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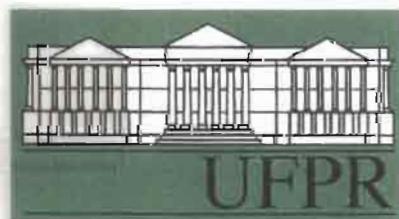
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Programme "Technologie Agroalimentaire
et Agroindustrialisation"

International Training Course on Solid State Fermentation

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INTERNATIONAL TRAINING COURSE ON SOLID STATE FERMENTATION



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- Dr. Ricardo PEREZ-CORREA (PUC-Santiago)
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The group of speakers during the course FMS97 in Curitiba .

From the left: Gérard Chuzel, Ricardo Pèrez, Carlos Soccol, Christopher Augur, Maurice Raimbault, José Rodriguez Leon, Sebastianos Roussos, Gerardo Saucedo, and Pierre Christen (not on the photo).

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FOREWORD

Solid Substrate Fermentation (SSF) is a microbial processes of growth on solid materials which contain water and soluble nutrients. Generally, solid substrates or supports are biodegradable heterogenous plant material. Starch, ligno-cellulose, natural fibers, inert polyurethane, matrices and chemical polymers are the most common material used for cultivating fungi and other microorganisms in SSF. Researches in this field were recently developed which indicated that does exist an alternative for new processes generating less effluents, recycling wastes or by-products in tropical agro-industry. This new technology represents a promising alternative for Latin American. Particularly, respirometry is of high interest for the *on line* control of the SSF and growth of mycelia biomass.

The general objectives of this courses consist in training professional or high level students in the area of agro-industry and fermentation. Theoretical and practical aspects of SSF, on solid substrates or impregnated supports, were studied with special attention to the practice of the technique of respiration measurement. Creation of a network of professional and academic researchers involved in Research and Development on SSF projects are of interest for Brazil and Latin American Countries.

Specific Objectives consisted in the presentation and discussion of direct experiences from Researchers and Professors on related topics to Microbiology, Biochemistry, Physiology and Scale-up of filamentous fungi cultivation by SSF. Revision of the Biological, Biochemical and Molecular Biology concepts involved in the bioconversion and respirometry aspects of the SSF was also studied. Finally, laboratory training and practice of SSF and respirometry analysis of fungal metabolism of *Aspergillus* and *Rhizopus* cultivated on Cassava by-product was performed.

This course was devoted to 30 professionals of the industrial sector, academic researchers, professors or students of Brazil (1/2) and from other countries of Latin American countries (1/2): Argentina, Mexico, Chile, Uruguay, Colombia and Cuba.

The Organizer Committee thanks sponsors Institutions: UFPR, ORSTOM, CNPq and French Cooperation for their financial support, and all invited speakers for their active participation in the course.

Maurice Raimbault,
Carlos Ricardo Soccol,
G rard Chuzel

GENERAL AND MICROBIOLOGICAL ASPECTS OF SOLID SUBSTRATE FERMENTATION

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Summary

We present at first some general considerations about specificity and characteristics of SSF, their advantages and disadvantages compared to LSF. We speak about micro-organisms involved in SSF processes, considering the better performances of filamentous fungi. The solid substrates and their basic macromolecular compounds are detailed in relation to this complex and heterogeneous systems. Biomass measurements are examined in detail, as so as environmental factors, both essential for studying and optimising SSF processes.

1. General considerations.

Aerobic microbial transformation of solid materials or "Solid Substrate Fermentation" (SSF) can be defined in terms of the following properties of the substrate to be transformed:

- A solid porous matrix which can be biodegradable or not but with a large surface area per unit volume, in the range of 10^3 to 10^6 m²/l for a ready microbial growth on the solid/gas interface.
- The matrix should absorb water once or several times its dry weight with a relatively high water activity on the solid/gas interface in order to allow fast rates of biochemical processes.
- Air mixture of oxygen with other gases and aerosols should flow under a relatively low pressure and mixing the fermenting mash.
- The solid/gas interface should be a good habitat for the fast development of specific cultures of moulds, yeasts or bacteria, either by isolated or mixtures of species.
- The mechanical properties of the solid matrix should stand compression or gentle stirring as required for a given fermentation process. This requires small granular or fibrous particles which do not tend to break or stick to each other.
- The solid matrix should not be contaminated by inhibitors of microbial activities and should be able to absorb or contain available microbial foodstuffs such as carbohydrates (cellulose, starch, sugars) nitrogen sources (ammonia, urea, peptides) and mineral salts.

Typical examples of SSF are traditional fermentations such as:

- Japanese "koji" which uses steamed rice as solid substrate inoculated with solid strains of the mould *Aspergillus oryzae*.
- Indonesian "tempeh" or Indian "ragi" which use steamed and cracked legume seeds as solid substrate and a variety of non toxic moulds as microbial seed.
- French "blue cheese" which uses perforated fresh cheese as substrate and selected moulds, such as *Penicillium roquefortii* as inoculum.

- Composting of lignocellulosic fibres, naturally contaminated by a large variety of organisms including cellulolytic bacteria, moulds and *Streptomyces sp.*

- In addition to traditional fermentations new versions of SSF have been invented. For example, it is estimated that nearly a third of industrial enzyme production in Japan which is made by SSF process and koji fermentation has been modernised for large scale production of citric and itaconic acids.

Furthermore, new applications of SSF have been suggested for the production of antibiotics (Barrios & al., 1988) or enriched foodstuffs (Senez et al., 1979).

Presently SSF has been applied to large scale industrial processes mainly in Japan. Traditional *koji*, manufactured in small wooden and bamboo trays, has changed gradually to more sophisticated processes: fixed bed room fermentations, rotating drum processes and automated stainless steel chambers or trays with microprocessors, electronics sensors and servomechanical stirring, loading and discharging. The usual scale in *sake* or *miso* factories is around 1 or 2 metric tons per batch but reactors can be made and delivered by engineering firms to a capacity as large as 20 tons (Fujiwara, Ltd.).

Outside Japan, Kumar (1987) has reported medium scale production of enzymes, such as pectinases, in India. *Koji* type processes are widely used in small factories of the Far East (Hesseltine, 1972) and *koji* fermentation has been adapted to local conditions of United States and other Western countries, including Cuba (III A). In France, a new firm (Lyven SA) was recently created to commercialise a process for pectinase production from sugarbeet pulp. Blue cheese production in France is being modernised with improvements on the mechanical conditioning of cheeses, production of mould spores and control of environment conditions.

Composting which was produced for small scale production of mushroom rooms has been modernised and scaled up in Europe and United States. Also, various firms in Europe and USA produce mushroom spawn by cultivating aseptically *Agaricus*, *Pleurotus* or *Shii-Take* on sterile grains in static conditions.

New versions for SSF reactors have been developed in France (Durand et al., 1988), Cuba (Cabello, 1985; Enriquez, 1983 and Rodriguez, 1984) and fundamental studies on process engineering are being conducted in Mexico (Saucedo, 1987).

SSF is usually a batch process using heterogeneous materials with various ages, (Raimbault, 1980 and Tengerdy, 1985), gibberellic acid (Agosin et al, 1987), pectinases (Kumar, 1987; Oriol, 1988), cellulases (Roussos, 1985), spores as biopesticides, flavours and fragrances and feed detoxification. All that points will be discussed during the course.

Generally, most of the recent research activity on SSF is being done in developing nations as a possible alternative for conventional submerged cultures which are the main process for pharmaceutical and food industries in industrialised nations.

SSF seems to have theoretical advantages over LSF. Nevertheless, SSF has several important limitations. Table 1 shows advantages and disadvantages of SSF compared to LSF.

Table 2 presents a list of SSF process in economical sectors of agro-industry, agriculture and Industrial fermentation. Most of the processes are commercialised in South-East Asian, African, and Latin American countries. Nevertheless, a resurgence of interest has occurred in Western and European countries over last 10 years. The future potentials and applications of SSF for specific processes are discussed in other sessions. But briefly, we can say :

TABLE 1. Comparison between Liquid and Solid Substrate Fermentations.

FACTOR	Liquid Substrate Fermentation	Solid Substrate Fermentation
Substrates	Soluble Substrates (sugars)	Polymer Insoluble Substrates: Starch Cellulose Pectines lignin
Aseptic conditions	Heat sterilisation and aseptic control	Vapor treatment. non sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited Consumption of water; low Aw. No effluent
Metabolic Heating	Easy control of temperature	Low heat transfer capacity
Aeration (O₂)	Limitation oby soluble oxygen High level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mecanical agitation	Good homogeneization	Static conditions prefered
Scale up	Industrial equipments Available	Need for Engineering & New design Equipment
Inoculation	Easy inoculation , continuous process	spore inoculation, batch
Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
Energetic consideration	High energy consuming	Low energy consuming
Volume of Equipment	High volumes and high cost technology	Low volumes & low costs of equipments
Effluent & pollution	High volumes of polluting effluents	No effluents, less pollution
Concentration S /Products	30-80 g/l	100/300 g:l

- Potentially many high value products as enzymes, metabolites, antibiotics. could be produced in SSF. But improvements in engineering and socio-economic aspects are required because processes must use cheap substrate locally available, low technology applicable in rural region, and processes must be simplified.

- Potential exists in developed countries, but require close cooperation and exchanges between developing and industrialised countries for further application of SSF.

- The greatest socio-economical potential of SSF is the raising of living standards through the production of protein rich foods for human consumption. Protein deficiency is a major cause of malnutrition and the problem will become worse with further increases in the world population. Two ways can be explored for that:

- Production of protein-enriched fermented foods for direct human consumption. This alternative involve starchy substrates for its initial nutritional calorific value. Successful production of such food will require demonstration of economical feasibility, safety, significant nutritional improvement, and cultural acceptability.

- The second alternative consists to produce fermented products for animal feeding. Starchy fermented substrates with protein enrichment could be fed to monogastric animals or poultry. Fermented lignocellulosic substrates by increasing in the fibre digestibility could be fed to ru-

minants. In this case, the economical feasibility should be decisive in comparison to the common model using protein of soybean cake, a by-product of soybean oil.

Table 2. Main applications of SSF processes in various economical sectors

Economical Sector	Application	Examples
<u>Agro-Food Industry</u>	Traditional Food Fermentations	Koji, Tempeh, Ragi, Allieke, Fermented cheeses
	Mushroom Production & spawn	Agaricus, Pleurotus, Shi-take
	Bioconversion By-products	Sugar cane Bagasse Coffee pulp, Silage Composting, Detoxication
	Food Additives	Flavours, Dyestuffs, Essential Fat and organic acids
<u>Agriculture</u>	Biocontrol , Bioinsecticide	Beauveria, Metarhizium, Trichoderma
	Plant Growth, Hormones	Gibberellins, Rhizobium, Trichoderma
	Mycorrhization, Wild Mushroom	Plant inoculation,
<u>Industrial Fermentation</u>	Enzymes production	Amylases, Cellulases, Proteases, Pectinases, Xylanases.....
	Antibiotic production	Penecillin, feed & Probiotics
	Organic acid Production	Citric acid Fumaric acid Gallic acid Lactic acid
	Ethanol Production	Schwanniomyces sp. Starch Malting and Brewing
	Fungal Metabolites	Hormones, Alcaloides,

Since 15 years, the Orstom group worked on solid fermentation process for improving protein content of cassava and other tropical starchy substrates using fungi, specially from *Aspergillus* group in order to transform starch and mineral salts into fungal proteins (Rainbault, 1981).

More recently, C. Soccol working at our Orstom laboratory in Montpellier, obtained good results with fungi of the *Rhizopus* group, of special interest in human traditional fermented foods (Soccol, 1993). These works are now continued in the view of increasing knowledge about specificity of strains of *Rhizopus* able to degrade the crude granules of starch, what could be simplify drastically the process of SSF.

In another hand, the ORSTOM group is collaborating since 1981 with the Mexican UAM group on the following aspects :

- Protein enrichment of Cassava and starchy substrates
- Production of organic acids or ethanol by SSF from starchy substrate and Cassava
- Digestibility of fibres and lignocellulosic materials for animal feeding

- Degradation of caffeine in coffee pulp and ensiling for conservation and detoxification
- Enzymes and fungal metabolites production by SSF using sugarcane bagasse

Main results will be discussed further in this course by the respective speakers . We are hoping that in the future, an extended collaborative program could be fitted for a best interconnection first with all other Latin-American groups of research involved in SSF, then tentatively, create an international network including American, Asian, European and Australian groups of research.

2. Micro-organisms

Bacteria, yeasts and fungi can grow on solid substrates, and find application in SSF processes. Filamentous fungi are the best adapted for SSF and dominate in research works. The Table 3 reports some examples of SSF processes for each category of micro-organisms involved.

Bacteria are mainly involved in composting, ensiling and some food processes (Doelle et al., 1992). Yeasts can be used for ethanol and food or feed production (Saucedo et al., 1991, 1992).

But filamentous fungi are the most important group of micro-organisms used in SSF process owing to their physiological, enzymological and biochemical properties.

The hyphal mode of fungal growth and their good tolerance for low A_w and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates.

Koji and *Tempeh* are the two most important applications of SSF with filamentous fungi. *Aspergillus oryzae* is grown on wheat bran and soybean for *Koji* production, which is the first step of soy sauce or citric acid fermentation. *Koji* is a concentrated hydrolytic enzymes required in further steps of the fermentation process. *Tempeh* is an Indonesian fermented food produced by the growth of *Rhizopus oligosporus* on soybeans. The fermented product is consumed by people after cooking or toasting. The fungal fermentation allows better nutritive quality and degrades some antinutritional compounds contained in the crude soybean.

The hyphal mode of growth gives to filamentous fungi a major advantage over unicellular micro-organisms in the colonisation of solid substrates and for the utilisation of available nutrients. The basic mode of fungal growth is a combination of apical extension of hyphal

tips, plus the generation of new hyphal tips through branching. An important feature is that although extension occurs only at the tip at a linear and constant rate, the frequency of branching make the growth of the total biomass at exponential kinetic pattern, mainly in the first steps of the vegetative stage. That point is of importance for the modelling of the growth, and we will be discussed further.

The hyphal mode of growth gives also the filamentous fungi the power to enter into the solid substrates. The cell wall structure attached to the tip and the branching of the mycelium ensure firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of LSF, that makes very efficient the action of hydrolytic enzy-

mes and allows penetration into most solid substrates. Penetration increases the accessibility of all available nutrients within particles.

Table 3. Main groups of micro-organisms involved in SSF processes.

Microflora	SSF Process
Bacteria	
<i>Bacillus sp.</i>	Composting, Natto, amylase
<i>Pseudomonas sp.</i>	Composting
<i>Serratia sp.</i>	Composting
<i>Streptococcus sp.</i>	Composting
<i>Lactobacillus sp.</i>	Ensiling, Food
<i>Clostridium sp.</i>	Ensiling, Food
Yeast	
<i>Endomycopsis burtonii</i>	Tape, cassava, rice
<i>Saccharomyces cerevisiae</i>	Food, Ethanol
<i>Schwanniomyces castellii</i>	Ethanol, Amylase
Fungi	
<i>Alternaria sp.</i>	Composting
<i>Aspergillus sp.</i>	Composting, Industrial, Food
<i>Fusarium sp.</i>	Composting, gibberellins
<i>Monilia sp.</i>	Composting
<i>Mucor sp.</i>	Composting, Food, enzyme
<i>Rhizopus sp.</i>	Composting, Food, enzymes, organic acids
<i>Phanerochaete chrysosporium</i>	Composting, lignin degradation
<i>Trichoderma sp.</i>	Composting, Biological control, Bioinsecticide
<i>Beauveria sp., Metharizium sp.</i>	Biological control, Bioinsecticide
<i>Amylomyces rouxii</i>	Tape cassava, rice
<i>Aspergillus oryzae</i>	Koji, Food, citric acid
<i>Rhizopus oligosporus</i>	Tempeh, soybean, amylase, lipase
<i>Aspergillus niger</i>	Feed, Proteins, Amylase, citric acid
<i>Pleurotus oestreatus, sajor-caju</i>	Mushroom
<i>Lentinus edodes</i>	Shii-take mushroom
<i>Penicilium notatum, roquefortii</i>	Penicillin, Cheese

Fungi can not transport the macromolecular substrate, but the hyphal growth allows a close contact between hyphae and substrate surface. The fungal mycelium synthesise and excrete high quantities of hydrolytic exoenzymes. The resulting contact catalysis is very efficient and the simple products are in close contact to the mycelium where they can enter across the cell membrane for biosynthesis and fungal metabolic activities. This contact catalysis by enzymes can explain the logistic model of fungal growth commonly observed (Raimbault, 1981). Also that point will be discussed further.

3. Substrates

All solid substrates have a common feature: their basic macromolecular structure. In general, substrates for SSF are composite and heterogeneous products from agriculture or by-products of agro-industry. This basic macromolecular structure (e.g. cellulose, starch, pectin, lignocellulose, fibres etc..) confers the properties of a solid to the substrate. The structural macromolecule may simply provide an inert matrix within which the carbon and energy source (sugars, lipids, organic acids) are adsorbed (sugarcane bagasse, inert fibres, resins). But generally the macromolecular matrix represents the substrate and provide also the carbon and energy source.

Preparation and pre-treatment represents the necessary steps to convert the raw substrate into a form suitable for use, that include:

- size reduction by grinding, rasping or chopping
- physical chemical or enzymatic hydrolysis of polymers to increase substrate availability by the fungus.
- supplementation with nutrients (phosphorus, nitrogen, salts) and adequation to pH and moisture content, through a mineral solution
- Cooking or vapour treatment for macromolecular structure pre-degradation and elimination of major contaminants. Pre-treatments will be discussed under individual applications.

The most significant problem of SSF is the high heterogeneity which makes difficult to focus one category of hydrolytic processes, and leads to poor trials of modelling. This heterogeneity is of different nature:

- non-uniform substrate structure (mixture of starch, lignocellulose, pectin)
- Variability between batches of substrates limiting the reproducibility
- Difficulty of mixing solid mass in fermentation, in order to avoid compactation, which causes non uniform cultivation, gradients of temperature, pH and moisture with virtual impossibility to obtain a representative sample.

Each macromolecular type of substrate presents different kind of heterogeneity:

Lignocellulose occurs within plant cell walls which consists of cellulose microfibrils embedded in lignin, hemicellulose and pectin. Each category of plant material contain variable proportion of each chemical compounds. Two major problems can limit lignocellulose breakdown:

- cellulose exists in four recognised crystal structures known as celluloses I,II,III and IV. Various chemical or thermal treatment can change the amorphous form of cristalinity.
- different enzymes are necessary in order degrade cellulose, e.g. endo and exo-cellulases plus cellobiase

Pectins are polymers of galacturonic acid with different ratio of methylation and branching. Exo-and endo pectinases and demethylases hydrolyse pectin in galacturonic acid and metha-

nol. Hemicellulases are divided in major three groups: xylans, mannans and galactans. Most of hemicellulases are heteropolymers containing two to four different types of sugar residue.

Lignin represents between 26 to 29% of lignocellulose, and is strongly bounded to cellulose and hemicellulose, hiding them and protecting them from the hydrolase attack. Lignin peroxidase is the major enzyme involved in lignin degradation. *Phanerochaete chrysosporium* is the most recognised fungi for lignin degradation.

So the lignocellulose hydrolysis is a very complex process. Effective cellulose hydrolysis requires the synergetic action of several cellulases, hemicellulases and lignin peroxidases.

But lignocellulose is a very abundant and cheap, natural, renewable material, so a lot of works were dedicated to micro-organisms breakdown, specially fungal species.

Starch is another very important and abundant natural solid substrate. Many micro-organisms are capable to hydrolyse starch, but generally the efficient hydrolysis requires previous gelatinization. Some recent works concern the raw (crude or native) starch like it occurs naturally.

The chemical structure of starch is relatively simple compared to lignocellulose substrates. Essentially starch is composed of two related polymers in different proportion following plant material: amylose (16-30%) and amylopectin (65-85%). Amylose is a polymer of glucose linked in a -1,4 bonds mainly in linear chains. Amylopectin is a large highly branched polymer of glucose including also a -1,6 bonds at the branch points.

Within the plant, cell starch is stored in the form of granules located in amyloplasts, intracellular organelles surrounded by a lipoprotein membrane. Starch granules are highly variable in size and shape depending on the plant material. Granules contain both amorphous and crystalline internal regions in respective proportions of about 30/70. During the process of gelatinization, starch granules swell when heated in the presence of water, which involves the breaking of hydrogen bonds, especially in the crystalline regions.

Many micro-organisms can hydrolyse starch, specially fungi which are suitable for SSF application involving starchy substrates. Glucoamylase, α -amylase, β -amylase, pullulanase and isoamylase are involved in the processes of starch degradation. Mainly α -amylase and glucoamylase are of importance for SSF.

α -amylase is an endo-amylase attacking α -1,4 bonds in random fashion which rapidly reduce molecular size of starch and consequently its viscosity and liquefaction. Glucoamylase occurs almost exclusively in fungi including *Aspergillus* and *Rhizopus* groups. This exo amylase produces glucose units from amylose and amylopectin chains.

Micro-organisms generally prefer gelatinised starch. But large quantity of energy is required for gelatinization, and it would be attractive to use organisms growing well on raw (ungelatinised) starch. Different works are dedicated to isolate fungi producing enzymes able to degrade raw starch, as has been done by Soccol et al (1991), Bergmann et al. (1988) and Abe et al. (1988).

In our lab we developed many studies concerning SSF of cassava, a very common tropical starchy crop, in the view of upgrading protein content, both for animal feeding using *Aspergillus sp.* or better for direct human consumption, using *Rhizopus*. Table 4 indicates the protein enrichment with different fungi.

Table 4. Protein enrichment of Cassava by various selected strains of fungi. (Raimbault et al., 1985)

Strain	Inoculum Source	Time (h)	Composition (% dry basis)	
			Protein	Total sugar
<i>Aspergillus niger</i> no. 10	Cassava	25	16.3	35.6
<i>Aspergillus niger</i> no. 12	Kopi	30	16.3	35.1
<i>Aspergillus niger</i> no. M190	Kopi	30	15.6	28.5
<i>Monilia stipitata</i> no. 27	Panal	42	15.1	32.3
<i>Rhizopus</i> sp. no. 7	Cassava	48	14.9	30.3
<i>Aspergillus oryzae</i> no. M84	Kopi	30	14.8	30.0
<i>Aspergillus</i> sp. no. B1	Banana	30	14.7	38.1
<i>Aspergillus</i> sp. no. T1	Tampak	30	14.3	34.0
<i>Aspergillus niger</i> no. 51	Cassava	30	14.3	34.5
<i>Aspergillus</i> sp. no. 14	Cassava	30	14.2	37.9
<i>Aspergillus terreus</i> no. R3	Kopi	30	14.1	40.9
<i>Aspergillus</i> sp. no. M101	Tampak	30	14.0	31.4
<i>Aspergillus</i> sp. no. 72	Banana	30	13.8	28.2
<i>Aspergillus niger</i> no. 13	Kopi	48	13.0	38.8
<i>Aspergillus</i> sp. no. M147	Kopi	30	12.7	32.4
<i>Aspergillus niger</i> no. 17	Cassava	30	12.0	45.2
<i>Aspergillus</i> sp. no. 39	Banana	30	11.1	40.0
<i>Aspergillus</i> sp. no. M82	Tampak	30	10.9	38.0
Raw cassava	—	—	2.50	90.00

Initial water content 50%, temperature of incubation 33°C.

Recently good results were obtained by Soccol for the protein enrichment of cassava and cassava bagasse using selected strains of *Rhizopus*, producing biotransformed starchy flours containing 10-12% of good protein, comparable to cereal. Such biotransformed Cassava flour can be used as cereal substitute for breadmaking until 20% without sensible change for the consumer.

4. Biomass Measurement

Biomass is a fundamental parameter in the characterisation of microbial growth. Its measurement is essential for kinetic studies on SSF. Direct determination of biomass in SSF is very difficult due to problems of separation of the microbial biomass from the substrate. This is especially true for SSF processes involving fungi, because the fungal hyphae penetrate into and bind the mycelium tightly to the substrate. On the other hand, for the calculation of growth rates and yields it is the absolute amount of biomass which is important. Methods that have been used for biomass estimation in SSF belong to one of the following categories.

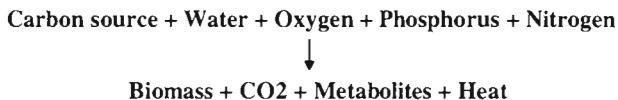
Direct evaluation of biomass

Complete recovery of fungal biomass is possible only under artificial circumstances in membrane filter culture, because the membrane filter prevents the penetration of the fungal

hyphae into the substrate (Mitchell et al, 1992). The whole of the fungal mycelium can be recovered simply by peeling it off the membrane and weighing it directly or after drying. This method obviously cannot be used in actual SSF. However, it could find application in the calibration of indirect methods of biomass determination. Indirect biomass estimation methods should be calibrated under conditions as similar as possible to the actual situation in SSF. The global mycelium composition could be appreciate through analysis of the mycelium cultivated in LSF in conditions as close as possible than SSF cultivation.

Microscopic observations can also represent good way to appreciate fungal growth in SSF. Naturally, optic examination is not possible at high magnitude but only at stereo microscope. Scanning Electron Microscope (SEM) is an useful manner to observe the mode of growth in SSF. New approach and researches are developed for image analysis by computing software in order to evaluate the total length or volume of mycelium on SEM photography. Another new approach very promising is the Confocal Microscopy based on specific reaction of fungal biomass with specific fluorochrome probes. Resulting 3D images of biomass can open new way to appreciate and may be measure biomass *in situ* in a near future.

Anyway, direct measurement of exact biomass in SSF is a very difficult question, then other approaches were preferred by workers. For that we can consider the global stoichiometric equation of the microbial growth:



Each component is under strict variation of others when all coefficients are maintained constants. For that measuring one of them can indicate the evolution of the others.

Metabolic measurement of the biomass

- Respiratory metabolism

Oxygen consumption and carbon dioxide release result from the respiration, the metabolic process by which aerobic micro-organisms derive most of their energy for growth. These metabolic activities are therefore growth associated and can be used for the estimation of biomass biosynthesis.

As carbon compounds within the substrate are metabolised, they are converted into biomass and carbon dioxide. Production of carbon dioxide causes the weight of fermenting substrate to decrease during growth, and the amount of weight lost can be correlated to the amount of growth that has occurred.

Growth estimation based on carbon dioxide release or oxygen consumption assumes that the metabolism of these compounds is completely growth associated, which means that the amount of biomass produced per unit of gas metabolised must be constant. Sugama and Okazaki (1979) reported that the ratio of mg CO₂ evolved to mg dry mycelia formed by *Aspergillus oryzae* on rice ranged from 0.91 to 1.26 mg CO₂ per mg dry mycelium. A gradual

increase in the ratio was observed late in growth due to endogenous respiration. Drastic changes can be observed for the respiratory quotient which commonly changes with the

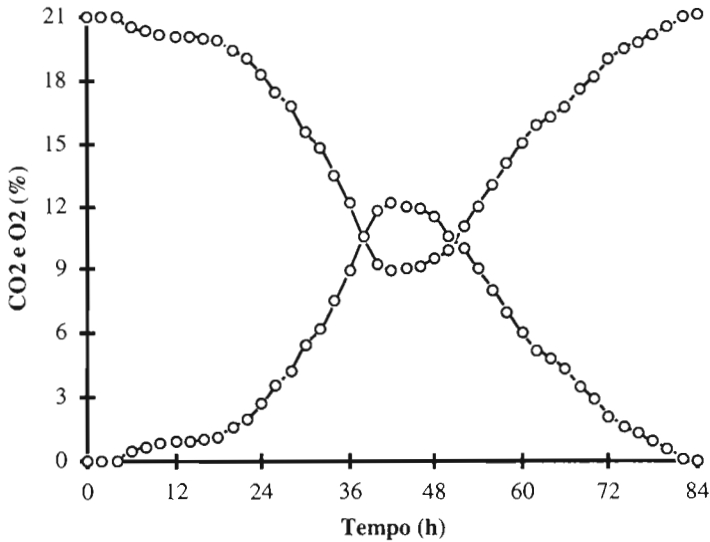


Fig. 1.- Kinetic evolution of CO₂ and O₂ in air flow during fermentation of *Rhizopus* on cassava.

growth phase, i.e: germination, rapid and vegetative growth, secondary metabolism, conidiation and degeneration of the mycelium.

The measurement of either carbon dioxide evolution or oxygen consumption is most powerful when coupled with the use of a correlation model. The term correlation model is used here to denote a model which correlates biomass with a measurable parameter. Correlation models are not growth models as such since they make no predictions as to how the measurable parameter changes with time. The usefulness of correlation models is that by following the profile for the change in the parameter during growth, a biomass profile can be constructed.

Application of these correlation models involving prediction of growth from oxygen uptake rates or carbon dioxide evolution rates requires the use of numerical techniques to solve the differential equations. A computer and appropriate software is therefore essential. If both the monitoring and computational equipment is available then these correlation models provide a powerful means of biomass estimation since continuous *on-line* measurements can be made. Other advantages of monitoring effluent gas concentrations with paramagnetic and infrared analysers include the ability to monitor the respiratory quotient to ensure optimal substrate oxidation, the ability to incorporate automated feedback control over the aeration rate, and the non-destructive nature of the measurement procedure.

The metabolic activity in SSF is so important that we have dedicated a special lecture to study all theoretical and practical aspects of respirometric measurement of fungal biomass cultivated

in SSF. Other speaker also will present a lot of data concerning lab and scale up experiences of respirometric measurement for several applications. More, during the practical training on the afternoons, you shall practice the laboratory methodology that we have specially design to study fungal growth on SSF based on the gas chromatography analysis of the effluent gas.

- Production of extracellular enzymes or primary metabolites

Another metabolic activity which may be growth associated is extracellular enzyme production. Okazaki and co-workers (1980) claim that the α -amylase activity was directly proportional to mycelial weight for *Aspergillus oryzae* grown in SSF on steamed rice. For growth of *Agaricus bisporus* on mushroom compost, mycelial mass was directly proportional to the extracellular laccase activity for 70 days (Wood, 1979). In our works we observed generally a good adequation between growth and hydrolytic enzymes as amylases, cellulases or pectinases (see annexed list).

In another hand, we observed frequently a good correlation between mycelial growth and organic acid production, which can be measured by the pH measurement or *a posteriori* correlated by HPLC analysis on extracts. In the case of *Rhizopus*, Soccol demonstrated a close correlation between fungal protein (Biomass) and organic acids (citric, fumaric, lactic or acetic).

Biomass Components

The biomass can also be estimated from measurements of a specific component, until the composition of the biomass is constant and stable and the fraction of the component be representative.

Protein content:

The most readily measured biomass component is protein. We used the protein content (as determined by the Lowry method) to measure the growth rate of *Aspergillus niger* on cassava meal (Raimbault and Alazard, 1980). For growth of *Chaetomium cellulolyticum* on wheat straw the TCA insoluble nitrogen was determined using the Kjeldahl method (Laukevics et al. 1984), biomass protein was then calculated as 6,25 times this value. In all cases the protein content of the biomass was assumed to be constant. Biomass protein contents measured by the biuret method were consistent with those measured by the Kjeldahl method. But unfortunately the biuret method was not suitable for application to SSF itself because of non-specific interference by the starch from the substrate. The Folin method is more sensitive and allowed a greater dilution of the sample which avoided interference from the starch in the substrate. For that we choose the Folin technique to measure protein enrichment in starchy substrates .

Nucleic acids

DNA production has been used to estimate the biomass of *Aspergillus oryzae* on rice (Bajracharya & Mudgett. 1980). The method was calibrated using the DNA contents of fungal mycelia obtained in submerged culture. DNA contents were higher during early growth and then decreased. levelling off as stationary phase was approached. The method was corrected for the DNA content of the rice, which did not change since *Aspergillus oryzae* did not produce extracellular DNases. DNA or RNA methods are reliable only if there is little nucleic acid in the substrate. and if no interfering chemicals are present .

Glucosamine

A useful method for the estimation of fungal biomass in SSF is the glucosamine method. This method takes advantage of the presence of chitin in the cell walls of many fungi. Chitin is a poly-N-acetylglucosamine. Interference with this method may occur with growth on complex agricultural substrates containing glucosamine in glucoproteins (Aidoo et al, 1981).

The accuracy of the glucosamine method for determination of fungal biomass depends on establishing a reliable conversion factor relating glucosamine to mycelial dry weight (Sharma et al, 1977). However, the proportion of chitin in the mycelium will vary with age and the environmental conditions. Mycelial glucosamine contents ranged from 67 to 126 mg per g dry mycelium. Another disadvantage of the glucosamine method is the tedious extraction procedures and processing times of over 24 hours which make it inconvenient to perform.

Ergosterol

Ergosterol is the predominant sterol in fungi. Glucosamine estimation was therefore compared with the estimation of ergosterol for determination of the growth of *Agaricus bisporus* (Matcham et al, 1985). In solid cultures directly proportional relationships for glucosamine and ergosterol against linear extension of the mycelium were obtained. Determination of ergosterol was claimed to be more convenient than glucosamine. It could be recovered and separated by HPLC and quantified simply by spectrophotometer, providing a sensitive index of biomass at low levels of growth. HPLC was necessary to separate the ergosterol from sterols endogenous to the solid substrate. However, Nout et al. (1987) showed that the ergosterol content of *Rhizopus oligosporus* varied from 2 to 24 micrograms per mg dry biomass, depending on the culture conditions. Ergosterol content was influenced by aeration, phase of growth and substrate composition. They concluded that it was an unreliable method for following growth of *Rhizopus oligosporus* in SSF.

Physical measurement of biomass

Peñaloza (1990) used another physical parameter to evaluate mycelial growth, based on the difference in the electric conductivity between biomass versus the substrate. Good correlation with biomass was obtained and a model was proposed.

Recently Auria et al. (1990) monitored the pressure drop in a packed bed during SSF of *Aspergillus niger* on a model solid substrate consisting of ion exchange resin beads. Pressure drop was closely correlated with protein production. Pressure drop is a parameter which is simple to measure and can be measured on-line. Further studies are required to determine whether the use of pressure drop in monitoring growth in forcefully aerated SSF bioreactors is generally applicable. An interesting point of this physical technique resides in the fact that it is sensible to the conidiation: early conidiophore stage make the pressure drop drastically and a breaking point can be easily observed.

In conclusion, the measurement of biomass in SSF is important to follow the kinetics of growth in relation to the metabolic activity. Measurement of metabolic activity by carbon

dioxide evolution or oxygen consumption can be generally applied, whereas extracellular enzyme production will only be useful when enzyme production is reasonably growth-associated.

Vital staining with fluorescein diacetate has potential in providing basic information as to the mode of growth of fungi on complex solid surfaces as this method can show the distribution of metabolic activity within the mycelium. But it can not be measured on line.

On the other hand, in the production of protein enriched feeds, the protein content itself is of greater importance than the actual biomass concentration, and the variation in biomass protein content during growth becomes less relevant.

Overall, oxygen uptake and carbon dioxide evolution methods are probably the most promising techniques for biomass estimation in aerobic SSF as they provide on-line information. The monitoring and computing equipment is relatively expensive and will not be suitable for low technology or rural applications. None method is ideally suited to all situations so the method most appropriate to the particular SSF application must be chosen on the basis of simplicity, cost and accuracy. The best choice could be to cross two or three, or more, techniques for measurement of various parameters, and the total balance could be highly correlated to the actual biomass.

5. Environmental Factors

Environmental factors such as temperature, pH, water activity, oxygen levels and concentrations of nutrients and products significantly affect microbial growth and product formation. In submerged stirred cultures environmental control is relatively simple because of the homogeneity of the suspension of microbial cells and of the solution of nutrients and products in the liquid phase.

The low moisture content of SSF enables a smaller reactor volume per substrate mass to be used for microbial cultivation than with submerged cultures and also simplifies recovery of the product (Moo-Young et al., 1983). Serious problems, however, are encountered in respect of mixing, heat exchange, oxygen transfer, moisture control and the localisation of pH gradients and nutrient and product levels as a consequence of the heterogeneity of the culture.

The latter characteristic of SSF renders the measurement and control of the above mentioned parameters difficult, laborious and often inaccurate, thereby limiting the industrial potential of this technology (Kim et al., 1985). Due to these problems, the micro-organisms that have been selected for SSF are more tolerant to a wide range of cultivation conditions (Mudgett, 1986).

Moisture content and Water activity (A_w)

SSF process can be defined as microbial growth on solid particles without presence of free water. The water present in SSF systems exists in a complexed form within the solid matrix or as a thin layer either absorbed to the surface of the particles or less tightly bound within the capillary regions of the solid. Free water will only occur once the saturation capacity of the solid matrix is exceeded. The moisture level at which free moisture becomes apparent varies

considerably between substrates, however, and is dependant upon their water binding characteristics. For example, free water is observed when the moisture content of solid substrates such as maple bark exceeds 40% and when it exceeds 50-55% in rice and cassava (Oriol et al, 1988). With most lignocellulosic substrates free water becomes apparent before the 80% moisture level is reached (Moo-Young et al, 1983).

The moisture levels in SSCF processes which vary between 30 and 85% has a marked effect on the growth kinetics, as shown on Figure 1 (Oriol et al, 1988). The optimum moisture level for the cultivation of *Aspergillus niger* on rice was 40%, whereas on coffee pulp the level was 80%, which illustrates the unreliability of moisture level as a parameter for predicting the growth of a micro-organism. It is now generally accepted that the water requirements of micro-organisms should be defined in terms of the water activity (A_w) of the environment rather than the water content of the solid substrate. This parameter is defined by the ratio of the vapour pressure of the water in the substrate (p) to the vapour pressure of pure water (p_0) at the same temperature, i.e $A_w = p/p_0$. This concept is related to other parameters such as relative humidity ($\%RH = 100 \times A_w$) and water potential ($\psi = RT/V \cdot \ln A_w$; where R is the ideal gas constant, T is the absolute temperature and V is the mol volume of water) (Griffin, 1981).

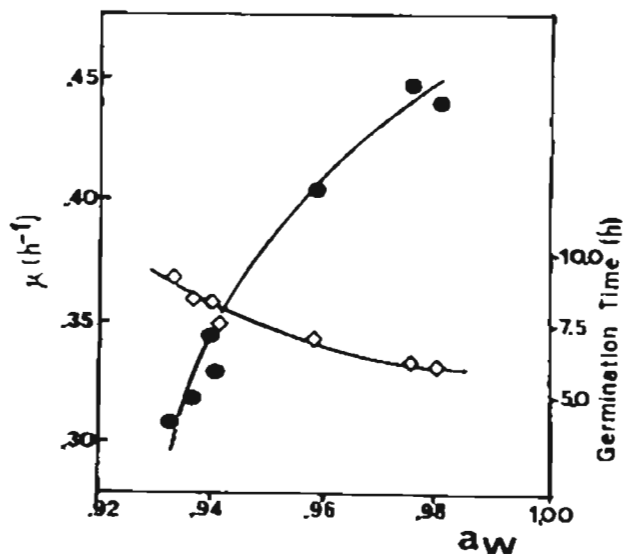


Fig. 2 Evolution of the specific growth rate (—●—) and of the germination time (—◇—) as a function of the initial water activity of the medium

The reduction of A_w has a marked effect on microbial growth. Typically, a reduction in A_w extends the lag phase, decrease the specific growth rate, and results in low amount of biomass

produced (Oriol et al. 1988) as it is shown in fig.2. In general, bacteria require higher values of A_w for growth than fungi, thereby enabling fungi to compete more successfully at the A_w values encountered in SSC processes. With the exception of halophilic bacteria, few bacteria grow at A_w values below 0.9 and most bacteria investigated show considerably higher minimum A_w values for growth. Some fungi, on the other hand, only stop growing at A_w values as low as 0.62 and a number of fungi used in SSC processes have minimum growth A_w values between 0.8 and 0.9.

The optimum moisture content for growth and substrate utilisation is between 40 and 70% but depended upon the organism and the substrate used for cultivation. For example, cultivation of *Aspergillus niger* on starchy substrates such as cassava (Raimbault & Alazard, 1980) and wheat bran (Nishio et al. 1979) was optimal at moisture levels considerably lower than on coffee pulp (Penaloza et al. 1985) or sugarcane bagasse (Roussos et al., 1989), possibly because of the greater water holding capacity of the latter substrate (Oriol et al. 1988). The optimum A_w for growth of a limited number of fungi used in SSF processes was at least 0.96 whereas the minimum growth A_w was generally greater than 0.9. This suggests that fungi used in SSF processes are not especially xerophilic. The optimum A_w values for sporulation by *Trichoderma viride* and *Penicillium roqueforti* were lower than those for growth (Gervais et al., 1988). Maintenance of the A_w at the growth optimum would permit fungal biomass to be produced without sporulation.

Temperature and Heat Transfer

Stoichiometric global equation of respiration is highly exothermic and heat generation by high levels of fungal activity within the solids lead to thermal gradients because of the limited heat transfer capacity of solid substrates. In aerobic processes, heat generation may be approximated from the rate of CO_2 evolution or O_2 consumption. Each mole of CO_2 produced during the oxidation of carbohydrates released 673 Kcal. That is for why it is of high interest to measure CO_2 evolution during a SSF process, because it is directly related to the risk of elevation of temperature. Detailed calculation of the relation between respiration, metabolic heat and temperature were discussed in early works on SSF with *Aspergillus niger* growing on cassava or potato starch (Raimbault, 1981). The overall rate of heat transfer may be limited by the rates of intra- and inter-particle heat transfer, by the rate at which heat is transferred from the particles surface to the gas phase. The thermal characteristics of organic material and the low moisture content in SSF are special difficult conditions for heat transfer. Saucedo-Castaneda and co-workers (1990), developed a mathematical model for evaluating the fundamental heat transfer mechanism in static SSF and more specifically to assess the importance of convection and conduction in heat dissipation. Saucedo will explain in his lecture how this model could be used as a basis for automatic control of static bioreactors.

Heat removal is probably the most crucial factor in large scale SSF processes, and conventional convection or conductive cooling devices are inadequate for dissipating metabolic heat due to the poor thermal conductivity of most solid substrates and result in non acceptable temperature gradients. Only evaporative cooling devices provide sufficient heat elimination. Although the primary function of aeration during aerobic solid state cultivations was to supply oxygen for cell growth and to flush out the produced carbon dioxide, it also serves a critical function in heat and moisture transfer between the solids and the gas phase. The most

efficient processes for temperature control consists in evaporating water, what needs in return to complete the loss to avoid desiccation.

Maintaining a constant temperature and moisture content in large scale solid substrate cultures is generally difficult, but as you will realise some alternative equipment begin to fit that function, and all that will be discussed by Perez and Saucedo. The reactor type can have a large influence on the quality of temperature control achieved. It depends highly of the type of SSF: static on clay or vertical exchangers, drums or mechanically agitated with parameters controls, all that aspect will be discussed in sessions about Engineering aspects of SSF.

pH control and risks of contamination.

The pH of a culture may change in response to microbial metabolic activities. The most obvious reason is the secretion of organic acids such as citric, acetic or lactic acids, which will cause the pH to decrease, in the same way than ammonium salts consumption. On the other hand, the assimilation of organic acids which may be present in certain media will lead to an increase in pH, and urea hydrolysis result in an alcalinisation. The changes in pH kinetics depends also highly on the micro-organism. With *Aspergillus sp.*, *Penicillium sp.*, and *Rhizopus sp.* the pH can drop very quickly until less than 3.0; for another type of fungi, like *Trichoderma*, *Sporotrichum*, *Pleurotus sp.* the pH is more stable between 4 and 5. Besides, the nature of the substrate influence highly pH kinetics, due to the buffering effect of lignocellulosic materials.

In our case we used a mixture of ammonium salt and urea to regulate the pH decrease during *A. niger* growth on starchy substrates (Raimbault, 1980). A degree of pH control may be obtained by using different ratios of ammonium salts and urea in the substrate. Hydrolysis of urea liberates ammonia, which counteracts the rapid acidification resulting from uptake of the ammonium ion (Raimbault & Alazard. 1980). In this manner, we obtained optimal growth of *Aspergillus niger* on granulated cassava meal when using a 3:2 ratio (on a nitrogen basis) of ammonium to urea. We observed that during the first stage of the cultivation the pH increased as the urea was hydrolysed. During the subsequent rapid growth ammonium assimilation exceeded the rate of urea hydrolysis and the pH decreased, but increased again in the stationary phase. During the cultivation the pH remained between the limits of about pH 5 to pH 6.2, whereas a lower urea concentration resulted in a rapid decrease in pH.

In a same way, pH adjustment during the cultivation of *Trichoderma viride* on sugar-beet pulp by spraying with urea solutions was effective due to the urease activity of the micro-organism causing an increase in pH at pilot plant level experimentation (Durand et al. 1988).

Finally, in a process of cultivation of filamentous fungi or yeasts, bacterial contamination may be minimised or prevented by employing a suitably low pH.

Aeration

Aeration fulfils four main functions in solid state processes, namely (i) to maintain aerobic conditions, (ii) for carbon dioxide desorption, (iii) to regulate the substrate temperature and (iv) to regulate the moisture level. The gas environment may significantly affect the relative levels of biomass and enzyme production. In aerobic submerged cultures oxygen supply is

often the growth limiting factor due to the low solubility of oxygen in water. In contrast, a solid state process allows free access of atmospheric oxygen to the substrate, aeration may be easier than in submerged cultivations because of the rapid rate of oxygen diffusion into the water film surrounding the insoluble substrate particles and also the very high surface of contact between gas phase, substrate and aerial mycelial. The control of the gas phase and air flow is a simple and practical mean to regulate gas transfer and generally no oxygen limitation are observed in SSF processes when the solid substrate is particular. It is important to maintain a good balance between the three phases gas, liquid and gas in SSF processes (Auria, 1989; Saucedo et al. 1984). Modelling mass transfer in SSF is a key to keep good conditions for the development of the mycelium. By this very simple aeration process, it is also possible to induce metabolic reaction, either by water stress, heat stress or temperature changes, all processes that can be drastically change biochemical, physiological or metabolic behaviour.

6. Conclusion

SSF is a well adapted process for cultivation of fungi on natural vegetal materials which are breakdown by excreted hydrolytic enzymes. In contrast with LSF, in SSF processes, water related to the water activity is a limiting factor, both parameters no involved in LSF where water is in large excess. On the other hand, oxygen is a limiting factor in LSF but not in SSF where aeration is facilitated by the porous and particular structure and high surface contact area which facilitate transfers between gas and liquid phases.

SSF are aerobic processes where respiration is a predominant processes for energy supply to the mycelium; but it can cause severe limitation of the growth when heat transfer is not efficient enough causing rapid elevation of the temperature.

Is the reason why it is so important to study and control respirometry in SSF. We developed a laboratory technique to measure CO₂ and O₂ *on line* in SSF. A special lecture will be dedicated to the theory, modelling and basic concept of respirometry. Also it will be organise training sessions at the lab, to practice respirometric measurement and kinetics analysis.

References

- Alazard, D. and Raimbault, M. 1981. Comparative study of amylolytic enzymes production by *Aspergillus niger* in liquid and solid state cultivation. *Eur. J. Appl. Microbiol. Biotechnol.* 12: 113-117.
- Auria, R., Hernandez, S., Raimbault, M. and Revah, S. 1990. Ion exchange resin: a model support for solid state growth fermentation of *Aspergillus niger*. *Biotechnol. Techniques.* 4: 391-396.
- Deschamps, F., Raimbault, M. and Senez, J.C. 1982. Solid state fermentation in the development of agro-food by-products. *Industry & Environ.* 5 (2): 27-30.
- Doelle H.W., Mitchell D.A. & Rolz C.E. (1992). Solid Substrate Cultivation. Elsevier Sci. Publ. Ltd; London & New York; 466 p.
- Durand A. & Chereau D. 1988. A new pilot reactor for solid state fermentation: application to the protein enrichment of sugar beet pulp. *Biotechnol. Bioeng.* 31: 476-486.

- Durand, A., Renaud, R., Maratray, J., Almanza, S. 1997. The INRA-Dijon Reactors: Designs and applications. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniégra-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, chapter 7 pp. 71-92.
- Moo-Young M., Moriera A.R. & Tengerdy R.P. 1983. Principles of solid state fermentation. In *The filamentous fungi*, Vol. 4, Fungal Biotechnology. Smith J.E, Berry D.R & Kristiansen B. Eds., Edward Arnold Publishers, London, pp. 117-144.
- Oriol, E., Schettino, B., Viniégra-Gonzalez, G. and Raimbault, M. 1988a. Solid- state culture of *Aspergillus niger* on support. *J. Ferment. Technol.* 66: 57-62.
- Oriol, E., Raimbault, M., Roussos, S. and Viniégra-Gonzales, G. 1988b. Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.*, 27: 498-503.
- Raimbault M. - (1981). "Fermentation en milieu solide: croissance de champignons filamenteux sur substrats amylacés". Edited by: ORSTOM-Paris; *Série Travaux et Documents* n° 127; 291 p.
- Raimbault, M. and Alazard, D. 1980. Culture method to study fungal growth in solid fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 9: 199-209.
- Raimbault, M., Revah, S., Pina, F. and Villalobos P. 1985. Protein enrichment of cassava by solid state fermentation using molds isolated from traditional foods. *J. Ferment. Technol.* 63: 395-399.
- Roussos, S., Olmos, A., Raimbault, M., Saucedo-Castañeda, G. and Lonsane, B.K. 1991. Strategies for large scale inoculum development for solid state fermentation system : Conidiospores of *Trichoderma harzianum*. *Biotechnol. Tech.* 5: 415-420
- Roussos, S., Raimbault, M., Viniégra-Gonzalez, G., Saucedo-Castañeda, G. and Lonsane, B.K. 1991. Scale-up of cellulases production by *Trichoderma harzianum* on a mixture of sugar cane bagasse and wheat bran in solid state fermentation system. *Micol. Neotrop. Apl.* 4 : 83-98.
- Roussos, S., Raimbault, M., Prebois, J-P. and Lonsane, B.K. 1993. Zymotis, A large scale solid state fermenter : Design and evaluation. *Applied Biochem. Biotechnol.* 42: 37-52.
- Saucedo-Castañeda, G., Gutierrez-Rojas, M., Bacquet, G., Raimbault, M. and Viniégra-Gonzalez, G. 1990. Heat transfert simulation in solid substrate fermentation. *Biotechnol. Bioeng.* 35: 802-808.
- Saucedo-Castañeda, G., Lonsane, B.K., Navarro, J.M., Roussos, S. and Raimbault, M. 1992a. Potential of using a simple fermenter for biomass built up, starch hydrolysis and ethanol production: Solid state fermentation system involving *Schwanniomyces castellii* , *Appl. Biochem. Biotechnol.* 36: 47-61.
- Saucedo-Castañeda, G., Lonsane, B.K., Krishnaiah, M.M., Navarro, J.M., Roussos, S. and Raimbault, M. 1992b. Maintenance of heat and water balances as a scale-up criterion for the production of ethanol by *Schwanniomyces castellii* in a solid state fermentation system. *Process Biochem.* 27: 97-107
- Senez, J.C., Raimbault, M. and Deschamps, F. 1980. Protein enrichment of starchy substrates for animal feeds by solid state fermentation. *World Animal Rev.* 35: 36-40.
- Soccol, C., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Potential of solid state fermentation for production of L(+) lactic acid by *Rhizopus oryzae*. *Appl. Microbiol. Biotechnol.* 41: 286-290.

- Trejo-Hernandez, M.R., Raimbault, M., Roussos, S. and Lonsane, B.K. 1992. Potencial of solid state fermentation for production of ergot alkaloids. *Let. Appl. Microbiol.* 15: 156-159.
- Trejo-Hernandez, M.R., Lonsane, B.K., Raimbault, M. and Roussos, S. 1993. Spectra of ergot alkaloids produced by *Claviceps purpurea* 1029c in solid state fermentation system: Influence of the composition of liquid medium used for impregnating sugar cane pith bagasse. *Process Biochem.* 28: 23-27.

CONTINUOUS ENZYMES AND FUNGAL METABOLITES PRODUCTION IN SOLID STATE FERMENTATION USING A COUNTER-CURRENT REACTOR

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Abstract:

This work presents the continuous production of fungal biomass and enzymes by solid state fermentation (SSF) in a counter-current reactor adapted for this purpose. Pre-germinated conidia of *Aspergillus niger* were used as an inoculum and sugarcane bagasse, embedded with a nutritive solution, was the solid support. The Solids residence time distribution (RTD) was carried out by feeding one impulse of blue-coloured humidified bagasse and this RTD was fixed at 20 hours. This study demonstrated that the values of the measured parameters (pH, moisture, biomass, glucoamylases production) were similar to those reported for batch SSF using the same solid support and micro-organism. A marked increase in biomass occurred from the progressive compartment (from compartment no.1 to no.9) into the reactor and the enzyme production was important (40 IU/g dry exit solids). No mycelium damage or sporulation was observed. The above results confirmed that the continuous production of enzymes by SSF under non-sterile conditions was successful. Inoculation with pre-germinated conidia shortened the processing time and allowed control of the age of the mycelium in each compartment. Aeration was accomplished by natural convection and moisture content had to be controlled. This process can be applied to the continuous production of fungal biomass and metabolites in SSF with industrial applications using environmentally friendly biotechnology.

Key words: Solid State fermentation, Continuous enzyme production, glucoamylases, *A.niger*, pre-germinated conidia, counter-current reactor, fungal metabolite.

Definition of SSF: Solid State Fermentation (SSF) is a microbial process occurring mostly on the surface of solid materials that have the property to absorb or contain water, with or without soluble nutrients (Viniestra Gonzalez 1997). The solid materials could either be biodegradable or not. For example, starch and cellulose are solid materials of the first type, whereas, amberlite or polyurethane belong to the second type (Alazard and Raimbault 1981; Moo-Young *et al.* 1983, Barrios Gonzalez *et al.* 1988, Oriol *et al.* 1988a, Auria *et al.* 1990, Gonzalez-Blanco *et al.* 1990, Roussos *et al.* 1991).

Advantages and disadvantages of SSF: Solid state fermentation offers various advantages in comparison with submerged ones (Aido *et al.* 1982, Lonsane *et al.* 1992). Aeration is facilitated through the spaces between the substrate (Lambraki *et al.* 1994, Soccol *et al.* 1994). Substrate agitation, when necessary, is discontinued (Senez *et al.* 1980, Deschamps *et al.* 1982). The absence of a liquid phase and a low water content permit a) reduction of fermentor volume of liquid effluents from the process, b) reagents saving during metabolites recovery, c)

reduction of bacteria contamination and d) use of no sterile solid substrate in some cases. Culture media are simple mainly composed of agro-industrial residues (Lonsane *et al.* 1985, Roussos *et al.* 1991). Culture growth conditions are close to those in the natural environment (Roussos *et al.* 1997). Its main disadvantages are the following: a) risks of high temperature rise (Saucedo-Castañeda *et al.* 1990, Rodriguez *et al.* 1991, Saucedo *et al.* 1992a), b) difficulty in parameter regulation (Durand *et al.* 1988), c) need of pre-treatment of solid material (Raimbault *et al.* 1985) d) high loss of humidity in fermentations lasting of long, e) necessity for high inoculation when natural microflora is not used (Roussos *et al.* 1991), and f) critical role of water and water activity (Oriol *et al.* 1988b, Gervais and Bensoussan 1995).

Metabolites production in SSF: There has been a considerable amount of attention given to the physiology of the micro-organisms involved and the characteristics of the metabolites produced (Trejo-Hernandez *et al.* 1993, Gutierrez-Rojas *et al.* 1995). Culture of filamentous fungi on solid supports has been applied to the production of enzymes, primary and secondary metabolites (Oriol *et al.* 1988b; Saucedo-Castañeda *et al.* 1992b; Trejo-Hernandez *et al.* 1992; Christen *et al.* 1995). It has also been used for the detoxification of a wide variety of materials (Aquiahuatl *et al.* 1988).

Bioreactors: Considering to all these aspects mentioned above, bioreactors have been developed traditionally for different purposes and SSF has been carried out as a batch process in laboratory scale (Raimbault and Alazard 1980; Lepilleur *et al.* 1997), in pilot plant scale (Deschamps *et al.* 1985; Lonsane *et al.* 1984; Durand *et al.* 1985; Pandey 1991, Roussos *et al.* 1993) and in industrial scale (Deschamps *et al.* 1982; Lonsane *et al.* 1992; Bandoor *et al.* 1997; Durand *et al.* 1997). However, continuous production of biomass and metabolites in SSF has not been reported yet.

What is a CCR? In the early 1980s, a new CCR has been developed by the Commonwealth Scientific and Industrial Research Organization (CSIRO) of Australia in cooperation with Bioquip Australia Pty Limited (Casimir, 1983). It has been demonstrated that this unit has a potential for high yields of soluble solids, flavours and colours. Leach (1993) assessed the effect of processing variables on the performance of this reactor in the extraction of apple juice. The performance was strongly influenced by temperature. The effects of draft ratio, screw speed and addition of pectinase enzyme were also investigated. More recently, this extractor has been successfully studied by Gutierrez-López *et al.* (1996) based on a chemical reaction engineering theory, where the extractor was divided into three different zones, according to the flow patterns present. The counter-current reactor has also been used as a solid-liquid extractor for processing fermented products such as enzymes, organic acids, antibiotics, phytohormones and salts (Greve and Kula 1991; Johansson *et al.* 1985; Klyueva and Zakharevich 1985; Kumar and Lonsane 1987; Likidis *et al.* 1989; Schwartzberg 1980; Srikanta *et al.* 1987).

Objective: The objective of this work is to describe the continuous production of glucoamylases by *Aspergillus niger*, a Gras fungus (Samson *et al.* 1997) grown in SSF using a CCR adapted for this purpose because there are not any bioreactors reported in the literature, capable of working in a continuous process for the production of fungal biomass or enzymes in SSF. Before the onset of fermentations it was necessary to study the flow of the solid

support (sugarcane pith bagasse) in the extractor in conditions similar to those used later during the fungal growth in SSF. It was important to know precisely the dependence of the mean residence time of the solid on the different possible programs of the extractor. It was also necessary to know the degree of mixing in different experimental conditions. It was desirable that during the fermentation two solid particles fed at the same time came out of the extractor approximately at the same time too. Otherwise, in each point of the bioreactor there would co-exist micro-organisms at different stages of development.

Residence time distribution

The counter-current extractor consists of a stainless steel ribbon flighted screw situated in a U-shaped stainless steel trough. The screw transports the solid material in both forward and backward directions. It is driven by an external motor which can work intermittently in both directions. In this way, the screw reverses its direction of rotation and that is the distinctive characteristic of the reactor. This reversing movement results in a very efficient solid-solid contact because the solid is constantly in movement between both sides of the CCR.

The reversing movement is controlled by four programmable timer switches located on the control panel of the extractor. One timer selects the desired time of forward movement and another timer, the time of backward movement. The other two timers control the stopping interval between the forward and backward motions. The screw is operated with a much more forward movement a backward. The solids are thereby given a net forward movement.

The screw speed of rotation is also selected in the control panel. Obviously the combination of a variable screw speed and variable forward/backward cycles enable a considerable flexibility in the control of the solid phase residence times¹ and mixing parameters.

The ideal unmixed flow pattern of the solid particles in a CCR is known as plug-flow. In this ideal flow no solid particle overtakes any other particle ahead or behind. Thus, all the particles take exactly the same time to go through the extractor. However, real reactors never fully follow this flow pattern. Usually each particle may take different routes through the reactor and, as a result, different lengths of time are required to reach the exit. In some cases, the deviation from ideality is considerable. This deviation always lowers the performance of the unit.

To determine the extent of deviation from the ideal flow we usually just need to know how long the individual particles stayed in the equipment or, more precisely, the residence time distribution (RTD) of the flowing stream. The RTD can be obtained experimentally using a stimulus-response technique. The system in study is disturbed somehow and the way the system responds to this stimulus gives us the desired information.

The nominal mean solids residence time is given by equation 1.

$$\tau = \frac{N}{n} \times \frac{t_F + t_B + 2 \cdot t_S}{t_F - t_B} \quad (1)$$

where τ = nominal mean residence time (in minutes)

N = number of flights of the screw

n = screw speed (in revolutions per minute, rpm)

t_F = time for which the screw is set to move forward

t_B = time for which the screw is set to move backward

¹ The residence time is the time necessary for the feed to travel from the feed-end to the discharge- end.

t_s = time for which the screw is set to be stationary.

In each fermentation the CCR was initially filled with the amount of non-inoculated bagasse corresponding to the hold-up. This bagasse was uniformly distributed throughout the compartments. Then, the screw movement was started at 1 rpm under the conditions presented in Table 1.

Table 1. Screw movement program for the continuous glucoamylases production in SSF using a CCR. Screw speed = 1 rpm.

Screw Movement				
Movement	Forward	Stopping	Backward	Stopping
Time (sec)	36.0	870.3	23.4	870.3

We knew from the results of the preliminary experiment that the dispersion should be low if there were 1.2 Kg per compartment. This corresponds approximately to a feeding rate of 0.5 Kg / h and that is the rate used in the first run. In the other two runs higher rates were tested. The residence time distributions obtained in these 3 runs are represented in Figures 1, 2 and 3. In this experiment, 3 runs with tracer were carried out in which we tested the effect of the feeding rate on the dispersion using the program of 36.0 seconds forward / 23.4 seconds backward².

The time needed to recover 90 % of the tracer fed gave us an idea of the dispersion. This value was calculated for each one of the runs and is given in Table 2. The variances of the residence time distributions were also calculated for each one of the runs and are presented in the same

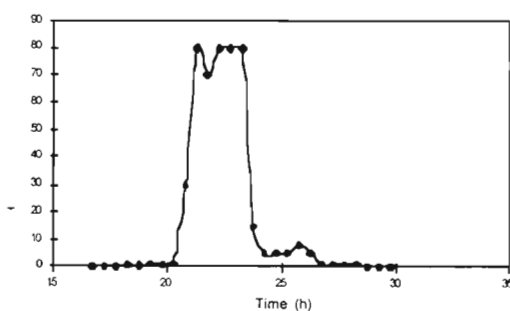


Figure 1.: RTD of the tracer in the first run. Feeding rate = 0.50 Kg / h.

² The objective was to work with exactly the same program used later in the fermentations. For practical reasons we were interested in having cycles of exactly half an hour during the fermentation. For this reason, it was necessary, to use a program in which the backward movement lasted 23.4 second in order to have a mean residence time of 20 h.

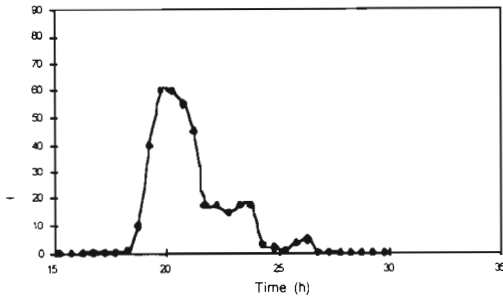


Figure 2 : RTD of the tracer in the second run. Feeding rate = 0.75 Kg / h.

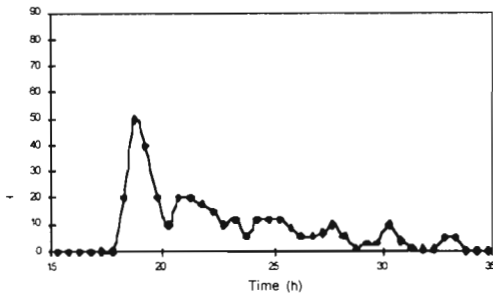


Figure 3 : RTD of the tracer in the third run. Feeding rate = 1.00 Kg / h.

table. These values were obtained using Equation 3. The experimental hold-up is also presented, as well as the hold-up calculated by multiplying the feeding rate by the residence time (Equation 1).

Table 2. Time necessary to recover 90 % of the tracer, variance of the RTD and experimental and calculated hold-ups.

Run	$t_{90\%}$ (h)	σ^2	Hold-up (Kg)	
			experimental	calculated
1	3	1.5	10.7	10
2	4.5	2.6	15.1	15
3	12	16	21.8	20

It can be seen from Figures 1, 2 and 3 and from the values of $t_{90\%}$ and σ^2 in Table 2 that, the dispersion increases proportionally with the feeding rate. This was expected and as explained before, this happened because as the feeding rate increased, the hold-up increased too and

hence so, the accumulation of bagasse on the ribbon was higher and it was easier for the bagasse to go over the ribbon, pass over the axis of the screw and finally fall in another compartment. Comparing the experimental with the calculated hold-ups a similarity was observed. Therefore, it was confirmed that the mean residence time was 20 h.

It is important to note that, although the hold-up and the feeding rate were expressed in units of mass, it was not the weight of the solid inside the equipment that influenced the dispersion. The dispersion occurred when the volume of the solids was so high that the solid passed over the axis to another compartment. In our case, as all the experiments were made with the same solid, we could always work just with the weight. However, if, for instance, the water content of the bagasse was changed, its density would change too and the results would be different. It would be interesting to carry out similar experiments with different solids and observe if experiments done with similar volumetric flow rates but different solids gave similar results in terms of dispersion.

Continuous glucoamylase production in SSF

Previously sterilised solid material was inoculated with 2×10^7 conidia of *A. niger* per gram of dry bagasse and all this material was incubated at 37 °C for 8 hours. Following that, this pre-germinated material was stored at 4°C for a few hours. At two hours intervals, one plastic bag was taken from the cold room to the incubator at 37°C and left for an hour before feed of the CCR. 28 feedings (0.5 Kg each) of the pre-germinated material were carried out each hour. Non-inoculated solid material was fed in the subsequent ten feedings.

The CCR screw movement was monitored as shown in Table 1. In this way the mean residence time of the solids in the reactor was 20 hours. During the fermentation no sterile conditions was followed. No special precautions have been taken to avoid the exchange of micro-organisms with the exterior of the reactor. Despite the metallic covers being used, there was contact at the discharge-end and at the feeder. No forced aeration has been used. The natural movement of the air would probably be sufficient enough to supply the necessary oxygen and remove the carbon dioxide produced during fermentation.

Samples from all the compartments (1 to 9) of CCR were collected at 16, 20, 24, 30 and 38 hours of fermentation. At the end of SSF in the CCR, analysis of the samples was carried out to observe the evolution of the main fermentation parameters (water content, pH, biomass, reducing sugars and glucoamylases). The results of these analysis are presented in Figure 4.

Water content: Kinetics of water content in the fermented solid material for each compartment of the CCR show that the humidity of the material decreased from 75 to 65 % from compartments 1 to 9. All kinetics present similar patterns (Figure 4.A). Only at 20 (compartment 9) and 38 hours (compartments 2 and 3) kinetics were different.

pH: The pH evolution was the same for all kinetics. At the beginning (compartments 1 and 2) the pH was stable and around 5.5. In compartments 3-6 it decreased and a value of 3 was reached. This acidic conditions were maintained in compartments 7-9 (Figure 4.B). Only at 38 a different pattern was shown. In compartments 1-6 the pH was always higher than the other kinetics. From compartment 7 onwards the pH changes were similar to the other kinetics.

Biomass: Biomass has only been analysed at 20, 24 and 30 hours of fermentation. Kinetics at 20 and 30 hours presented a similar pattern. At 24 hours kinetic was different. In this study, the change in biomass concentration pattern 20 and 30 hours only is described. At compartments 1-3 the biomass concentration was very low. Compartments 3-7 showed an exponential growth. The growth slowed down after compartment 7 and there was even a decrease in biomass (Figure 4.C)

The biomass change ranged between approximately 1 mg/ g of dry matter at the first compartment and 40 mg/g of dry matter at the 9th compartment, reaching a maximum of 55 mg/g of dry matter at compartments 7 and 8.

Reducing Sugars: The change in reducing sugars is heterogeneous for the different kinetics. However, in general an initial increase in their concentration could be observed, a maximum of 70 mg/g of dry matter being reached around the 4th compartment. Following that, a decrease occurred until compartment no.7 (Figure 4.D). Only at 38 hours the kinetics was clearly different.

Glucoamylases: Glucoamylases production was only analysed at 20 hours fermentation in the CCR. Compartments 1-4 presented a very low enzymatic activity (approx. 10 IU/g of dry matter). Then an increase in activity was predominant (compartments 5-9) until a maximum of 40 IU/g of dry matter was reached in the last compartment (Figure 4.E).

Fungal aspect during a continuous SSF process: Microscopically observations have also been carried out with samples at 20 and 30 hours. From each sample a very small particle of fermented solid material was taken and stained with a “blue Cotton” dye. This preparation was observed under 100x, 200x or 400x magnification.

At the 1 st compartment the conidia had already germinated and the germinative tube length was approx. 3 times the diameter of the conidia. At the 2nd compartment the germinative tubes were 5-10 times longer than the conidia diameter and at the 3rd compartment around 100 times. From compartment no.3 onwards the mycelium was vigorous, thick and presented ramifications.

At compartment no. 4 the mycelium was very well grown. There was a strong ramification of the hyphae and the surface of the solid material had been invaded. Compartments no. 4 showed a strong evolution. The surface of the solid material had been completely covered and the starch particles were degraded.

At compartment no.6 the mycelium replaced the hydrolysed starchy solid and offered a solid structure for the fermented substrate. Compartments no.7, 8 and 9 presented similar change to that of compartment no.6. The only difference was that in compartments no.8 and 9 the formation of a few asexual reproductive forms was detected. Phialides were formed however, these forms were still very young and no conidia were produced.

This first *A. niger* solid state fermentation in the CCR, a good fungal growth was present and a reasonable amount of glucoamylases was produced. The water content decreased significantly from the first to the last compartment. Hence, a second SSF was carried out (results not shown in this paper) with a higher initial water content and with no use of the heating jacket of the CCR. The feeding rate increased to 0.8 Kg/h because, with the feeding rate of 0.5 Kg/h used in the first fermentation, just a small part of the capacity of the reactor was in use.

The mycelium in each compartment was at a similar physiological state. At the first compartment, only germinated conidia were present. Successive compartments showed an increase in the colonisation of the substrate and at compartment no.5 the substrate was covered in dense mycelium. A marked increase in biomass occurred from compartment no.5 onwards. No mycelium damage or sporulation was observed.

In Table 1, the change of the measured parameters is shown for each compartment of the CCR. These values were similar to those reported for batch SSF using the same substrate and micro-organism (Oriol *et al.* 1987). However, moisture content dropped from about 74% in compartment no.1 to 64% in compartment no.9 due to the water evaporation effect caused by the high temperature of the fermented material.

In solid state fermentations several kinetic parameters have been analysed and, in general, all of them presented a similar behaviour. To facilitate the discussion of the results a representative kinetics has been calculated, from different values of the fermentation (Table 3). However, reference to the other kinetics was also made whenever necessary.

Parameters evolution in each CCR compartment during SSF

In this fermentation, 20 hours was selected as the most representative one. In Table 3, changes in water content, pH, biomass, reducing sugars and glucoamylases are shown at 20 hours fermentation.

Table 3: Parameters changes in each CCR compartment over 22 hours fermentation. Sugarcane bagasse moistened with a nutritive solution and containing 10 hours old pre-germinated conidia of *A. niger* was used for feeding in the continuous glucoamylases production under non-aseptic conditions during 38 hour SSF.

Parameter	Compartment								
	1	2	3	4	5	6	7	8	9
Temperature (°C)	30	35	37	39	40	38	39	39	36
Water content (% w.b.)	74	74	73	72	73	72	71	66	64
pH	5.1	5.3	4.9	4.4	3.1	3.0	2.9	2.8	2.9
Biomass (mg/g)	0.4	2.4	11	22	41	45	47	55	42
Reducing sugars (mg/g)	8.0	22	45	63	38	36	39	47	32
Glucoamylases (IU/g)	5.0	9.0	12	9.0	22	23	25	39	40

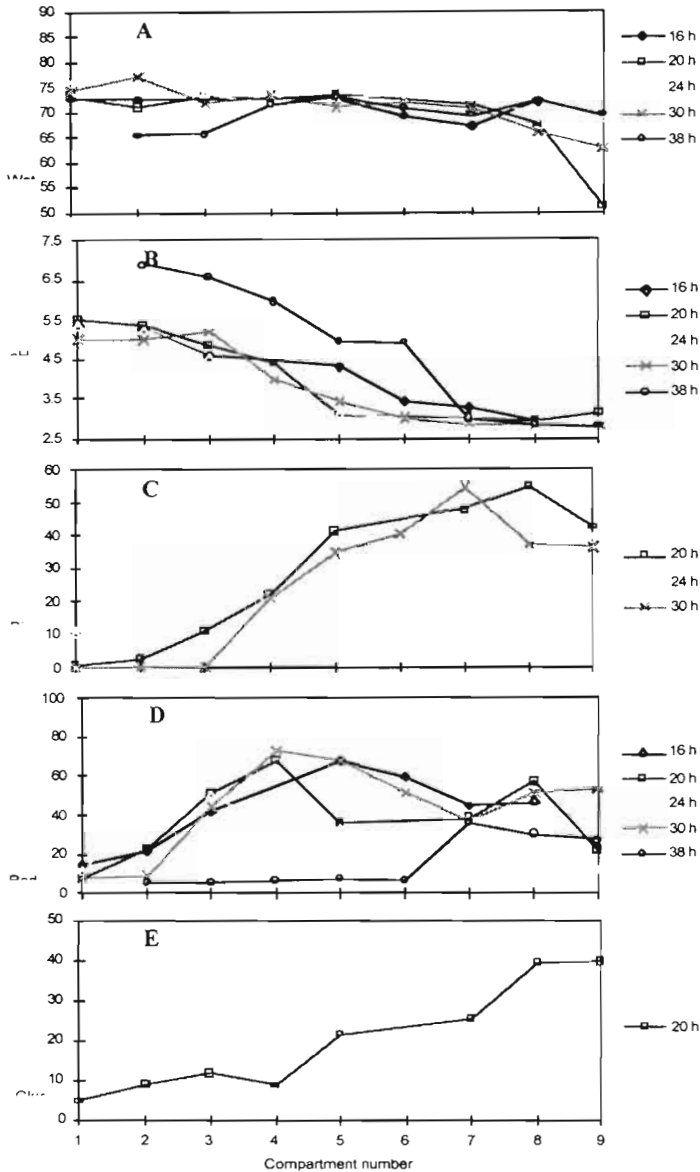


Figure 4 : Water content (A), pH (B), biomass (C), reducing sugars (D) and glucoamylases (E) evolution in each compartment of the CCR during the fermentation. Sugarcane bagasse media containing pre-germinated *A. niger* conidia was continuously fed. Residence time = 20 hours.

Water Content Evolution

Before culture solid medium sterilisation, the inert solid support was moistened to 50% with a nutritive solution. Then, the water content was adjusted to 74% with the spores suspension. The change in water content for the 40 hour SSF in a CCR is represented in Table 3.

A decrease from 74% in compartment no.1 to 64 % in compartment no.9, however is evident this amount of water is enough to ensure good conidia germination and mycelium growth. The last compartments were characterised by a low water content, but it is believed that it did not affect the enzymes production because other studies confirmed the production of glucoamylases in SSF with water content lower than 60 % (Raimbault and Alazard 1980), using starch material at 50% water content and had good glucoamylases production).

As long as it does not affect the fungal metabolism, a low water content is good because it avoids bacterial contaminations. On the other hand, when there is need to implement the process at an industrial scale, a high water content in the final fermented product could be helpful in the recovery of metabolites. For example, if this product had a water content of 75% ,80% of the fermented juice could be recovered by just hydraulically pressing the fermented solid material (Roussos *et al.*, 1992). This is one of the simplest and cheapest methods available to recover fungal metabolites.

Glucoamylases Continuous Production

Initially at 20 hours there was a slow increase in the concentration of glucoamylases between compartments no.1 and no.3. From compartment no.3 to no.4 a small decrease in concentration was observed but after the 4th compartment a sharp increase was evident.

We think that the glucoamylases present at the first four compartments are the glucoamylases that were contained inside the conidia and were liberated during germination. These glucoamylases were responsible for the initial increase in the reducing sugars concentration described above.

The biosynthesis of new glucoamylases started immediately after the 4th compartment and was clearly associated with the growth of mycelium. During this phase, a decrease in the reducing sugars concentration was observed because, although the glucoamylases production was high, it was not sufficient to compensate the reducing sugars consumption by the micro-organism. The micro-organism was on the exponential phase of growth and, as soon as the reducing sugars were produced, they were consumed by the mould.

It is very interesting to note that, although the reducing sugars concentration was high at compartments no.4-8, the biosynthesis of glucoamylases was not repressed. On the contrary, in submerged fermentations a strong catabolic repression in the biosynthesis of glucoamylases was observed when the reducing sugars concentration was high. This fact could be explained thinking of what happened in the surroundings of the mycelium in the solid state fermentation. The mycelium was surrounded by a thin layer of water. The glucoamylases produced by *A. niger* in SSF had to move toward this layer in order to reach the starch material. When the reducing sugars were liberated from the starch, they had to diffuse back through the water layer until they reach the micro-organism. As this diffusion process was

low, as soon as the sugars reached the micro-organism they were consumed by it. In this way, the concentration of sugars close to the micro-organism was very low and the micro-organism continued the production of glucoamylases in SSF, even if this concentration away from the mould was high.

In submerged fermentations, a layer of stagnant solution is also found around the micro-organism. However, due to the agitation of the fermentation broth, its thickness is much smaller and the micro-organism is better informed about the concentrations away from it (Favella-Torres *et al.* 1997).

Spore Germination and Mycelium Development.

Microscopy observations showed that the conidia were uniformly distributed throughout the solid material and the inoculation had been homogeneous. The amount of conidia used (2×10^7 conidia/g of dry bagasse) was enough to invade sugarcane bagasse during the mycelium growth, without being excessive. A high percentage of conidia was germinated.

However, when the pre-germinated solid material was fed to the CCR, the spores were still at the beginning of germination. Probably the temperature and time of incubation were not the most adequate.

At compartment no.3 the ramification of the mycelium was evident. At this phase, the exponential growth started (Figure 4.C). There was a clear association between the exponential increase of the biomass and the ramification and strong growth of the mycelium. After compartment no.8 the formation of some reproductive forms was observed and this corresponded to the slight decrease observed in biomass from compartment no.8 to no.9.

Conclusions

The continuous production of enzymes by SSF has been demonstrated. Inoculation with pre-germinated conidia shortened the processing time and allowed control of the age of the mycelium in each compartment. Aeration was accomplished by natural convection and moisture content was controlled. The fungal growth occurred in good conditions, and the movement of the screw no had caused any damage for the mycelium. The sugarcane bagasse was an excellent solid support used in this equipment. Similarities between batch fermentation and fermentation in CCR were observed. This process could be applied to the continuous production of fungal biomass and other metabolites (enzyme, organic acids, antibiotics), spores or biomass. However it is necessary to investigate thoroughly different aspects such as: a) The development of systems to measure and control fermentation parameters in SSF. b) The increase of capacity in the reactor, c) The use of the same biomass in successive cultures, d) The monitoring and control of the humidity , pH, temperature, oxygen transfer during the SSF in a CCR.

References

- Aido K., Hendry R. and Wood G.B. 1982. Solid substrate fermentation. *Adv. Applied Microbiol.* 28: 201-237
- Alazard, D. and Raimbault, M. 1981. Comparative study of amyolytic enzymes production by *Aspergillus niger* in liquid and solid state cultivation. *Eur. J. Appl. Microbiol. Biotechnol.* 12: 113-117.
- Auria, R., Hernandez, S., Raimbault, M. and Revah, S. 1990. Ion exchange resin: a model support for solid state growth fermentation of *Aspergillus niger*. *Biotechnol. Techniques.* 4: 391-396.
- Barrios-Gonzalez, J., Tomasini, A., Viniestra-Gonzalez, G. and Lopez, L. 1988. Penicillin production by solid state fermentation. *Biotechnol. Lett.* 10: 793-798.
- Byndoor, M.G., Karanth, N.G., Rao G.V. 1997. Efficient and versatile design of a tray type solid state fermentation bioreactor. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniestra-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, chapter 9: pp. 113-120.
- Casimir, D. J. 1983. Counter-Current Extraction of Soluble Solids from Foods. *CSIRO Food Research Quarterly.* 53: 38-43.
- Christen, P., Angeles, N., Corzo, G., Farres A. and Revah, S. 1995. Microbial lipase production on a polymeric resin. *Biotechnol. Techniques.* 9: 597-600.
- Deschamps, F., Raimbault, M. and Senez, J.C. 1982. Solid state fermentation in the development of agro-food by-products. *Industry & Environ.* 5 (2): 27-30.
- Deschamps, F., Giuliano, C., Asther, M., Huet, M.C. and Roussos, S. 1985. Cellulase production by *Trichoderma harzianum* in static and mixed solid state fermentation reactors under non-aseptic conditions. *Biotechnol. Bioeng.* 27: 1385-1388.
- Durand A. & Chereau D. 1988. A new pilot reactor for solid state fermentation: application to the protein enrichment of sugar beet pulp. *Biotechnol. Bioeng.* 31: 476-486.
- Durand, A., Renaud, R., Maratray, J., Almanza, S. 1997. The INRA-Dijon Reactors: Designs and applications. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniestra-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, chapter 7 pp. 71-92.
- Gervais, P. & Bensoussan, M. 1994. Solid-state fermentations of the genus *Aspergillus*. In: *Aspergillus*, Smith, J. E. (ed.), Plenum Press, N.Y., pp. 101-140.
- Gonzalez-Blanco, P., Saucedo-Castañeda, G. and Viniestra-Gonzalez, G. 1990. Protein enrichment of sugar cane by-products using solid-state cultures of *Aspergillus terreus*. *J. Ferment. Bioeng.* 70: 351-354.
- Greve A. & Kula M.R. 1991. Recycling of salts in partition protein extraction processes. *J. Chem. Technol. Biotechnol.* 50: 27-42
- Gutierrez G., Robles R. & Pyle L. 1996. Performance of a counter current solid-liquid extractor for food processing. *Fronteras en Biotecnología y Bioingeniería*. Galindo E. (Ed.), SMBB, México 245-255.

- Gutierrez-Rojas, M., Cordova, J., Auria, R., Revah, S. and Favela-Torres, E. 1995. Citric acid and polyols production by *Aspergillus niger* at high glucose concentration in solid state fermentation on inert support. *Biotechnol. Lett.* 17: 219-224.
- Johansson G., Joelsson M. & Akerlund H.E. 1985. An affinity-ligand gradient technique for purification of enzymes by counter-current distribution. *J. Biotechnol.* 2: 225-237.
- Klyueva L.M. & Zakharevich N.S. 1985. Study on gentamicin sorption in counter-current flow of liquid and solid phases. *Antibiot. Med. Biotechnol.* 30: 576-579.
- Kumar P.K.R. & Lonsane B.K. 1987. Extraction of gibberellic acid from dry mouldy bran produced under solid state fermentation. *Process Biochem.* 22: 139-143.
- Lambraki, M., Marakis, S. and Roussos, S. 1994. Effect of temperature and aeration flow on carob tannin degradation by *Aspergillus carbonarius* in solid state fermentation system. *Micol. Neotrop. Apl.* 7: 23-34.
- Leach, G. C. 1993. Studies on the Counter Current Diffusional Extraction of Apple Juice, Ph. D. Thesis, University of Reading, U.K.
- Lepilleur, C., De Araujo, A. A., Delcourt, S., Colavitti, P. and Roussos S. 1996. Laboratory scale bioreactors for study fungal physiology and metabolism in solid state fermentation system. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniegraz-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, p.93-111.
- Likidis Z., Schlichting E., Bischoff L. & Schuegerl K. 1989. Relative extraction of penicillin G from mycel-containing broth in a countercurrent extraction decanter. *Biotechnol. Bioeng.* 33: 1385-1392.
- Lonsane, B.K., Ghildyal, N.P., Budiatman, S., Ramakrishna, S.V. 1985. Engineering aspects of solid state fermentation. *Enzyme Microb. Technol.* 7: 258-265.
- Lonsane, B.K., Saucedo-Castañeda, G., Raimbault, M., Roussos, S., Viniegra-Gonzalez, G., Ghildyal, N.P., Ramakrishna, M. and Krishnaiah, M.M. 1992. Scale-up strategies for solid state fermentation systems. *Process Biochem.* 27: 259-273.
- Moo-Young M., Moriera A.R. & Tengerdy R.P. 1983. Principles of solid state fermentation. In *The filamentous fungi*, Vol. 4, Fungal Biotechnology. Smith J.E, Berry D.R & Kristiansen B. Eds., Edward Arnold Publishers, London, pp. 117-144.
- Oriol, E., Schettino, B., Viniegra-Gonzalez, G. and Raimbault, M. 1988a. Solid- state culture of *Aspergillus niger* on support. *J. Ferment. Technol.* 66: 57-62.
- Oriol, E., Raimbault, M., Roussos, S. and Viniegra-Gonzales, G. 1988b. Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.*, 27: 498-503.
- Pandey, A. 1991. Aspects of fermenter design for solid-state fermentations. *Process Biochem.* 26: 355-361.
- Raimbault, M. and Alazard, D. 1980. Culture method to study fungal growth in solid fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 9: 199-209.

- Raimbault, M., Revah, S., Pina, F. and Villalobos P. 1985. Protein enrichment of cassava by solid state fermentation using molds isolated from traditional foods. *J. Ferment. Technol.* 63: 395-399.
- Rodriguez, J.A., Torres, A., Echevarria, J. and Saura, G. 1991. Energy balance in solid state fermentation processes. *Acta Biotechnol.* 11: 9-14.
- Roussos, S., Olmos, A., Raimbault, M., Saucedo-Castañeda, G. and Lonsane, B.K. 1991. Strategies for large scale inoculum development for solid state fermentation system : Conidiospores of *Trichoderma harzianum*. *Biotechnol. Tech.* 5: 415-420
- Roussos, S., Raimbault, M., Viniegra-Gonzalez, G., Saucedo-Castañeda, G. and Lonsane, B.K. 1991. Scale-up of cellulases production by *Trichoderma harzianum* on a mixture of sugar cane bagasse and wheat bran in solid state fermentation system. *Micol. Neotrop. Apl.* 4 : 83-98.
- Roussos, S., Raimbault, M., Prebois, J-P. and Lonsane, B.K. 1993. Zymotis, A large scale solid state fermenter : Design and evaluation. *Applied Biochem. Biotechnol.* 42: 37-52.
- Roussos, S., Bresson, E., Saucedo-Castañeda, G., Martinez, P., Guymberteau, J. and Olivier, J-M. 1997. Production of mycelial cells of *Pleurotus opuntiae* on natural support in solid state fermentation. In Roussos, S., Lonsane, B.K., Raimbault, M and Viniegra-Gonzalez, G. (Eds.), *Advances in Solid State fermentation*. Kluwer Acad. Publ., Dordrecht, chapter 40: pp. 481-498.
- Samson, R. ... 1996. Mycol. Research.
- Saucedo-Castañeda, G., Gutierrez-Rojas, M., Bacquet, G., Raimbault, M. and Viniegra-Gonzalez, G. 1990. Heat transfert simulation in solid substrate fermentation. *Biotechnol. Bioeng.* 35: 802-808.
- Saucedo-Castañeda, G., Lonsane, B.K., Navarro, J.M., Roussos, S. and Raimbault, M. 1992a. Potential of using a simple fermenter for biomass built up, starch hydrolysis and ethanol production: Solid state fermentation system involving *Schwanniomyces castellii* , *Appl. Biochem. Biotechnol.* 36: 47-61.
- Saucedo-Castañeda, G., Lonsane, B.K., Krishnaiah, M.M., Navarro, J.M., Roussos, S. and Raimbault, M. 1992b. Maintenance of heat and water balances as a scale-up criterion for the production of ethanol by *Schwanniomyces castellii* in a solid state fermentation system. *Process Biochem.* 27: 97-107.
- Schwartzberg, H. G. (1980) Continuous Counter-Current Extraction in the Food Industry. *Chem. Engineer. Progress.* 76: 67-85.
- Senez, J.C. 1979. Solid fermentation of starchy substrates. *Food Nutr. Bull.* 1: 18-20.
- Senez, J.C., Raimbault, M. and Deschamps, F. 1980. Protein enrichment of starchy substrates for animal feeds by solid state fermentation. *World Animal Rev.* 35: 36-40.
- Srikanta S., Jaleel S.A., Ghildyal N.P., Lonsane B.K. & Karanth N.G. 1987. Novel technique for saccharification of cassava fibrous waste for alcohol production. *Starch* 39: 234-237.

- Socol, C., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Potential of solid state fermentation for production of L(+) lactic acid by *Rhizopus oryzae*. *Appl. Microbiol. Biotechnol.* 41: 286-290.
- Trejo-Hernandez, M.R., Raimbault, M., Roussos, S. and Lonsane, B.K. 1992. Potencial of solid state fermentation for production of ergot alkaloids. *Let. Appl. Microbiol.* 15: 156-159.
- Trejo-Hernandez, M.R., Lonsane, B.K., Raimbault, M. and Roussos, S. 1993. Spectra of ergot alkaloids produced by *Claviceps purpurea* 1029c in solid state fermentation system: Influence of the composition of liquid medium used for impregnating sugar cane pith bagasse. *Process Biochem.* 28: 23-27.
- Viniegra-Gonzalez, G. 1997. Solid state fermentation: Definition, characteristics, limitations and monitoring. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniegraz-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, chapter 2: pp. 5-22.

PRODUCTION OF ENZYMES BY SOLID SUBSTRATE FERMENTATION: RELATION SUBSTRATE/ENZYME AND INDUCTION/CATABOLIC REPRESSION

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Summary

Solid substrate cultures of moulds behave in quite a different way to conventional submerged cultures. Catabolic repression often observed in submerged fermentation can easily be overcome by using solid state fermentation. *A. niger* strains can be grown by SSF technique with the following advantages: enzyme titres and productivity are usually higher; there is a tendency to early enzyme excretion as compared to SmF technique.

Conventional enzyme production in a Stirred Tank Reactor (STR) by microbial organisms requires, an inexpensive and ready to use carbon source (sugar or starch derivative), a specific enzyme inducer, and a mixture of mineral salts and other organic compounds. Unfortunately, the presence of high levels of the carbon source often inhibit the production of many enzymes. For example, it has been reported that the addition of too much of sugar or even pectin to a STR inoculated with *Aspergillus niger* was detrimental to pectinase production [2, 3]. But continuous and controlled addition of small amounts of sugar (fedbatch reactor) increased four or five times pectinase productivity [2]. The explanation of this phenomenon was related to a dynamic balance between supply and demand of sugars in the SmF process leaving the sugar concentration at a low level, although the total supply of sugars was very high [2]. But, Ramesh and Lonsane [4] using *Bacillus subtilis* grown by SSF in fixed bed reactors and adding starch as inducer did not find the catabolic repression phenomenon commonly observed by the addition of high levels of carbohydrates.

Solis-Pereira *et al.* [5] confirmed that in shake flasks (SmF) inoculated with a strain of *Aspergillus niger*, called CH4, glucose levels of 30 g/L were inhibitory to pectinase production in the presence of 30 g/L of pectin (Table 1). But in SSF (packed bed reactors with bagasse) using the same strain and inducer, the addition of glucose enhanced pectinase production although the sugar level was 100 g/L in the absorbed broth (see Table 1).

TABLE 1. Effect of glucose addition to pectinase levels produced by SmF and SSF techniques [5].

Substrates	SmF (U/mL)	SSF (U/g)
30 g/L Pectin	0.75	5.00
A + 30 g/L glucose	0.00	11.0
A + 100 g/L glucose	N.D.	13.0

This observation has been confirmed in our laboratory (unpublished data) using different *A. niger* strains and inducers (sucrose, tannic acid) and assaying for the corresponding enzyme activities (invertase, tannase) as illustrated in figure 1.

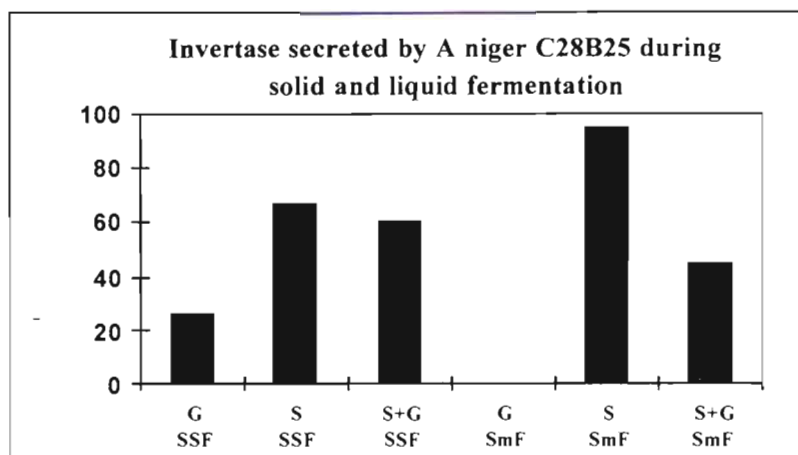


Figure 1. Invertase secreted by *A. niger* C28B25 during SSF and SmF. (G: Glucose, S: Saccharose).

Catabolic repression is observed in SmF at 40g/L of glucose (Fig. 1). The repression at the same concentration of glucose is much reduced in SSF. Glucose added to saccharose in SSF does not affect invertase production.

Production of other enzymes has also been tested in packed bed reactors with bagasse as solid support and in fixed bed reactors with polyurethane as solid matrix. In all those cases, enzyme production was increased using high levels of glucose or sucrose without much evidence of catabolic repression.

A consequence of such physiological behaviour is the increase of enzyme productivity by SSF. This was observed to a certain level. Garcia-Peña (11) studied the effect of adding increasing amounts of tannic acid as an inducer during an SSF process and observed that over and above 10% tannic acid, tannase production decreased dramatically, as shown in figure 2.

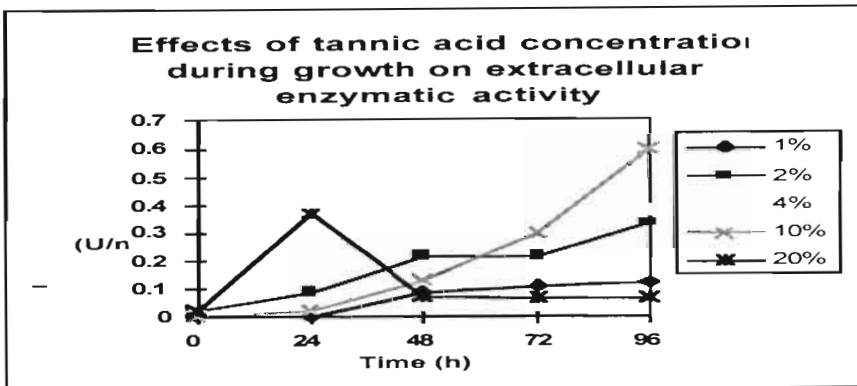


Figure 2. Effects of tannic acid concentration during growth on extracellular tannase activity

Another interesting observation is the fact that tannase has been reported to be bound to the hyphal biomass of *A. niger* when it is produced by SmF. Lekha and Lonsane (10) and Garcia-Peña (11) have found that this enzyme is mostly excreted to the culture medium of *A. niger* when produced by SSF (figure 3.). These intriguing differences between SSF and SmF techniques in the excretion of enzymes, seem to indicate that moulds can modulate the way to use enzymes, depending on the culture medium. Apparently, those organisms have "sensors" that pick up environmental signals and have also complex transducing systems that modulate their biochemical behaviour in order to adapt to a particular set of environmental variables (low or high a_w , good or bad mixing of substrates, low or high temperature, etc). This adaptation gives plasticity to those organisms in order to survive in changing culture conditions.

An unexplored solid state fermentation method using polyurethane foam (PUF) as inert carrier impregnated with a synthetic liquid medium was developed simulating the nutritional composition and culture conditions of solid state fermentation on sugar cane bagasse. with this system, biomass, the important parameter involved in SSF process, could be measured directly. Tannase production of various previously selected overproducing *A. niger* strains was tested (Figure 4.). The three strains produce maximum activity around 48 hours of incubation. The specific activities are similar to those obtained with bagasse [12]. However, maximum tannase production is observed much later (around 100 hrs) when sugar cane bagasse is used as solid support.

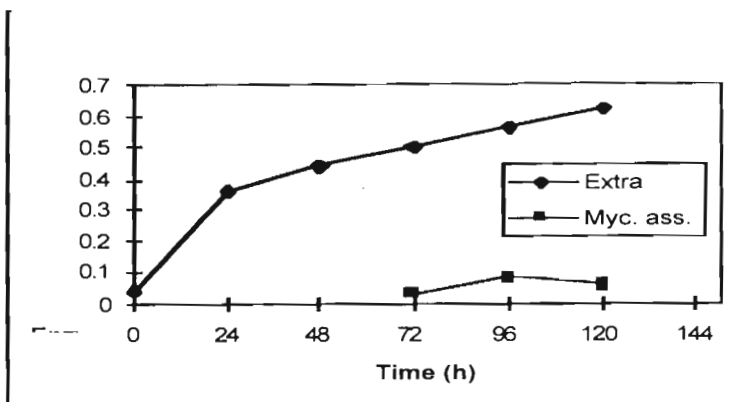


Figure 3. Comparison of tannase extracellular activity with activity associated with the mycelium.

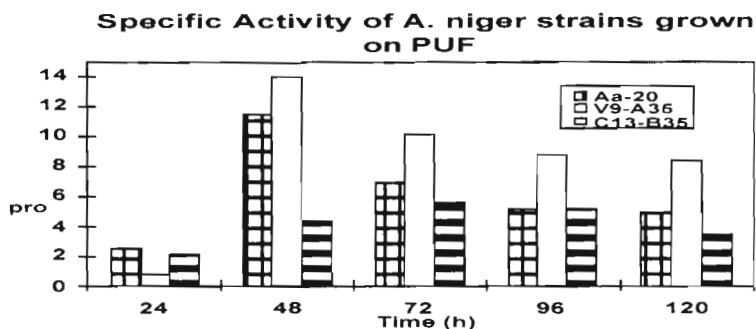


Figure 4. Specific activity of tannase from three *A. niger* strains during growth on PUF.

TABLE 2. Comparison of pectinase productivity by SmF and SSF cultures of *A. niger* CH4 [1].

Activity	Productivity ($U\ ml^{-1}\ h^{-1}$)		Ratio (SSF/SmF)
	SSF	SmF	
Endo-pectinase	0.006	0.001	6.0
Exo-pectinase	0.14	0.002	51.5
Pectin-lyase	0.008	0.0002	29.2

Acuña-Argüelles *et al.* [1] found that *A. niger* CH4 had a much higher pectinase productivity when cultured by SSF as shown in Table 2.

Clearly, SSF cultures were more productive for all pectinase activities assayed, such as endo-pectinase by viscometry, exo-pectinase by the production of reducing compounds and pectin lyase by the changes in UV absorbing material [1].

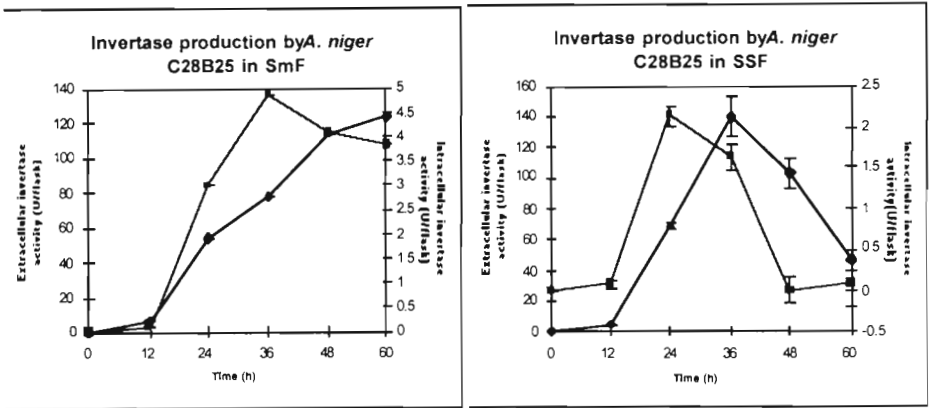


Figure 5. Invertase production of *A. niger* C28B25 in SmF and SSF. Squares represent extracellular invertase production and diamond represent intracellular invertase. [Note the difference in scales]

In SmF, intracellular accumulation is over twice that of SSF. Invertase is secreted earlier (maximum at 24 hrs) in SSF than in SmF (maximum at 36 hrs). Unlike tannase which is mycelium associated in SmF, invertase seems to be readily excreted in both SmF and SSF.

Such increases of productivity could have important economic consequences in the cost of enzyme production. Thus, Ghildyal *et al.* [6] have made an economic *pro forma* analysis of amylo-glucosidase production by SmF and SSF. Their calculations indicate that due to a higher yield of SSF using *A. niger* CFTRI 1105, which produced 10 times higher titres than by SmF, the overall economic picture was much better for SSF process. In fact there are reports of successful large scale production of pectolytic enzymes by SSF in India [7] and also of fungal amylase scale-up by SSF [8].

Pandey [9] has reviewed the reports of enzyme production by SSF including, cellulases, amylase, glucoamylase, beta glucosidase, pectinases, catalase and proteases with a list of 28 microbial species in which *Bacillus* and *Aspergillus* are the most frequently used genus.

Bibliography

- 1.- Acuña-Argüelles, M., Gutiérrez-Rojas, M., Viniegra-González, G., Favela-Torres, E.: Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid state fermentation. *Appl. Microbiol. Biotechnol.* 43 (1995), 1-6.
- 2.- Aguilar, G., and Huitrón, C.: Application of fed-batch cultures in the production of extracellular pectinases by *Aspergillus* sp. *Enzyme Microb. Technol.* 9 (1986), 541-545.
- 3.- Maldonado, M. C., Stresser A., and Callieri, D.: Regulatory aspects of the synthesis of polygalacturonases and pectinesterases by *Aspergillus niger* sp. *Sciences des aliments* 9 (1989), 101-110.
- 4.- Ramesh, M.V., and Lonsane, B.K.: Regulation of alpha-amylase production in *Bacillus licheniformis* M27 by enzyme end-products in submerged fermentation and its overcoming in solid state fermentation system. *Biotechnol. Lett.* 13 (1991), 355-360.
- 5.- Solís-Pereira, S., Favela-Torres, E., Viniegra-González, G., and Gutiérrez-Rojas, M.: Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentation. *Appl. Microbiol. Biotechnol.* 39 (1993), 36-41.
- 6.- Ghildyal, N.P., Lonsane, B.K., Sreekantiah, K.R., Sreenivasa-Murthy, V.: Economics of submerged and solid state fermentations for the production of amyloglucosidase. *J. Food Sci. Technol.* 22 (1985), 171-176.
- 7.- Ghildyal, N.P., Ramakrishna, S.V., Nirmaia Devi, P., Lonsane, B.K., Ashtana, H.N.: Large scale production of pectolytic enzyme by solid state fermentation. *J. Food Sci. Technol.* 18 (1985), 244-257.
- 8.- Jaleel, S.A., Ghildyal, N.P., Sreekantiah, K.R., Sreenivasa Murthy, V. Production and scale-up of fungal amylase by solid state fermentation. *Paper presented at 19th. Annual Conference, Association of Microbiologists*, India, Baroda (as cited in 17) (1978).
- 9.- Pandey, A.: Recent process developments in solid state fermentation. *Process Biochemistry.* 27 (1992), 109-117.
- 10.- Lekha, P.K., and Lonsane, B.K.: Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid state, liquid surface and submerged fermentations. *Proc. Biochem.* 29 (1994), 497-503.
- 11.- García-Peña, E.I.: *Producción, purificación y caracterización de tanasa producida por Aspergillus niger, en fermentación en medio sólido* M. Sc. thesis (Biotechnology). Universidad Autónoma Metropolitana. Iztapalapa, D.F. (Méx.) (1995).
- 12.- Ramírez, A. *Selección de cepas productoras de tanasa por fermentación en medio sólido*. Lic. thesis (Biotechnology) Universidad Autónoma Metropolitana. Iztapalapa, D.F. (Méx.) (1996).

FRUITY AROMAS PRODUCTION IN SOLID STATE FERMENTATION BY THE FUNGUS *Ceratocystis fimbriata*

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Abstract

Solid state fermentation (SSF) has been studied for enzymes, antibiotics, alcohol production or for protein enrichment, but few papers report the production of aromas by such a process. In this work, the study of the production of fruity aromas in SSF by the fungus *Ceratocystis fimbriata* is presented, with special interest in the nature of the support/substrate, the importance of added precursors in the medium and the aeration. The aromas were characterised by "sniffing" technique and GC headspace analysis; growth was followed by respirometry.

It was shown that wheat bran, cassava and sugarcane bagasse were adequate supports for growth and a detectable aroma production. Among the nutritive media tested, the synthetic medium previously defined, used with a higher glucose concentration (200 g/l) gave a strong apple aroma while those containing aminoacids precursors such as leucine and valine gave strong banana aroma. It was found that aroma production was growth dependent and the maximum aroma intensity was detected a few hours before or after the maximum respirometric activity, this varying between 0.8 and 1.6 ml CO₂ / h. g dry matter, after 24 hours). Experiments made under various aeration rates (0.05 and 0.005 ml/h. g dry matter) showed that this parameter was not limiting for growth even if the exit gas was very poor in oxygen at the lower aeration rate giving in this case the most intense aroma. For experiments made without forced aeration, the same aromas were also found with higher intensity. Fourteen compounds have been separated by GC headspace and 11 of them such as acetaldehyde, ethanol, ethyl acetate, isoamyl acetate and isoamyl alcohol were identified.

Introduction

Micro-organisms play an important role in the generation of natural flavouring compounds particularly in the field of food aromas. One can refer to the extensive reviews dealing with flavour generation by micro-organisms in the past few years (Larasse *et al.*, 1985; Welsh *et al.*, 1989; Janssens *et al.*, 1992). As pointed out recently by Bigelis (1992) and Christen (1995), filamentous fungi are especially interesting because they are able to produce flavouring compounds or flavours-related enzymes.

Some recent papers have reported the production of aromas in SSF: Yamauchi *et al.* (1989) obtained a fruity flavour growing a *Neurospora* strain on pregelatinized rice; Gervais and Sarrette (1990) studied the production of coconut aroma by *Trichoderma viride* on agar and Humphrey *et al.* (1990) patented a process where an *Aspergillus* strain grown on cellulose fibres produced methyl ketones from coconut oil. Moreover, the capacity of some moulds from the genus *Ceratocystis* to produce fruit-

like aromas has already been demonstrated (Hanssen and Sprecher, 1981; Senemaud 1988, Christen *et al.*, 1994).

In this work, the ability of *Ceratocystis fimbriata* to produce aromas in SSF was explored. It involved the study of the influences of the substrates/supports used, the aeration flow rates and the presence of precursors, on both growth and aroma production.

Organism and culture media. *Ceratocystis fimbriata* CBS 374-83 was used. It was periodically transferred onto Potato Dextrose Agar (PDA) slants and stored at 4°C. Four substrates / supports were used: wheat bran, sugarcane bagasse, cassava (donated by Pr. C. Soccol, UFPR, Brazil) and an anionic resin (Amberlite IRA-900, Rohm & Haas). They were prepared according to Christen *et al.* (1993). When forced aeration (packed bed) was used, the cultures were carried out in small columns placed in temperature controlled bath. For experiments without forced aeration (surface culture), they were made in 500 ml Erlenmeyer flasks covered with gauze or tight-sealed. They were filled with 7.5 g Initial Dry Matter (IDM) for wheat bran and Amberlite, and 5.25 g IDM for bagasse and cassava. For all experiments, initial conditions were: temperature, 30°C; pH, 6; inoculum size, 1×10^7 spores/g IDM. Aeration rates were 0.05 or 0.005 l/h.g IDM. Initial water content was calculated according to the maximum adsorption capacity of each support (wheat bran, 50%; Amberlite, 58%; sugarcane bagasse, 63% and cassava, 65%). Culture conditions are given in tables 1 and 2. SM (See table 1 and 2) refers to the synthetic medium optimised by Christen and Raimbault (1991). It was used with 200 g/l of glucose. For all cases, an oligoelement solution previously used by these authors was added. Urea, leucine and valine (167 mmol/l) were used as nitrogen source and/or precursor of the aroma.

Table 1. Culture conditions with forced aeration. *SM: synthetic medium

Run	Substrates/Supports	Nutritive media	Aeration rate (l/h.g)
1	Amberlite	Potato broth	0.05
2	Amberlite	Potato broth	0.005
3	Amberlite	SM*	0.005
4	Wheat bran	Urea	0.05
5	Wheat bran	-	0.005
6	Wheat bran	Urea	0.005
7	Wheat bran	Leucine	0.005
8	Wheat bran	Valine	0.005
9	Sugarcane bagasse	SM*	0.005
10	Sugarcane bagasse	Potato broth + glucose	0.005

Analytical procedures.

The odors of the cultures were determined by sensorial evaluation with a non-trained panel consisting of six members, with no restriction in descriptive terms.

Growth was characterised by respirometry measured by gas chromatography. For packed column experiments, this allowed the calculation of the carbon dioxide production rate (CDPR), the oxygen uptake rate (OUR) and the respiratory quotient (RQ) (Christen *et al.*, 1993). For surface cultures, O₂ and CO₂ concentrations evolu-

tion was followed. Water activity and pH were also determined at the end of the fermentation.

Table 2: Culture conditions without forced aeration. Each experiment was made with both gauze and tight-sealed.

Run	Substrates/Supports	Nutritive media
11	Wheat bran	-
12	Wheat bran	SM
13	Wheat bran	Leucine
14	Wheat bran	Urea
15	Cassava	-
16	Cassava	SM
17	Cassava	Leucine
18	Cassava	Urea
19	Sugarcane bagasse	SM + Leucine

Volatiles produced during the fermentation were characterised by gas chromatography (Hewlett-Packard 5890 equipped with a Megabore HP-1 column (length, 5m) and with a flame ionisation detector) of headspace vapour from the cultures (only for experiments without forced aeration). Conditions were: Temperatures, injector and detector: 210°C, oven held at 40°C during 2 minutes and then programmed at 10°C/min to 150°C. The nitrogen gas flow rate was 1.5 ml/min and split ratio 1:32.

Table 3: Results of aroma production in packed cultures and forced aeration. * - none, + weak, ++ medium, +++ strong. # tmax: time of maximum aroma perception. § in ml/h.g IDM.

Run	Aroma & Intensity *	tmax (h)#	CDPR max§	Aw final	pH final
1	-	-	2.58	0.996	6.76
2	banana +, then apple/pear +	67 / 91	0.95	0.973	3.05
3	banana +	91	0.89	0.990	2.65
4	pear/apple +	42	2.45	0.983	8.76
5	pear/apple ++	17	1.30	0.988	9.06
6	pear/apple ++	39	1.04	0.985	8.95
7	banana +++	17	0.72	0.989	9.03
8	banana +++	17	0.79	0.990	9.06
9	pear/apple +, then peach +++	20 / 91	0.84	0.993	2.71
10	pear/apple ++, then peach ++	68 / 116	0.70	0.998	6.24

Results are presented in two parts according to the modes of culture and aeration used.

Packed cultures experiments with forced aeration.

From the results presented in table 3, it can be seen that at the higher aeration rate (0.05 l/h.g IDM in runs 1 and 4), no or poor aroma was detected. It can be assumed that this rate swept away the volatiles produced and/or oxygenated conditions reduce the synthesis of such molecules. It is why a very low aeration was then used (0.005 l/h.g IDM). In that case, the overall aromas detected (pear/apple and banana) were stronger. In particular, it was clearly shown that leucine and valine, when added to the medium, played a precursor role for the development of the banana aroma (runs 7 and 8). This fruity aroma appeared very rapidly (before the first 24 hours) in these cases. When no precursor was used, pear/apple aroma was also detected (runs 4, 5, 6, 9 and 10). It must be pointed the observations made in runs 9 and 10 when 2 successive kinds of aromas were detected, first the pear/apple one and then at 5 days a strong peach one. With this aeration rate, lower CDPR max were observed (less than 1.3 ml/h.g IDM) which indicates maybe that, in poorly aerated media, growth was limited and volatile metabolites production favoured.

In terms of support evaluation, wheat bran (supplemented or not) and supplemented bagasse gave better result than Amberlite. In all cases, water activity was maintained at a satisfactory level, but pH sometimes at the end of the fermentation were alkaline (for wheat bran) or acid (for bagasse). In all cases, no compounds were detected in headspace analysis of the cultures.

As a conclusion, wheat bran and bagasse are adequate substrates and supports, very low aeration is recommended (0.005 l/h.g IDM), precursors like leucine or valine can shift fermentation to a particular aroma, although fruity aroma was also produced without them. From table 3 and figures 1 and 2, it can be seen that in both cases, the maximum aroma was detected just after the maximum in CDPR was attained. Seemingly, aroma production was found to be growth related.

Surface cultures experiments without forced aeration

In this case 9 combinations were tested, and in each case, experiments were made with gauze cover (static aerated culture) and tightly-sealed (without aeration). Sensorial evaluation was only possible in the case of aerated cultures. In the second case, respiration was characterised by O₂ (%) consumption and CO₂ (%) accumulation in the flask. Results are presented in Table 4.

All of the three substrates/support were found to allow growth and aroma production in aerated conditions. Aroma detection was more important than in experiments made with forced aeration. The strongest aroma detected (banana) corresponded to the media in which leucine was added (runs 13, 17, 19) while pear/apple aroma, with a lower intensity, was obtained with wheat bran completed with synthetic medium and urea (runs 12 and 14). These aromas were detected with major intensity between the first and the second day.

Table 4: Results of aroma production in surface cultures without forced aeration. * - none, + weak, ++ medium, +++ strong. # tmax: time of maximum perception of the aroma. § in ml/h.g IDM. 1 refers to aerated cultures and 2, to tight-sealed flask cultures.

Run	Aroma & Intensity *1	tmax (h)#1	CDPRmax §1	CO2 max (%)2
11	-	-	1.15	11.3
12	apple/pear ++	44.3	0.06	81.3
13	banana +++	35.8	0.16	31.5
14	apple/pear ++	41.3	1.23	22.3
15	banana ++	40	0.20	36.7
16	-	-	0.90	62.5
17	banana +++	40	0.20	29.6
18	-	-	0.45	7.3
19	banana +++	39.3	0.08	46.9

For tight-sealed flask cultures, growth was also observed. As growth and substrate fermentation evolved, CO₂ was produced and the internal pressure increased. This pressure was released during sampling which provoked the increase in CO₂ concentration up to 81%. These values of CO₂ were coupled with low O₂ (less than 2 % of residual oxygen) which channelled the metabolism toward the fermentative route. No sensorial evaluation was made for tight-sealed flask cultures but it can be seen in figure 3 that large amounts of volatile were also produced.

Separation and identification of GC detected compounds

For headspace chromatograms of aerated and tight-sealed cultures of run 19, fourteen compounds were detected. Eleven compounds were identified through retention time comparison with a standard and can be classified according to their relative quantity (peak area): ethanol, ethyl acetate, ethyl propionate and isoamyl acetate are important; acetaldehyde, isoamyl alcohol and isobutyl acetate are intermediate and 1-propanol, 2-propanol, 1-butanol and amyl acetate are in small amounts. Among them, isoamyl acetate and isoamyl alcohol are known to be important compounds in the aroma of banana, while acetaldehyde, ethanol and ethyl acetate are always present in fruit aromas. Other minor compounds like ethyl propionate and isobutyl acetate are also reported to participate in fruit aromas.

Some differences can be observed between aerated and tight-sealed cultures. Acetaldehyde peak is bigger, ethyl propionate peak is smaller and unknown peak #13 is absent in the second case. Unfortunately, it is not possible to evaluate directly the impact of these differences on the aroma.

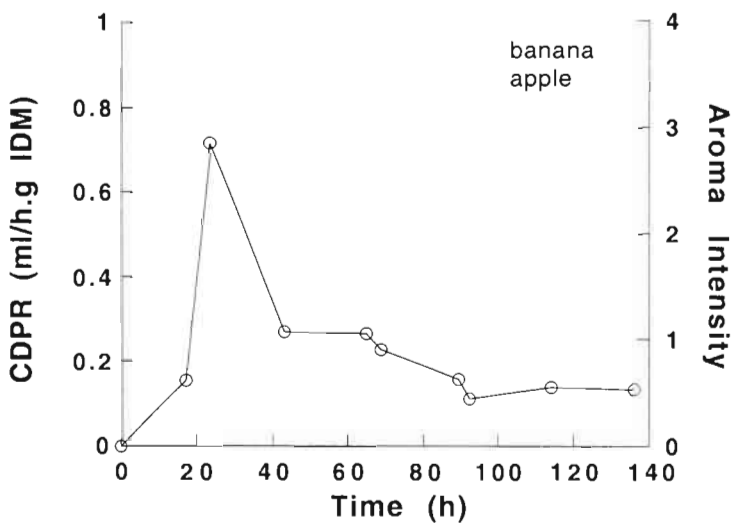


Figure 1. Evolution of Carbon Dioxide Production Rate (CDPR) during production of banana/apple aroma.

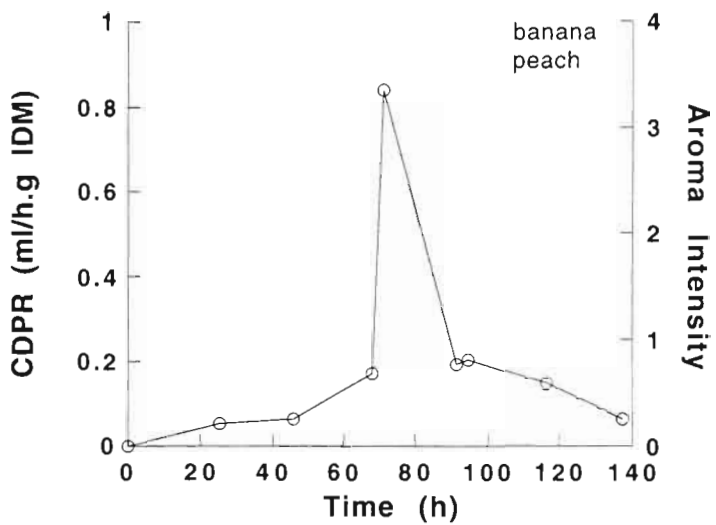


Figure 2. Evolution of Carbon Dioxide Production Rate (CDPR) during production of banana/peach aroma.

Conclusion

Wheat bran, cassava and sugarcane bagasse were found to be adequate substrates/support for aroma production by *C. fimbriata*. Amino acids like valine or leucine seemed to be direct precursors of banana-like aroma. Other aromas (peach, apple) were also detected without adding any precursor. The corresponding compounds of banana aroma (isoamyl alcohol and isoamyl acetate) were detected in the headspace of the culture at relatively important amounts. A total of 14 compounds were separated by GC and among them 11 were identified (1 aldehyde, 5 alcohols and 5 esters). Work is currently continued to identify the unknown peaks and to quantify the identified compounds.

Very low aeration (0.005 l/h.g IDM or passive diffusion) favoured the detection of strong aromas. Results were highly improved in these conditions in comparison with those obtained by Christen *et al.* (1994) at higher aeration rates. In the tight-sealed experiments, it was shown that the fungus was able to ferment the carbohydrates present in the medium (glucose in the case of bagasse, derivatives of starch in the case of wheat bran and cassava). The fact that very low or no aeration is required opens interesting technological perspectives for the production of fruity aromas by *C. fimbriata*.

References

- Bigelis, R. 1992. Flavor metabolites and enzymes from filamentous fungi. *Food Technol.* 46 (10) : 151-161.
- Christen, P., Raimbault, M. 1991. Optimization of culture medium for aroma production by *Ceratocystis fimbriata*. *Biotech. Lett.* 13 (7) : 521-526.
- Christen, P., Auria, R., Vega, C., Villegas, E., Revah, S. 1993. Growth of *Candida utilis* in solid state fermentation. *Biotech. Adv.* 11 : 549-557.
- Christen, P., Villegas, E., Revah, S. 1994. Growth and aroma production by *Ceratocystis fimbriata* in various fermentation media. *Biotech. Lett.* 16 (11) : 1183-1188.
- Christen, P. 1995. Producción de aromas en fermentación sólida. *Tópicos de Investigación y Docencia*. Submitted.
- Gervais, P., Sarrette, M. 1990. Influence of age of mycelium and water activity on aroma production by *Trichoderma viride*. *J. Ferment. Bioeng.* 69 (1) : 46-50.
- Hanssen, H.P., Sprecher, E. 1981. In: Schreier P. (ed.), *Flavour'81*, 547-556, W. de Gruyter, West Germany.
- Humphrey, M., Pearce, S., Skill, B. 1990. Biotransformation of coconut fat to methyl ketones. A commercial scale solid-state fermentation. In: *Abst. Symp. Bioformation of Flavours*. London.
- Janssens, L., de Pooter, H.L., Schamp, N.M., Vandamme, E.J. 1992. Production of flavours by microorganisms. *Process Biochem.* 27 : 195-215.
- Latrasse, A., Degorce-Dumas, J.R., Leveau, J.Y. 1985. Production d'arômes par les microorganismes. *Sci. Alim.* 5 : 1-26.
- Senemaud, C. 1988. Les champignons filamenteux producteurs d'arômes fruités. Etudes de faisabilité sur substrats agro-industriels. Ph. D. Thesis, Université de Bourgogne (France), 170p.
- Welsh, F.W., Murray, W.D., Williams, R.E. 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. *Crit. Rev. Biotech.* 9 (2) : 105-169.
- Yamauchi, H., Akita, O., Obata, T., Amachi, T., Hara, S., Yoshizawa, K. 1989. Production and application of a fruity odor in a solid-state culture of *Neurospora sp.* using pregelatinized polish rice. *Agric. Biol. Chem.* 53 (11) : 2881-2886.

FUNGAL BIOMASS ESTIMATION IN BATCH SOLID SUBSTRATE CULTIVATION USING ASYMPTOTIC OBSERVATION

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Abstract

Asymptotic observers for biomass estimation have been generalized for kinetic models that include mortality and maintenance coefficients, making them applicable to fermentation batch processes. The observer, which use CO₂ measurements and a non-linear model of the process, was applied to laboratory experiments with the fungus Gibberella fujikuroi. Model parameters were calibrated with laboratory data, using a non-degradable support to simplify biomass measurements. Convergence of the estimator is assured as long as the maintenance or mortality coefficients are non zero, and the sugars concentration is kept at a high level. However, the speed of convergence cannot be modified.

Introduction

The lack of on-line measurements of key variables is the main obstacle that hinders the development of proper control and optimization systems for fermentation processes. This is specially true for solid substrate cultivation (SSC), that lags behind submerged cultivation. To partially overcome this limitation of SSC processes, on-line observers of relevant variables such as biomass, secondary metabolites and nutrients concentration, can be developed. In fact, the high cost and scarcity of reliable sensors adapted to SSC systems, forces the development of softsensors (software + sensors) [1].

A large number of applications of softsensors has been reported in the literature. For example, the extended Kalman filter has been used successfully in the observation of variables in processes such as the effluent processing [2], foods drying [3] and vegetable cells cultivation [4]. The adaptive Kalman filter has been also used to determine the process states and, at the same time, certain time variant kinetic parameters [5, 6]. However these filters, given the non-linearity of the represented processes, loose the optimality character that has the linear Kalman filter. To overcome this problem, Ljung and Söderstrom [7] and then Goodwin and Sin [8] proposed an

algorithm called recursive predictive error. This algorithm was successfully used by Chattaway and Stephanopoulos [9] to detect contamination of bioreactors. However, these kind of estimators are difficult to implement, are sensitive to model parameters variations and are not generally applicable. The observability of the system model must be guaranteed throughout the whole process. These conditions are rarely met in batch fermentation processes, particularly in SSC.

To overcome the limitations of these Kalman based observers, Bastin and Dochain [10] proposed asymptotic estimators (without arbitrary convergence speed) especially adapted for fermentation processes. These estimators are useful in processes whose observability is not guaranteed, as are many batch fermentation non-linear processes. They have been successfully applied, given their implementation simplicity, in many biotechnology processes [11-15]. The observer developed in the present work, is heavily based on this kind of estimators.

First, the model of the SSC system is presented. Then, the observer is developed and conditions for convergence are analyzed. The application of the observer to laboratory data, with uncertainty in initial conditions, is discussed next. The work is concluded with some final remarks.

Model

The growth dynamics of Gibberella fujikuroi in a batch SSC process, can be represented by the next equation:

$$\frac{dX}{dt} = \mu \cdot X - K_d \cdot X \quad (1)$$

The variable X represents biomass concentration as gr. of dry biomass / gr. of dry matter (inert support). The term μ represents the specific growth rate and it is usually considered a function of temperature, pH, water activity and limiting substrate concentrations. The present model assumes that μ depends only on nutrients concentration, since the other variables are kept relatively constant, then:

$$\mu = \frac{\mu_m \cdot N}{[K_N \cdot X + N]} \cdot \frac{G}{[K_G + G]} \quad (2)$$

where N represents the substrate concentration in term of gr. of nitrogen / gr. of dry matter, G represents the hydrolyzed and non-hydrolyzed sugars concentration in gr. of sugars / gr. of dry matter, μ_m is the maximum specific growth rate, and K_N and K_G are kinetic parameters. It is assumed that the nitrogen consumption follows the Contois model, while sugars consumption follows a Monod model. This is justified since nitrogen is less available than sugars and its consumption is limited by diffusion.

Assuming that nitrogen consumption for maintenance and production is negligible, only the nitrogen used for biomass growth is taken into account:

$$\frac{dN}{dt} = -\mu \cdot X / Y_{X/N} \quad (3)$$

where $Y_{X/N}$ is the biomass/nitrogen yield coefficient.

Other authors [16, 17] used a different model that directly relates nutrients consumption with biomass growth kinetics:

$$\frac{dN_{ut}}{dt} = -\left[1/Y_{X/N_{ut}}\right] \frac{dX}{dt} \quad (4)$$

Then, introducing eq. (1), yields:

$$\frac{dN}{dt} = -\left[1/Y_{X/N}\right] \frac{dX}{dt} = -\left[1/Y_{X/N}\right] \left[\mu - K_d\right] \cdot X \quad (5)$$

This expression, which is different from eq. (3), is incorrectly used in the literature [18-20]. Then, eq. (4) is only applicable to the special case where autolysis is considered negligible.

The model used here for sugars consumption, follows the development of Acevedo [17]:

$$\frac{dG}{dt} = -\mu \cdot \left[X/Y_{X/G}\right] - m_G \cdot \frac{G}{K_G + G} \cdot X \quad (6)$$

where $Y_{X/G}$ corresponds to the biomass/sugars yield coefficient, and m_G to the maintenance coefficient.

The dynamics of CO_2 production and O_2 consumption rates are given by the following equations:

$$\frac{dCO_2}{dt} = \mu \cdot X / Y_{X/CO_2} + m_{CO_2} \cdot \frac{G}{K_G + G} \cdot X \quad (7)$$

$$\frac{dO_2}{dt} = -\mu \cdot X / Y_{X/O_2} - m_{O_2} \cdot \frac{G}{K_G + G} \cdot X \quad (8)$$

Here, CO_2 and O_2 are the accumulation of carbon dioxide formed and oxygen consumed, expressed in gr CO_2 / (gr dry matter) and gr O_2 / (gr dry matter) respectively. Y_{X/CO_2} and Y_{X/O_2}

are the biomass/carbon dioxide and biomass/oxygen yield coefficients respectively, and finally, m_{CO_2} and m_{O_2} are the maintenance coefficients for carbon dioxide and oxygen respectively.

In the above equations, X , N , G and CO_2 and O_2 are the state variables, where only carbon dioxide and oxygen outlet gas concentration are usually measured in SSC processes. Table 1 summarizes the model parameters estimated from laboratory data.

Table 1: Model Parameters

Parameter	value	units
CO_{20}	0.0000	[gr CO_2 /gr d.m.]
K_d	0.0012	[1/hr]
K_G	0.0800	[gr sugars/gr d.m.]
K_N	0.0500	[gr nitrog./gr biomass]
G_0	0.5000	[gr sugars./gr d.m.]
m_{CO_2}	0.0231	[gr CO_2 acc./gr biomass hr]
m_G	0.0420	[gr sugars/gr biomass hr]
N_0	0.0095	[gr nitrog./gr d.m.]
m_m	0.1650	[1/hr]
X_0	0.0060	[gr biomass/gr d.m.]
Y_{X/CO_2}	1.6700	[gr biomass/gr CO_2 acc.]
$Y_{X/G}$	0.5500	[gr biomass/gr d.m.]
$Y_{X/N}$	13.7000	[gr biomass/gr nitrog.]

Observer Development

The aim of the observer developed in this work, is to estimate biomass concentration based on carbon dioxide measurements. The use of exponential observers, as the Extended Kalman filter, emerges as the first alternative to be analyzed. However, the structure of the system, with state variables strongly interactive due to G and N influences, makes the observability and convergence analysis very cumbersome [21].

A much simpler and interesting alternative to exponential filters, are the asymptotic observers, as proposed in [10]. Unfortunately, the maintenance parameters in the model above, which depend on G , do not allow direct application of this method. In the above model, there is no auxiliary variable Z , which is only function of observed states. In fact, the model equations, excluding oxygen since it is not measured, written in vector form are:

$$\frac{dx}{dt} = K \cdot \Phi(x) + \Gamma(x) \quad (9)$$

with x the state vector,

$$x^T = [X, N, CO_2, G] \quad (10)$$

K a constants vector,

$$K^T = \left[1, -1/Y_{x|N}, 1/Y_{x|CO_2}, -1/Y_{x|G} \right] \quad (11)$$

with $\Phi(x)$ and $\Gamma(x)$, non-linear vector functions of the states, defined as,

$$\Phi(x) = \mu(N, G) \cdot X \quad (12)$$

$$\Gamma^T(x) = \left[-K_d \cdot X, 0, m_{CO_2}(G) \cdot x, -m_G(G) \cdot X \right] \quad (13)$$

This state representation can be split into two partitions:

$$\frac{dx_a}{dt} = K_a \cdot \Phi(x) + \Gamma_a(x) \quad (14)$$

$$\frac{dx_b}{dt} = K_b \cdot \Phi(x) + \Gamma_b(x) \quad (15)$$

Since $\text{rank}(K) = 1$, these states can be chosen in the following way:

$$x_a = X \quad (16)$$

$$x_b^T = [N, CO_2, G] \quad (17)$$

inducing the next partitions in K and G:

$$K_a = 1 \quad (18)$$

$$K_b^T = \left[-1/Y_{x|N}, 1/Y_{x|CO_2}, -1/Y_{x|G} \right] \quad (19)$$

$$\Gamma_a(x) = -K_d \cdot X \quad (20)$$

$$\Gamma_b^T(x) = \left[0, m_{CO_2} \frac{G}{K_G + G} \cdot X, -m_G \frac{G}{K_G + G} \cdot X \right] \quad (21)$$

According to the methodology proposed by Bastin and Dochain [10], this system can be transformed into another that do not include the specific kinetic vector, $F(x)$. For this purpose, an auxiliary variable, Z , can be defined:

$$Z = \begin{bmatrix} Z_1 \\ Z_2 \\ Z_3 \end{bmatrix} = \frac{-K_b}{K_a} \cdot x_a + x_b = \begin{bmatrix} 1/Y_{X/N} \\ -1/Y_{X/CO_2} \\ 1/Y_{X/G} \end{bmatrix} \cdot X + \begin{bmatrix} N \\ CO_2 \\ G \end{bmatrix} \quad (22)$$

whose dynamics is given by:

$$\frac{dZ}{dt} = \frac{-K_b}{K_a} \cdot \Gamma_a + \Gamma_b = \begin{bmatrix} 1/Y_{X/N} \\ -1/Y_{X/CO_2} \\ 1/Y_{X/G} \end{bmatrix} \cdot [-K_d \cdot X] + \begin{bmatrix} 0 \\ m_{CO_2} \cdot \frac{G}{K_G + G} \cdot X \\ m_G \cdot \frac{G}{K_G + G} \cdot X \end{bmatrix} \quad (23)$$

Finally, considering that the only observed variable is the CO_2 concentration, the vector Z can be reordered in the next form:

$$Z = \begin{bmatrix} Z_1 \\ Z_2 \\ Z_3 \end{bmatrix} = A_m \cdot x_m + A_n \cdot x_n = \begin{bmatrix} 0 \\ 1 \\ 0 \end{bmatrix} \cdot CO_2 + \begin{bmatrix} 1/Y_{X/N} & 1 & 0 \\ -1/Y_{X/CO_2} & 0 & 0 \\ 1/Y_{X/G} & 0 & 1 \end{bmatrix} \cdot \begin{bmatrix} X \\ N \\ G \end{bmatrix} \quad (24)$$

from where it is easy to obtain the non-observed state, x_n , in terms of the observed state, x_m , and the auxiliary variable, Z , yielding:

$$\begin{bmatrix} X \\ N \\ G \end{bmatrix} = \begin{bmatrix} 0 & -Y_{X/CO_2} & 0 \\ 1 & Y_{X/CO_2}/Y_{X/N} & 0 \\ 0 & Y_{X/CO_2}/Y_{X/G} & 1 \end{bmatrix} \cdot \begin{bmatrix} Z_1 \\ Z_2 - CO_2 \\ Z_3 \end{bmatrix} \quad (25)$$

Using the above matrix equations, the following asymptotic observer can be established:

$$\begin{bmatrix} d\hat{Z}_1/dt \\ d\hat{Z}_2/dt \\ d\hat{Z}_3/dt \end{bmatrix} = - \begin{bmatrix} 1/Y_{XF} \\ -1/Y_{XCO_2} \\ 1/Y_{XF} \end{bmatrix} \cdot [K_d \cdot \hat{X}] + \begin{bmatrix} 0 \\ m_{CO_2} \frac{\hat{G}}{K_G + \hat{G}} \cdot \hat{X} \\ m_G \frac{\hat{G}}{K_G + \hat{G}} \cdot \hat{X} \end{bmatrix} \quad (26)$$

with

$$\begin{bmatrix} \hat{X} \\ \hat{N} \\ \hat{G} \end{bmatrix} = \begin{bmatrix} 0 & -Y_{XCO_2} & 0 \\ 1 & Y_{XCO_2}/Y_{XF} & 0 \\ 0 & Y_{XCO_2}/Y_{XF} & 1 \end{bmatrix} \cdot \begin{bmatrix} \hat{Z}_1 \\ \hat{Z}_2 - CO_2 \\ \hat{Z}_3 \end{bmatrix} \quad (27)$$

using the following initial conditions for \hat{Z} :

$$\begin{bmatrix} Z_1(0) \\ Z_2(0) \\ Z_3(0) \end{bmatrix} = \begin{bmatrix} 0 \\ 1 \\ 0 \end{bmatrix} \cdot CO_2(0) + \begin{bmatrix} 1/Y_{XF} & 1 & 0 \\ -1/Y_{XCO_2} & 0 & 0 \\ 1/Y_{XF} & 0 & 1 \end{bmatrix} \cdot \begin{bmatrix} X(0) \\ N(0) \\ G(0) \end{bmatrix} \quad (28)$$

where $\hat{}$ means estimated variable.

Observer analysis

Here, the dynamics of \hat{Z} (eq. 26) is not independent of the non-observed states. Therefore, the above observer does not corresponds to the asymptotic observer as it was outlined in [10]. Then, it is necessary to analyze carefully the stability conditions of this new estimator. To do this, it is useful to consider the error estimate vector dynamics, given by:

$$\begin{bmatrix} dE_{z_1}/dt \\ dE_{z_2}/dt \\ dE_{z_3}/dt \end{bmatrix} = \begin{bmatrix} 0 & C_1 & 0 \\ 0 & -C_2 & 0 \\ 0 & C_3 & 0 \end{bmatrix} \cdot \begin{bmatrix} E_{z_1} \\ E_{z_2} \\ E_{z_3} \end{bmatrix} \quad (29)$$

with

$$\begin{bmatrix} E_{Z_1} \\ E_{Z_2} \\ E_{Z_3} \end{bmatrix} = \begin{bmatrix} Z_1 - \hat{Z}_1 \\ Z_2 - \hat{Z}_2 \\ Z_3 - \hat{Z}_3 \end{bmatrix} \quad (30)$$

and the functions C_1 , C_2 and C_3 , given by:

$$\begin{bmatrix} C_1 \\ C_2 \\ C_3 \end{bmatrix} = \begin{bmatrix} K_d \cdot Y_{X/CO_2} / Y_{X/N} \\ (K_d / Y_{X/CO_2} + m_{CO_2} \cdot G / (K_G + G)) \cdot Y_{X/CO_2} \\ (K_d / Y_{X/G} + m_G \cdot G / (K_G + G)) \cdot Y_{X/CO_2} \end{bmatrix} \quad (31)$$

From the characteristic polynomial of eq. (29), and assuming that:

$$\frac{G}{K_G + G} \equiv \frac{\hat{G}}{K_G + \hat{G}} \quad (32)$$

the eigenvalues of the error dynamics are: $\lambda_1 = \lambda_3 = 0$ and $\lambda_2 = -C_2$. The above assumption is valid only for sugars concentration values larger than $10 \cdot K_G$, that is, $G > 0.7$ (gr. / gr. d.m.), during all the course of the cultivation.

Therefore, the estimation error will disappear asymptotically for E_{Z_2} , as long as $C_2 > 0$. In the case of E_{Z_1} and E_{Z_3} , any initial error will not be drove to zero. In terms of the state variables, eq. (27) indicates that only X could be estimated asymptotically without error, since its estimation depends only on \hat{Z}_2 . On the other hand, the estimation of N and G will be always affected by the initial estimation errors.

Results and Discussion

Figure. 1 illustrates the above statements. In this simulated example, high sugars concentrations have been used. Different initial error estimates of N, G and X, have been considered; 10%, 10% and 500% respectively. As it was expected, the initial error remains in the estimation of N and G. On the other hand, the estimation of X converges to its true value.

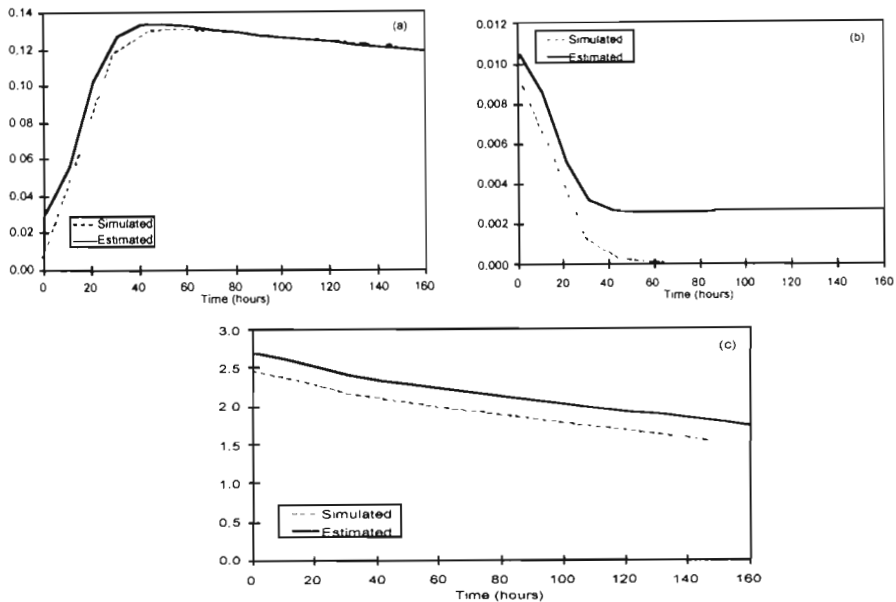


Figure 1: Estimation of biomass (a), nitrogen (b) and sugars (c). Initial estimation error of 10% for sugars and nitrogen concentrations and 500% for biomass. High initial sugars concentration, $G(0)=2.5$ (gr. / gr. d.m.). Symbols and discontinuous lines represent experimental measurements.

The instability of the estimator for low sugars concentrations ($G < 0.7$ gr. / gr. d.m.) can be appreciated in Figure 2. In this case, all the estimation errors growth without limit, even though the same initial estimate errors used in the previous figure were considered in this simulation.

It is worth noting that the developed observer for SSC processes converges in spite of being a batch process. Bastin and Dochain [10] have shown that the asymptotic estimator converges in continuous and fed-batch fermentation, as long as the dilution rate, D , is different from zero for long periods. Since in batch processes $D = 0$, the asymptotic estimator does not generally converges. However, the observer developed in the present work converges because the parameters K_d and m_{CO_2} , which define the value of C_2 in eq. (31), are not zero.

Finally, it must be pointed out that with asymptotic observers the speed of convergence cannot be modified. This is an even more serious limitation in batch processes, since in continuous and fed-batch processes the dilution rate can be used to speed up the convergence. Unfortunately, parameters as K_d and m_{CO_2} are intrinsic of the process and they cannot be modified to accelerate the estimation convergence.

In the example shown in Fig. 1, it is seen that such convergence is reached, for biomass, approximately after 100 hours. This corresponds to approximately 60% of the total time of the cultivation process, and once the exponential growth phase has already ended. This is not very good from the process control point of view, since it is in such phase where coherent actions must be exercised to attain the desired biomass concentration level.

The use of exponential or predictive observers, which provide faster convergence, stays as an alternative that will be dealt in future studies. To apply these observers to the model described in the present work, high values of sugars concentration are also required.

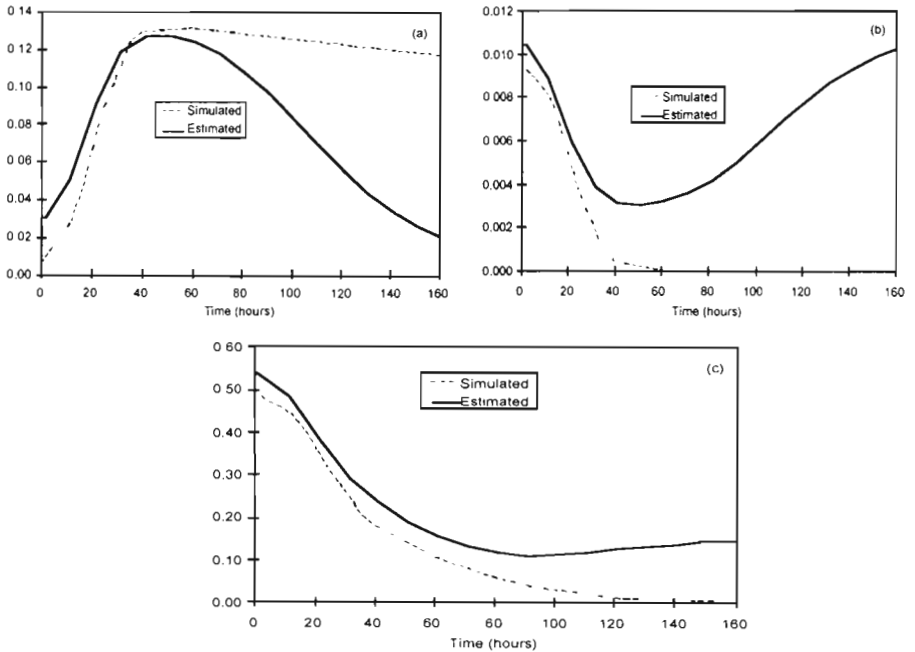


Figure 2: Estimation of biomass (a), nitrogen (b) and sugars (c). Initial estimation error of 10% for sugars and nitrogen concentrations and 500% for biomass. Low initial sugars concentration, $G(0)=0.5$ (gr. / gr. d.m.). Symbols and discontinuous lines represent experimental measurements.

Conclusions

The observers development method proposed by Bastin and Dochain [10], has been generalized for kinetic models that include mortality and maintenance coefficients, and applied to a SSC process. In addition, an analysis of the estimator concluded that it will converge for the main variable (biomass concentration), provided that the carbon source concentration is high enough and the mortality or maintenance coefficients are different from zero.

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References

- 1 Royce, P., 1993, A discussion of recent developments in fermentation monitoring and control from a practical perspective, *Crit. Rev. Biotechnol.*, 13:117-149.
- 2 Ayesa, E., Florez, J., Garcia-Heras, J.L., Larrea, L., 1991, State and coefficients estimation for the activated sludge process using a modified Kalman filter algorithm, *Wat. Sci. Tech.*, 24:235-247.
- 3 Kiranoudis, C.T., Dimitratos, J., Maroulis, Z.B., Marino-Kouris, D., 1993, State estimation in the batch drying of foods. *Drying Technol.*, 11:1053-1069.
- 4 Albiol, J., Robuste, J., Casas, C., Poch, M., 1993, Biomass estimation in plant cell cultures using an extended Kalman filter, *Biotechnol. Prog.* 9:174-178.
- 5 Stephanopoulos G., San, K.Y., 1984, Studies on on-line bioreactor identification I: Theory, *Biotechnol. Bioeng.*, 26:1176-1188.
- 6 Shimizu, H., Takamatsu, T., 1989, An algorithmic approach to constructing the on-line estimation system for the specific growth rate, *Biotechnol. Bioeng.*, 33:354-364.
- 7 Ljung, L., Söderstrom T., 1983, Theory and practice of recursive identification. MIT Press.
- 8 Goodwin, G.C., Sin, K.S., 1984, Adaptive filtering, prediction and control. Prentice-Hall, Englewood Cliffs, New Jersey.

- 9 Chattaway, T., Stephanopoulos, G.N., 1989, An adaptive state estimator for detecting contaminants in bioreactors, *Biotechnol. Bioeng.*, 34:647-659.
- 10 Bastin, G., Dochain, D., 1990, On-line estimation and adaptive control of bioreactors, Elsevier Science Publishing Co., Amsterdam.
- 11 Acuña, G., Ladrille, E., Béal, C., Corrieu, G., Chéruey, A., 1994, On-line estimation of biological variables during pH controlled lactic acid fermentations, *Biotechnol. Bioeng.*, 44:1168-1176.
- 12 Dochain, D., Pauss, A., 1988, On-line estimation of microbial specific growth-rates: an illustrative case study, *Can. J. Chem. Eng.*, 66:626-630.
- 13 Flaus, J.M., Chéruey, A., Pons, M.N., Engasser, J.M., 1989, Estimation de l'état physiologique des microorganismes dans un bioprocédé, *RAIRO-APII*, 23:211-220.
- 14 Lubenova, V., Ignatova, M., Tsonkov, S., 1993, On-line estimation of specific growth rate for a class of aerobic batch processes, *Chem. Biochem. Eng. Q.* 7:101-106.
- 15 Pomerleau, Y., Perrier, M., 1990, Estimation of a multiple specific growth rates in bioprocesses, *AIChE J.* 36:207-215.
- 16 Raimbault M. 1981. Fermentation en Milieu Solide, Croissance de Champignons Filamenteux sur Substrat Amylacé. Service des Publications de L' O.R.S.T.O.M. Paris, France, p 132- 132, 149-153, 166-167, 176, 222-223.
- 17 Acevedo F. 1988. Cinética de Fermentaciones. Tercer Curso Latinoamericano de Biotecnología, Octavo Curso Internacional de Ingeniería Bioquímica. Universidad Católica de Valparaíso, Escuela de Ingeniería Bioquímica, Organización de los Estados Americanos, Agosto 7-19 de 1988.
- 18 Menezes J. C. , Alves S. S. 1994 Mathematical Modelling of Industrial Pilot Plant Penicillin-G ; Fedbatch Fermentations. *J. Chem. Tech. Biotechnol.* 61, p 123-138.
- 19 Demain A. y Salomon N. 1988 Manual of Industrial Microbiology and Biotechnology. American Society for microbiology, Washinton D. C. , p 138.
- 20 Huang S. Y. Chow M. S. 1990 Jinetic Model for Microbial Uptake of Insoluble Solid-State Substrate. *Biotech. and Bioeng.* 35, p 547-558.
- 21 Ljung, L., 1979, Asymptotic behaviour of the Extended Kalman filter as a parameter estimator for linear systems. *IEEE Trans. autom. Control* 24, 36-50.

MUTAGENESIS AND ADAPTED STRAINS TO THE GROWTH IN LIQUID OR SOLID SUBSTRATES

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Summary

Mutation by UV light resulted in the isolation of *A. niger* strains geared towards high pectinase production in either SSF or SFM but never in both. Water activity plays an essential role on the levels of activity of specific enzymes. Mutated strains showed growth and sporulation patterns distinguishable by morphometric techniques, when cultured on defined media.

Many enzymes which break down complex polymers are produced commercially or are being developed for such use. Biosynthesis of these enzymes is often controlled by carbon catabolic repression. Selection for mutants resistant to analogues of glucose has been used to isolate mutants which are deficient in carbon catabolic repression of these enzymes. For example, resistance to 2-deoxyglucose has been used to isolate deregulated mutants of *Trichoderma*, which overproduce cellulase [17] or as we shall see below, to select for mutants overproducing pectinases in either SSF or SmF.

Shankaranand *et al.* [2] suggested that microbial strains selected for SSF processes should be different to those selected for SmF processes. They found, for example, that in a collection of nearly 30 bacterial strains, the majority of them were good enzyme producers either in SSF or SmF but seldom in both. Antier *et al.* [1, 3] isolated UV mutants of wild strain *A. niger* C28B25 [4].

The selection phenotype was 2-deoxy-glucose (DG) resistance (DG^R) but in culture media with two different water activities ($a_w = 0.99$ or 0.96) such phenotypes were labelled to belong to classes: a) AW99 and b) AW96 (when $a_w = 0.96$ after adding 15% ethylene glycol). Mutants AW99 had an inverse correlation between their ability to produce pectinase by SSF on coffee pulp ($a_w = 0.96$) as shown by solid bars in figure 1 with respect to the production of pectinase by SmF shown as clear bars in the same figure. Apparently there was a trade off relation between each kind of those phenotypes. Strain WT in that figure corresponds to wild type (C28B25) isolated by Boccas *et al.* [4]. Therefore, strain AW99-iii had seven times less potency for SSF pectinase production and more than three times more potency for SmF as compared to WT (see figure 1).

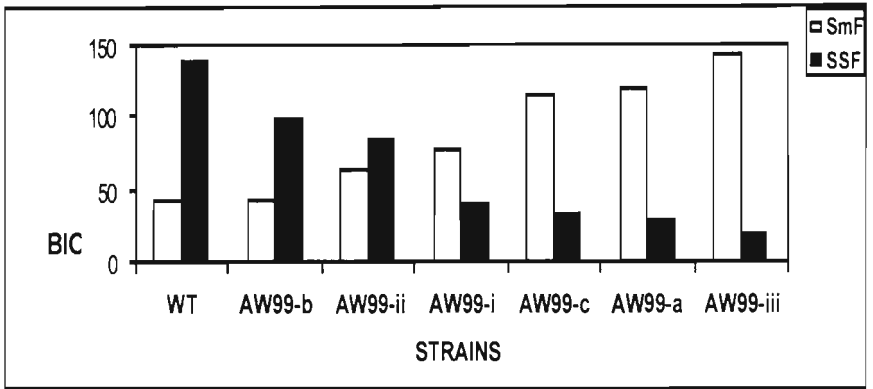


Figure 1. Comparison of pectinase activities of DG^R mutants of *A. niger* C28B25 isolated at high water activity ($a_w = 0.99$) and cultured in shake flasks (SmF) or coffee pulp packed bed columns (SSF) according to Antier *et al.* [1]. U PEC = arbitrary enzyme viscometry units expressed by g of solid substrate (SSF) or dry biomass (SmF).

In Figure 2 the same kind of results are shown for AW96 mutants. Here the inverse correlation of pectinase production by SSF and SmF is not as evident as in Fig. 1. Strain WT is the same wild type shown in the previous figure. All AW96 mutants had equal or higher potency in SmF than the wild type but some of them (strains AW96-1 and 3 in Fig. 2) increased their potency in SSF by nearly 40% over WT.

Antier *et al.* [3] showed that DG^R phenotype was independent from AW96 and AW99 phenotypes. Since a DG^R reverting strain (AW96-3 became DG^S) was found to retain high pectinase productivity in low water activity but became highly sensitive to DG as the wild type [3].

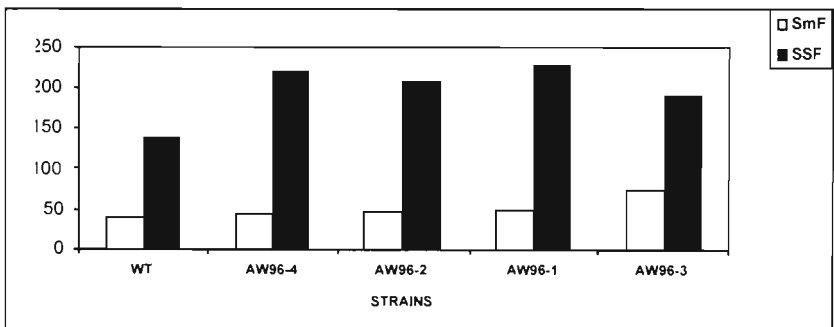


Figure 2. Comparison of pectinase activities of DG^R mutants of *A. niger* C28B25 (WT) isolated at low water activity ($a_w = 0.96$) and cultured in shake flasks (SmF) and coffee pulp packed bed columns (SSF) according to Antier *et al.* [1]. U PEC are arbitrary enzyme units by viscometry expressed by g of solid substrate (SSF) or dry biomass (SmF).

Recent work in our laboratory (Romero *et al.*, 1996 and 1997, unpublished data) has shown that strains AW96 and AW99 are derepressed for the production of pectinase, invertase and amylase, although they were selected on the basis of pectinase over production [1, 3].

Such observations support the existence of general regulatory mechanisms involved in the adaptation of moulds to liquid or solid environments and controlling the yield and quality of enzymes best suited for each kind of culture medium. As a consequence, the selection of strains for the production of enzymes by SSF technique requires the use of specific protocols including the survival to metabolic stress factors, namely, the presence of antimetabolites such as DG [1] or dinitro phenol [3] and also low levels of water activity.

Concerning the importance of water activity for the production of enzyme by SSF technique, Narahara *et al.* [13] proposed the use of mixtures of steamed rice particles with lignocellulosic particles in order to increase the moisture content of koji SSF fermentation for the production of amylases. Oriol *et al.* [11] used mixtures of steam cooked cassava granules with sieved fibres of sugar cane bagasse. This was based on the fact that bagasse can absorb four-fold its dry weight in water whereas cassava granules can only absorb one-fold its dry weight in water. The overall moisture content of those mixtures was a weighed average of the corresponding fractions of each material. Thus, initial moistures could be adjusted in the range from 42% up to 70%. A summary of their results is shown in Table 3. Similar results were obtained using different levels of glucose absorbed in bagasse, from 10 to 300 g/L [11].

The value of the specific growth rate was also an increasing function of a_w . In this system there was an optimum value for enzyme productivity (not shown in Table 2) because bagasse fibres did not contain starch nor were inoculated with mould spores, they were only used as a water reservoir. This experiment showed the presence of inter-particle mass transfer of moisture and suggested the use of mixed materials in order to correct for low moisture content in amylaceous substrates.

Table 3. Effect of initial water activity (a_w) on the maximal amount of biomass (X_{max}) and substrate conversion of *Aspergillus niger* No. 10 [11].

Initial a_w .	X_{max} g/g Initial Dry Weight	SC g/g Initial Dry Weight
0.933	0.123	0.233
0.940	0.172	0.344
0.958	0.251	0.417
0.957	0.324	0.477
0.980	0.339	0.558

Pandey [12] in his review of SSF processes comments "the types of micro-organisms that can grow in SSF systems are determined by the water activity factor a_w defined as the relative humidity of the gaseous atmosphere in equilibrium with the substrate"... "The micro-organisms, which can grow and are capable of carrying out their metabolic activities at lower

a_w values are suitable for SSF processes". Sarrete *et al.* [14] showed that addition of glycerol to a tempeh SSF fermentation by *Rhizopus oligosporus* changed the yield of various polysaccharidases having different optimal conditions, for example, polygalacturonases and xylanases were maximal for a_w values between 1.00 and 0.99 but endocellulase production was maximal when $a_w = 0.98$. Acuña-Argüelles *et al.* [15] used ethylene glycol as water depressor of SSF cultures of *A. niger* grown on bagasse as support. They found that decreasing values of a_w from 0.98 to 0.90 resulted in decreasing activities of exopectinase, as shown in Table 4.

Those results are in agreement with earlier observations [16] showing that the production of polygalacturonase and cellulase by *Geotrichum candidum* was very sensitive to a_w reduction, from 1.00 to 0.98, by the addition of KCl, mannitol or polyethylene glycol. Also, Grajek and Gervais [10] found that the production of polygalacturonase, D-xylanase and β -galactosidase by SSF cultures of *Tichoderma viridae* TS was influenced by water activity.

Table 4. Effect of water activity on the level of exopectinase produced by *A. niger* CH4, [15].

Water activity	Exopectinase activity (end point mg of reducing compounds per ml)
0.98	20
0.97	18
0.94	15
0.90	5

Minjares-Carranco *et al.*, [8] carried out physiological comparisons between pectinase-producing mutants of *A. niger* adapted either to SSF or SmF through morphometric analyses. Parent and mutant strains were grown on a specific medium and morphological measurements were performed with a digital image analyser. For the characterisation of each strain, two indexes were used Ib (growth rate of a colony on a given medium relative to maximum growth rate on medium DEX) and Ia (sporulated area over total area of colony after 72 hrs of growth). Each strain was then represented by a plot on the Ia versus Ib plane. Figure 3 shows the « mapping » of the wild type strain and the mutant strains. They map in three distinct regions.

Although the mutants described above were selected for their resistance to 2-deoxyglucose, the resulting pectinase-producing strains from both classes (AW99 and AW96) show distinct physiological and phenotypic patterns. The deoxyglucose resistance phenotype may not be directly involved with the complex patterns of physiological derepression and enzyme production.

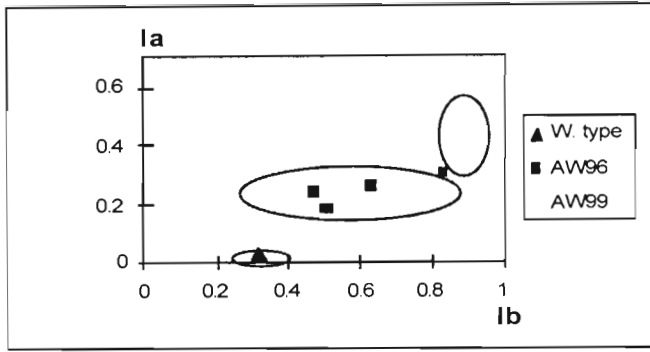


Figure 3.- Ia and Ib values obtained for wild type (triangles) and mutant strains of series AW96 (square) and AW99 (circle). Each plot represents the average of triplicate measurements on a given strain.

Bibliography

1. Antier, P., A., Minjares and G. Viniegra: Pectinase-hyperproducing mutants of *Aspergillus niger* C28B25 for solid state fermentation on coffee pulp. *Enzyme Microb. Technol.* **15** (1993a), 254-260.
2. Shankaranand, V., Ramesh, M.V. and Lonsane, B.K.: Idiosyncrasies of solid state fermentation systems in the biosynthesis of metabolites by some bacterial and fungal cultures. *Process Biochem.* **25** (1992), 33-36
3. Antier, Ph., Minjares, A., Roussos, S., and Viniegra-González, G.: New approach for selecting pectinase producing mutants of *Aspergillus niger* well adapted to solid state fermentation. *Biotechnol. Adv.* **11** (1993), 429-440.
4. Boccas, F., Roussos, S., Gutiérrez-Rojas, M., and Viniegra-González, G.: Production of pectinase from coffee pulp in solid state fermentation system: selection of wild fungal isolates of high potency by a simple three step screening technique. *J. Food Sci. Technol.* **31**(1) (1994), 22-26.
5. Loera-Corral, O.: *Estudios genéticos de las mutantes de Aspergillus niger C28B25 sobre productoras de pectinasas en sustratos líquidos y sólidos*. M. Sc. thesis in Biotechnology. Universidad Autónoma Metropolitana, Iztapalapa, D.F. (Méx.) (1994).
6. Allen, K.E., McNally, M.T., Lowendorf, H.S., Slayman, C.W., and Free, S.J.: Deoxyglucose-resistant mutants of *Neurospora crassa*: isolation, mapping, and biochemical characterization.. *J. Bacteriol.* **171** (1989), 53-58.
7. Alazard, D., Raimbault, M.: Comparative study of amylolytic enzyme production by *Aspergillus niger* in liquid and solid state cultivation. *J. Appl. Microbiol. Biotechnol.* **12** (1981), 113-117.
8. Minjares-Carranco, A., Trejo-Aguilar, B.A., Aguilar, G., and Viniegra-González, G.: Physiological comparisons between pectinase-producing mutants of *Aspergillus niger* adapted

- either to solid-state fermentation or submerged fermentation. *Enzyme Microb. Technol.* **21** (1997), 25-31.
9. Acuña-Argüelles, M., Gutiérrez-Rojas, M., Viniegra-González, G., Favela-Torres, E.: Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid state fermentation. *Appl. Microbiol. Biotechnol.* **43** (1995), 1-6.
- 10.-Grajek, W., and Gervais, P.: Influence of water activity on the enzyme biosynthesis and enzyme activities produced by *Trichoderma viride* TS in solid state fermentation. *Enzyme Microbiol. Technol.* **9** (1987), 658-662.
- 11- Oriol, E., Raimbault M., Roussos S., and Viniegra-González G.: Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **27**. (1988), 498-503.
- 12.- Pandey, A.: Recent process developments in solid state fermentation. *Process Biochemistry.* **27** (1992), 109-117.
- 13.- Narahara, H., Koyama, Y., Yoshida, T., Atthasampunna, P., Taguchi, H.: Control of water content in a solid-state culture of *Aspergillus oryzae*. *J. Ferment. Technol.* **62** (1984), 453-459.
- 14.- Sarrette, M., Nout, M.J.R., Gervais, P., and Rombouts, F.M.: Effect of water activity on the production and activity of *Rhizopus oligosporus* polysaccharidases. *Appl. Microbiol. Biotechnol.* **37** (1992), 420-425.
- 15.- Acuña-Argüelles, M., Gutiérrez-Rojas, M., Viniegra-González, G., Favela-Torres, E.: Effect of water activity on exo-pectinase production by *Aspergillus niger* CH4 on solid state fermentation. *Biotechnol. Lett.* **16** (1) (1994), 23-28.
- 16.- Davis, L.L., and Baudoin B.A.M.: Effect of osmotic potential on synthesis and secretion of polygalacturonase and cellulase by *Geotrichum candidum*. *Can. J. Microbiol.* **33** (1987), 138-141.
- 17.- Labudova, I., and Farkas, V. Enrichment technique for the selection of catabolite-repression -resistant mutants of *Trichoderma* as producers of cellulase. *FEMS Microbiol Letters* **20** (1983) -211-215

FUNGAL GENETICS, CASE OF ASPERGILLUS

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Summary

Classical genetic techniques enabled the selection of an arginine minus auxotroph from each of the mutant strains AW99 and AW96. When crossed, these two deoxyglucose resistant strains yielded a deoxyglucose sensitive diploid. Foreign DNA (via plasmids) can be introduced into the AW99 strain in a stable manner, by electroporation or by polyethylene glycol in the presence of protoplasts. Genes can be directed to a specific site of the genome by Random Enzyme Mediated Integration (REMI).

Pectinases play an important role in industry in the processing of fruit and vegetable juices as they can alter the viscosity and facilitate extraction, filtration and clarification processes [1]. A favourite organism for the production of pectinases is *A. niger* which has been modified by mutation or classical genetics [7] and through molecular genetics [6]. In both cases it was possible to increment production of specific pectinases. Antier *et al.* [3] devised a strategy to increase pectinase production using SSF (coffee pulp) to which the parent strain C28B25 was added. Following UV-induced mutagenesis, mutant strains were derived from *A. niger* C28B25 belonging either to series AW99 [producing maximum pectinase levels in submerged fermentations (SmF)] or series AW96 [producing maximum pectinase level in solid state fermentation (SSF)]. Mutants selected by Antier *et al* were resistant to catabolic repression by saccharose whereas the parent strain was sensitive.. In order to genetically characterise these strains, Loera *et al.*[4] decided to devise their own. It was decided to induce a mutation in the set of DG^R strains in the form of an auxotrophy to arginine. Furthermore, DNA could then be introduced by transformation into the auxotrophic strains of a plasmid pDHG25. This plasmid contained the *argB* gene which could complement a specific arginine deficient strain. For this study two pectinase hyperproducing strains were selected: AW99 and AW96 (Figure 2). These strains were mutagenized by irradiation with UV light. This step was followed by an enrichment step during which germinating spores on minimal medium were eliminated with the aim of picking up non germinated spores with a higher probability of being auxotrophic [5]. One arginine mutant was obtained from each of the two parent strains, resulting in the following strains : AW99arg⁻ and AW96arg⁻. These two strains were then crossed. Loera-Corral [4] obtained a dikaryon of the two DG^R strains (AW99arg⁻ x AW96arg⁻).

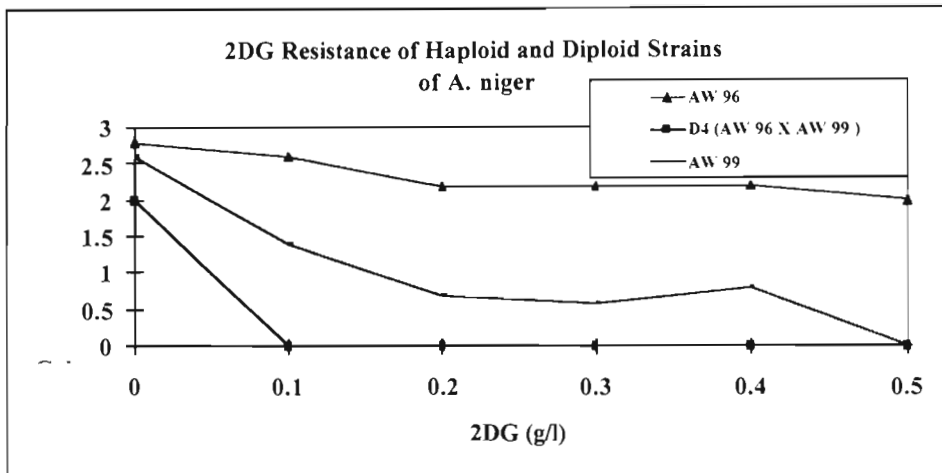


Figure 1. Deoxyglucose resistance of haploid and diploid strains of *A. niger*

The D4 dikaryon shown above in Figure 1 became deoxyglucose sensitive (DG^S); all other phenotypic characteristics were that of strain AW99 [4]. Phenotypes DG^R were complementary to each other (as seen when diploid D4 was obtained) but phenotype AW99 was dominant over AW96. The way in which the phenotype DG^R is associated to the selection of AW96 and AW99 phenotypes is still unknown. Allen *et al.*, [2] have found pleiotropic mutations of *Neurospora crassa* which are associated to DG^R phenotype, mapping as DG^R point mutants in four different loci, and at the same time, show derepressed and modified patterns of invertase and amylase production.

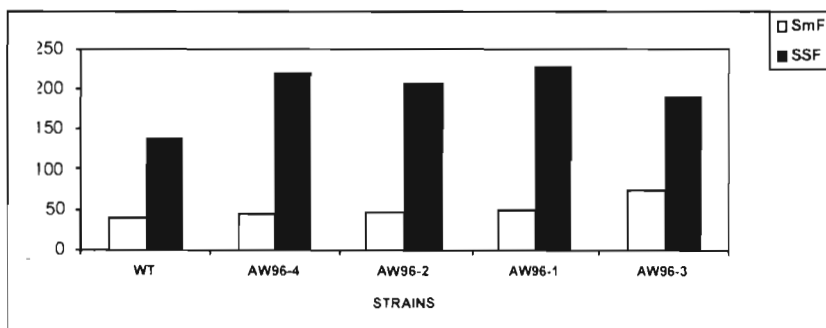


Figure 2. Comparison of pectinase activities of DG^R mutants of *A. niger* C28B25 (WT) isolated at low water activity ($a_w = 0.96$) and cultured in shake flasks (SmF) and coffee pulp packed bed columns (SSF) according to Antier *et al.* [3]. U PEC are arbitrary enzyme units by viscometry expressed by g of solid substrate (SSF) or dry biomass (SmF).

Recent work in our laboratory (Romero *et al.*, 1996 and 1997, unpublished data) has shown that strains AW96 and AW99 are derepressed for the production of pectinase, invertase and amylase, although they were selected on the basis of pectinase over production [3].

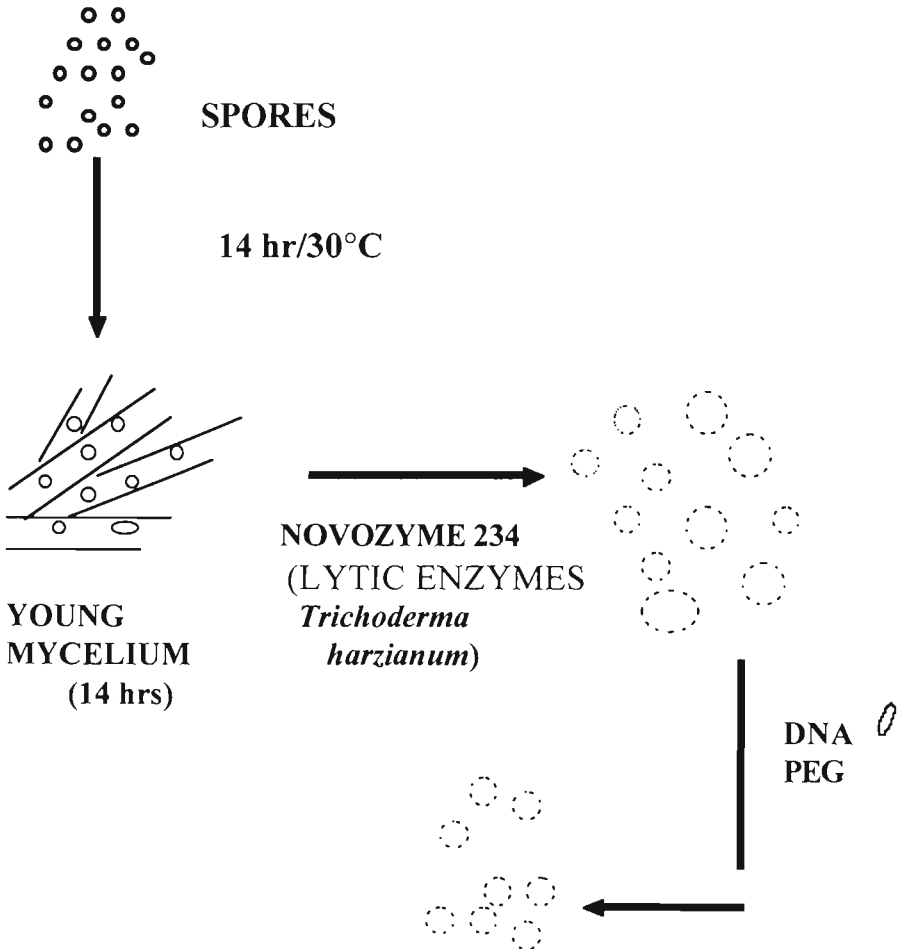
Such observations support the existence of general regulatory mechanisms involved in the adaptation of moulds to liquid or solid environments and controlling the yield and quality of enzymes best suited for each kind of culture medium. As a consequence, the selection of strains for the production of enzymes by SSF technique requires the use of specific protocols including the survival to metabolic stress factors, namely, the presence of antimetabolites such as DG or dinitro phenol and also low levels of water activity.

After development through classical genetics of strain AW99argB-, we used molecular genetic methods to test for the introduction of foreign DNA (via plasmids) into AW99arg-. The introduction of foreign genes into fungi has required the setting up of methods adapted from yeasts and bacteria. The arginine auxotroph AW99argB- was used as a host to study the introduction of two plasmids, pDHG25 (an autonomously replicating plasmid; see description in Annex) and pDC1 (an integrative plasmid, see description in Annex). Both plasmids were introduced into the host either by electroporation (see conditions in Annex) or by polyethylene glycol mediated introduction in protoplasts. A scheme of the latter technique is presented in annex. Additionally, the REMI (random enzyme mediated integration) technique was tested. This technique consists of adding a restriction enzyme (BamHI) along with the DNA to be introduced into the fungal cells. If the fungal genome contains within its DNA, the restriction site, the introduced DNA integrates within that site in the genome. *A. niger* is known to have only one BamHI site in its genome. Results of these experiments are presented in Annex.

The first conclusion to be made is that it is possible to insert foreign DNA into the AW99arg- strain either by electroporation or by polyethylene glycol (PEG) via protoplasts. In both cases, the number of transformants depends greatly on the type of DNA introduced (linear, circular). The integrative plasmid (pDC1) yields a greater number of transformants, irrespective of the method employed. REMI via electroporation of the integrative plasmid pDC1, seems to be the method of choice. Introduction of the linear pDC1 plasmid in the BamHI site of the genome induced a phenotypic mutation known as fluffy (data not shown) where no sporulation occurs. The integration of the plasmid therefore disrupted an essential gene involved in conidiation.

Foreign genes can therefore be introduced into the laboratory strains for study. The introduced genes are stable and can either replicate autonomously (if on the pDHG25 plasmid) or integrate into the genome either randomly or specifically (via REMI).

TRANSFORMATION BY PROTOPLAST FUSION



RESULTS FROM ELECTROPORATION

STRAIN: *A. niger dgrAW99arg* SPORE CONCENTRATION = 10^7

PLASMID	No. of TRANSFORMANTS / μ g DNA	λ PULSE (ms)
Negative control	-	6.7
pDHG25 (circular)	4	5.5
pDHG25 linear/BamHI	32	5.6
pDC1 (circular)	16	6.5
pDC1 linear/BamHI	40	6.3
pDC1 linear+ 1 U BamHI	68	6.5
pDC1 linear + 5 U BamHI	12	5.9
pDC1 linear + 10U BamHI	3	6.3

Conditions used for transformation:

Voltage (kv)	Resistance (Ω)	Concentration of DNA (μ g)	Recuperation time (hr)
1.0	400	0.25	2.5

RESULTS FROM PROTOPLAST TRANSFORMATION

STRAIN: *A. niger dgrAW99arg* SPORE CONCENTRATION = 10^7

TYPE OF DNA	No. TRANSFORMANTS/ μ g DNA
Negative control	2-3
Positive control pDHG25 linear BamHI TREATED AT 65°C	75
pDHG25 (circular)	24
pDC1 circular	120
pDC1 linear /BamHI	18
pDC1 linear /BamHI + 1 unit restriction enzyme	12
pDC1 linear /BamHI + 5 unit	6

restriction enzyme	
pDC1 linear /BamHI + 10 units restriction enzyme	2

STABILITY RESULTS

Strain: *A. niger dgrAW99arg*

transformation method: Electroporation

Type of plasmid	Percent stability
pDHG25 circular	60 %
pDHG25 linearized	68 %
pDC1 circular	75 %
pDC1 linearized	70 %
pDC1 linearized + 1 U BamHI	71 %
pDC1 linearized + 5 U BamHI	74 %
pDC1 linearized + 10 U BamHI	67 %

Strain: *A. niger dgrAW99arg*

Transformation method: Protoplasts

Type of plasmid	Percent stability
pDHG25 circular	70 %
pDHG25 linearized	65 %
pDC1 circular	79 %
pDC1 linearized	83 %
pDC1 linearized + 1 U BamHI	83 %
pDC1 linearized + 5 U BamHI	81 %
pDC1 linearized + 10 U BamHI	79%

Bibliography

- 1.- Ward, O. P. (1985). Hidrolytic Enzymes. In Blanch, H. W.; Drew, S.; Wang, D. I. (Des) *Comprehensive Biotechnology*. Pergamon Press. New York. **3**: 819-835.
- 2.- Allen, K. E.; MacNally, M. T.; Lowendorf, H. S.; Slayman, C. W.; Free, S. J. (1989) Deoxiglucose-Resistance Mutants of *Neurospora crassa*: Isolation, Mapping and Biochemical Characterisation. *J. Bacteriol.* **171** (1): 53-58.
- 3.- Antier, P.; Minjares, A.; Viniegra, G. (1993). Pectinase-Hyperproducing Mutants of *Aspergillus niger* C28B25 for Solid State Fermentation on Coffee Pulp. *Enzyme Microb. Technol.* **15**:254-260.
- 4.- Loera-Corral, O. (1994). Caracterización de Mutantes de *Aspergillus niger* Sobreproductores de Pectinasas. **Tesis de Maestría**. Universidad Autónoma Metropolitana. México, D. F.
- 5.- Bos, C. J.; Debets J. M.; Nachtegaal, H.; Slakhorst, S. M.; Swart, K. Isolation of Auxotrophic Mutants of *Aspergillus niger* by Filtration Enrichment and Lytic Enzymes. *Curr. Genet* (1992) **21**: 117-120.
- 6.- Bussink, HJD Van den Homberg, JPTW, Van den Ijssel and Visser, J. Characterization of polygalacturonase-overproducing *Aspergillus niger* transformants. *Applied Microbial Biotech.* (1992) **37**: 324-329.
- 7.- Leuchtenberger, A and Mayer, G. Changed pectinase synthesis by aggregated mycelium of some *Aspergillus niger* mutants. *Enzyme microbial technol.* (1992) **14**: 18-22



GROWTH AND PRODUCTION OF IMMOBILISED LI-PASE FROM *Rhizopus delemar* CULTIVATED IN SSF ON A SYNTHETIC RESIN (AMBERLITE)

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Abstract

In this work, a study of lipase production by *Rhizopus delemar* grown on a polymeric resin (Amberlite IRA 900) added with a medium previously optimised is presented. In the first part, the activity of several commercial preparations and the *R. delemar* lipase produced in liquid was compared. It also appeared that the resin can adsorb more than 24% of the lipase produced per g Amberlite. Desorption realised using 5g NaCl / g Amberlite at pH 5 allowed to recover 35 % of the adsorbed lipase. It was also shown that the resin displayed a thermo-protective effect since no loss in activity was observed when the adsorbed enzyme was heated at 80 °C for 24 hours. In solid state fermentation (SSF), the fungus was shown to produce high amounts of enzyme (93 U / g dry Amberlite against only 14 U /ml in submerged culture) when dextrin was used as carbon source after only 24 hours (against 48 hours in liquid culture). Significant activity was also detected with maltose and more surprisingly with glucose (68 and 57 U / g dry Amberlite respectively). The strong inhibitory effect of glucose observed in liquid culture was reduced in SSF.

Introduction

Lipases are widely used enzymes that can be obtained from animals, plants and micro-organisms. Microbial lipases have been used in the food industry, mainly in dairy products, and are also important in detergents, pharmaceutical, cosmetics and leather processing (Seitz, 1973). The enzyme modified cheeses (EMC) are also an interesting application involving lipases (Revah and Lebeault, 1989). New trends in that field are directed toward the use of immobilised lipases in organic solvent for ester synthesis, triglycerides hydrolysis, flavouring compounds synthesis (Christen and López-Munguía, 1994).

Solid state fermentation can be a suitable method for producing enzymes such as pectinases, amylases, or cellulases (Lonsane and Ghildyal, 1992), but few papers have dealt with lipases. Nevertheless, Yamada (1977) reported that, in Japan, most of the microbial lipases comes from *Aspergillus* strain cultivated in liquid culture (LC) and SSF. More recently, Rivera Muñoz *et al.* (1991), using *Penicillium candidum* grown on wheat bran, found that SSF has many advantages against LC for lipase production. To understand better fungal growth, inert supports impregnated with a nutritive solution have been reported. The aim of this work was to study the growth and

the lipase production by *Rhizopus delemar* on Amberlite, a well studied anionic resin (Auria *et al.*, 1990; Christen *et al.*, 1994). This include, characterisation of the lipolytic activity in LC, SSF experiments and interaction support/enzyme.

Micro-organisms and culture media

Two *Rhizopus delemar* strains were tested: CDBB H313 (CINVESTAV-MEX-ICO) and NRRL 1472. They were periodically transferred on Potato Dextrose Agar (PDA) slants and stored at 4°C. Spores were produced in Erlenmeyer flasks on PDA at 29°C during 6 days. The nutritive medium previously optimised by Martinez Cruz *et al.* (1993) was used both in LC and SSF. LC were made in 250 ml Erlenmeyer flasks placed on a rotatory shaker. Initial conditions were : temperature, 29°C; pH, 6; inoculum size, 1×10^7 spores/ml; agitation speed, 180 rpm. In solid state cultures, an anionic resin (Amberlite IRA-900, Röhm & Haas) was used and prepared according to Christen *et al.* (1993). Nutritive medium was added to the dried support to complete 58% final water content, the maximum absorption capacity of the resin. The cultures were carried out in small columns placed in temperature controlled bath. Initial conditions were: temperature, 29°C; pH, 6; inoculum size, 1×10^7 spores/g Initial Dry Matter (IDM) and aeration rate, 0.5 l/h.g IDM.

Analytical procedures

In LC, growth was followed by the evolution in dry weight. In SSF, respirometry was used which allowed to calculate CO₂ production rate (CDPR) as previously described (Christen *et al.*, 1993). Water activity, moisture content and pH were also determined at the end of the fermentation.

Lipolytic activity was assayed with the method used by Nahas (1988) with some modification. The substrate was a 5 % tributyrin emulsion prepared in a 1 % Tween solution in 2.5 M tris-maleate buffer (pH = 6) by homogenising with an Ultraturax apparatus (8000 rpm during 2 mn). The reaction mixture contained 18 ml substrate and 12 ml extract solution. In the case of adsorbed lipase, one gram of Amberlite was added to the reaction medium. The determination was achieved with a Mettler DL 21 pH stat, at 37°C and pH adjusted to 6. The butyric acid released was titrated with 5 mM NaOH solution during 5 min. One unit (U) was defined as the amount of enzyme releasing one mmol of free fatty acid per minute.

Adsorption/desorption study

R. delemar lipase adsorption study was achieved using entire, (average diameter : 0.53 mm), or ground Amberlite (average diameter: 0.10 mm). Amounts varying from 0.5 g to 6 g of Amberlite were contacted with 50 ml of enzymatic extract in 250 ml Erlenmeyer flasks placed in a rotatory shaker (150 rpm) during 24 h. Temperature was 29°C and pH adjusted to 6.

Desorption study was achieved using 2g of Amberlite in 50ml of chloride sodium concentrations ranging from 10 to 120 g NaCl /l and at different pH. Conditions were similar to those of the adsorption studies.

Results are reported as :

. Uads is defined as U0-Ures, where U0 was the initial and Ures the residual ac-

tivity of the extract after the adsorption experiment.

. **Uexp** which is measured and represents the activity shown by the Amberlite after the adsorption experiment.

. **Udes tot** : $100 \times (Udes/Uads)$ where Udes represents the desorbed lipase in the medium and is assayed as described previously. It is expressed as percentage of total adsorbed lipase.

. **Udes rel** : $100 \times (Udes/Uexp)$. It is expressed as percentage of total expressed lipase.

Results and Discussion

Evaluation of the R. delemar strains

Lipolytic activity of the 2 strains was evaluated according to Corzo (1993) by growing the mould in Petri dishes on PDA added with emulsified tributyrin (1%). The diameter of the halo around the colony, corresponding to the hydrolysis of the substrate was measured after 3 days. The CDBB H313 strain gave an average diameter, calculated from one hundred colonies, of 2.75 mm against 2.28 mm for the NRRL 1472 strain. The former was then used in all further experiments.

Growth and lipase production kinetics in LC

LC was used to produce the enzymatic extract needed to the adsorption/desorption experiments. Results are plotted in figure 1. It can be seen that maximum activity (14.07 U/ml) corresponds to maximum growth (12.3 mg/ml). These values were reached within 2 days and correspond to those obtained by Martinez Cruz *et al.* (1993). It was shown that a centrifugation at 5000 rpm during 5 mn was not recommended because a loss of more than 50% in lipolytic activity was observed, probably due to cell bound protein. The important decrease in lipolytic activity observed after 2 days may be due to proteolytic activity and/or denaturation of the protein. This was not observed when Amberlite (2g/50ml) was present in the medium (Angeles, 1995). In figure 2, it can be observed that the extract was more stable at pH 5 while the optimum activity was obtained at 6.5.

Growth and lipase production in SSF

Glucose is known to be a repressor of lipase production in LC Haas and Bailey (1993). One of the particular goal of the experiments in SSF was to see if this catabolic repression could be partially or totally overcome. Three carbon sources (20 g/l) were used in the SSF experiments: glucose, maltose and dextrin.

Results are presented in figures 3 and 4. The growth, followed by CDPR, did not display significative differences between the 3 substrates. Maxima of this parameter were between 15 and 20 hours - a very short time in SSF - and reached values of 3.5 ml/h.g IDM.. Maximum lipase production was found after 24 hours, just after the maximum of CDPR, corresponding to the lower pH in the medium. Best production was found for dextrin (95.6 U/g IDM) against 68.2 U/g IDM for maltose and 57.7 U/g IDM for glucose (See Figure 9). The equivalence in U/ml reactor is given in table 1 and

it is shown that SSF gave a higher productivity with the same substrate (dextrin) than LC. This activity decrease after this maxima for the 3 cases.

Fermentation Carbon source	LC	SSF		
	Dextrin	Glucose	Maltose	Dextrin
Time max. production (h)	48	18	18	15
CDPR max (ml/h.g IDM)	-	2.8	3.5	3.48
R.Q. (Range)	-	1-1.3	1-1.3	1-1.4
pH max. production	-	5.7	4.9	5.6
Aw	-	0.998	0.999	0.994
Lipolytic Act. (U/g IDM)	-	57.7	68.2	95.6
Lipolytic Act. (U/ml)	14.08	10.21	11.33	15.66

Table 1. Comparative data of LC and SSF lipase production.

In table 1 are summarised the most important data about LC and SSF for lipase production by *R. delemar*. It can be seen that the enzyme is produced faster and with a better productivity in SSF. Moreover, Amberlite is an adequate support for this purpose: it provided a good stability for pH, moisture content (which remained stable) and Aw, all three being key parameters in SSF. The best carbon source was dextrin as already observed in LC (Martinez Cruz *et al.*, 1993), but in SSF, the catabolic repression due to glucose was not as important as expected (only a decrease of 40% against dextrin). R.Q. observed are typical of the oxidative use of the carbon sources.

***R. delemar* lipase adsorption/desorption study**

. Adsorption study

Results for lipase adsorption on entire and ground Amberlite are represented in figures 5 and 6. It can be seen on figure 3 that the amount of lipase adsorbed per g of Amberlite decreased for higher amounts of resin, while the contrary trend is observed for the residual activity. Only 3% of the adsorbed enzyme was active when 0.5 g of resin were used, but this increased to 26% for 6 g. The important losses observed in expressed activity may be due to partial denaturation of the protein, inactivation of the active sites due to the anionic properties of the support or partial diffusion of the protein inside the resin.

In the case of ground Amberlite (Cf figure 6), as low as 0.5 g were sufficient to adsorb all the lipase present in the reaction medium. In the same way than for entire Amberlite, the relation between expressed and adsorbed lipase increased from 12.4% to 34% with the increase of amount of resin in the medium. Nevertheless, the values for adsorbed and expressed activities are higher than for entire Amberlite, probably due to the increase in the contact area in that case. It may also be explained by a decrease in the limitation of diffusion.

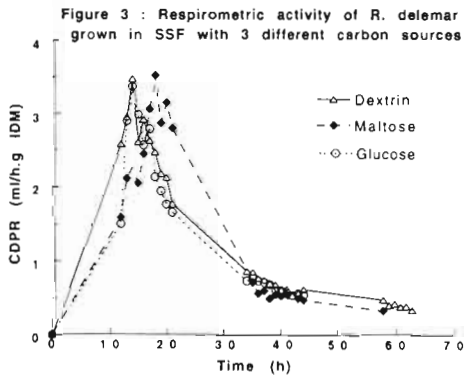
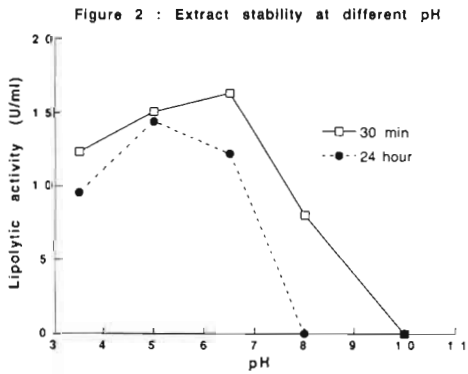
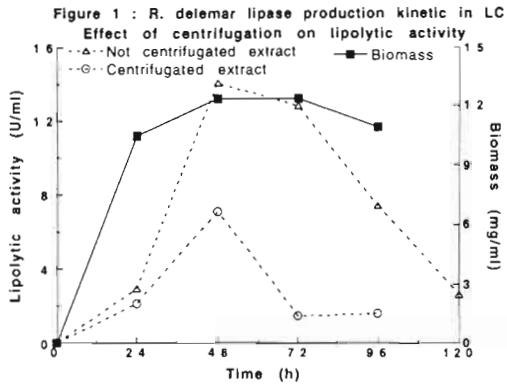


Figure 4 : Lipolytic activity for *R. delemar* grown in SSF with 3 different carbon sources

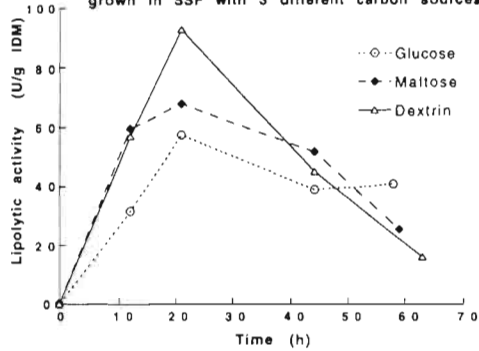


Figure 5 : *R. delemar* lipase adsorption on Amberlite

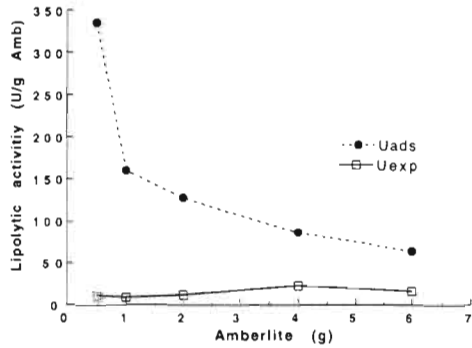


Figure 6 : *R. delemar* lipase adsorption on ground Amberlite

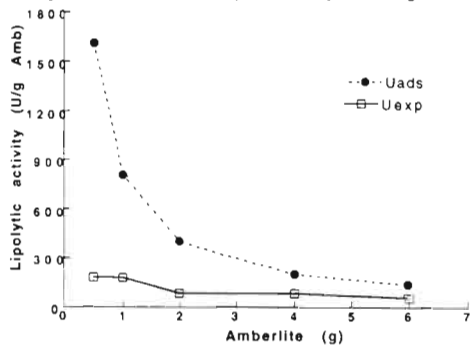


Figure 7 : R. delemar lipase adsorption kinetic on ground and entire Amberlite

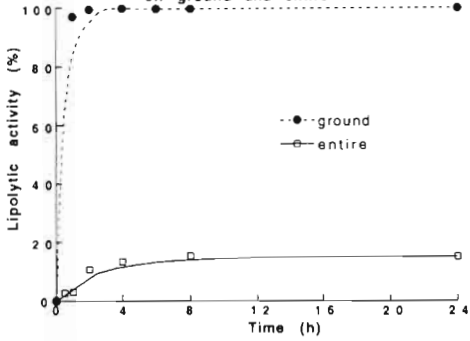


Figure 8 : influence of [NaCl] on lipase desorption (pH=6).

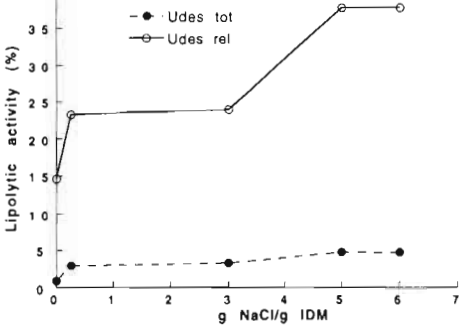
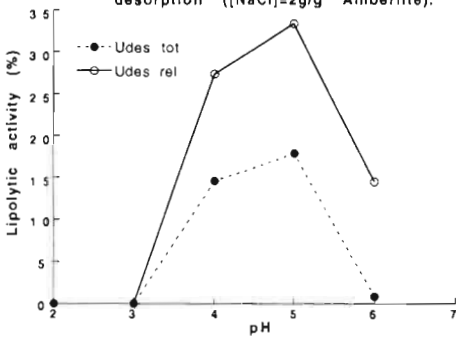


Figure 9 : Influence of pH on lipase desorption ([NaCl]=2g/g Amberlite).



In figure 7, it can be seen that all the lipase present in the medium is adsorbed on ground Amberlite within about 2 hours while, entire Amberlite was saturated after 8 hours, with only a little more than 20% of the lipase present in the reactive medium. These experiments showed that all the lipase was adsorbed on Amberlite in maximum 8 hours. The amount of adsorbed lipase and the adsorption dynamics depend strongly on the size of the resin. This confirmed the hypothesis presented above.

Moreover, the adsorption on Amberlite displayed a thermo protective effect since no loss in activity was observed after that a sample of adsorbed lipase stayed 24 hours at 80°C (Angeles, 1995). The adsorption of the produced enzyme on Amberlite during growth on SSF may serve as a method to concentrate it simultaneously.

Desorption study

To study the recovery of the adsorbed *R. delemar* lipase on Amberlite, the entire particle was used. The influence of NaCl concentration and pH were explored (See figures 8 and 9). These experiments were realised at 29°C; agitation, 150 rpm during 24 hours. Addition of NaCl, previously used by Corzo (1993), for lipase desorption allowed a 38 % of desorption at 100 g NaCl/l at an optimum pH of 5.

Conclusions

In this study:

. *R. delemar* CDBB H313 strain was selected for its better lipase production.

. In LC, the negative effect of centrifugation on lipase recovery, was demonstrated. It was established that the enzyme was more time stable at pH 5, while its optimum activity was at pH 6.

. In SSF, the mould showed a good capacity to grow on Amberlite with various carbon sources (dextrin, maltose and glucose). Best lipase production was found with dextrin (as in LC) while lower glucose repression was observed than in LC.

. In sorption/desorption experiences, it was evidenced that entire Amberlite was saturated with 24 % of the lipase while ground Amberlite was able to adsorb all the lipase present in the medium (about 700 U). There is an important difference between the "adsorbed" Amberlite (defined as initial rested from residual activity) and actual active lipase on Amberlite (only 26% and 34% for entire and ground support). The desorption experiments showed that the recovery of the adsorbed enzyme was uneasy (only 38% with 2 gNaCl/g IDM and pH 5). It will be preferable to use the enzyme adsorbed on Amberlite than to try to desorb it. Furthermore, such lipase displayed a good thermostability. The use of Amberlite as a support opens interesting possibilities to study simultaneous enzyme production and separation in SSF.

References

- Angeles, N. 1995. Producción de lipasa por *Rhizopus delemar* sobre una resina sintética (Amberlita IRA 900). Licence thesis, UNAM, México. In press.
- Auria, R., Hernandez, S., Raimbault, M., Revah, S. 1990. Ion exchange resin: a model support for solid state growth fermentation of *Aspergillus niger*. *Biotechnol. Techniques*, 4(6) : 391-396.
- Christen, P., Auria, R., Vega, C., Villegas, E., Revah, S. 1993. Growth of *Candida utilis* in solid state fermentation. *Biotech. Adv.* 11 : 549-557.
- Christen, P., Auria, R., Marcos, R., Villegas, E., Revah, S. 1994. Growth of *Candida utilis* on Amberlite with glucose and ethanol as sole carbon sources. *Adv. Bioprocess Eng.*, E. Galindo and O.T. Ramirez (Eds), Kluwer Acad. Pub. : 87-93.
- Christen, P., Lopez Munguía, A. 1994. Enzymes and food flavor - A review. *Food Biotechnol.*, 8(2&3) : 167-190.
- Corzo, G. 1993. Estudio comparativo de la producción de lipasas por *Yarrowia lipolytica* en sistemas líquido y sólido. Master thesis, UNAM, México, 94 Pp.
- Haas, M.J., Bailey, D.G. 1993. Glycerol as carbon source for lipase production by the fungus *Rhizopus delemar*. *Food Biotechnol.*, 7(1) : 49-73.
- Lonsane, B.K., Ghildyal, N.P. 1992. Exoenzymes. In: Solid substrate cultivation, H.W. Doelle, D.A. Mitchell, C.E. Rolz (Eds), 191-209, Elsevier Appl. Sci., London.
- Martinez Cruz, P., Christen, P., Farres, A. 1993. Medium optimization by a fractional factorial design for lipase production by *Rhizopus delemar*. *J. Ferment. Bioeng.* 76(2) : 94-97.
- Nahas, E. 1988. Control of lipase production by *Rhizopus oligosporus* under various growth conditions. *J. Gen. Microbiol.* 134 : 227-233.
- Revah, S., Lebeault, J.M. 1989. Accelerated production of blue cheese flavors by fermentation on granular curds with lipase addition. *Lait*, 69 : 281-289.
- Rivera-Muñoz, G., Tinoco-Valencia, J.R., Sanchez, S., Farres, A. 1991. Production of microbial lipases in a solid state fermentation system. *Biotechnol. Lett.*, 13(4) : 277-280.
- Seitz, E.W. 1973. Industrial applications of microbial lipases: A review. *J. Am. Oil Chem. Soc.*, 51 : 12-16.
- Yamada, K. 1977. Recent advances in industrial fermentation in Japan. *Biotechnol. Bioeng.*, 19 : 1563-1621.

RELATION BIOMASS / RESPIRATION: THEORETICAL AND PRACTICAL ASPECTS

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Summary

We present here some theoretical consideration on the correlation model between Biomass and respiration. The case of the growth of *Aspergillus niger* on starchy substrate is discussed as an example of the application of the calculation of fermentation parameters. It is specially possible to calculate from the respiratory metabolism the specific growth rate, the maintenance, yields in biomass, metabolic products, and also heat evolved during the fermentation on the basis of Oxygen Uptake Rate, CO₂ evolved and the consumed substrate. In a second part we detail the equipment and methodology of the lab standardised method we developed for all physiological studies and optimisation of the culture conditions for SSF of fungi. Finally we illustrate the technique by the case of *A. niger* and *Rhizopus oryzae* cultivated on starchy SSF for showing the software developed for automatic calculation from the on line data obtained by CPG.

I. Theoretical Aspects

1. General aspect of the fungal growth kinetics:

Exponential Model: Various models were proposed to fit up with the kinetics growth of micro-organisms. First of them were proposed by Monod for the unicellular growth of bacteria and can be written in the exponential equation:

$$dX / dt = \mu \cdot X$$

where X is Biomass; t is Time and μ the specific growth rate. μ represents the biomass produce per hour and by g of biomass; it means that the growth rate is proportional to the actual biomass. In the integrated form:

$$X = X_0 \cdot e^{\mu \cdot t}$$

and the logarithmic form:

$$\ln X = \mu \cdot t + \ln X_0$$

Then you can calculate the specific growth rate ($\mu = \ln 2 / t_d$) plotting the biomass on a log scale versus time. This type of exponential model is generally applied for unicellular bacteria or yeast when the number of cells growth exponentially.

Vegetative growth of mycelium: In the case of mycelium, the growth is of different mode without cellular division. The exponential form could be the result of a linear growth at the apex of the hyphae combined to the frequency of the branching point which increase the

number of hyphal apex. Trinci calculated that for *A. nidulans*, the apex grown exponentially until 120µm then the growth became linear and a new branching point appeared. Considering the biomass proportional to the total length of the mycelium, he global equations become :

$$X = k_1 \cdot L$$

$$dL/dt = k_1 \cdot n \quad \text{with} \quad n = k_2 \cdot L$$

where L= length of mycelium; k₁ and k₂ specific constants and n frequency of branching , which can be written :

$$dL/dt = k_1 \cdot k_2 (L)$$

$$dX/dt = k_1 \cdot dL/dt = (k_1 \cdot k_2) \cdot k_1 \cdot L = k_1 \cdot k_2 \cdot X$$

$$dX/dt = \mu \cdot X \quad (\text{with } \mu = k_1 \cdot k_2)$$

Thus, it is possible to explain the fungal growth as an exponential equation. This model fit well only in the first stage of the growth because rapidly, not all the total mycelium can grows without limitation, and after 3 to 5 generation-time, a part of the mycelial biomass can not participate to the growth rate.

Growth limitation:

The common cause of the growth limitation is the decrease of the substrate concentration. Monod (1942) proposed a relation between growth rate and substrate concentration:

$$\frac{dX}{dt} = \mu_{\max} \cdot \frac{S}{(k_S + S)} \cdot X$$

where the μ_{\max} is μ in the optimal condition, k_S the saturation constant of the substrate en S the substrate concentration.

Cubic Model of growth:

Pirt (1966) proposed a cubic root model to explain the fungal growth in pellet form:

$$X^{-3} = k \cdot t + X_0^{-3}$$

But this model fit up only in LSF and when the mycelium grow in pellet form and it do not fit well for SSF.

Logistic Model:

The mycelium is not homogeneous, and can depend of the distance to the apex, with vacuoles in oldest parts. The concept is based on the fact that the medium composition can produce a

defined maximum of biomass, when the biomass increase, the rate slow down in reason of appearing limiting factors. the general equation of logistic is as follows:

$$\frac{dX}{dt} = \mu \cdot X \left(1 - \frac{X}{X_{max}} \right)$$

This type of model generally is well correlated with fungal growth (Edwards & Wilke, 1968), particularly with batch cultures.

Maintenance concept:

When the growth stops and the biomass remains constant, the biomass needs to consume energy and substrate to maintain its viability and to realise its basic metabolic activities like respiration, secondary metabolisms., turnover of proteins and active transport (Pirt, 1965).

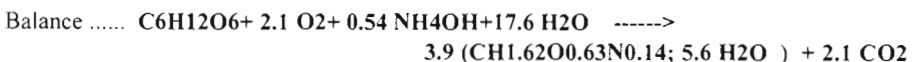
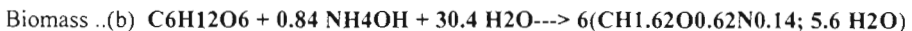
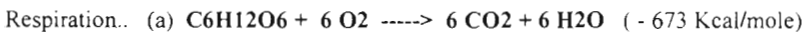
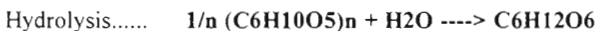
The general equation is:

$$\frac{dS}{dt} = \frac{1}{Y_s} \cdot \frac{dX}{dt} + m \cdot X$$

2. Stoichiometric equations of respiration and Biomass biosynthesis

General equations:

In the following, we suppose the growth of mycelium in the exponential form, and for constants coefficients, we used the data established for *Aspergillus niger* cultivated on starch substrate (Raimbault, 1981). The global equation for the biomass is the result of starch hydrolysis, respiration and biosynthesis:



In the present case of *A. niger* on starch, the proportion of glucose consumed for respiration (a) is 35% and 65% for biosynthesis.

The mycelium composition in CHON was determined on the basis of the composition of mycelium cultivated on liquid medium on starch substrate. The coefficients a and b were calculated on the basis of total glucose consumed and the oxygen uptake following the equation of respiration. where S is the substrate, Ys the cellular yield, and m the maintenance coefficient.

Starting from the global equation, it is possible to calculate the metabolic heat production considering the exothermic reaction of respiration:

$$\frac{dO_2}{dt} = K \cdot \frac{dQ}{dt} ; \quad \frac{dQ}{dt} = \frac{\Delta O_2\% \cdot F}{V O_2 \text{ mol}} \cdot 673 \text{ Kcal} ; \quad \frac{dQ}{dt} = k \frac{dT}{dt}$$

Kinetics of Biomass:

Considering the direct relation between CO₂ and O₂, it is possible to get on line the evolution of the biomass, capturing data of Oxygen Uptake Rate or CO₂ evolution:

$$\frac{dO_2}{dt} = \frac{1}{Y_{O_2}} \cdot \frac{dX}{dt} + m_{O_2} \cdot X ; \quad \frac{dCO_2}{dt} = \frac{1}{Y_{CO_2}} \cdot \frac{dX}{dt} + m_{CO_2} \cdot X$$

From this equation we can write considering $dX/dt = \mu \cdot X$:

$$\frac{dO_2}{dt} = \left(\frac{\mu}{Y_{O_2}} + m_{O_2} \right) \cdot X$$

Considering that $\Delta O_2\% = OUR$ and $F = \text{Air Flow}$

$$\frac{dO_2}{dt} = O_2\% \cdot F = Y_{O_2} \cdot X$$

The logarithmic form of this equation is:

$$\ln(O_2\%) = \ln X + \text{constant} ,$$

or

$$\ln(O_2\%) = \mu \cdot t + \text{constant}$$

Similarly we can write the same equation for the CO₂ evolution (CO₂%)

$$\ln(CO_2\%) = \mu \cdot t + \text{constant}$$

From the last two equation, it is thus possible to calculate the specific growth rate of the mycelium using on line data of gas composition evolving from the incubator, without

destroying the sample, observing the kinetic evolution of the same sample all the time of the fermentation. That represents a great advantage of the SSF.

3. Equipment & Methodology

In order to measure kinetics evolution of fungal biomass cultivated on SSF, we have developed a simple column incubator device which allows to control air flow and analysis on-line of the gas composition at the exit of the reactor (Raimbault, 1980; Alazard & Raimbault, 1981). The figure 1 shows the laboratory device designed to realise lab experimentation with 24 incubator with temperature and air flow control (Trejo-Hernandez, 1986 ; Oriol, 1987 ; Dufour, 1990 ; Saucedo-Castañeda, 1991 ; Socol, 1992).

Glass column reactors (5) of 2 or 4 cm diameter and 20 cm length are filled with the inoculated and moistened solid substrate (100-150 g of WM). Incubators are put on a humidificator (3) and installed in controlled water bath (2 & 4). The air flow, pre-saturated in water, is controlled by microvalves (6). So, the air flow can be controlled for each column.

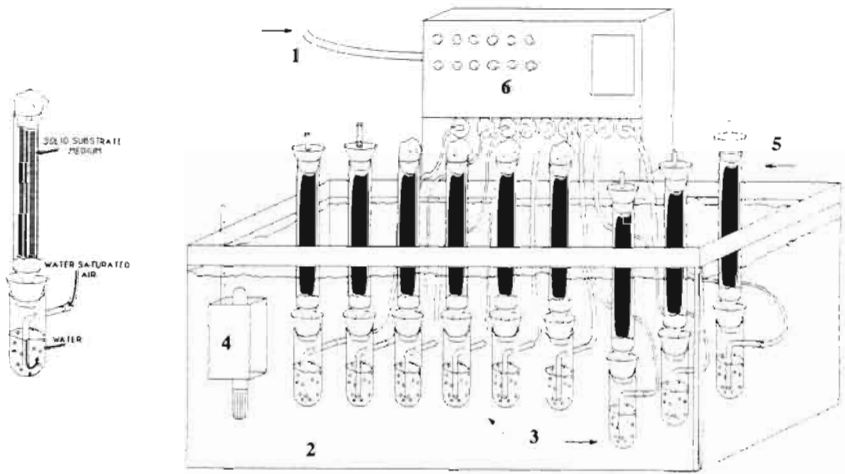


Figure 1 : Incubation device for aerobic SSF. (1): air input; (2) Thermoregulated water bath; (3) Humidificator; (4): Control heater; (5): Column incubator; 6: Microvalves.

To analyse gas composition, different techniques were developed including trapping CO₂ in alkaline solution, paramagnetic analysis for oxygen, Infra Red analysis for CO₂. We describe here (figure 2) the Gas Chromatography technique that we used with success during various years at the laboratory scale. Other alternative are also presented by other speakers during the course.

The equipment is composed by a gas chromatograph (CPG) equipped with thermal conductivity detector and Alltech column CTR1 (double concentric column : extern molecular screen 5 Å and internal Porapak as stationary phase .

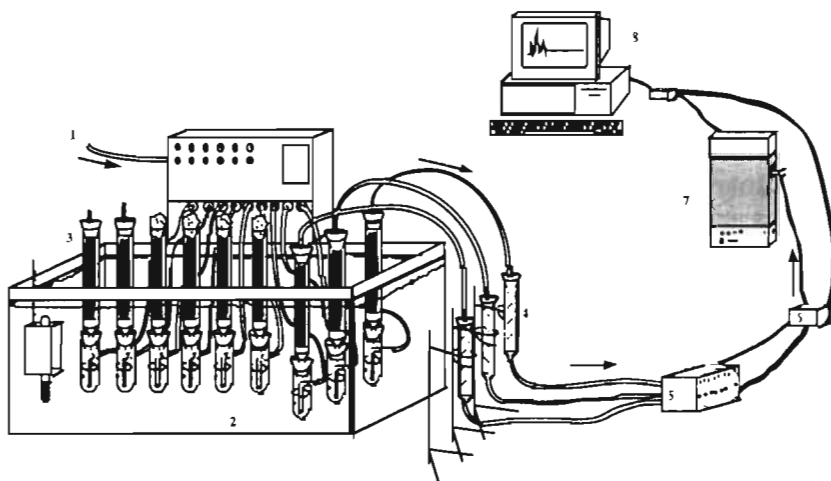


Figure 2: On line analysis equipment for gas measurement using CPG. 1 Air input; 2: Thermostatic; 3: Column incubator; 4 Silicagel ; 5: Sampler; 6 Interface for automatic injection.

The conditions of chromatograph are as follows:

- Detector: Thermal conductivity
- Detector temperature: 60°C
- Column temperature: 60°C
- Gas phase: Helium
- Gas phase flow: 40 ml.min⁻¹
- Catharometer current: 120 mA
- Helium pressure: 1 bar
- Loop injection Volume: 200 µl
- Gas for calibration: Air , CO₂ (0.0) / O₂ (21.0) / N (79.0)
- Mixture 1: CO₂ (5.0) / O₂ (5.0) / N (90.0)
- Mixture 2: CO₂ (10.0) / O₂ (15.) / N (75.0)

I. Experimental Aspects

The experimental part of the course was performed at the Laboratory of Biotechnological Processes of the Chemical Technology Department of the UFPR. So, the training part concerned practice cultivation of two filamentous fungi (*Aspergillus oryzae* and *Rhizopus formosa*) on Cassava Bagasse, a Brazilian by-product of the industrial extraction of Cassava Starch and flour production for human consumption.

Inoculum preparation:

The two strains of fungi were cultivated in erlen flask on the surface of PDA medium during a week at 35°C. Then, conidia were cropped with a platinum loop in a laminar flux cabinet, in sterile tubes containing 10 ml of water, 1% of Tween and glass balls. The suspension is homogenised 15 min and successively diluted as necessary ($10^{-1}/10^{-7}$) for direct microscopic count in a Neubauer cell. The number of conidia in the cell, allows calculate the number of conidia in the original suspension, using the formula:

$$N \text{ (conidia/ml)} = n \text{ (number of conidia in counting cell) } \cdot (1/\text{Dilution factor}) \cdot (25 \cdot 10^4)$$

The adequate dilution is then prepared in order to get a good concentration of conidia allowing final inoculation of 1×10^7 conidia/g of DM substrate. This suspension is kept at 4°C under constant agitation until utilisation (Soccol, 1991).

Substrate preparation:

In this experience, we used Cassava Bagasse, an industrial by-product obtained from the Lorenz Company (Quatro Pontes, SC, Brazil). The raw material was grounded in order to get a granulometry of 0,8-2,0 mm diameter particles. The material was dried at 55-60 °C in oven with circulating air during 12 hours.

This material was analysed in agreement to the recommended methods described in Analytical Normas of the Adolfo Lutz Institute (Sao Paulo, 1985). The starch was determined by the NS-00396/85 method (National Starch Chemical Corporation, 1985), using the Thermamyl commercial α -amylase (Thermamyl). Protein content was determined by the Stutzer method (Vervack, 1973). The following table shows the composition of the by-product:

Table 1. Physico-chemical composition of the Cassava Bagasse (Stertz, 1997)

Compound	Cassava Bagasse composition (g/100g DM)
Moisture Content	10.70
Proteins	1,60
Carbohydrates	63.40
Lipids	0,53
Fibres	22.20
Ashes	1.50

Preparation of the medium for Solid Substrate Fermentation:

The saline solution used to humidify flour contained:

- (NH₄)₂ SO₄..... 4,34 g
- KH₂PO₄ 1,7 g
- Urea 0,83 g
- Water 233 ml

The pH of the solution was adjusted to 5,8 with Na₂CO₃ (3N)

The volume of saline solution necessary to moisten the flour of cassava bagasse is calculated by the formula:

$$\text{Mass of Water (g)} = \frac{\text{M\%} \times \text{Mass of substrate (g)}}{(100 - \text{M\%})}$$

For example, for 100 g of Cassava Bagasse substrate, and for a Moisture content of 65%, it would be used 185 ml of saline solution.

Analytical procedure:

In order to characterise kinetics biotransformation of the material by the fungal strains, samples are picked up at regular duration of the fermentation and physical-chemical determinations are performed. For that purpose, samples are treated following the flow sheet showed on the figure 3.

All analysis (pH, Moisture content, Ashes, Lipid, Fibres, Total acidity and sugars) were performed as recommended by Normas Analíticas do Instituto de Adolfo Lutz (Sao Paulo, 1985).

pH:

pH was determined under agitation electronically with pH meter, after homogenisation of the suspension of 1g in 10 ml of distilled water.

Moisture content:

Moisture content was determined from 5 gm of moistened sample by drying in a controlled oven during 24 h at 105°C. The Moisture Content is calculated by the formula :

$$M \% = \frac{(P2-P1) \cdot 100}{P2}$$

with: M% = moisture content

P2 Weight of the pre-treated sample (5 g) ; P1 Weight after desiccation of the sample.

Carbohydrates and primary metabolites:

Sugar, organic acids, ethanol, were determined by H.P.L.C.

The starch was determined by the NS-00396/85 method (National Starch Chemical Corporation, 1985), using the NOVO Nordisk « Thermamyl 120L » (liquid commercial α -amylase (Thermamyl)). 4 grams of sample are added in 100 ml of water: autoclaved at room pressure during 1 h , and adjusted to pH 6,0-6,5 with a solution of NaOH (1N). When temperature is 95°C, 60 - 70 ppm of CaCl_2 and 1 ml of Thermamyl Novo were added , and kept 15 min at this temperature then filtered on Whatman paper, washed and centrifuged ; finally the residual material is dried at 105-110 °C during 1 h 30 and residual weight is determined. Starch % of residual dry matter was calculated by the formula:

$$\text{Starch \%} = \frac{(PT - PR) \cdot 100}{PT}$$

where PT = Total mass and PR = Residual mass

For true Protein content the Stutzer method was used (Vervack, 1973).

Solid State fermentation cultivation in column:

The device showed in figures 1 and 2 was used for incubation and respirometric analysis, under following conditions:

Temperature of 35 °C

Flow rate of air flux : 100 ml / min

The composition of air can be observed directly on the computer screen .

After 24 hours and 48 hours samples columns are pick up for analysis.

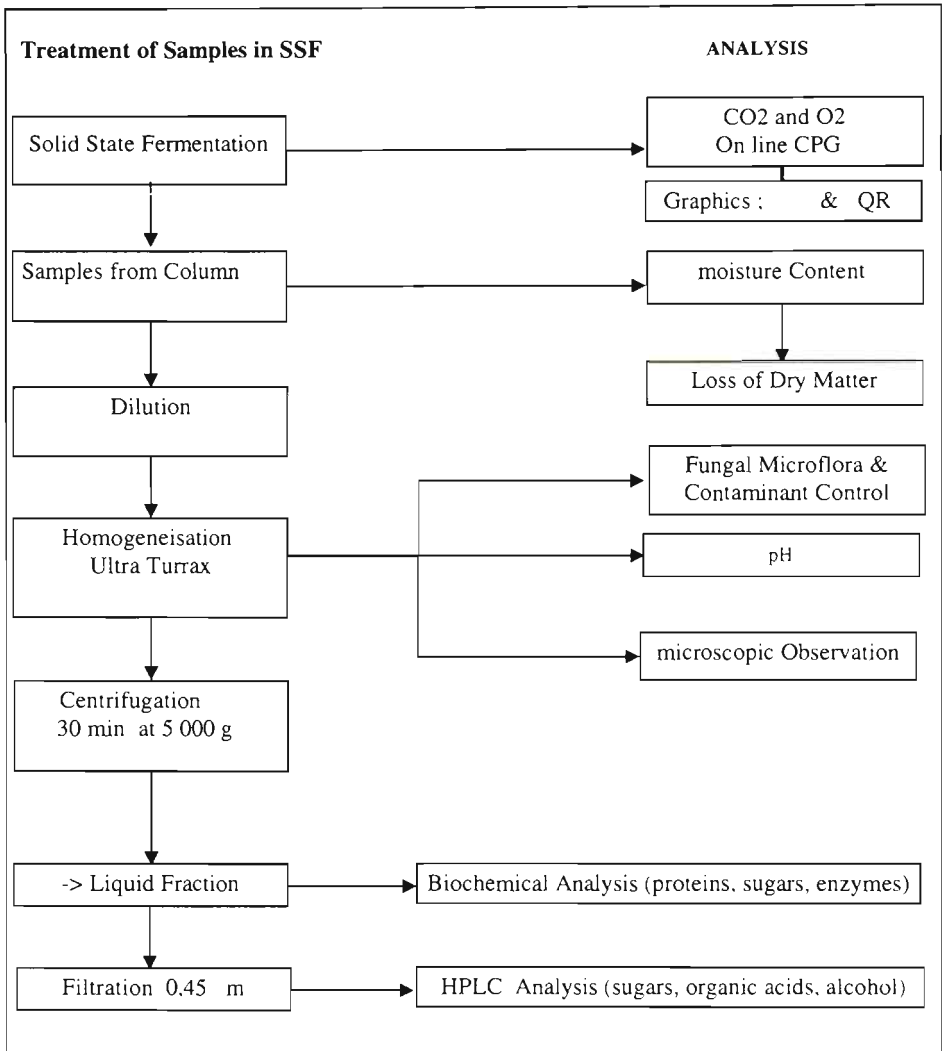


Figure 3. Flow sheet of samples treatment s for analysis in SSF

Typically the Table I represents results obtained from SSF cultivation of *Rhizopus oryzae* on Cassava. Figure 3 represent graphs obtained from *on line* data captured by the computer. It can be calculate directly the oxygen consumption and CO2 evolution, and also the specific growth rate. In addition, biochemical analysis allowed to correlate all fermentation parameters, and calculate all the balance of the biotransformation process. All that information is of importance for pilot and scale up further applications.

Table I.- Data of Solid Substrate Fermentation of *Rhizopus oryzae* MUCL 28168, cultivated on Cassava flour substrate. (Raimbault et al, 1995).

	INITIAL	FINAL
Total Mass of Material	142,20	128,40
Dry Matter Content	60,45	45,85
Air Flux of aeration	85,00	<-----
Total Dry Matter	85,96	58,87
Duration of the germinating	7 h	<-----
Time for Maximum Rate (16 - 30	<-----
Maximum O2 Uptake Rate(ml/H/g	4,65	<-----
VMaximum CO2 Evolved Rate (ml/H/g	4,91	<-----
Mean of Respiratory	1,05	<-----
Total O2 uptake (g/g	0,19	<-----
Total CO2 evolved (g/g	0,28	<-----
Duration of the exponential	9 h	<-----
Specific Growth Rate (μ)	0,263	<-----
Protein (% DM	9,96	20,08
Total Sugar (% DM	46,54	15,05
Loss in Dry Matter	31,51	<-----
Yield Protein / Sugar (Y	0,105	<-----
Yield	0,77	<-----

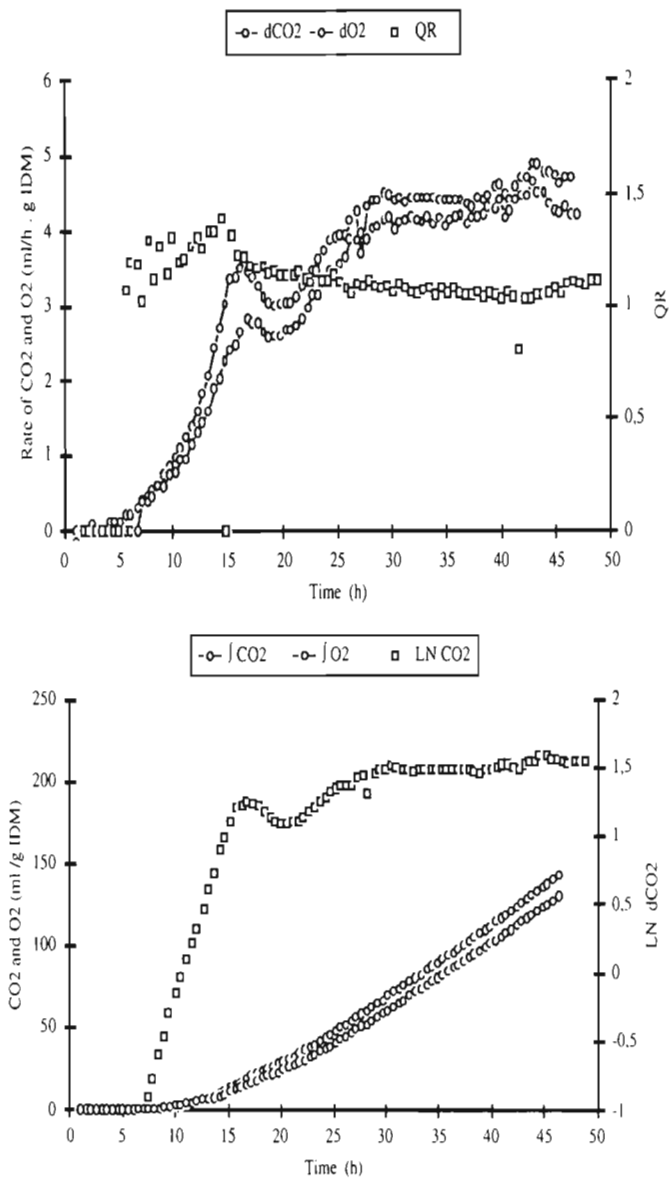


Figure 3. Kinetic of respiration characteristic during growth of *Rhizopus oryzae* on crude Cassava flour.

References :

- Alazard, D. and Raimbault, M. 1981. Comparative study of amylolytic enzymes production by *Aspergillus niger* in liquid and solid state cultivation. *Eur. J. Appl. Microbiol. Biotechnol.* 12: 113-117.
- Auria, R., Hernandez, S., Raimbault, M. and Revah, S. 1990. Ion exchange resin: a model support for solid state growth fermentation of *Aspergillus niger*. *Biotechnol. Techniques.* 4: 391-396.
- Deschamps, F., Raimbault, M. and Senez, J.C. 1982. Solid state fermentation in the development of agro-food by-products. *Industry & Environ.* 5 (2): 27-30.
- Doelle H.W., Mitchell D.A. & Rolz C.E. (1992). Solid Substrate Cultivation. Elsevier Sci. Publ. Ltd; London & New York; 466 p.
- Moo-Young M., Moriera A.R. & Tengerdy R.P. 1983. Principles of solid state fermentation. In *The filamentous fungi*, Vol. 4, Fungal Biotechnology. Smith J.E, Berry D.R & Kristiansen B. Eds., Edward Arnold Publishers, London, pp. 117-144.
- Lonsane, B.K., Saucedo-Castañeda, G., Raimbault, M., Roussos, S., Viniegra-Gonzalez. G., Gildyal, N.P., Ramakrishna, M. and Krishnaiah, M.M. 1992. Scale-up strategies for solid state fermentation systems. *Process Biochem.* 27: 259-273.
- Oriol, E., Schettino, B., Viniegra-Gonzalez, G. and Raimbault, M. 1988a. Solid- state culture of *Aspergillus niger* on support. *J. Ferment. Technol.* 66: 57-62.
- Prebois, J.P., Raimbault, M. and Roussos, S. 1985. Biofermenteur statique pour la culture de champignons filamenteux en milieu solide. *Brevet Français N° 85.17.934.*
- Raimbault M. - (1981). "Fermentation en milieu solide: croissance de champignons filamenteux sur substrats amylicés". Edited by: ORSTOM-Paris; *Série Travaux et Documents* n° 127; 291 p.
- Raimbault, M. and Alazard, D. 1980. Culture method to study fungal growth in solid fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 9: 199-209.
- Raimbault, M.; Ramirez Toro, C.; Giraud, E.; Soccol, C.R.; Saucedo, G. Fermentation in cassava bioconversion. In ; Dufour, D.; O'brien, G.M.; Best, R. (Eds) *Cassava Flour and Starch ; Progress in Research and Development - Session 4 - Bioconversion and Byproduct* us. CIAT Pub. N. 271, 1996. p187-196.
- Raimbault, M., Revah, S., Pina, F. and Villalobos P. 1985. Protein enrichment of cassava by solid state fermentation using molds isolated from traditional foods. *J. Ferment. Technol.* 63: 395-399.

- Raimbault, M., Roussos, S., Oriol, E., Viniestra, G., Gutierrez, M., Barrios-Gonzalez, J. 1989. Procédé de culture de microorganismes sur milieu solide constitué d'un support solide, absorbant, compressible et non fermentable. *Brevet Français* N° 89. 06558.
- Raimbault, M. and Viniestra-Gonzalez, G. 1991. Modern and traditional aspects of solid state fermentation. In . Chahal, D.S. (Ed.), *Food, feed and fuel from biomass*, Oxford & IBH Publis. Co. Pvt. Ltd. New Delhi, p. 153-163.
- Roussos, S., Olmos, A., Raimbault, M., Saucedo-Castañeda, G. and Lonsane, B.K. 1991. Strategies for large scale inoculum development for solid state fermentation system : Conidiospores of *Trichoderma harzianum*. *Biotechnol. Tech.* 5: 415-420
- Rodríguez León, J. A., Sastre, L., Echevarría, J., Delgado, G. & Bechstedt, W. (1988). A mathematical approach for the estimation of biomass production rate in solid state fermentation. *Acta Biotechnol.* 8, 307-310.
- São Paulo (1985) Instituto Adolfo Lutz. Normas Analíticas do Instituto Adolfo Lutz, São Paulo, Brazil, 523p
- Sato, K., Nagatani, M., Nakamuri, K. I. & Sato, S. (1983). Growth estimation of *Candida lipolytica* from oxygen uptake rate in a solid state culture with forced aeration. *J. Ferment. Technol.* 61, 623-629
- Saucedo-Castañeda, G., Gutierrez-Rojas, M., Bacquet, G., Raimbault, M. and Viniestra-Gonzalez, G. 1990. Heat transfert simulation in solid substrate fermentation. *Biotechnol. Bioeng.* 35: 802-808.
- Saucedo-Castañeda, G., Gutierrez-Rojas, M., Bacquet, G., Raimbault, M. and Viniestra-Gonzalez, G. 1990. Heat transfert simulation in solid substrate fermentation. *Biotechnol. Bioeng.* 35: 802-808.
- Saucedo-Castañeda, G., Lonsane, B.K., Navarro, J.M., Roussos, S. and Raimbault, M. 1992. Control of carbon dioxide in exhaust air as a method for equal biomass yields at different bed heights in column fermentor. *Appl. Microbiol. Biotechnol.* 37: 580-582.
- Saucedo-Castañeda, G., Trejo-Hernandez, M.R., Lonsane, B.K., Navarro, J.M., Roussos, S., Dufour, D. and Raimbault, M. 1993. On-line monitoring and control system for CO₂ and O₂ concentrations in aerobic and anaerobic solid state fermentations. *Process Biochem.* 29: 13-24.
- Soccol, C.R. 1992. Physiologie et métabolisme de *Rhizopus* en culture solide et submergée en relation avec la dégradation d'amidon cru et la production d'acide L(+) lactique. *Thèse de Doctorat*, Université de Technologie de Compiègne, France, 218 p.
- Soccol, C. R. Biotechnology products from cassava root by solid state fermentation. *Journal of Scientific and Industrial Research*, Vol. 55, may-june, 1996, pp 358-364.

- Soccol, C.R., Rodriguez-Leon, J., Marin, B., Roussos, S., Raimbault, M. 1993. Growth kinetics of *Rhizopus arrhizus* in solid state fermentation of treated cassava. *Biotechnol. Techniques*, 7: 563-568.
- Soccol, C., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation. *Appl. Microbiol. Biotechnol.* 41: 330-336.
- Soccol, C., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Potential of solid state fermentation for production of L(+) lactic acid by *Rhizopus oryzae*. *Appl. Microbiol. Biotechnol.* 41: 286-290.
- Soccol, C.R., Iloki, I., Marin, B., Roussos, S. and Raimbault, M. 1994. Comparative production of amylases and protein enrichment of raw and cooked cassava by *Rhizopus* strains in submerged and solid state fermentation. *J. Food. Sci. Technol.* 31: 320-323.
- Soccol, C. R.; Raimbault, M.; Pinheiro, L. I. Effect of CO₂ concentration on the micelium growth of *Rhizopus* species. *Arq. Biol. Tecnol.*, 37(1) : 203-210, 1994.
- Soccol, C. R.; Stertz, S. C.; Raimbault, M.; Pinheiro, L. I. Biotransformation of solid waste from cassava starch production by *Rhizopus* in solid state fermentation. Part I - Screening of strains. *Arq. Biol. Tecnol.*, 38(4) : 1303-1310, 1995.
- Soccol, C. R.; Stertz, S. C.; Raimbault, M.; Pinheiro, L. I. Biotransformation of solid waste from cassava starch production by *Rhizopus* in solid state fermentation. Part II - Optimization of the culture conditions and growth kinetics. *Arq. Biol. Tecnol.*, 38(4) : 1311-1318, 1995.
- Soccol, C. R.; Stertz, S. C.; Raimbalt, M.; Pinheiro, L. I. Biotransformation of solid waste from cassava starch production by *Rhizopus* in solid state fermentation. Part III - Scale-up studies in different bioreactors. *Arq. Biol. Tecnol.*, 38(4) : 1319-1326, 1995.
- Soccol, C. R.; Stonoga, V. I.; Raimbault, M. Production of L(+) lactic acid by *Rhizopus* species. *World J. Microbiol. Biotechnol.*, 10(4) : 433-435, 1994.
- Soccol, C. R.; Iloki, I.; Marin, B.; Roussos, S.; Raimbault, M. Comparative production of amylase and protein enrichment of raw and cooked cassava by *Rhizopus* strains in submerged and solid state fermentation. *J. Food Sci. Technol.*, 3: 320-323, 1994.
- Soccol, C. R.; Marin, B.; Raimbault, M.; Lebeault, J. M. Potential of solid state fermentation for production of L(+) lactic acid by *Rhizopus oryzae*. *Appl. Microbiol. Biotechnol.* 41: 286-290, 1994.
- Soccol, C. R.; Leon, J. R.; Marin, B.; Roussos, S.; Raimbault, M. Growth kinetics of *Rhizopus* in solid state fermentation of treated cassava. *Sci. Technol. Letters*, 7(8): 563-568, 1993.
- Vervack W (1973) Analysis des aliments, méthodes courantes d'analyses. Laboratoire de Biochimie de la Nutrition, UCL, Louvain-la-Neuve

KINETICS OF THE SOLID STATE FERMENTATION OF RAW CASSAVA FLOUR BY *Rhizopus formosa* 28422.

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The strain *Rhizopus formosa* 28422 was selected from a stock of ten stains from genera *Rhizopus*. for their capacity to attack raw cassava starch by solid substrate fermentation and showed the highest growth in this substrate. The optimal substrate composition, estimated by surface response design experiments, was 10 % cassava bagasse, 10 % soybean flour and 80 % cassava flour. Optimal fermentation conditions were temperature, 32 °C, moisture, 64 %, initial pH, 6.5 and inoculum rate, 10⁶ spores/g DM. These conditions were employed for studying the kinetics of the biotransformation of cassava flour considering the Oxygen Uptake Rate (OUR) and the CO₂ evolved during the process. The respiratory quotient was nearly 1, corresponding to an aerobic process in the first 24 h. Sporulation appeared after 26 hours of fermentation in which the respiratory quotient showed a trend to increase up to nearly 6. Biomass was estimated solving the OUR balance for a yield based on oxygen consumption ($Y_{v/o}$) of 0.531 g biomass /g consumed O₂ and a value for the maintenance coefficient (m) of the order of 0.068 g consumed O₂/g biomass⁻¹ h⁻¹. The corresponding value for the growth specific velocity at the exponential phase (μ) was 0.16 h⁻¹.

Keywords : Solid Substrate Fermentation, Cassava, *Rhizopus*, Protein Enrichment, Kinetics

Introduction

Cassava flour is a basic ingredient of Brazilian people diet. However, its protein content is low (2.1% w/w, on a dry matter basis) and of poor nutritional quality (El-Dash, 1994). A biotechnological alternative for improving content and quality of cassava flour proteins is the fermentation with filamentous fungi (Vanneste, 1982). Solid State Fermentation (SSF) of cassava is a relative simple procedure, which increases by four to five folds its protein content. On another hand, the protein quality of the final product is very acceptable when compared with FAO standards (Vanneste, 1982). *Rhizopus* are edible filamentous fungi, employed since thousand of years, mainly in Oriental Countries like Chine, Korea, Japan, Indonesia, Malaysian and others, for preparing fermented foods (Hesseltine, 1965; Raimbault & Alazard, 1981). *Rhizopus* fermentation leads to protein enrichment and digestibility improvement of foods (Soccol et al. 1992, 1993, 1994). Also *Rhizopus* fermentation can restrain toxic products formation, like aflatoxins (Ko, 1974; Zhu *et al.*, 1989), produce active biocides against bacteria (Wang et al., 1969) and detoxify cyanogenic glycosides of cassava (linamarin) (Padmaja & Balagopal, 1985). As cassava has a naturally high starch content, it would be interesting to identify mould strains able to utilise this carbon source in its native forms, i. e., without the energy consuming for

gelatinization step. Thus, this research aims to develop a biotransformed flour by solid state fermentation, in order to obtain a proper protein content, employing various strains of *Rhizopus*, able to attack native cassava starch and estimate the kinetics that describe such process.

Substrate preparation : Cassava flour was prepared in the laboratory from fresh commercial roots. Roots were cleaned and hand ground. The fraction passed through 2.0 mm sieve was dried at 55-60 °C and the new fraction retained in 0.8 mm sieve was employed for fermentation studies. Cassava bagasse was purchased from Yamakawa Industries (Paranavaí, PR - Brazil). It was further ground in a disc mill (Alpha) and the fraction 0.8 - 2.0 mm was retained for assays. Soybean flour was also prepared in the laboratory, from fresh commercial beans. Seeds were toasted (10 min/250°C), dehulled and ground. The fraction retained in 0.8 - 2.0 mm sieves was selected and employed for fermentation studies. The solid substrate was initially a mixture of 80 % cassava flour, 10 % cassava bagasse and 10% of soybean flour.

Strain: The *Rhizopus formosa* MUCL 28422 was employed due to their ability to growth in raw cassava. It was replicated in potato-dextrose agar medium, incubated during 10 days at 28-32 °C and then kept at 4 °C during six months maximum.

Inoculum preparation : Spores were first inoculated in Petri dishes containing cassava-agar medium and incubated at 28-32 °C for 10 days. Therefore, spores were collected with a platinum loop under laminar flow and diluted in test tubes with 10 mL of 1 % (v/v) Tween 80 in distilled water, previously sterilised. Spore suspension was diluted in distilled water and spores counted in a Malassez cell counter, before keeping at 4°C.

Cassava-agar medium preparation : 30 g of cassava flour were diluted in 1 L distilled water and cooked during 1 hour in autoclave. Resulting solution was then filtered and mixed with 2.93 g (NH₄)₂SO₄, 1.5 g KH₂PO₄, 0.72 g urea and 15 g agar. After dissolution by heating, pH was adjusted to 5.5 with Na₂CO₃ (3N) and the final solution sterilised at 121 °C during 20 minutes.

Solid substrate fermentation conditions : The initial inoculum rate was 10⁶ with an initial pH of 6.5 and an exit flow rate of 0.13 l / h g dried matter. The inlet air was saturated. The running fermentation time was 36 h at a temperature of 32 °C. The reactor was a column type with 3.5 inner diameter submerged in a water bath.

Analytical methods : Protein content was determined by the Stutzer method (Vervack 1973). Residual starch was measured by the NS-00396/85 method, employing commercial α-amylase (Thermamy). Other parameters, like pH and moisture, ash, lipid, protein, fiber and carbohydrate contents and were determined by the Institute Adolfo Lutz Recommended Analytical Procedures (São Paulo 1985).

Results

The kinetics of the solid fermentation was determined by measuring the Oxygen Uptake Rate (OUR), the CO₂ evolved and the respiration quotient (RQ) during the process.

A balance was made for the estimation of the OUR and the CO₂ evolved in terms of volumetric flow (l/h), considering an initial weight of 27 g of dry matter, the O₂ and CO₂ percentage composition of the exhausted air flow (F_{out}) which was 0.13 l / h g initial dried weight and the inlet air flow (F_{in}) to the fermentor. The following equations were considered:

$$V_{O_{2out}} = (\% O_{2out} / 100) F_{out}$$

$$V_{CO_{2out}} = (\% CO_{2out} / 100) F_{out}$$

$$V_{N_{2out}} = (100 - \% O_{2out} - \% CO_{2out}) / 100) F_{out}$$

and from the balance of O₂ and N₂ is obtained that:

$$V_{O_{2uptake}} = (20.9/100) F_{in} - (\% O_{2s} / 100) F_{out}$$

$$V_{N_{2in}} = V_{N_{2out}}$$

Relating the several equations considered, the following relationship for the inlet and outlet air flow is obtained:

$$F_{in} = \frac{(100 - \% O_2 - \% CO_2) F_{out}}{79.1}$$

For the estimation of the OUR and the CO₂ evolved in mass flow units (mmoles/h), it was considered that the air is an ideal gas, the respective volumetric flows (V_{O₂uptake} and V_{CO₂out}) and the proper corrections for temperature conditions, considering a temperature value of 32 °C. Table and Figure 1 show these results and the pattern of OUR and CO₂ evolved during the solid fermentation.

From Table 1 is observed that the process showed the characteristics of an aerobic system in the first 26 h with an acceptable RQ which has a mean value of 0.94 for this time interval. After 26 h this pattern holds no more and it was observed a sustained increase in the CO₂ evolved in relation to the OUR and therefor an increase in the RQ as is shown in Figure 2.

As is it observed from Table 2 there are not practically significant growth after the first 24 h with a very short lag phase of the order of only 1 h.

Table 1. OUR, CO₂ evolved and respiration quotient (RQ) pattern during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa* 28422 at 32 °C.

Time	% O ₂ in F _{out}	% CO ₂ in F _{out}	OUR	CO ₂ evolved	RQ
(h)			(mmoles/h)	(mmoles/h)	
0	20.90	0.00	0.000	0.000	
2	20.67	0.30	0.453	0.642	1.4
4	20.36	0.55	1.150	1.177	1.0
6	20.08	1.02	1.641	2.182	1.3
8	19.62	1.36	2.694	2.910	1.1
10	19.21	1.64	3.644	3.509	1.0
12	18.29	1.72	6.088	3.680	0.6
14	17.32	2.09	8.502	4.472	0.5
16	16.77	4.46	8.650	9.543	1.1
18	16.04	8.11	8.561	17.353	2.0
20	14.42	6.82	13.673	14.593	1.1
22	13.51	4.33	17.542	9.265	0.5
24	14.04	3.59	16.527	7.681	0.5
26	16.59	4.43	9.154	9.479	1.0
28	17.65	4.71	6.129	10.078	1.6
30	18.69	4.53	3.417	9.693	2.8
32	19.22	4.89	1.780	10.463	5.9
34	19.17	5.01	1.847	10.720	5.8
36	18.96	5.04	2.398	10.784	4.5

Table 2. Substrate and biomass characteristics during the solid state fermentation of raw cassava flour by *Rhizopus formosa* 28422 at 32 °C.

Fermentation time (h)	0 h	24 h	36 h
Humid substrate weight (g)	75.00	70.02	69.25
Humidity (%)	64.02	64.11	66.91
initial protein due the inoculum (%)	0.12	-	-
biomass protein content (%) (d.b.)	50.16	-	-
substrate protein content (%) (d.b.)	5.58	10.13	10.68
Total biomass (g)	0.065	1.809	1.881

Based in these results it was decided to proceed with the estimation of the biotechnological parameters considering the balance of the OUR. From this balance the following equation is obtained (Sato et. al., 1983):

$$X_n = \left(Y_{x/o} \Delta t \left(\frac{1}{2} \left(\left(\frac{dO_2}{dt} \right)_{t=0} + \left(\frac{dO_2}{dt} \right)_{t=n} \right) + \sum_{i=1}^{n-1} \left(\frac{dO_2}{dt} \right)_{t=i} \right) + \left(1 - \frac{a}{2} \right) X_0 - a \sum_{i=1}^{n-1} X_i \right) / \left(1 + \frac{a}{2} \right)$$

where: $a = m (Y_{x/o}) \Delta t$

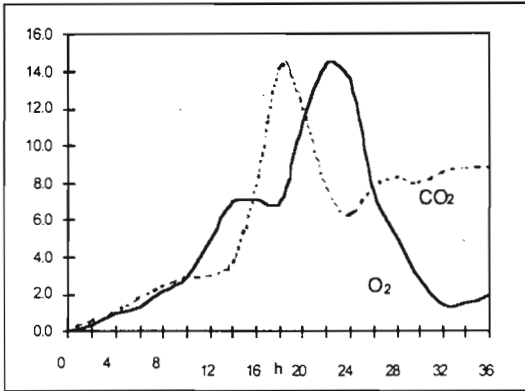


Figure 1. Kinetic pattern of the OUR and CO₂ evolved during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa* 28422.

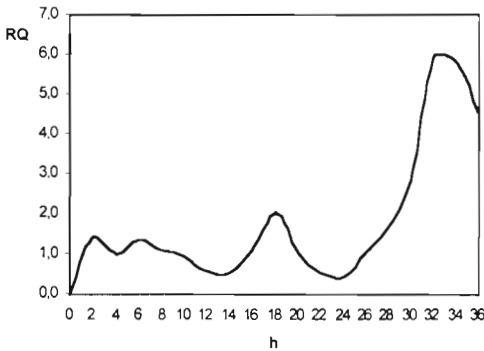


Figure 2. Respiration quotient pattern (RQ) during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa* 28422.

The procedure to estimate the biomass content in a particular moment (X_n) consist in make a trial and error estimation, assuming values for the biomass yield based in oxygen consumption ($Y_{x/o}$)

and for the maintenance coefficient (m), (Rodríguez León et al., 1988), considering in our case the biomass values analytically determined at 24 and 36 h until the values predicted by the equation here considered agree with those determined analytically. Using the data reported at Table 1 and Table 2 the system was solved for a value of 0.531 g biomass /g consumed O_2 for the biomass yield based in oxygen consumption ($Y_{x/o}$) and 0.068 g consumed O_2 / g biomass⁻¹ h⁻¹ for the maintenance coefficient (m).

In Table 3 are reported the biomass estimation for the different times considered, calculated via the equation for (X_n) reported before.

Table 3. Biomass estimated from the OUR during the solid state fermentation of raw cassava flour by the strain *Rhizopus formosa* 28422.

time (h)	biomass estimated (g)	biomass measured (g)	relative error (%)
0	0.065	0.065	0
2	0.067		
4	0.083		
6	0.115		
8	0.164		
10	0.237		
12	0.350		
14	0.520		
16	0.712		
18	0.891		
20	1.125		
22	1.462		
24	1.813	1.809	0.2
26	2.027		
28	2.089		
30	2.07		
32	1.996		
34	1.906		
36	1.830	1.881	2.4

Table 3 report too the comparison between the values estimates for 24 and 36 h with the values determined analytically. As it can be seen the error in the biomass estimation are lower than 2.5%. From the data of the biomass estimation it was calculated the specific growth velocity for the log phase (μ_{max}) by a regression of $\ln X_n$ vs t . The value obtained was 0.16 h⁻¹ with a regression coefficient of 0.997 considering the values between 2 and 24 h.

Discussion:

The behaviour of the system after 26 h could be related to a improper air flow distribution due to air flow canalisation after the mycelium biomass was fully developed. At the same time this is a point were initial sporulation start, therefor this pattern indicate that the mycelial growth lasted until 26 h. This fact is corroborated by the results reported in Table 2 where is shown the data that characterise the substrate and biomass synthesis during the fermentation.

The value of $Y_{x/o}$ considered for solving the OUR balance seems to be relatively low and at the same time the value for the maintenance coefficient (m) seems high. This could be due to the characteristics of the substrate employed, raw cassava flour, and the necessity of synthesis by the micro-organism of the proper amylases that allow the flour hydrolysis since the beginning of the process, considering that there is not lag phase in this system, as is shown by the kinetic pattern. In this sense is notable that the pattern of enzyme synthesis seems more a constitutive and not an inductive one taking into account that the lag phase is practically null. In other words, if the amylases synthesis is inductive as it could be expected, the process in this case, with raw cassava flour and strain of *Rhizopus formosa* 28422, is really fast, provoking, a high maintenance coefficient and therefor a low biomass yield based in oxygen consumption.

The results here discussed corroborate that the process in which raw cassava flour is fermented by a solid state fermentation process with the strain *Rhizopus formosa* 28422 is quite feasible and is developed in 24 h allowing the use of raw cassava flour without the necessity of previous pre-treatment as normally is done and attaching a level of protein of the order of 10% dried basis.

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References

- El-Dash A, Mazzari MR, Germani R (1994) Tecnologia de Farinhas Mistas - Uso de farinha mista de trigo e mandioca na produção de pães. vol I Embrapa/Spipp, Brasília. pp 17-18
- Hesseltine CW (1965) A millenium of fungi food and fermentation. *Mycologia* 57:149-197
- Ko SD (1974) Self protection of fermented foods against aflatoxin. Proceedings of the IV International Congress of Food Science and Technology v3 pp 244-253
- National Starch Quimical Corporation. *NS - 00396/85*. (1985).
- Padmaja G; Balagopal (1985) Cyanide degradation by *Rhizopus*. *Can J Microbiol* 35(8):663-69
- Phan-Tha-Luu R, Feneuille D. Mathieu D. (1983) Methodologie de la Recherche Experimentale. 4 volumes. LPRAIIVT. Marseille. France,
- Raimbault M & Alazard D (1980) Culture method to study fungal growth in solid fermentation. *Critical Reviews in Biotechnology* 4:199-209

- Rodríguez León, J. A., Sastre, L., Echevarría, J., Delgado, G. & Bechstedt, W. (1988). A mathematical approach for the estimation of biomass production rate in solid state fermentation. *Acta Biotechnol.* 8, 307-310.
- São Paulo (1985) Instituto Adolfo Lutz. Normas Analíticas do Instituto Adolfo Lutz, São Paulo, Brazil, 523p
- Sato, K., Nagatani, M., Nakamuri, K. I. & Sato, S. (1983). Growth estimation of *Candida lipolytica* from oxygen uptake rate in a solid state culture with forced aeration. *J. Ferment. Technol.* 61, 623-629.
- Soccol C R (1992) Physiologie et métabolisme de *Rhizopus* en culture solid et submergée en relation avec la dégradation d'amidon cru et la production d'acide L(+)lactique. PhD Thesis. Université de Technologie de Compiègne, Compiègne, France, 219 p.
- Soccol CR, Leon JR, Marin B, Roussos S, Raimbault M (1993) Growth kinetics of *Rhizopus* in solid state fermentation of treated cassava. *Sci Technol Letters* 7:563-568
- Soccol CR, Marin B, Raimbault M, Lebeault JM (1994) Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation *App Microbiol Biotechnol* 41:330-336
- Soccol CR, Marin B, Roussos S, Raimbault M (1993) Scanning electron microscopy of the development of *Rhizopus arrhizus* on raw cassava by solid state fermentation. *Micologia Neotropical Aplicada* 6:27-39
- Soccol C R (1994) Contribuição ao estudo da fermentação no estado sólido em relação com a produção de ácido fumárico biotransformação de residuo sólido de mandioca por *Rhizopus* e basidiomacomicetos do gênero *Pleurotus*. Titular Professor Thesis, Parana Federal University, Curitiba, Brazil
- Vanneste G (1982) Enrichissement proteique du manioc par fermentation fongique. *Rev Ferm Ind Aliment* 37:19-24
- Vervack W (1973) Analysis des aliments, méthodes courantes d'analyses. Laboratoire de Biochimie de la Nutrition, UCL, Louvain-la-Neuve
- Wang HL, Ruttle DI, Hesseltine CW (1969) Antibacterial compound from a soybean product fermented by *Rhizopus oligosporus*. *Proc Soc Exper Biol Med* 131:579-583
- Zhu C R; Du MJ; Lei DN; Wan LQ A study on the inhibition of aflatoxin b₁ induced hepatocarcinogenesis by the *Rhizopus delemar* *Mater Med Pol* 21(2) : 87-91 1989

VALORIZATION OF AGRO-INDUSTRIAL RESIDUES BY SOLID STATE FERMENTATION IN BRAZIL

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Abstract

In this work some experiments for the using agro-industrial residues as substrates in solid state fermentation is presented and discussed. Since Brazilian industry of cassava (*Manihoc esculenta*, Crantz) cultures produce a large amount of solid wastes, these residues were employed as model - systems. There were developed different processes such as protein enrichment for human and animal nutrition, production of mushroom and metabolites such as enzymes, organic acids with successful perspectives.

I- Biotransformation of residues from Cassava Starch production by *Rhizopus* genus

Parana State is nowadays one of the biggest Brazilian cassava producer, its annual production being about 3.2 millions tons of roots. The Northwest region concentrates a significant number of starch industries because of this high production, some of them processing about 500 tons/day of roots.

Although these industries grant economical development to the Northwest region, the produced wastes, being hundreds of tons of solid and liquids wastes every day, heavily polluted the environment, namely rivers and streams. Figure 1. shows a basic unitary operations used by one of these industries to produce cassava starch. This industry, which processing capacity is about 100 ton/day of roots, produces 0.47 tons of peel, 112 tons of solid waste (cassava bagasse) with 85% of moisture and 1060 m³ of liquid waste (manipueira). Through these data we can evaluate the pollutant charge and the damages against environment by each industrial unit. We believe that research efforts in biotechnological processes to take advantage of these wastes as substrates could interest the industries, in order to reduce the impact against the environment.

Table 1 shows the results of physicochemical determinations on dried cassava bagasse from Paraná State starch industry. Residual starch is about 52.45% and total reducing sugars 59.63%. These results show starch extraction processes deficiencies in these industries, producing high raw material losses, and therefore, reducing the process efficiency. The level of residual starch may be still higher than this: Cereda (1994), in samples from Paraná. So Paulo and Minas Gerais States reported levels of 60-70 % of residual starch. We believe that cassava bagasse residual starch different contents found by other authors could be explained by variance on each industrial process conditions. Protein, lipids, fibers and ashes percentages are respectively: 1.67 ; 0.53 ; 22.19 and 1.10%.

The main objective of this work was to select *Rhizopus* strains capable to attack raw cassava bagasse (ungelatinised starch) for the attainment of a high level protein flour to be used for human or animal feeding.

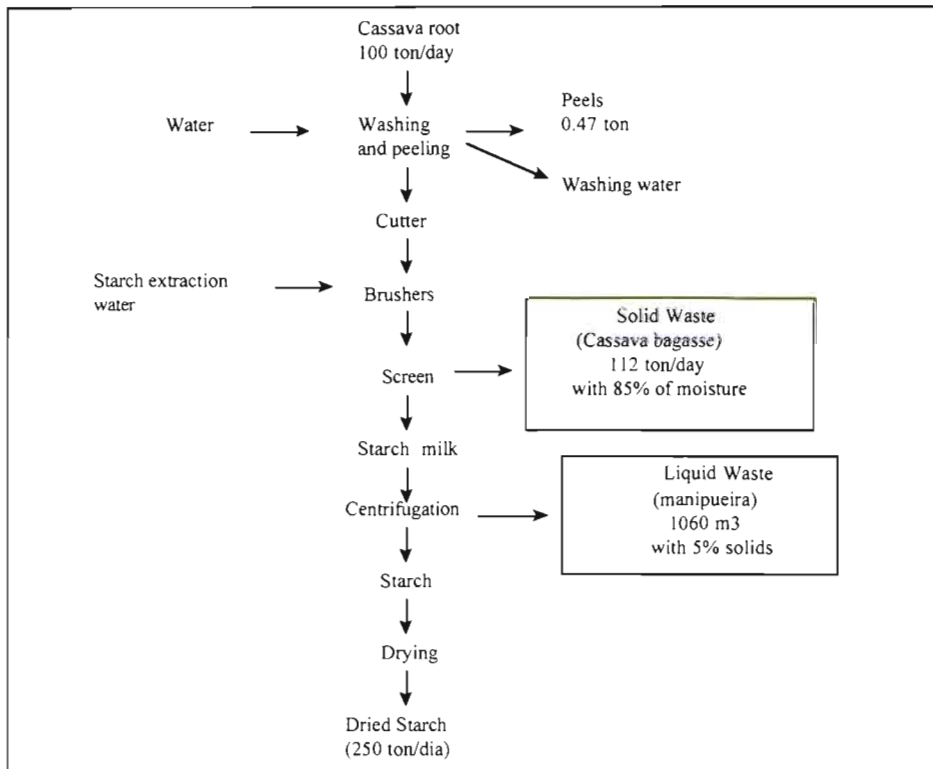


Figure 1 - Basic unitary operations utilised in cassava starch process in Parana State, Brazil.

Table 1 - Physicochemical composition of cassava bagasse

Composition	Content (%) ^a
Moisture	10.73
Proteins	1.67
Lipids	0.53
Starch	52.45
Total Sugar	59.63
Fibbers	22.19
Ashes	1.10

a) Dry basis

Table 2 shows the results of strain selection for different *Rhizopus* species able to attack and biotransform raw cassava bagasse. Considering the 19 studied strains, only 3 presented a significant development in raw cassava bagasse (*R. oryzae* 28627, *R. delemar* 34612 and *Rhizopus oryzae* 28168). Some of them presented a regular growth in this residue (*R. sp.* 25975 ; *R. formosa* 28422 ; *R. stolonifer* 28169 ; *R. oryzae* 22580 ; *R. oligosporus* 6203 ; *R. microsporus* 46436); and some showed a weak development (*R. arrhizus* 16179 ; *R. oryzae* 25976 ; *R. arrhizus* 1526 ; *R. oryzae* 395 ; *R. stolonifer* 28181 ; *R. arrhizus* 2582 ; *R. arrhizus* 28425). There were also those that did not grow on this substrate (*R. oligosporus* 2710 ; *R. circicans* 1475 and *R. delemar* 1472).

Table 2 – Screening for *Rhizopus* strains able to bio-transform cassava bagasse

strain	Growth	Residual starch g/100 g DM	Proteins g/100 g DM
<i>R. arrhizus</i> MUCL 16179	+	44.4	2.45
<i>R. oligosporus</i> NRRL 2710	-	52.3	1.8
<i>R. sp.</i> NRRL 25975	++	43	6.9
<i>R. oryzae</i> NRRL 25976	+	46	3.3
<i>R. circicans</i> NRRL 1475	-	50	1.8
<i>R. delemar</i> NRRL 1472	-	49	1.9
<i>R. arrhizus</i> NRRL 1526	+	46	3.8
<i>R. formosa</i> MUCL 28422	++	42	7.4
<i>R. oryzae</i> MUCL 28168	+++	32	10
<i>R. oryzae</i> NRRL 395	+	47	3.6
<i>R. oryzae</i> MUCL 28627	+++	30	10.5
<i>R. stolonifer</i> MUCL 28169	++	42	7.7
<i>R. oryzae</i> ATCC 22580	++	45	5.6
<i>R. oligosporus</i> ATCC 6203	++	43	5.9
<i>R. microsporus</i> ATCC 46436	++	42	6.3
<i>R. stolonifer</i> MUCL 28181	+	47	3.5
<i>R. delemar</i> ATCC 34612	+++	31	9.9
<i>R. arrhizus</i> NRRL 2582	+	48	3.5
<i>R. arrhizus</i> MUCL 28425	+	49	2.8
Raw cassava bagasse		53	1.7

ATCC = American Culture Collection (Rockville, Maryland, USA) ; MUCL = Catholic University of Leuven, Belgium) ; NRRL = Northern Regional Research Laboratory (U.S Department of Agriculture, Peoria, Illinois, USA)

- no growth, + weak growth, ++ regular growth, +++ excellent growth

Ideal growth conditions for *Rhizopus oryzae* 28627 in solid state fermentation using cassava bagasse were determined. These conditions were : temperature, 28-32 °C ; inoculation rate, 10⁵ spores/g dry bagasse; initial moisture, 70% ; C/N, 4.7-14 ; initial pH, 5.7-6.4.

Mould growth was evaluated through real synthesised protein determination. The results showed that its content varied from 1.67 g/100 g dry bagasse, in the beginning of the fermentation, to 12 g/100 g dry bagasse after 24 h of culture. Yield coefficient between produced protein and consumed starch and was about 0.5.

Kinetic of Growth

Figure 2A shows kinetic evolution of consumed starch and *R. oryzae* growth in cassava bagasse evaluated through synthesised protein in optimised conditions. During fermentation, about 42.23% of starch present in cassava was consumed. After 30h of fermentation the proteic level was 12.8 g/100g of dry cassava bagasse.

It is 7.7 fold of the initial value, in only 30 h of fermentation. 50% yield was obtained between consumed starch and synthesised protein, without considering that part of this starch was hydrolysed to reducing sugar equivalent in glucose (6.2 g/100g DM) and remained in cassava bagasse without use by the mould (Fig. 2B). It was observed a slight pH increase in the first 12 h of fermentation and an important decrease during the following hours, reaching pH 4.6 at the end of fermentation. This reduction would induce undesirable bacteria development.

Figure 2 C shows glucoamylase evolution during raw cassava bagasse bio-transformation. It was verified an important glucoamylase production increase after 12 h of fermentation, reaching its maximum after 30 h of fermentation (108 U/ g dry bagasse).

These numbers are similar to those obtained by SOCCOL (1992) and SOCCOL *et al.* (1994) working with raw cassava (ungelatinised) pellets. In the same figure, it can be observed a moisture increase from 69.14% at initial stage of fermentation to 72.77% after 30 h of culture. This moisture increase during fermentation is a good indicator of micro-organism growth.

Scale-up in Different Bio-Reactors

The global balance of the biotransformation of the raw cassava bagasse in different bioreactors it is presented in Table 3. We saw that it was possible to repeat and even to produce a significant increase in the protein richness, glucoamylase synthesis, as well as in starch consumption, in relation to the initial values on Petri plates. The bioreactors tray type showed an excellent performance in the transformation of cassava bagasse. The use of screens on the bottom of this type of bioreactor allowed a better aeration, as well as a more uniform growth of the fungi in the whole bagasse mass.

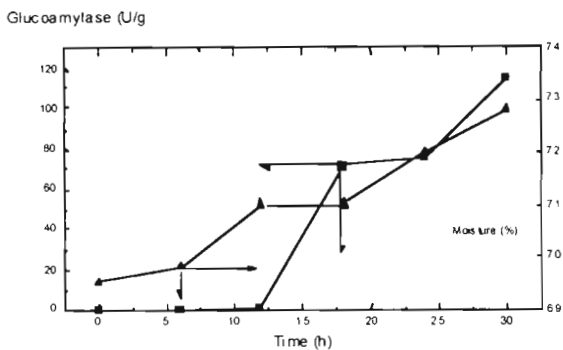
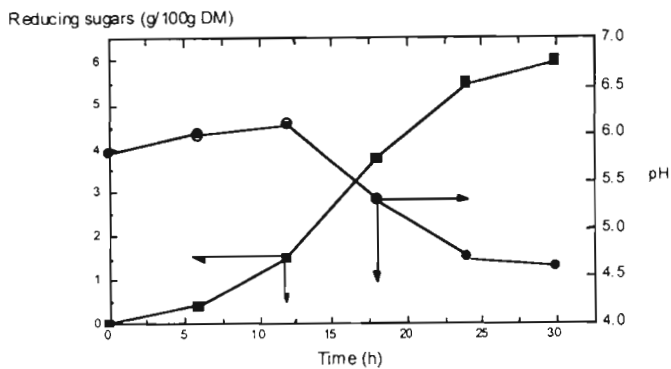
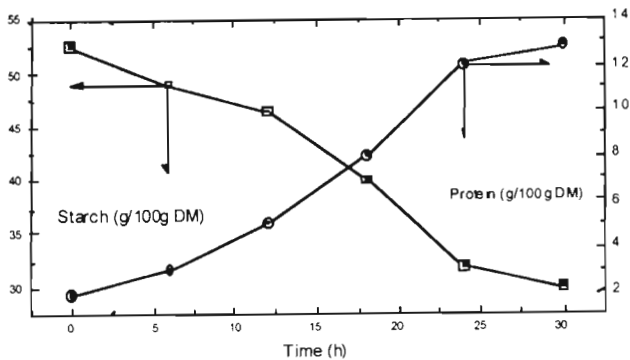


Figure 2. Kinetics of Cassava bagasse bio-transformation by *Rhizopus oryzae* 28.627. Evolution of A) Consumed starch and synthesised protein; B) pH and reducing sugars and C) Moisture and glucoamylase.

Table 3 – Global Comparison of Scale-Up Studies in Different Bioreactors

Bioreactors type	Residual Starch g/100b DM	Proteins g/100g DM	Glucosylase U/g DM	Reducing Sugar g/100g DM	Final pH	Final Moisture %	YP/S
Petri plate	30.3	12.8	108	5.7	4.6	73	0.5
Perforated small tray	29.6	13.44	94	4.7	4.4	71	0.52
Perforated big tray	28.7	13.5	103	5.2	4.3	0.5	0.5
Small column with forced aeration	27.9	11	80.5	4.2	4.8	74.5	0.38
Big column with forced aeration	31	9.4	74	3.54	4.9	77	0.36

DM : Dry matter ; P : Protein ; S : Starch ; Time of fermentation : 24 h ; Initial protein in bagasse 1.67 ; Initial starch in bagasse ; 52.45 ; YP/S : Yield coefficient

In such types of bio-reactors the protein synthesis was 44% superior to the values obtained in column bio-reactors. Even with forced aeration of 18 litres/hour used in the column type (big), the final concentration in real protein in the transformed bagasse was 9.4 g/100g of dried matter. The evolution of temperature during the bio-transformation of cassava bagasse by the fungus in column type bio-reactor with forced aeration is shown in Figure 3. The temperature rises at exponential rate after the first 10 hours of fermentation and reaches the maximum of 46 °C after 18 hours of fermentation. Physiologic studies showed that fungal growth can be affected when the temperature rises above 36°C. According to Raimbault (1980) and Crajek (1988) some fermentation can reach temperatures from 60-65°C due to the heat liberated by the micro-organism during its growth. This raising of the temperature could destroy the micro-organism and stop the fermentation, if not controlled. In our study the temperature did not affect the growth, although it was possible to notice a certain reduction in the development of the fungi. We believe that this problem could be minimised by a cooling cover around the column. Moisture level is superior at the end of bio-transformation in column type fermenters when compared to the tray type (Table 3). This air flow rate allows a more intense respiratory activity by the fungi, inducing an increasing in CO₂ and H₂O liberation. It was obtained superior values in glucosylase activity in tray type bioreactors when compared to the column type.

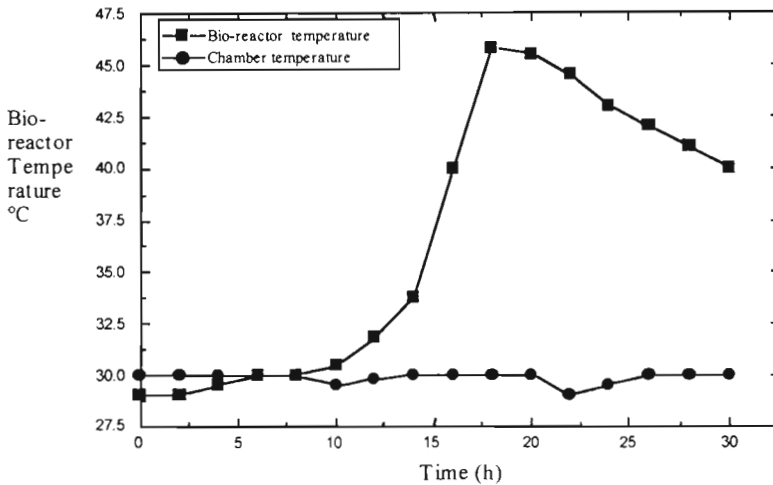


Figure 3. Evolution of the temperature in the core of the column type bio-reactor during the bio-transformation of cassava bagasse by *Rhizopus oryzae* 28.627.

Reducing sugars from the action of glucoamylase on starch cassava bagasse were found in every type of studied bio-reactors and represent an increase of nutritional value of the bio-transformed bagasse. Fermentation yield of 51% and 50% obtained in small and big tray are considered excellent when compared to the results obtained by Soccol (1992), working with pellets of raw cassava (38% yield). We believe that this high yield achieved with *R. oryzae* 28627 is due mainly to structural characteristic of the bagasse, though its less density and high fiber concentration could favour the aeration, as well microbial growth.

Thickness Effect of the Fermentation Bed in Bio-Reactor Tray Type

The effect of the thickness of the fermentation bed on the evolution of the temperature during the bio-transformation of cassava bagasse are shown in Figure 4. It was verified a raise of the fungus metabolism after 14 hours of fermentation. This activity reached its maximum between 22 and 24 hours of culture. The evolution of the temperature was proportional to the thickness of the fermentation bed in the bio-reactor. For a thickness of 8 cm, the inside temperature reached values up to 44 °C between 18 and 20 hours of fermentation. When the layer was reduced to 6 cm, the maximum temperature was 42.5 °C; with 4 cm, it was 37.5 °C and with 2 cm of thickness, it dropped to 31.5 °C. The values of the microbial growth, measured by synthesized protein, were superior for the thickness of 1.2 and 4.0 cm. These results showed that a rise in the temperature on the layers of 6.0 and 8.0 cm did not affect significantly the growth of the fungus. Yields in terms of synthesized proteins and consumed starch during the bio-transformation maintain themselves equally high to the different studied thickness (Table 4). We can see that the protein synthesis did not present a significant difference for all the studied thickness (12.0-13.7 g/100g DM). These results show the possibility of using almost the total practical volume of the bio-reactors tray type without any important losses in the protein synthesis.

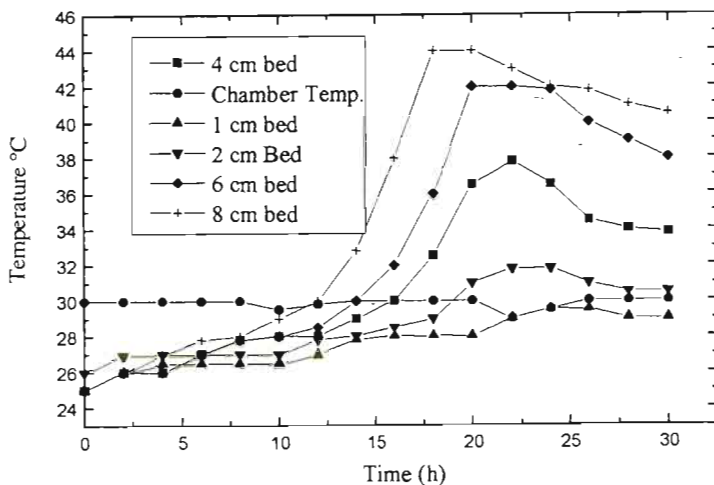


Figure 4. Effect of thickness on the temperature of the fermentation bed during the bio-transformation of cassava bagasse by *Rhizopus oryzae* 28.627 in tray type bio-reactor.

Table 4 - Effect of fermentation bed thickness on the bio-transformation of the cassava bagasse by *Rhizopus oryzae* 28627 in tray type bioreactor.

Fermentation Bed Thickness	Residual Starch g/100 g DM	Protein g/100g DM	Reducing sugar g/100 g DM	pH	YP/S
1	29.3	13.2	4.22	4.2	0.50
2	28.8	13.7	4.10	4.0	0.51
4	30.0	13.5	4.50	3.9	0.52
6	31.0	12.1	3.50	4.3	0.48
8	30.2	12.0	3.70	3.4	0.46

Bio-transformation conditions:

Time : 30 hours

Initial protein content in cassava bagasse : 1.67 g/100g DM

Initial starch in cassava bagasse : 53 g/100 g DM

YP/S : Yield coefficient (synthesised protein/consumed starch)

DM : Dried matter

Microbiological evaluation of the bio-transformed cassava bagasse

The results of the microbiological evaluation presented on Table 5 show that the bio-transformation of cassava bagasse allows the elimination almost completely of the microorganisms present in cassava bagasse before the bio-transformation (sample 1 and 2). The bio-transformed bagasse flour obtained showed an excellent sanitary condition (sample 2) and are perfectly inside the standards of the sanitary legislation, considering that the cassava bagasse was bio-transformed without any thermal process of sterilisation, being only dehydrated at 60 °C for about 24 h after fermentation. It can also be observed that the exposure of the flour under the ultraviolet rays (sample 3 and 4) for 5 and 10 min. eliminate completely the small number of yeast and moulds present at the sample 2. They reduced equally the account of mesophila bacteria, although did not eliminate it completely. The results showed that there was no growth of the undesirable bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* and faecal coliforms.

Table 5 – Microbiological Evaluation of the Bio-Transformed Cassava Bagasse Flour

Microbiological examinations	1	2	Samples 3	4	5
Mesophiles bacteria (NMP/g)	2 x 10 ⁵	3.3 x 10 ²	2.3 x 10 ²	2.1 x 10 ²	4.2 x 10 ²
Count total coliforms (NMP/g)	64	Negative	Negative	Negative	Negative
Moulds and Yeasts (CFU/g)	8 x 10 ³	2 x 10 ²	Negative	Negative	4 x 10 ²
<i>Staphylococcus aureus</i> (CFU/g)	Negative	Negative	Negative	Negative	Negative
<i>Bacillus cereus</i> (CFU/g)	Negative	Negative	Negative	Negative	Negative
<i>Salmonella</i> (in 25g)	Negative	Negative	Negative	Negative	Negative

Sample 1 - Cassava bagasse flour not bio-transformed

Sample 2 - Cassava bagasse flour bio-transformed

Sample 3 - Cassava bagasse bio-transformed and treated 5 min with UV

Sample 4 - Cassava bagasse bio-transformed and treated 10 min with UV

Sample 5 – Cassava flour selling in Curitiba City

CFU- Colony-forming units per g

MPN – Most probable number

The microbiological analysis of cassava bio-transformed bagasse flour and traditional cassava flour showed that the first one presented superior sanitary conditions. That is due probably to a bactericide action of *R. oryzae* 28627, associated to a reduction of pH that did not allow the development of a great number of bacteria, yeast and fungi. This results confirm those obtained by Wang *et al.* (1969), describing the capacity of certain strains of the genus *Rhizopus* to inhibit the growth of a large number of Gram (+) bacteria.

This study shows the high potential of some fungi strains like *Rhizopus* in bio-transforming solid amylaceous residues such as cassava bagasse. The performance of these strains was remarkable, considering that in only 24 hours of fermentation the protein content of the bagasse could be increased almost 8 times.

The efficiency in the synthesis of proteins together with the capacity of attacking the raw starch (not gelatinized) confer to these strains important characteristics, which could contribute significantly in the simplification of a large number of biotechnological processes that aim to the production of different metabolites.

II. EDIBLE MUSHROOMS *Pleurotus sajor-caju* PRODUCTION FROM CASSAVA BAGASSE

This work shows the feasibility of using cassava bagasse for production of edible mushrooms of the genus *Pleurotus*.

Selection of the strain

Table 6 shows the results of the five different strains of *Pleurotus* Spp. grew on the gelled medium prepared from cassava bagasse and agar. The strains *Pleurotus sajor-caju* CCB 19 and 20 showed the highest radial growth (13.3 and 11.3 mm/day, respectively) and also the highest biomass production (69.8 and 67.7 mg, respectively). These results led to the selection of strain *P. sajor-caju* CCB 19 to further experiments.

Table 6 – Radial growth and biomass production of five *Pleurotus* spp. strain cultivated in Petri dishes (8.5 cm) on agar-cassava fibrous residue medium (15g/l agar , 40 g/l cassava). Conditions: 6 days of culture, 25 °C and pH 7.0.

Strain	Radial growth (mm/day)	Biomass (mg/plate)
<i>Pleurotus ostreatus</i> CCB12	7.2 ± 1.27	27.3 ± 2.69
<i>Pleurotus ostreatus</i> LPB 22	9.5 ± 0.92	53.6 ± 4.52
<i>Pleurotus ostreatus</i> CCB 115	7.4 ± 0.28	9.8 0. ± 0. 64
<i>Pleurotus sajor-caju</i> CCB19	13.3 ± 0.0	69.8 ± 3.24
<i>Pleurotus sajor-caju</i> CCB 20	11.3 ± 0.71	67.7 ± 3.18

Fruit body production

In order to verify the feasibility of using agro-industrial residues for *Pleurotus sajor-caju* CCB19 development, several experiments with different ratios cassava fibrous waste : sugarcane bagasse were studied, as shows Table 7. Biological efficiency (BE) was utilized to measure to growth of the mushroom on the solid residues. The results showed that the best value (30.7%) was obtained for isolated cassava residue; the addition of sugarcane bagasse to cassava fibrous waste did not improve EB. Cassava residue had about 60% of carbohydrate (Barbosa, 1996).

Table 7 – Biological efficiency obtained with different cassava fibrous waste : sugarcane bagasse ratios for the fruit body production by *Pleurotus sajor-caju* CCB19. Conditions : spawn 10g, 55 days of culture (polyethylene bags) ; 24-28 °C, 70% relative humidity

Experiment	Cassava bagasse (g0)	Sugarcane bagasse	BE (%)
1	20	80	18.2
2	40	60	23.8
3	60	40	24.5
4	80	20	26.6
5	100	-	30.7
6	-	100	26.4

BE – Biological efficiency : fresh mushroom weight (g)/ substrate dry weight

The BE was obtained considering two flushes of fruit body production: the first at 45 days of culture (60% of the total yield) and the second at 55 days of culture This behavior was also observed by Chang et al (1981) and Nair (1989).

Effect of spawn concentration of fruit body production

Rajarithman and Bano (1987a) suggest 20% as the best value for seed-inoculum for *Pleurotus* spp. cultivation. However, Figure 5 shows that the best BE 38% obtained for *Pleurotus salor-caju* 10% of spawn inoculated to the medium

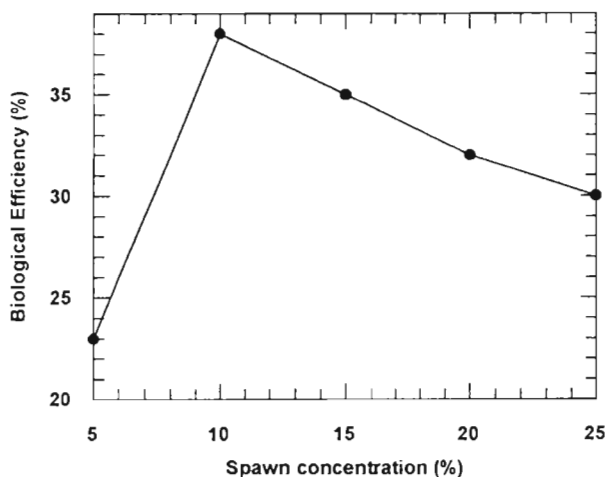


Figure 5. Effect of spawn concentration on biological efficiency (BE) obtained for the fruit body production by *Pleurotus sajor-caju* CCB19. Conditions: 55 days of culture (polyethylene bags) ; 24-28 °C, 70 % relative humidity.

Effect of soybean addition on fruit body production

The addition of ground soybean to cassava fibrous waste produced an improvement of *Pleurotus sajor-caju* development (maximum BE 69%) (Table 8), as reported by many authors (Rajaratnam *et al.*, 1987, Royse *et al.*, 1991, Royse, 1992, Belinski *et al.* . 1994). This result could be explained by the reduction of carbon:nitrogen ratio caused by soybean addition.

Table 8 – Biological efficiency obtained with different levels of soybean addition to cassava fibrous waste for the fruit production by *Pleurotus sajor –caju* CCB19. Conditions : spawn 10 g , 55 days of culture (polyethylene bags) ; 24-28 °C

Soybean (%)	Cassava bagasse (g)	BE (%)
0.0	100	29.80 ± 0.58
5.0	95	32.77 ± 1.80
10.0	90	37.10 ± 0.56
15.0	85	45.97 ± 3.37
20.0	80	69.00 ± 4.89
25.0	75	47.22 ± 2.46

BE – Biological efficiency : fresh mushroom weight (g)/substrate dry weight (g)

Scale -up of the fruit body production

Using the culture conditions established previously, the scale-up of the fruit body production by *Pleurotus sajor-caju* was carried out raising the quantities of the experiments 10 and 100 times higher. Table 9 shows that the EB value obtained for small scale was comparable to the results obtained for large scale, confirming the feasibility of mushroom cultivation in a larger scale.

Table 9 – Scale-up (1:10 ; 1:100) of the cultivation *Pleurotus sajor-caju* CCB19 on cassava bagasse.

Soybean	Cassava bagasse (g)	BE (%)
20	80	69.00 ± 4.89
200	800	63.43 ± 2.55
2000	8000	65.27 ± 5.21

B.E – Biological efficiency : fresh mushroom weight (g)/substrate dry weight (g)

Conclusions

Pleurotus sajor-caju CCB19 cultivated on cassava bagasse substrate showed a biological efficiency (BE) of 30%. The addition of soybean flour to the cassava bagasse raised the BE from 30 to 69% and the scale-up of process was successfully carried out. These results show that agro-industrial residues as cassava bagasse can effectively be used for commercial production of the edible mushroom by solid state fermentation. Similar advantageous results were also obtained for the production of *Lentinula edodes* with cassava bagasse.

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REFERENCES

- Barbosa, M.C.S. Tese de mestrado . Setor de Tecnologia, Universidade Federal do Paraná, Curitiba, 1996, 117p.
- Belinski , P. A . , Masaphy, S. , Levanon, D., Hadar, Y. and Dosoretz, C.G. *Appl. Microbiol. Biotechnol.*, 40, 629-633, 1994
- Cereda M . *Res'duos da industrialização da mandioca no Brasil*. São Paulo- Brasil, Editora Paulic_ia, 1994. P. 27.
- Chang S.T., Lau, O W and Cho, K.Y. *European J. Appl. Microbiol. Biotechnol.*, 12, 58-62, 1981.
- Grajek W. Cooling aspects of solid state cultures of mesophilic and thermophilic fungi. *J. ferment. Technol.*, 1988, 66 : 675-79.
- Nair, N.G. *Ann. App. Biol.*, 114, 167-176, 1989
- Raimbault M. Fermentation en milieu solide: croissance de champignons filamenteux sur substrat amylicé. Thèse de Doctorat es science, Univ. Paul Sabatier; Toulouse, 1980; 291 p.
- Rajarathnam, S. and Bano, Z.. *CRC. Critical Rev. Food Sci. Nutrition*, 26, 157-223, 1987a.
- Rajarathnam, S. and Bano, Z.. *CRC. Critical Rev. Food Sci. Nutrition*, 26, 243-311, 1987b.
- Royse, D.J., Fales, S.L. and Karunanandaa, K. *Appl. Microbiol. Biotechnol.*, 36,425-429, 1991.
- Royse, D.J. *Appl. Microbiol. Biotechnol.*, 38,179-182, 1992.
- Soccol, C. R., Raimbault, M. and Pinheiro, L. I. *Arq. Biol. Tecnol.*, 37, 203-210, 1994.
- Soccol C.R . Physiologie et métabolisme de *Rhizopus* en culture solid et submerg_e en relation avec la dégradation d'amidon cru et la production d'acide L(+) lactique. These de Doctorat. Mention/Genie enzymatique, Bioconversion et microbiologie, Universite de Technologie de Compiègne, Compiègne- France, 218p, 1992.
- Wang H.L., Ruttle D.I., Hesseltine C.W.. Antibacterial compound from a soybean product fermented by *Rhizopus oligosporus*. *Proc. Soc. Exper. Biol. Med.*, 1969; 131 : 579-583.

CITRIC ACID AND GLUCOAMYLASE PRODUCTION FROM CASSAVA BY-PRODUCTS IN SOLID STATE FERMENTATION

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I - Citric acid production from Cassava Bagasse

Six strains of *Aspergillus niger* were screened in liquid medium and strain LPB 21 was selected for further studies on 3 different agro-industrial residues for production of citric acid by solid state fermentation. Cassava bagasse was found to be a better substrate than vegetal sponge and sugar cane bagasse. Citric acid production and yields were respectively 13.64 g/100g dried substrate and 41.78 % of acid citric produced by starch consumed. In the studies under optimized conditions, the production of citric acid was 280 g/Kg of dry bagasse at 120 h, which corresponds to a yield 70%, based on starch consumed. Kinetics studies on of pH changes, moisture level, loss of weight of the substrate, starch utilization and α -amylase production allowed an insight in the process.

Effect of initial moisture content of cassava bagasse

The water content of solid support has been reported as an important limiting factor for solid state fermentation (Pandey,1992 , Lonsane *et al.*, 1992). Consequently, the effect of moisture present in cassava bagasse was investigated in the range 45-65% initial moisture. Results showed that the production of citric acid at 8 days increased with an increase in initial moisture content of the medium up to 50% (Table 1).

Table 1. Effect of Initial Moisture of Cassava Bagasse on Citric Acid Production by *Aspergillus Niger* LPB 21

Moisture %	Citric acid produced (g/100 g dried bagasse)	Sugar consumed (g/100g dried bagasse)	Yield %
45	16.35	36.92	44.28
50	27.25	40.1	67.96
55	25.32	36.83	68.75
60	22.15	39.63	55.89
65	19.39	37.19	52.14

Initial sugar : 46.5 g/100 g dried support, Yield : g produced citric acid / g consumed sugar

A further increase in the initial moisture of cassava bagasse of 10% had an adverse effect on the synthesis of citric acid. Thus, the initial moisture content of 50% could be considered as ideal to produce high quantity of citric acid (272g/Kg cassava bagasse). In this case, fermentation yield was approximately 70%.

Effect of Initial pH

Table 2 presents the results of production of citric acid by SSF using different initial pH values. It can be seen that very low values (1.0) reduced the production of the metabolite; higher values favored the production and the maximum was obtained at pH 2.0; in this case, the concentration of produced citric acid was 246 g/Kg dried cassava bagasse with a yield of 71.45 %. These results confirm those reported by many authors, that the initial pH of the medium for citric acid production is in the range of 1.6 to 3.5 (Kolichski, 1995, Meers *et al.* 1991 ; Miller, 1981).

Table 2- Effect of Initial pH of the Medium on Citric Acid Production by *Aspergillus Nige* LPB 21 on Cassava Bagasse by Solid State Fermentation

pH	Citric acid production (g/100 g dried bagasse)	Consumed Sugar (g/100 g dried bagasse)	Yield (%)
1	18.12	28.32	63.98
2	24.64	34.54	71.45
3	21.93	34.19	64.14

Initial sugar : 46.5 g/100 g dried support, Yield : g produced citric acid / g consumed sugar

Aeration Effect

Aeration is an important factor for citric acid production in column type bio-reactors, since the air flow helps to dissipate metabolic heat and also provide necessary oxygen for the growth of the microorganism. Table 3 shows that the optimal aeration flow was between 50 and 60 ml/min. Aeration rates above or below these values lead to a decrease in citric acid production.

Temperature Effect

The optimal temperature for fermentation varies according to the microorganism and is usually between 25 and 35°C (Prescott and Dunn, 1959). In this work the highest production of citric acid occurred at 26 °C (Table 4), confirming those reported above.

Table 3 – Effect of Aeration Rate on Citric Acid Production by *Aspergillus Niger* LPB 21 Grown on Cassava Bagasse in Solid State Fermentation.

Aeration (ml/min/column)	Citric acid produced (g/100 g dried support)	Sugar consumed (g/100 g dried support)	Yield (%)
40	13.6	38.36	35.45
50	25.42	36.13	73.36
60	27.12	37.54	72.24
70	19.45	37.51	51.85
80	17.32	32.61	53.11

Initial sugar : 46.5 g/100 g dried support, Yield : g produced citric acid / g consumed sugar

Table 4. Effect of Temperature on Citric Acid Production by *Aspergillus niger* LPB 21 Grown on Cassava Bagasse in Solid State Fermentation.

Temperatures °C	Citric acid production (g/100 g dried bagasse)	Consumed sugar (g/100 g dried bagasse)	Yield (%)
24	15.64	37.81	41.36
26	28.45	37.66	75.54
28	23.91	36.88	64.83
30	20.80	35.97	57.83
32	19.73	37.67	51.96

Initial sugar : 46.5 g/100 g dried support, Yield : g produced citric acid / g consumed sugar

Kinetics of Citric Acid Production

Figure 1A shows the evolution of consumption of starch and citric acid production during the fermentation. The production of citric acid started in the initial period of fermentation although its concentration reached 74 g/kg dried cassava bagasse at 48 h of culture. Between 48 and 72 h, citric acid productivity achieved its highest point (201.34 g/Kg dried cassava bagasse) and from 72 to 120 h, the final concentration was 280 g/Kg dried cassava bagasse, which corresponds to a yield of 70% in relation with the consumed starch. These results are higher than those reported by some authors working with different substrates (Hang *et al.*, 1987 ; Omar *et al.*, 1992 ; Shakaranand and Lonsane, 1993).

Production of α -amylase was higher during the first 72 h fermentation (192 IU/g dried cassava bagasse) (Figure 1B) and is associated with the higher consumption of starch in this period (Figure 1A). pH is gradually reduced as citric acid is accumulated in the medium and reaches its lowest value (pH 0.5) after 96 h fermentation (Figure 1B).

Figure 1C shows the increase in medium moisture and the support loss of weight of during the culture. An increase of moisture from 60.32 to 64.18 % is most likely due to fungal metabolism (Soccol, 1992 and 1994) and the loss of weight of the support is due to utilization of starch by micro-organism.

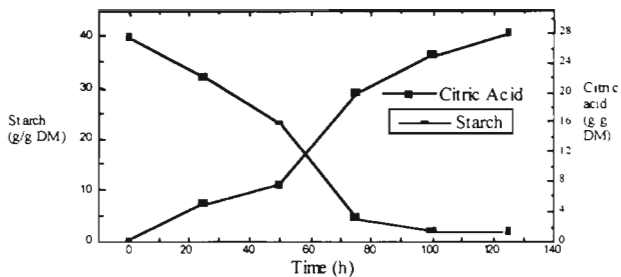


Figure 1A - Kinetics of citric acid production by *Aspergillus niger*. Evolution of starch consumption and citric acid production

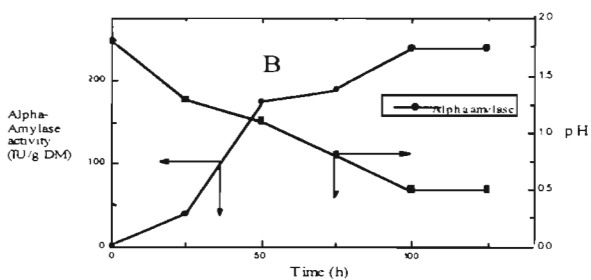


Figure 1B - Kinetics of citric acid production by *Aspergillus niger*. Evolution of alpha-amylase and pH during the fermentation

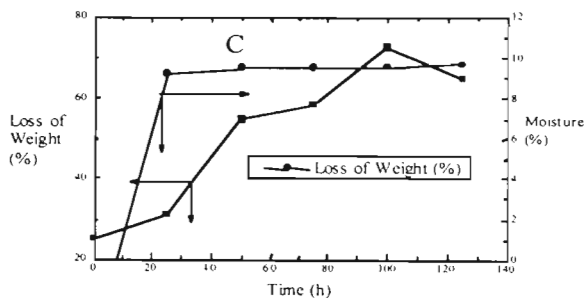


Figure 1C. Kinetics of citric acid production by *Aspergillus niger*. Evolution of moisture and loss of weight during the fermentation

II - Glucoamylase production from Cassava Bagasse by Solid State

Two strains of *Rhizopus oryzae* (MUCL 28627 and MUCL 28168) and one of *Rhizopus delemar* (ATCC 34612) were investigated for their ability to produce glucoamylase able to attack granular starch. Cassava (*Manihoc esculenta*, Crantz) bagasse was employed as substrate in solid state fermentation (SSF). Scanning electron microscopy was used to follow granular starch degradation by the fungal enzyme. An experimental design was used to optimize the fermentation time and the most suitable pH condition to extract the enzyme from SSF medium. Enzyme yield was evaluated as specific activity. *R. oryzae* MUCL 28627 presented most important specific activity after fermentation of crude bagasse. Higher specific activity values (> 10 kat/kg) were obtained after 72 hours fermentation at 32 °C and extraction of the enzyme activity with pH 4.5 200 mM acetate buffer from the whole fermented biomass.

Screening of strain

Previous screening work realized by SOCCOL (1992) showed that three strain of *Rhizopus* were able to attack granular crude starch: *Rhizopus oryzae* MUCL 28627 and MUCL 28168 and *Rhizopus delemar* ATCC 34612. In this work, these selected strains were assayed to growth in cassava bagasse, an agroindustrial waste still having starch as the main component (50 %, dry basis), by solid state fermentation. Actually, the three strains presented a marked development in the substrate, characterized by the colonization of the entire surface of the Petri dish by mold mycelium, after 32 hours fermentation (results not shown). The specific activity recovery is low in the first 12 hours of fermentation (Figure 2). In fact, the initial 8-12 hours correspond to the final of the latency phase and the start of spore germination. Later the specific amyolytic activity enhances as a function of fermentation time until 32 hours for all strains investigated (Figure 2). However, the activities of *Rhizopus delemar* ATCC 34612 and *Rhizopus oryzae* MUCL 28168 remain almost constant after this time period whereas the activity of *Rhizopus oryzae* MUCL 28627 shows a continuous increase in the studied period of 48 hours. On another hand, the absolute values observed for specific activity of *R. oryzae* 28627 were always superior to those observed for the two other strains.

As specific activity of glucoamylase from *R. oryzae* MUCL 28627 seemed to increase as a function of time (Figure 2), an experimental design with superior time periods was employed to follow the activity yield enhancement (Table 5). Other factors, like bagasse initial moisture content and extraction pH were also investigated. In fact, a high humidity content may affect enzyme production by favoring contaminant development or altering enzyme and oxygen diffusion. The correct choice of the pH for enzyme activity extraction, on the other hand, may improve further purification procedures.

The experimental design was run twice, totaling 34 assays. The protein content and the activities of glucoamylase and α -amylase were measured (Table 5). In experimental conditions of glucoamylase determination (60 °C temperature, pH 4.5 and 2-5 min. reaction time) no significant activity of α -amylase was detected, since the amount of reducing sugars determined

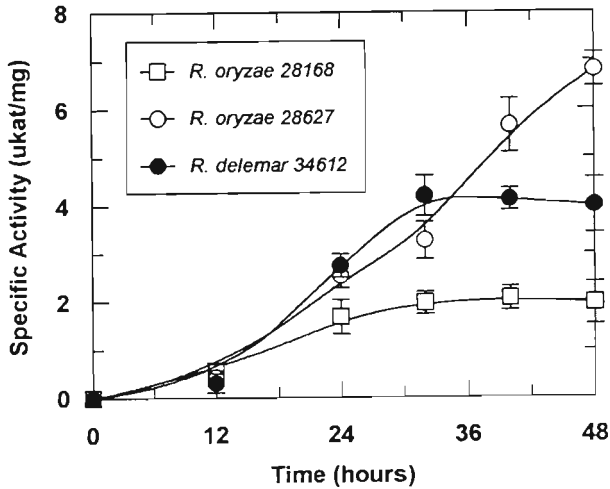


Figure 2. Glucoamylase specific activity as a function of the selected *Rhizopus* strain and the time of solid state fermentation.

by Somogyi-Nelson method were coincident with the content of α -D-glucose measured enzymatically by glucose-oxidase/peroxidase method.

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Experimental results of soluble protein content and specific amylolytic activity were submitted to ANOVA (Table 6 and 7). For soluble proteins, the correlation coefficient ($R^2 = 0.938562$) and the very low p-value for the lack-of-fit ($p = 0.0019$) indicate that the mathematical model explains the experimental variation observed (Table 6). Fermentation time and extraction pH caused significant effects ($p < 0.0005$) on the response content of proteins/mL of crude extract, but not the initial moisture content. Indeed, the levels of added

Table 5. Experimental design employed to optimize the extraction of glucoamylase activity from cassava bagasse fermented by *Rhizopus oryzae* 28627 and the respective experimental responses.

Run	Time (hours)	Extraction pH	Initial moisture (mL H ₂ O/g dry bagasse.)	Soluble proteins (mg/mL)	Activity (nkat/mL)	Specific activity (mkat/mg)
1	53	6.5	2.8	177	281	1.59
2	34	4.5	1.9	163	386	2.39
3	72	4.5	1.9	50	462	9.25
4	34	8.5	1.9	372	168	0.45
5	72	8.5	1.9	296	273	0.92
6	34	4.5	3.7	182	431	2.37
7	72	4.5	3.7	37	461	12.46
8	34	8.5	3.7	354	142	0.40
9	53	6.5	2.8	164	291	1.77
10	72	8.5	3.7	316	301	0.95
11	27	6.5	2.8	181	175	0.97
12	79	6.5	2.8	104	288	2.77
13	53	3.8	2.8	53	256	4.83
14	53	9.2	2.8	321	125	0.39
15	53	6.5	1.5	189	244	1.29
16	53	6.5	4.1	157	367	2.34
17	53	6.5	2.8	186	285	1.53
18	53	6.5	2.8	204	259	1.27
19	34	4.5	1.9	175	418	2.39
20	72	4.5	1.9	41	481	11.74
21	34	8.5	1.9	390	161	0.41
22	72	8.5	1.9	282	305	1.08
23	34	4.5	3.7	194	395	2.04
24	72	4.5	3.7	53	495	9.34
25	34	8.5	3.7	367	124	0.34
26	53	6.5	2.8	109	301	2.76
27	72	8.5	3.7	280	304	1.09
28	27	6.5	2.8	198	190	0.96
29	79	6.5	2.8	74	324	4.38
30	53	3.8	2.8	59	290	4.92
31	53	9.2	2.8	296	148	0.50
32	53	6.5	1.5	221	258	1.17
33	53	6.5	4.1	171	310	1.81
34	53	6.5	2.8	193	306	1.59

water presently investigated are correspondent to 65 up to 79% of initial moisture. Soccol (1994) verified that this is the optimum range for protein production by *R. oryzae* 28627 by SSF, which explains why its effect does not significantly affects protein responses. In the same way, interaction effects of initial moisture with other variables and quadratic effects of time variable were not significant ($p > 0.10$). They were eliminated of the analysis and the resulting regression equation was employed to plot Figure 3. The maximal response zone is located in the direction of minor fermentation time periods and higher pH values of the extraction buffer (Figure 3). The decrease on protein content as a function of time is supposed to be related to secretion of protease during fermentation and it is presently under investigation in our laboratory. The effect of the pH of extraction buffer is outstanding: the basic buffer (pH 8.5) extract 2 up to 6 fold more protein than the acidic one (pH 4.5), depending on fermentation time course. In fact, a great part of biosynthesized proteins present pI near to pH 4.5 and they are very soluble at basic pH (pH > 8) what could contribute to the observed increase of the extraction yield. Conversely, acidic pH acts as a selective extraction factor, since only proteins presenting net charge in acidic pH will be solublized (Chiarello et al. 1996).

Table 6. ANOVA for experimental responses of soluble protein content (mg/mL of crude extract)

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F. ratio	p value
A: Time	53205.19	1	53205.19	118.11	0.0000
B: pH	256374.64	1	256374.64	569.13	0.0000
C: Initial Moisture	403.05	1	403.05	0.89	0.3661
AB	3136.00	1	3136.00	6.96	0.0162
AC	25.00	1	25.00	0.06	0.8188
BC	225.00	1	225.00	0.50	0.4958
AA	108.28	1	108.28	0.24	0.6347
BB	9294.00	1	9294.00	20.63	0.0002
CC	10181.90	1	10181.89	22.60	0.0001
Lack-of-fit	13236.23	5	2647.25	5.88	0.0019
Pure error	8558.83	19	450.46		
TOTAL	354748.03	33			
$R^2 = 0.938562$					

Table 7. ANOVA for experimental responses of specific glucoamylase activity (mkat/mg of soluble proteins).

Effect	Sum of Squares	Degrees of Freedom	Mean Square	F. ratio	p value
A: Time	81.44	1	81.44	157.32	0.0000
B: pH	134.88	1	134.88	260.55	0.0000
C: Initial Moisture	0.26	1	0.26	0.50	0.4939
AB	58.53	1	58.53	113.05	0.0000
AC	1.02	1	1.02	1.97	0.1765
BC	0.01	1	0.01	0.01	0.9407
AA	83.05	1	83.05	11.69	0.0029
BB	10.49	1	10.49	20.27	0.0002
CC	1.50	1	1.50	2.90	0.1051
Lack-of-fit	19.06	5	3.81	7.36	0.0005
Pure error	9.84	19	0.52		
TOTAL	323.07	33			
$R^2 = 0.910551$					

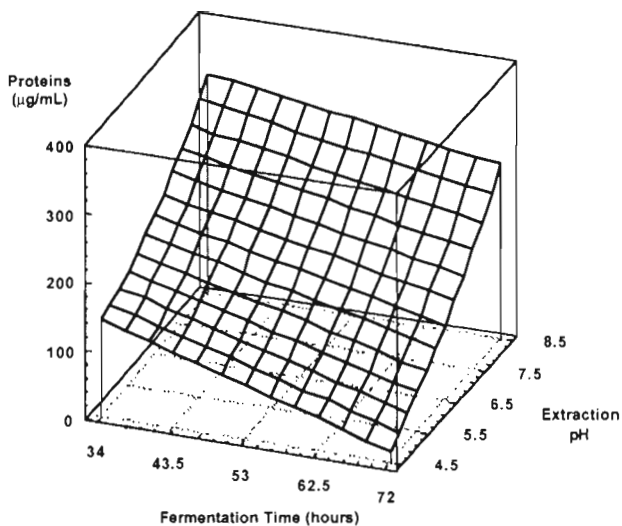


Figure 3. Response surface to soluble proteins as a function of fermentation time and extraction pH. Soluble proteins (mg/mL) = $157.42 - 47.76A + 104.84B - 4.16C + 14AB + 26.32B^2 + 27.56C^2$, where A is coded units of time, B, coded units of extraction pH and C coded units of initial moisture. $R^2 = 0.937552$

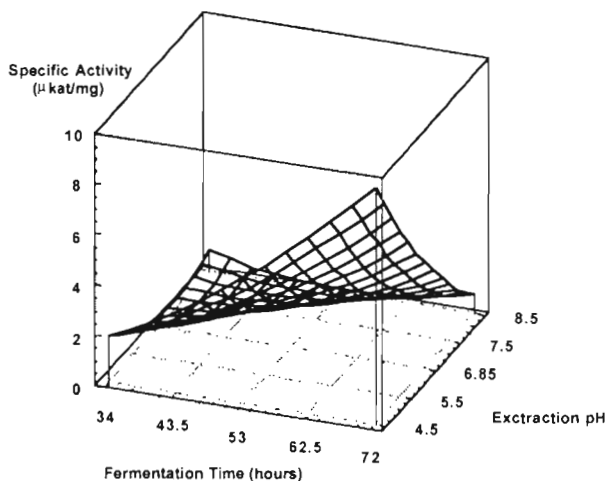


Figure 4. Response surface to specific glucoamylase activity as a function of fermentation time and extraction pH. Specific activity (mkat/mg) = $1.67 + 1.87A - 2.41B + 0.11C - 1.91AB + 0.67A^2 + 0.88B^2$, where A is coded units of time, B coded units of extraction pH and C coded units of initial moisture. $R^2 = 0.902743$.

As observed for soluble protein responses, only the fermentation time and the extraction pH showed a significant effect on the glucoamylase specific activity responses (Table 7). Statistical analysis confirmed that the mathematical model fits well to experimental data ($R^2 = 0.910551$ and p value < 0.0005 for lack-of-fit). Since the initial moisture variable, its interactions and quadratic effects were also not significant, they were eliminated of the analysis and the resulting regression equation was employed to plot Figure 4.

The best yield of specific activity was obtained with fermentation time of 72 hours and extraction pH of 4.5 (Figure 4). The acidic pH is supposed to protect enzyme against aspartic proteinases attack during a long period fermentation, since the pI of these fungal hydrolases is below pH 5.1 (Campos & Felix, 1995). On another hand, the low solubility of non enzymatic proteins at pH 4.5 seemed to contribute to the selective extraction of glucoamylase.

In fact, glucoamylase is a widely spread enzyme, found mainly in fungi and less often in bacteria and generally in more than one isoform in the same species (Ali et al. 1994 ; Fitatusuji et al. 1993). Depending on the enzyme source, the pI is also variable. Mold glucoamylases can display pI from 4.1 up to 8.4 (Speck et al. 1991 ; Yamasaki, 1978) and, probably, the glucoamylase from *R. oryzae* 28627, able to attack granular starch, has a pI located on neutral or alkaline zone, what explains its selective extraction at pH 4.5.

Conclusion

Glucoamylases of *Rhizopus oryzae* 28627 were able to attack and perforate granular cassava starch as observed by scanning electron microscopy. Higher yields of specific activity were obtained after 72 hours of solid state fermentation of cassava bagasse provided an extraction of the enzymatic protein with 200 mM pH 4.5 acetate buffer. Ongoing studies in this field are in progress to purify glucoamylases and to elucidate the action of proteases on glucoamylolytic activity losses.

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REFERENCES

- Ali, S.; Malek, ; Hossain, Z. (1994) Purification and characterization of a thermostable glucoamylase from a *Myrothecium* isolate. *J. App. Bacteriol.*, 76:210-215.
- Barret, A. J. (1986). An introduction to the proteinases. In: Barret, A. J. & Salvesen. *Proteinase Inhibitors*. Amsterdam, Elsevier, 1986. p. 1-21.28.
- Campos & Feliz, C.R. (1995). Purification and characterization of a glucoamylase from *Humicola grisea*. *Appl. Environ. Microbiol.*, 61(6):2436-2438.
- Chiarelo, M. D.; Larre, C.; Kedzior, M.; Gueguen, J. (1996). Pea seedling extract catalysis amine-binding and cross-linking. I. Evidences for the role of a diamine oxidase. *J. Agric. Food Chem.*, 44(11<):3717-3722.

- Fitatsuji, M.; Ogawa, T.; Fukuda, H. (1993). Purification and properties of two forms of glucoamylase from *Saccharomyces fibuliger*. *J. Ferm. Bioeng.*, 76(6):. 521-523.
- Raimbault M & Alazard D. Culture method to study fungal growth in solid fermentation. *European J. Appl. Microbiol.*, 1981, 9 : 199-209.
- Soccol, C. R. Tese, Professor Titular em Biotecnologia e Tecnologia de Alimentos - Setor de Tecnologia, Universidade Federal do Paraná, Curitiba, 1994, 228 p.
- Soccol C.R. Physiologie et métabolisme de *Rhizopus* en culture solide et submergée en relation avec la dégradation d'amidon cru et la production d'acide lactique L(+). Thèse de Doctorat. Mention/Genie enzymatique, Bioconversion et microbiologie, Université de Technologie de Compiègne, Compiègne- France, 218p, 1992.
- Specka, U.; Mayer, F.; Antranikian, G. (1991). Purification and properties of a thermoactive glucoamylase from *Clostridium thermosaccharolyticum*. *Appl. Environ. Microbiol.* 57(8):2317-2323.
- Yamasaki, Y. & Suzuki, Y. (1978). Purification and properties of α -glucosidase and glucoamylase from *Lentinus edodes* (Berk) Sing. *Agric. Biol. Chem.*, 42(5):917- 980.
- Ayala, L. A. C.; Soccol, C. R.; Santos, H. R.; Todeschini, M. L. Produção de esporos de fungo entomopatogênico (*Beauveria bassiana*) por fermentação no estado sólido utilizando como substrato refugo de batata. *Anais do XI SINAIFERM*, Sociedade Brasileira de Microbiologia, Vol 2, pp. 574-579, 1996.
- Barbosa, M.C.S.; Soccol, C.R.; Todeschini, M.L.; Tonial, T.; Flores, V. Cultivation of *Pleurotus sajor-caju* in cassava waste. In : G.M. Zanin & F.F. Moraes (Eds). *Anais IV Seminário de Hidrólise Enzimática de Biomassas*. Editora Sthampa, 1994, p 179-181.
- Barbosa, M.C.S.; Soccol, C.R.; Krieger, N.; Chiarello, M. D. *Pleurotus sajor-caju* production from cassava waste by solid state fermentation. *Fifth Brazilian Symposium on The Chemistry of Lignins and Other Wood Components*. Curitiba (Pr) Brasil, 31/8 to 05/09, 1997, p 557-569.
- Beux, M.R.; Soccol, C.R.; Raimbault, M. Use cassava bagasse on cultivation edible fungus *Lentinus edodes* by solid state fermentation. In : G.M. Zanin & F.F. Moraes (Eds). *Anais IV Seminário de Hidrólise Enzimática de Biomassas*. Editora Sthampa, 1994, p 190- 198.
- Beux, M. R. & Soccol, C. R. - Cultivo do fungo comestível *Lentinula edodes* em resíduos agroindustriais do Paraná através do uso de fermentação no estado sólido. *Boletim do CEPPA*, vol. 14 (1) p. 11-21, 1996.
- Beux, M. R.; Soccol, C. R.; Marin, B.; Tonial, T.; Roussos, S. Cultivation of *Lentinus edodes* on mixture of cassava and sugar cane bagasse. In: Roussos, S.Lonsane, B. K.; Raimbault, M.; Viniegra-Gonzalez, G. (Eds) *Advances in Solid State Fermentation - Edible mushrooms/ fungi*. Dordrecht, Kluwer Acad. Pub., c. 41, 1997. p501-514.
- Kolicheski, M. B.; Soccol, C. R.; Marin, B.; Medeiros, E.; Raimbault, M. Citric acid production on three cellulosic supports in solid state fermentation. In: Roussos, S.Lonsane, B. K.; Raimbault, M.; Viniegra-Gonzalez, G. (Eds) *Advances in Solid State Fermentation - Secondary metabolites, aroma, pigment and biopesticides*. Dordrecht, Kluwer Acad. Pub., c. 36, 1997. p449-462.
- Kolicheski, M. B. & Soccol, C. R. Produção de ácido cítrico por fermentação estado sólido (FES) utilizando como substrato bagaço de mandioca. *Anais do XI SINAIFERM*, Sociedade Brasileira de Microbiologia, Vol 1. pp 369-375, 1996.
- Raimbault, M.; Giraud, E.; Soccol, C.R.; Saucedo, G. Fermentation in cassava bioconversion. In ; Dufour, D. ; O'brien, G.M. ; Best, R. (Eds) *Cassava Flour and Starch ; Progress in*

Research and Development - Session 4 - Bioconversion and By-products. CIAT Pub. N. 271, 1996. p187-196..

- Soccol, C. R. Agroindustrial Applications of Solid State Fermentation Processes. In: E. Galindo (Ed.), *Fronteras en Biotecnología y Bioingeniería*, pp. 349-358. *Sociedade Mexicana de Biotecnología y Biengeneria*, 1996.

- Soccol, C. R. Aplicações da fermentação no estado sólido na valorização de resíduos agroindustriais. *França -Flash Agricultura*, 4(2): 3-4, 1995.

- Soccol, C. R. Contribuição ao estudo da fermentação no estado sólido em relação com a produção de ácido fumárico e biotransformação de resíduo sólido de mandioca por *Rhizopus* e Basidiomacromicetos do gênero *Pleurotus*. Tese de Professor Titular. UFPR, Curitiba. 1994. 228p.

- Soccol, C. R.; Marin, B.; Raimbault, M.; Lebeault, J. M. Potential of solid state fermentation for production of L(+) lactic acid by *Rhizopus oryzae*. *App. Microbiol. Biotechnol.* 41: 286-290, 1994.

- Soccol, C.R. ; Cabrero, M.A. ; Roussos, S ; Raimbault, M . Selecion de cepas de *Rhizopus* em base a su capacidade de crecimiento en yuca. *Sociedade Mexicana de Biotecnología e Bioengennieria*, V.3 ; N 1-2, p. 61-64,1993.

YEAST CULTIVATION IN SSF: CONTROL OF METABOLISM OF *Schwanniomyces castellii* DURING SOLID CULTIVATION ON STARCH

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Abstract *

Cultivation reports of yeasts in SSF are scarce, there are some work done on ethanol production from sweet sorghum and few papers on protein enrichment. Filamentous fungi are the most used microorganism in SSF. Nevertheless, yeasts systems provide an interesting experimental model for studies concerning control of metabolism in SSF systems. Other advantage of yeasts is that cells can be easily separated from support, then biomass determination can be carried out using standard methods of submerged fermentation.

SSF by Schw. castellii was selected as experimental model to study a fermentation with two phases: an aerobic stage required for biomass build up, amylase synthesis and an anaerobic phase for the transformation of hydrolyzed starch into ethanol.

Switching of phases can be easily done by modifying the gas phase in the fermentor. Data on conversion of starch into biomass and ethanol indicated that the overall ethanol conversion was of 57 % of the theoretical values, nevertheless, the alcohol yield in the anaerobic phase was 94 % of the theoretical value. The biomass formation is confined to aerobic stage.

Solid state cultivation of yeasts was used also to develop a automated monitoring and control system for SSF. It provides on line data of carbon dioxide and oxygen in real time without disturbing the fermentation. In this sense, the estimation of specific growth rate by direct biomass determination and gas analysis were similar. In the recent years, our research team have supported development on these equipments using specific transducers and gas chromatography. To our knowledge this kind of in line automated systems appears to be a very useful feature for SSF systems.

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SUGARCANE USED IN SOLID STATE FERMENTATION FOR CELLULASES PRODUCTION

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Cellulases production and applications

Cellulases, the enzymes capable of hydrolyzing cellulosic compounds, find extensive use in extraction of green-tea components, modification of food tissues, removal of soybean seed coat, improving cattle feed quality, recovering juice as well as other products from plant tissues and as component of digestive aid (Toyama, 1969; Lonsane and Ghildyal, 1991). Cellulases can be produced by submerged or solid state fermentations.

The latter technique is generally preferred as it offers many advantages such as two-three times higher enzyme production as well as protein rate, higher concentration of the product in the medium, direct use of air-dried fermented solids as source of enzyme which lead to elimination of expenses on downstream processing, employment of natural cellulosic wastes as substrate in contrast to the necessity of using pure cellulose in submerged fermentation (SmF) and the possibility of carrying out fermentation in nonaseptic conditions (Chahal 1983; Toyama and Ogawa, 1978; Pamment *et al.*, 1978; Deschamps *et al.*, 1985; Allen, 1983; Sternberg, 1976). The biosynthesis of cellulases in SmF process is strongly affected by catabolic and end product repressions (Gallo *et al.*, 1978; Ryu and Mandels, 1980) and the recent reports on the overcoming of these repressions to significant extent in solid state fermentation (SSF) system (Ramesh and Lonsane, 1991 a, b), therefore, are of economic importance.

The amenability of SSF technique to use upto 20-30% substrate, in contrast to the maximum of 5% in SmF process, has been documented (Pamment *et al.*, 1978). It is, therefore, not surprising that cellulases to the extent of 45 tonnes/annum and worth about 170×10^6 yen were produced by SSF in Japan as early as in 1975-76 (Yamada, 1977). About 7.2 tonnes of cellulases were exported to West Germany and Australia from Japan in 1967-68 (Toyama, 1969), thereby indicating its leading status in cellulase production by SSF system.

An important potential application of cellulases is in the production of glucose, ethanol, high fructose syrup and other feedstocks from agro-industrial cellulosic residues and wastes (Emerts and Katzen, 1980; Lonsane and Ramakrishna, 1989). Extensive R&D efforts have been put up in last 20 years to produce the enzymes by microbial fermentations and thousands of publications as well as patents are available on the production and application of the cellulases (Ryu and Mandels, 1980; Mandels, 1982; Frost and Moss, 1987). However, no commercial exploitation has emanated from these efforts because of high cost of cellulases even when these are produced by SSF system. For example, the cost of the enzyme was shown to comprise nearly 50% of the outlay required to produce sugar from corn stover (Perez *et al.*, 1980).

Lignocellulosic residues/wastes solid substrat

The agro-industrial lignocellulosic residues/wastes form a most important renewable reservoir of carbon for a variety of vitally important chemical feedstocks and fuel in the overall economy of any country. Their unlimited availability and environmental pollution potential, if not disposed-off properly, dictate renewed efforts for their efficient and economic utilization. It is well known that the selection of appropriate and highly potent microorganism, use of cheaper and efficient substrate, selection of bioreactor, employment of standardized process parameters, characteristics of the enzyme produced, extent of downstream processing as well as waste treatment, inoculum development technique, degree of colonization of the substrate, and efficiency of each unit operations of the process are of vital importance in determining the economics of the process (Kumar and Lonsane, 1989; Ramesh and Lonsane, 1990; Mitchell and Lonsane, 1991; Roussos *et al.* 1991 a,b,c; Lonsane and Krishnaiah, 1991).

Criteria for cellulolytic microorganisms selection

Efforts were, therefore, initiated for screening of potent and most appropriate microorganisms for cellulase production in SSF system. A large number of cultures from various fungal genera and species were screened and *Trichoderma harzianum* CCM F-470 was selected based on four different criteria, i.e., rapid apical growth which leads to higher degree of colonization of the substrate, good sporulation capability which is vital for uniform distribution of the culture in the moist solid medium during inoculation, rapid growth of the culture which facilitates fermentation in non-aseptic conditions and higher enzyme production ability (Roussos and Raimbault, 1982). The kinetics and the ratios of two different cellulolytic enzyme activities on various solid substrates in column fermenter were investigated in the continuation of these renewed efforts. These results are reported in the present communication due to their importance in effecting economy in the enzyme production cost. The data also allow the production of tailor-made activities of different cellulolytic enzymes which may prove useful in efficient hydrolysis of different lignocellulosic materials.

Natural Microflora of sugarcane bagasse

The microbial loads on fresh bagasse and after storage for 15 days in the normal conditions of storage in the sugar mill yard are presented in table 1. The data indicate tremendous increase in the microbial population in the bagasse stored for 15 days. For example, the total bacterial, total fungal and cellulolytic fungal counts were about 715, 917 and 2218 times over those of the fresh bagasse. Such high microbial counts will be disastrous in the fermentation process without sterilization. It is, therefore, essential to sterilize the substrate before use in the fermentation process.

The pretreatment of the substrate also leads to many advantages. For example, it is found to be efficient in killing a larger microflora present naturally on the substrate. No contamination of the medium by bacteria, yeasts and other fungi was observed during the entire course of fermentation in the cases when the medium is based on pretreated substrate, in spite of the use of non-aseptic conditions beyond the moist medium autoclaving stage. The contamination control was probably aided by the use of large inoculum (3×10^7 spores / g SDM) which probably allowed preferential growth of *T. harzianum* and imparted it the status of

dominance. Other beneficial changes due to pretreatment of the substrate are : 1) reduction in crystallinity of the cellulose due to formation of amorphous celluloses, 2) gelatinization of starch present in the substrate, 3) swelling of the substrate, 4) hydration of the substrate. 5) homogeneous distribution of mineral-salt media and a horde of other benefits (Tanaka and Matsuno, 1985).

Table 1. Natural microflora of sugarcane bagasse.

Sample	Moisture %	Microbial load / g material		
		Total bacteria	Total fungi	Cellulolytic fungi
Immediately as it exit from sugar mill	44.4	2.63×10^6	1.08×10^4	2.66×10^3
After 15 days storage in sugar mill yard	59.6	1.88×10^9	9.90×10^6	5.90×10^6

Untreated and pretreated substrates

The necessity for pretreating the lignocellulosic residues and wastes to improve the accessibility of cellulose to microbial attack has been well established (Tanaka and Matsuno, 1985). A number of different physical and chemical pretreatments, either individually or in combination, have been developed and include ball milling, compression milling, grinding, cryomiling, gamma ray dosage, microwave irradiation, steam explosion, rapid depressurization and autohydrolysis by various chemicals such as acids, alkalies, solvents, gaseous ozone etc.. (Tanaka and Matsuno, 1985). Most of these pretreatment methods are impractical at larger scale and highly cost-intensive due to various reasons such as longer pretreatment time, high energy requirement, need for using specific equipments or machinery and occurrence of undesirable side reactions.

The possibility of combining sterilization of the substrate and its pretreatment was, therefore, conceived in the present studies. The moist solids with 50% moisture were transferred in 100 g moist weight quantity in beaker for autoclaving at 121°C for 20 min.

The results of the comparative enzyme production on untreated and pretreated substrates in column fermenters are depicted in table 2. The CMCase and FPA fractions were present right from 0-21 h in the medium without any pretreatment probably due to their presence in the substrate used for fermentation. These might have been formed during the storage of sugarcane bagasse in the sugar mill and institute premises before employing it for fermentation in the present studies. These enzymes, in contrast, were totally absent upto 21 h in the medium based on pretreated substrate. Obviously, the enzymes initially present on the substrate were destroyed during autoclaving of the moist medium. The enzyme production beyond 21 h was, however, at faster rate in both the media. The peaks in enzyme production were achieved at 30 h in untreated medium as compared to those at 48 h in the pretreated medium. However, the peak values of the enzymes in case of pretreated substrate were higher by about 2.7 and

Table 2. Comparative production of cellulases in column fermenter by *T. harzianum* on treated and untreated substrates

Fermentation time, h	Medium pH		CMCase production		FPA production	
	A	B	A	B	A	B
0	6.2	5.4	1.1	0	1.1	0
6	6.2	5.4	0.7	0	1.1	0
21	4.8	5.3	0.1	0	2.8	0
30	5.9	4.2	45.9	31.6	7.1	3.4
44	7.4	5.1	35.4	124.6	5.4	10.9
48	7.7	5.1	14.1	125.8	3.5	12.8
67	8.3	5.5	7.6	12.4	2.8	11.2

A: Untreated substrate, B : treated substrate.
The enzyme titres are expressed as IU / g SDM.

Table 3. A typical fermentation data on the production of cellulases by *T. harzianum* at laboratory scale in column fermenter under standardized parameters

Attribute	Unit	Value
Peak value in CMCase	IU/g SDM	204.4
Peak value in FPA	IU/g SDM	16.1
CMCase : FPA at peak level	Ratio	1 : 0.08
Peak enzyme production time	h	48
Range of moisture content of the medium during fermentation	%	68.3-73.9
Lowest pH in growth phase (at 28 h)	-	4.5
Highest pH during enzyme synthesis and liberation (at 48 h)	-	6.3
Contamination during fermentation	-	Absent

1.8 times as compared to those on the untreated substrate. These values at 48 h fermentation were higher by about 8.9 and 3.7 times in case of the pretreated substrate.

The microscopic examination of the samples from both the fermentations, involving untreated and pretreated substrates, revealed extensive contamination by bacteria and yeast in the former case right from the beginning of the fermentation. The growth rate of some of these contaminants, especially the bacterial cultures, was much faster than that of *T. harzianum* as the samples from latter phases of fermentation showed many bacterial cells and very few fungal mycelia or spores. Moreover, the mycelial cells of *T.harzianum* were noticed to be lysed probably by the contaminants or their products. The contamination of the order of 10^{11} cells of bacteria and yeast was also recorded by Pepe (1984) when sugar beet cosset was used without any autoclaving in large fermenter for protein upgradation.

The changes in the pH of the media, based on the use of untreated and pretreated substrates, showed interesting pattern (Table 2). The pH dropped to 5.9 by 30 h and then increased to 8.3 by 67 h fermentation in case of untreated substrate. The general trend in pH drop and rise was similar in the medium based on pretreated substrate but the values were 4.2 and 5.5 at 30 and 67 h, respectively. It is interesting to note that the initial pH was much higher in case of the medium involving untreated substrate as compared to that of the medium based on pretreated substrate.

Many times higher production of the enzymes by the culture in the medium based on pretreated substrate indicates that the heat treatment of the substrate in moist condition modifies it physically for imparting better accessibility of the cellulose to microbial attack. Consequently, the substrate becomes more amenable to microbial growth and leads to improved production of the enzymes. The particle size reduction due to chopping of the bagasse might also have resulted in exposing larger surface area of the substrate to heat action and thus is partially responsible in making the substrate more accessible to the microorganism.

Sugarcane bagasse alone as substrate for *T.harzianum* growth

The manufacture of sucrose from cane sugar in the tropical countries results in the generation of large quantity of bagasse which are generally used as fuel in the sugar mill. In another usage, the bagasses are depithed and the fibres thus obtained are used in the manufacture of paper. The sugarcane bagasse forms an excellent substrate in SSF processes.

The results of the growth and metabolism of *T.harzianum* on bagasse in column fermenter for 64 h under SSF system revealed that the conidiospores started germinating at about 10 h and the spore germination was 100 % by 20 h. The mycelial cells enveloped the substrate particles more or less fully by about 30 h. The moisture content of the medium during the course of fermentation was quite stable and ranged between 70.5 - 72.9 % (Fig. 3). Similar was the case for the kinetics of pH changes which were in the range of 5.9 - 6.3 during first 52 h fermentation. This is the fermentation period which led to maximum enzyme titres. Further continuation of fermentation beyond 52 h resulted in increasing the pH of the medium to 6.9 at 58 and 64 h fermentation.

The data on the production of CMCase and FPA fractions indicated that no CMCase was produced upto 24 h, in contrast to the production of FPA at slower rate right from the start of fermentation (Fig. 4). The rates of production of these enzymes were, however, faster between 24 to 44 h and about 80 % of the total enzyme was formed during this period. The peaks in enzyme titres were achieved at 52 h for both the enzyme and their levels decreased if

Table 4. Large scale production of cellulases by *T.harzianum* in Zymotis and laboratory scale column fermenter run in parallel

Fermentation time, h	Moisture content of the medium, %		pH of the medium		CMCase production		FPA production	
	A	B	A	B	A	B	A	B
0	71.0	71.3	5.5	5.6	0	0	0	0
10	72.6	70.5	5.6	5.5	0	0	0	0
22	71.5	70.4	4.8	5.3	0	0	0	0
26	71.4	71.5	4.2	4.7	0	0	0	0
30	72.4	71.6	4.5	4.4	9.3	3.1	1.2	0.8
34	72.6	72.1	5.0	4.7	29.9	15.1	3.4	3.2
46	73.3	73.1	5.7	5.6	74.1	62.2	5.6	4.4
48	72.7	76.6	5.8	5.8	74.2	71.8	8.1	5.5

A : Zymotis, B : laboratory scale column fermenter. The enzyme titres are expressed as IU/g SDM

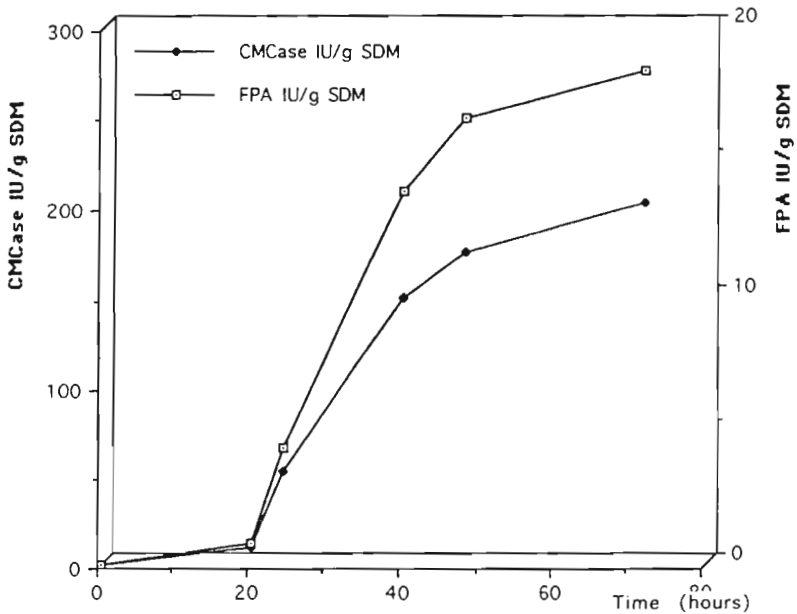


Figure 4. CMCase and FPA cellulase production by *T. harzianum*

the fermentation was continued further (Fig. 4). The ratios of CMCase and FPA ranged between 8.3-10.0 during 44-52 h fermentation.

Combination of bagasse and wheat bran as substrate

The use of sugarcane bagasse and wheat bran at the ratio of 80 : 20 in the column fermenter and under the fermentation parameters such as 20 g medium in the column, 72 % initial moisture content, $28 \pm 1^\circ\text{C}$ incubation temperature and aeration at 5 l air / h / column in the production of cellulolytic enzymes by *T. harzianum* showed entirely different patterns in the enzyme synthesis during initial period of fermentation. The CMCase and FPA fractions were not formed upto 20 h fermentation. Subsequently, the rate of production of these enzyme activities was at faster rate between 28-48 h (Fig. 6). The peak in enzyme titres was attained at 48 h in both the cases. The ratios of these activities were about 6 upto 28 h but these changed to 11.0-12.3 in the subsequent period.

The kinetics of moisture content of the medium showed that it increased gradually during the entire course of fermentation and was 4-5 % higher at the end of fermentation as compared to the initial value at 0 h (Fig. 7). The kinetics of the pH changes are interesting. The pH decreased sharply from 5.8 to 4.8 between 12-28 h, the period which involves active growth of the culture. The bagasse appears to have strong buffering action as the pH was not reduced to a value less than 4.8. Subsequently, the pH increased to 6.3 between 28-65 h fermentation, the period which corresponds to enzyme biosynthesis and its release in the medium. In contrast, the pH was nearly stable at the initial value of 5.8 during 0-12 h, the period which involves the germination of the conidiospores. The data indicate that there is no need for pH control during the fermentation as the above kinetic changes in the pH seems to be helpful.

Typical fermentation data at laboratory scale

The production of cellulases by *T.harzianum* in pretreated moist medium in laboratory scale column fermenter of 18 g working capacity under standardized parameters indicated that the maximum enzyme production was achieved at 48 h (Table 3). The peak values of enzymes were 204.4 and 16.1 IU/ g SDM of CMCase and FPA fractions, respectively, thereby leading to the ratio of 1 : 0.08 at 48 h. These values are much higher than those in the pretreated moist medium under non-standardized parameters (Table 2), thereby indicating the efficacy of parameter standardization performed in this process (Roussos, 1987). The continuation of the fermentation under standardized parameters beyond 48 h. however, resulted in reduction in the titres of the enzymes, which was more drastic in case of FPA fraction. The moisture content of the medium during the course of fermentation ranged between 68.3 - 73.9% (Table 3). The pH of the medium decreased gradually in the initial 28 h fermentation from the initial value of 5.8 to 4.5. In the subsequent fermentation period, it started increasing and reached the value of 6.3 at 48 h. This confirms the trend of pH changes during growth and enzyme production phases and its utility in monitoring the fermentation as stressed earlier (Roussos *et al.*, 1991 a). The microscopic examination of the fermenting solids at different intervals during the entire fermentation period has not revealed any contamination by bacteria, yeast and fungi other than *T. harzianum*. The growth of the culture was found to be uniform throughout the solid mass in the fermenter.

The higher production of the enzymes at 48 h without the need for maintaining aseptic conditions during fermentation, the ratio of CMCase : FPA fractions at 1 : 0.08, an absence of any contamination during the fermentation due to combination of substrate pretreatment with autoclaving of the medium, the cheapness of the substrate and the homogeneous growth of the culture in the medium probably due to uniform distribution of the spore inoculum during inoculation collectively indicate the high potential of the system for economic exploitation at industrial scale. Hence, the scale-up trials were undertaken.

Scale-up in Zymotis

The data on the production of cellulases in Zymotis charged with 41.4 kg moist medium and in laboratory scale column fermenter run in parallel, along with the changes in pH as well as % moisture of the media are presented in Table 4. The titres of CMCase and FPA fractions were at peak values at 48 h in both the fermenters except for that of CMCase in Zymotis at 46 h. The production of both these components of the cellulolytic enzyme were initiated at 30 h and their accumulation increased steadily till the peak values were attained. The ratios of CMCase and FPA fractions at peak levels were 1 : 0.075 and 1 : 0.077 in Zymotis and parallel column fermenter, respectively. The ratio increased to 1 : 0.11 at 48 h in Zymotis due to increase in FPA production between 46-48 h but no change in CMCase titre. The production of both of these enzymic fractions was higher and also at a faster rate in Zymotis as compared to those in the parallel column fermenter (Table 4), thereby indicating that the conditions were more favorable at the large scale than those at smaller scale fermentation. Similar results were also reported earlier for other products in SSF system (Lonsane *et al.*, 1991 ; Saucedo-Castaneda *et al.*, 1991).

The moisture content of the medium during entire period of fermentation was similar in both the fermenters and ranged between 71.0 - 73.3 and 70.4 - 73.1 % in Zymotis and parallel column fermenter, respectively (Table 4). The pH of the medium at the start and also at the end of fermentation was same in both these fermenters. However, the drop in pH during the initial growth phase and the increase in pH during the subsequent enzyme production phase were faster in Zymotis (Table 4). This probably explains the faster rate of enzyme production as well as its accumulation in Zymotis as compared to those in parallel column fermenter.

Comparison of enzyme production at laboratory and large scales

The production of CMCase and FPA fractions at larger scale in Zymotis was 36.28 and 50.03 % of those produced by the culture in column fermenter at laboratory scale under standardized parameters (Tables 3 and 4). The production of such low enzyme at larger scale in Zymotis indicates some lacunae or deficiency at larger scale fermentation.

The close similarity in the profiles of fermentation parameters, such as moisture content and pH of the medium, in the laboratory scale column fermentation under standardized parameter and Zymotis rules out the possibility of any role played by fermentation parameters in giving lower yields at larger scale. The temperature, medium compositions, inoculum quality, inoculum ratio are also similar in both the cases. The deficiency in the performance of Zymotis or its design features in obtaining lower enzymes at larger scale is also ruled out as

the production of the enzyme in Zymotis and column fermenter, which was run in parallel to Zymotis, were also 35.15 and 34.27 % as compared to those in the laboratory scale column fermentation under standardized parameters (Table 3 and 4). In addition, the enzyme titres in Zymotis and the parallel column fermenter were also similar (Table 4).

The analysis of the whole process and process methodology indicate that the only difference between laboratory scale column fermentation in Zymotis and the parallel fermentation in column is the change in the substrate pretreatment method. The substrate pretreatment was carried out by charging the moist medium in 100 g quantity in a beaker for autoclaving at 121°C for 20 min in case of laboratory scale column fermentation under standardized parameters, in contrast to the use of 6 kg moist solid medium in cylindrical aluminium vessel at 121°C for 60 min. The depth of the medium in the aluminium vessel during autoclaving was much higher (60 cm) as compared to that in the beaker (10 cm) with the use of 100 g moist medium in the laboratory scale process. In fact, it is for this reason that the autoclaving time was extended from 20 to 60 min in the larger scale process for giving more time for heat transfer.

The results, however, indicate that the heat transfer during autoclaving at larger scale is less than that achieved at laboratory scale. Probably the temperature achieved at the centre of the moist medium held in the cloth sac during autoclaving at large scale was less than 121°C or the heating of each particle of the medium was not for 20 min at 121°C. The autoclaving of the medium has also been specified as problematic unit operation during scale-up of submerged fermentation processes (Bank, 1984).

It is felt that the same productivity would be possible to achieve at larger scale either by increasing the autoclaving temperature or time. Both of these approaches are, however, energy and cost intensive. Use of perforated aluminium trays for substrate pretreatment-cum-autoclaving and the bed depth of about 10 cm, as generally employed in tray fermentation processes (Lonsane *et al.*, 1985; Ghildyal *et al.*, 1981), may provide a simple and economic approach to overcome the problem.

Bibliography

- ACEBAL, C., M.P. CASTILLON, P. ESTRADA, I. MATA, E. COSTA, J. AGUADO, D. ROMERO and F. JIMENEZ, 1986. Enhanced cellulase production from *Trichoderma reesei* QM 9414 on physically treated wheat straw. *Appl. Microbiol. Biotechnol.* 24 : 218-223.
- ACEBAL, C., M.P. CASTILLON, P. ESTRADA, I. MATA, J. AGUADO and D. ROMERO, 1988. Production of cellulases by *Trichoderma reesei* QM 9414 in batch and fed-batch cultures on wheat straw. *Acta Biotechnol.* 6 : 487-494.
- ALLEN, A.L., 1983. Enzymic hydrolysis of cellulose to fermentable sugars. In : Wise, D.L. (Ed.). *Liquid fuel developments*. pp. 49-64. CRC Press, Boca Raton, Florida.
- BANK, G.T., 1984. Scale-up of fermentation processes. In : A. Wiseman (Ed.), *Topics in Enzyme and Fermentation Biotechnology*, Vol. 3, pp. 170-266, Ellis Horwood Ltd., Chichester.
- CASTANON, M. and C.R. WILKE, 1980. Adsorption and recovery of cellulases during hydrolysis of newspaper. *Biotechnol. Bioeng.* 22 : 1037-1053.
- CHAHAL, D.S., 1983. Growth characteristics of microorganisms in solid state fermentation for upgrading of protein value of lignocelluloses and cellulase production. In : Blanch, H.W., E.T. Poputsakis and G. Stephanopoulos (Eds.), *Foundation of biochemical engineering kinetics and thermodynamics in biological systems*, ACS Symp. Series No. 207. pp. 421-442. American Chemical Society, Washington, D.C.

- DESCHAMPS, F., C. GIULIANO, M. ASTHER, M.C. HUET, and S. ROUSSOS, 1985. Cellulase production by *Trichoderma harzianum* in static and mixed solid state fermentation reactors under non-aseptic conditions. *Biotechnol. Bioeng.* 27 : 1385-1388.
- EMERT, G.H. and R. KATZEN, 1980. Gulf's cellulose-to-ethanol process. *Chemtech* 10 (Octo) : 610-615.
- ENARI, T.M. and P. MARKKANEN, 1977. Production of cellulolytic enzymes by fungi. *Adv. Biochem. Eng.* 5 : 1-24.
- ESTRADA, P., C. ACEBAL, M.P. CASTILLON, I. MATA and D. ROMERO, 1988. Adsorption of cellulase from *Trichoderma reesei* on wheat straw. *Biotechnol. Appl. Biochem.* 10 : 49-58.
- FAN, L.T., Y.H. LEE and D.H. BEARDMORE, 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. *Biotechnol. Bioeng.* 23 : 419-442.
- FROST, G.M. and D.A. MOSS, 1987. Production of enzymes by fermentation. In : Kennedy, J.F. (Ed.), *Biotechnology, Vol. 7a, Enzyme technology*, pp. 65-211. VCH, Weinheim.
- GALLO, B.J., R. ANDREOTTI, C. ROCHE, D. RYU, and M. MANDELS, 1978. Cellulase production by a new mutant strain of *Trichoderma reesei* MCG 77. *Biotechnol. Bioeng. Symp.* 8 : 89-101
- GHILDYAL, N.P., S.V. RAMAKRISHNA, P.N. DEVI, B.K. LONSANE and H.N. ASTHANA, 1981. Large scale production of pectolytic enzyme by solid state fermentation. *J. Food Sci. Technol.* 18 : 248-251.
- GHOSE, T.K., 1977. Cellulase biosynthesis and hydrolysis of cellulosic substances. *Adv. Biochem. Eng.* 6 : 39-76.
- GONG, C.S. and G.T. TSAO, 1979. Cellulase and biosynthesis regulation. In : G.T. TSAO (Ed.), *Annual reports on fermentation processes. Vol. 3*, pp. 111-140, Academic Press, New York.
- GONZALEZ-BLANCO, P., G. SAUCEDO-CASTANEDA and G. VINIEGRA-GONZALEZ, 1990. Protein enrichment of sugarcane by-products using solid-state cultures of *Aspergillus terreus*. *J. Ferment. Bioeng.* 70 : 351-354.
- GRAJEK, W., 1987. Comparative studies on the production of cellulases by thermophilic fungi in submerged and solid-state fermentation. *Appl. Microbiol. Biotechnol.* 26 : 126-129.
- HOFFMAN, R.M. and T.M. WOOD, 1985. Isolation and partial characterization of a mutant of *Penicillium funiculosum* for the saccharification of straw. *Biotechnol. Bioeng.* 27 : 81-85.
- KUMAR, P.K.R. and B.K. LONSANE, 1989. Microbial production of gibberellins : state of the art. In : Neidleman, S.A. (Ed.), *Advances in applied microbiology, Vol. 34*, pp. 29-139, Academic Press, Inc. New York
- LONSANE, B.K., N.P. GHILDYAL, S. BUDIATMAN and S.V. RAMAKRISHNA, 1985. Engineering aspects of solid state fermentation. *Enzyme Microb. Technol.* 7 : 258-265.
- LONSANE, B.K., A. DURAND, R. RENAUD, S. ALMANZA, J. MARATRAY, C. DESGRANGES, P.S. CROOKE, K. HONG, C.W. MALONY and R.D. TANNER (1991). Reactor design. In : H.W. Doelle, D.A. Mitchell and C. Rolz (Eds), *Solid substrate cultivation*, Elsevier Science Publishers, Essex, UK, pp;
- LONSANE, B.K. and N.P. GHILDYAL, 1991. Exoenzymes. In : Doelle, H.W., D.A. Mitchell and C.E. Rolz (Eds.), *Solid substrate cultivation*, Elsevier Science Publishers, Essex, UK, In : H.W. Doelle, D.A. Mitchell and C. Rolz (Eds), *Solid substrate cultivation*, Elsevier Science Publishers, Essex, UK, pp.
- LONSANE, B.K. AND M.M. KRISHNAIAH, 1991. Leaching of the product and further downstream processing. In : Doelle, H.W., D.A. Mitchell and C.E. Rolz (Eds.), *Solid substrate cultivation*, Elsevier Science Publishers, Essex, U.K., pp.
- LONSANE, B.K. and M. RAMAKRISHNA, 1989. Microbial enzymes in food processing industry : present status and future prospects in India. *Indian Fd. Industry* 8 : 15-31.
- LONSANE, B.K. and M.V. RAMESH, 1990. Production of bacterial thermostable α -amylase by solid state fermentation: A potential tool for achieving economy in enzyme production and starch hydrolysis. In : Neidleman, S.A. (Ed.), *Advances in Applied Microbiology, Vol. 35*, pp. 1-56, Academic Press, Inc., New York.
- MANDEL, M. 1982. Cellulases. In : G.T. Tsao (Ed.), *Annual reports on fermentation processes. Vol. 5*, pp. 35-38, Academic Press, New York.
- MANDEL, M. and J. WEBER, 1969. The production of cellulases. *Adv. Chem. Ser.* 95 : 391-414.
- MANDELS, M., R. ANDREOTTI and C. ROCHE, 1976. Measurement of saccharifying cellulase. *Biotechnol. Bioeng. Symp.* 6 : 21-33.

- MITCHELL, D.A. and B.K. LONSANE, 1991. Definition, characteristics and potential. In : Doelle, H.W., D.A. Mitchell and C.E. Rolz (Eds.), Solid substrate cultivation. Elsevier Science Publishers, Essex, UK, pp.
- MONTENECOURT, B.S. and D.E. EVELEIGH, 1977. Semiquantitative plate assay for determination of cellulase production by *Trichoderma viride*. Appl. Environ. Microbiol. 33 : 178-183.
- OOSHIMA, H., M. SAKATA and Y. HARANO, 1983. Adsorption of cellulase from *Trichoderma viride* on cellulose. Biotechnol. Bioeng. 25 : 3103-3114.
- PAMMENT, N., C. ROBINSON, J. HILTON and M. MOO-YOUNG. 1978. Solid state cultivation of *Chaetomium cellulolyticum* on alkali pretreated sawdust. Biotechnol. Bioeng. 20 : 1735-1744.
- PEPE, P.A., 1984. Enrichissement protéique de la pulpe de betterave par culture de *Trichoderma album* en milieu solide. Evolution des populations microbiennes au cours du procédé. Mémoire ingénieur ENSBANA, Université de Dijon, 33 p.
- PÉREZ, J., C.R. WILKE and H.W. BLANCH, 1980. Economics of sugar production with *Trichoderma reesei*. Rutgers C 30, Lawrence Berkeley Report 11, 489. Paper presented at the 2nd Chemical Congress of the North American Continent, Las Vegas, Nev. August 1980.
- RAIMBAULT, M. and D. ALAZARD, 1980. Culture method to study fungal growth in solid fermentation. Eur. J. Appl. Microbiol. Biotechnol. 9 : 199-209.
- RAMESH, M.V., and B.K. LONSANE, 1991 a. Regulation of α -amylase production in *Bacillus licheniformis* M27 by enzyme end-products in submerged fermentation and its overcoming in solid state fermentation system. Biotechnol. Lett. 13 : 355-360.
- RAMESH, M.V., and B.K. LONSANE, 1991b. Ability of solid state fermentation technique to significantly minimize catabolic repression of α -amylase production by *Bacillus licheniformis* M27. Appl. Microbiol. Biotechnol. in press.
- RAO, M.N.A., B.M. MITHAL, R.N. THAKUR and K.S.M. SASTRY, 1982. Solid state fermentation for cellulase production by *Pestalotiopsis versicolor*. Biotechnol. Bioeng. 25 : 869-872.
- REESE, E.T., R.G.H. SIU and H.S. LEVINSON, 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59 : 485-497.
- ROUAU, X. and E. ODIER. 1986. Production of extracellular enzyme by the white rot fungus *Dichomitus squalens* in cellulose containing liquid culture. Enzyme Microb. Technol. 8 : 22-26.
- ROUSSOS, S. 1987. Croissance de *Trichoderma harzianum* par fermentation en milieu solide : Physiologie, sporulation et production de cellulases. Etudes et Thèses, Université de Provence, France, ORSTOM édition n° 857-3. 193 p.
- ROUSSOS, S. and M. RAIMBAULT, 1982. Hydrolyse de la cellulose par les moisissures. I. Screening des souches cellulolytiques. Ann. Microbiol. 133B : 455-464.
- ROUSSOS, S., M. RAIMBAULT, G. SAUCEDO-CASTANEDA, G. VINIEGRA-GONZALEZ and B.K. LONSANE, 1991 a. Kinetics and ratios of carboxy-methyl cellulase and filter paper activities of the cellulolytic enzymes produced by *Trichoderma harzianum* on different substrates in solid state fermentation. Micol. Neotrop. Apl. 4: 19-40.
- ROUSSOS, S., M. RAIMBAULT, G. SAUCEDO-CASTANEDA and B.K. LONSANE, 1991 b. Efficient leaching of Cellulases produced by *Trichoderma harzianum* in solid state fermentation. Biotechnol. Techniques, 6: 429-432.
- ROUSSOS, S., M. RAIMBAULT, G. SAUCEDO-CASTANEDA and B.K. LONSANE, 1991 c. Potential of ensiling for efficient management of spent residue from solid state fermentation system. Biotechnol. Techniques 6: 87-90.
- ROUSSOS, S., A. OLMOS, M. RAIMBAULT, G. SAUCEDO-CASTANEDA, and B.K. LONSANE, 1991 d. Strategies for large scale inoculum development for solid state fermentation system : Conidiospores of *Trichoderma harzianum*. Biotechnol. Techniques. 5: 415-420.
- RYU, D.D.Y. and M. MANDELS, 1980. Cellulases : Biosynthesis and applications. Enzyme. Microb. Technol. 2 : 91-102.
- SAUCEDO-CASTANEDA, G., B.K. LONSANE, M.M. KRISHNAIAH, J.M. NAVARRO, S. ROUSSOS and M. RAIMBAULT, 1991. Maintenance of heat and water balances as a scale-up criterion for the production of ethanol by *Schwanniomyces castellii* in a solid state fermentation system. Process Biochem. 27: 97-107.
- STERNBERG, D., 1976. Production of cellulases by *Trichoderma*. Biotechnol. Bioeng. Symp. 6 : 35-53.

- TANAKA, M. and R. MATSUMO, 1985. Conversion of lignocellulosic materials to single-cell protein (SCP) : Recent developments and problems. *Enzyme Microb. Technol.* 7 : 196-206.
- TOYAMA, N., 1969. Application of cellulases in Japan. In : Gould, R.E. (Ed.), *Cellulases and their applications*. Advances in Chemical Series, Vol. 95, pp. 259-390. American Chemical Society Publications, Washington, D.C.
- TOYAMA, N., 1976. Feasibility of sugar production from agricultural and urban cellulosic wastes with *Trichoderma viride* cellulase. *Biotechnol. Bioeng. Symp.* 6 : 207-219.
- TOYAMA, N. and K. OGAWA, 1978. Cellulase production of *Trichoderma viride* in solid and submerged culture methods. In : Ghose, T.K. (Ed.), *Bioconversions of cellulosic substrates for energy, chemicals and microbial protein* - Symp. Proc. (1 st), 1977, Indian Institute of Technology, New Delhi.
- VIDAUD, C., S. ROUSSOS, M. RAIMBAULT and F. DESCHAMPS. 1982. Effet de divers prétraitements sur l'accessibilité de la cellulose de la paille de blé aux cellulases de *Trichoderma harzianum*. *Cah. ORSTOM sér. Biol.* 45 : 17-23.
- YAMADA, K., 1977. Bioengineering report - Recent advances in industrial fermentations in Japan. *Biotechnol. Bioeng.* 19 : 1563-1621

BIOTECHNOLOGICAL MANAGEMENT OF COFFEE PULP

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General introduction

Agro-industrial residues/wastes are generated in large quantities throughout the world. Their non-utilization results in loss of valuable nutrients and environmental pollution (Zuluaga, 1989). Their better utilization by biotechnological means assumes social, economic and industrial importance. Considering these facts, ORSTOM participated into a scientific collaboration with Universidad Autonoma Metropolitana (UAM), Mexico, for the development of biotechnological processes for better utilization of agro-industrial byproducts/wastes, especially the coffee pulp (Viniestra *et al*, 1991). Coffee pulp, generated to the extent of 40% in the fermentation of coffee berries (Zuluaga, 1989), poses many problems in the coffee producing tropical countries. Its disposal in nature, without any treatment, causes severe environmental pollution, due to putrefaction of organic matter (Zuluaga, 1989). Hence, the possibility of utilizing coffee pulp in the biotechnological processes for production of different metabolites was investigated through by Roussos *et al* (1993).

Coffee pulp is the main byproduct on coffee exploitation industry. Two tons of green coffee produces one ton of coffee pulp (dry matter). Its production on world scale rised 2.400.000 tons (two million four hundred thousand tons) in the harvest cycle from 1986 to 1987. Coffee pulp is essentially composed of carbohydrates, proteins, aminoacids, mineral salts, tannins, poly phenols and caffeine. The last two compounds are reported to be antiphysiological factors on animal feed. Hence, coffee pulp has to follow a preliminary treatment before is used. Moreover, this byproduct can occurs in the nature and spoiling hardly the environment.

Research on this field is been carried out to study its further utilization in biotechnological processes. Several alternatives were founded and developed these decades to avoid and minimized the environmental impact due to coffee pulp. Previous assays of yeast culture on coffee pulp were realized by The Colombian Research Center CENICAFE in 1951, conducted by Dr. Calle. Later, INCAP and ICAITI in Guatemala also reported research on this matter. However, the most important contribution was the happening of the First International Conference on Coffee Byproducts Utilization for Animal Feed and its further Industrial Applications, wellcomed by the Costarican Center CATIE in 1974. More recently, in 1989 INMECAFE in Veracruz, Mexico and in 1991 CENICAFE, Manizales, Colombia, there are organized an International Symposium on Biotechnology of Agroindustry of Coffee, called SIBAC; where sessions and contributions presented original research on the potential of coffee pulp byproducts (aquaculture and feed).

Aquaculture : Fish farming could be a way to produce animal protein using locally available feedstuffs but taking into account present market limitations (Ramos-Henao, 1988). Some

work has been done on feeding coffee pulp to Tilapia (Garcia and Baynes, 1974), carp and catfish (*Clarius mossenbicus*) as indicated by Christensen (1981). The level of coffee pulp used in the experimental diets was closed to 33% without negative effects on the growth rate and yields of the fish. Lagooning may be also used as a secondary water treatment process, after an anaerobic primary treatment of spent waters in the coffee mill.

Ruminant nutrition: Proximate composition of coffee pulp shows a relative low nutritive value due to high level of wall materials, lignin and also due to the presence of caffeine, tanins and chorogenic acid. Therefore, the use of raw coffee pulp is been suggested to be lower than 20% in ruminant diets (Ruiz and Ruiz, 1977; Vargas et al. 1982; Abate and Pfeffer, 1986). High raw coffee pulp intake has been associated to negative nitrogen balance because of the caffeine diuretic effect (Cabezas, et al. 1974). Coffee pulp silage seems to correct this problem probably because of caffeine leaching in the silage liquor (Cabezas et al. 1976). On the other hand, solid-state culture of fungal organisms such as *Penicillium roquefortii* or *Aspergillus niger* may reduce to less than 10% the level of caffeine in coffee pulp, leaving a probiotic activity in the fungal biomass as indicated above (Tapia et al. 1989; Campos-Montiel, 1995). Therefore, despite the nutritional limitations of raw coffee pulp, solid-state fungal culture and ensiling (the two step fermentation process discussed above) may increase the ruminant nutritional and market value of this material. This is an interesting feature which remains to tested *in vivo*.

Recent work done in the Biotechnology Laboratory of Centre ORSTOM, Montpellier (France) and UAM (Mexico) has shown that it is possible to keep and improve the biochemical quality of coffee pulp by using a mixture of selected strains of lactic bacteria and filamentous fungi. Solid-state fermentation of this material yields a decaffeinated product which can be dried or rensiled (Roussos et al., 1989; Perraud-Gaime, 1995) Some HPLC measurements suggest that a major fraction of phenolic compounds is broken down (Perraud-Gaime, 1995). On the other hand, work by Antier et al. (1993 a,b), has shown that coffee pulp is an excellent substrate for pectinase production by selected strains of *Aspergillus niger* (Boccas et al. 1994). The solid residue after such fermentation is done has been found to have *probiotic* effect when assayed *in vitro* by Tapia et al. (1988). This probiotic effect is apparently linked to a water soluble enhancement growth factor present in fungal biomass and acting on rumen cellulolytic bacteria (Campos-Montiel and Viniegra-Gonzalez, 1995, Islas et al. 1995).

In the first transparency the New alternative for the Biotechnological Upgradation of Coffee Pulp is presented. Fresh Coffee Pulp is subjected to Lactic Acid Fermentation and Coffee Pulp Silage thus obtained can be used as substrate for solid State Fermentation system for the production of Probiotics, enzymes, animal feeds and phytohormona.

Content: During these presentation I will be covering mainly about production of coffee pulp, its biochemical composition, natural microflora, oriented silage, caffeine degradation and pectinases production by filamentous in SSF system by filamentous fungi like *Aspergillus* and *Penicillium* species.

Word production: The data on the World Green coffee and Coffee Pulp Production for the period 1989-90 are presented in Table 1. The World Coffee Production is five and a half

million tons. For every ton of coffee produced half a ton of coffee pulp is generated in humid process. This is applicable only when the coffee is processed by humid process. In India the total production of coffee during this period was one hundred thirty thousand (130,000) tons. the exact quantity of coffee pulp produced is not definitely known because both humid and dry process are used.

Coffee pulp chemical composition: The chemical composition of coffee pulp is presented in Table 2. Coffee pulp is essentially composed of carbohydrates, protein, amino acids, mineral salts, tannins, and caffeine (Zuluaga 1989). The last two compounds are reported to be antiphsiological factors for animal consumption (Bressani et al. 1972)..

The composition of soluble sugars present in coffee pulp are presented in Table 3. These represents about 23% of total solids by dry weight (Zuluaga, 1981). The presence of protein, sugars, minerals and water in coffee pulp obtained by humid process, offers itself as an excellent substrate for the growth of microorganisms. If it is not utilized immediately it causes environmental pollution particularly in the rivers surrounding the factory processing area. In order to conserve the nutritional factors present in the coffee pulp and to maintain its quality throughout the year we have used silage process (Perraud-Gaime and Roussos, 1997).

Natural Microflora of coffee pulp: In the first instance the natural microflora of coffee pulp was evaluated and the data is shown in Table 4. Bacteria represents nearly 95% of the microflora whereas filamentous fungi and yeast population was only about 5% (Gaime-Perraud et al. 1993). Figure 1 gives an idea of groups of microorganisms (bacteria, yeast molds) present in Mexican and Columbian coffee pulp with its nutritional capacities such as amyolytic, cellulolytic, pectinolytic and lactic acid bacterial population.

LONG TIME CONSERVATION OF COFFEE PULP

1- Preservation of coffee pulp by ensilage: Influence of biological additives

Coffee pulp, as it is generated, contains 80-85% moisture (Bressani *et al*, 1972), in addition to appreciable quantities of sucrose, proteins, amino acids and other nutrients. All these factors and nutrients allow various microflora to develop quickly on the coffee pulp and the development of the microorganisms cause the putrefaction of coffee pulp (Gaime-Perraud *et al*, 1993 ; Roussos et al, 1995). It is also not practicable to utilize the coffee pulp immediately, after its generation during coffee berry treatment mainly because the season of coffee berry processing lasts for 3-5 months. During this season, the industry cannot divert attention to this waste, as its priority is focused on the quality of coffee seeds during the entire season. Moreover, quick dehydration of the coffee pulp is impracticable, considering the huge quantity of the waste, high energy requirement, larger capacity of machinery needed and heavy investment on space and building, not only for dehydration, but also for stocking of the dehydrated pulp, till its utilization.

Ensilage of coffee pulp, for its preservation and improvement of feed value, is one of the avenues for value-added utilization of coffee pulp. Ensilage, a quick anaerobic process involving lactic acid bacteria, has been extensively used for preservation of forage in the

temperate regions. It allows the prevention of putrefaction of the forage with minimum degradation of organic matter. The process is quicker and it also improves the nutritive quality of the forage (Mc Donald *et al*, 1991).

Ensilage factors: A number of factors are of vital importance in obtaining a good silage. The substrate to be ensiled should have 30-40% dry matter, should be compactable to the desired level, amenable for anaerobiosis and contain utilizable sugars in sufficient quantities (Bertin, 1986). It must also have the colour, which is most nearer to the raw material, the fruity aroma and slightly acidic taste. In terms of chemical characteristics and achievement of the organic matter stability, the ensilage should involve a minimum loss of dry matter and the resulting silage should have a pH value lower than 4.5, higher than 3% lactic acid, but less than 0.5 and 0.3% acetic and butyric acid, respectively (Mc Donald *et al*, 1991).

A number of chemical and biological additives are mixed with the substrate for improving silage or reducing fermentation time. In the case of biological additives, a lactic acid bacterial inoculum is added, as a minimum of 10^5 lactic acid bacteria per g dry matter is required (Gouet, 1994) to convert the carbohydrates into lactic acid, but not into butyric acid. Enzymes are also added, when the rate of assimilation of sucrose by the endogenous lactic acid bacteria is slower (Bertin, 1986).

Ensilage is also practiced in tropical countries, despite the problems in terms of temperature, humidity and rains. Consequently, the rate of ensilage is slower, putrefaction is common and there is need to use a number of additives.

A number of reports have been produced on ensilage of coffee pulp (Bohkenfor and Fonseca, 1974; Murillo, 1978; Carrizalez and Gonzalez, 1984). But, most of these are associated with the development of the ensilage technique or the effect of chemical additives on the process. For example, Murillo (1974) compared the silage of coffee pulp, obtained by natural microflora based fermentation, with that involving the use of molasses or organic acids as additives. After 90 days of ensilage, the loss of dry matter was as high as 26.8%, in the case of the use of organic acids as additive, though it allowed to attain a pH of less than 4.0. Caffeine content of the drained water was reported to increase significantly, in the case of the use of organic acids, probably because it became more soluble in acidic pH.

Table I. Comparative physico-chemical characteristics of the coffee pulp ensiled using natural microflora and biological additives (Perraud-Gaime, 1995).

Parameters	Ensilage of coffee pulp (28 days)				
	Initial pulp	Without additives	Natural microflora	<i>L. plantarum</i> A6	Commercial inoculum
Moisture	62,55	61,63	66,78	66,78	66,19
pH	4,44	3,90	3,91	3,92	4,11
DM losses (%)	-	0,80	1,73	1,41	0,38
Lactic acid (%DM)	0,00	2,39	3,35	2,14	0,08
Acetic acid (%DM)	0,00	0,29	0,68	0,48	0,05
Reducing sugars (%DM)	4,72	4,85	4,56	3,67	8,32
Caffeine (%DM)	1,04	0,95	1,02	0,93	0,90

The ensilage of coffee pulp was investigated by Perraud-Gaime (1995) with respect to the microbiology and biochemistry of the process, along with the evaluation of biological additives, for improving the process and also the quality of the silage. Accordingly, the studies involved a) allowing the endogenous lactic microflora to grow on coffee pulp for using the fermented mass as inoculum for the next batch, b) use of monoculture of *Lactobacillus plantarum* A6 as a biological additive (Giraud et al. 1991) and c) the use of commercial inoculum as yet another biological additive. The latter contained two lactic bacteria and an enzyme complex.

Data on the influence of three biological additives on the ensilage of coffee pulp for its preservation show that the endogenous microflora of the coffee pulp is efficient enough to produce good quality silage, with acceptable levels of organic acid, dry matter loss and final pH. The use of inoculants, as biological additives, showed the efficiency of natural microflora grown on coffee pulp and the monoculture of *Lactobacillus plantarum* A6 in improving the physico-chemical characteristics of the silage, though commercial inoculum was not efficient, due to several reasons (Perraud-Gaime, 1995). Degradation of caffeine was absent in all the cases. Cellulases as a biological additive showed increased sugar production during ensilage. The results on the kinetics of different microflora development and physico-chemical characteristics during ensilage provide the insight into the microbiology and physiology of the process and point out a number of possibilities for improving the ensilage process as well as the quality of the silage (Perraud-Gaime & Roussos, 1997).

Data allow us to conclude that ensilage is a good technique for preservation of wet coffee pulp. The endogenous lactic acid flora of dry coffee pulp is sufficient enough to produce a good quality of silage (Table 5). However, addition of biological additives, such as lactic acid bacterial inoculants and enzymes, allows the improvement of the quality of the silage, in terms of increasing of lactic acid production, without concomitant production of volatile organic acids and ethanol. Caffeine is not degraded during the silage and hence it is necessary to decaffeinate the coffee pulp with appropriate fungi by solid state fermentation (Perraud-Gaime and Roussos, 1997), if the ensiled coffee pulp is to be used for animal feeding, as caffeine has antiphsiological effects (Bressani *et al.*, 1972).

2.- Selection of filamentous fungi for coffee pulp decaffeination in SSF

It is of economic and industrial importance to note that only 5.8% of the solids of the coffee berry result in the ultimate coffee drink and the remaining 94.2% forms water and various byproducts (Zuluaga, 1989). Among the latter, the coffee pulp is the maximum and represents 40% of the coffee berry in wet form (Tauk, 1986), corresponding to 29% of dry matter (Bressani *et al.*, 1972). This large quantity of the coffee pulp poses problems of disposal to coffee berry producers, due to putrefaction and causes environmental pollution if not disposed after appropriate treatment (Zuluaga, 1989). Due to its high organic matter content, coffee pulp can be utilized for beneficial purposes and intensive research on this topic has been carried out at ORSTOM (Roussos *et al.*, 1995) and also in collaboration with Universidad Autonoma Metropolitana (UAM-I), Mexico (Viniegra-Gonzalez *et al.*, 1991).

Direct use of coffee pulp in animal feeding poses problems, due to its chemical composition (Viniestra-Gonzalez *et al*, 1991). For example, the coffee pulp of *Coffea arabica* contains approximately 1% caffeine and has antiphenological effects on the animals (Braham *et al*, 1973; Cabezas *et al*, 1974, 1976; Vargas *et al*, 1982). It is, therefore, necessary to decaffeinate the coffee pulp, before its use as animal feed. Moreover, the coffee pulp gets putrified, because of its high content of water and, hence, needs preservation by appropriate economic technique. At ORSTOM, Montpellier, the techniques of ensilage and fungal degradation of caffeine by solid state fermentation (SSF) have been selected for preservation and decaffeination of the coffee pulp, respectively, because of their economic character. If these two techniques are applied in succession, it is of vital importance that the decaffeination by fungi is achieved before the formation of conidiospores. In the case of conidiospore formation, it will be essential to sterilize the decaffeinated coffee pulp, before ensiling. However, mycelial cells of fungi can be eliminated during ensiling and hence sterilization step can be avoided to achieve economy (Perraud-Gaime, 1995).

Isolation of new fungi strains: Isolation, purification and conservation of filamentous fungi capable of degrading caffeine was carried out as shown in figure 2.

A total of 350 fungi have been isolated from coffee domains (coffee plants, soils of coffee plantation, coffee byproducts, fermenting coffee berries, etc.) during the research at ORSTOM and UAM (Aquiahuatl *et al*, 1988; Viniestra-Gonzalez *et al*, 1991; Roussos *et al*, 1995). From this collection, a total of 8 filamentous fungi, representing two strains of *Penicillium* and 6 strains of *Aspergillus*, were selected for use in the present studies, based on their higher capacity to degrade caffeine to the extent of 90 to 100% in liquid culture (Roussos *et al*, 1989). One of the *Penicillium* strains selected (V33A25) showed negative effect on caffeine degradation, upon the addition of inorganic nitrogen to the medium in SSF process (Roussos *et al*, 1994).

Selection of filamentous fungi to Caffeine degradation: The objective of this study was to select one or more of the filamentous fungi to grow in SSF and to degrade caffeine to the extent of 80%, before the initiation of conidia formation. Work was also carried out to develop a simple criterion, to correlate growth of the fungi, degradation of caffeine and sporulation time, so that it can be used to stop fermentation at the most appropriate stage. The ability of seven fungal isolates which can degrade caffeine totally is shown in table 6. They belong to the genus of *Aspergillus* and *Penicillium*. Of these, only two strains are belonging to the genus of *Aspergillus* species (V12A25) and *Penicillium* species (V33A25) was selected to study the kinetic and biochemical pathway of caffeine degradation.

Decaffeination of coffee pulp in Solid State Fermentation, to eliminate its antiphenological effects on animals, was studied by aerobic fungal solid state fermentation, prior to the stage of initiation of conidiospore formation (Perraud-Gaime, 1995). Comparative data on performance of two strains of *Penicillium* and six strains of *Aspergillus* spp., selected for their high ability to degrade, indicated the potential of *Penicillium* sp V33A25 for caffeine degradation in aerobic solid state fermentation, before the initiation of sporulation by the culture. Kinetic studies pointed out that the evolution of CO₂ is the reliable criterion for the determination of the phase of fermentation, caffeine degradation, increase in medium pH and initiation of sporulation, without taking sample and subjecting it to analyses or disturbing the

fermentation. These advantages are not available, if rise in pH the medium is selected as a criterion. Amongst 7 different factors, the fermentation temperature, level of CaCl₂ in the medium and autoclaving or non-autoclaving of the medium exhibited strong effects on the initial time of sporulation, extent of CO₂ evolution, pH of the medium and caffeine degradation (Figure 3). The data allow to envisage the use of mixed culture of lactic acid bacteria and filamentous fungi for decaffeination and ensilage of the coffee pulp, or in two stage fermentation, involving any of the simpler order.

Table II. Comparative data on growth and metabolism of the filamentous fungal cultures in column fermenters under solid state fermentation (Perraud-Gaime. 1995).

Strains	Lag phase h	CO ₂ production ml/g MSI	Respirometry coefficient h ⁻¹	Caffeine degradation at 30 h. %	Time of initiation of sporulation
<i>Penicillium</i> V26A25	<i>sp.</i> 12,5	115	0,34	91	32 h
<i>Penicillium</i> V33A25	<i>sp.</i> 11,5	95	0,34	94	30 h
<i>Aspergillus</i> C16A25	<i>sp.</i> 13,0	100	0,30	80	32 h
<i>Aspergillus</i> V12A25	<i>sp.</i> 10,5	130	0,29	82	28 h
<i>Aspergillus</i> C17B25	<i>sp.</i> 17,0	65	0,34	87	32 h
<i>Aspergillus</i> C11B25	<i>sp.</i> 20,0	65	0,26	12	42 h
<i>Aspergillus</i> C28B25	<i>sp.</i> 11,0	100	0,30	94	32 h
<i>Aspergillus</i> C23B25	<i>sp.</i> 11,0	85	0,34	79	30 h

It can be concluded that it is possible to decaffeinate the coffee pulp in 30 h under aerobic conditions by using selected fungal culture, i.e., *Penicillium* sp. V33A25 in solid state fermentation, before initiation of the sporulation by the strain. It is also not necessary to sterilize the substrate. It is, therefore, possible to envisage the inoculation of the coffee pulp with mixed culture of lactic acid bacteria, for the ensilage preservation of coffee pulp, along with the selected filamentous fungi, for degradation of the caffeine. It can lead to decaffeinated and stabilized coffee pulp, which is suitable for animal feeding (Perraud-Gaime, 1995).

It is also possible that the stages of the fermentation can be observed visually on the computer, through respirometric parameters, without removing the sample and subjecting it to analyses and also without disturbing the culture medium. This factor of CO₂ evolution permits to reliably estimate different phases of the development of *Penicillium* sp. V33A25, in terms of degradation of caffeine and time of the sporulation of the filamentous fungi.

Biochemical pathway for caffeine degradation by filamentous fungi: In order to understand the biochemical pathway for caffeine degradation by filamentous fungi, synthetic caffeine was used under submerged fermentation system with defined synthetic medium. Metabolic pathway of caffeine degradation by *Pseudomonas putida* is shown in figure 4. The caffeine is degraded to urea as indicated in this figure. The same pathway may not hold good for filamentous fungi.

The kinetic of caffeine degradation by *Penicillium* and *Aspergillus* species is presented in figure 5. In both cases the degradation of caffeine is total in 50 hours, but the intermediate metabolites produced are different (Denis, 1996). In the case of *Penicillium* only theophylline appeared, whereas with *Aspergillus* species, Theobromine, paraxanthine and 3-methyl xanthine appeared as intermediates. The proposed pathway for the degradation of caffeine by filamentous fungi is shown in Figure 6.

Pectinases production from coffee pulp in SSF: Another utilization of coffee pulp is for the production of enzymes (Antier et al. 1993). Coffee pulp is an excellent substrate for pectinase production (Boccas et al. 1994, Augur et al. 1997). The ability of four wild fungal isolates capable of producing pectinase in solid state fermentation system using coffee pulp is shown in table 9. Caffeine degradation by *Aspergillus oryzae* and *Penicillium roquefortii* was also studied (Denis, 1996).

Conclusion

In conclusion it may be said that by selecting proper filamentous fungi it is possible to detoxify coffee pulp (caffeine degradation) and upgrade the coffee pulp for animal feed. The silage of coffee pulp permit to conserve the good potentialities of the pulp for various uses as indicated in the trophic chain (figure 9). The silage of coffee pulp under anaerobic conditions inhibits polyphenol oxidation. Under aerobic conditions the caffeic acid, chlorogenic acid and tannic acid forms polyphenols which can be further oxidized in presence of air to form quinones (fig. 10). These quinones in presence of proteins and free amino acids form a black water insoluble product (fig. 11). In order to overcome this anaerobic fermentation of coffee pulp with selected lactic acid bacteria is most efficient for the total detoxification of coffee pulp, polyphenols.

Bibliography

- Antier, P., Minjares, A., Roussos, S., Raimbault, M. and Viniegra-Gonzalez G. 1993. Pectinases hyperproducing mutants of *Aspergillus niger* C28B25 for solid state fermentation of coffee pulp. *Enzyme Microbiol. Technol.* 15: 254-260.
- Aquihuatl, M.A., Raimbault, M., Roussos, S. and Trejo, M.R. 1988. Coffee pulp detoxification by solid state fermentation : Isolation, identification and physiological studies. In , Raimbault, M. (Ed), *Proceedings of the seminar on solid state fermentation in bioconversion of agroindustrial raw materials*, ORSTOM, Montpellier, France, pp 13-26.
- Augur, C., Minjares, A., Viniegra-Gonzalez. 1997. Comparative studies of pectinase production by *Aspergillus niger* in solid state and submerged fermentation. In Roussos, S., Lonsane, B.K., Raimbault M., Viniegra-Gonzalez, G. (Eds), *Proceeding of Advances in Solid State Fermentation*, Kluwer, Dordrecht, pp. 347- 353.

- Bertin, G. 1986. Utilisation des enzymes polysaccharolytiques dans les milieux d'ensilage. Moyen de sélection et résultats pratiques. *Thèse d'Etat en Sciences*. Université Paul Sabatier. Toulouse, France, 209 p.
- Boccas, F., Roussos, S., Gutierrez, M., Serrano, L., Viniestra, G. 1994. Fungal strain selection for pectinases production from coffee pulp in solid state fermentation system. *J. Food Science Technol.* 31: 22-26.
- Bohkenfor, B. and Fonseca, H. 1974. Calidad de ensilado con pulpa de café conteniendo diferentes niveles de humedad y varios aditivos. In Primera Reunión Internacional sobre la Utilización de Subproductos del Café en la Alimentación Animal y otras Aplicaciones Agrícolas e Industriales, CATIE, Turrialba, Costa Rica, 41 p.
- Braham, J.E., Jarquin, R., Gonzalez, J.M. and Bressani, R. 1973. Pulpa y pergamino de café. 3. Utilización de la pulpa de café en la alimentación de rumiantes. *Turrialba* 23: 41-47.
- Bressani, R., Estrada, E. and Jarquin, R. 1972. Pulpa y pergamino de café. 1. Composición química y contenido de aminoácidos de la proteína de la pulpa. *Turrialba* 22: 299-304.
- Cabezas, M.T., Gonzalez, J.M. and Bressani, R. 1974. Pulpa y pergamino de café. 5. Absorción y retención de nitrógeno en terneros alimentados con raciones elaboradas con pulpa de café. *Turrialba* 24: 90-94.
- Bressani, R., Estrada, E. and Jarquin, R. 1972. Pulpa y pergamino de café. 1. Composición química y contenido de aminoácidos de la proteína de la pulpa. *Turrialba* 22: 299-304.
- Cabezas, M.T., Estrada, E., Murillo, B., Gonzalez, J.M. and Bressani, R. 1976. Pulpa y pergamino de café. 12. Efecto del almacenamiento sobre el valor nutritivo de la pulpa de café para terneros. *Archivos Latinoamericanos de Nutrición* 26: 203-215.
- Campos, R., Viniestra-Gonzalez, G. 1995. Utilization of a consortium of cellulolytic bacteria grown on a continuous anaerobic digester as a bioassay of probiotic extracts from fungal origin. *Biotechnol. Tech.* 9: 65-68.
- Carrizales, V. and Gonzalez, J. 1984. Aprovechamiento de la pulpa de café. Estudio Experimental, Reporte final. Fundación CIEPE, San Felipe, Venezuela.
- Christensen, M.S. 1981. Preliminary tests on the suitability of coffee pulp in the diets of Common Carp (*Cyprinus carpio* L.) and catfish (*Clarias mossambicus* Peters). *Aquaculture*, 25, 235-242.
- Denis, S. 1992. La dégradation de la caféine par deux champignons filamenteux : *Aspergillus oryzae* et *Penicillium roqueforti*. *Rapport de DEA*, Sciences des Aliments. Université de Montpellier II, France, 30 p.
- Denis S. 1996. Dégradation de la caféine par *Aspergillus* sp. et *Penicillium* sp. Etude physiologique et Biochimique. *Thèse de Doctorat*, Université de Montpellier II. France. 194 p.
- Gaime-Perraud, I., Roussos, S. and Martinez-Carrera, D. 1993. Natural microorganisms of the fresh coffee pulp. *Micol. Neotrop. Apl.* 6: 95-103.
- García, R.C., Baynes, D.R. 1974. Cultivo de *Tilapia aurea* (Staindachner) en corrales de 100m², alimentada artificialmente con gallinazas y un alimento preparado con 30 % de pulpa de café. Ministerio de Agricultura y Ganadería, Dir. Gen. de Pecursos Nat. Ren., El Salvador, 23p.
- Giraud, E., Brauman, A., Keleke, S., Lelong, B. and Raimbault, M. 1991. Isolation and physiological study of amylolytic strain of *Lactobacillus plantarum*. *Appl. Microbiol. Biotechnol.* 36: 379-383.
- Gouet, P. 1994. Bactériologie des ensilages. In De Roissart, H. and Luquet, F.M. (Eds.), *Bactéries lactiques*. vol. 2, Loriga, Grenoble, France, pp 257-270.

- Luis, L. and Ramirez, M. 1985. Estudio de los principales grupos de microorganismos presentes en los ensilados de pastos de estrella de jamaicano (*Cynodon nlemfuensis*). Silos y su relación con parámetros bioquímicos. *Pastos y Forrajes* 8: 141-155.
- McDonald, P., Henderson, A.R. and Heron, S.J.E. 1991. *The biochemistry of silage*. 2nd ed., Chalcombe Publications, Marlow, England. 340 pp.
- Murillo, B 1974. Composición química y fraccionamiento de los componentes celulares de la pulpa de café ensilada con aditivos. In Reunión Internacional sobre la Utilización de Subproductos del Café en la Alimentación Animal y otras Aplicaciones Agrícolas e Industriales, CATIE, Turrialba, Costa Rica, 41 p.
- Murillo, B. 1978. Ensilaje de pulpa de café. In Braham, J.E. and Bressani, R. (Eds.), *Pulpa de café : Composición, tecnología y utilización*, INCAP, Guatemala, pp 97-110.
- Perraud-Gaime, I. 1995. Cultures mixtes en milieu solide de bactéries lactiques et de champignons filamenteux pour la conservation et la détoxification de la pulpe de café. *Thèse de Doctorat*, Université de Montpellier II, France, 209 p.
- Perraud-Gaime, I. and Roussos, S. 1995. Preservation of coffee pulp by ensiling : Influence of biological additives. In Roussos, S., Lonsane, B.K., Raimbault M., Viniegra-Gonzalez, G. (Eds), *Proceeding of Advances in Solid State Fermentation*, Kluwer, Dordrecht 193-207.
- Roussos, S., Hannibal, L., Aquiahuatl, M.A., Trejo, M. and Marakis, S. 1994. Caffeine degradation by *Penicillium verrucosum* in solid state fermentation of coffee pulp : Critical effect of additional inorganic and organic nitrogen sources. *J. Food Sci. Technol.* 31: 316-319.
- Roussos, S., Aquiahuatl, M.A., Cassaigne, J., Favela, E., Gutierrez, M., Hannibal, L., Huerta, S., Nava, G., Raimbault, M., Rodriguez, W., Salas, J.A., Sanchez, R., Trejo, M., Viniegra-Gonzalez, G. 1989. Detoxificación de la pulpa de café por fermentación sólida. In Roussos, S., Licon, F.R. and Gutierrez, R.M. (Eds.), *I Seminario Internacional de Biotecnología en la Industria Cafetalera.*, Memorias I SIBAC, Xalapa, Veracruz, p. 121-143.
- Roussos, S., Aquiahuatl, M.A., Trejo-Hernandez, M.R., Gaime-Perraud, I., Favela, E., Ramakrishna, M., Raimbault, M. and Viniegra-Gonzalez, G. 1995. Biotechnological management of coffee pulp: Isolation, screening, characterization, selection of caffeine-degrading fungi and natural microflora present in coffee pulp and husk. *Appl. Microbiol. Biotechnol.* 42: 756-762.
- Tapia, I.M., Herrera-Saldaña, R., Viniegra-Gonzalez, G., Gutierrez-Rojas, M. and Roussos, S. 1989. Pulpa de café fermentada: su uso como aditivo en la fermentación de rumiantes. In Roussos, S., Licon, F.R. and Gutierrez, R.M. (Eds.), *Seminario Internacional de Biotecnología en la Industria Cafetalera*. Memorias, Jalapa, Veracruz, Mexico, p. 153-175.
- Tauk, S.M. 1986. Estudo da decomposição da polpa de café a 45°C através do uso de microorganismos isolados da polpa. *Turrialba* 36: 271-280.
- Trejo-Hernandez, M.R. 1992. Physiologie de croissance de souches de *Claviceps*: Production d'alcaloides par fermentation en milieu solide. *Thèse de Doctorat*, Université de Provence, Aix-Marseille I, France, 161 p.
- Vargas, E., Cabezas, M.T., Murillo, B., Braham, J.E. and Bressani, R. 1982. Efecto de altos niveles de pulpa de café deshidratada sobre el crecimiento y adaptación de novillos jóvenes. *Archivos Latinoamericanos de Nutrición* 32: 973-989.
- Viniegra-Gonzalez, G., Roussos, S. and Raimbault, M. 1991. Fermentations en milieu solide comme moyen de valorisation des produits agricoles tropicaux au Mexique. *ORSTOM Actualités* 34: 23-25.

- Vitzthum, O.G., Barthel, M. and Kwasny, H. 1974. Détermination rapide de la caféine dans le café décaféiné ou non par chromatographie en phase gazeuse avec détecteur d'azote. *Zeitschr. f. Lebensm. Unters. u. Forsch (Munich)* 154: 135-140.
- Weinberg, Z.G., Ashbell, G., Azrieli, A. and Brukental, I. 1993. Ensilage peas, ryegrass and wheat with additives of lactic acid bacteria (LAB) and cell wall degrading enzymes. *Grass Forage Sci.* 48: 70-78.
- Zuluaga, J. 1981. Contribution à l'étude de la composition chimique de la pulpe de café (*Coffea arabica* L.). Thèse de doctorat es Sciences. Faculté des sciences, université de Neuchatel-Suisse
- Zuluaga, J. 1989. Utilización integral de los subproductos del café. in Roussos S., Licona R. y Gutierrez M. (Eds), *Memorias I Sem. Intern. Biotecnol. Agroindust. Café* (I SIBAC), Xalapa, Mexico, pp 63-76.

MOLECULAR TECHNIQUES APPLIED TO FUNGAL STRAIN UPGRADATION CAPABILITY RELATED TO SSF CULTURES

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Summary

Mutant strains derived from *A. niger* C28B25 and belonging to series AW99 [producing maximum pectinase level in submerged fermentation (SmF)] and series AW96 [producing maximum pectinase level in solid state fermentation (SSF)] were compared concerning pectinase properties and thermal stability. Pectinases produced by AW96 exhibited increased thermal stability compared to AW99. Results support the idea that not only the quantity but also the quality of the enzymes can be modified when SSF or SmF techniques are used. The use of differential display polymerase chain reaction may enable the isolation of solid state fermentation-specific genes.

Our group is trying to use molecular methods in order to study a fundamental problem of fungal fermentation, that is, the way fungi adapt to grow and reproduce in liquid or solid media. With ever growing new uses for enzyme production in SSF, there is a need to improve the basic understanding of the physiology of molds used to produce enzymes by SSF. This way, over fifteen years of SSF studies can help to give a qualitative jump on the physiology and biochemistry of filamentous fungi with specific reference to their interaction with a specific solid substrate. In fact, filamentous fungi in general (whether perfect or imperfect, but growing as filamentous structures or mycelia) have a remarkable adaptability to the specific type of substrate. Such kind of adaptations are most surely controlled by the transcription and expression of different sets of genes. Identification of the « trigger » genes controlling those fungal adaptations will help to use modern techniques of genetic engineering to clone, transfer and express new genes from any biological source coding for enzymes and other useful products to be produced by different fermentation techniques.

Previous work in our laboratory [1] with a wild-type strain of *A. niger* called C28B25 led to the isolation of two different kinds of mutants resistant to 2-deoxyglucose (2-DG), namely the DG^R AW96 class of strains and DG^R AW99 class of strains. Interestingly, these mutant strains behave in an opposite manner to each other that is, DG^R AW96 proved to be endopectinase hyperproducers when cultivated by SSF but not as much when cultivated by SmF whereas DG^R AW99 hyperproduced endopectinase when cultivated by SmF but were poor pectinase producers when cultivated by SSF [3]. Thus it can be considered that DG^R AW96 strains are « adapted » to produce pectinase on SSF and DG^R AW99 are « adapted » to SmF production.

Alazard and Raimbault [6] found that amylase activity produced by *A. niger* using SSF technique was more heat tolerant than the one produced by SmF technique. Acuña-Argüelles

and coworkers [2] confirmed such observation by measuring pectinase activities produced by SSF and SmF and using an *A. niger* CH4 strain.

In an effort to approach at a molecular level, the nature of the differences observed, zymographic patterns of pectin hydrolases and pectin esterases produced by a wild strain *A. niger* C28B25 and mutants AW96 and AW99 were obtained.

For the detection of *in situ* pectinolytic activity, and to study the effect of heat, extracts from the growth of each strain in either SSF or SmF were obtained and divided into two samples. Only one sample was heated at 90° C for 60 s. The samples were electrophoresed and pectinolytic activity was detected *in situ*. AW99 lacked a pectin esterase band at 70 Kd when compared to the other strains. Neither the AW96 nor the wild-type strains hydrolytic activities could be detected before heating and only the AW99 mutant exhibited a slight hydrolytic activity band. Nevertheless, heating of the SSF extracts resulted in an apparent **activation** of an additional hydrolytic activity.

Only two low molecular weight pectin esterase activities could be detected in SSF extracts but none showed the 70kDa activity that had previously been observed in SmF extracts. All esterase activities were lost by heating the extracts.

The SSF culture technique, thus produced pectin hydrolases that had the remarkable property of needing a brief thermal treatment in order to show catalytic activity and were different from the ones produced by the SmF culture technique which led to thermal-sensitive pectin hydrolases. Apparently, thermal sensitivity of this latter activity was greater than for those of the wild type or AW96 strains which provides additional evidence for the presence of discreet differences at the molecular level. The exact nature of the differences, whether resulting from modifications of the polypeptide chain or differences in glycosylation patterns, has yet to be elucidated.

Furthermore, thermal stability of *in vitro* enzyme activity was studied by viscometry [mainly endopolysaccharuronase (endoPG) activity]. EndoPG activities from AW96 strain produced by either SmF or SSF techniques declined slowly with thermal treatment whether in the presence or absence of substrate (Figure 1.)

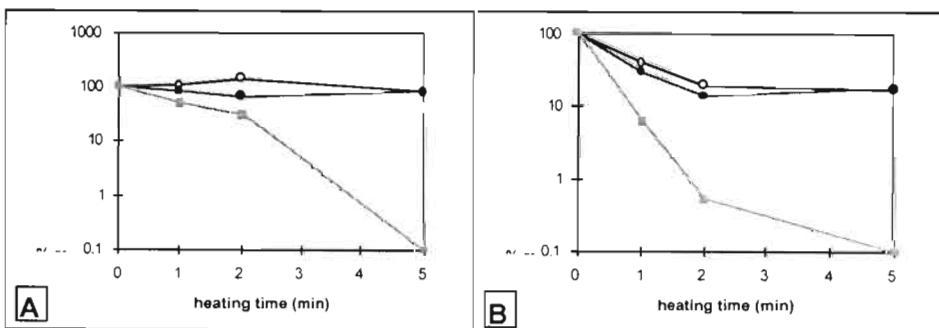


Figure 1. Thermal stability of *in vitro* endopectinase activity of SmF extracts (A) and SSF extracts (B) in the presence (closed symbols) or absence (open symbols) of pectin (5g/L). AW96=square symbols, AW99=round symbols. T=92°C. Note the logarithmic scale on the axis.

Activity from AW99 produced by SmF showed a trend without any significant difference from that of AW96 extracts when heated in buffer. In contrast, the same extract

exhibited a much more pronounced inactivation slope when heated in the presence of substrate (Figure 1A). Samples from SSF exhibited a more pronounced inactivation slope especially in the case of AW99 (Figure 1B). In this case, no significant differences were found in the presence or absence of substrate. In all cases, endoPG activity produced by AW96 strain was more thermostable than that produced by AW99 strain.

Exopectinase activities (Figure 2) produced by both types of mutants in SmF had similar trends when heated in the presence or absence of substrate (Figure 2A).

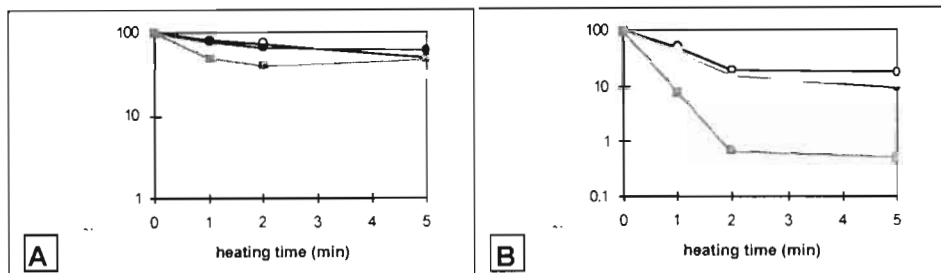


Figure 2. Thermal stability of *in vitro* exopectinase activity of SmF extracts (A) and SSF extracts (B), in the presence (closed symbols) or absence (open symbols) of pectin (5g/L). AW96=square symbols, AW99=round symbols. T=92°C. Note the logarithmic scale on the axis.

Likewise, exopectinase activities had similar trends in extracts obtained from both strains by SSF when heated in buffer (Figure 2B) however, extracts from the AW96 strain showed and increased thermal sensitivity when heated in the presence of substrate. Results therefore indicated that pectinolytic enzymatic complexes produced by each type of mutant strain were different when produced by SmF or SSF.

Pectin hydrolases produced by SSF technique were more resistant to heat denaturation than those produced by SmF technique. Pectin esterases were, instead heat labile in a similar way when produced either by SSF and SmF techniques. Pectinase activity produced by AW96 mutants was more heat tolerant than that produced by AW99 [2].

Thermal tolerance of pectin hydrolases is an interesting property when analyzed by electrophoretic zymography. Zymographic differences were related to the use of SSF or SmF techniques and the nature of each given strain. For example, the SSF technique produced a pectin hydrolase band requiring previous heating in order to have activity in the gel. The nature of the heat tolerance of the protein produced by SSF requires further basic work. Perhaps this could be related to the activation of a zymogen or the inactivation of a thermolabile inhibitor associated with the native protein.

In relation to pectin esterase activities, SmF produced a distinct band at 70 kDa which was absent in cultures obtained by SSF. These results give further support to the idea that each given fermentation technique is responsible for the production of different pectinase patterns [2, 7]. On the other hand, pectinase activities measured in crude extracts by viscometry showed very important differences between culture techniques and strains. For example, pectinase activities produced by the AW96 strain were more thermostable than those

produced by the AW99 strain. This could be related to earlier reports which suggested that enzymes produced by SSF are more thermostable than those produced by SmF [2,6,7].

The effect of heating on pectinase activities measured by viscometry did not seem to be the same as that revealed by the zymograms, but this may be related to the interaction in the former among several enzymes and soluble materials present in the reaction mixture; nevertheless, thermal stability analysis of enzyme extracts helps to distinguish among different enzymatic phenotypes produced by different strains and culture techniques.

The molecular basis of those differences would require purification and detailed biochemical characterization of each given enzyme but opens some interesting questions on the way the molds adapt to solid and liquid culture techniques using perhaps a different set of genes or modifying their expression in a differential way.

In brief, although both types of mutant strains (AW96 and AW99) were selected for their resistance to 2-deoxyglucose (DG^R), the results showed that both classes are in fact different and also that the DG^R phenotype may not be directly involved with the complex patterns of physiological derepression and enzyme production which is in agreement with the hypothesis of having pleiotropic mutations associated with the DG^R phenotype.

As stressed in the introductory comments, identification of the « trigger » genes controlling fungal adaptations to SSF would greatly help in the understanding of the mechanisms that control the adaptability of molds. We intend to test in the near future, a relatively new technique known as differential display polymerase chain reaction (DD-PCR) in order to determine whether it is possible to identify genes that are specific for solid state fermentation. The technique has been successfully used in our laboratory to identify genes that regulate caffeine degradation in *A. niger*. Differential display is a powerful screening technique used for the detection and identification of differentially expressed genes [4] related to solving developmental, environmental [5] and hormonal problems. In this method, two or more RNA's (from SSF and SmF cultures) are used as templates to generate cDNA. Subsequently the cDNA fragments are amplified by PCR using an arbitrary primer in the presence of a radiolabeled nucleotide (dNTP). After separation by denaturing polyacrylamide gel electrophoresis the gels are fixed and dried. Differentially amplified cDNA's are identified by autoradiography. To produce sufficient DNA for further analysis, the differentially expressed DNA is eluted from the gel and reamplified and then cloned. Northern analysis then confirms that a given clone is SSF-specific.

The above mentioned results seem to support the idea that not only the quantity but the quality of enzymes can be modified when SSF or SmF techniques are used. This may be accomplished either by turning on and off different sets of genes coding for different polypeptides, by controlling the edition of the same polypeptides (i.e., differences in glycosylation) or by a combination of both kinds of mechanisms. Distinction between such hypotheses the use of techniques such as DD-PCR which is part of the present research program in our laboratory.

Bibliography

- 1.- Antier, P., A., Minjares and G. Viniegra: Pectinase-hyperproducing mutants of *Aspergillus niger* C28B25 for solid state fermentation on coffee pulp. *Enzyme Microb. Technol.* **15** (1993), 254-260.
- 2.- Acuña-Argüelles, M., Gutiérrez-Rojas, M., Viniegra-González, G., Favela-Torres. E.: Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid state fermentation. *Appl. Microbiol. Biotechnol.* **43** (1995), 1-6.
- 3.- Minjares-Carranco (1992) Obtención de mutantes de *Aspergillus niger* C28B25 hiperproductoras de pectinasas por fermentation en medio solido de la pulpa de cafe. Tesis de maestria. Universidad Iberoamericana. Mexico D.F.
- 4.- Liang, P., and Pardee, A.B. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, (1992) 967-971.
- 5.- Sharma, Y.K. and Davis, K. R. Isolation of a novel Arabidopsis ozone-induced cDNA by differential display *Plant Mol. Biol.* **29**: (1995) 91-98.
- 6.- Alazard, D., Raimbault, M.: Comparative study of amylolytic enzyme production by *Aspergillus niger* in liquid and solid state cultivation. *J. Appl. Microbiol. Biotechnol.* **12** (1981), 113-117.
- 7.- Solís-Pereira, S., Favela-Torres, E., Viniegra-González, G., and Gutiérrez-Rojas, M.: Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentation. *Appl. Microbiol. Biotechnol.* **39** (1993), 36-41.

THEORY AND STRATEGY FOR SCALE UP: DEVELOPMENT OF SCALE-UP CRITERIA FOR BIO-REACTORS OF SOLID STATE FERMENTATION (SSF)

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Abstract*

Scale up is the crucial link in transferring a laboratory scale process to a pilot plant and then to a commercial production scale. During this exercise multidisciplinary groups involving specialists from biological and engineering sciences, are required. In this paper biological and engineering aspects concerning scale-up of bioreactor of solid state fermentation are discussed. The problems associated with scale up of bioreactor of solid state fermentation are not necessary the same than those found in submerged fermentation. In some cases particular, those can be listed as follows: Variation in the biomass due to an excessive subculturing, medium sterilization, inoculum production, heat removal and moisture maintenance are particularly important because of the heterogeneity of materials in solid cultivation. The general methodology used to develop criteria for scale up involves principles of geometric, thermal and biochemical similarity as well as heat and mass balances. In the sense some cases can be described. In SSF, inoculum is used at a very high ratio, consequently inoculum production is a particular unit operation in large scale SSF fermentors. In this sense, the production of conidiospores of *T harzianum* has been reported before indicating a large production level in a pilot plant reactor. In another experience, the combination of appropriate sterilization and treatment of substrate resulted in significant increase of cellulase activities.

Probably the most important problem during SSF processes is heat removal and moisture control. To overcome this problem several strategies have been proposed including maintenance of heat and water balances and utilization of evaporative cooling as criteria for scaling - up. In our research team we believe that fundamentals of mathematical modeling are an alternative for scaling-up of bioreactors. Coming from energy and mass balances dimensionless numbers could be found to be useful in scale-up processes. The application of dimensionless numbers (Biot and Peclet) were first proposed for solid state fermentation for the case of cultivation of *A. niger* on cassava, since in those ratio are described the operating conditions, geometry and size of bioreactor, leading to improve solid state reactors systems.

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MULTIVARIABLE MODEL PREDICTIVE CONTROL OF A SOLID SUBSTRATE PILOT BIOREACTOR: A Simulation Study

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Abstract

This work deals with the modelling and control of a Solid Substrate Cultivation pilot bioreactor (50 kg of capacity) for gibberellic acid production with *Gibberella fujikuroi*. A simplified physical lumped parameters model was developed. The model can reproduce the observed qualitative behaviour of the main process variables, such as bed temperature and bed water content. In addition, the performances of several control strategies using classic algorithms (Proportional/Integral/Derivative, PID) and a predictive algorithm (Dynamic Matrix Control, DMC) were analyzed by simulation. In these control strategies, temperature and relative humidity of the input air, and also fresh water and nitrogen additions were defined as manipulated variables. On the other hand, bed temperature, bed water content and biomass concentration were defined as controlled variables.

It was verified that the employment of a DMC algorithm simplifies the design and start up of the control system. DMC possesses a flexible structure that can be adapted to the process facilities and limitations. In this application, the effort devoted to tuning the temperature control loop was significantly reduced and a better closed loop response was achieved with a DMC algorithm compared with a PID.

Keywords: DMC, temperature control, water content control, solid substrate fermentation.

Introduction

Modelling and control in solid substrate cultivation systems (SSC) are critical aspects that must be solved before scaling up the process to industrial level. SSC systems are difficult to understand due to the strong interaction among solid, liquid, gaseous and biotic phases. In addition, the lack of adequate sensors limits the identification of the process states.

Cultivation in solid substrate has been traditionally used by humankind for several centuries. In this process, the microbial growth and the formation of products occur in the interior of a solid matrix, under low water content conditions. High yields, concentrated products and low operation costs are usually achieved with this kind of cultivation. However, despite its advantages, SSC has a series of drawbacks [1] that make it difficult to scale up processes that are promising at laboratory scale. Many of the difficulties are related with the regulation of the temperature and water content of the solid bed, and the control of the biomass concentration.

In fixed bed bioreactors with periodic agitation, water content control is particularly complex. Part of the water is strongly bound to the solid, while the rest can exist in free form in the capillary areas of the material. Care must be taken to avoid saturation of the solid matrix capacity [2]. On the other hand, there is a lack of appropriate and unexpensive sensors for on line water content monitoring. Then, the water content regulation is difficult, slow and not precise.

For temperature control, the difficulty is mainly found in the limited dissipation capacity of the metabolic heat generated during the cultivation. This heat cannot be removed by vigorous agitation, since this affects negatively the growth of the microorganism.

The lack of general and reliable biokinetic models for SSC processes is the main problem for the control of the biomass concentration in the bioreactor. Fungi grow preferably in the interparticular voids and in the solid surface that is used as support. Then, a heterogeneous substrate is generated that changes throughout the cultivation cycle [1], inhibiting direct biomass measurements [3, 4, 5]. On the other hand, substrate degradation, heat buildup and water content variations, are directly related with the microorganism activity [6].

Discrete PID and on/off control algorithms are currently used in pilot and industrial SSC bioreactors. Given the process difficulties mentioned above, these controllers are difficult to tune and demand human supervision during the whole cultivation process to operate it effectively [7].

In the present work, the performance of an advanced model predictive control algorithm is assessed and compared with a classic PID control. The study is based on simulations with a physical model of a pilot SSC bioreactor for gibberellic acid (GA_3) production using Gibberella fujikuroi.

MODELLING

In figure 1, the SSC pilot bioreactor used for GA_3 production is described. This bioreactor has a nominal capacity of 50 kg and has been designed for an aseptic operation in batch or fed -batch mode. The bed agitation system is mechanical and operates like a plough, where the basket, which contains the solid bed, rotates and the blades are static. Sterile water and specific nutrients can be added sporadically. The input air is previously filter-sterilized and then conditioned before entering the bioreactor. The bioreactor operates with a semi-automatic strategy for bed temperature regulation and water content control. Details of the instrumentation and control strategy can be seen in [7].

A simplified SSC process can be represented as a two phase's system. These are the solid bed that contains the substrate, the inert support, the liquids (water for the most part) and the microorganism; and the gas that flows through the bed.

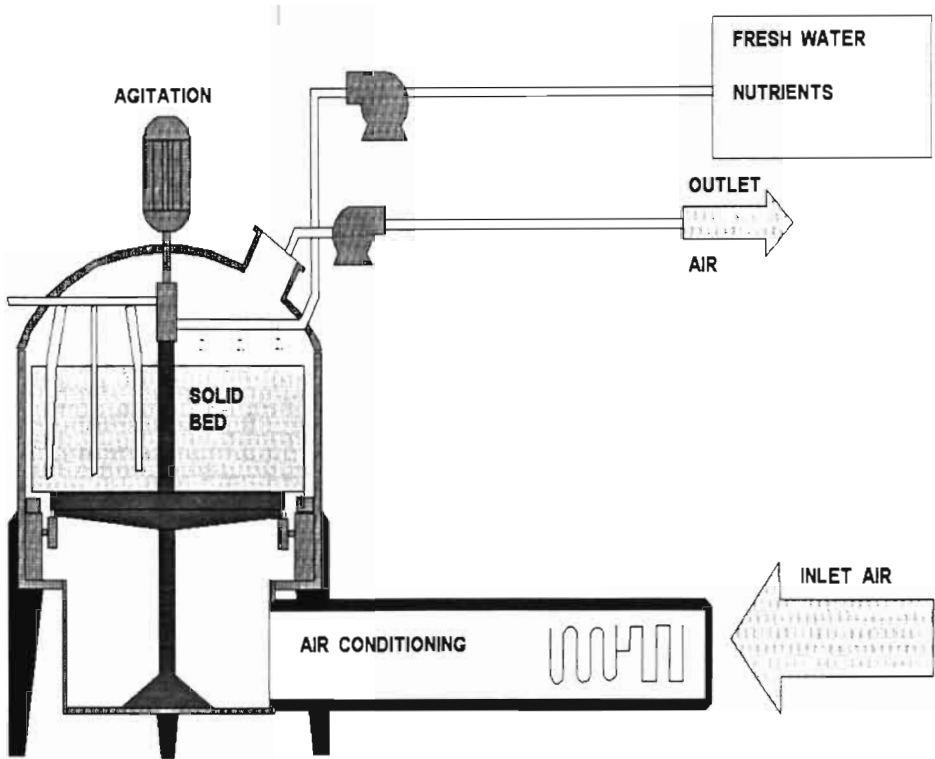


Figure 1: Diagram of the solid substrate cultivation pilot reactor

The main model assumptions are:

- a) homogeneous temperature and component's concentration within the bioreactor
- b) biomass growth limited by nitrogen only
- c) the outlet gas is saturated
- d) negligible accumulation of the gas phase in the reactor

The following set of equations can be derived through mass and energy balances in the solid and gaseous phases. These equations describe the evolution of the main bioreactor variables.

First, the fungus growth kinetics can be represented by :

$$\frac{dx}{dt} = (\mu - K_D) \cdot x \quad (1)$$

where x is the biomass concentration, K_D is a death constant, and m is the specific growth rate estimated from the Contois model. This structure was used because it has been argued that this model behaves well both at high and low biomass concentrations. In addition, it considers diffusional limitations of nutrients [8].

$$\mu = \frac{\mu_M \cdot N}{K_N \cdot x + N} \quad (2)$$

The value of m_M , maximum specific growth rate, is considered constant in the above equation, since temperature and water activity of the solid bed are kept regulated. In equation 2, N is the nitrogen concentration.

From this kinetic model, the consumption rates of oxygen and nutrients, and the production rate of carbon dioxide can be derived, which are described below:

$$\frac{dN}{dt} = -\frac{\mu \cdot x}{Y_{xN}} \quad (3)$$

Here, Y_{xN} is the biomass/nitrogen yield coefficient.

$$\frac{dG}{dt} = -\left(\frac{\mu}{Y_{xG}} + m_G\right) \cdot x \quad (4)$$

where Y_{xG} is the biomass/glucose yield coefficient, and m_G the maintenance coefficient.

$$\frac{dO_2}{dt} = -\left(\frac{\mu}{Y_{xO_2}} + m_{O_2}\right) \cdot x \quad (5)$$

$$\frac{dCO_2}{dt} = \left(\frac{\mu}{Y_{xCO_2}} + m_{CO_2}\right) \cdot x \quad (6)$$

In equations 5 and 6, Y_{xO_2} and Y_{xCO_2} are the biomass yield coefficients for O_2 and CO_2 respectively and m_{O_2} and m_{CO_2} the maintenance coefficients; dO_2/dt and dCO_2/dt the production rates, for oxygen and carbon dioxide, respectively.

All these rates (eqs. 3 to 6) are referred to the total dried mass within the bed, whose evolution can be estimated from the CO_2 production rate according to,

$$\frac{dM_S}{dt} = -k_G \cdot \left[\frac{(12/44)}{0.44}\right] \cdot M_S \cdot \frac{dCO_2}{dt} \quad (7)$$

where, M_S is the dried solid mass and k_G is a constant that accounts for the weight loss caused by the production of carbon-containing compounds different than CO_2 . The factor (12/44) represents the ratio between the molecular weights of Carbon and CO_2 , while 0.44 is the elemental carbon composition in the organic solid mixture.

To estimate the bed water content evolution, appropriate mass balances were defined, yielding after rearrangement:

$$\frac{dX}{dt} = \frac{[F_2 + z - w - X \cdot \frac{dM_S}{dt}]}{M_S} \quad (8)$$

where X is the solid water content and F_2 the fresh water addition rate, w is the water evaporation rate, estimated from a water balance in the gas phase. On the other hand, z represents the water production rate, which is estimated from the stoichiometric equations that describe the aerobic metabolism during the growth phase of the microorganism [9],

$$z = \frac{18 \cdot M_S}{44} \cdot \frac{dCO_2}{dt} \quad (9)$$

where 44 and 18 are the molecular weights of the carbon dioxide and water respectively.

The evolution of the bed temperature is obtained from an energy balance, yielding:

$$\frac{dT_S}{dt} = \frac{[q + (F_1 \cdot H_1 - F_3 \cdot H_3) - Cp_S \cdot T_S \cdot \frac{dM_S}{dt} - M_S \cdot T_S \cdot Cp_{H_2O} \cdot \frac{dx}{dt}]}{M_S \cdot Cp_S} \quad (10)$$

T_S is the bed temperature, F_1 and F_3 are the inlet and outlet gas flowrates, and H_1 and H_3 the inlet and outlet gas enthalpies. The specific heat of the wet solid (Cp_S) can be expressed in terms of the specific heats of the dried solid and the water (Cp_{H_2O}) [1, 10]. The metabolic heat generation, q , is estimated using the methodology proposed in [11]. Then:

$$q = M_S \cdot \frac{dQ}{dt} \quad (11)$$

where the specific heat generation rate, dQ/dt , is estimated through the next equation,

$$\frac{dQ}{dt} = - \left(\frac{\mu}{Y_{x/Q}} + m_Q \right) \cdot X \quad (12)$$

m_Q is the maintenance coefficient, and $Y_{x/Q}$ is the biomass/heat coefficient, obtained experimentally from the heats of combustion of the substrate and of the biomass [11].

Constants, parameters and Initial Conditions

The values of the coefficients and parameters model are shown in table 1. Yield and maintenance coefficients for *Gibberella fujikuroi* were experimentally measured in the laboratory, using urea and starch as nutrients, and vermiculite (mean particle size mesh 16) as inert support. These values were

Table 1: model parameters

Parameter	Value	Units
k_G	1.1000	[-]
K_D	0.0012	[h ⁻¹]
K_N	0.2500	[kg / kg d.m.]
m_G	0.0110	[kg / kg d.m. h]
m_Q	$3.3 \cdot 10^5$	[J / kg d.m. h]
m_{O_2}	0.0130	[kg / kg d.m. h]
m_{CO_2}	0.0140	[kg / kg d.m. h]
$Y_{x/Q}$	$6.5 \cdot 10^{-8}$	[kg d.m. / J]
$Y_{x/G}$	0.5500	[kg / kg]
$Y_{x/N}$	14.3970	[kg / kg]
Y_{x/O_2}	0.9510	[kg / kg]
Y_{x/CO_2}	0.6250	[kg / kg]
m_M	0.2200	[h ⁻¹]

then readjusted with data obtained in the pilot bioreactor, using sterilized wheat bran as substrate and inert support. The values of K_D and m_M were estimated from the biomass growth curves. The constants K_N and k_G were estimated by least square error minimization, comparing the biomass growth and weight loss experimental curves with the model. The specific heat of the dried solid was measured at 28°C, employing a differential scanning calorimeter (DSC) Perkin Elmer, Series 1020, DSC7.

Equations (1) to (12) were integrated to simulate a typical cultivation run of 150 hours. The initial conditions employed in the simulation were: $M_S = 25.19$ [kg]; $N = 0.0166$ [kg/kg d.m.]; $G = 0.6595$ [kg/kg d.m.]; $X = 1.5$ [kg/kg d.m.]; $T_S = 27$ [°C] and $x = 0.005$ [kg/kg d.m.]. The initial dried solid is composed of 20 kg of sterilized wheat bran, 5 kg of starch, 0.068 kg of urea and 0.1253 kg of dried biomass. Nitrogen and total glucose in this substrate are 0.4161 and 16.6160 kgs, respectively.

CONTROL

The performance of classic and DMC controllers was analyzed based on MATLAB simulations using the above physical model. Bed temperature (T_s), bed water content (X) and bed biomass concentration (x) were defined as controlled variables, while inlet air temperature (T_i) and relative humidity (R_{H1}), and also fresh water (F_2) and nitrogen addition (N), were defined as manipulated

variables. Transfer functions for bed temperature, water content and biomass concentration were obtained from step input responses [12], and are computed as the differences between a defined nominal trajectory and the disturbed trajectory. Bed temperature response is first order for inlet air temperature and relative humidity disturbances. On the other hand, bed water content and biomass concentration respond as pure integrators for fresh water and nitrogen addition respectively. Other transfer functions relating these variables are negligible.

Recent studies have defined the optimum operating conditions for achieving high productivity of gibberellic acid (GA₃), when Gibberella fujikuroi is used in an SSC bioreactor. Temperature must be kept at 28°C throughout all the cultivation cycle. On the other hand, water content should be increased at a constant rate during the growth phase and then maintained at about 70% w/w for the rest of the cultivation cycle [13]. Finally, biomass concentration must be regulated by the nitrogen sources to optimize GA₃ production [14]. Based on these studies, in our simulations bed temperature set point is kept constant at 28°C. In addition, bed water content set point was programmed to follow a linear trajectory from an initial condition of 1.5 [kg/kg d.m.] up to 2.5 [kg/kg d.m.], in 40 hours, then the set point is kept constant; this is the way the SSC pilot reactor is currently operated [7]. Finally, for bed biomass concentration, the set point is kept constant at a high previously defined value.

Classic Control

The bed temperature was controlled with the inlet air relative humidity (as in the pilot reactor) using a discrete PI, since the transfer function is first order. The controller pulse transfer function is given by :

$$G_C = \frac{[q_0 + q_1 \cdot z^{-1}]}{[1 - z^{-1}]} \quad (13)$$

where z is the discrete transformed variable, and q_0 and q_1 are related with the standard PI tuning parameters (proportional gain, K_c , and reset time, t_1) through:

$$q_0 = K_c \cdot \left[1 + \frac{T}{\tau_1} \right] \quad (14)$$

$$q_1 = -K_c \quad (15)$$

where T is the sampling time.

Control of water content and biomass concentration was achieved with a proportional algorithm, since both relevant transfer functions were pure integrators. Here, the pulse transfer function of the controller is simply:

$$G_c = \frac{q_j \cdot z^{-1}}{[1 - z^{-1}]} \quad (16)$$

DMC Control

The Dynamic Matrix Control algorithm, DMC, has been presented and discussed widely in standard process control texts [15], then only a basic description will be given here. This discrete controller uses a multivariable step response model (Dynamic Matrix) to compute future manipulated variables moves that would minimize a given quadratic cost function. This prediction is achieved using current output values, accumulated past errors and two matrices in the cost function that weight independently the output errors and the control moves. If input and output restrictions are included in the minimization problem, the algorithm is usually known as QDMC (Quadratic Dynamic Matrix Control).

The cost function, J , of the DMC algorithm is given by the product of the weight matrix, τ , and the square of the normalized error vector, e , plus the product of the weight matrix, λ , and the square of the normalized future control moves, Δm . Here, it is considered all future vector errors up to time h_p (prediction horizon), and all future moves up to time h_c (control horizon). Then, J can be represented by:

$$J = \sum_{i=1}^{h_p} e_i \cdot \tau \cdot e_i' + \sum_{i=1}^{h_c} \Delta m_i \cdot \lambda \cdot \Delta m_i' \quad (17)$$

In this algorithm, the matrices τ and λ , and the horizons h_p and h_c , are tuning parameters. Usually, h_p is defined to include 90% of the step response and h_c is set to $h_p/2$. The weighting matrices are diagonal, and their elements can be set to get tighter control in certain outputs and to move more some desired inputs.

Results and Discussion

In all simulations, the same sampling time of 90 s was used for the three output variables. This is the value used in the temperature control loop in the pilot reactor [7].

Figure 2 shows the results for bed temperature control with PI and DMC. Here, the system was subjected to inlet air flowrate and temperature (PI only) disturbances. When a PI controller is used, the inlet air humidity is manipulated, while with DMC, inlet air temperature and relative humidity are manipulated simultaneously. The PI controller was tuned by trial and error, where the values of $K_c = 20$ and $t_i = 10$ gave the best overall performance. The values of the tuning parameters of the

DMC were set to $h_p = 40$, $h_c = 20$, $l_i = 1$ and $t_i = 1$ (for all i). As seen in the figure, when the classic algorithm PI is used, the inlet flowrate and temperature disturbances strongly affect the control performance. In this case, response is oscillatory, slow (20 hours to reach 28°C), presents large deviations and even fail when an inlet air temperature disturbance is applied at time 90 hr. On the other hand, the DMC seems a more adequate algorithm for this process. A fast response can be achieved (2 hours), with practically no oscillations and with a simple tuning procedure. Moreover, with the DMC algorithm, the bed temperature can be controlled without requiring extremely low values of the manipulated variables, which is difficult and expensive to attain in a real system.

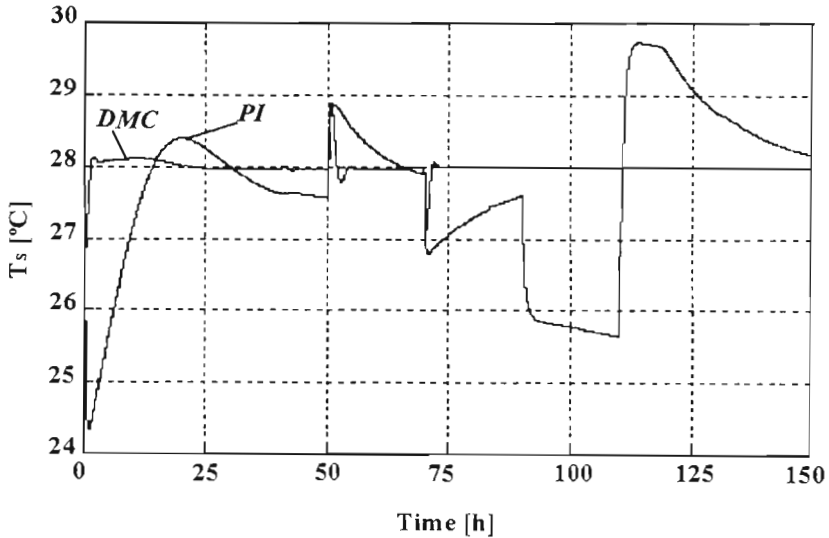


Figure 2: Bed temperature closed loop response with PI and DMC control under inlet air flowrate and temperature disturbances.

The control of the bed water content and biomass concentration was practically the same with both algorithms. Here, a perfect tracking was obtained (Figs. 3 and 4) and the manipulated variables behave similarly (not shown).

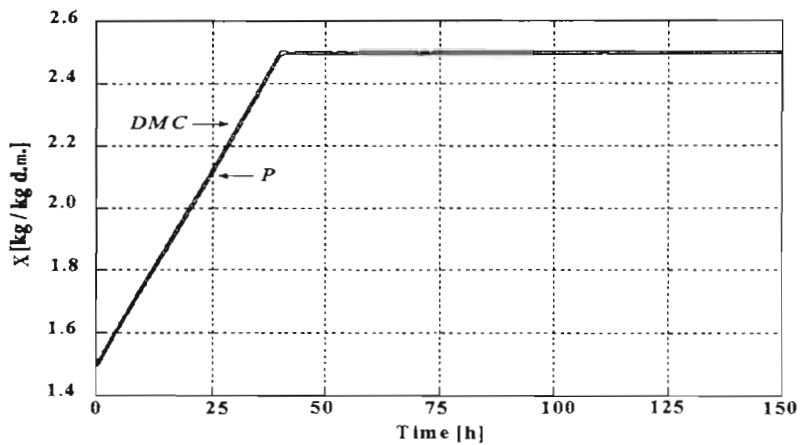


Figure 3: Bedwater content tracking with PI and DMC control.

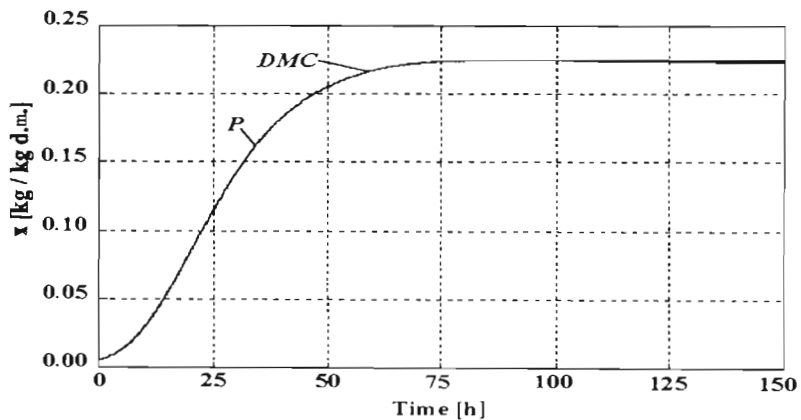


Figure 4: Bed biomass concentration tracking with PI and DMC control.

Conclusions

A physical model of an SSC pilot bioreactor was developed. Simulations with this model showed the same qualitative behavior as experimental results in the reactor. Based on simulations with this model, it was concluded that a DMC controller behaves better than a simple PI in the control of the bed temperature, a critical operating variable in this kind of reactors. Furthermore, PI controller is difficult to tune and demands more control effort; these are severe limitations in SSC processes. The fact that the DMC used in this application includes more manipulated than observed variables, explains the good performance achieved.

Although PI and DMC gave the same performance for water content and biomass concentration, the P controllers were more difficult to tune. Moreover, in the simulations shown here, it was considered that fresh water and nitrogen addition were continuously added to the reactor. In a real process this addition must be done by pulses, since the reactor is agitated only periodically. Then, it would be easier to adapt a DMC than a PI to this kind of manipulation.

Acknowledgements

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References

- 1 Sargantanis, J., Karim, N., Murphy, V. and Ryoo, D. 1993. Effect of operating conditions on solid substrate fermentation. *Biotechnology and Bioengineering*, 42:149-158.
- 2 Mudgett, R. E. 1986. Solid-state fermentations. In: Demain, A. and Solomon, N. *Manual of Industrial Microbiology and Biotechnology*. American Society for Microbiology, Washington D.C.
- 3 Mitchell, D., Duong, D., Greenfield, P. and Doelle, H. 1991. A semimechanistic mathematical model for growth of *Rhizopus oligosporus* in a model solid-state fermentation system. *Biotechnology and Bioengineering*, 38:353-362.
- 4 Auriá, R., Ortiz, I., Villegas, E. and Revah, S. 1995. Influence of Growth and high mould concentration on the pressure drop in solid state fermentations. *Process Biochemistry*, 30:8:751-756.
- 5 Colin, R.T. 1992. Image analysis: putting filamentous microorganisms in the picture. *Tibtech* October (Vol 100), 343-348.
- 6 Saucedo-Castañeda G., Gutiérrez-Rojas, M., Backet and G., Raimbault, M. 1990. Heat transfer simulation in solid substrate fermentation. *Biotechnology and Bioengineering*, 35:802-808.

- 7 Fernández, M., Pérez-Correa, R., Solar, I., and Agosín, E. 1996. Automation of a Solid Substrate Cultivation Pilot Bioreactor, *Bioprocess Engineering* 16:1-4.
- 8 Menezes, J. and Alvez, S. 1994. Mathematical modelling of industrial pilot-plant Penicillium-G fed batch fermentations. *J. Chem. Tech. Biotechnol.*, 61:123-138.
- 9 Raimbault, M. 1981. Fermentation en milieu solide, croissance de champignons filamenteux sur substrat amy lacé. Service des publications de L'O.R.S.T.O.M., France.
- 10 Sweat, V. 1989. Thermal properties of foods. Agricultural Engineering Department, Texas A & M University College station, Texas, 75-79.
- 11 Bailey, J. and Ollis, D. 1986. *Biochemical Engineering Fundamentals*. McGraw-Hill Publishing Company.
- 12 Paján, H. 1996. Modelación, Simulación y Control de un Bioreactor Piloto de Cultivo de Sustrato Sólido. MSc thesis, Chem. and Biopro. Eng. Dept., Pontificia Universidad Católica de Chile. (in spanish)
- 13 Corona, A. 1995. Actividad de agua: un parámetro fundamental en cultivo sobre sustrato sólido. MSc thesis, Chem. and Biopro. Eng. Dept., Pontificia Universidad Católica de Chile. (in spanish)
- 14 Pastrana, L., González, M., Torrado, A. and Murado, M. 1995. A fed-batch culture model for improved production of gibberellic acid from a waste medium. *Biotechnology Letters*, 17:263-268.
- 15 Luyben, W. 1990. *Process Modelling, Simulation and Control for Chemical Engineers*. McGraw-Hill Publishing Company, 281-288.

NOMENCLATURE

C_{ps}	heat capacity, wet solids	[J/kg \times $^{\circ}$ C]
C_{pH_2O}	heat capacity, liquid water	[J/kg \times $^{\circ}$ C]
CO_2	concentration, carbon dioxide	[kg/ kg d.s.]
e	error vector	
F_1	mass flowrate, inlet dry air	[kg/ h]
F_2	mass flowrate, inlet fresh water	[kg/ h]
F_3	mass flowrate, outlet dry air	[kg/ h]
G	concentration, glucose	[kg/ kg d.s.]
hc	control horizon	
hp	prediction horizon	
H_1	enthalpy, inlet air	[J/ kg d.a.]
H_3	enthalpy, outlet air	[J/ kg d.a.]
R_{H1}	relative humidity, inlet air	[%]
J	cost function, DMC	
k_G	weight loss factor	
K_C	proportional gain	
K_D	death factor	[h $^{-1}$]
K_N	Contois constant	[kg/ kg d.s.]
m_G	maintenance coefficient, nitrogen	[kg/ kg d.s. h]
m_Q	maintenance coefficient, metabolic heat	[J/ kg d.s. h]
m_{O_2}	maintenance coefficient, oxygen	[kg / kg d.s. h]
m_{CO_2}	maintenance coefficient, carbon dioxide	[kg CO $_2$ / kg d.s. h]
M_S	dry solid mass	[kg d.s.]
N	concentration, nitrogen	[kg/ kg d.s.]
O_2	concentration, oxygen	[kg/ kg d.s.]
q	metabolic heat, rate	[J/h]
q_0, q_1	constants, PI pulse transfer function	
Q	metabolic heat, generation	[J/ kg d.s.]
t	time	[h]
T	sample time	[h]
T_S	temperature, solid bed	[$^{\circ}$ C]
T_1	temperature, inlet air	[$^{\circ}$ C]
w	evaporation rate	[kg/ h]
X	bed water content	[kg/ kg d.s.]
x	concentration, dry biomass	[kg/ kg d.s.]
y	controlled variable	
y	set point	
$Y_{x/N}$	yield coefficient, biomass-nitrogen	[kg/ kg]
$Y_{x/G}$	yield coefficient, biomass-glucose	[kg/ kg]

Y_{x/O_2}	:	yield coefficient, biomass-oxygen	[kg/ kg]
$Y_{x/Q}$:	yield coefficient, biomass-metabolic heat	[kg/ J]
Y_{x/CO_2}	:	yield coefficient, biomass-carbon dioxide	[kg/ kg]
z	:	water generation	[kg/ h]
z^{-1}	:	shift operator	

GREEK SYMBOLS

μ	:	specific growth rate	[h ⁻¹]
μ_M	:	maximum specific growth rate	[h ⁻¹]
l	:	outputs weight matrix, DMC	
t	:	inputs weight matrix, DMC	
t_I	:	PI integral time	
Dm	:	input increments, DMC	

AN ASEPTIC PILOT BIOREACTOR FOR SOLID SUBSTRATE CULTIVATION PROCESSES

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Abstract

An SSC general purpose aseptic pilot reactor has been developed and evaluated for the production of gibberellic acid (GA₃), a secondary metabolite. This article describes the reactor, its instrumentation and control system, and its main operation steps. It is shown that the reactor can be used to obtain high yields of gibberellic acid in a reproducible way.

Keywords: gibberellic acid, control, monitoring.

Introduction

Solid Substrate Cultivation processes (SSC) are characterized by the growth of microorganisms within a porous support without free water. This condition favors the development of filamentous fungi, given their unique capacity to colonize the interparticular spaces of solid matrices. In addition, the risk of bacterial contamination is reduced due to the low water activity (lower than 0.98) of the solid medium. Some interesting advantages of SSC processes compared with submerged cultivation are: higher productivity, lower operation costs and higher products concentration [1].

Most of the research and development work on SSC has been carried out at laboratory scale, and only few processes have been scaled up to industrial level. Then, a general purpose SSC pilot reactor can simplify the scaling up of laboratory studies. This reactor must operate aseptically to deal with different kind of processes. Currently, industrial, semi-industrial or pilot reactors, reported so far, do not operate aseptically [2, 3]. However, aseptic operation has been obtained at laboratory scale, on a rotary reactor of 2 kg capacity [4]. In this work, it is described a new pilot SSC bioreactor of 50 kg nominal capacity, which has been operated successfully for the production of gibberellic acid by the filamentous fungus *Gibberella fujikuroi* [5, 6].

REACTOR DESCRIPTION

General features

The structure of the stainless steel reactor is shown in Fig. 1, where the following parts can be identified:

a) A raisable lid that allows a hermetic close through a rubber seal and a handle as in an autoclave. It has perforations for air sampling and nutrients feeding, and observation windows. The agitation system (motor, double arm and blades) and the feeding system are mounted on the lid.

b) The principal body has a double jacket system for sterilization.

c) An air chamber, located in the lower part of the reactor, is used for inlet air homogenization. Here, air temperature and relative humidity are measured.

d) A rotating basket, of 1.15 m of diameter and 0.28 m height, which has a nominal capacity of 50 kg of wet solid. The basket base contains perforations of 2 mm of diameter with a separation of 3.5 mm.

Air Conditioning

As shown in Fig. 2, the air is forced into the reactor by a fan (a) with a prefilter (b). The speed of the air can be regulated manually between 0.25 and 7 m/s through a purge system (c). An absolute filter (d) insures the purity of the air. It is able to retain particles larger than 0.3 μm with a minimal efficiency of 99.99%. A heating system (e), made of electric resistances with 6 kW maximum capacity, can heat the air for reactor sterilization and process needs. In addition, the air can be cooled up to 0 °C with a fins cooler (f) and a refrigeration system (g). The relative humidity is controlled by vapor addition through a solenoid valve (h) connected to a steam boiler (i) with a working pressure of 5-10 psi.

Agitation

The double arm bed agitation system is shown in detail in Fig. 3. One arm contains blades (a) that operate like a plough. The other arm has curved paddles (b) that scrape the basket base. Both arms can be manually regulated with cranks (c and d), according to process needs. The basket movement is provided by a reducing motor of 1.5 kW. The motor shaft is connected to the basket shaft (e). An inverter driver, CDS 150, controls the rotation speed, achieving a minimum of 5 rpm with a torque of 5500 Nm. An aseptic water seal (f) insures that all the inlet air pass through the solid bed. The basket is supported on roller bearings (g) to get a uniform and soft movement.

Water and nutrient feeding

A set of 3 sprinklers (not seen in the figures) are mounted on the lid. They are used to add water and dissolved nutrients periodically, according to process needs. The solution is forced into the reactor with a peristaltic pump with a maximum flow of 10 l/min. To distribute the solution all over the solid bed, the agitation system is turned on each time the solution is fed.

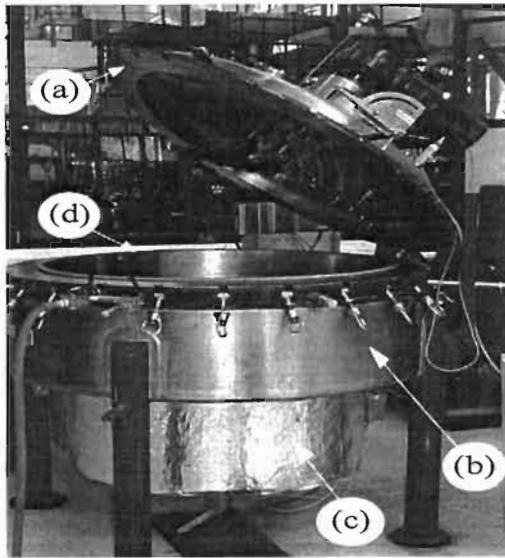


Figure 1: General view of the pilot reactor for aseptic SSC processes. (a) Lid; (b) Main body; (c) Air chamber; (d) Basket

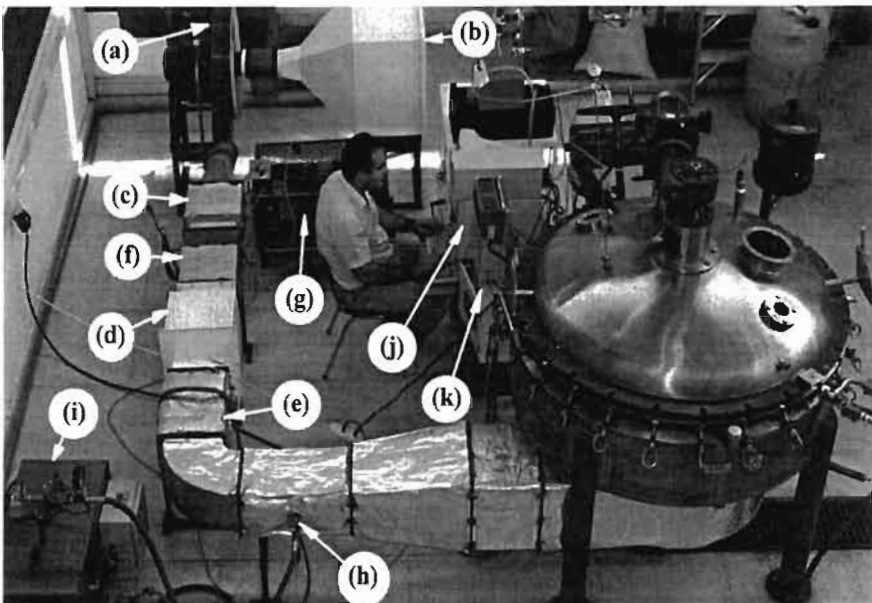


Figure 2: Air conditioning system. (a) Fan; (b) Prefilter; (c) Air purge; (d) Absolute filter; (e) Electric heaters; (f) Fins cooler; (g) Cooling system; (h) Solenoid valve; (i) Boiler; (j) PC ; (k) Instrumentation cabinet

Table 1: Main features of the SSC pilot reactor

CHARACTERISTICS	VALUE	UNITS
External height	2150	mm
External diameter	1440	mm
Basket height	279	mm
Basket diameter	1150	mm
Basket weight	93	kg
Maximum load	25	kg (d.m.)
Useful volume	0.65	m ³
Normal rotation speed	5	rpm
Maximum torque	5500	Nm
Agitation power	2	HP
Air flow	0.25-7.1	m/s
Air Filter Efficiency (0.3mm)	99.99	%
Flow area through basket base	51.3	%
Air chamber height	550	mm
Air chamber diameter	1000	mm
Maximum heating power (operation)	3	Kw
Maximum cooling power (operation)	6.6	Kw

Process Monitoring

A Programmable Logic Controller (PLC), Hitachi series EM-II, is used for on-line data acquisition. The PLC is provided with modules for input/output analog and digital signals and an interface with a PC IBM compatible (j in Fig. 2). The process is observed through the following measuring devices (as shown in the Process and Instrumentation Diagram, P&ID, in Fig. 4.):

a) Temperature (TT in Fig. 4) is measured in three different points in the solid bed: close to the rotation shaft, in the middle and close to the basket wall. In addition, temperatures of inlet, outlet and ambient air are also measured. K type thermocouples are used as sensing devices and a 45C-THM PLC module is used for signal conditioning.

b) A Vaissala sensor/transmitter provides continuous monitoring of inlet air relative humidity (HT in Fig. 4) in the range 0-100%. The output signal (4-20 mA) from the Vaissala device is

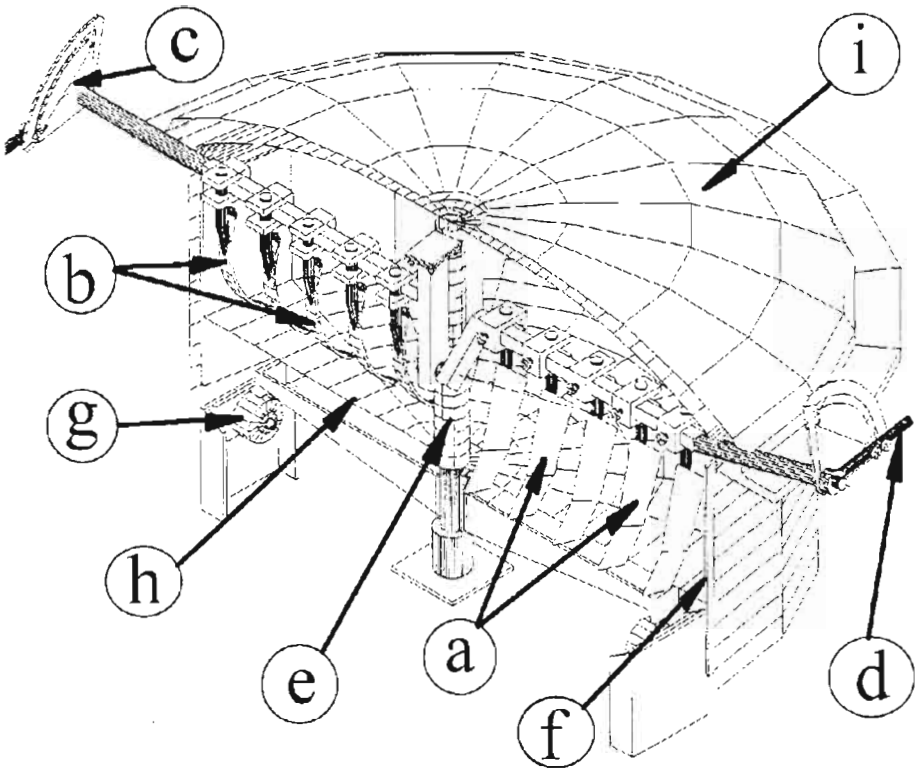


Figure 3: Agitation system. (a) Blades; (b) Curved paddles; (c) Crank for curved paddles arm regulation; (d) Crank for blades arm regulation; (e) Basket shaft; (f) Water seal; (g) Roller bearing; (h) Basket base; (i) Lid.

connected to an indicator in the instrumentation cabinet (k in Fig. 2) and to the analog module of the PLC (ACTANA-S2).

c) Outlet air CO₂ concentration (CO2T in Fig. 4) is measured by infrared detection with a Horiba PIR 2000 device, which gives a 0-1 V output signal that is connected to the analog module. This measurement technique requires a constant air flowrate, which is controlled manually.

d) The air pressure drop (DPT in Fig. 4) is measured with a differential pressure transmitter Modus Instr. T30, which provides an output signal of 4-20 mA connected to the analog module.

Process Control

The main objectives of the control system are the regulation of temperature and water activity of the solid bed. The bed temperature is regulated by manipulating the set point of the inlet air relative humidity (RH); a PID discrete algorithm is used to compute the control action. To control the inlet air RH, an ON/OFF regulation algorithm drives a solenoid valve that introduces vapor into the air stream. Inlet air temperature is kept constant through the cultivation process, manipulating simultaneously the cooling and heating system. The water activity is controlled by periodic addition of sterile fresh water. Water demand is computed from a mass balance based on off-line bed humidity measurements.

A special purpose control software was developed. It allows, through a graphic interface, direct intervention in the process (similar to a command console) and visualization of trend curves of process variables. Using this interface, the reactor can be operated in manual or automatic form. The control algorithms are executed in the PC, according to options and parameters selected from the keyboard. Different ON/OFF and PID algorithms have been implemented. Details of the design and performance of the control loops have been published elsewhere [5, 6].

Reactor Operation

During the reactor operation for gibberellin production, the following main steps can be distinguished: a) sterilization, b) substrate loading, c) inoculation, d) cultivation, e) substrate unloading, and f) cleaning. All these steps, except cultivation, are carried out manually.

a) The sterilization process, performed with steam and forced hot air, lasts two hours. The minimum temperature inside the reactor during this process is 100 °C. Then, the reactor is cooled with forced chill air until the reactor reaches 28 °C; this lasts another two hours.

b) The humidified wheat bran, previously sterilized in an autoclave, is loaded manually in few minutes into the reactor. During substrate loading, the agitation system is turned on, to achieve an even substrate distribution in the basket. To keep a positive pressure, minimizing the risk of contamination, the air fan is also turned on.

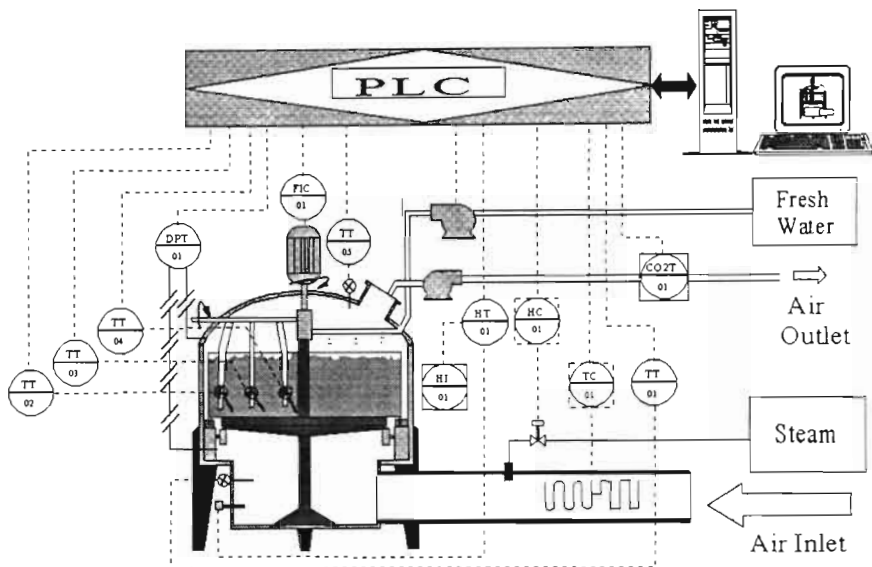


Figure 4: Process and Instrumentation Diagram, P&ID. (a) TT: temperature measurement; (b) DPT: differential pressure measurement; (c) HT: relative humidity measurement; (d) CO2T: Carbon dioxide air concentration measurement; (e) TC: temperature controller; (f) HC: humidity controller; (h) HI: humidity indicator; (i) FIC: Rotation speed controller.

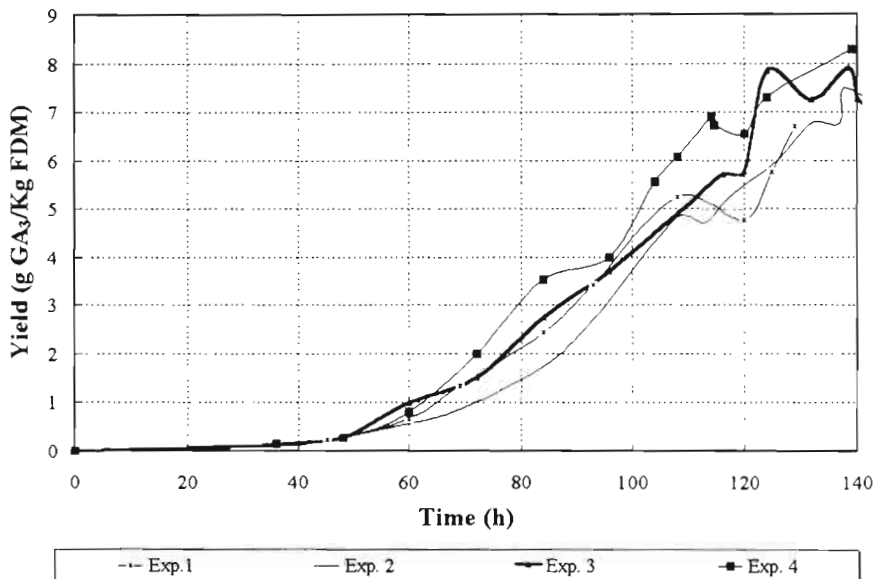


Figure 5: Kinetics of gibberellic acid production in the pilot SSC bioreactor, from several cultivations. (FDM = Final Dry Matter; Exp.= Experiment).

- c) With the agitation system on, the inoculation is carried out through the sprinklers. For this purpose, vegetative mycelium of a non-sporulating strain of Gibberella fujikuroi propagated in submerged culture was added at a level of 0.2% (w/w). Then, the solution of nutrients is fed. Finally, the water content of the solid bed is measured to adjust it to the optimum initial content.
- d) Cultivation time is 5 to 6 days. During this period, the reactor is operated semi-automatically. Periods of agitation are of approximately 2 hours, since more frequent agitation seriously injures the mycelium. This situation favors the appearance of agglomerations of bran and mycelium, which are difficult to break by agitation without damaging too much the rest of the mycelium.
- e) At the end of cultivation, the substrate is cooled to 15°C forcing cool and dry air through the fermented bed. Then, the substrate is manually harvested from the reactor and stored at 4°C (or -20°C) before GA₃ extraction.
- f) Reactor cleaning is carried out with hot water containing a quaternary ammonium compound (cationic detergent) for equipment disinfection.

The above procedure was used to cultivate Gibberella fujikuroi on solid substrate for gibberellic acid (GA₃) production. The process is quite reproducible as shown in Fig. 5, which illustrates the evolution of GA₃ concentration in different cultivation runs. Yields obtained at pilot scale do not differ much from those obtained at laboratory scale (data not shown).

Conclusions

Although the reactor is not completely automatic, a semi-automatic control system for the cultivation step has been developed. This step requires just one operator with a low level of dedication, due to the alarm's system and the process visualization in the graphic interface.

The reactor cannot operate with frequent agitation since the mycelium can be damaged. In addition, solid agglomeration reduces the overall yield. A new 200 kg pilot reactor is now operating with an improved agitation system, which will minimize these difficulties.

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References

- 1 Doelle, H.W.; Mitchell, D.A. and Rolz, C.E., 1992. Solid Substrate Cultivation. Elsevier Applied Science, London, 466 pp.
- 2 Deschamps F.; Meyer F. and Prebois J.P., 1980. Mise au Point d'une Unité Pilote de Fermentation Aerobie en Milieux Solides. Colloque Soc. Fr. Microbiol., Toulouse, pp. 135- 147.

3 Durand A. and Chereau D., 1988. A New Pilot Reactor for Solid-State Fermentation: Application to the Protein Enrichment of Sugar Beet Pulp. *Biotechnol. Bioeng.* 31: 476-486.

4 Ryoo D. Murphy V.G., Karim M.N. and Tengerdy R.P., 1991. Evaporative Cooling and Moisture Control in a Rocking Reactor for Solid Substrate Fermentation. *Biotechnol. Techniques*, 5: 19-24.

5 Fernández M.; Ananías J., Solar, I.; Pérez. R.; Chiang, L. and Agosin, E., 1997. Advances in the Development of a Control System for a Solid Substrate Pilot Bioreactor. In: *Advances in Solid Substrate Fermentation*, Roussos, Lonsane, Raimbault & Viniestra-González (Editors), Kluwer Academic Publish., Dordrecht, pp. 155-168.

6 Fernández. M., J.R. Pérez-Correa, I. Solar, E. Agosin, 1996. Automation of a Solid Substrate Cultivation Pilot Reactor. *Bioprocess Engineering*, 16:1-4

ANNEXES

List of papers published by the group of invited speakers on SSF

- Alazard, D. and Raimbault, M. 1981. Comparative study of amylolytic enzymes production by *Aspergillus niger* in liquid and solid state cultivation. *Eur. J. Appl. Microbiol. Biotechnol.* 12: 113-117.
- Alazard, D. and Baldensperger, J. 1982. Amylolytic enzymes from *Aspergillus hennebergii* (*Aspergillus niger* group): Purification and characterization of amylases from solid and liquid cultures. *Carbohydr. Res.* 107: 231-241.
- Antier, P., Minjares, A., Roussos, S., Raimbault, M. and Viniegra-Gonzalez G. 1993. Pectinases hyperproducing mutants of *Aspergillus niger* C28B25 for solid state fermentation of coffee pulp. *Enzyme Microbiol. Technol.* 15: 254-260.
- Antier, P., Minjares, A., Roussos, S. and Viniegra-Gonzalez G. 1993. New approach for selecting pectinase producing mutants of *Aspergillus niger* well adapted to solid state fermentation. *Adv. Biotechnol.* 11: 429-440.
- Aquiahuatl, M.A. 1992. Detoxificación de la pulpa de café: morfología, fisiología y bioquímica de hongos filamentosos que degradan la cafeína. *Tesis de Maestría en Biología.* UNAM, Mexico, 72 p.
- Aquiahuatl, M.A., Raimbault, M., Roussos, S. and Trejo, M.R. 1989. Coffee pulp detoxification by solid state fermentation: isolation, identification and physiological studies of filamentous fungi. In Raimbault, M. (Ed.), *Proceedings of the seminar Solid State Fermentation in Bioconversion of Agroindustrial Raw Materials*, Montpellier 24-28 July 1988. ORSTOM, France, pp. 13-26.
- Aufeuvre, A.M. and Raimbault, M. 1982. Etude au microscope électronique à balayage du développement d'*Aspergillus niger* Van Tieghem sur milieu solide. *CR Acad. Sc. Paris T 294*: 949-956.
- Augur, C. and Viniegra-Gonzalez, G. 1996. Comparatives studies of pectinases production by *Aspergillus niger* in solid state and submerged fermentations. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniegraz-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, p.347-353.
- Auria, R., Hernandez, S., Raimbault, M. and Revah, S. 1990. Ion exchange resin: a model support for solid state growth fermentation of *Aspergillus niger*. *Biotechnol. Techniques.* 4: 391-396.
- Auria, R., Palacios, J. and Revah, S. 1992. Determination of the interparticular effective diffusion coefficient for CO₂ and O₂ in solid state fermentation. *Biotechnol. Bioeng.* 39: 898-902.
- Auria, R., Morales, M., Villegas, E., Revah, S. 1993. Influence of mold growth on the pressure drop in aerated solid state fermentors. *Biotechnol. Bioeng.* 41: 1007-1013.
- Auria, R. and Revah, S. 1994. Pressure drop as a method to evaluate mold growth in solid state fermentors. In: Galido E. and Ramirez, O.T. (Eds.), *Advances in Bioprocess Engineering* Kluwer Acad. Publ., Dordrecht, p. 289-294.
- Auria, R., Ortiz, I., Villegas, E. and Revah, S. 1995. Influence of growth and high mold concentration on the pressure drop in solid state fermentors. *Process Biochem.* 30: 751-756.

- Ayala, L. A. C.; Soccol, C. R.; Santos, H. R.; Todeschini, M. L. Produção de esporos de fungo entomopatogênico (*Beauveria bassiana*) por fermentação no estado sólido utilizando como substrato refugo de batata. *Anais do XI SINAIFERM*, Sociedade Brasileira de Microbiologia, Vol 2, pp. 574-579, 1996.
- Ayala, L. A. C. ; Soccol, C.R. ; Santos, H. R. ; Viscarra, R.V. Biotechnological improvement of potato (*Solanum tuberosum*) refuses to produce enthomopathogenic fungus *Beauveria bassiana* by solid state fermentation. In : G.M. Zanin & F.F. Moraes (Eds). *Anais IV Seminário de Hidrólise Enzimática de Biomassas*. Editora Sthampa, 1994, p 182-189.
- Baldensperger, J., Le Mer J., Hannibal, L. and Quinto, P.J. 1985. Solid state fermentation of banana wastes. *Biotechnol. Lett.* 7: 743-748.
- Barbosa, M.C.S. ; Soccol, C.R. ; Todeschini, M.L. ; Tonial, T. ; Flores, V. Cultivation of *Pleurotus sajor-caju* in cassava waste. In : G.M. Zanin & F.F. Moraes (Eds). *Anais IV Seminário de Hidrólise Enzimática de Biomassas*. Editora Sthampa, 1994, p 179-181.
- Barbosa, M.C.S ; Soccol, C.R ; Krieger, N ; Chiarello, M. D. *Pleurotus sajor-caju* production from cassava waste by solid state fermentation. *Fifth Brazilian Symposium on The Chemistry of Lignins and Other Wood Components*. Curitiba- Pr- Brazil, 31/8 to 05/09, 1997, p 557-569.
- Barbosa, M. C. S.; Soccol, C. R.; Marin, B.; Todeschini, M. L.; Tonial, T.; Flores, V. Prospecto production of *Pleurotus sajor-caju* from cassava fibrous waste. In: Roussos, S.Lonsane, B. K.; Raimbault, M.; Viniestra-Gonzalez, G. (Eds) *Advances in Solid State Fermentation - Edible mushrooms/ fungi*. Dordrecht, Kluwer Acad. Pub., 41, 1997 p 515-528.
- Barrios-Gonzalez, J. and Anaya, S. 1987. Desarrollo de un sistema para el estudio de la germinación de esporas de *Aspergillus niger*. *Rev. Mex. Mic.* 3: 9-18.
- Barrios-Gonzalez, J., Tomasini, A., Viniestra-Gonzalez, G. and Lopez, L. 1988. Penicillin production by solid state fermentation. *Biotechnol. Lett.* 10: 793-798.
- Barrios-Gonzalez, J., Martinez, C., Aguilera, A. and Raimbault, M. 1989. Germination of concentrated suspensions of spores from *Aspergillus niger*. *Biotechnol. Lett.* 11: 551-554.
- Barrios-Gonzalez, J., Rodriguez, G.M. and Tomasini, A. 1990. Environmental and nutritional factors controlling aflatoxin production in cassava solid state fermentation. *J. Ferment. Bioeng.* 70: 329-333.
- Bensoussan, M., Tisserand, E., Kabbaj, W. and Roussos, S. 1995. Partial characterization of aroma produced by submerged culture of morel mushroom. *Cryptog. Mycol.* 16: 65-75.
- Beux, M.R. ; Soccol, C.R. ; Raimbault, M. Use cassava bagasse on cultivation edible fungus *Lentinus edodes* by solid state fermentation. In : G.M. Zanin & F.F. Moraes (Eds). *Anais IV Seminário de Hidrólise Enzimática de Biomassas*. Editora Sthampa. 1994, p 190- 198.
- Beux, M. R. & Soccol, C. R. - Cultivo do fungo comestível *Lentinula edodes* em resíduos agroindustriais do Paraná através do uso de fermentação no estado sólido. *Boletim do CEPPA*, vol. 14 (1) p. 11-21, 1996.
- Beux, M. R.; Soccol, C. R.; Marin, B.; Tonial, T.; Roussos, S. Cultivation of *Lentinus edodes* on mixture of cassava and sugar cane bagasse. In: Roussos, S.Lonsane, B. K.; Raimbault, M.; Viniestra-Gonzalez, G. (Eds) *Advances in Solid State Fermentation - Edible mushrooms/ fungi*. Dordrecht, Kluwer Acad. Pub., c. 41, 1997. p501-514.

- Boccas, F., Roussos, S., Gutierrez, M., Serrano, L., Viniegra, G. 1994. Fungal strain selection for pectinases production from coffee pulp in solid state fermentation system. *J. Food Science Technol.* 31: 22-26.
- Chiarello, M.D., Soccol, C.R., Stertz, S.C., Furlaneto, L., Krieger, N. 1997. Rhizopus glucoamylase able to attack cassava granular starch by solid state fermentation. *Arq. Biol. Tecnol.* 40 (3) : 310-322.
- Carta, F.S ; Soccol, C.R ; Prado A.C ; Ramos L.P ; Machado, L .. Production of fumaric acid by fermentation of cassava bagasse. *Fifth Brazilian Symposium on The Chemistry of Lignins and Other Wood Components*. Curitiba -Pr - Brasil, 31/8 to 05/09, 1997, p 583-591.
- Christen, P. and Raimbault, M. 1991. Optimization of culture medium for aroma production by *Ceratocystis fibriata*. *Biotechnol. Lett.* 13: 521-526.
- Christen, P., Auria, R., Vega, C., Villegas, E., Revah, S. 1993. Growth of *Candida utilis* in solid state fermentation. *Biochem. Adv.* 11: 549-557.
- Christen, P., Auria, R., Marcos, R., Villegas, E. and Revah, S. 1994. Growth of *Candida utilis* on amberlite with glucose and ethanol as sole carbon sources. In: Galido E. and Ramirez. O.T. (Eds.), *Advances in Bioprocess Engineering*. Kluwer Acad. Publ., Dordrecht, p. 87-93.
- Christen, P., Villegas, E. and Revah, S. 1994. Growth and aroma production by *Ceratocystis fibriata* in various fermentation media. *Biotechnol. Lett.* 16: 1183-1188.
- Christen, P., Angeles, N., Corzo, G., Farres A. and Revah, S. 1995. Microbial lipase production on a polymeric resin. *Biotechnol. Techniques.* 9: 597-600.
- Christen, P. 1995. Producción de aromas en fermentación sólida. *Tópicos de Investigación y Posgrado.* 4: 102-109.
- Christen, P., Meza, J.C. and Revah, S. 1997. Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: Influence of the substrate type and the presence of precursors. *Mycological Research.* 101: 911-919.
- Cruz, C.T., Oriol, E., Schettino, B., Gutierrez-Rojas, M. and Viniegra-Gonzalez, G. 1987. Determinación del grado de gelatinización en la harina de yuca para fermentación sólida. *Tecnol. Alim. (Mexico)* 22: 4-7.
- Denis, S. 1996. Dégradation de la caféine par *Aspergillus* sp. et *Penicillium* sp. : Etude physiologique et biochimique. *Thèse de Doctorat*, Université Montpellier II, France, 200 p.
- Deschamps, F., Raimbault, M. and Senez, J.C. 1982. Solid state fermentation in the development of agro-food by-products. *Industry & Environ.* 5 (2): 27-30.
- Deschamps, F., Giuliano, C., Asther, M., Huet, M.C. and Roussos, S. 1985. Cellulase production by *Trichoderma harzianum* in static and mixed solid state fermentation reactors under non-aseptic conditions. *Biotechnol. Bioeng.*, 27: 1385-1388.
- Favela, E., Huerta, S., Roussos, S., Olivares, G., Nava, G., Viniegra, G. and Gutierrez-Rojas, M. 1989. Producción de enzimas a partir de la pulpa de café y su aplicación a la industria cafetalera. In Roussos, S., Licon, F.R. and Gutierrez, R.M. (Eds.), *I Seminario Internacional de Biotecnología en la Industria Cafetalera*. Memorias, Xalapa. Veracruz, p. 145-151.
- Gaimpe-Perraud, I., Hannibal, L., Trejo, H.M., Raimbault, M. and Roussos, S. 1991. Resultados preliminares sobre el ensilaje de la pulpa de café. Poster. II SIBAC, Manizales, Colombia, 4-7 Nov. 1991, CENICAFE.
- Gaimpe-Perraud, I., Roussos, S. and Martinez-Carrera, D. 1993. Endogenous microflora of fresh coffee pulp. *Micol. Neotrop. Apl.* 6: 95-103.
- Gonzalez, P.C., Delgado, G., Antigua, M., Rodriguez, J., Larralde, P., Viniegra, G., Pozo, L., and Perez, M.C. 1994. Some aspects of *Gibberella fujikuroi* culture concerning gibberellic acid

- production. In: Galido, E. and Ramirez, O.T. (Eds.), *Advances in Bioprocess Engineering*, Kluwer Acad. Publ., Dordrecht, p. 425-430.
- Gonzalez-Blanco, P., Saucedo-Castañeda, G. and Viniestra-Gonzalez, G. 1990. Protein enrichment of sugar cane by-products using solid-state cultures of *Aspergillus terreus*. *J. Ferment. Bioeng.* 70: 351-354.
 - Gutierrez-Rojas, M., Huerta-Hochoa, S., Lopez-Ulibari, R., Saucedo-Castañeda, G., Favela-Torres, E. and Viniestra-Gonzalez, G. 1989. Solid state fermentation: scaling up prototypes and strategies. In Raimbault, M. (Ed.), *Proceedings of the seminar Solid State Fermentation in Bioconversion of Agroindustrial Raw Materials*, Montpellier 24-28 July 1988, ORSTOM, France, pp. 61-65.
 - Gutierrez-Rojas, M., Auria, R., Benet, J.C. and Revah, S. 1995. A mathematical model for solid state fermentation of mycelial fungi on inert support. *Biochem. Eng. J.* (in press).
 - Gutierrez-Rojas, M., Cordova, J., Auria, R., Revah, S. and Favela-Torres, E. 1995. Citric acid and polyols production by *Aspergillus niger* at high glucose concentration in solid state fermentation on inert support. *Biotechnol. Lett.* 17: 219-224.
 - Huerta-Ochoa, S. 1984. Efecto de la transferencia de masa y la acumulación del calor metabólico en la fermentación de cultivos sólidos. *Tesis de Maestría en Ciencias*, Ingeniería Química, UAM-Iztapalapa, Mexico, p. 147.
 - Kabbaj, W., Bensoussan, M. and Roussos, S. 1996. Factors affecting physiology of mycelial growth and aroma production in solid state fermentation of mushrooms. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniestra-Gonzalez, G.(Eds.), *Advances in Solid State fermentation.*, Kluwer Acad. Publ. Dordrecht, chap.
 - Kolichski, M.B., Soccol, C.R. ; Medeiros, E. ; Raimbault, M. Citric acid production by solid state fermentation with different cellulosic supports. In : G.M. Zanin & F.F. Moraes (Eds). *Anais IV Seminário de Hidrólise Enzimática de Biomassas*. Editora Sthampa, 1994, p 213-220.
 - Kolichski, M. B.; Soccol, C. R.; Marin, B.; Medeiros, E.; Raimbault, M. Citric acid production on three cellulosic supports in solid state fermentation. In: Roussos, S. Lonsane, B. K.; Raimbault, M.; Viniestra-Gonzalez, G. (Eds) *Advances in Solid State Fermentation - Secondary metabolites, aroma, pigment and biopesticides*. Dordrecht, Kluwer Acad. Pub., c. 36. 1997. p449-462.
 - Kolichski, M. B. & Soccol, C. R. Produção de ácido cítrico por fermentação estado sólido (FES) utilizando como substrato bagaço de mandioca. *Anais do XI SINAFERM*, Sociedade Brasileira de Microbiologia, Vol 1. pp 369-375, 1996.
 - Kolichski, M. B. & Soccol, C. R. Otimização do meio e condições de cultura na produção de ácido cítrico por fermentação submersa. *Bol. CEPPA*, 13(2) : 111-118, 1995.
 - Lambraki, M., Marakis, S. and Roussos, S. 1994. Effect of temperature and aeration flow on carob tannin degradation by *Aspergillus carbonarius* in solid state fermentation system. *Micol. Neotrop.* Apl. 7: 23-34.
 - Lambraki, M., Marakis, S., Hannibal, L. and Roussos, S. 1996. Effects of sugar and mineral salts on the growth of *Aspergillus carbonarius* in carob pod solid state fermentation. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniestra-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, p.245-255.
 - Lepilleur, C., De Araujo, A. A., Delcourt, S., Colavitti, P. and Roussos S. 1996. Laboratory scale bioreactors for study fungal physiology and metabolism in solid state fermentation

- system. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniegraz-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, p.93-111.
- Lonsane, B.K., Saucedo-Castañeda, G., Raimbault, M., Roussos, S., Viniegra-Gonzalez, G., Gildyal, N.P., Ramakrishna, M. and Krishnaiah, M.M. 1992. Scale-up strategies for solid state fermentation systems. *Process Biochem.* 27: 259-273.
- Lozano, P., Pioch, D. and Roussos, S. 1995. Procédé de préparation d'un arôme, notamment d'un arôme de coco, par fermentation en milieu solide et application de ce procédé. *Brevet Français* N° 95. 01.713.
- Marin, B. and Raimbault, M. 1992. Quelques approches pour les pays tropicaux? *Biofutur.* 113: 58-65.
- Martinez-Cruz, P., Christen, P. and Farres, A. 1993. Medium optimization by a fractional factorial design for lipase production by *Rhizopus delemar*. *J. Ferment. Bioeng.* 2: 94-97.
- Morales, M., Perez, F., Auria, R. and Revah, S. 1994. Toluene removal from air stream by biofiltration. In: Galido, E. and Ramirez, O.T. (Eds.), *Advances in Bioprocess Engineering*. Kluwer Acad. Publ., Dordrecht, p. 405-411.
- Oriol, E. Croissance de *Aspergillus niger* sur milieu solide: Importance de l'eau et de l'activité de l'eau. *Thèse de doctorat*, Université Paul Sabatier, Toulouse, 115 p.
- Oriol, E., Contreras, C. and Raimbault, M. 1987. Use of microcalorimetry for monitoring the solid state culture of *Aspergillus niger*. *Biotechnol. Techniques*, 1: 79-84.
- Oriol, E., Schettino, B., Viniegra-Gonzalez, G. and Raimbault, M. 1988a. Solid- state culture of *Aspergillus niger* on support. *J. Ferment. Technol.* 66: 57-62.
- Oriol, E., Raimbault, M., Roussos, S. and Viniegra-Gonzales, G. 1988b. Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.*, 27: 498-503.
- Perraud-Gaime, I. 1995. Cultures mixtes en milieu solide de bactéries lactiques et de champignons filamenteux pour la conservation et la décaféination de la pulpe de café. *Thèse de Doctorat*, Université Montpellier II, Frane, 209 p.
- Prebois, J.P., Raimbault, M. and Roussos, S. 1985. Biofermenteur statique pour la culture de champignons filamenteux en milieu solide. *Brevet Français* N° 85.17.934.
- Raimbault, M. 1980. Fermentation en milieu solide. Croissance de champignons filamenteux sur substrat amylicé. *Thèse d'Etat*. Université Paul Sabatier, Toulouse, France, 291 p.
- Raimbault, M. 1989. Enzymes production by solid state fermentation. In Raimbault, M. (Ed.), Proceedings of the seminar Solid State Fermentation in Bioconversion of Agroindustrial Raw Materials, Montpellier 24-28 July 1988, ORSTOM, France, pp. 5-12.
- Raimbault, M. 1989. Solid state fermentation in bioconversion of agroindustrial raw materials. Proceedings *Seminar Solid State Fermentation in Bioconversion of Agroindustrial Raw Materials*, Montpellier 24-28 July 1988. ORSTOM-Montpellier, France, p. 143.
- Raimbault, M. and Germon, J.C. 1976. Procédé d'enrichissement en protéines de produits comestibles solides. *Brevet Français* N° 76.06.677.
- Raimbault, M. and Alazard, D. 1980. Culture method to study fungal growth in solid fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 9: 199-209.
- Raimbault, M.; Ramirez Toro, C.; Giraud, E.; Soccol, C.R.; Saucedo, G. Fermentation in cassava bioconversion. In: Dufour, D.; O'brien, G.M.; Best, R. (Eds) *Cassava Flour and Starch; Progress in Research and Development - Session 4 - Bioconversion and By-products*. CIAT Pub. N. 271, 1996. p187-196.

- Raimbault, M., Revah, S., Pina, F. and Villalobos P. 1985. Protein enrichment of cassava by solid state fermentation using molds isolated from traditional foods. *J. Ferment. Technol.* 63: 395-399.
- Raimbault, M. and Roussos, S. 1985. Procédé de production de spores de champignons filamenteux. *Brevet Français* N° 85.08555.
- Raimbault, M., Roussos, S., Oriol, E., Viniegra, G., Gutierrez, M., Barrios-Gonzalez, J. 1989. Procédé de culture de microorganismes sur milieu solide constitué d'un support solide, absorbant, compressible et non fermentable. *Brevet Français* N° 89. 06558.
- Raimbault, M.; Viniegra-Gonzalez, G. 1991. Modern and traditional aspects of solid state fermentation. In: Chahal, D.S. (Ed.), *Food, feed and fuel from biomass*, Oxford & IBH Publis. Co. Pvt. Ltd. New Delhi, p. 153-163.
- Revah, S. 1995. Treatment of polluted gaz from cellophane and rayon plants. *Proceeding of Seven European Congress on Biotechnology*, Nice, 19-23 February, France.
- Rodriguez, J.A., Torres, A., Echevarria, J. and Saura, G. 1991. Energy balance in solid state fermentation processes. *Acta Biotechnol.* 11: 9-14.
- Roussos S. 1982. Mise au point d'une méthode pour la taxonomie numérique de certains genres de champignons imparfaits. I- Les caractères morphologiques et biochimiques. *Cah. ORSTOM, ser. Biol.*, 45: 25-34.
- Roussos, S. 1985. Croissance de *Trichoderma harzianum* par fermentation en milieu solide: Physiologie, sporulation et production de cellulases. *Thèse d'Etat*, Université de Provence, Marseille, 193 p.
- Roussos, S. and Raimbault, M. 1982. Hydrolyse de la cellulose par les moisissures. I- Screening des souches cellulolytiques. *Ann. Microbiol.*, 133: 455-464.
- Roussos, S. and Raimbault M. 1982. Hydrolyse de la cellulose par les moisissures. II- Production de cellulases de *Trichoderma harzianum* par fermentation en milieu liquide. *Ann. Microbiol.*, 133: 465-474.
- Roussos S., Garcia J-L., Raimbault M. 1982. Valorisation de la cossette de betterave par culture de *Trichoderma harzianum*. *Rev. Ind. Agric. Alim.*, 100: 449-452.
- Roussos, S., Aquiahuatl, M.A., Brizuela, M.A., Olmos, A., Rodriguez, W. and Viniegra, G. 1989. Production, conservation and viability of filamentous fungi inoculum for solid substrate fermentation. *Micol. Neotrop. Apl.* 2: 3-17.
- Roussos, S., Aquiahuatl, M.A., Cassaigne, J., Favela, E., Gutierrez, M., Hannibal, L., Huerta, S., Nava, G., Raimbault, M., Rodriguez, W., Salas, J.A., Sanchez, R., Trejo, M., Viniegra-Gonzalez, G. 1989. In Roussos, S., Licon, F.R. and Gutierrez, R.M. (Eds.), *I Seminario Internacional de Biotecnología en la Industria Cafetalera.*, Memorias I SIBAC, Xalapa, Veracruz, p. 121-143.
- Roussos, S., Olmos, A., Raimbault, M., Saucedo-Castañeda, G. and Lonsane, B.K. 1991. Strategies for large scale inoculum development for solid state fermentation system : Conidiospores of *Trichoderma harzianum*. *Biotechnol. Tech.* 5: 415-420
- Roussos, S., Raimbault M., Saucedo-Castaneda, G., Viniegra-Gonzalez, G. and Lonsane, B.K. 1991. Kinetics and ratios of carboxy-methyl cellulase and filter paper activities of the cellulolytic enzymes produced by *Trichoderma harzianum* on different substrates in solid state fermentation. *Micol. Neotrop. Apl.* 4 : 19-40.
- Roussos, S., Raimbault, M., Viniegra-Gonzalez, G., Saucedo-Castañeda, G. and Lonsane, B.K. 1991. Scale-up of cellulases production by *Trichoderma harzianum* on a mixture of

- sugar cane bagasse and wheat bran in solid state fermentation system. *Micol. Neotrop. Apl.* 4 : 83-98.
- Roussos, S., Raimbault, M., Saucedo-Castañeda, G. and Lonsane, B.K. 1992. Efficient leaching of cellulases produced by *Trichoderma harzianum* in solid state fermentation. *Biotechnol. Techniques*, 6: 429-432.
 - Roussos, S., Raimbault, M., Geoffroy, F., Saucedo-Castañeda, G. and Lonsane, B.K. 1992. Potential of ensiling for efficient management of spent residue from solid state fermentation system. *Biotechnol. Techniques*, 6: 87-90.
 - Roussos, S., Raimbault, M., Prebois, J-P. and Lonsane, B.K. 1993. Zymotis. A large scale solid state fermenter : Design and evaluation. *Applied Biochem. Biotechnol.* 42: 37-52.
 - Roussos, S., Gaime-Perraud, I., Denis, S., Marin, B., Marakis, S., Viniegra G., Trejo M., Aquiahuatl A., Gutierrez M. 1993. Biotechnological advances on coffee byproducts utilization. *IFCON*, Mysore (India), 7-12 september 1993.
 - Roussos, S., Hannibal, L., Durand, A., Diez, M., Saucedo-Castañeda, G., Montet, D. and Graille, J. 1994. Enrichissement en protéines du tourteau de coprah: sélection de champignons filamenteux en Fermentation en milieu solide. *Oléagineux*. 49: 235-247.
 - Roussos, S., Hannibal, L., Aquiahuatl, M.A., Trejo, M., Marakis, S. 1994. Caffeine degradation by *Penicillium verrucosum* in solid state fermentation of coffee pulp: critical effect of additional inorganic and organic nitrogen sources. *J. Food Science Technol.* 31: 316-319.
 - Roussos, S., Aquiahuatl, M.A., Trejo-Hernandez, M., Gaime-Perraud, I., Favela, E., Ramakrishna, M., Raimbault, M. and Viniegra-Gonzalez, G. 1995. Biotechnological management of coffee pulp - Isolation, screening, characterization, selection of caffeine degrading fungi and natural microflora present in coffee pulp and husk. *Appl. Microbiol. Biotechnol.* 42: 756-762.
 - Roussos, S., Bresson, E., Saucedo-Castañeda, G., Martinez, P., Guymberteau, J. and Olivier, J-M. 1995. Production of mycelial cells of *Pleurotus opuntiae* on natural support in solid state fermentation. In Roussos, S., Lonsane, B.K., Raimbault, M and Viniegra-Gonzalez, G. (Eds.), *Advances in Solid State fermentation*. Kluwer Acad. Publ., Dordrecht, p. chapter 40: 481-498.
 - Roussos, S. and Bossis, C. 1995. Dispositif et méthodes pour étudier l'effet d'huiles essentielles sur les moisissures. *Brevet Français* N° 95.02.204.
 - Sahry-Bagnon, V., Lozano, P., Pioch, D. and Roussos, S. 1996. Coconut-like aroma production by *Trichoderma harzianum* in solid state fermentation. In Roussos, S., Lonsane, B.K., Raimbault, M and Viniegra-Gonzalez, G. (Eds.), *Advances in Solid State fermentation*. Kluwer Acad. Publ., Dordrecht, chapter 31: 379-391.
 - Saucedo-Castañeda, G. 1991. Contrôle du métabolisme de *Schwanniomyces castelli* cultivé sur support solide. *Thèse de Doctorat*, Université Montpellier II, France. 212 p.
 - Saucedo-Castañeda, G., Raimbault, M. and Viniegra-Gonzalez, G. 1990. Energy of activation in cassava silages. *J. Sci. Food Agric.* 53: 559-562.
 - Saucedo-Castañeda, G., Gonzalez, P., Revah, S., Viniegra-Gonzalez, G. and Raimbault, M. 1990. Effect of Lactobacilli inoculation on cassava (*Manihot esculenta*) silage: fermentation pattern and kinetic analysis. *J. Sci. Food Agric.* 50: 467-477.
 - Saucedo-Castañeda, G., Gutierrez-Rojas, M., Bacquet, G., Raimbault, M. and Viniegra-Gonzalez, G. 1990. Heat transfert simulation in solid substrate fermentation. *Biotechnol. Bioeng.* 35: 802-808.

- Saucedo-Castañeda, G., Lonsane, B.K., Navarro, J.M., Roussos, S. and Raimbault, M. 1992. Potential of using a simple fermenter for biomass built up, starch hydrolysis and ethanol production: Solid state fermentation system involving *Schwanniomyces castellii* , *Appl. Biochem. Biotechnol.* 36: 47-61.
- Saucedo-Castañeda G., Lonsane, B.K., Navarro, J.M., Roussos, S. and Raimbault, M. 1992. Importance of medium pH in solid state fermentation system for growth of *Schwanniomyces castellii* . *Appl. Biochem. Biotechnol.* 15: 164-167.
- Saucedo-Castañeda, G., Lonsane, B.K., Krishnaiah, M.M., Navarro, J.M., Roussos, S. and Raimbault, M. 1992. Maintenance of heat and water balances as a scale-up criterion for the production of ethanol by *Schwanniomyces castellii* in a solid state fermentation system. *Process Biochem.* 27: 97-107.
- Saucedo-Castañeda, G., Lonsane, B.K., Navarro, J.M., Roussos, S. and Raimbault, M. 1992. Control of carbon dioxide in exhaust air as a method for equal biomass yields at different bed heights in column fermentor. *Appl. Microbiol. Biotechnol.* 37: 580-582.
- Saucedo-Castañeda, G., Trejo-Hernandez, M.R., Lonsane, B.K., Navarro, J.M., Roussos, S., Dufour, D. and Raimbault, M. 1993. On-line monitoring and control system for CO₂ and O₂ concentrations in aerobic and anaerobic solid state fermentations. *Process Biochem.* 29: 13-24.
- Senez, J.C. 1979. Solid fermentation of starchy substrates. *Food Nutr. Bull.* 1: 18-20.
- Senez, J.C. 1979. Pour une politique nationale en matière de protéines alimentaires. *Le progrès Scientifique.* 203: 5-32.
- Senez, J.C., Raimbault, M. and Deschamps, F. 1980. Protein enrichment of starchy substrates for animal feeds by solid state fermentation. *World Animal Rev.* 35: 36-40.
- Soccol, C.R. 1992. Physiologie et métabolisme de *Rhizopus* en culture solide et submergée en relation avec la dégradation d'amidon cru et la production d'acide L(+) lactique. *Thèse de Doctorat*, Université de Technologie de Compiègne, France, 218 p.
- Soccol, C.R., Rodríguez-Leon, J., Marin, B., Roussos, S., Raimbault, M. 1993. Growth kinetics of *Rhizopus arrhizus* in solid state fermentation of treated cassava *Biotechnol. Techniques.* 7: 563-568.
- Soccol, C. R.; Marin, B.; Roussos, S.; Raimbault, M. Scanning electron microscope of the development of *Rhizopus arrhizus* on raw cassava by solid state fermentation. *Micologia Neotropical Aplicada*, 6: 27-39, 1993.
- Soccol, C.R. ; Cabrero, M.A. ; Roussos, S ; Raimbault, M . Seleccion de cepas de *Rhizopus* em base a su capacidad de crecimiento en yuca. *Sociedade Mexicana de Biotecnologia e Bioengennieria*, V.3 ; N 1-2, p. 61-64,1993.
- Soccol, C., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation. *Appl. Microbiol. Biotechnol.* 41: 330-336.
- Soccol, C. R.; Raimbault, M.; Pinheiro, L. I. Effect of CO₂ concentration on the micelium growth of *Rhizopus* species. *Arq. Biol. Tecnol.*, 37(1) : 203-210. 1994.
- Soccol, C. R.; Stonoga, V. I.; Raimbault, M. Production of L(+) latic acid by *Rhizopus* species. *World J. Microbiol. Biotechnol.*, 10(4) : 433-435, 1994.
- Soccol, C. R.; Iloki, I.; Marin, B.; Roussos, S.; Raimbault, M. Comparative production of amylase and protein enrichment of raw and cooked cassava by *Rhizopus* strains in submerged and solid state fermentation. *J. Food Sci. Technol.*, 3: 320-323, 1994.
- Soccol, C. R. Contribuição ao estudo da fermentação no estado sólido em relação com a produção de ácido fumárico e biotransformação de residuo sólido de mandioca por

- Rhizopus* e Basidiomacromicetos do gênero *Pleurotus*. Tese de Professor Titular. UFPR, Curitiba, 1994. 228p.
- Soccol, C., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Potential of solid state fermentation for production of L(+) lactic acid by *Rhizopus oryzae*. *Appl. Microbiol. Biotechnol.* 41: 286-290.
 - Soccol, C.R., Iloki, I., Marin, B., Roussos, S. and Raimbault, M. 1994. Comparative production of amylases and protein enrichment of raw and cooked cassava by *Rhizopus* strains in submerged and solid state fermentation. *J. Food. Sci. Technol.* 31: 320-323.
 - Soccol, C. R.: Stertz, S. C.; Raimbault, M.; Pinheiro, L. I. Biotransformation of solid waste from cassava starch production by *Rhizopus* in solid state fermentation. Part I - Screening of strains. *Arq. Biol. Tecnol.*, 38(4) : 1303-1310, 1995.
 - Soccol, C. R.: Stertz, S. C.; Raimbault, M.; Pinheiro, L. I. Biotransformation of solid waste from cassava starch production by *Rhizopus* in solid state fermentation. Part II - Optimization of the culture conditions and growth kinetics. *Arq. Biol. Tecnol.*, 38(4) : 1311-1318, 1995.
 - Soccol, C. R.: Stertz, S. C.; Raimbault, M.; Pinheiro, L. I. Biotransformation of solid waste from cassava starch production by *Rhizopus* in solid state fermentation. Part III - Scale-up studies in different bioreactors. *Arq. Biol. Tecnol.*, 38(4) : 1319-1326, 1995.
 - Soccol, C. R. Aplicações da fermentação no estado sólido na valorização de resíduos agroindustriais. *França -Flash Agricultura*, 4(2): 3-4, 1995.
 - Soccol, C. R. Agroindustrial Applications of Solid State Fermentation Processes. In: E. Galindo (Ed.), *Fronteras en Biotecnología y Bioingeniería*, pp. 349-358. *Sociedade Mexicana de Biotecnología y Biengeneria*, 1996.
 - Soccol, C. R. Biotechnology products from cassava root by solid state fermentation. *Journal of Scientific and Industrial Research*, Vol. 55, may-june, 1996, pp 358-364.
 - Soccol, C.R.; Ayala, L.A.; Thomaz-Soccol, V.; Krieger, N.; Santos, H. R.. Spore production by entomopathogenic fungus *Beauveria bassiana* from declassified potato by solid state fermentation. *Revista de Microbiologia*. 28(1) : 34-42, 1997.
 - Sturtza, R.C.M.C. ; Soccol, C.R. ; Freitas, R.J.S. ; Kubicki, D. ; Medeiros, E. Biotechnological improvement of apple juice extraction pomace. In : G.M. Zanin & F.F. Moraes (Eds). *Anais IV Seminário de Hidrólise Enzimática de Biomassas*. Editora Sthampa, 1994, p 172-178.
 - Tapia, M.N., Herrera, R.S., Gutierrez, MR., Roussos, S., Viniegra, G.G. 1988. The effect of four fungal compounds as probiotics on in vitro dry matter disappearance of different feedstuffs. *J. Anim. Sci.* 1260 (abstr.)
 - Tapia, I.M., Herrera-Saldana, R., Viniegra-Gonzalez, G., Gutierrez-Rojas, M. and Roussos, S. 1989. Pulpa de café fermentada: su uso como aditivo en la fermentacion de rumiantes. In Roussos, S., Licon, F.R. and Gutierrez, R.M. (Eds.), *Seminario Internacional de Biotecnología en la Industria Cafetalera*. Memorias, Jalapa, Veracruz, Mexico, p. 153-175.
 - Torres, A. 1995. Dégradation et biodégradation de polymères d'acide lactique. *Thèse de Doctorat*, Université Montpellier I, 170 p.
 - Torres, A., Roussos, S., Vert, M., Li, S.M and Soccol, C.R. 1994. Bioconversion de l'amidon en acide L(+)-lactique, synthèse chimique du polylactique et biodegradation par les microorganismes. In Gueguen, J. (Ed.), *Valorisation non-Alimentaire des grandes productions Agricoles*, Nantes (France), 18-19 mai 1994, INRA. Les Colloques N° 71, Paris, pp. 249-257.

- Trejo-Hernandez, M. 1992. Physiologie de croissance de souches de *Claviceps*: Production d'alcaloïdes par fermentation en milieu solide. *Thèse de Doctorat*, Université de Provence, Aix-Marseille I, 164 p.
- Trejo-Hernandez, M.R., Oriol, E., Lopez-Canalez, A., Roussos, S., Viniegra-Gonzalez, G. and Raimbault, M. 1991. Producción de pectinasas de *Aspergillus niger* en fermentación sólida sobre soporte. *Micol. Neotr. Apl.* 4 : 49-62.
- Trejo-Hernandez, M.R., Raimbault, M., Roussos, S. and Lonsane, B.K. 1992. Potencial of solid state fermentation for production of ergot alkaloids. *Let. Appl. Microbiol.* 15: 156-159.
- Trejo-Hernandez, M.R., Lonsane, B.K., Raimbault, M. and Roussos, S. 1993. Spectra of ergot alkaloids produced by *Claviceps purpurea* 1029c in solid state fermentation system: Influence of the composition of liquid medium used for impregnating sugar cane pith bagasse. *Process Biochem.* 28: 23-27.
- Trejo-Hernandez, M.R., Lonsane, B.K., Raimbault, M. and Roussos, S. 1993. Solid substrate mediated changes in ergot alkaloid spectra in solid state fermentation system. *Chem. Mikrobiol. Technol. Lebensm.* 15: 643-646.
- Vidaud C., Roussos S., Raimbault M., Deschamps F. 1982. Effets de divers prétraitements sur l'accessibilité de la cellulose de la paille de blé aux cellulases de *Trichoderma harzianum*. *Cah. ORSTOM, ser. Biol.*, 45: 17-23.
- Villegas, E., Aubague, S., Alcantara, L., Auria, R. and Revah, S. 1993. Solid state fermentation: acid protease production in controlled CO₂ and O₂ environments. *Biotech. Adv.* 11: 389-397.
- Viniegra-Gonzalez, G. 1989. Perspectives and limitations of solid state fermentation in Mexico. In Raimbault, M. (Ed.), Proceedings of the seminar Solid State Fermentation in Bioconversion of Agroindustrial Raw Materials, Montpellier 24-28 July 1988. ORSTOM, France, pp. 67-72.
- Viniegra-Gonzalez G., Roussos S. and Raimbault M. 1991. Fermentations en milieu solide comme moyen de Valorisation des Produits Agricoles Tropicaux au Mexique. *ORSTOM Actualités*, 34: 23-25.
- Viniegra-Gonzalez, G. 1996. Solid state fermentation: Definition, characteristics, limitations and monitoring. In Roussos, S., Lonsane, B.K., Raimbault, M. and Viniegra-Gonzalez, G. (Eds.), *Advances in solid state fermentation*, Kluwer Acad. Publ., Dordrecht, p.5-22

Program FMS-97

Monday 6th of October:

9:00-09:30 Opening ceremony

I. General aspects of SSF (9:30-12:00)

Chairman: C. Augur

09:30-10:00 General and microbiological aspects of SSF (M. Raimbault)

10:00-10:30 Continuous enzymes and fungal metabolites production in SSF using a counter-current reactor (S. Roussos)

10:30-10:45 Coffee Break

10:45-11:30 Probiotics from Solid State Fermentation (G. Saucedo-Castañeda)

11:30-12:00 Fruity aromas production in SSF by *Ceratocystis fimbriata* (P. Christen)

12:00-14:00 Lunch

14:00-18:00 Practice in the laboratory

Tuesday 7th of October: II Biological Aspects of fungal growth in SSF

Chairman: S. Roussos

9:00-9:30 Fungal biomass estimation in batch solid substrate cultivation using asymptotic observation (R. Perez)

09:30-10:00 Mutagenesis and adapted strains to the growth in liquid or solid substrates (C. Augur)

10:00-10:30 Coffee Break

10:30-11:15 Fungal genetics, case of *Aspergillus niger* (C. Augur)

11:15-12:00 Growth and production of immobilized lipase from *Rhizopus delemar* cultivated in SSF on Amberlite (P. Christen)

12:00-14:00 Lunch

14:00-18:00 Practice in the laboratory

Wednesday 8th of October: III Kinetics aspects in SSF processes

Chairman: G. Saucedo-Castañeda

9:00-9:30 Relation between Biomass and Respiration: Theoretical aspects (M. Raimbault)

09:30-10:00 Kinetics of the solid state fermentation of raw cassava flour by *Rhizopus furmosa* 28422 (J. Rodriguez-Leon)

10:00-10:30 Coffee Break

10:30-11:15 Biotransformation of by-products and agro-industrial wastes by SSF:

Part I: Biotransformation of solid wastes (C. Soccol)

Part II: Production of industrial metabolites (C. Soccol)

12:00-14:00 Lunch

14:00-18:00 Practice in the laboratory

Thursday 9th of October: IV Applications in SSF

Chairman: R. Perez-Correa

9:00-9:30 Yeasts cultivation in SSF: control of metabolism of *Schwanniomyces castelii* during SSF on starch (G. Saucedo-Castañeda)

09:30-10:15 Mushroom mycelium cultivation and *Pleurotus* aroma production on sugarcane bagasse (S. Roussos and W. Kabbaj)

10:15-10:30 Coffee Break

10:30-11:15 Biotechnological management of coffee pulp (S. Roussos and I. Gaime)

11:15-12:00 Molecular techniques applied to fungal strain upgradation capacity related to SSF cultures, (C. Augur)

12:00-14:00 Lunch

14:00-18:00 Practice in the laboratory

Friday 10th of October: V Models and strategies for scale-up

Chairman: J. Rodriguez-Leon

9:00-9:30 Theory and strategy for scale-up in SSF. (G. Saucedo-Castañeda)

09:30-10:00 Multivariable model predictive control of a solid substrate pilot bioreactor: a simulation study (R. Perez-Correa)

10:00-10:30 Coffee Break

10:30-11:15 An aseptic pilot bioreactor for SSF processes (R. Perez-Correa)

11:15-12:00 Inoculum production for solid substrates

12:00-14:00 Lunch

14:00-18:00 Practice in the laboratory

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A group of trainees in the FMS97 course on Solid State Fermentation in Curitiba