliest method of obtaining reliable leavens was to keep back a piece of fermenting dough to be used as additive to the new dough for the folowing production. The piece of dough kept for the next production is called the sour ferment, and tradicionally is designated by "isco" in some regions of Minho.

From an economic point of view, the bread made of maize has a great importance in our country due the significant number of small farmers who produce this traditional product. This type of bread also plays an important social function via helping fixation of people in rural areas and preventing desertification towards urban areas. Finally, this product has a great potential to address the issues of modern consumers with respect to natural, balanced foods.

However, such promising future will require a greater quality and a more constant qualities of the product. This quality can only be legally enforced by certification, which occurs via definition of *Appéllation d'Origine Protegée* (AOP) regions. To this goal, several microbiological analyses of samples made available by traditional producers in two different periods and from different geographical locations were done using 12 different media and 24 different growth conditions. Total viable counts were performed after inoculation on such media, as well as viable counts of Enterobacteriaceae, yeasts and molds, *Bacillus*, *Lactobacillus*, *Pediococcus*, *Staphylococcus*, *Micrococcus*, Pseudomonas, *Clostridium*, *Streptococcus*, Lactococci, Enterococci and *Leuconostoc*. Strains were further characterized via 7 different appropriate APIs.

Yeasts and Lactic Acid Bacteria (LAB) were the predominant microbial groups in the products analysed. The sourdough had the highest viable count levels of *Enterobacteriaceae*, yeasts, *Clostridium*, *Streptococcus* and *Leuconostoc*, and no spore forms were found. Maize flour contained a significant number of viable molds. In both flours, spores of the genus *Bacillus* were present at highest numbers, followed by yeasts and *Clostridium*.

Optimização de Condições da Coagulação dos Leites de Vaca e Cabra através da Utilização das Flores do Cardo (*Cynara cardunculus* L.)

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Palavras-chave: cardo, Cynara cardunculus *L., leite, cabra, coagulação.*

A espécie vegetal *Cynara cardunculus* L., vulgarmente designada por cardo ou cardo do coalho, devido à utilização das suas flores como agente coagulante do leite, tem possibilitado, no nosso país, o fabrico artesanal de queijo de cabra e de ovelha, particularmente o queijo de cabra da serra algarvia.

Com este trabalho pretendeu-se caracterizar e optimizar algumas condições da coagulação dos leites de vaca e cabra através da utilização das flores do cardo, provenientes de diferentes regiões do Algarve.

Procedeu-se para tal à caracterização dos parâmetros de coagulação dos leites com um extracto bruto de flores de cardo (temperatura, pH, concentração de cálcio e concentração de cardo), a fim de se obter um tempo óptimo de coagulação para cada um dos dois tipos de leite estudados.

Constatou-se que os parâmetros de coagulação estudados apresentaram diferenças entre os leites de vaca e cabra, evidenciando uma maior afinidade para o leite de cabra. Assim tem-se para o leite de vaca: temperatura de 50ºC, pH normal do leite (6,6-6,7), concentração de $CaCl_2$ $2H_2O$ de 10 mM e concentração de cardo de 0,03% (p/v). E para o leite de cabra: temperatura de 55ºC, pH de 5,5 e concentração de cardo de 0,03% (p/v).

Searching for *Listeria* spp. by Following Marked Salmon along its Cold-smoking Processing Chain

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***Key words**: Cold-smoked salmon;* Listeria *spp.*

The occurrence of *Listeria* spp. and *Listeria monocytogenes* along the process line of three cold-smoking Portuguese producers has been ascertained already (Duarte *et al*, 1995)). That survey was done by sampling, in a random basis, fish and environmental samples, just to evaluate the presence of the organisms in each food plant and in each type of fish. Traditional and impedimetric analytical techniques were used. *Listeria* spp. was found in fish products as well as in environmental samples. In all the factories *Listeria* spp. was found in samples of sliced and vacuum packed cold-smoked salmon.

In this study, two different fishes, (*Salmo salar* species, as they are processed in a regular basis were easier to be evaluated) were marked and followed along the process line by collecting the samples in each step of the processing chain. In each plant the experience was performed twice. The ISO 11290-1 protocol was used, together with the miniVidas/ Biomérieux, an Enzyme Linked Fluorescent Assay. Oxford and Palcam agars were used as isolation media.

A total of 72 fish salmon samples were analysed. *Listeria* spp. was found in the steps before smoking in all the factories. Only in one sample from Plant B the organism has been found at the end of the process. On the other factories the smoking process showed to be successful on the reduction of *Listeria* spp. levels, despite no changes (no listericidal steps) had been introduced in the processes when compared to the first survey mentioned above. *Listeria* spp. was never found in the 53 environmental samples of the salmon processing line analysed during this survey. Environmental samples included water, containers, processing boards, knives, working tables, smoker trays, clean brine, filleting machine, packaging material.

Presence of *Listeria* spp. along cold-smoking salmon processing line

Process line	Samples	Plant A		*Plant B		Plant C	
Raw material	Sample 1	-	-	-	L.monocyto.	-	L.seeligeri
	Sample 2	-	L.seeligeri	-	L.seeligeri	-	L.seeligeri
Fillets after washing	Sample 1	-	-	-	-	-	-
	Sample 2	L.monocyto.	-	-	L.seeligeri	-	-
Fillets after salting	Sample 1	-	-	-	-	-	-
	Sample 2	-	-	-	L.seeligeri/L .innocua	-	-
Fillets after smoking	Sample 1	-	-	-	-	-	-
	Sample 2	-	-	-	L.seeligeri	-	-
Fillets after slicing	Sample 1	-	-	-	-	-	-
	Sample 2	-	-	-	L.seeligeri	-	-
Final product	Sample 1	-	-	-	-	-	-
	Sample 2	-	-	-	L.seeligeri	-	-

* Wet salting is used in this plant

Dominant Microflora of *Picante* Cheese: Independent Role upon Proteolysis and Lipolysis in Model Systems

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Key Words: Picante *cheese, proteolysis, lipolysis, microflora*

Four species of bacteria (two species of enterococci, *Enterococcus faecium and Enterococcus faecalis*, and two species of lactobacilli, *Lactobacillus plantarum* and *Lactobacillus paracasei*) and three species of yeasts (*Debaryomyces hansenii*, *Yarrowia lipolytica* and *Cryptococcus laurentii*), previously isolated from *Picante* cheese were assayed for biochemical performance in proteolysis and lipolysis. In addition to the difference of the microbiological strains, the milk type (caprine or ovine), the ripening time (0 to 65 days) and the concentration of NaCl (0 to 14%(w/v)) have been deliberatly fixed *in vitro* curdled milk (previously prepared from heat-sterilized milk, coagulated with animal rennet and inoculated with each strain) and subject to 12 ºC. High proteolytic activity was demonstrated by *Y. lipolytica* and by all the other strains to a lesser extent; *Y. lipolytica* produced ca. 85% of WSN by 65 days of ripening whereas *E. faecium*, *D. hansenii* and *C. laurentii* produced levels of WSN ranging in 40-50%, and *E. faecalis*, *L. plantarum* and *L. paracasei* in 30-40%. In terms of peptidolytic activity, measured by NPN contents and by release of free amino acids, once again *Y. lipolytica* presented the highest activity, followed by *L. plantarum*, *L. paracasei*, *E. faecium* and *E. faecalis*. Milk type, ripening time, and content of NaCl revealed to be statistically significant processing factors in terms of proteolysis; caprine milk, 65 days of ripening and lower contents of NaCl led to the highest values. The lipolytic activity, assessed by the release of butyric acid from tributyrin, was strong for *Y. lipolytica* and *C. laurentii*, whereas release of free fatty acids was observed at different rates for all strains under study. Ripening time proved to be a statistically significant factor for lipolysis, whereas milk type was not; lipolytic activities, measured as fat acidity index, were strongly affected by NaCl content and, as happened with release of free amino acids, the extent of fat hydrolysis was much more affected by the increase of NaCl from 0 to 7% than by its increase from 7% to 14%. Although it is not possible to directly compare results obtained *in vitro* using pure, single cultures with those obtained *in loco* using actual cheese, our results suggest that a mixed-strain starter for *Picante* cheese including *L. plantarum*, *E. faecium* (or *E. faecalis*) and *D. hansenii* (and/or *Y. lipolytica*) would be of potential interest.

Studies on Degradation of Aspartic Proteinases from Fresh Flowers of *Cynara cardunculus* L.

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***Keywords**:* Cynara cardunculus*, aspartic proteinase, degradation*

Aspartic proteinases can be found in monocotyledonous and dicotyledonous species, as well as in gymnosperms.

Milk clotting enzymes from plant origin are rarely used for cheese making. One of the most successful plant rennets, used in Portugal and some regions of Spain since Roman times is obtained from dried flowers of *Cynara cardunculus* L., for the manufacture of traditional cheeses from raw ovine milk.

Here we report some studies on purification and protein degradation of milk clotting proteases isolated from fresh flowers of *C. cardunculus*.

Aqueous extracts of dried flowers of *C. cardunculus* showed to possess three aspartic proteinases each composed of two sub units with Mr of 30 and 15 KDa respectively, when analysed by SDS-PAGE [1]. More recently, two additional aspartic proteinases were isolated from fresh stigmata of *C. cardunculus* extracted at pH 3.0. On SDS-PAGE these two aspartic proteinases showed a Mr close to 30 and 15 KDa [2]. A 67 KDa band was also obtained from a fresh flowers of *C. cardunculus* suggesting this protein band represents the precursor form of the enzyme [3]

Knowing that aspartic proteinases from plant contain an insert of about 100 amino acids that should be processed away and, that these proteinases, in general, synthesised as zymogens, are activated at acidic pH, studies on proteinase degradation were done by incubating purified extracts at pH 3.0. Aspartic proteinases from fresh flowers, were extracted at pH 8.3 as referred above, further purified by salt precipitation and ionic exchange chromatography, and incubated at pH 3.0.
After incubation at pH 3.0 only the bands with Mr around 30 and 15 KDa were observed Working at basic pH and starting with fresh material, higher bands, similar to those reported by Ramalho-Santos (1996), were obtained on this work. Working at conditions far from those reported to be the optimal for aspartic proteinases activation, higher bands obtained probably represent the proenzyme form subsequently converted to converted to mature protein, by a proteolytic process, as described for *Centaurea calcitrapa* aspartic proteinases [4].

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Caracterização de Queijo Serpa Proveniente de Três Genótipos Ovinos

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***Keywords**: queijo, ovelha, Queijo Serpa, raça ovina*

O Queijo Serpa, tradicionalmente manufacturado com leite proveniente de ovelhas das raças Merina e Campaniça, passou gradualmente a ser produzido a partir de leite de ovelhas de raças estrangeiras e nacionais, nomeadamente Lacaune e Serra da Estrela, mais especializadas na produção de leite.

A legislação elaborada para a defesa da qualidade do Queijo Serpa (Dec. Reg. nº 39/87) não considera o factor "raça ovina" como condicionante na atribuição da Denominação de Origem "Queijo Serpa".

Neste trabalho utilizaram-se ovelhas das raças Merina, Serra da Estrela e Lacaune, submetidas ao mesmo tipo de exploração e maneio. Pretendeu-se com o estudo dos queijos produzidos a partir do leite de cada genótipo ovino, avaliar a influência da raça nas características físicas, químicas e sensoriais dos queijos e comparar estas características ao longo da época de fabrico.

Analisaram-se queijos, produzidos quinzenalmente na Herdade da Abóbada segundo tecnologia tradicional, com aproximadamente 40 dias e 55 dias de maturação. Estes queijos foram submetidos a avaliação sensorial pelo Painel de Provadores do Queijo Serpa (ACOS), tendo sido previamente determinado o seu peso, diâmetro e altura. Seguidamente foram efectuadas análises microbiológicas (contagens de microrganismos totais mesófilos, coliformes totais, *Escherichia coli*, *Staphylococcus aureus* e pesquisa de *Salmonella*) e análises químicas (humidade, matéria gorda, acidez, cloretos, pH, azoto total e azoto solúvel). Os dados obtidos foram submetidos a análise multivariada, em particular foram aplicadas as técnicas de análise de correlações, análise em componentes principais normadas e análise classificatória hierárquica. Foi ainda utilizada a análise de variância.

Concluiu-se que a tecnologia artesanal de fabrico do Queijo Serpa origina queijos muito heterogéneos. Verificou-se que a raça da ovelha não influencia significativamente as características sensoriais, físicas e químicas dos queijos. Ao longo da época de fabrico verificaram-se variações significativas na pontuação total da avaliação sensorial ($P \leq 0.05$); humidade, matéria gorda, cloretos, pH e azoto total ($P \leq 0.001$) e acidez ($P \leq 0.05$). O prolongamento da cura de 40 para 55 dias conduz a uma diminuição da qualidade dos queijos. O coeficiente de maturação revelou-se muito abaixo do valor legalmente exigido e não indicativo da qualidade do queijo. A falta de higiene na ordenha e fabrico dos queijos, é patente nos teores elevados de flora contaminante encontrada.

Estrela Cheese: Chemical Considerations

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Keywords: Cheese, flavour, casein degradation

Development of specific aromas in cheeses is the result of a complex set of interdependent biochemical reactions. After casein full degradation, and depending on the microflora present, free amino acids may be further transformed to yield volatiles, among which amines and sulphur-containing compounds contribute considerably to the overall characteristic aroma. The complex structure of the cheese matrix raises difficulties in extraction of flavour compounds, and problems also develop with the analytical assays.

The main aim of this study was to test and fit techniques for flavour assessment applicable in differenciation among flavour patterns at several ripening stages of an artisanal Portuguese cheese. Changes in the flavour profile of Serra de Estrela cheese throughout ripening up to four months have been assessed using solid phase micro extraction by adsorbing volatiles onto a polyacrylate fiber under dynamic headspace conditions and subsequent analysing using a gas chromatograph. This method, adopted from Chin et al. has been further improved to fit our requirements. Whereas other isolation methods failed, this adsorption technique provided yields of volatiles of interest that are sufficiently good to allow identification by mass spectrometry. Initially, identification has been done via comparison with 50 reference compounds in terms of their retention times in gas chromatography.

Preliminary results indicate, that typical fermentation products (e.g. ethanol, acetone, 2,3-butanediol and ethyl esters of short chain fatty acids) dominate during the first month of ripening. Late stages of ripening are characterized by domination of free short chain fatty acids, of which butyric, caproic, caprylic and capric acids play major roles in the characteristic, pleasant aroma of this cheese.

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Separation and quantification of milk proteins

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Key-words: milk proteins, caseins, β-lactoglobulina, α-lactalbumina, HPLC

Protein content of cow's milk is a complex mixture. About 80 % of these proteins consist of caseins, a heterogeneous fraction which is insoluble at its isoelectric pH (pH 4.6). The casein fraction can be subdivided into the α_{s1}, α_{s2}, β and κ-casein components, which in milk occur as a micelar complex in the approximate proportions 4:1:4:1, respectively. The remaining 20% of the milk protein fraction is composed of the whey proteins (soluble at pH 4.6) of which β-lactogloblin and α-lactalbumin are the main components (ratio ca. 3:1). The molecular weight of the individual milk protein monomers ranges from 15.500 to 67.000 [1].

Quality control of milk and its derivatives is presently a very demanding field and urges the need for economic, time-saving and accurate methods to be developed. Therefore the aim of our work was to develop a single method capable of separating and quantifying the two protein fractions simultaneously.

Our initial work on polyacrylamide gradient gel electrophoresis showed that the separation of the subunits of these proteins was possible, but quantification of the individual proteins was difficult because the proteins separated in very close bands, the gradient gel varied in thickness and staining and destaining of the protein bands was not reproducible. Subsequently, we applied chromatographic methodologies known as useful tools available for protein purification and quantification.

The present work describes the simultaneous HPLC separation of casein, β-lactogloblin and α-lactalbumin in milk, achieved with a Chrompack 300 RP chromatographic column. Gradient elution was carried out with a mixture of two solvents. Solvent A was trifluoroacetic acid in water and solvent B was acetonitrile-water-trifluoroacetic acid. The effluent was monitored by a UV detector set at 280 nm.

The separation of major milk proteins can be performed rapidly and reliably with this method. Because most protein quantification and separation techniques are too inconvenient for dairy plants, payments for milk at present are mostly based on the fat content instead of the protein content. By using this HPLC system, the dairy industry may grade raw milk by its nutritional value based on protein content. This technique can also be applied with success to the detection of adulteration of fresh milk with cheaper powdered milk.

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Aroma Compound Recovery by Pervaporation Process

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key words: Pervaporation, Aroma, Biotechnology, Esthers.

The objective of this study was the recovery of aroma compounds, that can be produced by Biotechnology, using the pervaporation process (one type of membrane process). The aroma compounds chosen were ethyl propionate (aroma of rum), ethyl isovalerate (apple aroma) and ethyl hexanoate (pineapple aroma).

In this work was selected a comercial composite membrane of polydimethylsiloxane - GFT 1060. The pervaporation showed to be a selective process to recovery of investigated compounds exhibiting liquid-liquid separation in the permeate after its condensation (one organic fase and one aqueous fase), that is a advantage for this process because it was possible to get a high value product. The selectivity of this process, to ethyl propionate was about 243, 480 to ethyl isovalerate and 797 to ethyl hexanoate.

Experiments with binary mixtures of aroma compounds / water and multicomponent mixtures of aroma compounds / water were realized. The results showed that there wasn't difference between the permeate fluxes of chosen compounds in binary and multicomponent mixtures, that was confirmed by the calculation of activities of studied substances in these mixtures. Thus, coupled permeate fluxes were not known.

In the real fermentation media there are non-volatiles compounds, like proteins, salts, lipids and other substances. So it´s necessary to investigate the influence of composition of the fermentation media on the fluxes of the aroma compounds. It was known that the non-volatiles compounds don't interfere in permeate fluxes of aroma compounds. Therefore, the pervaporation can be a promising alternative for the recovery of nature aroma compounds produced by fermentation process.

Distribuição Diferencial das Cardosinas A e B ao Longo do Pistilo de *Cynara cardunculus* L – Implicações no Fabrico do Queijo

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***Palavras Chave**: Cardosina A, Cardosina B,* Cynara cardunculus *L., Proteases aspárticas*

Os pistilos de cardo (*Cynara cardunculus* L.) são usados tradicionalmente em Portugal na manufactura de queijos de elevada qualidade. A actividade coagulante desta flor deve-se à presença de duas proteases aspárticas denominadas por cardosina A e cardosina B [1], que tinham sido já isoladas e extensivamente caracterizadas [2].

Com vista a avaliar o conteúdo em cardosinas ao longo dos pistilos, estes foram seccionados em quatro fracções e efectuada a extracção e quantificação das cardosinas de cada fracção. Por técnicas electroforéticas, imunológicas e cromatográficas, mostrou-se existir uma expressão diferencial de ambas as cardosinas ao longo do pistilo de cardo.

As cardosinas encontram-se predominantemente na porção superior do pistilo, diminuindo a sua expressão nas zonas mais basais. Ao longo do pistilo ocorre também uma variação na distribuição relativa das duas cardosinas. A razão entre a quantidade de cardosina A e a quantidade de cardosina B, diminui ao longo do pistilo, existindo apenas cardosina B na zona mais basal do pistilo.
Uma vez que os pistilos de *Cynara cardunculus* L. são utilizados no processo tradicional de manufactura do queijo como agente coagulante, os extractos obtidos a partir das quatro fracções dos pistilos foram também avaliados sob o ponto de vista das actividades coagulantes (C) e proteolíticas (P). A razão entre estas actividades (C/P), dá-nos uma boa informação acerca da qualidade do coalho [3].

Os resultados mostraram ser o extracto superior o que se apresenta mais indicado a ser usado na manufactura do queijo, uma vez que possuía uma razão de actividades (C/P) nove vezes superior à razão do extracto mais basal. Estes resultados indicam uma eventual possibilidade de a qualidade dos coalhos ser afectada pelo modo de colheita dos pistilos. Quando arrancados dos capítulos (processo denominado popularmente por ripagem), são obtidos pistilos quase completos, em oposição, o corte dos pistilos leva à obtenção apenas das porções superiores dos mesmos, material este que apresenta melhores qualidades como agente coagulante.

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Brevibacterium linens: Molecular Biology and Biochemistry of Physiological Activities Relevant to Cheese Processing

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Key words: Brevibacterium linens*; protease; bacteriocin; genetic fingerprinting*

The species *Brevibacterium linens* belongs to the phylogenetic branch of high % GC Gram positive bacteria, being classified as a coryneform bacteria. The strains deposited on culture collections were mostly isolated from the surface of cheeses. Until recently this species was poorly studied, but during the last three years the number of publications with its name on the title as grown exponentially. This is a consequence of the recognised relevance of members of *B. linens* on cheese ripening, giving to this species a status similar to the lactic acid bacteria.

The action of *B. linens* strains on cheese ripening seems to be important at three different levels: proteolytic activity, methanetiol production and biosynthesis of compounds with anti microbial activity. Proteolytic activity is well documented for lactic acid bacteria, but the knowledge about substrate specificities and biochemical properties of *B. linens* proteases is still very limited. The action of proteases produced by cheese flora produces small peptides which seem to be important on the final product flavour and aroma. Several sulphur compounds of variable complexity contribute also for the modulation of flavour and aroma. One of those compounds is methanethiol, produced by fungi and some bacteria. *B. linens* is recognised as a methanetiol producer and the study of the physiological aspects of this activity is still beginning. Recent publications refer the detection of proteins with lytic activity against *Listeria spp* and other Gram positive bacteria. Those proteins belong to the bacteriocin group of anti-microbial compounds and are useful tools as growth inhibitors of potentially pathogenic bacteria on food products. The physiology of these activities as well as the genetic regulation of the enzymes involved is a point of great interest for the industry as the knowledge acquired could be a great contribute for the development of starter cultures with improved capabilities.

Taking advantage of the previous experience on corynebacteria, we started at our laboratory the physiologic and genetic characterisation of *B. linens* strains. One of our strains produces a peptide with anti-bacterial activity. From the supernatants of liquid medium cultures a protein of 30 KDa was purified and is now under characterisation. Another strain has a protease partially bound to the membrane. This strain is now subjected to intensive study and efforts are being made to purify this protease.

The number of new isolates referred on the literature is growing enormously. Many of those strains are not well characterised and some of them could be misclassified. An important effort is being made for the fingerprinting of *B. linens* strains at the molecular level. Using Pulsed-field Gel Electrophoresis analysis and RFLPs at the rRNA genes we were able to give an indisputable identity to several strains. Information about genome complexity and stability were also obtained.

Production of Cheese Flavours (methyl ketones) by *Aspergillus niger*

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***Keywords**: methyl ketones, fungi,* Aspergillus niger*, β-oxidation.*

Methyl ketones have a variety of applications in the food industry, such as in blue cheeses (e.g. Roquefort, Stilton, Danish Blue) and fruit flavours. They are the result of enzymatic and microbial processes and, because they comply with the definition of "natural", they can be used as additives in food products [1].

These conversions are confined to certain fungi: the most relevant belong to the genera *Aspergillus* and *Penicillium* and their related teleomorphic genera *Trichoderma*, *Cladosporium* and *Fusarium* [2]. Methyl ketone formation by fungi has been known for more than 70 years, however, the details are still obscure [3]. It is generally accepted that methyl ketones arise from incomplete β-oxidation of medium chain fatty acids [4]. It is possible that an incomplete fatty acid oxidation system, for example lacking one of the enzymes, would be in the origin of formation of intermediates. It is not certain where the conversion takes place but it is assumed that it is more likely to be peroxisomal.

Aspergillus niger does not grow easily on medium chain fatty acids. Thus, incubation with these substrates was performed by transference. High concentrations of medium chain fatty acids proved toxic to the cells, especially glucose-grown cells. This toxicity was evident when these cells were observed under the electron microscope. Similar levels of methyl ketones were obtained with both glucose- and fatty acid-grown cells (triolein). It does not seem that growth on fatty acids (formation of peroxisomes induced) is an advantage in respect to methyl ketone formation. Methyl ketone formation was quantified by GLC analysis.

The principal enzymes of fatty acid oxidation and their substrate specificity were investigated in *A. niger* cell-free extracts. Lipase activity was below the levels of detection in glucose-grown cells but induced on incubation with fatty acids. No acyl-CoA oxidase was detected whereas an acyl-CoA dehydrogenase activity was measured and was inducible. The low activity of this enzyme suggested it is rate-limiting in β-oxidation. There was an active and stable 3-ketoacyl-CoA thiolase present in these cell extracts. It does not appear that lack of 3-ketoacyl-CoA thiolase would be the explanation for methyl ketone formation. Methyl ketone formation by cell extracts was attempted but it seems that the system responsible is not functional in cell extracts of *A. niger*. This is supported by the existence of an active 3-ketoacyl-CoA thiolase in the cell-free extracts.

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Enological Diversity of *S. cerevisiae* populations at the Last Stages of Wine-Making in Albariño Grape Musts (Salnés Region)

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Key words: metabolic variability, S. cerevisiae*, chromosomal polymorphism.*

Seven representative cellars from the Salnés Region (D.O. Rías Baixas) were chosen to get to know the *S.cerevisiae* yeasts diversity at the last wine-making stages. Ending the alcoholic fermentations, several must samples were collected and cultured into must-agar petri dishes. Forty five colonies, with different macroscopical morphology, were then isolated and identified as *S.cerevisiae*. Their chromosomal and enological profiles were respectively made by karyotyping (PFGE) and microvinification assays. The populations of *S.cerevisiae* species fermenting these musts are characterised by a high chromosomal polymorphism and metabolic variability. In addition, the alcoholic fermentation is not ended by a only strain, but two or more different enological varieties conduce simultaneously the last periods of vinification. Methods of isolation and molecular characterization were useful to detect several wine strains in a same cellar. The utility of chromosomal polymorphism for search the *S.cerevisiae* enological varieties is discussed.

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In Vitro Culture and Genetic Transformation of Almond

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Keywords: Almond, adventitious regeneration, genetic transformation

Genetic improvement of almond cultivars has traditionally been carried out by classical breeding methods requiring long periods of time. Recent biotechnological approaches, such as gene transfer techniques based on tissue culture protocols, have the potential for introducing specific genetic changes in selected genotypes within a shorter period of time. The aims of this work were (1) *in vitro* establishment and micropropagation of several portuguese almond varieties, (2) development of a protocol for adventitious regeneration and (3) study of the conditions for genetic transformation and recovery of transgenic almond plants.
Actively growing shoots and seeds from cultivars 'Boa Casta', 'Molar da Fuzeta', 'Duro da Estrada Grado', 'Bonita de S. Brás' and 'Peneda' were collected, and axillary shoot cultures derived from adult and seed materials were established in vitro and micropropagated as previously described [1]. Rooting of micropropagated shoots was accomplished by brief induction periods on highly concentrated solutions (0.08-1 g/l) of IAA or IBA followed by culture on medium devoid of growth regulators. After thorough testing of culture conditions for adventitious shoot regeneration a protocol was established yielding high regeneration rates from leaves of juvenile origin [1] and, therefore it was used for *Agrobacterium*-mediated genetic transformation experiments and for DNA transfer using an electric discharge particle acceleration device. In the particle bombardment experiments several plasmids were tested carrying different selection markers. The surviving regenerated shoots are currently under evaluation. In *Agrobacterium*-mediated transformations EHA105 (p35SGUSINT / pFAJ3003) or LBA4404 (p35SGUSINT) strains were tested. Strain EHA105 was selected for further use due to its effectiveness evaluated by transient GUS expression assays. Shoot induction was performed on 0 or 10-20 mg/l kanamycin, followed by transfer to shoot elongation medium with 50 mg/l kanamycin. Kanamycin resistant shoots were subjected to PCR analysis using specific primers for the introduced genes (*npt*II and *uid*A). Amplification was obtained for 4 of the analysed shoots. After propagation of these shoots for a larger scale DNA isolation only one of the 4 above mentioned clones gave a positive signal in Southern blot hybridisation and showed strong GUS expression. This is the first report of recovery of transgenic almond plants and opens new perspectives for the genetic engineering of almond for various agronomic traits including virus resistance.

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Biochemical Oxygen Demand of *Rhizobium tropici* Pre-incubated with *Canavalia brasiliensis* Lectin

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Keywords: Rhizobium, Lectin, Agglutination, BOD, Oxygen.

The *Rhizobium tropici* bacteria establishes with *Phaseolus vulgaris* plants an N_2-fixing symbiosis. The legumes lectins has been showing participation in the establishment of that symbiosis. However, the cell wall of bacteria not only precludes any interation between the glycoconjugades on their membrane and carbohydrates-binding proteins but also prevents these proteins from penetrating the cytoplasm, and still, the plants lectins cannot alter the struture and/or permeability of the membrane or distrub the normal intracelular processes of the membrane invading microbes [1,2]. In order to establish condition to improve this symbiosis we study the effect of lectin in the oxygen metabolism of *R. tropici*. The strains of *R. tropici CIAT899 e F35* were obtained from the CNPAF/EMBRAPA-Goiânia/Brasil. Lectins used in the experiments were as following: *Vatairea macrocarpa*-VmaL, *Dioclea violacea*-DviL, *Canavalia brasiliensis*-Con Br, *Cratylia floribuntia*-CrfL. Cells of *Rhizobium* cultivated in YM (4 days, 30°C), were centrifuged (8.000g, 15min, 20°C) 3x in buffer MES (0.01M, pH 5.5 and 6.8) and added to the same buffer up to OD_{610}=0.6. Agglutinating activity of lectins were performed using serial 2-fold dilutions test samples in flat microtiter U-plates. Bacterial agglutination test was carried out in pH 5.5 and 6.8 by incubating (1h, 30°C) the lectins in buffer MES. Biochemical oxygen demand-BOD were measured by a Clark electrode (Dissolved Oxygen Meter; YSI Model 58/Clandon) using *Rhizobium* cells in pH 6.8. The treatment assay was carried out as following: 1 - without lectin (20min) + Manitol (55mM, 48min); 2 - lectin (0-400 µg. ml^{-1}) + Manitol. The BOD measures were made in mixture under agitation with air flow constant (30± 0,2°C). The agglutinating activity of lectins on strain *Rhizobium* shown to be pH dependent, been higher with pH 5.5 (>30 µg. ml^{-1}) the majorities of the tested lectins. Activity was reduced to pH 6.8 in majorities of the lectins and others was not detected (DviL and Con Br in CIAT899). The Con Br was selected by showing agglutinative activity pH dependent with both strains (agglutinates in >250 µg. ml^{-1}, pH 5.5). The BOD for both strains was different. In the pre-treatment (without lectin), at CIAT899 and F35, both removes 13% and 22% of initial BOD (6.71 mg. l^{-1} and 5.98 mg. l^{-1} of O_2), respectively. After that, was added 400 µg. ml^{-1} of Con Br, this increased 45% of initial BOD in both strains. However, applying 50, 100 and 200 µg. ml^{-1} of lectin in both strains plus Manitol, the BOD increased 51% for CIAT899 and 19% for F35 strain. Pre-incubation with Con Br (400 µg. ml^{-1}) increased 70% and 30% in BOD for CIAT899 and F35, respectively.

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Identification of the Human Lewis[a] Carbohydrate Motif in a Secretory Peroxidase from a Plant Cell Suspension Culture (*Vaccinium myrtillus* L.)

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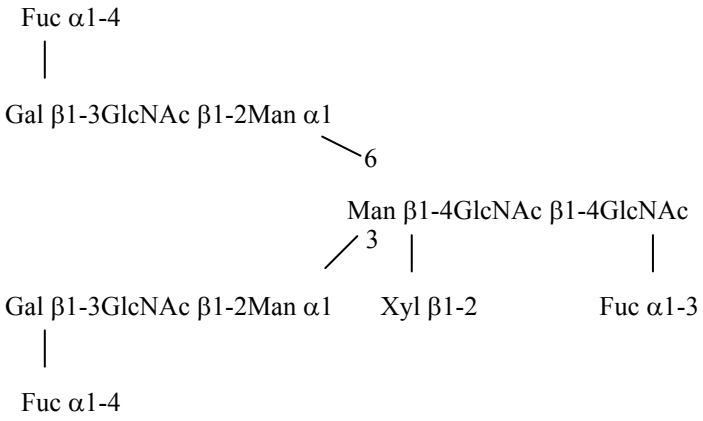
Key words: *Extracellular glycoproteins, Lewis[a], peroxidase, plant complex oligosaccharides,* Vaccinium myrtillus

In this work we report for the first time the presence of the human Lewis[a] type determinant in glycoproteins secreted by plant cells [1]. A single glycopeptide was identified in the tryptic hydrolysis of the peroxidase VMPxC1 from *Vaccinium myrtillus* L. by HPLC/ESI-MS. The oligosaccharide structures were elucidated by ESI-MS-MS and by methylation analysis before and after removal of fucose by mild acid hydrolysis. The major structure determined is of the biantennary plant complex type and contains the outer chain motif Lewis[a].



A corresponding fucosyltransferase activity catalyzing the formation of Lewis[a] type structures *in vitro* was identified in cellular extracts of the suspension cultures.

Lewis[a] motifs are mostly found on the surface of cancer cells of the digestive system and seem to be directly involved in metastasis. Molecules containing these motifs at their surface can be efficiently used for the inhibition of cellular interactions, and may be helpful in the therapeutic treatment of metastasis.

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Study of Ion Accumulation in Sunflower Plants and *Calli* Exposed to High Levels of NaCl and PEG by X-Ray Microanalyses, Capillary Electrophoresis and ICP

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Keywords: sunflower, tissue culture, salt stress, capillary electrophoresis, electron microscopy

Several areas in South Europe suffer from at least one kind of salt or water stress, and this is a problem that is increasing every year. Salt stress induces not only osmotic stress in plants, but they also have to face ion toxicity and nutrient deficiency. Several works show the negative effects of NaCl on Ca, N, K and Mg levels in plants, and Ca and N may become limiting factors. On the other hand, the effects of NaCl on P accumulation are more complex, depending on the concentration of P in the soil. The effect of NaCl and osmotic stress on micronutrient levels in plants is also controversy, depending on genotype and tissue and on the microelements in study. Different results found previously for the behaviour of elements in the same species under stress may be due to different analytical techniques used.

In this report we analyse changes in macro and microelement levels induced by NaCl using Capillary Electrophoresis (CE), Inductively Coupled Plasma (ICP) and microanalysed by Scanning Electron Microscope coupled with an X-ray Detection System (SEM-EDS). Sunflower (*Helianthus annuus* L. cv. Girapac SH 222) *calli* were induced and maintained on solid MSmod medium (1). Plants and *calli* were exposed to NaCl moderate salt stress (50 mM NaCl), to severe salt stress (100 mM NaCl) and to stress induced by Polyethylene Glycol (50 g dm^{-3}). *Calli* had an ID_{50} of 183 mM for NaCl, whereas plants had an ID_{50} of 66 mM. Mineral analysis by EC and by ICP showed an increase in Na and Cl contents, and a decrease in nitrate, sulphate and potassium in whole plant. Calcium and Mg levels decreased only in shoots while phosphate was not affected. In *calli* only nitrate, K and Ca levels decreased, while Na and Cl increased. Ultrastructural analyses of stem medullar cells by SEM-EDS also showed an increase in the contents of sodium, chloride and phosphorus and a decrease in Ca, Mg and sulfur levels. From this work we established a pattern of accumulation of macro and micronutrients in plants and *calli* exposed to NaCl stress. This pattern was confirmed by the different methods we used. These results are particularly important in establishing a pattern for nutrient behaviour in sunflower cells exposed to high levels of NaCl and for further analysis of salt tolerance mechanisms in sunflower plants.

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Pharmaceutical Interesting Compounds Produced by *in vitro* Cultures of *Hypericum perforatum*

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Keywords: Hypericum perforatum, in vitro *cultures, xanthones, hypericins*

Hypericum perforatum L. (St. John´s wort) has been used as a medicinal plant since middle age due to its reputation as an anti-inflammatory and healing agent. Nowadays plants of this species are used by their anti-depressive and anti-viral properties. According to some authors hypericin and its derivatives, natural anthraquinones of *H. perforatum*, have anti-viral activity (1, 2), namely against AIDS (3). Although *in vitro* cultures may contribute to the study of natural product biosynthesis and to the obtention of biomass with high contents on compounds of pharmaceutical interest, references concerning to *in vitro* cultures of *H. perfotratum* are scanty.

We have established *in vitro* cultures of shoots, *calli* and suspended cells of these species on MS medium supplemented with auxins and citokinins under a photoperiod of 16h light/8h dark. We have analysed, qualitatively and quantitatively, the phenolic compounds accumulated in these type cultures and we have compared them with those produced by *in vivo* plants.

The HPLC-DAD profiles obtained in the course of this work clearly evidenced a distinct phenolic production between *in vivo* and *in vitro* biomass. For example, *calli* and suspended cells produced mainly xanthones while in *in vivo* plants species these compounds were not detected. The major xanthones were identified, after isolation, as 1,3,6,7 oxygenated xanthones with methoxy and prenyl groups. Xanthones with this oxygenation pattern are very interesting pharmacological compounds possessing anti-depressive activity, due to inhibition of MAO, and having anti-bacterial and anti-fungic properties. Hypericins were detected in *in vivo* plants and in *in vitro* shoots of *H. perforatum* but not in *calli* or suspended cells of the same species. The content of hypericins in *in vitro* shoots is ten times higher then in *in vivo* plants. It seems, therefore, that hypericin production is dependent of tissue differentiation beeing the shoot cultures a good source of these type compounds.

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Astaxanthin Production by Yeast Growing in Solutions Containing Glucose and Cellobiose Derived from Enzymatic Hydrolysates of Wood

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Keywords*: Astaxanthin,Carotenoids, Enzymatic Hydrolysates,* Phaffia rhodozyma*, Wood*

INTRODUCTION

The red yeast *Phaffia rhodozyma* is one of the most promising microorganisms for the comercial production of astaxanthin, a carotenoid found in nature having a typical orange-pink colour that is responsible for its applications in food and feed products. *Eucalyptus* wood, a cheap and largely available hardwood with favorable characteristic for chemical processing, was subjected to an alkaline treatment under selected conditions for obtaining a solid residue susceptible to enzymatic sacharification. Enzymatic hydrolysates containing glucose or glucose and cellobiose (a disaccharide that has been reported as astaxanthin inducer in yeasts) were used to make fermentation media for *Phaffia* proliferation. An inorganic nitrogen source (KNO_3) was also added enhanced carotenogenesis. The main purpose of this work was to study the potentiality of enzymatic wood hydrolysates for making suitable culture media for astaxanthin production by *Phaffia rhodozyma*, with special attention to the improvements caused by the presence of cellobiose or KNO_3.

MATERIALS AND METHODS

Eucalyptus wood samples were submitted to alkaline treatments under selected operational conditions (10% NaOH, 130°C, 10 g alkaline solution/g dry wood, 2 hours). The solid residues from alkaline treatments were hydrolyzed in media containing eiter cellulases or a mixture of cellulase and β-glucosidase. Enzymatic activities were 0.3 U. Filter Paper/ml and 4 IU of cellobiase/ml. The enzymatic assays were performed during 48 h at 48.5 °C and pH 4.85 using a liquor/solid ratio of 10 g/g. Enzymatic hydrolysates or comercial sugar solutions were supplemented with nutrients, inoculated with *Phaffia rhodozyma* ATCC 24228 and incubated in a batch fermentor (Biostat B, Braun Biotech). The experimental conditions for fermentation were: agitation speed controlled to provide 40% oxigen saturation, pH=6 and temperature=22°C. The biomass generation was followed by dry cell mass measurements, and the carotenoid contents of cells was analysed by HPLC-DAD after DMSO extraction.

RESULTS AND DISCUSSION

The maximum carotenoid concentrations were reached after 40-70 hours in media made from hydrolysates, in comparison with 90-200 hours in synthetic media. Because of this, the mean volumetric rate of carotenoid production during the exponential phase of growth was 5-6 times higher for hydrolysate-based media than for synthetic ones. The presence of cellobiose significanty affected the carotenoid profile, increasing the astaxanthin percent of total hydrolysates (from 61.7 up to 68.5%). Significant increases in total carotenoid concentration (17-24.5%) were caused by the inorganic nitrogen source.

Bioconversion of Xylose-Containing Hydrolysates: Fed-batch Production of *Phaffia* Biomass with Improved Pigmentation

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Keywords: Astaxanthin,Carotenoids, Hemicellulose hydrolysates, *Phaffia rhodozyma*, Wood

INTRODUCTION

The pink yeast *Phaffia rhodozyma* is one of the most promising microbial sources of astaxanthin, a pigment used in salmonid or poultry feed. *Phaffia rhodozyma* can grow from pentoses. In this field, studies in fermentation media formulated with commercial xylose have been reported in literature. However, the practical interest of these studies would be enhanced if the fermentation media are prepared by processing cheap raw materials instead of pure sugars.

This work deals with the production of highly-pigmented Phaffia biomass by fed-batch cultures of microorganisms in media derived from acid hydrolysates of *Eucalyptus* wood.

MATERIALS AND METHODS

Acid hydrolyses of wood were performed under the following conditions: 3% aqueous sulphuric acid, 130 ºC, 1 hour, liquor:solid ratio = 8/1 g/g. Neutralized hydrolysates were detoxified by contact with charcoal (90 min, room temperature, mass ratio hydrolysate:charcoal=50/1 g/g). Culture media were supplemented with 0.2 g KNO_3/L and 3 g peptone/L, and used as fermentation media. The parent strain *Phaffia rhodozyma* NRRL Y-17268 used in this work was kindly provided by the Agricultural Research Service Culture Collection (Peoria, Illinois, USA). Experiments were performed in a 2- liter Braun Biostat B bioreactor (Melsungen, Germany) under the following conditions: temperature, 22 ºC; air flow rate, 3 L/min; agitation speed, 400 rpm; constant illumination (300 lux) provided by cool white fluorescent lamps. The dissolved O_2 concentration was above 75% of saturation during all the experiments.. Wood hydrolysates and carotenoids in biomass were assayed by HPLC (with IR and DAD detection, respectively).

RESULTS AND DISCUSSION

Two fed-batch strategies (continuous and multi-step feeding) were assayed to improve the carotenoid production from xylose-containing hydrolysates. Both strategies performed better than single batchs. The most favourable conditions were determined for multi-step fed-batch operation. This procedure led to culture media containing high volumetric concentrations of pigments (up to 33.5 mg total carotenoids/L with 30.5 mg astaxanthin/L), with remarkable concentrations of pigments in dry cell mass basis (up to 1094 mg carotenoids/kg dry cell mass with 996 mg astaxanthin/kg dry cell mass). The volumetric productivities of pigment generation reached 0.101 mg total carotenoids/L.h with 0.091 mg astaxanthin/L.h. These results compare well with reported data.

Fractionation and Characterization of Proteins from *Rosa Moschata* Seeds

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***Keywords**:* Rosa moschata*, vegetable protein*

INTRODUCTION

Dog rose (*Rosa moschata*) seed is a low-oil content seed of economical relevance for the excellent cosmetic properties of their oil [1]. The solid residue resulting after oil extraction is rich in crude fiber [2]. Alternatively the deffated meal and could be used as a potential source of both dietary fiber and low-cost protein. Incorporation of these proteins in processed food for human consumption require further characterization.

In this work, *Rosa moschata* proteins were fractionated by solubility and the molecular weight distribution in each fraction was studied by electrophoresis and gel filtration chromatography.

MATERIALS AND METHODS

Seeds. *Rosa moschata* seeds were provided by Forestal Casino Ltda. (Santiago, Chile). The seeds were ground, defatted at room temperature with hexane and stored at 4 °C.

Protein fractionation. Sequential extraction of seed proteins was carried out with five different solvents: deionized water, 0.5 M NaCl, 70 (v/v) ethanol, 50% (v/v) glacial acetic acid and 0.1 M NaOH.

Analytical methods. Nitrogen content of the each solubilized fraction and defatted meals was determined by Kjeldahl, using the factor 6.25 to convert this value to protein. Protein content was also analysed by the Lowry method. SDS-PAGE was carried out with 12% (w/v) acrylamide gels according to Laemmli in both presence and absence β-mercaptoethanol. Gel filtration chromatography was performed in a fast protein liquid chromatography system equipped with a Sephacryl® S200 column, both from Pharmacia.

RESULTS AND DISCUSSION

The five soluble fractions obtained from the deffated meal contained 90% of the initial protein in the seed as it could be inferred from the Kjeldahl determinations, being the major extracted with 50% (v/v) glacial acetic acid (glutelins). A good correlation was found with the protein content analysed by the Lowry method. The five protein group fractionated were the characterized by SDS-PAGE and gel filtration chromatography in order to determined their molecular weight profile.

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The Water Stress Produces a Significant Effect on Pungency of "Padrón" Pepper Fruits

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Keywords: pepper, soluble phenol, lignin, capsaicin, dihydrocapsaicin

Pungency is the most outstanding property of peppers, resulting from the accumulation of capsaicin and other related compounds, associated with a particular developmental stage of a single organ of the plant, the fruit. However the level of capsaicinoids varies depending on the different pepper cultivar examined [1].

This results are in keeping with the fact that pungency level in pepper fruit is the result of two factors: the genetics of the plant and how it interacts with the environment. The genetic control of pungency is not fully understood. However, environmental factors that warm weather, poor soil, dry climates, and high night temperatures are suggested to favor the production of high capsaicin concentrations within the pods, althouhg no research supporst this suggestion. Recently, mineral supplementation has been identified as a factor affecting the capsaicin content in "Padrón" pepper fruit [2].

In order to determine how capsaicin biosynthetic pathway is affected by water stress, "Padrón" pepper plants were grown using three different water supplies (control, low and high water treatment) to determine the effect of water stress with pungency levels and others phenylpropanoid compounds.

Control and stressed pepper fruits were harvested every 7 days from 14 to 35 days after flowering, weighed, oven-dried at 60ºC for 2-5 days, and stored at room temperature. Whole or sliced fruits were ground to a fine powder and pungency was quantified using HPLC analysis of capsaicinoids [3] using a Spherisorb ODS2 C_{18} column with a photodiode array detector Waters 996 reading at 280 nm. The mobile phase was isocratic, with 50% solvent A (100% acetonitrile-HPLC grade) and 50% B (10% acetonitrile).

Water stress had no significant effect on fresh fruit or dry weight. The amounts of soluble phenolics and lignin decreased with water stress, as a similar form in both treatments. The capsaicinoids content (capsaicin and dihydrocapsaicin) in Padrón pepper fruits of water stressed plants, were found to be higher than those of control plants, specially, at the low water treatment.

The results of the present experiments suggest that environmental conditions (like water stress) in "Padrón" pepper fruits, has a profound effect upon the accumulation of capsaicinoids, resulting of the competition between capsaicinoids biosynthesis and the others phenylpropanoid metabolites.

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Effect of Dietary Lipid Content on Astaxanthin Serum Absorption and on Flesh Pigmentation of Rainbow Trout Fed Diets Suplemented with Haematococcus and with Synthetic Astaxanthin

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Key words: Microalgae, Haematococcus, Carotenoid, Astaxanthin, Rainbow Trout

Salmonids cannot synthesize the carotenoids de novo and therefore depend entirely on dietary supplement to achieve a natural pigmentation. Synthetic astaxanthin (3,3«-dihydroxi-b,b-carotene-4,4«-dione) and canthaxanthin (b,b-carotene-4,4«-dione) are commonly used in the diets of farmed salmonids to produce coloration similar to wild fish so as to gain market acceptance. Astaxanthin is preferable because it produces nature-identical pigmentation and is more efficiently deposited.

The freshwater, biflagellate green alga Haematococcus pluvialis has recently received much attention due to its capability to accumulate large amounts of astaxanthin (> 1% of dry weight).

This experiment was designed 1) to compare astaxanthin absorption in the serum and flesh pigmentation of rainbow trout fed diets supplemented with astaxanthin from Haematococcus and synthetic astaxanthin, and 2) the effect of two different dietary lipid levels on this absorption and pigmentation since carotenoids are liposoluble compouds.

Rainbow trout were fed a diet supplemented with microalgal astaxanthin (136 mg Kg^{-1}) and synthetic astaxanthin (115 mg Kg^{-1}) each one with two different lipid levels (9 and 24%)

1) There was a higher absorption of astaxanthin in the serum for fish fed diets supplemented with algal biomass when lipid level was low (9%). No differences in the absorption were found for fish fed diets supplemented with either natural or synthetic astaxanthin, when the lipid level was high (24%).

2) Astaxanthin concentration in the serum was higher for fish fed high lipid level diet whatever the astaxanthin source.

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Biological Formation of Food Acidulants - Improvement in Raw Material Utilization

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***Keywords**: citric acid, malic acid, cleaner production.*

Food acidulants are important bulk commodities produced either by biological or chemical methods. The main food acidulant, citric acid, is produced mainly by fermentation. The theoretical yield for citric acid formation is 192 gram per liter of acid for 1 mol of glucose (180 g).

The formation of L-malic acid is theoretically 268 gram per liter, due to the different biochemistry in the formation of this acid. The reductive route of the tricarboxylic acid cycle is active, with the incorporation of CO_2, resulting in a potential molar yield of 200 percent.

Optimization of the fermentation conditions resulted in an actual yield of 113 gram per liter of L-malic acid (126 mol percent). Better utilization of raw materials, higher product yields and less waste can potentially be obtained in the manufacture of L-malic acid, as compared to citric acid.

Identificação Molecular de Clones de Castas da Região Demarcada do Douro

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Palavras-chave: Casta, Clone, RAPD

Na região de Trás-os-Montes e Alto Douro encontra-se a mais antiga e importante zona vitivinícola portuguesa - a Região Demarcada do Douro. As castas Touriga Nacional, Touriga Francesa e Tinta Roriz são algumas das castas de excelente qualidade cultivadas nesta região e, por isso, têm sido objectivo do Programa Nacional de Selecção Clonal da Videira. Por tal motivo foram objectivo do nosso estudo.

O conhecimento da variabilidade genética e a identificação molecular das diferentes castas de videira é de relevante importância dado que não só permite formular hipóteses acerca da origem, evolução e antiguidade das castas, como também conhecer o material disponível para a selecção massal e clonal.

Para a caracterização das castas em estudo utilizou-se a técnica de RAPD (Random Amplified Polymorphic DNA); assim, após a extracção e quantificação do DNA, procedeu-se à sua amplificação com *primers* aleatórios. Os produtos de amplificação foram posteriormente submetidos a electroforese em geis de agarose e os diferentes fragmentos separados de acordo com o seu tamanho. Obtiveram-se diferentes padrões de bandas tanto entre castas, como era de esperar, como entre clones pertencentes à mesma casta, o que comprova variabilidade genética dentro destas castas.

Este método revela-se assim de grande importância tanto na caracterização de castas como na distinção entre clones, por vezes difícil por outros meios.

Effect of the Transformation with *Agobacterium rhizogenes* on *in vitro* Shoots of *Eucalyptus globulus* Labill to Improve Rooting

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Keywords: Agrobacterium rhizogenes; Eucalyptus; transformation; opines

This report results from a collaboration between the University of Aveiro and a Pulp Mill, Stora Celbi, concerning *Eucalyptus* improvement. Conventional vegetative propagation of *Eucalyptus* species has mostly been done by stem cuttings, but in *Eucalyptus,* as well as in other trees, the success of cuttings is limited by problems concerning rooting ability. *In vitro* plant regeneration from *Eucalyptus* shoots also faces the same problem.

Agrobacterium rhizogenes is a soil Gram-negative bacteria (Rhizobiaceae) which causes hairy root formation at wound sites in compatible host plants by means of the natural system they possess to transfer oncogenic DNA to plant cells. We report here the use of Ri plasmid of *Agrobacterium rhizogenes* to improve rooting and achieve successful propagation of *Eucalyptus globulus.* Transformation of *E. globulus* was achieved by inoculating the cut surface of shoots with different wild strains of *Agrobacterium*: LBA 9404, R 1601, A4 and TR 8/3. Inoculated shoots were then incubated for 7 days on half-strength MS in a growth chamber at 22ºC with 16h photoperiod. Shoots were then placed, for seven days, on hormone-free medium containing 500mg/l cefotaxime to kill bacteria. Thereafter they were transferred to hormone-free MS, for another 4 weeks, and then hardened off. Two controls were established: one set of un-inoculated shoots was transferred to hormone-free MS and another set was transferred to MS supplemented with 1mg/l IBA. Rooting percentage was dependent on the bacterial strains used, with best results obtained with strain TR 8/3. A substantial improvement in root quality of chimeric plants was observed 6 weeks after inoculation.

Only roots regenerated in inoculated shoots grew as root culture in hormone-free liquid medium confirming that roots were effectively transformed. Thereafter, the presence of opines was also tested in transformed roots. Electrophoretic analysis did not detect the presence of nopaline and agropine in roots transformed with strains LBA 9404, R 1601 and TR 8/3. Roots transformed with strain A4 showed no presence of nopaline but the absence of agropine was not consistent and more studies are being undertaken. This study suggests that gene transfer of *A. rhizogenes* can be used to substantially improve rooting of *in vitro E. globulus* seedlings from seeds of open field pollinated trees. When chimeric plants were hardened off extensive lateral roots developed. No morphological abnormalities were observed in plants with transformed roots suggesting that this procedure could be used to improve rooting in *Eucalyptus globulus.* These results led us to believe that transformation of *Eucalyptus* (and other woody plants) with the strains used in our laboratory may be a useful tool for breeding programs probably without harmful consequences to environment. To test this hypothesis we are studying if growing transformed roots in pots increases the population of *Agrobacterium.*

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Comparison of Microbial Spoilage on Portuguese and Imported Cold-smoked Fish Stored in Chilled Vacuum Packs

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Key words: *Cold-smoked fish, Spoilage, Shelf-life*

Microbiological quality of smoked fish products is continually being improved by the application of more severe sanitary controls. However, there are few studies of the microflora developing on vacuum packaged cold-smoked fish products. Salmon-trout (*Oncorhyncus mykiss*) and salmon (*Salmo salar*) are the main raw material for cold smoking. Studies about the occurrence of *Listeria* spp. in products from Portuguese smokeries has already been investigated by Vaz-Velho *et al.* (1997). Studies on the shelf-life and microbial spoilage on final products, sliced and vacuum-packaged, from Portuguese producers and imported samples, were the objective of this study. Four different imported samples taken from a supermarket and samples from two Portuguese smokeries stored in chill and frozen conditions were sampled weekly for microbiological status and sensory analyses.

Total viable counts were performed on spread plates of Long and Hammer's medium (LH) (1% NaCl) and by pour plates of Iron agar (IR) (Lyngby) incubated at 15ºC for 5-7 days and 21ºC for 3 days, respectively. Counts of lactic acid bacteria (LAB) were done by spread plates of Elliker pH 6.8 medium incubated at 21ºC for 5 days. Enterobacteriaceae counts were done by pour plates of 5 ml Tryptone Soya Agar (TSA), which after 2h at 20-25ºC were overlaid by 12-15 ml of Violet Red Bile Glucose Agar (TSA/VRBGA). Typical Enterobacteriaceae colonies were counted after 2 days of incubation at 30ºC. To assess the suitability of the different media, representative colonies were picked from plates and the following test were performed: cell morphology, gram stain, catalase and oxidase tests. The general appearance, colour, smell and texture were noted at each sampling time.

Results indicated that counts in LH were always higher than on IA. This was probably due to the extreme heat sensitivity of marine *vibrio/Photobacterium* group of bacteria, which would be killed by the temperature of molten, cooled (ca. 45ºC) agar. Higher counts on Elliker medium indicated a larger proportion the developing flora were lactic acid bacteria in all samples. From TSA/VRBGA plates, only a very few colonies were detected, indicating that Enterobacteriacea are not a predominant group in these products. Portuguese samples showed <1 log CFU/g differences from imported samples of cold-smoked fish during storage. These preliminary results indicate that the predominant flora in these products consists of lactic acid bacteria and the remaining flora are Gram negative and other Gram positive species.

Expression of Glutathione Peroxidase during *Zantedeschia aethiopica* Spathe Senescence and Regreening

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***Keywords**: oxidative stress enzymes, senescence, regreening,* Zantedeschia aethiopica

Even under optimal conditions many metabolic processes in plants produce active oxygen species (AOS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH°). In leaf senescence the breakdown of the photosynthetic apparatus could lead to the formation of AOS which are produced as a consequence of both inefficient transfer of electrons through the photosystems and breakdown of thylakoid components due to lipoxygenation. As a result, uncontrolled free radical chain reactions take place damaging unsaturated lipids, proteins and DNA, which are senescence promoters. However, plants are well-adapted to minimising the oxidative damage unless very extreme conditions occur. Several protection mechanisms have evolved that scavenge AOS, like the occurrence of several antioxidant compounds (ascorbic acid or carotenoids) in high concentrations or the increasing of antioxidant systems, which includes glutathione peroxidase among different enzymes [1]. Glutathione peroxidases (GPX, EC 1.11.1.9) are a family of key enzymes involved in scavenging oxyradicals in animals. The existence of this enzyme in plants has been reported only recently. This enzyme catalysis the reduction of H_2O_2, organic hydroperoxydes and lipid hydroperoxydes by reduced glutathione [2].

In order to be able to study the expression of this enzyme during senescence and regreening processes of calla lily (*Zantedeschia aethiopica* Spreng., Araceae) we have screened a a young leaf cDNA library for GPX. After sequence analysis we studied the genetic expression of this enzyme during the senescence and regreening processes. These results will be discussed considering the alterations suffered by photosynthetic electron chain during spathe develpment.

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Construction of a cDNA library from *Zantedeschia aethiopica*

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***Keywords**: cDNA library, senescence, regreening,* Zantedeschia aethiopica

Calla lily - *Zantedeschia aethiopica* (L.) Spreng. (Araceae) - exhibits leaf-like structures (spathes) enveloping the inflorescences. Soon after its formation the photosynthetic spathe undergoes senescence and will die. However, if fructification occurs, spathe senescence is inhibited occurring its regreening [1]. These features let us to consider this plant as a good biological model for the study of natural foliar senescence and regreening mechanisms. Several metabolic studies were performed, including photosynthetic rate determination, PSI and PSII activities quantification and pigment-protein complexes analysis [2,3]. The reductor metabolism of carbon was also studied in what concerns ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and concentration, suggesting that during the regreening process there is *de novo* synthesis of Rubisco.

In order to perform the identification and characterisation of genes involved in either senescence or regreening of *Z. aethiopica* spathe a cDNA library was constructed from young leaves of this species. Here we report the construction of the cDNA library in Lambda ZAP Express and sequence analysis of nearly 40 clones. The cDNA synthesis was initiated using the Stratagene "Zap Express cDNA synthesis kit" and the total number of recombinant clones achieved was 6.9×10^5, which is considered a good representational primary library size. Several clones were picked at random and, after minipreparation of plasmid DNA, the average size of the insert was determined. Of the nearly 40 clones picked, the great majority had a insert size higher then 1500 pb (>60%).

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Determinação de Propriedades Térmicas de Frutos Tropicais: Polpa e Néctar de Cupuaçu (*Theobroma grandiflorum*) e de Açaí (*Euterpe oleracea*)

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Keywords: conductividade térmica, frutos tropicais, cupuaçu, açaí

O cupuaçu (*Theobroma grandiflorum*) é um fruto exótico Brazileiro, muito ácido (pH_3.2), com um baixo teor em açucares e um aroma bastante forte [1]. É esta última propriedade que lhe confere características únicas, sendo utilizado na confecção de néctares, compotas, yogurtes e doces. Devido ao seu valor de pH bastante baixo um processamento térmico suave, pasteurização, é suficiente para estabilizar o produto à temperatura ambiente [2]. Um outro fruto da mesma região, Sul e Sudoeste do Estado do Pará e região pré-amazónica do Maranhão no Brazil, muito apreciado pelas populações locais, é o açaí (*Euterpe oleracea*). Este fruto é muito rico em gordura e proteína, sendo o alimento principal em algumas zonas da referida região. O pH deste fruto é de aproximadamente 6, sendo necessária uma esterilização para ser possível a sua estabilização à temperatura ambiente. Ambos os frutos, cupuaçu e açaí, devido às suas características exóticas, oferecem potencialidades para comercialização e exportação a partir do Estado do Pará - Brazil. No entanto, é necessário encontrar as condições de adequadas para a sua estabilização à temperatura ambiente.

No projecto e controlo de processos de produção de alimentos, envolvendo operações térmicas, é fundamental o conhecimento dos parâmetros do processo e em especial a difusividade e conductividade térmicas que variam com a temperatura e com a composição química do alimento [3]. Existem descritos vários, sendo o mais frequentemente utilizado um método transiente que recorre a uma sonda de conductividade térmica acoplada a uma fonte de calor. A teoria baseia-se no facto do aumento de temperatura num ponto próximo da fonte de aquecimento, num sólido semi-infinito sujeito a um aumento em degrau na potência de aquecimento, ser uma função da potência aplicada, do tempo e de dois parâmetros: a conductividade térmica e a difusividade térmica [4].

Neste estudo experimental, estudou-se o comportamento da sonda de conductividade térmica ISOMET 104, tendo-se obtido uma boa correlação entre os valores determinados para vários produtos alimentares e os correspondentes valores disponíveis na literatura [5]. A conductividade e difusividades térmicas de polpas e néctares de dois frutos tropicais, cupuaçu e açaí, para a gama de temperatura de 20 a 100 °C, foi determinada. Verificou-se que a sonda utilizada apresenta uma boa reprodutibilidade e precisão para temperaturas inferiores a 50 °C. Acima de 80°C a sonda apresentou resultados menos precisos devido à mudança de fase da amostra.

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Photoperiod Influence on Inorganic Polyphosphate content in the Microalgae *Tetraselmis chui*

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***Keywords**: Polyphosphates, Microalgae, Photoperiod*

Inorganic linear polyphosphates (polyP) are energy-rich polymers of up to several hundreds of orthophosphate (Pi) residues. Polyphosphates have been found in bacteria, fungi, protozoa, plants and mammals. Their biological function is still very unclear in spite of the several proposed roles including phosphate and energy reserve, chelator for divalent cations, protection against osmotic stress, pH stress, and bacterial cell competence. Research on Polyphosphate synthesis and degradation in microalgae is of great importance not only to understand its metabolism and function but also to evaluate the potential ability of these cells to actively remove phosphate from phosphate rich effluents.

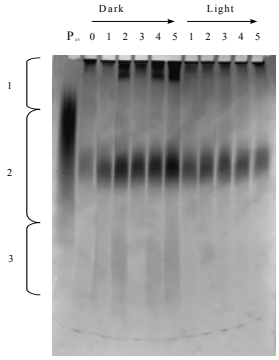

Fig. 1 - PolyP in *T.chui* exposed for 5 days to constant dark and then for 5 days to constant light. **1** High molecular weight fraction of Polyphosphates **2** Medium molecular weight fraction of Polyphosphates. **3** Low molecular weight fraction of Polyphosphates. **P.** Polyphosphate glass (average size - 45 residues) used as a molecular marker.

Acknowledgements: M. P. Oliveira was a recipient of a JNICT/DAAD fellowship grant. H.C. Schöder & J. M. Leitão were recipients of CRUP/DAAD fellowship grants for mobility.

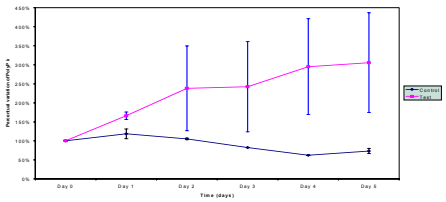

Fig. 2 - Evolution of PolyP content in *T.chui* subjected to constant light (Control) and darkness (Test).

Microalgae cell cultures of *Tetraselmis chui*. were used in this study. PolyP were isolated by phenol-chloroform extraction after resuspension of algae cells in 50 mM Tris-HCl buffer containing 25 mM EDTA, 250 mM NaCl and 1%SDS. PolyP were concentrated by ethanol precipitation. Algae cells were grown under constant light or darkness for several days. Control cells were grown under a 16:8 light/darkness regime. The quantification of polyP was performed by measuring the metachromatic shift induced in Toluidine Blue and by PAGE analysis in 15% gels under denaturing conditions. PolyP increased significantly during the first days of growing in the dark (Fig 1 and 2). Qualitative-quantitative analysis by PAGE revealed that the total increase on polyP is due to the simultaneous augmentation of its concentration in the three main fractions: (Fig.1). On the other hand when exposing *T. chui* cells to constant light the result was a continuous decrease of polyP content (Fig. 1). Further work is now being performed to evaluate the possibility of using this phenomena to control the orthophosphate uptake by algae cells.

Assessement of Genetic Variability among Populations of *Cymodocea nodosa* (Ucria) Anderson in Ria Formosa by RAPD markers

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***Keywors**: Cymodocea nodosa, RAPDs, genetic variability*

Cymodocea nodosa (Ucria) Anderson is an aquatic angiospermic species of marine environments that has an important role in the dynamic ecosystem of Ria Formosa, Algarve, Portugal. This species can reproduce by vegetative propagation of subterraneous rhizomes or by seeds. The latter form of reproduction is not the most common, however.

Four populations from four distinct locations of the Ria Formosa were analysed by RAPD markers to evaluate the degree of genetic variability of this species in this particular ecosystem.

In each population samples of fifteen individuals were collected. Plant tissues were homogenized in a mortar with a pestle under liquid nitrogen. DNA was isolated by phenol chloroform extraction and purified by RNase A treatment. Genomic DNA of ten individuals per population was mixed in equal quantities forming four DNA-bulks used for further molecular analysis.

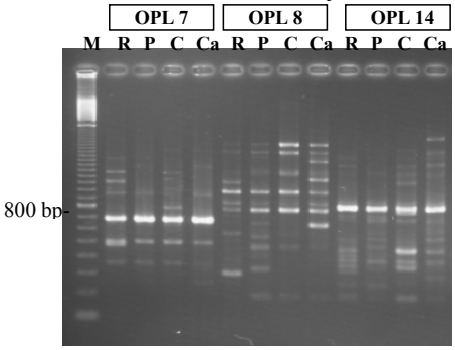

Fig.1 RAPDs of *Cymodocea nodosa*. Boxes above lanes identify the respective primers. Letters above lanes identify the four populations, Ramalhete (R); Ponte de Faro (P); Culatra (C) and Cações (Ca) and 100 bp marker (M).

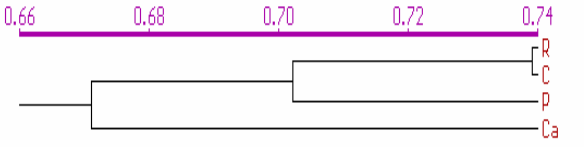

Fig.2. Dendrogram showing the genetic relatedness among the four populations of *Cymodocea nodosa*.

As assumed in other works [1,2] herein we conceived DNA bulks as a representative sample of the DNA pool of the population and the obtained RAPD markers as a fingerprint of the most common genetic characteristics of the population. One hundred 10-mer primers were assayed for their suitability to generate clearly defined markers. Eleven primers generated 119 amplification products which discriminated among the four populations of (Fig1).

Genetic similarities among populations were estimated by the DICE coefficient and the UPGMA cluster analysis was performed to generate a dendrogram (Fig.2), using the NTSYs package. Although no phenotypic differences could be recorded DNA-markers revealed clear genetic differences among populations. This fact suggest that RAPD markers are useful to study natural populations of *Cymodocea nodosa*. Studies are now been carried out with RAPD markers in order to clarify the intrapopulation genetic structure of this species.

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Selection and Definition of the Operational Conditions for the Removal of Lactose from Whey by Ion Exchange

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Keywords: Cheese Whey, Lactose removal, protein concentrate

Whey is a by-product obtained from milk after casein curd separation during cheese manufacture, representing 90% of the milk volume used in the process – to produce 1 kg of cheese 10 litres of milk are used, being produced 9 litres of whey. Due to its composition, this effluent has a high BOD and consequently its environmental impact is considerable [1]. In Portugal 1 000 000 litres of cheese whey are produced daily. One of the most attractive ways to minimise this effect is to recover the main components of the whey – lactose (5% w/v) and protein (1% w/v) –, thus adding value to a food industry by-product [2, 3]. Cheese whey proteins - α-lactalbumin and β-lactoglobulin – due to its excellent functional and nutritional properties have a wide range of industrial applications such as sport drinks, baby foods, fortified fruit juices, meat products, desserts and other confectionery products [4]. To take full advantage of its properties, protein concentrates with low fat and lactose contents are needed. So far, two main alternatives have been considered for lactose removal – ultrafiltration coupled with diafiltration and ionic exchange processes [3]. In this work, several ion exchange resins were assayed for whey deproteinization. Since protein charge is, mainly, controlled by pH, results on optimal pH for proteins sorption and desorption are presented as well as data on contact time between cheese whey and resin and on the volume ratio between cheese whey and resin.

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Reacção de Explantes de *Eucalyptus globulus* Labill a Diferentes Tipos de Meios de Indução de Embriogénese: Efeito da Idade e da Origem do Explante

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Palavras chave: Eucalyptus globulus*, embriogénese*

Este trabalho é fruto da colaboração entre o Departamento de Biologia de Universidade de Aveiro e uma empresa de pasta de papel, Stora Celbi tendo em vista várias estudos para o melhoramento de *Eucalyptus globulus.*

O *Eucalyptus globulus* é uma árvore muito importante na produção de pasta de papel dada a excelente morfologia apresentada pela sua fibra. Trata-se de uma das espécies de eucalipto mais plantadas em Portugal desde a sua introdução no século XIX.

Neste trabalho descreve-se a reacção de vários tipos de explantes como folha, caule, cotilédones e hipocótilos a diferentes tipos de meios de indução de embriogénese. Dado que a nível industrial, o enraízamento por estaca e a propagação *in vitro* apresentam ainda bastantes problemas, a embriogénese somática apresenta-se como um sistema importante de regeneração de plantas e em termos florestais constitui um sistema potencial para a produção de plantas em larga escala. Para além disso apresenta-se como um sistema valioso na regeneração de plantas transgénicas, pelo que o domínio desta técnica, no que diz respeito ao eucalipto, é de extrema importância.

Sementes esterilizadas provenientes de árvores polinizadas em campo foram colocadas a germinar em meio MS sem hormonas e com 30g/l de sacarose. Usaram-se oito estádios de germinação diferentes (3, 6, 8, 10, 14, 17, 20 e 22 dias após colocação no meio de germinação) de onde se retiraram assepticamente cotilédones e hipocótilos. Folhas e caules eram provenientes de plantas germinadas *in vitro* com cerca de 6 semanas de idade. Os vários meios de indução de embriogénese testados baseiam-se no meio MS variando em concentração de 2,4-D, concentrações e tipo de açucares, leite de côco, PVP e diferentes fontes de azoto.

Para avaliar a produção de *calli* nos diferentes meios foram realizadas pesagens verificando-se um aumento do peso quer em folha, caule, hipocótilo e cotilédones e para todos os meios (excepto os enriquecidos com nitratos que não produziram nenhum tecido caloso). Dentro do tipo de explantes usados, o maior aumento de peso verificou-se para hipocótilos e cotilédones. Foram também contabilizados os números de raízes e de porções aéreas formados por explante. Dependendo do meio de cultura, desenvolveram-se diferentes tipos de tecido caloso, uns mais friáveis e outros mais compactos. Neste momento estão em curso estudos histológicos e ultraestruturais para averiguar o processo morfogénico que deu origem às estruturas aéreas entretanto formadas.

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Challenge of *Ulmus minor*, Mill. with *Ophiostoma ulmi sensu lato* Spores and Culture Filtrate

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Key Words: Infection, Spores, Culture filtrate, Senescence

Ulmus minor is the only native elm species that occurs extensively in Portugal and, like all elm species and populations in Europe and North America, it is being severely attacked by the fungus *Ophiostoma ulmi*, causative agent of Dutch Elm Disease (DED). Biochemical and molecular studies of the disease require that plants or plant tissues are subjected to the pathogen, or to any substance produced by it that can cause symptoms similar to those of the disease, preferably in a relatively controlled environment (like a greenhouse or *in vitro* conditions). Here we report the assay of different methods of *in vitro* and *ex vitro* challenge of *Ulmus minor* with *Ophiostoma ulmi* and their effects in leaf senescence and/or growth.

In vitro inoculation of shoots with spores of *O. ulmi*, produced evident symptoms of disease like the typical vascular discoloration, chlorosis and depressed growth rate in 2 to 3 weeks. The progression of the disease was followed during 3 weeks by analysis of chlorophyll and protein content, fluorescence parameters and membrane permeability. In greenhouse plants infected in the same manner the symptoms appeared latter than *in vitro* and were not so evident. Ultrastructural studies are in course.

Culture filtrates of *O. ulmi* have been referred to contain a substance, probably the protein cerato-ulmin, that causes symptoms similar to those of DED[(1)]. To determine whether the culture filtrate of *O. ulmi* can be responsible for the development of disease, *callus* and shoot cultures of *U. minor* were cultivated in media[(2,3)] supplemented with different concentrations of culture filtrate. As controls we used the same media supplemented with the equal concentrations of the fungus culture medium not inoculated, and the regular media. After 2 months in culture shoots cultivated in medium with 10% of culture filtrate did not reveal significant differences from the ones cultivated in control media. Shoots cultivated in media with 50% of culture filtrate where chlorotic and their growth depressed. However shoots cultivated in medium with 50% of the fungus culture medium not inoculated also showed the same symptoms. As to *callus* cultures, no evident depression of growth was detected in media with culture filtrate.

In vitro inoculation of shoots with culture filtrate or a 30 minutes immersion of its basal end in the culture filtrate (before transferring to fresh culture medium) had no significant effect on the parameters analysed.

In conclusion, up to now, we have found no evidence of the involvement of culture filtrate of *Ophiostoma ulmi* in the development of DED.

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Micropropagation and regeneration of *Ulmus minor*, Mill.

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Key Words: Micropropagation, Organogenesis, Rooting, Acclimatisation

Micropropagation of *Ulmus minor* was accomplished using, as starting material, sprouts from stem cuttings taken from adult individuals in the field, during dormancy, and maintained in the greenhouse. Shoot tips and nodes were cultivated in Driver and Kuniyuki Walnut medium supplemented with 1 mg/l BAP and 0,01 m/l IBA[(1)], and subcultivated at 4 weeks intervals. The number of shoots regenerated for each regenerative explant was of 1.7 three months after the initial culture but it increased with successive subcultures. Each explant (shoot tip or node) also developed *callus* at it's basal end. From this *callus* numerous shoots with few millimetres arose after 3 to 4 weeks in culture. When the basal *callus* was transferred to fresh medium some of those shoots elongated and were themselves transferred to fresh medium. This procedure was responsible for a great increase in the number of shoots obtained from one explante.
Leaves obtained from the sprouts in the greenhouse or from micropropagated shoots were also cultivated in the micropropagation medium or in a variation of that medium where the BAP concentration was altered from 1 to 4 mg/l. In this case they were transferred to the original micropropagation medium after 1 week. Within 3 weeks shoots were visible in the petiole area and also in the cuts made across the ribs and *callus* was also forming abundantly. The two different treatments and different ways of making and placing in culture the leave explants resulted in different percentages of regenerative explants. Although efficient in inducing organogenesis in leaves and in the basal *callus* developed in shoot tip and node explants, the micropropagation medium was not suitable for inducing organogenesis in *callus* previously obtained from leaves cultivated on MS medium supplemented with 2,5 μM 2,4-D and 1 μM Kinetin[(2)]. Histological studies to determine the origin of the shoots are in course.
Shoots maintained in the micropropagation medium for two months often regenerated roots. When transferred to *ex vitro* conditions a considerable percentage of the shoots was acclimatised successfully. However the long time in culture also is responsible for extensive axilary branching. To avoid this a more rapid *in vitro* rooting system was necessary. Several media based in the DKW formulation supplemented with NAA or IBA, or without growth regulators were tested and all showed high efficiency. The rooted shoots obtained in this assay were successfully acclimatised to *ex-vitro* conditions. Shoots obtained by organogenesis from leaves also rooted easily in those media and were acclimatised. In a separate assay rooting and acclimatisation in a single step was attempted. Shoots of about 5 to 7 cm (from micropropagation) were transferred to a sterilised mixture of peat and perlite (3:1) after treatment with IBA or with no treatment and successfully acclimatised. This will reduce time and cost of rooting and acclimatisation processes.

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Regulation of Rubisco Activase Expression during *Zantedeschia aethiopica* Spathe Development: Consequences in Rubisco Activity

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***Keywords**: Rubisco activase, senescence, regreening,* Zantedeschia aethiopica

The spathes from the ornamental plant commonly known as calla lily - *Zantedeschia aethiopica* (L.) Spreng., Araceae - suffer a senescing process soon after its formation. The spathe (floral bud stage) exhibits reduced photosynthetic activity and rapidly changes into a non-photosynthetic organ (white stage). When fructification occurs the white spathe develops into a green leaf-like structure (regreened spathe) which completely envelopes the spadix. This regreening takes place only if fructification occurs; otherwise the spathe dies [1]. Previous works allows to postulate that spathe senescence inhibition and regreening occur as consequence of the action of endogenous cytokinins produced at time of fructification. During spathe development, several metabolic studies were performed [2, 3], including Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and concentration, suggesting that during the regreening process there is *de novo* synthesis of Rubisco. The regulation of Rubisco activity, under physiological conditions, is influenced by several factors, like CO_2, O_2 and Mg^{2+} concentration and enzyme activation by a stromal protein (Rubisco activase, Rca) [4]. The expression of *Rca* genes is regulated by light, with both phytochrome and blue-light receptors involved.

In this work we report the identification and sequence of a *Rca* gene from *Z. aethiopica*, wich was performed by screening a cDNA library with an *Rca* probe from tobacco. Expression analysis during spathe development were performed by Northern blotting followed by hybridisation with an homologous ^{32}P-labelled *Rca* probe. The results will be discussed considering the expression pattern of both *rbc*S and *rbc*L (Rubisco) genes and the action of endogenous cytokinins.

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In Vitro Multiplication of *Bougainvillea* cvs. "Mrs O. Perry" and "Rubiana"

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Key words: Bougainvillea*, micropropagation*

In vitro vegetative propagation of *Bougainvillea* cvs. "Mrs O. Perry" and "Rubiana", has been obtained from mature field-grown plants. Shoot explants taken from selected plants were induced to multiply in vitro. Explants were cultured on MS medium supplemented with 0.5 $mg.l^{-1}$ benzylaminopurine (BAP). During multiplication phase different cytokinin and cytokinin / auxin ratios were assayed at different concentrations. The multiplication rate increased with the increasing concentration of BAP, and shoot elongation was strongly improved by adding indolacetic acid to the multiplication medium, in both cultivars. Basal callus was observed with high incidence in all media. The effect of different auxins and various concentrations on the in vitro and ex vitro rooting was studied. Best results were observed ex vitro. The results observed constitute a promising step towards large-scale in vitro propagation of these economically important cultivars.

Variações na Composição e Concentração de Compostos Lipídicos Associadas à Embriogénese Somática de *Linum usitatissimum* L.

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Palavras-chave: Hidrocarbonetos, ácidos gordos livres, embriogénese somática, linho.

A embriogénese somática de *Linum usitatissimum* L. foi induzida a partir de segmentos de hipocótilo em meio MS suplementado com 0,4 mg/l de 2,4-D e 1,6 mg/l zeatina. De modo a estudar a variação ontogénica ocorrida na fracção lipídica associada ao processo de embriogénese somática, fizeram-se 4 colheitas durante o período de ensaio: no início, segmentos de hipocótilo (t_0); ao fim de 2 semanas, explants com início de diferenciação de callus mas sem indução de embriões somáticos (t_1); ao fim de 5 semanas, calli sem embriogénese aparente (CA), calli embriogénico (CE) e plântulas obtidas nestes últimos (ES) (t_2); e no final de 7 semanas (máxima expressão embriogénica), calli não embriogénico (CNE), CE e ES (t_3). A fracção lipídica obtida por extracção do material liofilizado com *n*-hexano foi sujeita a partição com fase alcoólica, em meio alcalino, seguido de meio ácido, tendo-se obtido respectivamente uma fracção neutra, ácida e polar. As amostras foram analisadas em GC e GC-MS. Neste trabalho são apresentados os resultados obtidos para a fracção neutra não derivatizada e fracção ácida metilada (FAME). Procedeu-se a análise quantitativa dos compostos identificados pelo método do padrão interno corrigindo para a discriminação através da analise de séries padrão nas mesmas condições de operação.

Os resultados obtidos revelaram que as amostras t_0 a t_2 apresentam curvas de distribuição de *n*-alcanos de tipo gaussiana com máximos nos compostos hexa- (C16) e heptadecano (C17). O teor relativo dos *n-alcanos* varia com o tipo de tecido e fase da cultura. O padrão de distribuição dos *n*-alcanos alterou-se significativamente nas amostras t_3 havendo uma redução muito significativa. Relativamente aos ácidos gordos livres foram identificados maioritariamente ácidos saturados de cadeia par (C14:0 (ácido tetradecanóico) ao C24:0), o ácido penta- e heptadecanóico e ainda os insaturados C18:1, C18:2 e C18:3. A sua variação temporal nos diferentes tecidos é igualmente discutida.

Enzymatic Hydrolysis of Whey Proteins brought about by Extract of *Cynara cardunculus*

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Keywords: *Proteases, dairy products, thistle, proteolysis*

Upgrading of whey by enzymatic hydrolyzis of its protein inventary may increase the return of cheesemaking processes, in fact, enzymatically-hydrolyzed whey proteins are potential ways to improve taste, color, structure, viscosity and digestibility of foodstuffs when used as additive.
The major whey proteins from cow's milk, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), were hydrolyzed by various amounts of *Cynara cardunculus* aqueous extracts at 37 and 45 ºC, and at pH 5.2 and 6.0 using Fast Protein Liquid Chromatography (FPLC) and SDS-PAGE to monitor progress of reaction.
The action of the enzymatic extract on either α-La and β-Lg was, as expected, shown to depend on the amount of enzyme added as well as incubation temperature and pH. The electrophoretic pattern of the hydrolyzates exhibited qualitative differences in the number and intensity of bands, and the FPLC analysis showed a decrease in concentration of native whey proteins and concomitant appearance of low molecular weight peptides. In general, β-Lg was degraded to a much lower extent than α-La by 24 h hydrolyzis, i.e., β-Lg is hydrolysed more gradually. Incubation at 45 ºC promotes a more rapid hydrolysis of both α-La and β-Lg than at 37 ºC. It could slso be shown that hydrolysis of α-La at pH 6.0 was slightly slower than at pH 5.2, whereas the reverse was observed for β-Lg.

Detection of Skeletal Abnormalities in Hatchery Reared *Solea senegalensis* (Kaup, 1858) by a Whole Mount Double Staining Technique

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The sole, *Solea senegalensis*, is a species with high comercial value, commonly exploited in extensive aquaculture in Portugal, Spain and other southern European countries. In our University several kind of studies have been carried out trying to develop the species for mass production aquaculture. However, there are few informations on the developmental biology of this species, in particular about development and malformations at skeletal level, which would be useful to improve the rearing potential of hatchery produced larvae and juveniles. The aim of this study was to detect skeletal malformations with a bone-cartilage double staning technique in hatchery reared soles. Wild broodstock of *S. senegalensis* has been adapted to captivity in our facitities spawning naturally in the normal period for this species (March-July). Larvae were reared in 80l fiberglass cylindroconical tanks until juvenile stage; samples were collected along development, fixed in paraformaldeyde and dyed with alcian blue and alizarin red. Cartilagenous structures were dyed blue and osseous tissues were dyed red. Several malformations were detected in vertebral column and fins. Eventual relations with rearing techniques are discussed.

Micropropagação de *Melissa Officinalis* L.

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PALAVRAS CHAVE: Melissa officinalis*, micropropagação, fitoreguladores*

Segmentos caulinares de plântulas assépticas de *Melissa officinalis* foram cultivados em diferentes variantes do meio MS. Aproximadamente uma semana após o inicio da cultura, em meio MS isento de fitoreguladores, foi registada reacção nos inóculos primários que culminou na regeneração, por organogénese, de plantas completas e vigorosas. A regeneração manteve-se nas subsequentes subculturas de segmentos caulinares das plantas regeneradas para meio de igual composição, à razão de 1,56+-0,47 rebentos caulinares por inóculo. A regeneração de rebentos caulinares vigorosos, à razão de 1,71+-0,49 por inóculo, foi mantida com adição de baixas concentrações de ácido 3-naftaleno-acético (NAA) ao meio MS. Porém a diferenciação de raíz a partir destes rebentos caulinares só ocorreu à superfície do meio. Adicionalmente foram obtidos calli, friáveis e acastanhados. O aumento da concentração de NAA resultou na diminuição da capacidade regenerativa e no aumento da formação e crescimento de calli a partir dos quais se diferenciavam raizes. A adição de doses crescentes de benziladenina (BA) resultou na diminuição progressiva do tempo de reacção dos inóculos e no aumento crescente da taxa de regeneração acompanhado por um decréscimo do crescimento dos rebentos caulinares individuais e por um decréscimo na rizogénese. Com a suplementação do meio MS com zeatina (ZEA) acompanhada de NAA foram obtidas taxas de multiplicação de 2,50+-0,84 rebentos caulinares por inóculo. A suplementação do mesmo meio com ZEA e 2,4-diclorofenoxiacético (2,4-D) proporcionou taxas de multiplicação de 3,5+-0,73 rebentos caulinares por inóculo. Em ambos os casos a regeneração foi acompanhada por indução de calli. Verificou-se também que a rizogénese diminuia com o aumento da concentração de ZEA, sendo necessário transferir os rebentos caulinares para meio MS isento de fitoreguladores ou meio MS suplementado com baixas concentrações de NAA para que fosse induzida a formação de raizes.

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Two Novel Lectins from Retama monosperma L. and Pancratium maritimum L.

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Keywords: Lectins, Retama, Pancratium.

Lectins are proteins or glycoproteins of non-immune origin which specifically and reversibly bind to complex carbohydrates and cells. Plant lectins were found in seeds, leaves, roots, bark and in symbiosis associated tissues such as nodules. The interest in lectins has greatly intensified during the last decade, as they proved to be excellent and versatile macromolecular tools, especially in biomedical sciences. In this work we report on two plant lectins from *Pancratium maritimum L*. and *Retama monosperma L.,* both specific to D(+)-glucosamine and D(+)-galactosamine.

The *Retama monosperma* lectin is calcio-dependent, needing a minimum of 2mM of Ca^{2+} for hemagglutination activity. Extracts of *Retama* seeds remain active, with a titer of 2^{-2} and a protein content of 0.531 mg/ml, after precipitation with acetic acid and water-bath treatment at 70ºC during 20. Attempts to purify this lectin demonstrate that in the presence of at least 100mM NaCl the activity of prepurified extracts is suppressed when mixed with Sepharose matrixes.

The isolation of *Pancratium maritimum* L. lectin has already been reported [1].This lectin was isolated from an acid treated crude extract of *Pancratium maritimum* L. seeds by affinity chromatography on N-acetyl-glucosamine and N-acetyl-galactosamine-Sepharose 4B. The lectin is calcium-dependent (2mM $CaCl_2$) and precipitates sheep and horse red blood cells as well as the four human blood cell types (A, B, O, AB). The lectin is thermostable, remaining active after one hour at 100°C. Dissolving a precipitate induced by $CaCl_2$ addition to the prepurified extract (0.986 mg/ml; 101.3 U/mg specific activity) resulted in a solution with 0.979 mg/ml protein content and 1633 U/mg specific activity and enables the purification procedure to be more effective (Table 1).

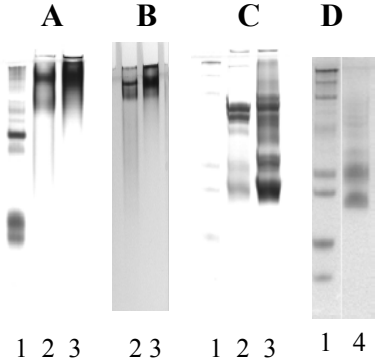

Fig.1 - Prepurified extracts of *Pancratium maritimum* L.: **A-**Native-PAGE 8%; **B-**Native-PAGE 12%;**C-**SDS-PAGE 12%; **D-**SDS-PAGE 12; **1-**Molecular marker; **2-**Acid and heat treated extract (titer 2^{-2}); **3-**Precipitation pellet induced by adition of $CaCl_2$; **4-***Pancratium maritimum* lectin.

Table 1-Purification of *Pancratium maritimum* lectin.

Purification step (20g of cotiledons)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification yield (%)	lectin recov. (%)
Crude extract (100ml; 6.45 mg/ml)	930	5000	5.4	50	100
Prepurified extract (100ml; 0.986 mg/ml)	98.6	9985	101.3	99.8	69.4
Conc. Solution (1.25ml; 0.979 mg/ml)	1.225	2000	1633	80	0.13

U-Hemagglutination units.

Both lectins have shown affinity in very specific conditions to D(+)-Glucosamine and D(+)-Galactosamine among 32 sugars tested for hemagglutination inhibition.

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Cultura *in vitro* de *Thevetia peruviana*. Optimização do Meio de Cultura e Cinética de Crescimento.

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Palavras Chave: Thevetia*, Cultura* in vitro*, Hadamard, Cinética de crescimento*

Os metabolitos secundários produzidos pelas plantas constituem recursos naturais que podem ser aproveitados no desenvolvimento de estratégias de protecção das culturas (1).

A *Thevetia peruviana* e o *Nerium oleander*, conhecidas pela sua capacidade de produção de glucósidos cardíacos, são plantas com potencialidades de serem utilizadas como biopesticidas (2)(3)(4).

Com o objectivo a curto-médio prazo de se estudar as vias metabólicas envolvidas na produção desses glucósidos cardíacos, como a neriifolina, o peruvosídeo e a oleandrina, foram estabelecidas culturas de células em suspensão de *Thevetia peruviana* e *Nerium oleander.*

As células cultivadas em meio líquido foram obtidas a partir de *calli* produzidos por cultura *in vitro* de explantados foliares destas plantas em meio MS suplementado com reguladores de crescimento.

Para a optimização do meio de cultura das células em suspensão, tendo em vista a produção da biomassa vegetal e a friabilidade dos *calli*, utilizou-se o delineamento experimental baseado nas matrizes de Hadamard (5).

A partir destas culturas foram determinados os parâmetros de crescimento, o consumo de glícidos e a concentração de proteína extracelular.

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Regeneração de Plantas a Partir da Cultura *in vitro* de Sementes e Embriões de *Thevetia peruviana*

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Palavras Chave: Thevetia*, Cultura* in vitro*, Regeneração, Micropropagação*

A *Thevetia* spp., vulgarmente designada por chapéus de Napoleão, é uma planta oleaginosa pertencente à família da Apocináceas. Considera-se originária das Índias Ocidentais e do México, encontrando-se espalhada pelo continente americano e africano, podendo ser considerada sub-espontânea em Angola e Moçambique. Esta planta é conhecida pela sua toxicidade devido à capacidade de produzir glucósidos cardíacos como a neriifolina e o peruvosídeo [1].

Devido à dificuldade que encontramos na obtenção de material vegetal, quer em quantidade quer em qualidade, passível de ser utilizada em ensaios de cultura *in vitro*, e dada a grande importância de que se reveste a regeneração da planta de forma eficiente reprodutível e rápida, com vista à sua utilização biotecnológica (ex. produção de glucósidos cardíacos) [2, 3], procedeu-se à cultura de sementes e embriões de *Thevetia*, em meio base MS.

Parte das plantas regeneradas foi transplantada para vaso contendo uma mistura de turfa e perlite. As restantes plantas foram utilizadas como matéria prima para ensaios de micropropagação, em meio MS com diferentes concentrações de reguladores de crescimento.

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Resistência das Culturas de Células em Suspensão de *Ficus carica* a Diferentes Concentrações de Cloreto de Sódio (0,25 g/l, 0,5 g/l, 5 g/l e 10 g/l) ao Longo do Tempo

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Key Words: *Culturas em suspensão, figueira,* stress*, salinidade*

Ficus carica (figueira) é uma espécie termófila cultivada, por vezes subespontânea, que cresce em terrenos secos e pedregosos das regiões costeiras [1], sendo relativamente tolerante à salinidade [2].
O trabalho aqui apresentado refere-se ao efeito do cloreto de sódio em culturas de células em suspensão de *F. carica* mantidas em meio B5 [3], suplementado com 20 g/l sacarose, 0,5 mg/l 2,4-D e 0,2 mg/l Kin estabilizadas há pelo menos 6 meses nas novas condições. Todas as culturas foram mantidas às escuras, a 24 ºC±1 ºC, sob agitação orbital a 160 rpm.
As culturas suplementadas com 5 g/l e 10 g/l de cloreto de sódio apresentavam-se homogéneas, com muito poucos agregados de dimensões reduzidas, de cor creme. Contrariamente, as culturas sem o cloreto de sódio ou com 0,25 g/l e 0,5 g/l de cloreto de sódio, apresentavam agregados mais ou menos irregulares castanhos claros.
A produção de biomassa nas culturas suplementadas com as concentrações mais elevadas de cloreto de sódio (5 g/l e 10 g/l) foi significativamente inferior relativamente às restantes culturas. A produção de biomassa nas culturas sem sal, ao fim de 435 horas, era de 0,64 g (p. s.) ao passo que as suplementadas com 10 g/l era de 0,15 g, partindo de um inóculo inicial de 0,10 g (p. s.). A produção de biomassa nas culturas suplementadas com 0,25 g/l e 0,50 g/l de cloreto de sódio foi bastante semelhante às culturas sem o sal. A mobilização de alguns nutrientes nas culturas suplementadas com o cloreto de sódio nas concentrações mais elevadas foi inferior comparativamente às restantes. Enquanto as culturas sem cloreto de sódio ao fim de 965 horas tinham no meio 0,11 g/l de nitrato, as culturas suplementadas com 5 g/l e 10 g/l de cloreto de sódio tinham ainda 1,34 e 1,65 g/l de nitrato.
As culturas de células em suspensão de *F. carica* são resistentes à presença de cloreto de sódio quando em concentrações relativamente baixas. Concentrações mais elevadas limitam o crescimento celular.

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In Vitro Initiation of *Rhododendron ponticum* Subsp. *baeticum*

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Key words: "adelfeira", in vitro initiation, micropropagation, Rhododendron

Rhododendron ponticum subsp. *Baeticum*, commonly known as "adelfeira" in Algarve, is an endemic plant confined to the mountain of Monchique and Caramulo. This plant has been protected since 1971 in Reserva Botânica do Cambarinho, and it is very interesting for ornamental purposes, due to its beautiful flowers and foliage. During this work procedures were developed in order to attain in vitro establishment of selected plants growing in the field (Foia, Monchique). The initiation of cultures starting from material directly taken from the field resulted in 100% of contaminants, for all the sterilization methods assayed. In order to solve this problem field material was pre-treated as follows: cuttings, 20-30 cm long, were collected and pre-sterilized in sodium hypochlorite solution followed by immersion in benlate solution. These cuttings were maintained under high humidity in controlled conditions, to force buds to grow out. During this pre-conditioning period the stem segments were sprayed weekly with BAP. After fifteen days the developed sprouts were collected for in vitro establishment. Different sterilizing agents as well as sterilizing procedures were tested. Explants were cultured on MS medium containing 0.5 $mg.l^{-1}$ benzylaminopurine. Establishment was very difficult due to high incidence of contaminants and reduced viability of the explants. The best results were observed with sodium hipochlorite, 15%, 15 min. (81% of clean explants and 40% of viable explants). In spite of these difficulties, culture establishment was achieved, and we are now studying the following phases of the micropropagation process.

Pigment Analysis of Dysmorphococcus globosus

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Key words: *Microalgae,* D. globosus, *pigments*

Carotenoids are a wide spread group of naturally occuring pigments, present almost without exception in photosynthetic tissues. They are also found in fungi, bacteria and microalgae. Among microalgae, several species are known to produce significant quantities of carotenoids as *Dunaliella*, *Haematococcus*, *Euglena*, *Chlamydomonas*; biochemical studies of their pigments have been published.

In a previous study, Dawson (1977) identified some pigments in microalgae of the genus *Dysmorphococcus*: chlorophylls a and b, neoxanthin, violaxanthin, lutein and carotenes.

In this work, biomass of the microalgae specie *Dysmorphococcus globosus* (Phacotacea: Chlorophyta) in different life stages was analysed in order to identify its major pigments, in particular its carotenoids. Chromatographic techniques as TLC, HPTLC and HPLC, were used as separation and analytical methods. The pigment composition of this microalgae is intrinsically dependent on the age of the cells and growth conditions of the culture. Green exponential cultures exhibited the presence of chlorophyll a and b and xanthophylls while deep orange stationary cultures also contained ß-carotene, echinenone, astaxanthin esters and canthaxanthin.

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In situ hybridization for rye segments detection in wheat landrace Barbela

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Keywords: Landraces, in situ *hybridization, wheat-rye introgression.*

The fluorescent *in situ* hybridization is a powerful tool for detection of alien genetic material introgression for a wild relative in crop. In cereal breeding, the small alien chromosome segments are desirable for the transfer of useful characteristics into wheat lines, without including the deleterious characters form alien species.
Barbela wheat is an aggregate of old Portuguese landraces that has been known in the literature for more than one century [1,2]. Its wide adaptation to different edapho-climatic conditions was one of its main characteristics, why it was grown in environments as different as the Northern Interior Highlands and the Southern dry regions.
The University of Trás-os-Montes and Alto Douro was undertaken several studies to collect and evaluate this threatened germoplasm and, more recently, to enter it in breeding programs in order to reinclude it eventually in the National Varieties Catalogue. These studies have shown a positive interaction between soils with poor fertility and the dry maturation of the kernel [3], and a genetic x environment interaction similar to that of regional rye populations [4]. The fact that this wheat landrace was grown frequently side by side with regional ryes, led to the hypothesis that if it could have a spontaneous introgression of rye DNA.
Because of the unknown number, size and genetic composition of rye segments potentially present in the landrace, genomic *in situ* hybridization was considered to be an efficient method to search for rye chromatin introgression, in preference to methods using molecular markers needing many tests per line.
The fluorescent *in situ* hybridization used the protocol of Schwarzacher [5] with minor modifications. The results showed that Barbela wheat lines contain small, spontaneously occurring rye chromosome segments. Three of lines examined showeda small rye chromosome segment representing up 5 per cent of chromosome, localized in the terminal region of the short arm of a submetacentric chromosome. A fourth wheat line showed one pair of submetacentric chromosomes with a small terminal labeled segment on the short arm and another pair of a interstitial labeled segment. It occupied 3.5 per cent of the chromosome and was located 75 per cent of the length of the arm from the centromere on the long arm of a chromosome with unequal arms. Results from *in situ* hybridization with the rDNA probe showed that no major NOR was present on either translocated chromosome pair. In the other wheat lines, no segments were seen to be labeled by the rye DNA probe, indicating this rye chromosome segments were either absent or too small to be detected conclusively with this technique described above.
The results presented here show that translocations resulting in small, alien introgression - whether terminal or interstitial - may occur spontaneously and are of such high agronomic value that they were selected by farmers as landraces.

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Essencial Oils Produced by the Hepaticae *Targionia lorbeeriana* in *In Vitro* Culture and in its Natural Habitat

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Key words: *Bryophyte, hepaticae, liverwort,* Targionia lorbeeriana*, essencial oils*

The bryophytes, in particularly the hepaticae, produce terpenoids of high cientific interest due to the large diversity of their chemical structures and their biological activity [1].

Targionia genus(Targionaceae) includes three species [2]. In this genus, only a reference was found in the literature on the isolation of two monoterpens from *Targionia hypophylla* [3].

Targionia lorbeeriana is widely distributed in Portugal [4] and has a pleasant fragrance when it is crushed.

In this work, a comparison was carried out between the essencial oils produced by hepaticae *Targionia lorbeeriana*, in its natural habitat and *in vitro* culture in Gamborg B5 medium.

The plant material was submitted to hydrodestilation and the destilated recovered in a small portion of n-hexane, was afterwords analysed by GC-MS.

From the performed analysis it was observed that although the essencial oils produced *in vitro* do not show the diversity of those produced in the natural *habitat* a significante part of their production is maintain *in vitro*.

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Molecular Characterization of Portuguese Olive Trees (*Olea europaea* L.) Cultivars

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Key words: Olea europaea*, molecular characterization, RAPD markers, AFLP markers.*

Selection and identification of olive clones for the production of olive oil or table olives is fundamental in Portugal due to its profitable exploitation. The olive oil production and exportation has reached a maximum in the 50s, but has been decreasing until these days. One of the reasons is the use of old-fashion techniques, the other the lacking of renewal of the old orchards.

One important region for the production of olive oil is Moura-Serpa, that relies in three olive tree cultivars for its quality. These are Galega vulgar, Cordovil de Serpa and Verdeal alentejana. With an annual renewal of 80 000 trees, it is of the utmost importance to guarantee that the young trees are indeed of the right cultivar.

Therefore, one of the main problems is to find ways of identifying the several cultivars. Our strategy is to use molecular characterization based on DNA to identify and distinguish among the three cultivars. Random Amplified Polymorphic DNA (RAPD) analysis is one of the methods being developed. DNA extraction from young olive leaves has been performed according with [1] and PCR conditions have been optimized. DNAs from more than 30 trees belonging to the same cultivar have been bulked together and a sample of this DNA mixture has been used as template. Several primers from Operon Technologies have been selected for giving discriminating profiles between the three cultivars (figure 1). Another aim of this work is to select primers that discriminate trees among each cultivar. After gel electrophoresis the band pattern is photographed using a Gel-Doc 1000 from Bio-RAD and the image processed using the Gel Compar from Applied Maths.

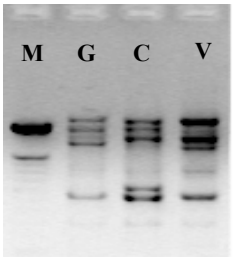

Figure 1. An example of a RAPD profile using one primer that distinguishes between the three cultivars. Lanes: 100 bp ladder (M); G- Galega; C - Cordovil; V - Verdeal.

In order to achieve the same goals, another molecular technique is being assayed. This relatively new technique known as AFLP for Amplified Fragment Length Polymorphism [2] is being tested in the same cultivars and trees. The results obtained will be compared with the results achieved with the RAPD technique.

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Carbon Dioxide and Microalgae: Its Influence on Growth and Fatty Acid Composition of *Phaeodactylum tricornutum*

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Key words: microalgae, lipids, photosynthetic metabolism

Microalgae that grow chymo- or photolitotrophycally use carbon sources of inorganic nature, usually soluble CO_2 or HCO_3^-, to synthesize *de novo* their organic carbon compounds. Nevertheless, the relationships between increase in the concentration of available inorganic carbon and the consequent change in biomass productivity are different between species. Furthermore, saturation of the endocellular fatty acid residues can be induced by increases in CO_2 concentration during growth in some (but not all) species.

In the present study, the effects of CO_2 upon biomass yield and fatty acid profile of *Phaeodactylum tricornutum*, a marine microalga, were ascertained via supplementation of a buffered culture medium with 0, 1 and 2% (v/v) CO_2. Biomass yields were increased proportionally to increases in CO_2 concentration, although statistically significant differences (at the 5% significance level) could only be observed after 15 days of incubation and between cultures with and without CO_2, irrespective of the concentration of gas added. Analysis of the fatty acid profile revealed consistent trends in the fractional concentration of some fatty acids (especially of the polyunsaturated type) and the amount of CO_2.

Molecular Characterization of Strawberry (*Fragaria* x *Ananassa*, Duchesne) cultivars by Isozymes and RAPD markers

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Keywords: Strawberry, Fragaria, Isozymes, RAPDs.

Strawberry (*Fragaria* x *Ananassa*, Duchesne) is the most important small fruit crop in Portugal with a cultivated area of 500-600 hectares. Plants are propagated in 20 authorized nurseries, which produced 35 millions of plants in 1993. The nursery industry is often faced with a lack of fast and reliable methods for unequivocal identification of cultivars. Among the commercial varieties grown in Portugal, attention should be focused on the Californian cultivars, economically important as a result of their good adaptation to the ecological conditions of the country which results in higher yields and early ripening. Herein, we report the results of a preliminary study on the identification of six strawberry Californian varieties: Camarosa, Cuesta, Chandler, Irvine, Oso Grande and Sweet Charlie, using isozyme and RAPD markers. Six enzymatic systems were analysed after starch gel electrophoresis of leaf extracts: PGI, PGM, MDH, IDH, GOT and LAP.

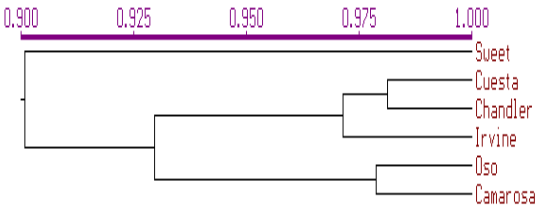

Fig.2. Genetic similarity tree of the six strawberry cultivars.

Acknowledgments: We thank the nursery "Multiplantas" for the offered strawberry plants.

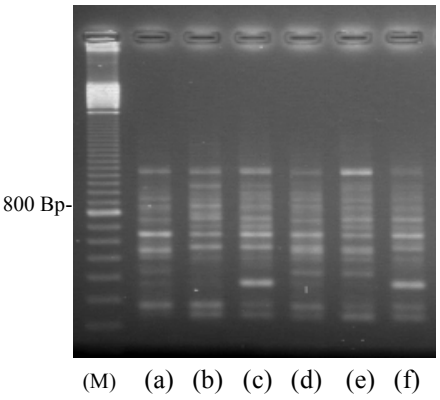

Fig.1. RAPD patterns of strawberry cultivars: (a) Sweet Charlie; (b) Cuesta; (c) Chandler; (d) Oso Grande; (e) Camarosa; (f) Irvine, generated by primer OPM 12. (M) One hundred base pairs molecular marker.

Among them, only four: PGI, PGM, LAP and MDH, showed clearly resolved zymograms, useful for cultivar discrimination. For RAPD analysis, DNA was isolated from leaves by a fast procedure, avoiding phenol or chloroform extraction and was further quantified by agarose gel electrophoresis. Forty two 10-mer primers were tested for their ability to generate clear RAPD polymorphic markers. Fifteen primers were shown to generate polymorphic patterns that discriminated among all the cultivars (Fig.1). A binary matrix of presence vs. absence of markers was built up. The NTSYS program was used for statistical treatment of the data. A dendrogram of the six strawberry cultivars was elaborated using the UPGMA method after estimation of genetic similarities among cultivars by the coefficient DICE (Fig2).

Microalgal Biomass as a Natural Purveyor of Carotenoids in Aquaculture

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Key words: pigmentation, carotenoids, Chlorella vulgaris*,* Sparus aurata

Microalgæ are important components of hatchery feeds because of the uniqueness of the components which they purvey and despite the practical difficulty and expense at which they are produced. On the other hand, hues obtained in a series of aquacultured products by means of carotenoids such as astaxanthin and canthaxanthin administered in feed, strongly contribute to their consumer acceptability, image, market size and value.
Gilthead sea bream, along with other animals, are thought not to be able to perform a *de novo* synthesis of carotenoids. Carotenoid biosynthesis is specific of photosynthetic organisms and in the aquatic food chain microalgæ are the original purveyors of these pigments.

Some microalgæ, namely *Chlorella* sp. when subject to stress, will accumulate lipidic components which will generally include carotenoids [1].

Gouveia *et al.* [2,3,4] were able to demonstrate that the use of microalgal carotenoids as they are present in dry *Chlorella* biomass was effective in colouring the muscle of rainbow trout (*Oncorhyncus mykiss*), when mixed into normal feed instead of commercial pigment and at comparable total carotenoid content.

The fact that use of synthetic astaxanthin may face regulatory problems in some countries on the one hand and progress in microalgal production engineering on the other may soon drastically improve the usefulness of the work which was reported.

The main purpose of this work was to evaluate dry biomass obtained from stressed cells of *C. vulgaris* as a pigmentation source for gilthead seabream (*S. aurata*) in comparison to commercially available synthetic pigments

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Repetitive somatic embryogenesis in the pasture legume *Medicago truncatula*

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***Key Words**:Repetitive somatic embryogenesis,* Medicago truncatula

Medicago truncatula Gaertn. is an annual pasture legume capable of nitrogen fixation which is important both for grazing and for the subsequent growth of grain crops. Our purpose is to extend the drought tolerance of this legume through genetic engineering so that it can occupy drier regions in the South of Portugal and for a larger period along the year. Since regeneration in *Medicago truncatula* is strongly genotype dependent, we have tried to develop a somatic embryogenesis protocol applicable to our local varieties.

Figure: Secondary embryos of *Medicago truncatula* in different development stages

The subspecies Narbonensis and four *in vitro* propagated clones of *Medicago truncatula* CV Jemalong were tested for their somatic embryogenesis potential using a two step protocol: In the first step, primary somatic embryogenesis was induced in folioles isolated from shoots grown *in vitro*. These folioles were cultured on Murashige & Skoog medium (MS) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and Zeatin. In the second step, somatic embryos were allowed to develop from the induced callus in MS medium without growth regulators. Individual somatic embryos were then isolated and transferred again to MS growth regulator free medium where they formed secondary somatic embryos in a repetitive manner. Conversion of somatic embryos into plantlets was achieved by isolating late torpedo phase somatic embryos with distinct cotyledons and re-culturing them onto growth regulator free medium.

Environmental Benefits of Genetically Modified Crops in Mediterranean Countries

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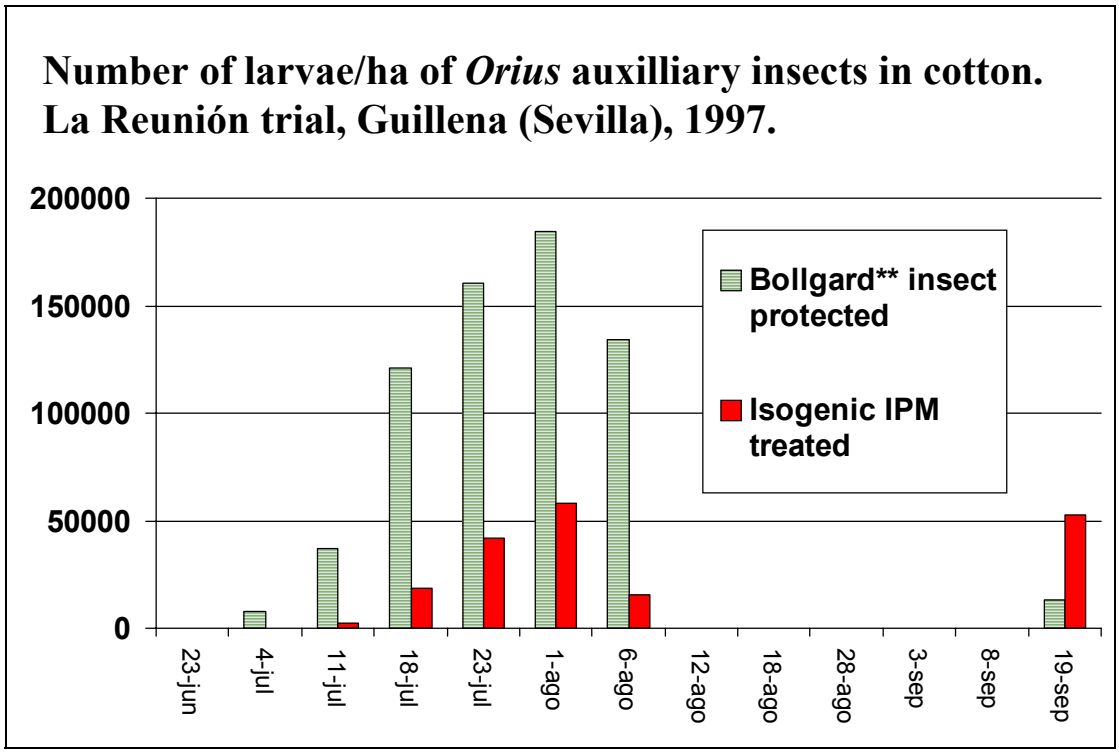
KEY WORDS: cotton, maize, soybeans, sugarbeets, transgenics.

ABSTRACT: The reasons for the quick adoption in America of genetically modified crops - such as Roundup Ready** soybeans, insect protected cotton, insect protected maize, etc. - will be discussed, together with expectations about introduction of these traits in Spain and Portugal.
Data from field trials in Spain with RoundupReady varieties of sugarbeets, cotton, maize and soybeans will be summarized, offering a promise to reduce soil erosion, CO_2 emissions and better farming efficiency.
Additional spanish field data from insect protected cotton and maize varieties have shown an excellent compatibility with auxilliary insects (see chart below) and Integrated Pest Management techniques, with substantial reduction of insecticide applications and improving farmer efficiency.
Finally, the effectiveness of different communication approaches is reviewed.



**Roundup Ready and Bollgard are registered trademarks of Monsanto.

Mapping QTLS Controlling Vegetative Propagation Traits in *Eucalyptus*

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Keywords : QTLs, AFLP markers, Vegetative propagation, Eucalyptus

The competence for vegetative propagation and the quality of the root system, varies within and across *Eucalyptus* species,and can be a bottleneck in genetic improvement programas. Genomic mapping using molecular markers is a powerful tool to investigate genetic architecture of polygenic traits. Very little is known about the genetic basis of rooting and sprouting ability in eucalypts.

We have used the pseudo-testcross mapping strategy and AFLP markers to map quantitative trait loci (QTL) controlling vegetative propagation traits in an *Eucalyptus globulus* and *E. tereticornis* clone. Both a selective genotyping (to increase power to detect QTL) and a random sampling approach (to provide better estimates of QTL effects) were adopted for QTL discovery. Rooting and sprouting ability data were colleted in two years. Phenotypic data were not normally distributed, even after several tranformations were attempted.

Conventional genetic improvement strategies will differ for a polygenic model of inheritance (many unlinked genes with small effects) or an oligogenic model (few loci with relatively large effects). In the first case, phenotypes are handled at the population level. In the second, breeding stategies should focus on identifying and manipulating key QTLs in specific breeding lines.

Molecular Characterization of Portuguese Almond Varieties and Study of their Self-incompatibility

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Keywords: Almond, molecular characterization, Prunus dulcis*, self-incompatibility*

Almond (*Prunus dulcis* Mill.) is a Portuguese traditional culture well adapted to the Mediterranean clime, that grows in the South (Algarve and South Alentejo), and in the North (the hot land of Trás-os-Montes). Portuguese local varieties were studied in the forties, but nearly forgotten since then. Some of the problems we are now facing with Portuguese varieties are related to the absence of a program for genetic improvement, to insufficient characterization and precise identification and to a self-incompatibility system, that in almond may reach 100%, which has not been studied for Portuguese genotypes in the last half century. To face these problems, we have started a program for molecular characterization of local genotypes and for the study of self-incompatibility.
RAPD (Random Amplified Polymorphic DNA) is being used for the molecular characterization of almond collections maintained at the Regional Agricultural Services. Leaf samples were collected from the selected trees, shortly after bloom, DNA was isolated using a CTAB-based protocol and amplification was accomplished by PCR, with synthetic oligonucleotides obtained from Operon Technologies.
To study self-incompatibility variety-related S-allele identification is being performed by S-protein analysis while controlled pollination experiments were just initiated. The molecular identification of S-alleles is also being studied by reverse transcriptional PCR of mRNA isolated from almond styles. A cDNA fragment putatively coding for an S-protein, was isolated and sequenced, but it proved to correspond to an S-like RNase. Pollen/pistil interaction in compatible and incompatible crosses is being evaluated in light, fluorescence and confocal microscopy of whole pistils or cryo-sections.

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Identification of New Storage Proteins Alleles using 1D and 2D Electrophoresis In Barbela Wheat

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Key words: Triticum aestivum *L., SDS-PAGE, IPG x SDS-PAGE, storage proteins, alleles.*

The storage proteins constitute about 85% of the endosperm proteins in wheat kernels, are traditionally classified into gliadins and glutenins, according to their solubility properties [1] .. The two protein groups impart different properties to the dough: glutenin bestows elasticity, whereas gliadin is viscous and confers extensibility [2]. Glutenin is subdivided according to molecular weight into high-molecular-weight (HMW) and low-molecular-weight (LMW) subunits. The HMW-GS (high-molecular-weight glutenin subunits) are further subdivided into high M_r x-type and low M_r y-type subunits. Two genes, which are inherited as tightly linked pairs encoding an x-type and a y-type subunit, are present on the 1A, 1B and 1D chromosomes of hexaploid bread wheat [3].

Observation of HMW-GS, LMW-GS, and ω-gliadins has been carried out by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) for 155 collected Barbela lines (a portuguese wheat collection). The sequential method of Singh et al. [4] was employed for gliadins and glutenins extraction from crushed wheat grains or flour. Nomenclature of the HMW-GS and gliadin bands corresponds to the terminology of Payne and Lawrence [5] and Khelifi et al. [6], respectively. Two new alleles, each having only one band named 1.1 and 13 encoded at Glu-A1 and Glu-B1, respectively, were identified. Five new ω-gliadin group of bands were described usin g the SDS-PAGE technique, four were attributed at Gli-A1 and one at Gli-B1 loci [7,8]. Two-dimensional electrophoresis using immobilized pH gradient gel electrophoresis followed by sodium dodecyl sulphate-polyacryl amide gel electrophoresis (IPG x SDS-PAGE) were carried out in order to characterize the subunits found in some Barbela lines. The 1.1 subunit encoded at Glu-A1x was compared to subunits 2* and 1 of the same locus. The 13 subunit of Barbela was also compared to band 13 of the 13-16 group of subunits corresponding to allele Glu-B1f.

The verified correlations between bread-making quality and specific HMW-GS bring advantage to wheat breeders, using SDS-PAGE of proteins as a screening test for quality. In view of the importance of HMW-GS for the bread-making quality of flours, several laboratories have tried to obtain antibodies specific to these polypeptides in order to quantify these proteins or to screen them for particular subunit combinati ons in breeding programs. Studies for baking quality parameters and molecular analysis of the new alleles of storage proteins detected are now being carried out.

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Healthcare Biotechnology

Stimulatory Effect of Spermadhesin PSP-I/PSP-II On Neutrophil Migration To the Peritoneal Cavity of Rats

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***Keywords**: Spermadhesin PSP-I/PSP-II, neutrophil migration*

Spermadhesins are low-molecular-mass (12-16 kDa) (glyco)proteins built by a single CUB domain architecture found in the seminal plasma of various domestic animals, i.e., boar, bull, and stallion [1]. Members of this protein family display heparin- and β-galactoside-binding activities, become bound to the sperm surface at ejaculation, and are thought to play a role in sperm capacitation and the initial binding of spermatozoa to glycoconjugates of the zona pellucida of homologous oocytes [2]. On the other hand, PSP-I/PSP-II, a heterodimeric lectin of glycosylated spermadhesins and the major component of boar seminal plasma, does not bind to the sperm surface and its function remains elusive.

In many instances, animal mating elicits damage of female genital tract tissues, and infection of the lower reproductive tract with a variety of pathogens have been documented. Rapid recruitment of leukocytes to the site of infection would be adventageous for the animal and for the success of the fertilization process. Neutrophil migration in acute inflammatory reactions involves sequential interactions between these leukocytes and endothelial cells and extracellular matrix components mediated by adhesion molecules such as selectins and integrins. Pro-inflammatory activity of plant lectins has been demonstrated by intraperitoneal inyection [3]. Here we have investigated the effect of spermadhesin PSP-I/PSP-II on neutrophil migration to the peritoneal cavity of rats.

1 ml of sterile PSP-I/PSP-II saline solutions at three different concentrations (100, 200, and 300 μg/cavity) were inyected intraperitoneally in six male Wistar rats., and the effect compared to a control group of rats, which were subjected to the same treatment but omitting the protein. After four hours, cells were recovered by lavage of the cavity, and total and differential cell counts were performed. It was found that PSP-I/PSP-II stimulated in a dose-dependent manner neutrophil migration to the peritoneal cavity. Maximal stimulation ($p < 0.05$) occurred with 200 μg/ml PSP-I/PSP-II (3608 ± 573 cells/ml compared to 1840 ± 249 cells/ml in control animals). Studies aiming to elucidate the mechanism of PSP-I/PSP-II stimulatory activity will be presented.

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Construction and Expression of a Transmissible Gastroenteritis Coronavirus Self-Replicating RNA

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Key words: coronavirus, TGEV, self-replicating RNA, vector

Transmissible gastroenteritis coronavirus (TGEV) is a member of the *Coronaviridae* family with a positive-stranded RNA genome of 28.5 kb. As a consequence of the large size of its genome it has not yet been possible to obtain a full-length cDNA clone, what has made necessary the characterization of defective interfering (DI) RNAs to study viral gene function. These DI RNAs will also be useful as a tool to develop a safe expression system.

Three TGEV defective interfering RNAs of 21, 10.6 and 9.7 kb (DI-A, DI-B and DI-C, respectively) were isolated. By Northern blot analysis it was shown that DI-A RNA conserved the complete polymerase gene although it lacked the structural genes. Dilution experiments showed that DI RNA was synthesized following one-hit kinetics, implying that its replication is independent of a helper virus, in contrast to other minigenomes of smaller size.

In order to engineer a cDNA encoding the RNA replicon a strategy based on the cloning of DI-C cDNA has been developed. A cDNA complementary to DI-C RNA was cloned under the control of the cytomegalovirus (CMV) promoter (pDI-C-CMV). In ORF 1a and ORF 1b of the polymerase gene pDI-C-CMV contained a 10 kb and a 1.1 kb deletion, respectively. The consensus sequences corresponding to the deleted regions were cloned, and the deletions in pDI-C-CMV were replaced to yield a complete cDNA clone of DI-A, pDI-A-21-CMV, containing a full-length TGEV polymerase, driven by a CMV promoter. At the 3' end the replicon was flanked by the bovine growth hormone termination and polyadenylation sequences. Using the two-layer expression system, i. e. cellular transcription from the CMV promoter followed by amplification by the helper virus, DI-C RNA and minigenome M39 carrying the ß-glucuronidase (GUS) reporter gene under a TGEV subgenomic promoter have been expressed in the absence of detectable splicing. The production of an active replicase is being investigated at the single-cell level by cotransfecting virus-susceptible cells with the cDNA encoding the replicon and a minigenome carrying the GUS reporter gene under a TGEV subgenomic promoter.

Two strategies have been developed to provide the replicon with the structural proteins in the absence of a helper virus. A packaging cell line that provides the TGEV structural proteins is being made under control of a doxycycline-mediated inducible system. On the other hand, the most 3'-end eight kilobases of TGEV genome, that include all the structural genes required for encapsidation and tropism, were cloned and inserted in M39 minigenome. Cotransfection of this construction and the DI-A cDNA clone is being accomplished to check for replication and encapsidation of both RNAs, what would demonstrate that DI-A is a self-replicating RNA.

Mapeo de Epítopos en la VP5 del Virus de la Peste Equina Africana. Identificación de un Epítopo Neutralizante Conservado en otros Orbivirus

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Palabras claves: virus de la peste equina africana, VP5, epítopo, neutralización, vacunas.

El virus de la peste equina africana (VPEA) pertenece a la familia *Reoviridae*, género Orbivirus. El VPEA es el agente responsable de una enfermedad altamente virulenta en caballos, que ha originado graves brotes en España y Portugal. El virión está formado por una doble envuelta proteica en cuyo interior se encuentra el material genético, constituido por 10 segmentos de ARN de doble cadena. La envuelta externa está formada por dos proteínas, VP2 de 124 Kda y la VP5 de 56 Kda. La presencia de epítopos neutralizantes en la VP2 ha sido demostrada previamente en los tres orbivirus económicamente más importantes, esto es, VPEA, virus de la lengua azul de las ovejas (BTV) y virus de la enfermedad hemorrágica epizoótica de los ciervos (EHDV). Sin embargo, el papel de la VP5 en el proceso de neutralización no ha sido determinado todavía.

En este trabajo, se ha investigado la presencia de epítopos neutralizantes en la VP5 y se han identificado y localizado los determinantes inmunodominantes de la VP5 del VPEA serotipo 4, responsables de la capacidad neutralizante. Por inmunización con virus completo y purificado y VP5 recombinante expresada en baculovirus, se desarrollaron 2 anticuerpos monoclonales anti-VP5, 10AC6 y 10AE12, y sueros de conejo anti-VP5, respectivamente. Los AcMs no compitieron en la unión al antígeno definiendo, por tanto, 2 sitios antigénicos distintos. El 10AE12 fue capaz de reducir el número de placas de lisis en un 80%, mientras que el 10AC6 neutralizó al VPEA-4 en un 68%. Por otra parte, la VP5 del VPEA-4 expresada en baculovirus indujo en conejos anticuerpos capaces de neutralizar *in vitro* al virus homólogo en ensayos de protección de monocapa. Los títulos de neutralización alcanzados fueron del orden de 10^2. Esta es la primera vez que se describe en un orbivirus que la VP5 es capaz de inducir anticuerpos neutralizantes.

El siguiente paso fue la localización e identificación de los dominios antigénicos en la VP5 mediante PEPSCAN y mediante la expresión en *E. coli* de 17 fragmentos solapantes que abarcan la totalidad de la secuencia de la proteína. Antisueros de conejo y AcMs definieron 8 sitios antigénicos lineales los cuales se localizaron en la mitad amino terminal de la proteína, mientras que en la región carboxilo terminal no se localizó ningún dominio antigénico, sugiriendo una posible localización interna de esta región. Los epítopos de los AcMs neutralizantes 10AE12 y 10AC6 se localizaron entre los aa 85 y 92 (PDPLSPGE) y 179 y 185 (EEDLRTR), respectivamente. El epítopo PDPLSPGE presentó otra propiedad interesante, además de la capacidad de inducir anticuerpos neutralizantes, como es el alto grado de conservación entre diferentes orbivirus. Anticuerpos dirigidos a esta secuencia son capaces de reconocer también a las VP5 de BTV y EHDV por *inmunoblotting*. El uso de este epítopo como reactivo específico de grupo en diagnóstico se encuentra en fase de prueba. Asimismo, nos encontramos realizando las pruebas de inmunogenicidad de los diferentes péptidos y regiones antigénicas identificados.

Superoxide Dismutase Immobilized on Hyaluronic Acid

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Key words: Superoxide dismutase, Hyaluronic acid and Biopolymers.

Oxygen radicals are capable of reversibly or irreversibly damaging compounds of biochemical classes, including nucleic acids, protein and free amino acids, lipids and lipoproteins, carbohydrates, and connective tissue macromolecules [1]. The superoxide radical (O_2^-) is generated within aerobic biological systems during both enzymatic and nonenzymatic oxidations. It is eliminated, by conversion to O_2 plus H_2O_2, by a family of metalloenzymes called superoxide dismutases [2]. Superoxide dismutase (SOD) seems effective in the protection against reperfusion damage after ischemia in several systems [1], and has been proposed as an antiinflammatory agent for use in rheumatoid arthritis [3]. Hyaluronan (hyaluronic acid) is a glycosaminoglycan made up of a repeating disaccharide unit of N-acetylglucosamine and glucuronic acid, exhibits extraordinary biocompatibility and unique rheological properties. Hyaluronic acid (AH) and hyaluronan derivatives have been developed as topical, injectable and implantable vehicles for controlled and localized delivery of biologically active molecules [4]. In the present work we evaluated the SOD activity in four different forms of association: 100μg or 200μg of SOD with 1mg AH carbodiimide activated (SOD100-AHA and SOD200-AHA) or non activated (SOD100-NAH and SOD200-NHA). Also, it has been investigated the influence of two forms of associations (SOD100-AHA and SOD100-NHA) with hyaluronic acid specific viscosity (ηsp). The experiments were carried out for five weeks, in which the preparations were stored at 4° C.

Activity of SOD in all forms of association. Values represent mean ± SD:

	SOD200 (U/mg)	SOD100-AHA (U/mg)	SOD200-AHA (U/mg)	SOD100-NHA (U/mg)	SOD200-NHA (U/mg)
1st week	2.000±83	2.200±244	2.750±0	1.800±0	2.500±306
5th week	1.500±71	3.800±292	2.733±210	3.000±141	1.750±0

The findings showed that association with hyaluronic acid increased superoxide dismutase activity from 10% for SOD100-AHA until 37,5% for SOD200-AHA, in the first week, and from 17% for SOD200-NHA until153% for SOD100-AHA in the fifth week. All preparations were related to SOD200 (enzyme free). The initial viscosity (ηsp) of SOD100-AHA, SOD100-NHA preparation showed to be 13% higher than the free HA preparation ($p<0,05$), however, after five weeks stored at 4^oC this vsicosity was practically the same. This property could be useful for clinical application.

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Comparative Study of Conjugated Dienes in Erythrocytes Membrane and Superoxide Ânion Production in Neutrophils with Healthy Individuals and non Insuline Dependent Diabetes Mellitus Carriers

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Key words: Conjugated dienes, Lipid peroxidation and Superoxide.

Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids, and the eventual destruction of membrane lipids, producing a variety of breakdown products, including alcohols, ketones, aldehydes, and ethers Lipid peroxidation usually begins with the abstraction of a hydrogen atom from an unsaturated fatty acid, resulting in the formation of a lipid radical. The rearrangement of the double bonds results in the formation of conjugated dienes[1].
The respiratory burst is a metabolic event induced in phagocytes by a variety of natural and artificial stimuli. In this event, molecular oxygen undergoes a partial enzymatic reduction to produce a range of microbicidal oxidants. The key reaction in the respiratory burst is the reduction of oxygen to O_2^- at expense of NADPH [2]:

$$2\ O_2 + NADPH \rightarrow 2\ O_2^- + NADP$$

Fluxes of O_2^-, generated enzymatically or photochemically, have been shown to inactivate virus, induce lipid peroxidation, damage membranes and kill cells [3]. Were studied the production of conjugated dienes, in erythrocytes membrane with in healthy individuals and non insuline dependent diabetes mellitus(niddm) carriers as a indicator of damage provocated for this pathology. The measurement of O_2^- production by human neutrophils, was carried out, during twelve minutes, according by Markert *et al.* [4], with the same groups. Statistical analysis (Anova-one way) of the results showed that there was a significative difference of O_2^- concentration at six minutes (p= 0,020) and eight minutes (p= 0,0438) after neutrophils activation. However, there was no significative difference about the production rate of O_2^-.The group healthy (n=10) presented 0,971 $\pm$0,393 μmol/ml the erythrocytes and the group non insuline dependent diabetes mellitus (niddm), also with ten (n=10) individuals, showed 0,583 $\pm$ 0,321 μmol/ml the erythrocytes. These results were significantly differents ($p<0.05$) between diabetics and healthy in the dienes conjugated production.

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Production of Pharmaceutical-Grade Plasmid DNA for Gene Therapy

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***Keywords**: Gene Therapy, Plasmid DNA, Chromatography*

Gene therapy is a promising process for the prevention, treatment and cure of diseases like cancer, acquired immunodeficiency syndrome (AIDS) or cystic fibrosis. The transport of therapeutic genes towards the nuclei of target cells can be carried out by viral or non-viral vectors. While the use of genetically modified retroviruses, adenoviruses and other viral systems presents some problems and raises safety concerns, synthetic formulations of genes inserted in plasmids are regarded as safer and can be used much like a conventional pharmaceutical, for instance by intramuscular injection [1]. By the end of 1996, around 24 % of the clinical trials under way had used palsmids as vectors [2]. This mode of treatment will require repeated treatments, and therefore considerable amounts of pharmaceutical-grade plasmid DNA since the efficacy and duration of gene expression in the target tissues is presently relatively low [1].
This communication places the problem of large scale production of plasmids in the context of gene therapy applications. A brief introduction on gene therapy and plasmid structure is followed by a more detailed description of the problems and bottlenecks associated with the design and operation of large scale processes for the manufacture of plasmid DNA for gene therapy. As with recombinant proteins, the production of plasmids follows through the usual steps of fermentation, primary isolation and purification. However, and apart from usual issues, very specific problems, uncommon to recombinant proteins await the Biochemical Engineer.

Representative results from the primary isolation, anion-exchange [3] and gel chromatography [4] steps are shown as an illustration.

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Scale-up of a Baby Hamster Kidney Clone that Produces a Recombinant Protein for Tumour Therapy

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Key words: Animal cells, Scale-up, Recombinant protein, Tumour therapy, Aeration, Agitation

Recombinant antibody-cytokine fusion proteins have considerable potential for target tumour therapy in a variety of carcinomas including those from the colon, pancreas, ovary, breast and bladder. In this study a recombinant BHK clone was used to produce a fusion protein. A scaleable, cost effective and reproducible production process is required for the eventual industrial application of this BHK clone. This was the main goal of this study, performed in the Pilot Plant of IBET, using stirred tank bioreactors with volumes ranging from 3 to 42 Lt (from B. Braun B.I., Germany).

The initial studies of the most critical operational variables (i.e., sparger aeration and agitation rate ranges) were performed in 3 Lt. vessels, using the Universal Bio-Process Control (UBICON) System (ESD, Hanover, Germany) for data control and acquisition and Mass-Spec (from Micro-Mass, UK) system for gas mixer and gas mixture analysis.

The used strategy was to maintain pO2 constant at 15% by using a constant gas mixture composition, controlled by one of the variables, whereas the other was maintained constant. In the agitation rate study a surface aeration with 0.1 vvm was used, and in the sparger aeration study the agitation was maintained constant at 60 rpm. As the cell growth occurred, the oxygen consumption increased, requiring gradual increases of agitation or aeration rates in order to concomitantly increase oxygen transfer. During these studies all other nutrients and toxic metabolites were maintained at non-critical ranges, not interfering with the experiments. Eventually at a certain critical value of agitation or aeration the shear stress exerted on the cells was so high that the cells started to die, determining the maximum sustainable value of the variable under study.

It was possible to conclude that the cells sustain 270 rpm and 0.03 vvm but higher agitation or aeration rates lead to cell growth arrest and death.

From these results a double cascade control strategy was planned for the optimal operation of large scale stirred tanks, based on the agitation rate increase up to the defined maximum value (based on a constant paddle shear stress scale-up strategy), followed by the increase of aeration rate up to its maximum and, if necessary, followed by an enrichment of the gas mixture with oxygen. Results will be presented with the application of this strategy and consequences upon the cells, production and product quality will be depicted.

Application of 3D-QSAR in the development of new drugs: Carbohydrate Processing Inhibitors (CPI's)

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Keywords: *Drug design, 3D-QSAR, Carbohydrate Processing Inhibitors*

The design, development and commercialization of a new drug is a tedious, time-consuming and expensive process. In the last decade, the use of computer-aided drug design has appeared to be an attractive alternative to accelerate the design of potent and specific drugs. This methodology implies the use of an explicit or implicit knowledge of the three-dimensional arrangement of the participating molecules, the target receptor and the ligand.

3D-QSAR (Three-dimensional Quantitative Structure-Activity Relationship) is one of the new methods which have emerged from the efforts to improve the lead optimization process, that is, the conversion of lead compounds into drug candidates with improved potency and specificity. This approach, based on the correlation of the measured inhibitory power against an enzyme with the computed molecular and structural properties, might allow the prediction of the biological activity of an analogue well before envisaging its synthesis and hence weeding out poor candidates early in the drug development cycle.

We report here an application of the 3D-QSAR method to the design of a 'second generation' of Carbohydrate Processing Inhibitors (CPI's) for Mannosidases. Information on the molecular structure of the participants is crucial to understanding the molecular mechanism of the interactions of drugs with their biological targets. Therefore, in the approach currently used at GlycoDesign Inc., the molecular structure and molecular properties such as electron densities, atomic charges, HOMO, LUMO are predicted by high level *ab initio* molecular orbital calculations and then used for the correlation with the measured inhibitor activity. Such an approach has led to the establishment of 3D-QSAR models which have proved to be useful in our drug development strategy.

Enthalpy of Captopril-Angiotensin I-Converting Enzyme Binding

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***Keywords**: Angiotensin I-converting enzyme, captopril, microcalorimetry, binding*

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) plays an important role in blood pressure regulation. It is a dipeptidyl carboxypeptidase which converts angiotensin I into the potent vasopressor peptide angiotensin II and inactivates the vasodepressor peptide bradykinin [1]. The molecular cloning revealed that ACE is a glycoprotein that consists of a single polypeptide chain containing two homologous domains called the N and C domains, each bearing a potencial catalytic site [2]. A lot of highly potent inhibitors of ACE which can be taken orally have been developed during the past two decades [3]. The first of these, captopril (D-[3-mercapto-2-methyl propanoyl]-L-proline) was designed with the help of a theoretical model of the active site of ACE, which was based on its presumed similarity to the known active site of carboxypeptidase A and thermolysin.

High-sensitivity titration calorimetry is used to characterize the binding of captopril to the angiotensin converting enzyme in buffer cacodylate at pH 7 over a temperature range of 16ºC to 30ºC. A concomitant net uptake of protons with the binding of captopril was detected carrying out calorimetric experiments in two buffer systems with different heats of ionization. ACE was prepared from bovine lung by the method described by García-Fuentes *et al.* [4].

Captopril binds to the two potential active sites identificated in the sequence of somatic ACE [2]. The enthalpy changes are positive within the range of temperature studied and decrease linearly with the temperature, given a large negative value of ΔC_p.. The Gibbs energy change for ACE complex formation is entropy-controlled and although the enthalpy and entropy changes exhibit strong temperature dependencies, arising from a significant negative heat-capacity change, the binding process remains dominated by entropy throughout the physiological temperature range. Thus, the thermodynamic parameter values suggest that the driving force for the binding of captopril to ACE is provided by electrostatic interactions and hydrophobic effects related to buried apolar surface with liberation of water molecules from both the protein and the drug, showing that the hydrophobic character of the groups in the drug is an important factor in the binding of an inhibitor to ACE.

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Production of Monoclonal Antibodies Against Human Immunodeficient Virus Type 2 Antigens

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Keywords:*Monoclonal antibody, HIV, p26, gp 105, Reverse Transcriptase.*

Since the production of first monoclonal antibodies (Mabs) by Köhler and Milstein [1], their use *in vivo* and *in vitro* diagnosis, as well in therapeutic applications, did not stop to increase. Advances in antibody engineering have allowed the cloning of genes encoding antibodies enabling the creation of many antibody-derived molecules. The single-chain antibodies (ScFv) has a heavy and light chain variable region that are linked by a flexible 15 amino acid linker. With a molecular weight of 27 KDa they are the smallest molecules that retain full (monovalent) antibody-binding activity.
Mabs against HIV core protein p26, surface gp105 and reverse transcriptase (RT) would be useful for serological diagnosis of HIV infection and to predict progression of disease.
Hybridoma secreting Mabs against RT (HIV-1), rgp105 (HIV-2ROD) and rp26 (HIV-2ROD), were obtained. The Mab that recognizes HIV-2 ROD (R26.1) also reacts with the p26 of a set of HIV-2 strains. The R26.1 mab does not cross react with HIV1LAI p24. The Mab against HIV-2 RODgp105 (R105.1) was tested, by immunofluorescence microscopy, to detect HIV-2 infected peripheral blood mononuclear cells from seropositive individuals and the inhibition of syncicium formation.
After mRNA isolation and cDNA synthesis from each hybridoma, the VH and VL fragments were specifically amplified by PCR. In all cases the expected band sizes in agarose gel electrophoresis for VH (340 bp) and for VL (325 bp) fragments were obtained, however, the amount of PCR product for VL fragment showed to be low. When ligation of equimolar amounts of VH and VL was performed, followed by PCR amplification of ligated fragments, no band with the expected size of 720 bp was obtained. Studies are in progress to improve the yield of VL cDNA amplification, in order to assay new ligations with increased amounts of VH and VL fragments.
Described Mabs has being tested in our laboratories to detect HIV-2 antigens in blood samples.

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The use of a filamentous phage library for a monoclonal antibody epitope determination

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***Keywords**: epitope mapping, filamentous phage library,* Plasmodium chabaudi chabaudi*, Bacteriophage fd, FUSE 5*

In this work a filamentous phage library was used in which genome had been cloned DNA sequences that display on its surface 10^8 different hexapeptides [1] for the identification of peptide epitopes recognised by two monoclonal antibodies (Pc97 and Pc99) produced against antigens of *Plasmodium chabaudi chabaudi*. Both antibodies in indirect imunofluorescence assays, showed specific fluorescence against antigens from the erythrocyte schizont stage parasites. Three rounds of panning were carried out to select phage clones with affinity to each antibody. The phages were amplified by infecting them into their host cells (bacteria). The antibody-reactivity of individual purified clones from a panning, recognised by monoclonal antibodies Pc97 and Pc99, was determined by dot blot analysis, plaques immunoblotting and tested by inihibition assays. Sequencing the inserts of the single-stranded phage DNA from a number of imunopositive colonies has made possible the identification of the aminoacids codified by the insert DNA from the recognised epitopes.The screening made by monoclonal antibody Pc99 led to the selection of 12 similar clones (according to Taylor's classification) with the predominance of the aminoacid sequence SRL (33.3%), which when compared with MSP-1 protein of the Gene Bank, using GCG package (University of Wisconsin), showed 100% homology with the regions between aminoacids 1162-1164 and 1246-1256.

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Aplicación de la Catálisis Enzimática a la Síntesis de Nuevos Fármacos Colinérgicos

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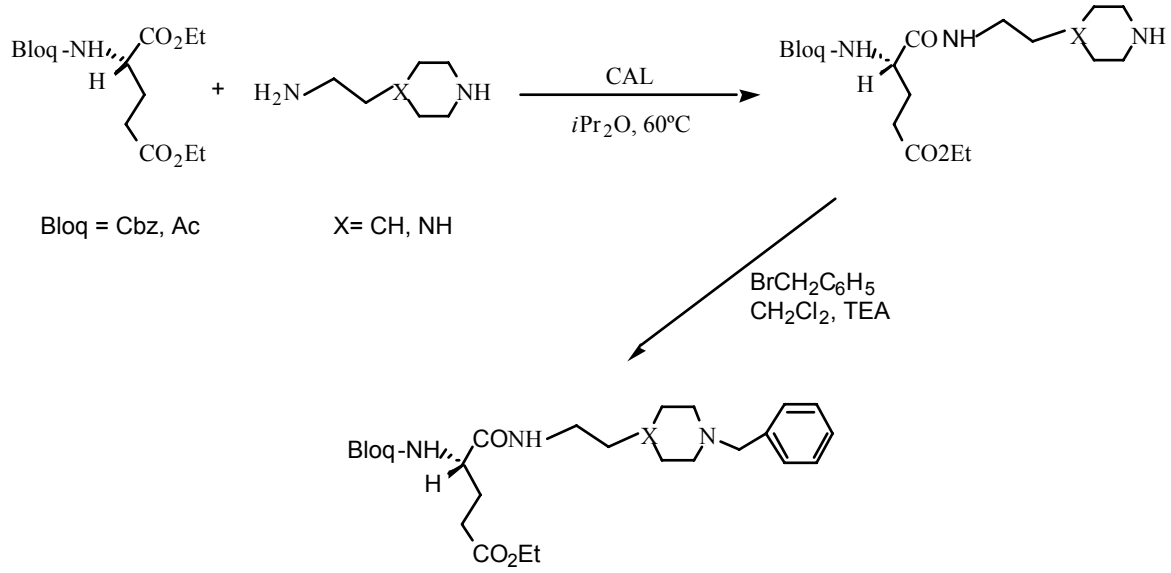
Palabras llave: Alzheimer, colinérgico, lipasa, amidación, glutamato

La enfermedad de Alzheimer es un proceso neurodegenerativo que afecta a una parte significativa de la población de edad avanzada, caracterizada por un deterioro de la función cognitiva, con pérdida progresiva de memoria, desorientación y demencia. Se han observado en los pacientes disminuciones de numerosos neurotransmisores, sobre todo del sistema colinérgico.
En la búsqueda de nuevos fármacos colinérgicos, nuestro equipo de investigación está aplicando la catálisis enzimática a la síntesis de moléculas con potencial actividad inhibidora de acetilcolinesterasa. Las reacciones enzimáticas muestran unas características de regioselectividad difíciles de encontrar por vía química orgánica clásica. En esta comunicación se presenta una reacción doblemente selectiva: la amidación del grupo α–carboxilo de derivados del dietiléster del ácido glutámico con la amina primaria de 4-(2-aminoetil)-piperazina, catalizada por la lipasa B de *Candida antarctica* (Novozim 435).



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Replication and Expression of Synthetic Minigenomes Derived From Transmissible Gastroenteritis Coronavirus Defective rRNAs

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A helper virus dependent expression system is being engineered based on transmissible gastroenteritis coronavirus (TGEV) defective minigenomes. The vector has been designed to target gene expression to enteric and respiratory tissues and is based on cDNAs encoding RNA minigenomes. *In vitro* transcribed minigenome RNAs were replicated *in trans* upon transfection to helper virus infected cells. The minimal sequences required for minigenome replication were analyzed by deleting internal fragments. These minigenomes have been reduced from 28.5 to 3.3 kb. The results suggested that TGEV may have two packaging signals located in the 5' and 3' ends of the ORF 1b or that other limitations for minigenome packaging, such as secondary structure or RNA size restrict the encapsidation. Rescue of synthetic minigenomes has been improved by using two amplification steps, the first under the control of cytomegalovirus (CMV) promoter and the second under the control of the viral replicase. This system yielded higher RNA levels than systems based on the T7 polymerase. The levels of heterologous proteins were also reached earlier when the amplification mediated by the CMV promoter was included.

Constructs based on two defective particles with diferent size, M39 and M54, that facilitate the exchange of genes and promotors have been constructed in order to optimize the expression levels of the heterologous antigen. The expression of the ß-glucuronidase (GUS) as a reporter gene, and viral spike protein (S) have been studied to optimize expression. The aim is to evaluate whether minigenome size affects the expression levels, and the possible differences between homologous and heterologous gene expression in this system.

The expression levels have been studied under the control of the nucleocapsid (N), the integral membrane (M), and the spike (S) gene promotors. The highest expression levels detected were 2 µg per 10^6 cells. Immune responses specific for the heterologous protein were elicited by immunizing swine with minigenomes encoding the spike protein of an enteric virus using a porcine respiratory coronavirus as helper.
The engineered minigenomes will be very helpful to understand the mechanism of coronavirus replication and to improve a family of vectors for the tissue specific expression of antigen, antibody or virus interfering molecules in order to control viral diseases.

Development of Packaging Cell Lines to Encapsidate Transmisible Gastroenteritis Coronavirus Genomes

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Key words: coronavirus, TGEV, packaging cells, tetracycline-responsive expression system.

The construction of packaging cell lines to encapsidate transmisible gastroenteritis virus (TGEV) genomes is being developed to study TGEV gene function and to design a new recombinant vector to induce mucosal inmunity based on a self-replicating RNA (replicon) defective in the structural proteins.

The development of TGEV packaging cell lines is being accomplished by expressing the TGEV structural proteins using a doxycycline (dox) inducible system, based on the *Escherichia coli* Tn 10 tetracycline (tc) operon. The system is based on a fusion protein (rtTa) which contains the DNA-binding tc-repressor (tetR) and the C-terminal domain of the herpes simplex virus VP16 transactivator. In the presence of doxycicline rtTa binds and trans-activates minimal promoters carrying multiple tet-operator sites. In the absence of doxycycline, DNA binding does not occur and the gene of interest is silent.

The swine testis (ST) cell line susceptible to TGEV has been stably transformed with plasmids encoding the reverse transactivator (rtTa). To select transformed ST cell lines inducing high expression levels, 117 cloned cell lines expressing the rtTa transactivator were transiently transfected with a plasmid carrying the luciferase gene under the control of the minimal promotor. The transformed cells were tested for luciferase expression. The objective was to identify a cell line producing high levels of luciferase activity in the induced state and low expression levels in the absence of induction. Four clones showing induction ratios higher than one thousand were selected. Luciferase expression levels of 1-10 ng/10^6 cells were obtained. The stability of the selected clones using the tetracycline-responsive expression system was tested by analyzing luciferase expression levels in at least 16 subclones of each cell line and by comparing the expression levels between the subclones and the parental cell line. Two stable clones were selected in which more than the 90% of the subclones analyzed maintained expression levels similar to those shown by the parental clone.

The essential proteins for coronavirus assembly are the N, E and M; S protein is not essential for virion assembly but it is necessary for infectivity. To study the assembly of TGEV the structural proteins of the virus (M, E, N and S) were transiently expressed by cotransfecting plasmids expressing the different structural proteins using the tetracycline-responsive expression system. The same plasmids are being used to stably transform a ST cell line expressing the transcription transactivator. Protein expression has been screened by Western-blot and immunoadsorption and by studying the assembly of viral structures and the results will be discussed in the meeting.

Construction of Transgenic Animals Secreting Neutralizing Antibodies Against Transmissible Gastroenteritis Coronavirus

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Transmissible gastroenteritis virus (TGEV) and related coronaviruses infect enteric and respiratory mucosal areas. Animals are infected very early in life, when their immune system is unmature, producing mortality rates approaching 100% during the first 10 days of life. At this time protection can only be provided passively through lactogenic immunity.
Transgenic mice secreting TGEV neutralizing monoclonal antibodies (MAbs) in milk during lactation have been constructed by microinjecting DNA into pronuclei of one cell fertilized eggs ((C57BL/6 x CBA)F1). Immunoglobulin (IMG) gene fragments encoding the VH and VL modules of a TGEV neutralizing MAb, which recognizes a highly conserved virus epitope and does not lead to the selection of MAb escape mutants, have been cloned and sequenced. Chimeric immunoglobulin genes with the variable modules from the murine MAb and constant modules of human (gamma 1 and kappa chains) and porcine (alpha and kappa chains) origin were constructed using RT-PCR. The chimeric immunoglobulin genes were initially cloned and expressed under the control of an immunoglobulin enhancer, SV-40 promoter and polyadenylation coding sequences. These plasmids have been used to express the chimeric immunoglobulins in murine myelomas, to test for secretion of TGEV neutralizing MAb. Recombinant antibodies had titers against TGEV, both in RIA and neutralization assays, comparable to those of the original hybridoma. Specific activity of recombinant IgA against TGEV resulted 50-fold higher than that of recombinant IgG, as expected for a dimeric immunoglobulin. The chimeric immunoglobulin genes have been cloned into different exons of the whey acid protein (*WAP*), and into β-lactoglobulin (*BLG*) genes. Mouse mammary gland cells (HC11), stably transformed with these constructs, expressed chimeric MAb following the induction by peptide and steroid hormones, which promote tissue specific expression in mammary gland. These results imply that epithelial cells from the mamary gland are able to sinthesize, assemble and secrete virus neutralizing antibodies. Transgenic mice have been generated by microinjection of either *WAP*-IMG constructs together with *MAR* (matrix attachment regions) or BLG-IMG constructs together with *BLG* sequences. MAR or BLG sequences frequently flank genetic units increasing their transcription. Transgenic animals carrying light and heavy chain genes have been obtained and both genes were transmited to the progeny. Concentrations of TGEV neutralizing MAb of 6 mg/ml have been obtained in the milk of the WAP-IMG and BLG-IMG transgenic animals. The recombinant antibodies secreted into the milk had RIA titers ranging 10^5-10^6 and reduced virus infectivity more than 10^5-fold. Probably, these MAb expression levels should suffice *in vivo* to protect against TGEV infection.

Clonaje y Expresión del Gen de la Nucleoproteína del Virus del Moquillo Canino en Celulas de Insecto

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Palabras claves: Moquillo canino, Morbillivirus, Baculovirus, Nucleoproteína, diagnostico.

El virus del moquillo canino (VMC) es un agente infeccioso que produce alteraciones respiratorias, digestivas y del sistema nervioso central en perros y otras especies relacionadas. El VMC es un morbillivirus que contiene un ARN de polaridad negativa. El ARN se encuentra estrechamente asociado a la proteína de la nucleoproteína que da forma helicoidal al virus. Dado que los animales suelen ser vacunados, en muchas ocasiones es necesario diferenciar los perros vacunados de los infectados. Tras la infección los primeros anticuerpos que se detectan son IgM, posteriormente aparecen los anticuerpos neutralizantes e IgG. Existe una buena correlación entre el título de IgG y el título de seroneutralización. No existen en la actualidad buenos métodos de diagnóstico rápidos, fiables y de fácil automatización para el VMC, teniendo que recurrir a la valoración de sueros por inmunofluorescencia. Una de las razones para la ausencia de buenos métodos de diagnóstico es la extrema labilidad del virus. Con objeto de paliar este problema se decidió utilizar antígenos recombinantes y más específicamente uno de los antígenos mas inmunodominantes, la nucleoproteína.

Para desarrollar un ELISA que permita detectar el nivel de anticuerpos en sueros de perro, se clonó mediante RT-PCR el gen de la nucleoproteína del virus del moquillo. Se infectaron células VERO con la cepa Onderstepoort del VMC. A las 48 horas post-infección se recogieron las células y se extrajo el ARN citoplásmico, este se amplificó mediante RT-PCR con oligonucleótidos específicos para el gen de la nucleoproteína. El fragmento de 1600 pb así obtenido, se clonó en pMTL25 en extremos romos. Mediante digestiones parciales con *BamHI* se introdujo el inserto en dos vectores de transferencia a baculovirus: pAcYM1 (bajo el promotor de la poliedrina) y pAcAs3 (bajo el promotor de la proteína p10). Por cotransfección de las células de insecto con cationes lipídicos se obtuvieron baculovirus recombinantes que contienen en su genoma el gen de la nucleoproteina del VMC. Se analizó el producto de expresión de este gen en células Sf9 mediante inmunoblotting y ELISA con sueros de perro infectados con VMC y con el anticuerpo monoclonal (cedido por el Dr Orvël, Estocolmo, Suecia). La nucleoproteína fue detectada por los métodos previamente mencionados tanto en la fracción citoplásmica como secretada en el sobrenadante del cultivo. Por inmunoblotting con sueros de perro se detectaron dos bandas de 53 KDa y 40 KDa en las células de insecto que aparecen también en células VERO infectadas con VMC. Por inmunobloting con anticuerpo monoclonal solo se detecta la banda de 53 KDa. La proteína de 40 KDa podría ser un producto proteolitico de la nucleoproteina.

El producto de expresión en Sf9 es capaz de formar estructuras particuladas similares a la nucleoproteina viral, propiedad que permite su rápida purificación tanto mediante colchón de sacarosa como por precipitación con sulfato amonico al 20 %. La proteína obtenida en Sf9 ha permitido desarrollar un ELISA indirecto para la detección de anticuerpos en sueros de animales infectados, así como de animales vacunados tanto con virus del moquillo como con virus atenuado de sarampión.

Determination of Dopamine in Pharmaceutical Formulations with "Graviola" (*Annona Muricata*, L.) Polyphenol Oxidase (Ec 1.10.3.2) using an Amperometric Flow Injection Analysis

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Keywords: Dopamine; Polyphenol oxidase; amperometric biosensor

Polyphenol oxidase is an enzyme found in fruits, vegetables and fungi, which catalyses the oxidation of phenolic compounds to quinones, such as dopamine. It is an adrenergic neurotransmitter related to Parkinson's desease when its level is high.

This work described a catalytic microsensor to determine dopamine concentration carried out in the following composition: 1- polyphenol oxidase 25% (w/w) from "graviola" tissue, 2 - 30% (w/w) graphite; 3- 30% (w/w) silicone wax and; 4 - 15%(w/w) TCNQ (mediator). The biosensor showed a good stability, reproducibility and life time higher than 30 days, after 250 assays. Also, its response was linear in the range 0.1mM up to 1mM showing a variance coefficient of 3.5% after 100 injections, using 0.3M phosphate buffer as carrier at flow rate of 2.5 mL/min and 250 μL of sample. This Flow Injection Analusis system was tested with success to determine dopamine concentration in medicines, REVIVAN-ZAMBON and DOPAMIN-Cristalia, showing a relative error, compared to theoretical values, 3.9% and 3.3% respectively.

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Comparative Genomic Analysis, Exopolysaccharide Biosynthetic Ability and Antibiotic Multi-Resistance of *Burkholderia cepacia* Isolates From a Portuguese Cystic Fibrosis Center

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Keywords: Burkholderia cepacia*, cystic fibrosis, molecular typing, antibiotic multirresistance, exopolysaccharide*

Burkholderia cepacia has recently emerged as a multi-drug-resistant nosocomial pathogen [1]. The acquisition of *B. cepacia* by cystic fibrosis (CF) patients constitutes a real threat to them because pulmonary colonization with this bacterial species is frequently associated with increased morbidity and rapid and fatal deterioration of pulmonary functions and septicemia (the cepacia syndrome) [1]. Increasing evidences of the importance of person-to-person transmission, whether direct or indirect [1], led some CF centers to introduce hygiene guidelines and segregation policies designed to prevent transmission between *B. cepacia*-positive and negative CF patients [1]. Contrasting with the extensive information on the virulence determinants of *Pseudomonas aeruginosa*, the most frequently reported respiratory pathogen in CF patients, virulence factors of *B. cepacia* remain largely unknown [1], notably the possible contribution of the polysaccharide produced extracellularly by some isolates [2,3] in the development of pulmonary infections. However, in the case of *Pseudomonas aeruginosa* alginate, the exopolysaccharide produced by some CF isolates, was pointed out to play a critical role in the colonization and the persistance of the microorganism in the respiratory tract of CF patients [1].

In Portugal, the first clearly identified *B. cepacia* isolate recovered from the sputa of a CF patient attending the CF care center at the S.ta Maria Hospital, in Lisbon, took place in 1992. Since then, more than 20 isolates were recovered from 8 of the 137 patients followed in this unit (an occurrence in approximately 6 % of the patients). *B. cepacia* isolates obtained from this CF center were compared by genomic fingerprinting using ribotyping, RFLP-PFGE and PCR-based techniques, envisaging their epidemiological chararacterization. Results of this first study within part of the portuguese CF population, during the last 5 years, as well as the profiles of antibiotic resistance and of exopolysaccharide biosynthetic ability of the isolates will be presented.

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A Homogeneous Method to Measure Aminoacyl-tRNA Synthetase Activities Using Scintillation Proximity Assay Technology

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Over the last years, the emergence of bacterial resistance to known antibiotics and its spread through populations of clinically relevant pathogens has become a critical issue. Aminoacyl-tRNA synthetases (ARS) play an essential role in the biosynthesis of proteins. The known differences between prokaryotic and eukaryotic ARS, make these enzymes attractive targets to look for novel antibacterial agents, as proved by the marketed antibiotic mupirocin, a specific inhibitor of bacterial isoleucyl-tRNA synthetase.

In our efforts to find new antibiotics with novel mechanisms of action, all 19 ARS from *S. aureus* have been cloned, expressed, purified, and utilised for High-Throughput Screening against large collections of file compounds, combinatorial chemistry libraries and natural products at SmithKline Beecham. In order to facilitate the screening process, a new method to measure ARS activities has been developed using the scintillation proximity assay technology (SPA) from Amersham Pharmacia Biotech. The incorporation of a radiolabelled aminoacid to its cognate tRNA, catalysed by its specific ARS is detected in an end-point mode, by addition of nude yttrium silicate beads under acidic conditions, and subsequent counting in a liquid scintillator counter.

This new assay method was validated by several means, using different bacterial ARS as model enzymes. The results obtained using the classic filtration assay after acidic precipitation, and the novel SPA technology, showed a perfect correlation in all cases studied. Furthermore, the net proximity counts observed with the SPA method were very reproducible, with coefficients of variation below 5%.
In order to study the best isotope for this type of assay methionyl-tRNA synthetase was used as model enzyme. ^{3}H, ^{14}C and ^{35}S radiolabeled methionine were utilised for this study. The results indicated that the most appropriate labels are ^{14}C and ^{35}S, ^{3}H showing a very low efficiency (15 %) with respect to the filtration assay.

The nature of the binding of tRNA to the beads was also studied. Results indicated that the tRNA aggregates formed under acidic conditions, settle slowly and stick to the dense YSi beads in a non-specific way. Bare YSi beads, but also poliLys-YSi beads and even polystyrene are able to bind these tRNA aggregates.
In contrast to the classical filtration assay, no separation step is required, and so this new assay format is faster and more amenable for High-Throughput Screening purposes.

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