Universidade do Minho
Escola de Engenharia

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Biotransformation of ricinoleic acid into γ-decalactone by *Yarrowia lipolytica:* Bioprocess optimization

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"Sit down before fact as a little child, be prepared to give up every preconceived notion, follow humbly wherever and to whatever abyss nature leads, or you shall learn nothing."

Thomas Huxley

LIST OF PUBLICATIONS

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, this thesis is based on the following original articles:

- Goméz-Díaz, D.; **Gomes, N.**; Teixeira, J.A.; Belo, I. (2009) Oxygen mass transfer to emulsions in a bubble column contactor. *Chemical Engineering Journal* 152:354-360 [CHAPTER 4].

- Goméz-Díaz, D.; **Gomes, N.**; Teixeira, J.A.; Belo, I. (2010) Gas–liquid interfacial area in the oxygen absorption to oil-in-water emulsions in an airlift reactor. *The Canadian Journal of Chemical Engineering* 88:561-564 [CHAPTER 4].

- **Gomes, N.**; Teixeira, J.A.; Belo, I. (2010) The use of methyl ricinoleate in lactone production by *Yarrowia lipolytica*: Aspects of bioprocess operation that influence the overall performance. *Biocatalysis and Biotransformation* 28(4):227-234 [CHAPTER 5].

- **Gomes, N.**; Waché, Y.; Teixeira, J. A.; Belo, I. (2011) "Oil-in-water emulsions characterization by laser granulometry and impact on γ-decalactone production by *Yarrowia lipolytica*". Biotechnol. Lett. DOI 10.1007/s10529-011-0593-9 [CHAPTER 5].

- **Gomes, N.**; Teixeira, J.A.; Belo, I. (2011) Empirical modeling as an experimental approach to optimize lactones production. *Catalysis and Science Technology* 1: 86-92 [CHAPTER 6].

Throughout the practical work that underlies this thesis, some analysis methods were optimized which, in turn, resulted in the following publications:

- **Gomes, N.**; Gonçalves, C.; García-Román, M.; Teixeira, J. A.; Belo, I. (2011) Optimization of a colorimetric assay for yeast lipase activity in complex systems. *Analytical Methods* 3: 1008-1013 [CHAPTER 3].

- Gonçalves, C.; Rodriguez-Jasso, R.; **Gomes, N.**; Teixeira, J. A.; Belo, I. (2010) Adaptation of dinitrosalicylic acid method to microtiter plates". *Analytical Methods* 2: 2046-2048 [CHAPTER 3].

ABSTRACT

The biotechnological production of γ-decalactone (a peach-like aroma compound) by biotransformation of ricinoleic acid carried out by microorganisms is an interesting process to produce the aroma with a "natural" label, which is valuable, considering the preference of consumers. Although there are many works described in the literature about this subject, several factors in the process remain to fully understand and, consequently, to optimize. One of these factors is the effect of oxygen in the overall process. Thus, this work initially aimed to study the oxygen mass transfer phenomenon from gas to the biotransformation medium, an oil-in-water emulsion stabilized by a non-ionic surfactant, Tween 80. The oil is simultaneously the substrate of the process and it works as an oxygen carrier, since the solubility of this compound is higher in the oil than in the aqueous phase. The influence of each operation parameter (aeration rate and presence and concentration of surfactant agent and organic phase) on the variables involved in the oxygen transfer (gas-liquid interfacial area, a; liquid-side mass transfer coefficient, k_L; and volumetric mass transfer coefficient, k_La) was analyzed in a bubble column and in an airlift reactor. Results demonstrated that in the bubble column the increase of aeration rates is positive for both gas-liquid interfacial area and mass transfer due to the increase of turbulence and gas hold-up. The surfactant concentration had a positive effect on the interfacial area since it reduced the gas bubbles size and it had a negative effect upon k_L because its molecules are located at the gas-liquid interface, obstructing the oxygen mass transfer. Regarding the oil concentration, it had a negative effect upon the interfacial area but it improved k_L, since it causes a new distribution of surfactant in the medium, decreasing its concentration in the gas-liquid interface. The overall result was a negative effect of the organic phase upon k_La. In the airlift reactor, it was observed that the increase of the aeration rates had a negative effect on k_L. This was attributed to differences in the liquid distribution inside the airlift reactor.

Since the main goal of this work was to optimize the production of γ-decalactone, two different ricinoleic acid sources (methyl ricinoleate, MR, and castor oil, CO) were tested, in different concentrations, as substrates of the process. Moreover, different cell inoculation strategies were attempted, differing among each other in the washing or not of the cells. The results revealed that the use of non-washed cells is more beneficial for the aroma production, independently of the substrate used; and a concentration of 30 g L^{-1} MR was the most adequate among the range tested, since it allowed the highest γ-decalactone productivity (14.9 mg L^{-1} h^{-1}). This substrate revealed also to be a lipase inducer.

The use of CO as substrate of the process allowed to achieve almost 2 g L^{-1} of aroma but the process was rather slow, resulting in low productivities. It was then hypothesized an insufficient oil hydrolysis and an enzymatic hydrolysis was attempted with different commercial enzymes and operating conditions

(temperature and pH). Lipozyme TL IM, pH 8 and 27 °C were selected as the most efficient lipase and operating conditions, respectively, to hydrolyze CO. The results obtained using CO previously hydrolyzed by the selected lipase were compared with the results obtained in experiments in which the enzymatic hydrolysis occurred during the biotransformation and in experiments without adding lipase, indicating that the process was faster when lipase was involved in any form, but the aroma concentrations were lower, resulting in similar productivities.

The droplets size of both oils was characterized by laser granulometry in emulsions with different oil concentrations. The impact of the presence of cells on droplets size was also analyzed as well as the relevance of washing inoculum cells. The granulometry of emulsions was related with γ-decalactone production and it was observed that, in the presence of non-washed cells, the smaller droplets disappeared, with both oils, which increased γ-decalactone concentration, suggesting that the access of cells to the substrate occurs by their adhesion around larger oil droplets.

Experiments in a stirred bioreactor using 30 g L^{-1} MR (concentration at which the highest aroma productivity was achieved) and different aeration and agitation rates demonstrated the direct influence of oxygen transfer rate on the production of γ-decalactone and of another compound, 3-hydroxy-γ-decalactone, that can also accumulate in the medium. The accumulation of this compound indicates a deviation in the metabolic pathway of γ-decalactone production, decreasing its yields.

A response surface methodology was used to optimize pH (6.17) and dissolved oxygen concentration (44.4%) for the aroma production. These operating conditions were applied in two fed-batch strategies: with constant medium feeding rate and with intermittent feeding. Both strategies were compared with the traditional batch mode in terms of overall productivity and yield in respect to the substrate. Although the productivity was considerably higher in the batch mode, the level of substrate conversion to both lactones was greater in the intermittent fed-batch, allowing the accumulation of high aroma concentrations (6.8 g L^{-1} γ-decalactone and 10.0 g L^{-1} 3-hydroxy-γ-decalactone).

Finally, the production of aroma was attempted in an airlift bioreactor due to the advantages of this type of bioreactor, mainly in terms of high power economies, the non-mechanical agitation which avoids damage to cells and the higher mass transfer coefficients attained. The highest γ-decalactone production was obtained at an air flow-rate of 1 L min^{-1}. The aeration rate increase of 5-fold lead to lower aroma concentrations. However, the time needed to reach the peak of production was also reduced, resulting in higher productivities.

RESUMO

A produção biotecnológica de γ-decalactona (composto com aroma a pêssego) através da biotransformação de ácido ricinoleico por microrganismos é um processo interessante para produzir o aroma com um rótulo de "natural", o que é uma mais-valia, considerando as actuais preferências dos consumidores. Embora existam muitos trabalhos na literatura sobre este tema, vários factores do processo ainda permanecem por compreender totalmente e, consequentemente, por optimizar. Um desses factores é o efeito do oxigénio no processo global. Assim, este trabalho teve inicialmente como objectivo estudar o fenómeno de transferência de O_2 do gás para o meio de biotransformação, uma emulsão do tipo óleo-em-água estabilizada pelo surfactante não-iónico Tween 80. O óleo é simultaneamente o substrato do processo e actua também como transportador de O_2, uma vez que a solubilidade deste composto no óleo é maior do que na fase aquosa. A influência de cada parâmetro de operação (arejamento, presença e concentração de surfactante e fase orgânica) nas variáveis envolvidas na transferência de O_2 (área interfacial gás-líquido, a; coeficiente de transferência de O_2 na fase líquida, k_L; e coeficiente volumétrico de transferência de massa, k_La) foi analisada numa coluna de bolhas e num reactor airlift. Os resultados demonstraram que na coluna de bolhas o aumento da taxa de arejamento é positivo tanto para a área interfacial como para a transferência de massa devido ao aumento da turbulência e do gás hold-up. A concentração de surfactante teve um efeito positivo na área interfacial, uma vez que reduziu o tamanho das bolhas de gás e teve um efeito negativo no k_L porque as suas moléculas localizam-se na interface gás-líquido, dificultando a transferência de O_2. Relativamente à concentração de óleo, esta teve um efeito negativo na área interfacial mas melhorou o k_L, uma vez que provocou uma nova distribuição do surfactante no meio, diminuindo a sua concentração na interface gás-líquido. O resultado global foi um efeito negativo da fase orgânica no k_La. No reactor airlift, observou-se que o aumento do arejamento produziu um efeito negativo no k_L. Este resultado foi atribuído às diferenças na distribuição do líquido dentro do reactor airlift.

Como o principal objectivo deste trabalho era optimizar a produção de γ-decalactona, duas fontes de ácido ricinoleico (ricinoleato de metilo, RM, e óleo de rícino, OR) foram testadas, em diferentes concentrações, como substratos do processo. Diferentes estratégias de inoculação de células foram também testadas, diferindo entre elas na lavagem ou não das células. Os resultados revelaram que o uso de células não lavadas é mais vantajoso para a produção do aroma, independentemente do substrato usado; e uma concentração de 30 g L^{-1} RM foi seleccionada como a mais adequada de entre a gama testada, uma vez que foi a que permitiu a obtenção da maior produtividade de γ-decalactona (14.9 mg L^{-1} h^{-1}). Este substrato revelou-se também um indutor de lipase.

O uso de OR como substrato do processo permitiu obter quase 2 g L^{-1} de aroma mas o processo foi bastante lento, resultando em baixas produtividades. Hipotetizou-se então uma insuficiente hidrólise do óleo e testou-se uma hidrólise enzimática com diferentes enzimas comerciais e condições operatórias (temperatura e pH). Lipozyme TL IM, pH 8 e 27 °C foram seleccionadas, respectivamente, como a enzima e as condições operatórias mais eficientes na hidrólise do OR. Os resultados obtidos usando OR previamente hidrolisado pela enzima seleccionada foram comparados com os resultados obtidos em ensaios em que a hidrólise enzimática ocorreu durante a biotransformação e em ensaios em que não se adicionou lipase, indicando que o processo foi mais rápido quando a lipase esteve envolvida de alguma forma, mas as concentrações de aroma foram inferiores, resultando em produtividades idênticas.

O tamanho das gotas de ambos os óleos em emulsões com diferentes concentrações de óleo foi caracterizado por granulometria laser. O impacto da presença de células no tamanho das gotas também foi analisado, assim como a relevância de se lavarem as células do inóculo. A granulometria das emulsões foi relacionada com a produção de γ-decalactona e observou-se que, na presença de células não lavadas, as gotas mais pequenas desapareceram, com ambos os óleos, aumentando a produção do aroma e sugerindo que o acesso das células ao substrato ocorre pela sua adesão à volta das gotas de óleo de maior tamanho.

Ensaios num bioreactor agitado com 30 g L^{-1} RM (concentração à qual se obteve a maior produção de aroma) e diferentes arejamentos e agitações demonstraram a directa influência da taxa de transferência de oxigénio na produção de γ-decalactona e de outro composto, a 3-hidroxi-γ-decalactona, que também pode acumular no meio. A acumulação deste composto indica um desvio na via metabólica de produção de γ-decalactona, diminuindo o rendimento.

A metodologia de superfície de resposta foi utilizada para optimizar o pH (6.17) e a concentração de O_2 dissolvida no meio (44.4%) na produção do aroma. Estas condições foram aplicadas em duas estratégias semi-contínuas: com alimentação contínua de meio e com alimentação intermitente. Ambas as estratégias foram comparadas com o modo descontínuo tradicional em termos de produtividade global e rendimento em relação ao substrato. Embora a produtividade fosse consideravelmente superior no modo descontínuo, o nível de conversão de substrato em lactonas foi superior na estratégia semi-contínua intermitente, acumulando-se elevadas concentrações de aroma (6.8 g L^{-1} γ-decalactona e 10.0 g L^{-1} 3-OH-γ-decalactona).

Por fim, a produção de aroma foi testada num bioreactor airlift devido às vantagens deste tipo de reactor, nomeadamente no que diz respeito à poupança energética, à agitação não mecânica que evita danos nas células e aos elevados coeficientes de transferência de massa que se obtêm. A maior produção de γ-decalactona foi obtida com um caudal de arejamento de 1 L min^{-1}. O aumento do caudal de arejamento em 5 vezes resultou numa diminuição da concentração do aroma. Porém, o tempo necessário para se atingir o pico de produção também foi reduzido, resultando em produtividades mais elevadas.

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LIST OF ABBREVIATIONS

a	Gas-liquid interfacial area (m^{-1})
A	Absorbance at 600 nm at the beginning of the MATH test
A_0	Absorbance at 600 nm at the end of the MATH test
$A_{\gamma\text{-decalactone}}$	Area of γ-decalactone
$A_{3\text{-OH-}\gamma\text{-decalactone}}$	Area of 3-hydroxy-γ-decalactone
Aoxip	Acyl-CoA oxidase
CMC	Critical micellar concentration
CO	Castor oil
C_{oil}	Oil concentration in the sample (mg mL^{-1})
d	Bubble diameter
d_B	Diameter of the equivalent sphere
df	Degrees of freedom
d_i	Equivalent diameter
d_{32}	Sauter mean diameter
DO	Dissolved oxygen
e	Minor axe of the bubble projected ellipsoid
E	Major axe of the bubble projected ellipsoid
F	Medium feeding rate (L h^{-1})
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
GRAS	Generally Regarded As Safe
K	Response coefficient
k_L	Liquid side mass transfer coefficient (m h^{-1})
k_La	Volumetric mass transfer coefficient (h^{-1})
L	Concentration of lactone (mg L^{-1})
M	Molarity (mol L^{-1})
MM	Molar mass (g mol^{-1})
MR	Methyl ricinoleate
NAD^+	Nicotinamide adenine dinucelotide
NADH	Reduced nicotinamide adenine dinucelotide
n_i	Number of bubbles
O	Dissolved oxygen concentration within the reactor (mg L^{-1})

OD	Optical density (nm)
O_0	Dissolved oxygen concentration when the aeration is resumed (mg L^{-1})
O_i	Dissolved oxygen concentration in the beginning of the biotransformation (mg L^{-1})
O^S	Oxygen saturation concentration (mg L^{-1})
OTR	Oxygen transfer rate (g L h^{-1})
OUR	Oxygen uptake rate (g L h^{-1})
P	Productivity (mg L^{-1} h^{-1})
P_g	Power input of the aerated bioreactor (W)
POXi	Genes codifying acyl-CoA oxidase
Q_g	Gas flow-rate (L h^{-1})
q_{O2}	Specific oxygen uptake rate (g g h^{-1})
R^2	Correlation coefficient
R^2_{adj}	Correlation coefficient adjusted for degrees of freedom
RSD	Relative standard deviation
S_f	Concentration of substrate in the feeding medium (g L^{-1})
SV	Saponification value (mg g^{-1})
t	Time (h)
t_0	Time when the aeration is resumed (h)
U_g	Surface gas velocity (m s^{-1})
U_L	Liquid velocity (m s^{-1})
V	Bioreactor working volume (m^3)
V_B	KOH volume dispended in the blank titration (mL)
V_L	Ungassed liquid volume (m^3)
V_S	Titrated sample volume (mL)
vvm	Volume of air per volume of reactor per minute
X	Cell density (g L^{-1})
x_i	Dimensionless coded value of the independent variable
X_i	Independent variable
X_0	Value of the independent variable at the central point
X_{ORG}	Organic fraction in the medium (L L^{-1})
X_{Tween}	Tween 80 fraction in the medium (L L^{-1})
$Y_{L/S}$	Yield of lactone in respect to the substrate (mg g^{-1})

Subscripts:

f	Final conditions
0	Initial conditions
si	Sample conditions

Greek letters:

α	Numerical constant
β	Numerical constant
δ	Numerical constant
ϖ	Numerical constant
ϕ	Numerical constant
γ	Interfacial tension (mN m^{-1})
ε_g	Gas hold-up
γ	Numerical constant
τ	Probe response time (s^{-1})
ΔV	Volume expansion after gas dispersion
ΔX_i	Step change

Notes:

In general, the International System of Units (SI) was used in this work. However, sometimes multiples and sub-multiples of the fundamental units (which are not SI) were used since their use is so common that is allowed by that system.

Some units not recognized by the SI were also used to express some variables, such as the volume percent (% v/v) to denote the composition of some solutions, the revolutions per minute (rpm) to indicate the agitation rates and the volume of air per volume of reactor per minute (vvm) to designate the aeration rates.

1 INTRODUCTION, RESEARCH AIM AND THESIS OUTLINE

This chapter introduces the background information about the theme of the work, as well as its objectives.

The outline of the thesis is also presented.

1.1 INTRODUCTION AND RESEARCH AIM

The demand for fragrances increases yearly as a result of the increasing production of processed foods. Most fragrances are produced by chemical means and natural aromas are obtained from plant materials in small amounts, being very expensive. Chemical synthesis of flavor compounds generally requires numerous steps and often lacks stereoselectivity (Gopinath *et al.*, 2008). Moreover, climates, seasonal variation, political and socioeconomic factors, often lead to a constant shortfall of product supply of natural fragrances from plant sources. In addition, due to the preferences of consumers, it is important that the flavors used can be designated as "natural".

Lactones are well-known aroma compounds found in a variety of foods and beverages, reason why the food industry has a high interest in their biotechnological production (Gatfield, 1997).

The conventional routes of chemical synthesis or isolation from plants are still viable, but the biotechnological generation of aroma compounds is becoming more attractive. Microbial fermentation is regarded as a potential means to produce aroma compounds classified as "natural" by the European and the US food legislation. This label represents a strong marketing advantage (Krings and Berger, 1998), reason why this technology has attracted a great deal of research interest.

The most widely biotechnologically produced lactone is γ-decalactone, a peach-like flavor that can be obtained from the biotransformation of ricinoleic acid, catalyzed by various microorganisms. γ-Decalactone is then considered as a GRAS (Generally Regarded As Safe) food additive by the U.S. Food and Drug Administration (Arctander, 1969).

In the present work, the aerobic yeast *Yarrowia lipolytica* was used to produce γ-decalactone from two different ricinoleic acid sources: methyl ricinoleate and castor oil. γ-Decalactone production from methyl ricinoleate by *Yarrowia lipolytica* strains has been studied by several authors, concerning the improvement of the knowledge about the lipidic metabolism that leads to lactones production (Waché *et al.*, 2003; García, 2008), the interactions of cells with the substrate (Aguedo, 2002), the toxicity of the metabolites produced (Feron *et al.*, 1996b; Aguedo *et al.*, 2003b) and the selection of over producing mutants (Groguenin *et al.*, 2004). More recent studies have shown the importance of oxygen mass transfer in the overall process (Aguedo *et al.*, 2005a; Gomes *et al.*, 2007). Therefore, the characterization of the oxygen mass transfer from the gas (air) to the biotransformation biphasic medium is of great importance in order to optimize the process.

Moreover, the fact that the yeast used in this work, *Y. lipolytica*, is an aerobic microorganism also contributes to the interest of that knowledge. With this objective, the influence of operating conditions (gas flow-rate, surfactant and organic phase composition) upon the gas-liquid interfacial area and mass transfer coefficient was analyzed. The results allowed to describe the mechanism of oxygen transfer from the gas phase into the biphasic medium.

Considering the main aim of this dissertation which is the improvement of γ-decalactone production in a biphasic medium containing a ricinoleic acid source, by the yeast *Yarrowia lipolytica*, several parameters were studied and/or optimized: ricinoleic acid source (methyl ricinoleate and castor oil), substrate concentration, role of lipases in the process, pH and dissolved oxygen concentration. Also different fermentation strategies were tested: batch, fed-batch with constant feeding rate and with intermittent feeding. Finally, the production of γ-decalactone was assayed in an airlift bioreactor, due to its known advantages concerning high power economies resulting from the absence of mechanical agitation, which also prevents damage to the cells and to the high gas-liquid mass transfer coefficients usually attained in this type of bioreactor.

1.2 OUTLINE OF THE THESIS

The thesis is structured in 8 chapters:

The present section (**CHAPTER 1**) introduces the background information about the biotechnological production of γ-decalactone and the motivations of finding new strategies to optimize the process

A review on the state of art of the biotechnological production of γ-decalactone as well as on the oxygen mass transfer mechanism in biphasic media and on fermentation strategies is presented on **CHAPTER 2**.

CHAPTER 3 describes the general materials and methods used.

In **CHAPTER 4** the results concerning the oxygen mass transfer in the biphasic medium are reported.

CHAPTER 5 contains the results obtained when two different ricinoleic acid sources (methyl ricinoleate and castor oil) were used as substrates for γ-decalactone production. The influence of oil droplets upon the process is also described.

CHAPTER 6 presents information about the optimization of operating conditions (pH and dissolved oxygen concentration) in a stirred bioreactor and the application of two fed-batch strategies using the optimal conditions previously established. Moreover, the production of γ-decalactone in an airlift bioreactor was also a subject of study.

CHAPTER 7 encloses final conclusions as well as suggestions for future work in this field of research.

CHAPTER 8 gathers all the references used in the elaboration of this work.

2 LITERATURE REVIEW

In a first instance, this chapter characterizes the aroma compound g-decalactone and the yeast used in this work to produce it, Yarrowia lipolytica. The metabolic pathways of lactone production and degradation are also discussed.

A brief overview about the biotransformation process on biphasic media and the oxygen transfer in these systems is presented.

2.1 THE AROMATIC COMPOUND γ-DECALACTONE

The demand for fragrances increases yearly as a result of the increasing production of industries, such as food and beverages, soaps, cosmetics, chemicals, pharmaceuticals, detergents and toiletries, more than ever, which rely on aroma additives to replenish or add flavor to their products (Marasco and Schmidt-Dannert, 2003).

Essential oils of higher plants, fruit juices, vegetable extracts and a very few products of animal origin were, for a long time, the sole sources of natural flavors (Krings and Berger, 1998). However, plants frequently produce small amounts of aroma and its isolation is often difficult, resulting in very expensive compounds. Moreover, climates, seasonal variation, political and socioeconomic factors, often lead to a constant shortfall of product supply of natural fragrances from plant sources (Longo and Sanromán, 2006). To liberate the aroma industry from a dependence upon those factors and to allow large-scale production of aroma compounds, chemical synthesis techniques were developed, producing most of the today's existing flavors. However, chemical synthesis of flavor compounds generally requires numerous steps and often results in an environmentally unfriendly production process and in undesirable racemic mixture compounds (Gopinath *et al.*, 2008; Vandamme and Soetaert, 2002).

The increasing sensitivity of the ecological systems supports the choice of environmentally friendly processes and consumers have developed a preference for "natural" or "organic" products, hence developing a market for flavors of biotechnological origin (Serra *et al.*, 2005).

Biotechnological options for flavor synthesis comprise single-step biotransformations, bioconversions and *de novo* synthesis, using microorganisms, plant cells or enzymes (Vandamme and Soetaert, 2002; Aguedo *et al.*, 2004). Aroma compounds produced by enzymatic activities or fermentation are considered as "natural", according to the US Food and Drug Administration Code of Federal Regulations (Code of Federal Regulations 21, 1993) which states: "The term natural flavor or natural flavoring means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product or roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice fruit, juice, vegetable, or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavoring rather than nutritional". In the European Union there is also a legal definition of natural substances described

as: “a flavoring substance which is obtained by appropriate physical processes (including distillation and solvent extraction) or enzymatic or microbiological processes from material of vegetable or animal origin either in the raw state or after processing for human consumption by traditional food-preparation processes (including drying, torrefaction and fermentation)” (The Council of the European Communities, 1988).

Microbial fermentation is thus regarded as a potential means to produce natural flavor substances and has attracted a great deal of research interest (Longo and Sanromán, 2006). Lactones are very interesting aroma compounds especially for the food industry because of their fruity aroma. Those compounds can be found naturally in a wide variety of foods, such as fruits, milk and dairy products, meats and some fermented foods (Lin *et al.*, 1996).

The microbial production of lactones results in the same enantiomeric configuration of the lactones found in peaches and other fruits (Schrader *et al.*, 2004).

The most biotechnologically produced aromatic lactone is γ-decalactone ($C_{10}H_{18}O_2$), with a market volume of several hundred tons per year. It has an oily-peachy, extraordinarily tenacious odor and a very powerful, creamy-fruity, peach-like taste in concentrations below 5 ppm (Rabenhorst and Bertram, 2004). In the early eighties, γ-decalactone was a natural rare aroma and extremely expensive (> US$ 10 000 kg^{-1}). The subsequent introduction and optimization of microbial processes resulted in a decline of the price to approximately US$ 62 kg^{-1} (data obtained in November 2010 from Penta Manufacturing Company, New Jersey, USA).

Most of the industrial processes use ricinoleic acid, the main fatty acid (about 90%) of castor oil, or esters thereof, for γ-decalactone biotechnological production. This aroma can be obtained from the biotransformation of ricinoleic acid, catalyzed by microorganisms with GRAS (Generally Regarded As Safe) status, conferring a natural label to the compound. The production of γ-decalactone from that fatty acid by yeasts of the genus *Candida* was originally observed by Okui *et al.* (1963).

Although several lactone compounds are produced by various microorganisms, the processes with higher yields use *Yarrowia lipolytica* strains. For example, using an uracil auxotrophic *Y. lipolytica* strain genetically modified, designated PO1D, Nicaud *et al.* (1996) obtained a high production of γ-decalactone from methyl ricinoleate: at the end of the growth phase, these authors transferred the concentrated biomass into a biotransformation uracil-limited medium. After 75 hours, a concentration of 9.5 g L^{-1} γ-decalactone was achieved.

In a production process established by Haarmann & Reimer GmbH (H&R), in Germany, the production of 11 g L^{-1} γ-decalactone in 65 hours was reported, using a non-genetically modified strain and castor oil as raw substrate (Rabenhorst and Gatfield, 2000).

Other organisms performing the same process but with much lower product yields were *Monilia fructicola*, *Sporobolomyces odorus* and *Rhodotorula glutinis* (Schrader *et al.*, 2004).

The production of γ-decalactone was investigated using different species of *Sporidiobolus*, as *Sporidiobolus salmonicolor*, *Sporidiobolus ruinenii*, *Sporidiobolus johnsonii* and *Sporidiobolus pararoseus* (Dufossé *et al.*, 1998). These strains proved to be very sensitive to γ-decalactone. On the contrary, the open form, i.e., acid-4-hydroxydecanoic, proved to be less toxic to the cells. The concentrations of γ-decalactone obtained from fermentations with these yeasts were low (< 1 g L^{-1}). Similar yields were obtained by Cardillo *et al.* (1990) using *Aspergillus niger*, *Pichia etchelsii* and *Cladosporium suaveolens*.

Kumin and Munch (1998) used *Mucor circillenoides* to produce γ-decalactone from ethyl decanoate. A production of 10.5 g L^{-1} γ-decalactone after 60 hours was reported.

Table 2.1 presents a summary of the microorganisms mentioned in the literature, capable of producing γ-decalactone.

Table 2.1 - Microorganisms able to produce γ-decalactone.

	Microorganisms	Authors
Bacteria	*Lactococcus lactis*	Ambid *et al.* (1999)
Yeasts	*Candida albicans, C. guilliermondii, C. krusei, C. parakrusei, C. pseudotropicalis, C. rugosa, C. stellatoidea, C. tropicalis*	Farbood and Willis (1983); Farbood *et al.* (1990)
	Candida boidinii	Boog *et al.* (1990)
	Candida molischiana, C. rhagii, C. intermedia, C. nemomendra	Endrizzi-Joran (1994)
	Candida tenuis	Dufossé *et al.* (1998); Feron *et al.* (1996a)
	Cladosporium suavolens	Cardillo *et al.* (1990)
	Debaromyces hansenii	Boog *et al.* (1990)
	Geotrichum fragrans, Geotrichum sp.	Neto *et al.* (2004)
	Hansenula saturnus	Farbood and Willis (1983); Meyer (1993)
	Malassezia furfur	Endrizzi-Joran (1994)
	Pichia etchellsii	Cardillo *et al.* (1990)
	Pichia guilliermondii	Pagot (1997); Endrizzi-Joran *et al.* (1993); Iacazio *et al.* (2002)
	Pichia ohmeri	Ercoli *et al.* (1992)
	Pityrosporum sp.	Labows *et al.* (1979)
	Rhodotorula glutinis	Cheetham *et al.* (1988); Cheetham *et al.* (1993)
	Saccharomyces cerevisiae	Ambid *et al.* (1999); Boog *et al.* (1998); Gatfield (1995)
	Sporidiobolus sp.	Blin-Perrin *et al.* (2000)
	Sporidiobolus ruinenii, Sp. pararoseus, Sp. johnsonii	Dufossé *et al.* (1998); Feron *et al.* (1996a)
	Sporidiobolus salmonicolor	Dufossé *et al.* (1998); Endrizzi-Joran (1994); Feron *et al.* (1996b); Feron *et al.* (1997); Lee *et al.* (1998); Lee *et al.* (1999a)
	Sporobolomyces odorus	Ambid *et al.* (1999); Cheetham *et al.* (1988); Cheetham *et al.* (1993); Haffner and Tressl (1996); Lee and Chou (1994); Lee *et al.*

		(1995); Lin *et al.* (1996)
	Yarrowia lipolytica	Aguedo (2002); Ambid *et al.* (1999); Farbood and Willis (1983; 1985); García *et al.* (2009); Gomes (2005); Gomes *et al.* (2010) Groguenin *et al.* (2004); Meyer (1993); Nicaud *et al.* (1996); Pagot *et al.* (1998); Rabenhorst and Gatfield (2000); Rabenhorst and Gatfield (2001); Reis *et al.* (2006); Waché *et al.* (1998); Wang *et al.* (1999)
Filamentous Fungi	*Aspergillus niger*	Cardillo *et al.* (1990)
	Aspergillus oryzae, Geotrichum klebahni	Farbood and Willis (1983); Meyer (1993)
	Ceratocystis moniliformis	Lanza *et al.* (1976)
	Claviceps purpurea, Fusarium monolilifonne, Penicillium sp., *Streptomyces dimorphogenes*	Ambid *et al.* (1999)
	Fusarium poae	Sarris and Latrasse (1985); Latrasse *et al.* (2004); Spinnler *et al.* (1994)
	G. ramanniana var. angulispora, Syncephalastrum racemosum	Meyer (1993)
	Mortierella sp.	Han and Han (1995)
	Mucor sp.	Page and Eilerman (1989); Kumin and Munch (1998)
	Phanerochaete chrysosporium	Cardillo *et al.* (1990)
	Phoma, Thermomyces, Neurospora sp.	Nozaki (1994)
	Piptoporus soloniensis	Okamoto *et al.* (2002)
	Polyporus durus	Berger *et al.* (1986)
	Trichoderma harzianum	Serrano-Carréon *et al.* (1997)
	Tyromyces sambuceus	Pakula and Freeman (1996); Hädrich-Meyer and Berger (1994)

2.2 THE YEAST *YARROWIA LIPOLYTICA*

Yarrowia lipolytica is an aerobic microorganism, eukaryotic, non-pathogenic, belonging to the Fungi kingdom (class Ascomycetes, subclass Hemiascomycetes). It was originally classified as *Candida*

lipolytica, since no sexual state was described, and then reclassified as *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica*, and finally, *Yarrowia lipolytica* (Barth and Gaillardin, 1997).

This microorganism is one of the more intensively studied "non-conventional" yeast species (Barth and Gaillardin, 1997). This yeast is quite different from the well-studied yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* with respect to its phylogenetic evolution, physiology, genetics and molecular biology (Wolf, 1996).

Y. lipolytica is a dimorphic yeast. This characteristic refers to the ability of fungi to grow in two distinct morphological forms, usually as single oval cells or as filamentous hyphae, being reversible between the two forms. It is believed that the dimorphism of this yeast, as well as other species, provides a mechanism for responding to adverse conditions (Kawasse *et al.*, 2003). The morphology of the cells is determined by both growth conditions (aeration, carbon and nitrogen sources, pH, dissolved oxygen concentration in the medium, etc.) and by the genetic characteristics of the strain (Cruz *et al.*, 2000).

This microorganism is considered to be an appropriate model for the study of dimorphism in yeasts as well as other areas of cell and developmental morphology, because it is susceptible to genetic manipulation and has an efficient transformation system (Szabo, 2001).

Because of its ability to produce several biotechnologically important metabolic intermediates (including organic acids like citric and isocitric acids (Finogenova *et al.*, 2002), lysine (Furui *et al.*, 1996), extracellular proteases (Davidow *et al.*, 1987), lipases (Lopes *et al.*, 2008), esterases (Zhang *et al.*, 2009), phosphatases (Galabova *et al.*, 1993) and RNAses (Cheng and Ogrydziak, 1986)), *Y. lipolytica* has been studied since the mid-twentieth century, justifying the efforts for its use in industry as biocatalysts, in molecular biology and genetic studies (Szabo *et al.*, 2001). Moreover, since this yeast is generally regarded as safe (GRAS) by the American Food and Drug Administration, it has been widely used in biotechnological applications also considered as GRAS, such as the production of single cell protein (Beckerich *et al.*, 1998), γ-decalactone (Waché *et al.*, 2001) and citric acid (Papanikolaou *et al.*, 2008).

Y. lipolytica uses a large range of substrates, including oils, alkanes, fats and fatty acids (Fickers *et al.*, 2005), alcohols, organic acids, proteins and some sugars (mainly glucose), which contributes to the high interest on this yeast. Although some articles have reported the inclusion of sucrose in

the range of substrates (Nicaud *et al.*, 1989), some strains have no detectable invertase activity (Pereira-Meirelles *et al.*, 1997; Förster *et al.*, 2007).

Y. lipolytica is able to transform ricinoleic acid into γ-decalactone. This biotransformation involves the β-oxidation pathway and requires the lactonization at the C10 (Blin-Perrin *et al.*, 2000; Gatfield *et al.*, 1993; Waché *et al.*, 2003). Like most of the yeasts capable of performing this biotransformation, *Y. lipolytica* is also able to degrade the lactone produced.

2.3 PATHWAY OF γ-DECALACTONE PRODUCTION: PEROXISOMAL β-OXIDATION

The yeast metabolic pathway involved in the biotransformation of ricinoleic acid into γ-decalactone was firstly proposed by Okui *et al.* (1963). Working with *Candida* species, these authors found intermediates derived from the catabolism of ricinoleic acid, containing 18 to 8 carbons (Figure 2.1). They hypothesized that the degradation of ricinoleic acid into 4-hydroxydecanoic acid (the direct precursor of γ-decalactone) could be carried out by enzymes of the β-oxidation pathway. This pathway is exclusively peroxisomal in yeasts. The same intermediates were later identified in *Y. lipolytica* (Gatfield *et al.*, 1993) and in *Sporidiobolus ruinenii* (Spinnler *et al.*, 1996).

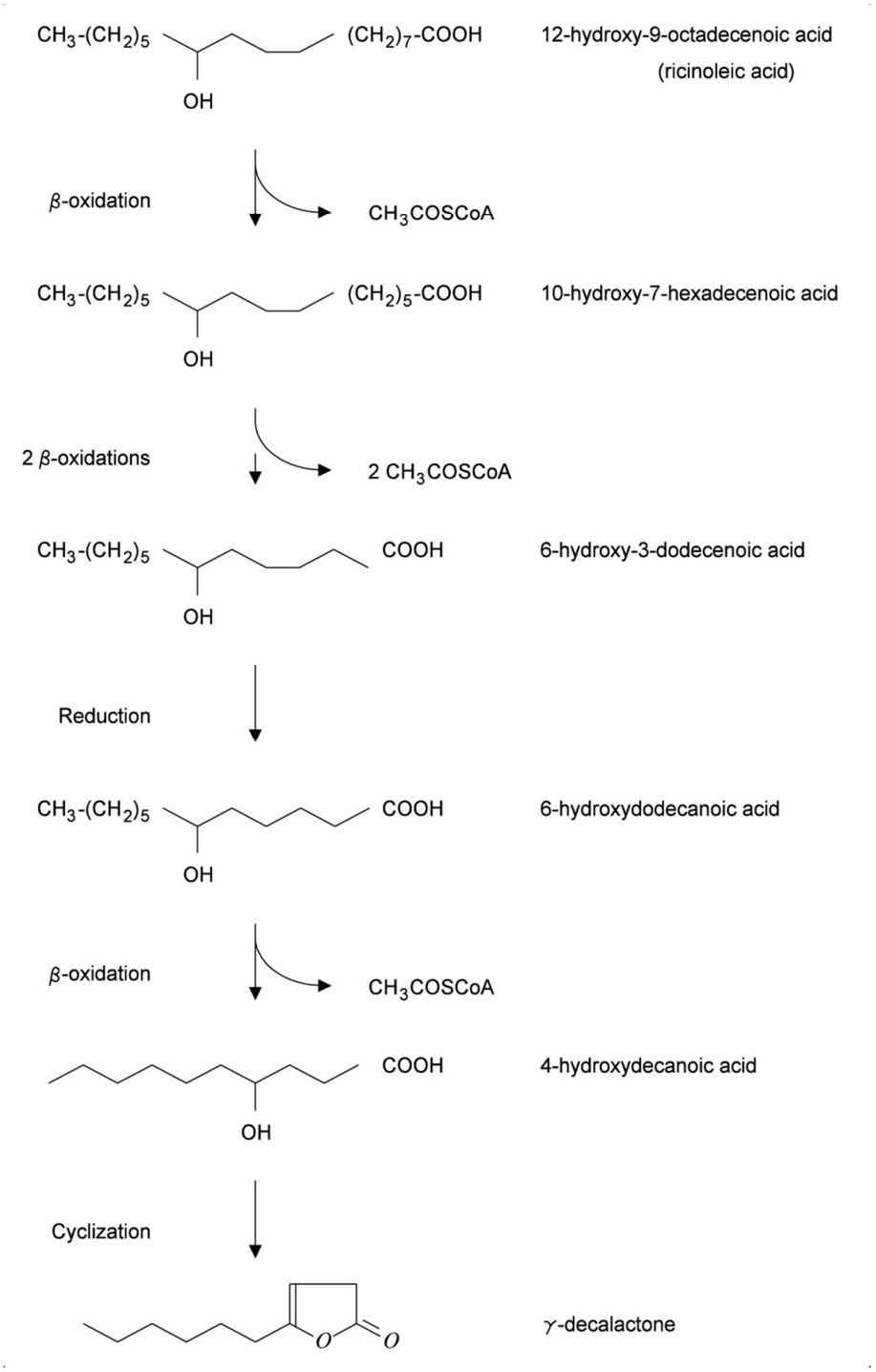

Figure 2.1 - Intermediates of ricinoleic acid degradation by β-oxidation and γ-decalactone synthesis (adapted from Blin-Perrin et al., 2000).

β-Oxidation is a system of cyclic oxidation of fatty acids consisting in a sequence of four steps, repeated several times, usually until the degradation of the substrate into acyl-CoA. Each cycle ends with the decrease of 2 carbons in β position of the molecule and the release of an acyl-CoA.

Before entering the peroxisomes, fatty acids are activated in the cytoplasm into esters of coenzyme A, by action of an acyl-CoA synthetase. β-Oxidation is catalyzed, in yeast peroxisomes, by acyl-CoA oxidase and then, by two activities of a multifunctional enzyme, 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, and by 3- ketoacyl-CoA thiolase (Figure 2.2).

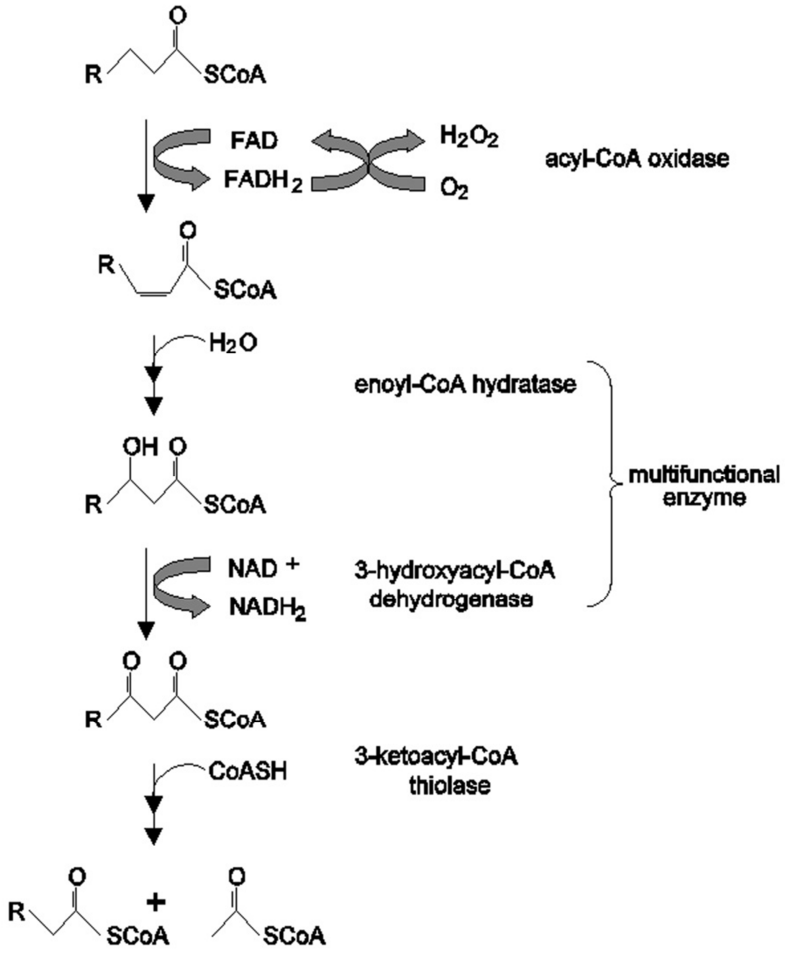

Figure 2.2 - Enzymes involved in the yeast peroxisomal β-oxidation (adapted from Waché et al., 1998).

Y. lipolytica has the peculiarity of possessing a family of six members of acyl-CoA oxidases (Aox1p to Aox6p, encoded by the genes POX1 to POX6), the enzyme that catalyzes the first step of β-oxidation (García *et al.*, 2007a). Some acyl-CoA oxidases are specific for long chains (Aox2p) (Wang *et al.*, 1999) or specific for short chains (Aoxp3) (Luo *et al.*, 2000). The specificity derives only from a small number of amino acids.

The role of each of these enzymes in the degradation of fatty acids can be studied using mutant strains in which one or more genes have been removed or amplified. Understanding the specific roles of each acyl-CoA oxidase is important to "construct" a yeast strain growing at a good rate and producing γ-decalactone, without degrading it (Groguenin *et al.*, 2004).

2.4 γ-DECALACTONE DEGRADATION PATHWAY

During γ-decalactone production by biotransformation of ricinoleic acid, its concentration in the medium achieves a maximum and then progressively diminishes, due to a re-consumption. This decrease in the aroma compound concentration may be extremely prejudicial to the yields of the process and it has been observed with different yeast strains, among which: *Candida guilliermondii* (Okui *et al.*, 1963), *Sporidiobolus salmonicolor* (Dufossé, 1993), *Candida intermedia* (Endrizzi-Joran and Belin, 1995) and *Y. lipolytica* (Aguedo *et al.*, 2005a). According to Endrizzi-Joran (1994), the yeasts ability to re-consume γ-decalactone exists during the whole biotransformation and its concentration in the medium is the difference between what is produced and degraded.

Many hypotheses have been proposed to explain the re-consumption, all involving the β-oxidation and some including ω-oxidation or de-lactonization. The β-oxidation pathway is not yet well understood. In peroxisomal β-oxidation, which is used by yeasts, depending on the substrate and the concentrations of CoA, several compounds (3-hydroxy-γ-decalactone, dec-2-enolide and dec-3-enolide) derived from 4-hydroxydecanoic acid, the direct precursor of γ-decalactone, are occasionally detected in the medium (Waché *et al.*, 2001) (Figure 2.3).

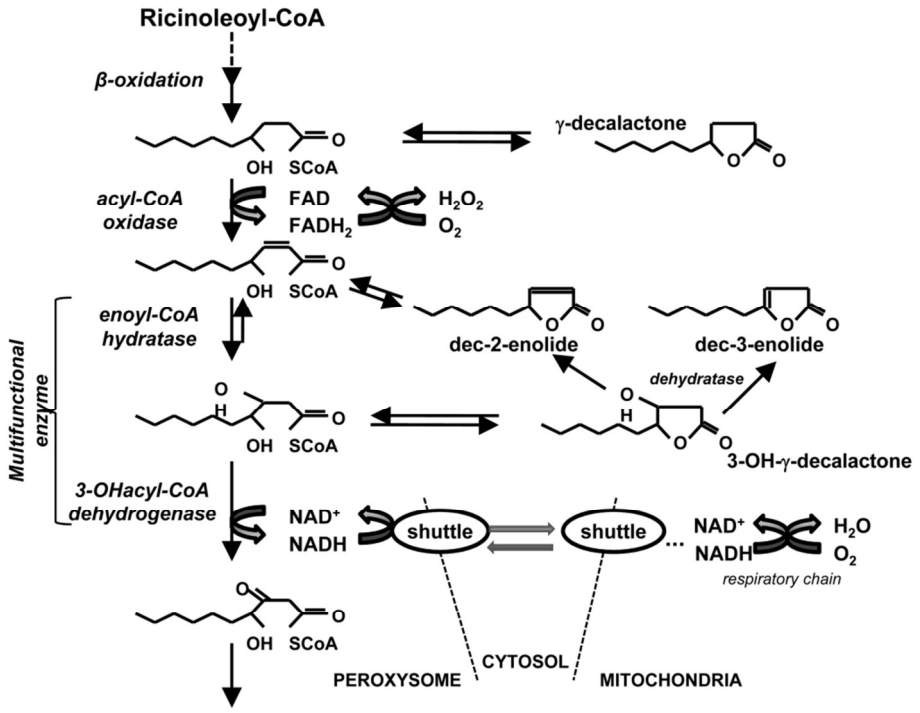

Figure 2.3 - β-Oxidation cycle from the direct precursor of γ-decalactone (4-hydroxydecanoic acid) during the degradation of ricinoleoyl-CoA. Shuttle mechanisms leading to oxidation of NADH and the link with the mitochondrial respiratory chain have been simplified (adapted from Bakker et al., 2001).

Gatfield *et al.* (1993) observed with *Y. lipolytica* the concomitant disappearance of γ-decalactone and accumulation of 3-hydroxy-γ-decalactone and dec-2-enolide, reducing the yields. Those authors hypothesized that 4-hydroxydecanoic acid would initially experience an oxidation followed by an hydration, leading to the 3,4-dihydro-decanoic acid which is then cyclized into 3-hydroxy-γ-decalactone (compound without sensory properties). Dehydration of the latter compound would allow the formation of two decenolides with aroma properties: dec-2-enolide (mushroom notes) and dec-3-enolide (fruity notes). Due to their flavoring properties, these two molecules arise as interesting compounds to be produced and used in the flavoring industry (García *et al.*, 2009). The concentrations of these compounds in the medium vary mainly according to the activity of acyl-CoA oxidase and 3-hydroxyacyl-CoA dehydrogenase. Thus, when one of these enzymes has low activity, it will limit the β-oxidation pathway flow, controlling it. Consequently, the accumulation of those compounds, which are directly related to their activity, will occur, namely γ-decalactone, whose accumulation is related to the activity of acyl-CoA oxidase and 3-hydroxy-γ-decalactone, whose accumulation is related to the activity of 3-hydroxyacyl-CoA dehydrogenase. The formation of dec-2-

enolide may even result from the direct action of enoyl-CoA hydratase (Figure 2.3). Similarly, if this enzyme has low activity, it will control the pathway flow at this level, becoming the limiting enzyme of the pathway, which will allow the accumulation of dec-2-enolide (Waché *et al.*, 2001).

2.5 BIOTRANSFORMATION IN BIPHASIC MEDIA

The biotransformation medium for lactone production is biphasic, consisting of an aqueous-liquid phase and an organic-liquid phase of castor oil or methyl ricinoleate (sources of ricinoleic acid), forming an emulsion stabilized by the emulsifier Tween 80.

2.5.1 Organic phase

Castor oil is a natural oil obtained from the seeds of castor plant (*Ricinus communis*). Chemically, castor oil is a triglyceride of fatty acids. Approximately 90% of the fatty acid content is ricinoleic acid, an acid with 18 carbons having a double bond at position C9-C10 and a hydroxyl group at C12. This combination of hydroxyl group and unsaturation occurs only in castor oil and is responsible for the ricinoleic acid and castor oil to be unusually polar, also allowing chemical derivatization that is not practical with other biological oils. The other most relevant fatty acids in castor oil are linoleic acid (4%), oleic acid (2%), stearic acid (1%), palmitic acid (1%), dihydroxystearic acid (0.7%), linoleic acid (0.3%) and eicosanoic acid (0.3%).

Ricinoleic acid is the precursor used in γ-decalactone production. In some cases castor oil hydrolysates, fatty acids or esters of these compounds (Page and Eilerman, 1996), like methyl ricinoleate (MR), are used as substrates.

In this work, castor oil and methyl ricinoleate were used as substrates of the process.

2.5.2 Surfactants

Surfactants constitute an important class of chemical compounds widely used in various industrial sectors (Nitschke and Pastore, 2002).

The emulsifying agents or surfactants can have a synthetic or a biological origin, in which case they are naturally synthesized by the cells (Amaral *et al.*, 2006a).

Surfactants are amphipathic molecules consisting of a non-polar hydrophobic group and a polar hydrophilic group. The non-polar group is often a hydrocarbon chain, while the polar group can be ionic (anionic or cationic), non-ionic or amphoteric (Nitschke and Pastore, 2002).

Surfactants tend to be distributed at the interface between liquid phases with different degrees of polarity (oil-in-water and water-in-oil). The formation of a molecular film, placed at the interface, reduces the interfacial and surface tensions, being responsible for the unique properties of surfactants (Nitschke and Pastore, 2002).

Aguedo (2002) tested several surfactants (Tween 80, Triton X-100 and Saponin with neutral character; SDS, with anionic character; and CTAB, with cationic character) to determine their effects on the viability of yeasts, on the interaction with their membranes, on the emulsion, on the hydrophobicity of yeasts surface and on the biotransformation. The author concluded that Tween 80, a non-ionic surfactant derived from olyethoxylated sorbitan and oleic acid, with a molecular weight of 1310 g mol^{-1}, was the ideal surfactant, since it was the compound that allowed achieving higher γ-decalactone concentrations. In addition, it had no effect on the viability of cells and did not interact with their membranes, providing a greater interfacial surface to the medium and the largest relative surface hydrophobicity to the cells, among all compounds tested. The production of γ-decalactone obtained with concentrations of 1 g L^{-1} and 2 g L^{-1} Tween 80 was identical, using the same methyl ricinoleate concentration: 10 g L^{-1}. However, the hydrophobicity of cells was higher in the second case. The interfacial surface of the emulsion was very similar in both cases.

Due to the presence of the lipophilic group, when in solution, the surfactants preferentially occupy the surface of the liquid, reducing the cohesive force between the solvent molecules and, consequently, lowering the surface tension. However, after saturating the surface, the addition of new molecules of surfactant has little effect on the surface tension value, especially after reaching a certain critical concentration (CMC), where molecular aggregates of colloidal dimensions, called micelles, are formed spontaneously. The CMC depends on the structure of the surfactant (more specifically on the size of the hydrocarbon chain) and on the experimental conditions (ionic strength, counter ions, temperature, etc.) (Behring *et al.*, 2004).

2.5.3 Lipids-yeast interactions

The cells growth in a medium containing lipids occurs directly on the surface of droplets since the solubility of oil in water is limited (Gutierrez and Erickson, 1977) and it is, thus, influenced by the size of the oil droplets (Bakhuis and Bos, 1969).

The interfacial surface of the oil droplets and consequently the surface between the two liquid phases is a determining factor for the degradation of the hydrophobic substrate and, thus, for the cells growth and aroma production (Aguedo *et al.*, 2005b). The lipidic droplets size is influenced by numerous parameters, which may be related to physical and chemical characteristics of the medium (pH, ionic strength, presence of surfactants, etc.) (Wilde, 2000) or to the presence of microorganisms (inoculum concentration, cells surface properties, etc.). During the biotransformation of ricinoleic acid into γ-decalactone, there is a direct contact between the cells surface and the substrate droplets, which may occur by adhesion of cells to the larger droplets surface or by adsorption of micro-droplets at the cells surface (Bakhuis and Bos, 1969). The assimilation of lipids may occur by diffusion or active transport at the contact points between cells and lipids. For the adhesion to be possible, the cell surface must present an affinity for the organic phase, i.e., having globally or partially a hydrophobic character, or the medium must contain emulsifiers which will be located at the oil/water interface, as it is the case of the emulsifier Tween 80. Thus, the role of emulsifying agents is very important for an optimal degradation of lipids. The hydrophobicity of the cell surface is considered one of the most important factors in the assimilation of hydrophobic compounds (Kim *et al.*, 2000). In the case of diffusion, several mechanisms may be involved including Van der Waals, Lewis acid-base, hydrophobic and electrostatic interactions (Aguedo *et al.*, 2003a).

2.6 GAS-LIQUID MASS TRANSFER

The processes involving the cultivation of microorganisms and accumulation of products are often influenced by the oxygen transfer rate (*OTR*) to the culture medium, especially in the case of aerobic microorganisms, such as the yeast *Y. lipolytica*.

In aerobic processes, oxygen is a key substrate and, due to its low solubility in aqueous solutions; a continuous oxygen transfer from the gas phase to the liquid phase is required in order to maintain

the respiratory activity of cells (Sauid and Murthy, 2010). In general, the dissolved oxygen concentration variation in aerobic reactors is described by the following mass balance equation (Eq. 2.1).

$$\frac{dO}{dt} = OTR - OUR$$

Eq. 2.1

Where: O represents the dissolved oxygen concentration within the reactor, t, the time and OUR, the oxygen uptake rate.

While the oxygen transfer rate to the medium exceeds the oxygen uptake rate and no other nutrient is limiting, cellular growth continue at an exponential rate.

The oxygen uptake rate by cells depends on cell density (X) and on the specific oxygen uptake rate (q_{O2}), as shown in Eq. 2.2.

$$OUR = q_{O_2} \cdot X$$

Eq. 2.2

At a critical cell concentration, oxygen may no longer be supplied to the medium fast enough to meet the oxygen requirements. Under these conditions, oxygen becomes the limiting nutrient for cell growth and is responsible for low cell densities and low yields (Junker *et al.*, 1990). Due to this critical impact on productivity, several solutions have been proposed to increase the oxygen transfer rates from the gas phase to the liquid medium, e.g., by projecting different reactor geometries, through agitation and aeration rates or by using different types of spargers (Junker *et al.*, 1990). The increase of the partial pressure of the gas phase has also been used to improve the oxygen transfer to the medium. That increase may be achieved by supplying pure oxygen to the bioreactor or by increasing the total air pressure (Yang and Wang, 1992; Belo *et al.*, 2003; Aguedo *et al.*, 2005a). Under these conditions, the oxygen requirements of a given culture can be satisfied

with low volumetric mass transfer coefficient (k_La) values and therefore with reduced hydrodynamic friction, resulting from shear stresses caused by high agitation speeds (Henzler and Kauling, 1993). Several researchers have demonstrated the existence of limits of tolerance of microorganisms to shear stress (Toma *et al.*, 1991; Märkl *et al.*, 1991).

The oxygen transfer rate in a bioreactor depends on the fluid physical properties (viscosity, superficial tension, etc.), temperature, pressure, solution composition, agitation, gas superficial velocity and configuration of the reactor (type of bioreactor, distribution of stirrer design, etc.) (Puthli *et al.*, 2005). The oxygen transfer rate in a system is a function of the volumetric mass transfer coefficient, which is the product of the liquid side mass transfer coefficient (k_L) by the gas-liquid interfacial area (a), and the driving force resulting from the difference between the dissolved oxygen concentration and the oxygen saturation concentration in the liquid phase at the gas-liquid interface (O^S) (Tribe *et al.*, 1995; Rols *et al.*, 1990) (Eq. 2.3).

$$OTR = k_L a\left(O^S - O\right)$$

Eq. 2.3

For a specific medium and bioreactor with an optimized design, it is possible to increase k_La values and, consequently, OTR, by using high agitation and aeration rates. These variables can be correlated according to Eq. 2.4.

$$k_L a = \delta \cdot \left(\frac{P_g}{V}\right)^{\alpha} \cdot \left(U_g\right)^{\beta}$$

Eq. 2.4

Where P_g represents the power input of the aerated bioreactor, V the bioreactor working volume, U_g the superficial gas velocity through the bioreactor and δ, α and β are numerical constants.

However, the use of high agitation and aeration rates causes high power consumption, significantly increasing operation costs. High stirring rates also present the limitation associated to its impact to

cells sensitive to hydrodynamic stress. This problematic can be overtaken by the addition of a second, water-immiscible phase, in which oxygen has a higher solubility. This system has been proposed by several authors as an alternative mean to improve *OTR* (Sauid and Murthy, 2010; Galaction *et al.*, 2004; Nielsen *et al.*, 2003). Most of the works described in the literature use an additional inert and external compound (like hydrocarbons (Rols *et al.*, 1990; Ho *et al.*, 1990), perfluorocarbons (Yoshida *et al.*, 1970; Amaral *et al.*, 2008) or silicone oils (Quijano *et al.*, 2010), among others) as the second liquid phase (organic phase), with the sole purpose of improving mass transfer from the gas to the aqueous phase. The effects of adding this organic phase on k_La may vary, and it was observed that k_La values may increase, decrease or remain constant, depending on the nature of the organic compound and on the operating conditions.

In order to take into account the effect of the organic phase (X_{ORG}) on k_La, empirical correlations have been proposed (Eq. 2.5), assuming that the two liquid phases behave as a single homogeneous phase (Nielsen *et al.*, 2003).

$$k_L a = \delta \cdot \left(\frac{P_g}{V} \right)^{\alpha} \cdot (v_s)^{\beta} (1 - X_{ORG})^{\varpi}$$

Eq. 2.5

ϖ, representing a numerical constant.

In previous works from our research group, the impact of Tween 80 presence on the overall k_La values was also taken into account, a series of correlations were developed, among which, a global one that contemplates the presence and effect of both organic and surfactant (X_{Tween}) fractions in the medium (Gomes *et al.*, 2007)(Eq. 2.6).

$$k_L a = \delta \cdot \left(\frac{P_g}{V} \right)^{\alpha} \cdot (v_s)^{\beta} (1 - X_{ORG})^{\varpi} (1 - X_{Tween})^{\phi}$$

Eq. 2.6

ϕ, representing a numerical constant.

The coexistence of an organic phase and an aqueous phase in the culture medium where the nutrients and cells are present turns the system more complex concerning oxygen transfer. To study the effect of adding an immiscible liquid phase upon the gas transfer rate in a gas-liquid-system, it is necessary to separate the effects of interfacial area, *a*, from those of the mass transfer coefficient in the liquid phase, k_L (Cents *et al.*, 2001).

The determination of *OTR* and k_La is essential for the characterization of bioreactors in aerobic processes. Through these parameters it is possible to determine the optimum aeration and to quantify the effect of each of the factors influencing oxygen transfer (Stanbury and Whitaker, 1984).

Ideally, *OTR* should be evaluated in a bioreactor with the culture medium and the cell population (Bailey and Ollis, 1977). However, not all methods available can be applied to active cultures.

There are several ways to determine k_La in a bioreactor. The most used traditional methods are divided in two groups, based on the state in which they are performed: steady-state or stationary-state (Stanbury and Whitaker, 1984). In this work only steady-state methods were applied, justifying the merely reference made to the other methods.

The steady-state methods, also known as “degassing methods”, are based on the introduction of a disturbance in the aeration conditions of the system, monitoring the subsequent alteration on the dissolved oxygen concentration.

The steady-state methods imply a reduction of the oxygen concentration up to a certain low value. There are two techniques that allow achieving that reduction: the static gassing-out and the dynamic gassing-out. Both techniques are described in detail in the next chapter (section 3.3)

2.7 FERMENTATION STRATEGIES: BATCH VERSUS FED-BATCH SYSTEMS

The fermentation by microorganisms is fundamental in biotechnological industry. Fermentations can be operated in batch, fed-batch or in continuous systems. In a batch system, all components,

except gaseous substrates, pH-controlling substances and antifoaming agents, are placed in the bioreactor at the beginning of the fermentation. During a process there is no input nor output movements. In a fed-batch system, nothing is removed from the reactor during the process but one substrate component is added in order to control the reaction rate by its concentration. Finally, in a continuous system there are both input and output flows, but the reaction volume is kept constant (Enfors and Häggström, 2000).

Considering that the primary goal of fermentation research is the cost-effective production of the products, it is important to develop a culture method that allows the production of the desired products at concentrations as high as possible, and with highest productivity and yield (Lee *et al.*, 1999b).

Fed-batch techniques are widely used in industrial applications (such as production of biomass and various bio-products including primary and secondary metabolites, proteins and other biopolymers) since they combine the advantages of both batch and continuous strategies. The process starts as batch but when the initial substrate (usually glucose) is consumed, substrate starts to be fed to the bioreactor. The inlet substrate feed should be as concentrated as possible to minimize dilution and to avoid process limitation caused by the reactor size. In a fed-batch process, the dilution rate refers to the components rate of dilution due to the volume increase caused by the inlet feed.

The main advantages of the fed-batch operation over the batch process are the possibilities to control both reaction rate and metabolic reactions by substrate feeding rate, allowing to achieve high cell density, which is often necessary for high yield and productivity of the desired product (Yamane and Shimizu, 1984).

Fed-batch strategy is especially interesting for γ-decalactone production, considering the toxicity of ricinoleic acid for the yeast cells (Feron *et al.*, 1996b; Lee *et al.*, 1998; Lin *et al.*, 1996). With this approach, it is possible to supply more substrate to the cells, preventing the inhibition effects of high concentrations of ricinoleic acid.

Several bioprocesses involving *Y. lipolytica* use the fed-batch operation mode successfully (Fickers *et al.*, 2009; Kim *et al.*, 1999; Kyong and Shin, 2000; Nicaud *et al.*, 2002; Rymowicz *et al.*, 2009; Turki *et al.*, 2010). However, there are very few works using this culture strategy for γ-decalactone production (Kapfer *et al.*, 1989; Kumin and Munch, 1998; Lee *et al.*, 1995) and none about its application for γ-decalactone production from methyl ricinoleate by *Y. lipolytica*. Thus, the

exploration of the fed-batch operation strategy to increase the yields of γ-decalactone production is of great interest.

Moreover, since the substrate of the process is ricinoleic acid, added in the form of an oil (usually methyl ricinoleate or castor oil), experimental problems arise when trying to add the substrate in a continuous mode, due to the viscosity of the oil that makes the feed difficult through the tubing with the aid of peristaltic pumps. Thus, the addition of that substrate by pulses (intermittent feeding) is a way of circumventing the problem (Lee *et al.*, 1995; Kim and Hou, 2006). In fact, the feeding profile of the fed-batch is approximated by periodical feedings.

3 GENERAL METHODOLOGY

The general methods, operating conditions and equipment used in this work are presented in this chapter.

Some specific developed methods presented in this Chapter were published in:

Gomes, N.; Gonçalves, C.; García-Román, M.; Teixeira, J. A.; Belo, I. (2011) Optimization of a colorimetric assay for yeast lipase activity in complex systems. Anal. Methods 3: 1008-1013.

Gonçalves, C.; Rodriguez-Jasso, R.; **Gomes, N.**; Teixeira, J. A.; Belo, I. (2010) Adaptation of dinitrosalicylic acid method to microtiter plates". Anal. Methods 2: 2046-2048.

3.1 MICROORGANISM, MEDIA AND CULTURE CONDITIONS

3.1.1 Microorganism

The microorganism used in the present work was the yeast strain *Yarrowia lipolytica* W29 (ATCC20460).

3.1.2 Yeast preservation

Y. lipolytica W29 was stored at -80 °C in cryogenic tubes (Microbank, Pro-Lab Diagnostics, Canada). After thawing, the strain was cultured for 48 h on YPDA medium (30 g L^{-1} agar, 20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract) at 27 °C. The colonies were stored at 4 °C, to be used posteriorly to inoculate the culture media.

3.1.3 Biomass growth medium

Unless otherwise stated, the cultures previously prepared were used to inoculate (at an optical density at 600 nm (OD_{600}) of 0.5, equivalent to 1.2×10^8 cells mL^{-1}) a 500-mL baffled Erlenmeyer flask containing 200 mL of glucose medium (YPD - Yeast Peptone Dextrose: 20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract).

The medium was sterilized in an autoclave, at 121 °C during 20 minutes.

Flasks were shaken at 140 rpm and 27 °C for 24 h until the cultures reached the late logarithmic growth phase, with a final OD_{600} of 4 (equivalent to 2×10^9 cells mL^{-1}), corresponding to total glucose consumption (confirmed by an adaptation of the DNS method described in the section 3.4.2). That suspension was used to inoculate the biotransformation medium.

In the experiments carried out in bioreactor, the cultures preserved in YPDA medium were used to inoculate 1.7 L of YPD medium in the bioreactor, with an OD_{600} of 1 (equivalent to 2.4×10^8 cells mL^{-1}). Cellular growth occurred at 27 °C, 500 rpm and 1.76 vvm (volume of air per volume of reactor per minute) for 19 h until the culture reached a final OD_{600} of 4, and the glucose was completely consumed.

Throughout this work, all spectrophotometry measurements were done on a modular absorbance microtiter plate reader (Sunrise, Tecan, Switzerland).

3.1.4 Biotransformation medium

After the biomass production phase, the biotransformation takes place. Throughout this work, two methodologies were tested for inoculation of the biotransformation medium: with or without the cell washing step. In the first case, cells from the YPD medium were washed three times in sterile water, centrifuged (6000 g, 5 min) and transferred to the biotransformation medium. In the second case, the components of the biotransformation medium were directly added as an emulsion to the YPD medium containing the cells, in order to start the biotransformation phase.

According to the results of Aguedo (2002), a medium composition of 10 g L^{-1} MR and 1 g L^{-1} Tween 80 was adopted to use in the experiments. In some experiments, different oil concentrations were used and therefore the concentration of Tween 80 had to be modified, but the ratio between the two compounds was kept constant.

The biotransformation medium composition is presented on Table 3.1.

Table 3.1 – Biotransformation medium composition.

Compound	Concentration (g L^{-1})
Ricinoleic acid source (castor oil or methyl ricinoleate)	10, 30 or 50
Tween 80	1, 3 or 5
NH_4Cl	2.5
Yeast Nitrogen Base (YNB) with amino acids	6.7

All chemicals were purchased from Sigma-Aldrich (Portugal), except for methyl ricinoleate that was kindly supplied by Stéarinerie Dubois (France) and by Biotor (India) and castor oil, which was purchased from Interfat, S.A. (Spain).

3.2 BIOREACTORS

3.2.1 BIOLAB bioreactor

A 2-L bioreactor (BIOLAB, B. BRAUN, Germany) with 22.5 cm height and 12 cm diameter was used to investigate the influence of oxygen in the lactones production process using 30 g L^{-1} MR as substrate and non-washed cells (Figure 3.1). In the experiments performed with this bioreactor, different agitation (400 and 600 rpm) and aeration rates (0.6, 1.8 and 3 vvm) were tested.

Figure 3.1 - BIOLAB bioreactor with biotransformation medium.

The medium was agitated with two six-blade turbine impellers. Air was supplied to the bioreactor with a sparger located at the base of the agitator shaft. The aeration flow was automatically controlled by a flow meter (Alicat Scientific, Inc., USA).

Dissolved oxygen concentration was measured with a polarographic-membrane probe (12/220 T, Mettler Toledo, USA) and monitored with a computer interface (CIODAS08JR, Computer Boards, USA) at 8 min intervals using the LABtech Notebook software (Datalab Solution, USA).

3.2.2 RALF PLUS SOLO bioreactor

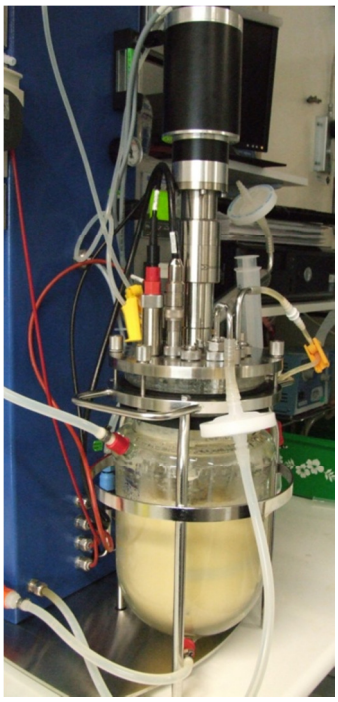

Figure 3.2 - RALF PLUS SOLO bioreactor with biotransformation medium.

A 3.7-L bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with 31 cm height and 17 cm diameter was used to carry out experiments on the optimization of γ-decalactone production, using 30 g L^{-1} MR as substrate and non-washed cells (Figure 3.2).

In a first step, a full factorial design with two factors at three levels (3^2), with an additional central point to estimate the intrinsic variability (pure error) in the data, was performed, in order to investigate the effect of important variables such as pH and dissolved oxygen concentration (DO) in the biotransformation medium, on the production of γ-decalactone and 3-hydroxy-γ-decalactone. A series of ten experiments was carried out in random order.

The pH values tested were: 4.5, 5.6 and 6.7 for pH; and the dissolved oxygen concentrations in the medium were 10%, 30% and 50%.

The setpoint values for pH and DO were automatically controlled by a control unit coupled to the bioreactor. The pH control was performed by addition of 5N KOH or 21% (v/v) orthophosphoric acid, through Peripex peristaltic pumps (Bioengineering, Switzerland). The DO concentration was controlled by manipulating the agitation and aeration rates, through a cascade control mode.

After defining the optimal operating conditions for γ-decalactone production, different fed-batch strategies were performed under those conditions, in order to optimize the process: fed-batch with

constant medium feeding rate and intermittent feeding. In the first case, the cellular growth occurred in 1 L of glucose medium, at which the biotransformation medium components were added. After reaching the maximum peak of γ-decalactone production, 1.5 L of biotransformation medium began to be fed to the bioreactor at a constant rate of 1 mL min^{-1} (initial dilution rate = 0.06 h^{-1}). In the second approach, 30 g L^{-1} MR were added to the bioreactor, in two cycles. The beginning of each cycle occurred when no more MR was detected in the biotransformation medium.

3.2.3 Bubble column

A square bubble column contactor built in *Perspex* (polymethylmethacrylate – PMMA) with a working volume of 0.9 L and geometrical characteristics of 4 cm inside side-length and height of 65 cm was used in the oxygen mass transfer studies (Figure 3.3).

Figure 3.3 – Bubble column contactor.

Air was used as gas stream in the gas-liquid contactor and it was fed at the bottom of the bubble column using a five holes sparger. The inlet gas flow-rate was measured and controlled with a mass flow controller (Alicat Scientific, USA).

3.2.4 Airlift bioreactor

Figure 3.4 - Airlift bioreactor with biotransformation medium.

An airlift bioreactor was also used for oxygen mass transfer studies and to carry out biotransformation essays (Figure 3.4).

This bioreactor was also constructed in *Perspex* with a working volume of 4.5 L and 7 cm inside diameter. The height of the riser-tube was 0.37 m with an inside diameter of 0.032 m. Air was used as gas stream in the gas-liquid contactor and it was fed at the bottom of the bioreactor using a five holes sparger. The inlet gas flow-rate was measured and controlled with a mass flow controller (Alicat Scientific, USA).

3.3 GAS-LIQUID MASS TRANSFER DETERMINATION

3.3.1 Static gassing-out technique

For the experimental k_La determination in experiments without cells, the static gassing-out technique was used.

Originally proposed by Wise in 1951, this technique allows evaluating the effect of operational variables (like agitation and aeration rates) on the oxygen transfer efficiency.

The oxygen present in the medium is initially removed by gassing the liquid out with compressed nitrogen, so that the medium is free of oxygen. Aeration is then initiated at a constant air flow rate until saturation and the dissolved oxygen concentration is measured with a polarographic-membrane probe (12/220 T, Mettler Toledo) and monitored with a computer interface

(CIODAS08JR, Computer Boards, USA) at 5 s intervals using the software LABtech Notebook, Datalab Solution.

To estimate the probe response time (τ), the method describing the response of the probe to a step in dissolved oxygen concentration by a first-order system was used (Tribe *et al.*, 1995). The obtained τ value at 27 °C, was thus 7 s, and it was used to correct k_La values.

The technique is based on the oxygen mass balance equation (Eq. 3.1) which, in the absence of cells and in batch mode, is simplified to the equality between the time variation of the dissolved oxygen concentration (dO/dt) and the oxygen transfer rate from the gas to the liquid.

$$\frac{dO}{dt} = k_L a\left(O^S - O\right)$$

Eq. 3.1

The integration of this equation gives k_La, which is equal to the symmetrical slope of the line resulting from the graphical representation of the logarithm of $1 - \frac{O}{O^S}$ as function of time (Stanbury and Whitaker, 1984).

Figure 3.5 presents, for illustrative purposes, the evolution of dissolved oxygen concentration in the medium without cells and the graphical representation of the logarithm of $1 - \frac{O}{O^S}$ as function of time.

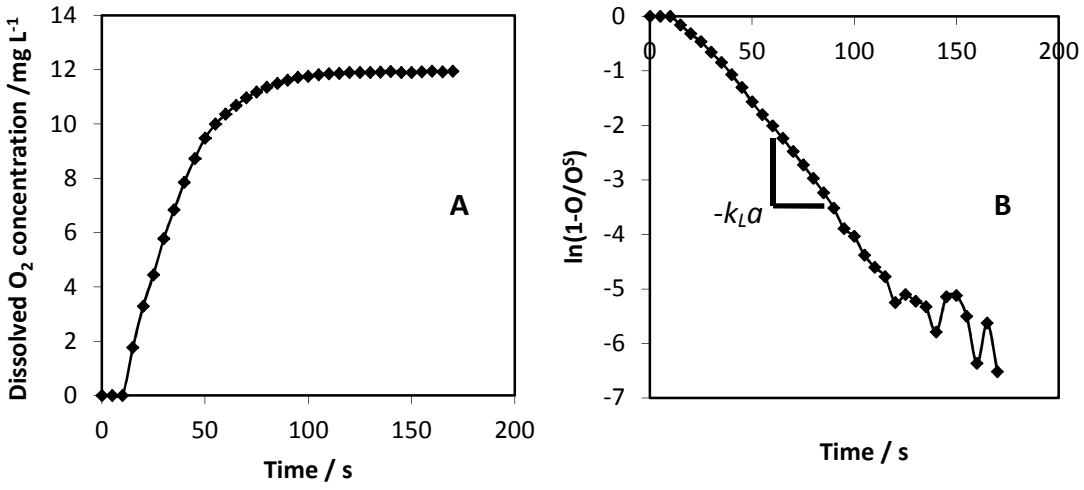

Figure 3.5 – Time course of dissolved oxygen concentration in the biotransformation medium without cells after re-aeration (A) and logarithmic representation of 1-(O/O^S) (B).

3.3.2 Dynamic gassing-out technique

For the experimental k_La determination in essays with cells, the dynamic gassing-out technique was used.

In the presence of active cells and in the absence of aeration, Taguchi and Humphrey (1966) used the respiratory activity of microorganisms to remove oxygen from the medium. This technique has the advantage of being performed during a fermentation, thus, providing more realistic k_La values.

The procedure involves two steps: one to stop aeration and one of resumption of aeration in the operating conditions. Thus, in the first step, monitoring the decrease of dissolved oxygen concentration will allow to determine the specific oxygen uptake rate (*OUR*) by Eq. 3.2.

$$\frac{dO}{dt} = -OUR$$

Eq. 3.2

Aeration is resumed before reaching the critical dissolved oxygen concentration value, below which oxygen consumption is limited. According to Tribe *et al.* (1995), the aeration should be resumed

when dissolved oxygen concentration is 1.5 times the critical concentration value (the amount of oxygen in the liquid phase above in which the microbial growth is not oxygen limited) for a given strain. This limits the applicability of the method to cultures with high oxygen requirements, near the maximum oxygen transfer capacity of the system, where the dissolved oxygen concentration is near the critical concentration.

The oxygen requirements of each microorganism depend on the biochemical nature of the cell and its nutritional environment. However, when the dissolved oxygen level in the medium is lower than the critical concentration, the specific oxygen uptake rate is also dependent on oxygen concentration. In opposition, when the oxygen concentration in the liquid phase is higher than the oxygen critical concentration, the oxygen uptake rate is independent of oxygen concentration.

The oxygen critical concentration value depends on the microorganism and substrate used. According to Bailey and Ollis (1977), the oxygen critical concentration for yeast is 0.12 mg L^{-1} at 20 °C and 0.15 mg L^{-1} at 34.8 °C.

After the resumption of aeration (second step), the oxygen mass balance in the liquid phase is expressed by Eq. 3.3.

$$\frac{dO}{dt} = k_L a\left(O^S - O\right) - OUR$$

Eq. 3.3

Considering the pseudo-steady state immediately before the determination, *OUR* can be replaced by (Eq. 3.4):

$$k_L a\left(O^S - O_i\right) = OUR$$

Eq. 3.4

Where O_i is the dissolved oxygen concentration in the beginning of determination.

By integration of this last equation, results Eq. 3.5:

$$\ln\left(\frac{O_i - O}{O_i - O_0}\right) = -k_L a\left(t - t_0\right)$$

Eq. 3.5

O_0 and t_0 being, respectively, the dissolved oxygen concentration and the time, when aeration is resumed.

The graphical representation of the term on the left side of Eq. 3.5, as a function of time, gives a line whose slope is $-k_La$ (Figure 3.6).

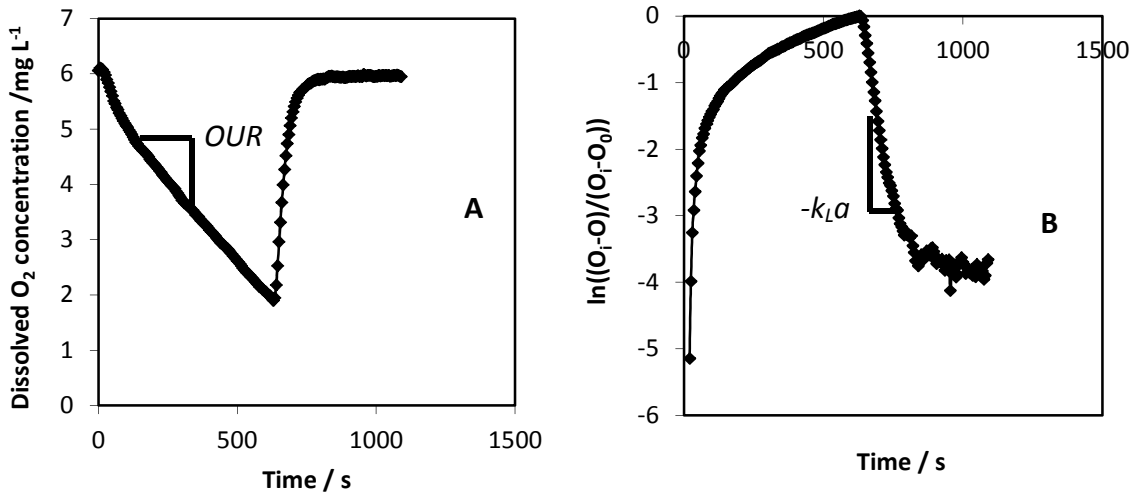

Figure 3.6 – Time course of dissolved oxygen concentration in the biotransformation medium with cells after cessation of aeration, followed by its resume (A) and logarithmic representation of (O-O_i)/(O-O_0) (B).

Tribe *et al.* (1995) demonstrated that the use of Eq. 3.5 upon k_La calculation introduces less error than the use of other arrangements of Eq. 3.3, involving the derivative of the oxygen probe response time.

3.4 ANALYTICAL METHODS

Samples were collected at appropriate intervals for analysis of cell concentration and viability and for lactones quantification. In some experiments, lipase and protease activities were also determined.

3.4.1 Cell concentration

3.4.1.1 Dry weight

The biomass determination was determined by dry weight, using a microwave oven (Olsson and Nielsen, 1997). The method consists in the following steps:

- Drying the filter (pore size 0.45 μm, Orange Scientific, Belgium) in the microwave oven on 150 W for 10 min. Then, the filter is placed in a desiccator during 10-15 min and weighed.
- The cell suspension is filtered and the cells are washed with distilled water.
- The filter is dried again in the microwave oven for 15 min at 150 W, placed in a desiccator where it is allowed to cool for 10-15 min and then it is weighed.

3.4.1.2 Cell counting

Cell concentration was determined by direct counting under the microscope, using a Neubauer-improved counting chamber (Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany) (Mather and Roberts, 1998).

3.4.1.3 Cell viability

For viability, the methylene blue method was used (Bonora and Mares, 1982).

Methylene blue is blue in its oxidized form, becoming colorless when reduced. According to Postgate (1967) when the methylene blue enters viable cells, it is reduced by a dehydrogenase which is inactive in the dead cells. Another explanation of the method is the assumption that the methylene blue penetrates only cells whose selective permeability of the plasma membrane is seriously compromised (Bonora and Mares, 1982).

The method consists in adding to a given sample volume, an equal volume of methylene blue solution (0.1 g L^{-1}) and waiting 5 minutes. The stained suspension is then placed in a Neubauer-improved chamber and observed under a microscope. The fraction of viable cells is the ratio between the number of cells not stained and the total number of cells.

3.4.2 Reduced sugars quantification

Reduced sugars were measured by an adaptation of the dinitrosalicylic acid (DNS) method. In the presence of reducing sugars, 3,5-dinitrosalicylic acid is reduced into 3-amino-5-nitrosalicylic acid, a brownish compound that strongly absorbs light at 540 nm, allowing a quantitative spectrophotometric measurement of the amount of reducing sugars present in a given sample.

The reaction was carried out in wells of 340 μL, adding 25 μL of DNS reagent to 25 μL of sample previously filtered (pore size 0.20 μm, Orange Scientific, Belgium) or distilled water (blank). The microtiter was subsequently placed, with cap, in an oven during 5 minutes, then it was placed on ice and 250 μL of distilled water were immediately added to each well (Gonçalves *et al.*, 2010).

The absorbance values obtained in each essay were converted to a reducing sugar concentration expressed in grams per liter (g L^{-1}), using a calibration curve previously obtained. This calibration curve was achieved by preparing six glucose solutions with concentrations between 0.05 and 1 g L^{-1} and analyzing them according to the technique previously described and representing graphically OD as a function of the known concentrations of each solution.

3.4.3 Lactones extraction and quantification

For the quantification of lactones, 2-mL medium samples were removed and their pH was lowered to 2 with HCl to promote the total lactonization of 4-hydroxydecanoic acid. The extraction of lactones was performed with 2 mL of diethyl ether by 60 gentle shakings, after addition of γ-undecalactone as internal standard. After the complete separation of the liquid phases, the ether phase was separated and analyzed by gas chromatography (Varian 3800 instrument, Varian, Inc., USA) with a TR-WAX capillary column (30 m × 0.32 mm × 0.25 μm, Teknokroma, Spain).

Operating conditions:

Temperature of the split injector: 250 °C

Temperature of the detector: 300 °C

Oven temperature: programmed to increase from 60 °C to 145 °C, at a rate of 5 °C min^{-1} and then to 180 °C, at a rate of 2 °C min^{-1}.

Pressure of the carrier gas (He) = 50 kPa

Data were analyzed using the acquisition and integration software Star Chromatography Workstation v. 6.30 (Varian, Inc., USA) and to the calibration curves previously obtained for γ-decalactone and γ-undecalactone.

The average of various concentration/area ratios was determined, for both compounds, which allowed determining the response coefficient (*K*), which is obtained from the ratio between the averages corresponding to γ-decalactone and γ-undecalactone.

The determination of *K* allows quantifying γ-decalactone using Eq. 3.6.

$$[\gamma - decalactone] = [\gamma - undecalactone] \times \frac{1}{K} \times \frac{A_{\gamma - decalactone}}{A_{\gamma - undecalactone}}$$

Eq. 3.6

Where:

[γ-decalactone] represents the concentration of γ-decalactone, in mg L^{-1};

[γ-undecalactone] is the concentration of γ-undecalactone, in mg L^{-1};

$A_{\gamma decalactone}$ refers to the area of γ-decalactone;

$A_{\gamma undecalactone}$ corresponds to the area of γ-decalactone.

3.4.4 Enzymatic activities

Total lipase (lipase adhered to cell surface and extracellular) activity was determined by a spectrophotometric method based on the method that uses *p*-nitrophenyl butyrate (*p*-NPB) as substrate (Morin *et al.*, 2003), which was further adapted and validated. The reaction mixture was composed of 950 µL of substrate (2.63 mM *p*-nitrophenyl butyrate in 50 mM sodium acetate buffer, pH 5.6, with 4% (v/v) Triton X-100) and 50 µL of broth sample. The mixture was incubated for 15 minutes at 37 °C and the reaction was stopped by the addition of 2 mL of acetone. The absorbance was measured at a wavelength of 405 nm. One unit of activity was defined as the amount of enzyme that produces 1 µmol of *p*-nitrophenol per minute under assay conditions.

Total protease activity was quantified at 37 °C for 40 min, as described elsewhere (Pinto, 1998), using 0.5% (*w*/v) azocasein in acetate buffer (50 mM, pH 5.0) as substrate. The reaction mixture was composed of 500 µL of substrate and 500 µL of broth sample. After incubation, 10% (w/v) trichloroacetic acid was added to precipitate residual protein not hydrolyzed by the proteolytic enzymes. The sample was then centrifuged (3000 rpm, 5 min) and 1 mL of 5 N potassium hydroxide, was added to the supernatant, producing a pinky-orange color, characteristic of the azo groups in alkaline pH. The intensity of this coloration was measured at a wavelength of 428 nm. One unit of activity was defined as the amount of enzyme that caused an increase of 0.01 of absorbance relatively to the blank per minute, under assay conditions.

4 OXYGEN MASS TRANSFER

In this chapter, the process of oxygen mass transfer to the biotransformation medium used to produce γ-decalactone, was analyzed. That medium is a complex system consisting of an oil-in-water emulsion stabilized by a non-ionic surfactant. Due to the large number of components in the liquid phase, it is of great importance to analyze the way in which gas transfer occurs and the effect of each component in the process. These experiments were carried out in a bubble column and/or in an airlift bioreactor.

The results presented in this Chapter were published in:

Gómez-Díaz, D.; **Gomes, N.**; Teixeira, J. A.; Belo, I. (2009) Oxygen mass transfer to emulsions in a bubble column reactor. Chem. Eng. J. 152: 354-360.

Gómez-Díaz, D.; **Gomes, N.**; Teixeira, J. A.; Belo, I. (2010) Gas-liquid interfacial area in the oxygen absorption to oil-in-water emulsions in an airlift reactor. Can. J. Chem. Eng. 88: 561-564.

4.1 OXYGEN MASS TRANSFER TO OIL-IN-WATER EMULSIONS IN BUBBLE CONTACTORS

Adapted from *Chemical Engineering Journal* (2009) 152: 354-360 and from *The Canadian Journal of Chemical Engineering* (2010) 88: 561-564.

Chemical Engineering Journal 152 (2009) 354–360

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Oxygen mass transfer to emulsions in a bubble column contactor

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GAS–LIQUID INTERFACIAL AREA IN THE OXYGEN ABSORPTION TO OIL-IN-WATER EMULSIONS IN AN AIRLIFT REACTOR

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4.1.1 Introduction

Reactions involving three-phase systems are frequently found in different industrial processes. In general, the third phase (solid or liquid) dispersed in a gas-liquid system can be a reagent, substrate or a heterogeneous catalyst. Although the majority of works are focused on gas-liquid-solid systems (Mena *et al.*, 2005; Zhang *et al.*, 2005; Dagaonkar *et al.*, 2003), gas-liquid-liquid systems are gaining importance due to the increase of this type of application in bioprocess and homogeneous catalysis systems (Dumont and Delmas, 2003).

Nowadays reactors or bioreactors involving two liquid phases (organic and aqueous phases) are used as an alternative to other systems because they may improve the overall process efficiency

(Dumont and Delmas, 2003). Two different fields of application have been considered for these reactors: (i) reactors for the cleaning of gaseous streams by means of the capture of pollutant gases (for instance, the presence of an organic phase could enhance the capture of volatile organic compounds due to the hydrophobic nature of these gases) (Dumont *et al.*, 2006a); (ii) bioreactors that enhance the oxygen mass transfer rate by the addition of an organic phase (Dumont *et al.*, 2006b). Usually this organic phase is an inert and external compound (like perfluorocarbons and silicone oils, among others), added to the system with the purpose to improve mass transfer from the gas to the liquid aqueous phase (Dumont and Delmas, 2003). These systems are encountered in some biochemical applications where a hydrophobic compound with higher oxygen solubility than water is used to improve the oxygen transfer rate to the culture medium. However, some biotechnological processes are based on the development of microorganisms within a biphasic medium formed by an oil-in-water emulsion where the oil is the substrate to be degraded. In these cases, the organic substrate can retard the gas-liquid mass transfer, thus limiting the overall productivity.

The system herein presented is an emulsion of methyl ricinoleate in water, stabilized by Tween 80. This system has a great interest because it constitutes the medium used to produce γ-decalactone by the yeast *Y. lipolytica* (Aguedo *et al.*, 2005a) and where the organic phase is the substrate of the process and not an inert compound added only with the aim of enhancing oxygen mass transfer.

The biochemical pathway leading to the aroma production has been extensively studied. However, crucial steps controlling the production ratio of γ-decalactone and other C10 lactones (Figure 2.3) are not totally understood. The oxygen is believed to influence these side-reactions, but this point remains to elucidate. Since *Y. lipolytica* is an obligate aerobe, oxygen is a crucial operational parameter for the control of processes involving this yeast. Previous studies (Gomes *et al.*, 2007) have analyzed the influence of operating conditions on oxygen mass transfer and on γ-decalactone production in a bubbling stirred bioreactor.

The aim of this work is to study the oxygen mass transfer mechanism in the mentioned biphasic medium, especially the influence of operating conditions and emulsion composition to develop the absorption process and the way oxygen is transferred to the biphasic system.

4.1.2 Materials and methods

4.1.2.1 Medium

The biphasic medium used in this work consists of an aqueous-liquid phase and an organic-liquid phase of MR (Stéarinerie Dubois, Boulogne, France), forming an emulsion stabilized by Tween 80. Different concentrations of MR (from 0 to 5 g L^{-1}) and Tween 80 (from 0 to 1 g L^{-1}) were tested, as well as air flow-rates from 0.25 to 1.34 L min^{-1}. Silicon anti-foaming was employed.

The experiments were carried out in a bubble column (section 3.2.3) and/or in an airlift (section 3.2.4) reactor.

4.1.2.2 Liquid velocity

The velocity of liquid circulation was determined in the airlift reactor with the neutral buoyancy flow follower technique. A small square piece of plastic (side length, 0.5 cm; density, 1030 Kg m^{-3}) was used as a flow follower. The time taken by the follower to traverse a known vertical distance (0.2 m) in the downcomer was noted and the flow velocity was calculated from an average of 10 measurements. The density of the follower was very close to that of the liquid (1010 Kg m^{-3}).

4.1.2.3 Gas-liquid interfacial area

For determination of the gas-liquid interfacial area, a bubble column with geometrical characteristics of 4 cm inside side-length and height of 65 cm, was used due to the need of plane reactor walls to enable the determination of bubbles size by a photographic method. Since the diameter and height of the bubble column are similar to those of the riser's airlift reactor, results can be extrapolated.

Bubbles diameter was measured using a photographic method based on images of the bubbles taken along the height of the column, from the bottom to the top. A Sony (DCR-PC330E) video camera was used to obtain the images. The column height was divided in three sections and a minimum number of 50 well-defined bubbles along the column were used to evaluate the size distribution of the bubbles in the liquid phase for the different gas flow-rates investigated. The

Image Tool v3.0 software was used to carry out the required measurements of the bubbles geometrical characteristics.

4.1.2.4 Oxygen mass transfer

Oxygen mass transfer studies in the medium were carried out in a square bubble column and in an airlift reactor (section 3.2.3 and 3.2.4).

For the experimental k_La determination, the static gassing-out technique was used (sub-chapter 3.3.1).

Other experiments of oxygen absorption in emulsions were performed in a stirred cell (Figure 4.1). In this contactor, the oxygen transfer occurs through a planar gas-liquid interface. The gas-liquid interfacial area was determined from the geometrical characteristics (internal diameter = 8 cm and height = 15 cm) of the stirred cell. Four baffles have been placed in its internal wall to improve mixing and prevent vortex formation during stirring. The liquid phase volume used in these experiments was 250 mL. The experimental procedure consisted in the addition of different quantities of oil to the aqueous phase in the absence of Tween 80. The absorption rate was studied with basis on the variation of oxygen concentration in the liquid phase.

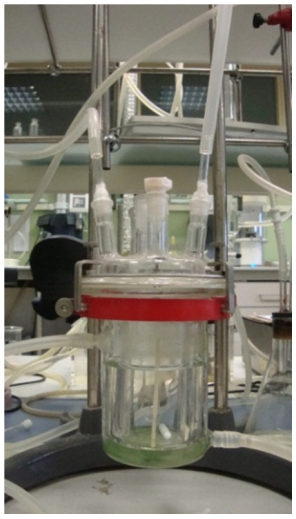


Figure 4.1 – Stirred cell used in the experiments of oxygen absorption in emulsions.

4.1.2.5 Interfacial tension and viscosity

Liquid-liquid interfacial tension was determined with a Krüss K-11 tensiometer using the Wilhelmy plate method. The plate employed was a commercial platinum plate supplied by Krüss. The platinum plate was cleaned with water and acetone and flame-dried before each measurement.

The experimental set-up to carry out viscosity measurements was a capillary viscosimeter (Schott Gerate AVS 350), connected to a thermostated bath. An electronic stopwatch with an accuracy of ± 0.01 s was used for measuring efflux times.

4.1.3 Results and discussion

4.1.3.1 Liquid velocity

Figure 4.2 shows that the increase of the air flow rate leads to an improvement of the liquid velocity, although it seems to be a trend to become constant at the higher aeration rates tested.

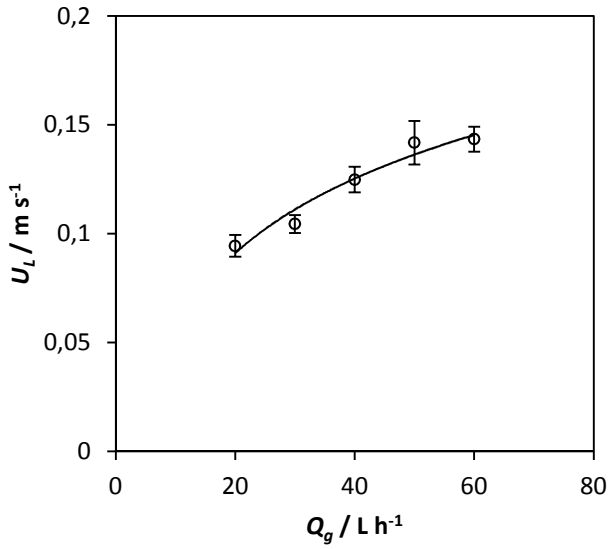


Figure 4.2 - Liquid velocity (U_L) generated into the air-lift reactor at different aeration rates (Q_g).

4.1.3.2 Gas-liquid interfacial area

The use of bubble contactors involves the analysis of the gas-liquid interfacial area generated under the different experimental conditions employed in this study, in order to obtain useful information that allows the understanding of the mass transfer mechanism in this complex system based on the values of the mass transfer coefficient and on the influence of the gas flow-rate and liquid phase composition upon this parameter. For this reason, the study of gas-liquid interfacial area generated in the bubble column is the first step necessary to analyze the oxygen absorption process.

The photographic method used in this work for the gas-liquid interfacial area determination was proposed in previous works (Sotiriadis *et al.*, 2005; Couvert *et al.*, 1999). The geometrical characteristics of air bubbles produced in the contactor enable to calculate the value of the gas-liquid interfacial area. Figure 4.3 shows an example of the photographs taken from the bubble column.

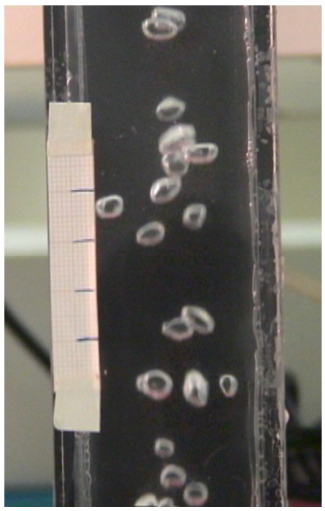


Figure 4.3 - Photograph of the bubble column using an aqueous solution of Tween 80 as liquid phase. Q_g = 0.5 L min^{-1}; [Tween 80] = 1 g L^{-1}.

The bubbles produced in the contactor have ellipsoidal shape and for this reason, major (E) and minor (e) axes of the projected ellipsoid (in two dimensions) must be determined. The diameter of the equivalent sphere (d_B) was taken as the representative bubble dimension.

Through Eq. 4.1 it is possible to determine the diameter distribution of a bubble present along the gas-liquid contactor.

$$d_B = \sqrt[3]{E^2 \cdot e}$$

Eq. 4.1

An example of the accumulative bubble diameter distribution and the influence of surfactant concentration upon this kind of distribution are shown on Figure 4.4.

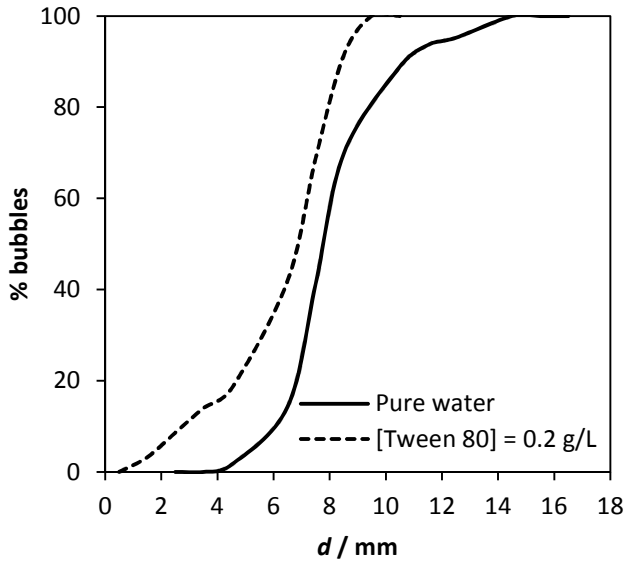


Figure 4.4 - Influence of Tween 80 concentration in the liquid phase upon the bubble size. Q_g= 0.5 L min^{-1}.

According to the results displayed on Figure 4.4, the presence of Tween 80 in the liquid phase produces a clear decrease in the bubble diameter formed in the bubble column, in agreement with Sardeing *et al.* (2006).

The bubble diameter distribution along the bubble contactor has a direct influence on the value corresponding to the gas-liquid interfacial area. The interfacial area determination is developed on the basis of two important parameters: the Sauter mean diameter and the gas hold-up.

Different authors recommend the use of the Sauter mean diameter (d_{32}) (Shah *et al.*, 1982), that can be determined by Eq. 4.2 using the data calculated for the equivalent diameter.

$$d_{32} = \frac{\sum_i \left(n_i \cdot d_i^3\right)}{\sum_i \left(n_i \cdot d_i^2\right)}$$

Eq. 4.2

Where n_i is the number of bubbles that have an equivalent diameter (d_i).

The gas hold-up (ε_g) is an important parameter that brings information about the flow behavior into the bubble column. It corresponds to the volume fraction of gas phase in the gas-liquid dispersion and it was determined in the square bubble column, due to the difficulty to measure the variations on the height of the liquid in the airlift reactor. The use of this methodology is possible due to the absence of bubbles in the downcomer of the airlift reactor.

The gas hold-up was thus calculated using the difference between the liquid height in the absence of gas in the bubble column and under the different experimental conditions of gas flow-rate fed to the contactor (Vasconcelos *et al.*, 2003) (Eq. 4.3).

$$\varepsilon_g = \frac{\Delta V}{\Delta V + V_L}$$

Eq. 4.3

Where V_L is the ungassed liquid volume and ΔV is the volume expansion after gas dispersion, calculated from the liquid level change and the cross sectional area. The change of volume in the bubble column was calculated based on the change observed in the liquid level and the increase of this value after gassing.

The experimental results obtained for the gas hold-up in the bubble column are reported in Figure 4.5.

An increase in the gas flow-rate (or surface gas velocity) produces a clear increase in the gas hold-up value with a linear trend. It can also be observed that there are no significant differences between the different liquid phases compositions tested, although for higher gas flow-rates it seems that the gas hold-up becomes greater for the aqueous medium. This makes sense since bubbles increase their size in water and therefore, its residence time decreases.

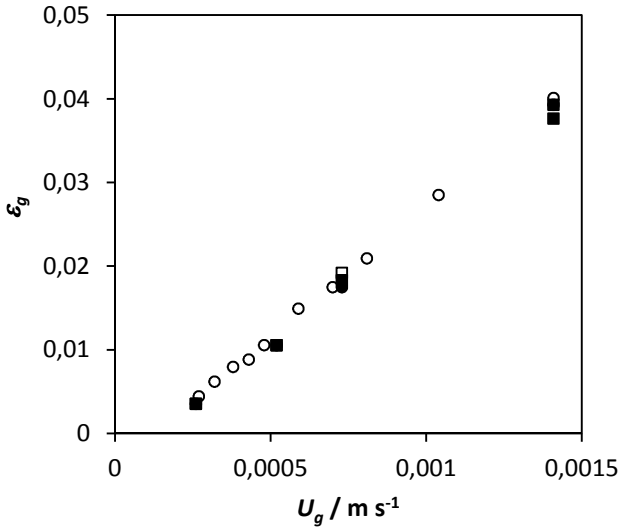


Figure 4.5 - Influence of surface gas velocity (U_g) upon gas hold-up (ε_g). (○) Pure water; (●) 1 g L^{-1} Tween 80; (□) 1 g L^{-1} MR and 1 g L^{-1} Tween 80; (■) 5 g L^{-1} MR and 1 g L^{-1} Tween 80.

The Sauter mean diameter and the gas hold-up values allowed the calculation of the specific gas-liquid interfacial area using Eq. 4.4 (van't Riet and Tramper, 1991).

$$a = \frac{6 \cdot \varepsilon}{d_{32} \cdot \left(1 - \varepsilon_g\right)}$$

Eq. 4.4

This experimental methodology allows the determination of the gas-liquid interfacial area under the different experimental conditions (liquid phase composition and gas flow-rate) assayed in this work.

Experimental results shown in Figure 4.6 indicate the influence of Tween 80 concentration and gas flow-rate upon the specific interfacial area generated in the bubble column. When the gas flow-rate increases, the interfacial area also increases due to the previously observed behavior caused by this variable upon the gas hold-up (Figure 4.5). The gas hold-up increases with the gas flow-rate because a higher number of bubbles are generated in the sparger, thus increasing the interfacial area. Moreover, since a homogeneous regime is maintained, an increase in the gas flow-rate also increases the bubble size due to low degree of coalescence, but with lower effect on interfacial area than on gas hold-up.

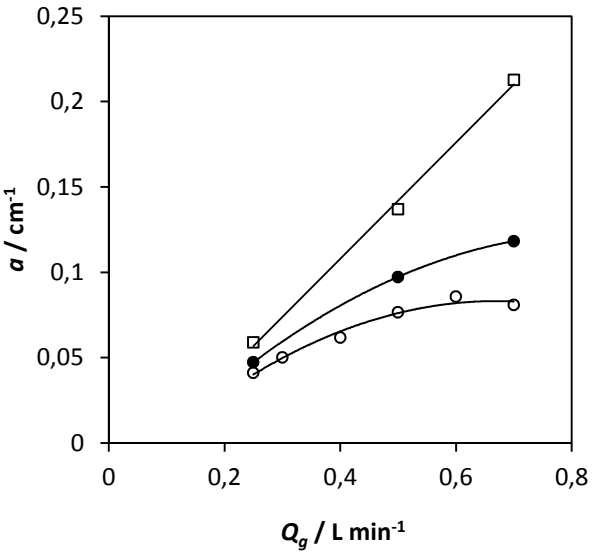


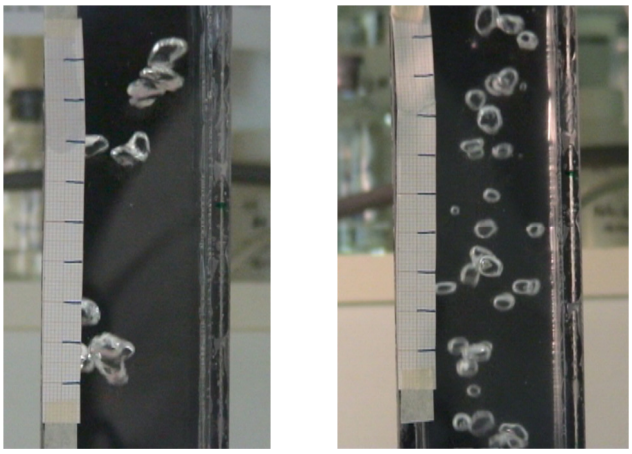
Figure 4.6 - Influence of gas flow-rate (Q_g) and Tween 80 concentration in the liquid phase upon gas-liquid interfacial area (a). (○) [Tween 80] = 0 g L^{-1}; (●) [Tween 80] = 0.02 g L^{-1}; (□) [Tween 80] = 1 g L^{-1}.

When pure water was employed as liquid phase in the bubble contactor at high values of gas flow-rate, such an increase was not observed because, at high values of gas flow-rate, there is a great number of bubbles that collide producing coalescence and then an increase in the bubbles diameter. This increase in the bubbles size has a negative effect upon the gas-liquid interfacial area

and reduces the positive effect caused by the gas hold-up. Thus, for pure water, the effect caused by coalescence upon gas-liquid interfacial area is higher than the influence caused by gas hold-up.

The presence of surfactant in the liquid phase produces a clear increase in the value of the specific interfacial area (Figure 4.6) and the increase regarding the corresponding value for pure water is higher when the gas flow-rate increases. This behavior may have different causes. One of them is the clear decrease in the liquid phase surface tension when the surfactant concentration increases (Shah *et al.*, 1982) as it causes the formation of bubbles with minor size, increasing the interfacial area (Vogler *et al.*, 1993). Also, the presence of surfactant molecules in the liquid phase produces a decrease in the bubbles effective collisions and then the bubbles small size remains constant along the bubble column (Lee *et al.*, 2005).

Photographs in Figure 4.7 confirm that the presence of Tween 80 in the liquid phase produces a decrease in the bubbles size, implying an increase in the gas-liquid interfacial area.

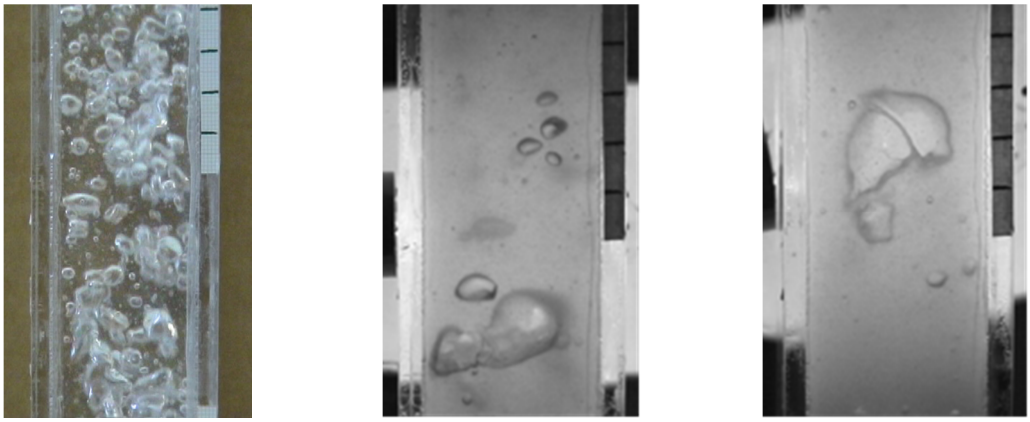


Pure water [Tween 80] = 1 g L^{-1}

Figure 4.7 - Photographs from the middle section of the bubble column. Q_g = 0.5 L min^{-1}.

After studying the influence of Tween 80 upon the bubbles diameter, gas hold-up and gas-liquid interfacial area, the effect of organic phase addition to the aqueous solutions was also analyzed. For this study, concentrations of methyl ricinoleate from 0 to 5 g L^{-1} were added. The operating conditions regarding the gas flow-rate were kept in the range of 0.25 to 1.34 L min^{-1}. Surfactant concentration remained constant at its higher value (1 g L^{-1}) in order to maintain the stability of the

emulsion. The concentration of organic phase was modified to analyze its influence upon the interfacial area. Figure 4.8 and Figure 4.9 show the influence of the organic phase presence upon the bubbles size.



A B C

Figure 4.8 - Photographs from the bubble column at an air flow-rate of 1.34 L min^{-1} for different liquid phase compositions: (A) aqueous solution with 1 g L^{-1} Tween 80; (B) emulsion with 3 g L^{-1} MR and 1 g L^{-1} Tween 80; (C) emulsion with 5 g L^{-1} MR and 1 g L^{-1} Tween 80.

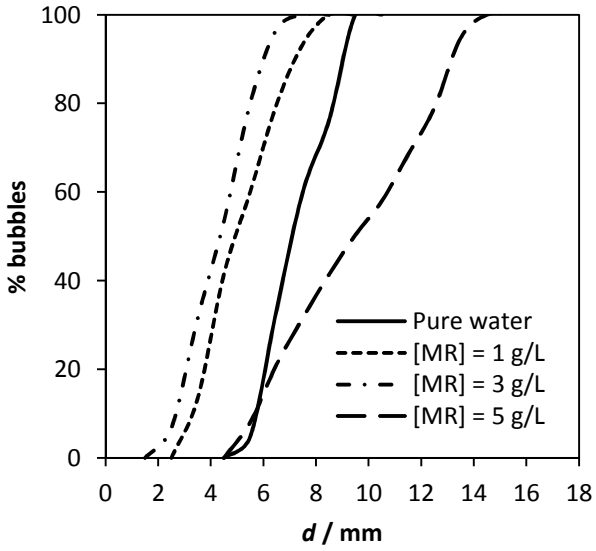


Figure 4.9 - Influence of methyl ricinoleate concentration upon the bubbles size distribution for water and emulsions. Q_g = 0.5 L min^{-1}.

The images of Figure 4.8 clearly indicate that an increase of the methyl ricinoleate concentration in the liquid phase produces an increase in the bubbles size.

As previously mentioned, the addition of Tween 80 to pure water produces a clear decrease in the bubbles size, however when oil is added to the aqueous phase, a continuous increase in the bubbles diameter is observed and, for the highest oil concentration, the bubbles diameter are higher than the corresponding ones for pure water.

Using the same procedure than the previously employed for aqueous solutions of Tween 80, the gas-liquid interfacial area was calculated for oil-in-water emulsions. The experimental results obtained for these systems are presented in Figure 4.10 that shows a decrease in the specific interfacial area value when the oil concentration increases in the liquid phase. This effect is negligible for the lowest gas flow-rate analyzed but when the values of this variable increase, the presence of the organic phase has an important contribute to the values of interfacial area.

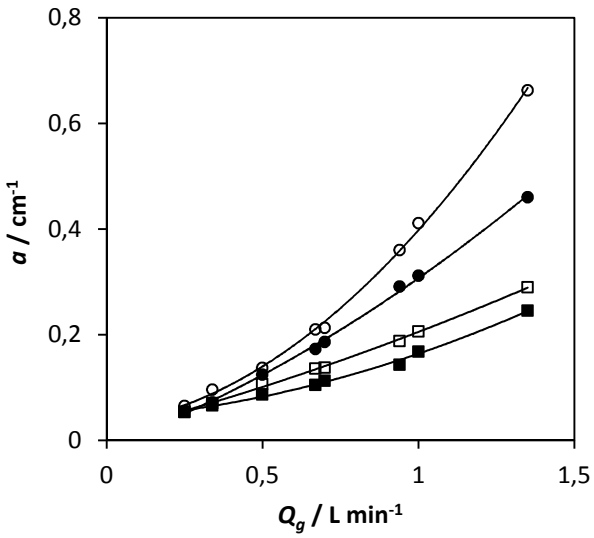


Figure 4.10 – Influence of the gas flow-rate and methyl ricinoleate concentration on the interfacial area in the airlift bioreactor. All systems had 1 g L^{-1} Tween 80 in their composition: (○) 0 g L^{-1} MR; (●) 1 g L^{-1} MR; (□) 3 g L^{-1} MR; (■) 5 g L^{-1} MR.

This behavior is assigned to a change in the surfactant concentration at the gas-liquid interface because when an organic phase is added to aqueous solutions with Tween 80, a new distribution of surfactant between interface and bulk phases is originated.

Interfacial tension measurements between water and methyl ricinoleate allowed to confirm this assumption, because a decrease in the value of interfacial tension was observed when Tween 80 was added to the liquid-liquid biphasic system (Figure 4.11). This distribution affects the value of the liquid phase surface tension and consequently bubbles produced under these conditions have higher diameters than the ones formed in the absence of the organic phase.

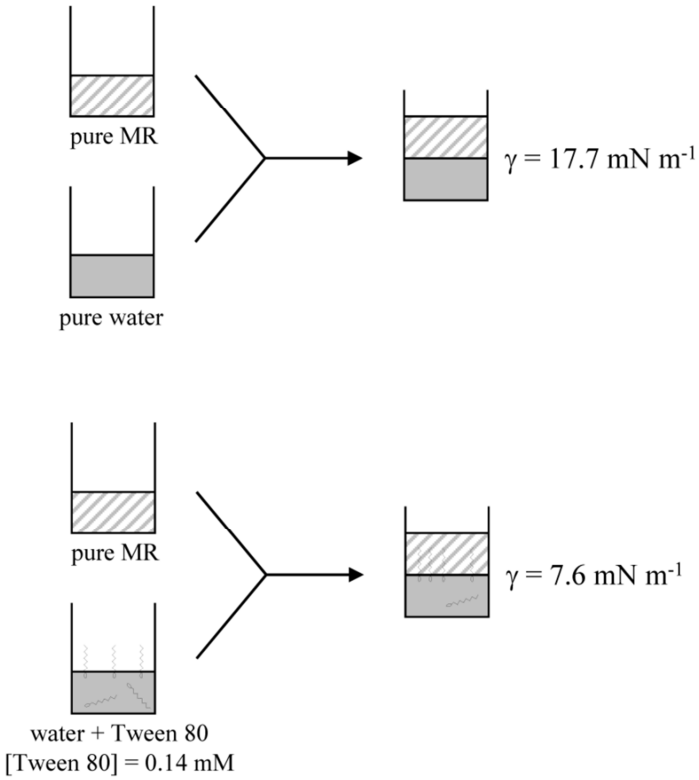


Figure 4.11 – Effect of Tween 80 upon the interfacial tension in water-methyl ricinoleate system.

Moreover, the presence of the surfactant in the aqueous phase reduced the coalescence process but in this case, the concentration of surfactant in the gas-water interface decreased and

consequently the reduction in coalescence was minor and the bubbles increased their diameter in the ascension along the bubble column.

4.1.3.3 Gas-liquid mass transfer

Semi-continuous regime has been employed in the present work and Eq. 4.5 was used to determine the volumetric mass transfer coefficient (k_La) obtained, using oxygen concentration data over time.

$$\frac{dO}{dt} = k_L a(O^S - O)$$

Eq. 4.5

To use this equation, it is assumed that the mixture is homogeneous within the reactor and the volumetric mass transfer coefficient was determined taking into account the time constant of the oxygen probe.

Liquid side mass transfer coefficient was calculated as the quotient between the volumetric mass transfer coefficient and the specific gas-liquid interfacial area determined previously (section 4.1.3.2).

The oxygen mass transfer to the biphasic medium was investigated in the bubble column and in the airlift bioreactor.

4.1.3.3.1 Bubble column

The influence of surfactant concentration on the value of the mass transfer coefficient is shown in Figure 4.12.

An addition of Tween 80 to the liquid phase produced an important decrease in the mass transfer coefficient due to the presence of surfactant molecules at the gas-liquid interface (Vasconcelos *et al.*, 2003). The presence of surfactant inhibits the mass transfer because its accumulation at gas-

liquid interface causes a decrease in the liquid surface elements renewal (turbulence near the interface) and it entails that the oxygen concentration in the liquid phase near to the gas-liquid interface is higher than the oxygen concentration in the bulk of the liquid phase. Therefore, the driving force of the absorption process decreases producing the same decrease in the absorption rate.

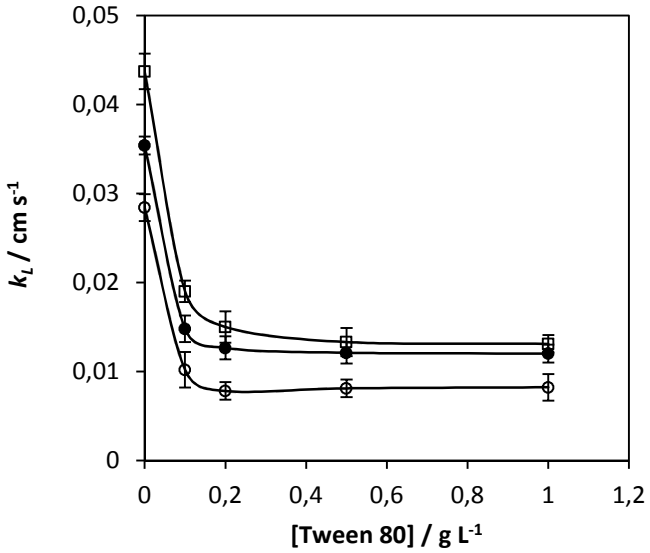


Figure 4.12 - Influence of surfactant concentration on the liquid side mass transfer coefficient. (○) Q_g = 0.25 L min^{-1}; (●) Q_g = 0.5 L min^{-1}; (□) Q_g = 0.7 L min^{-1}.

A similar behavior has been found by other researchers when they analyzed the influence of surfactant concentration in the liquid phase in an absorption process (Painmanakul *et al.*, 2005). An important decrease in the mass transfer coefficient at low surfactant concentrations is observed and then a slight decrease is produced when the surfactant concentration increases in the liquid phase. This slight decrease in the higher concentration range, is related to the critical micelle concentration of the surfactant in water ($CMC_{Tween\ 80}$ = 0.012 mM) (Haque *et al.*, 1999).

Figure 4.12 also shows that an increase in the air flow-rate leads to an increment in the mass transfer coefficient. This effect is due to an increase in the turbulence caused by the bubbles ascension along the bubble column.

The effect of the organic phase on the mass transfer coefficient in this bubble contactor was also analyzed and the experimental results are shown in Figure 4.13.

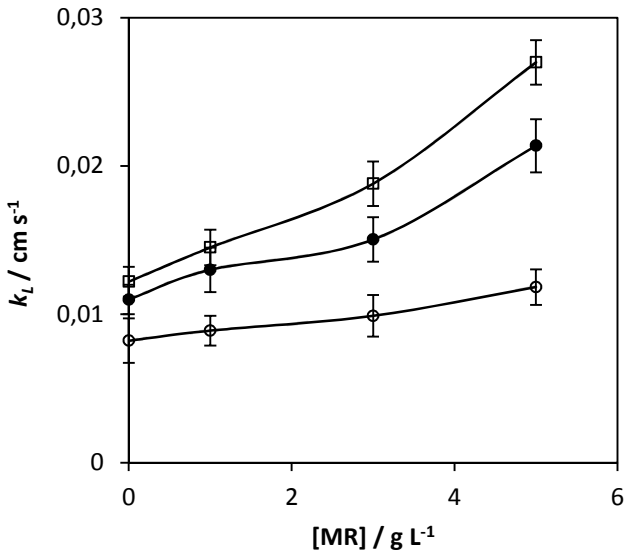


Figure 4.13 - Influence of organic phase concentration on the mass transfer coefficient. (○) Q_g = 0.25 L min^{-1}; (●) Q_g = 0.5 L min^{-1}; (□) Q_g = 0.7 L min^{-1}.

The presence of methyl ricinoleate in the liquid phase increased the mass transfer coefficient which was more pronounced at high values of gas flow-rate. These results are in agreement with a previous study carried out in a stirred tank contactor with this same system, where an increase in the volumetric mass transfer coefficient in the presence of organic phase was observed (Gomes *et al.*, 2007).

The explanation used for the influence on interfacial area can also be used to justify the behavior observed regarding the mass transfer coefficient. Thus, the presence of methyl ricinoleate in the liquid phase produced a new distribution of the surfactant molecules and then a decrease in Tween 80 concentration in the gas-liquid interface occurred. The presence of surfactant molecules produced a negative effect on the mass transfer coefficient (Figure 4.12) due to a reduction of the liquid renewal at interface. However, the presence of methyl ricinoleate caused an increase in the driving force and it improved the oxygen transfer to the bulk of the liquid phase. This new situation caused by the presence of the organic phase was positive for mass transfer.

Other authors have studied the absorption process in biphasic systems and they have observed a maximum in the value of the oxygen mass transfer rate, but for higher organic phase concentrations (Nielsen *et al.*, 2003).

The influence of the gas flow-rate on the mass transfer coefficient value for the emulsion system was similar to the previous results for aqueous solutions of Tween 80; an increase in its values occurred with an increase in the gas flow-rate. This is caused by the enhancement of turbulence in the liquid phase and at the gas-liquid interface.

Several studies (Cents *et al.*, 2001; Lekhal *et al.*, 1997) show that the influence of an organic phase on absorption processes can be due to the higher absorption capacity of the organic liquid, enhancing the mass transfer. For this reason, additional experimental work was developed in a flat stirred vessel. The results obtained (Figure 4.14) show that an increase in the presence of the organic phase over the water interface produces a decrease in the volumetric mass transfer coefficient (the interfacial area remains constant). This behavior is due to the higher viscosity of the organic phase (ν_{MR} = 68 mm^2 s^{-1}) when compared with water (ν_{water} = 0.9 mm^2 s^{-1}), producing a negative effect upon the mass transfer rate since it reduces the value of gas diffusivity in the liquid phase. Therefore, we consider that the positive effect on the mass transfer coefficient caused by the presence of methyl ricinoleate in this contactor is due to the reduction in Tween 80 concentration at the gas-liquid interface.

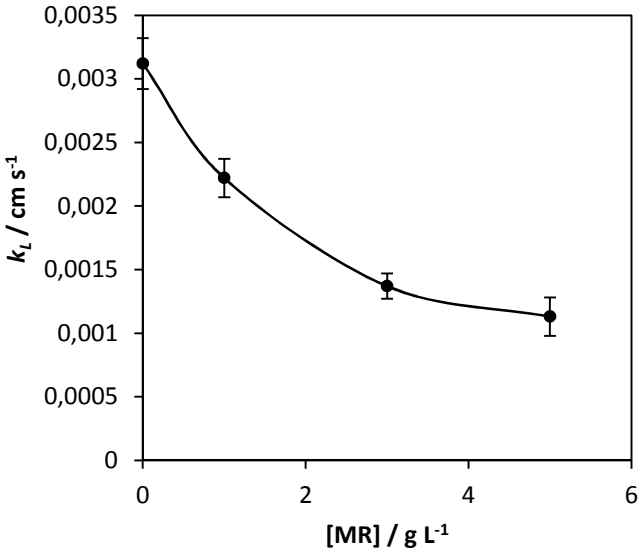


Figure 4.14 - Effect of organic phase on mass transfer coefficient, in a flat stirred vessel. Q_g = 0.333 L min^{-1}.

4.1.3.3.2 Airlift

The experimental results obtained for the different systems studied, as well as for the different gas flow-rates investigated, are displayed in Figure 4.15.

The positive effect of the gas flow-rate increase on the volumetric mass transfer coefficient was clear, as previously obtained. An increase in the gas flow-rate augmented the interfacial area and usually also produces an increase of the turbulence between the two liquid phases, which results in an overall mass transfer improvement.

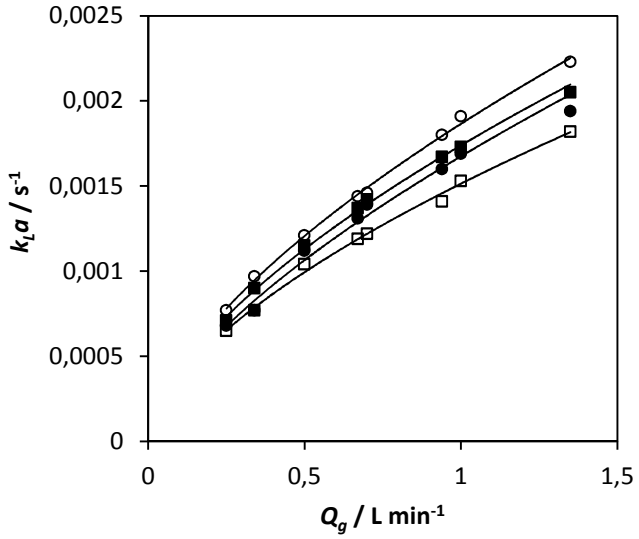


Figure 4.15 - Influence of the gas flow-rate (Q_g) and MR concentration in the liquid phase upon the volumetric mass transfer coefficient (k_La). (○) 0 g L^{-1} MR; (●) 1 g L^{-1} MR; (□) 3 g L^{-1} MR; (■) 5 g L^{-1} MR.

As the MR concentration increases in the emulsion, a decrease in the volumetric mass transfer coefficient is observed. However, for the highest oil concentration there is an increase. This behavior must be highly influenced by the liquid side mass transfer coefficient since the presence of MR decreases the interfacial area, as previously demonstrated.

The influence of the gas flow-rate and the organic phase upon the liquid side mass transfer coefficient is displayed in Figure 4.16.

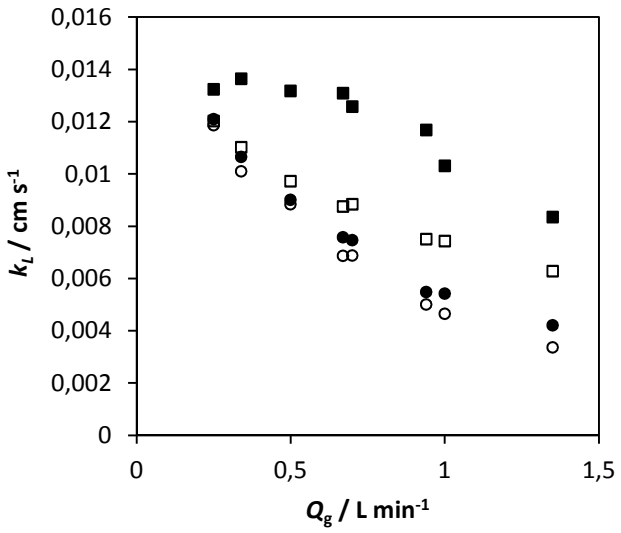


Figure 4.16 - Influence of the gas flow-rate (Q_g) and MR concentration in the liquid phase upon the mass transfer coefficient (k_L). (○) 0 g L^{-1} MR; (●) 1 g L^{-1} MR; (□) 3 g L^{-1} MR; (■) 5 g L^{-1} MR.

Increasing concentrations of the oil phase produced an increase in the mass transfer coefficient. The same comportment was observed in studies with the square bubble column, so the explanation for this behavior is identical.

Regarding the influence of gas flow-rate on the mass transfer coefficient, there is a decrease of this coefficient as the gas flow-rate increases. Since the effect of the interfacial area has been eliminated by dividing the volumetric mass transfer coefficient by the specific interfacial area, a discussion of these results should not be taken into account.

Usually, an increase in the gas-flow rate increases turbulence and therefore, the value of mass transfer coefficient. This was the behavior observed in the square bubble column. However, in the airlift, the effect is completely different.

In a bubble column the liquid circulates in a different way than how it does in the airlift, since in the latter, the gas pushes the liquid in the riser and then the liquid moves down the downcomer. In the case of the bubble column, the liquid is always contained in the same space. These differences are highly significant since they control the liquid flow.

Given an increased flow of the gas in the bubble column, the gas pushes the liquid up until that same liquid must come down in countercurrent with the gas phase. In this case it produces a high

turbulence, resulting in high renewal of elements in the liquid interface and therefore an increase in mass transfer.

In the case of the airlift, by increasing the gas flow, it pushes harder the liquid through the riser. In this type of reactor, the liquid then descends through the downcomer, reducing turbulence. This prevents the mixing between both phases, resulting in a decrease in the mass transfer coefficient.

4.1.4 Conclusions

Present work analyzed the absorption process of oxygen from an air stream towards a liquid heterogeneous phase composed of an aqueous and an organic phase, forming an emulsion, in a bubbling contactor. The complex nature of this kind of liquid phase implies the knowledge of the influence of each one of the components on the gas-liquid mass transfer process.

Hereby, the presence of the surface active substance used to stabilize the emulsion has a significant effect both on the interfacial area generated in the bubble column and on the mass transfer coefficient due to the accumulation trend of this kind of compound at the gas-liquid interface. The presence of the organic phase has a positive effect on the oxygen mass transfer rate to the liquid phase, but on the other hand, it produces a very clear decrease of the interfacial area, which has a negative effect on the global absorption process.

The knowledge acquired from this work about the oxygen transfer to the biotransformation medium will be applied in the subsequent chapters referring to aroma production strategies.

5 EFFECT OF SUBSTRATES ON BIOTRANSFORMATION

Two main sources of ricinoleic acid are referred in literature as commonly used in the biotechnological production of γ-decalactone: castor oil and methyl ricinoleate. Previous works (Gomes *et al.*, 2007; Aguedo *et al.*, 2005a) used 10 g L^{-1} MR in the process but small aroma concentrations were achieved (up to 300 mg L^{-1}). Thus, it was decided to test higher substrate concentrations and to compare both substrates. Moreover, different inoculation strategies were also approached as well as the role of extracellular lipases on the process.

In this chapter, the use of methyl ricinoleate will be firstly investigated, followed by the use of castor oil as substrate for γ-decalactone production.

Finally, the oil droplets size is characterized in emulsions with different substrate concentrations and the impact on the aroma production is described.

The results presented in this Chapter were published in:

Gomes, N.; Teixeira, J. A.; Belo, I. (2010) The use of methyl ricinoleate in lactone production by *Yarrowia lipolytica*: Aspects of bioprocess operation that influence the overall performance. Biocatal. Biotransf. 28(4):227-234.

Gomes, N.; Waché, Y.; Teixeira, J. A.; Belo, I. (2011) Oil-in-water emulsions characterization by laser granulometry and impact on γ-decalactone production by *Yarrowia lipolytica*. Biotechnol. Lett. DOI 10.1007/s10529-011-0593-9.

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5.1 THE USE OF METHYL RICINOLEATE IN LACTONE PRODUCTION BY YARROWIA LIPOLYTICA: ASPECTS OF BIOPROCESS OPERATION THAT INFLUENCE THE OVERALL PERFORMANCE

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ORIGINAL ARTICLE

The use of methyl ricinoleate in lactone production by *Yarrowia lipolytica*: Aspects of bioprocess operation that influence the overall performance

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5.1.1 Introduction

Interactions of the lipid substrate with the cells may influence the overall performance of the process. These interactions depend not only on emulsion characteristics and the presence of surfactant (Aguedo *et al.*, 2005b), but also on the surface properties of the cells (sub-chapter 2.5.3). Manipulations of the cell, such as washing after pre-culture, prior to inoculation of the biotransformation medium, may alter the hydrophilic character of the cells, affecting subsequent interaction with the oil influencing the substrate uptake.

Besides differences in substrate uptake, accumulation of γ-decalactone in the medium results from the balance of rates of production and degradation by the cells.

Previous studies have shown the importance of oxygen mass transfer in the biotechnological γ-decalactone production (Aguedo *et al.*, 2005a; Gomes *et al.*, 2007). Since oxygen is more soluble in the organic phase (MR) than in the aqueous phase, increasing oil concentration improves oxygen mass transfer, which is important for the process efficiency. Oxygen transfer rate from the gas to the liquid medium can also be improved by increasing aeration and stirring rates.

The yeast used in this work, *Y. lipolytica*, is also known to be a lipase producer (Lopes *et al.*, 2008). Lipases (EC 3.1.1.3, triacylglycerol hydrolases) catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Sharma *et al.*, 2001), and their presence in the biotransformation medium may improve the availability of fatty acid substrates resulting from oil hydrolysis. However, lipase activity may be influenced by the activity of proteases since these enzymes may cause changes in lipase characteristics and also degrade lipases (Kulkarni and Gadre, 1999). In the present work, the induction of lipase and protease activities during biotransformation was monitored.

Previous studies on the biotransformation of ricinoleic acid into γ-decalactone using *Y. lipolytica* typically involved washing the cells from the preculture, prior to inoculation of the biotransformation medium (Aguedo *et al.*, 2005a; García *et al.*, 2007a). An alternative approach, without the washing step, was examined in the present work. For this, the components of the biotransformation medium were added to a culture of cells at late-logarithmic phase in glucose medium. In order to explain the differences obtained using washed or non-washed cells, the relative surface hydrophobicity of the cells was evaluated after both procedures.

The influence of several concentrations of a ricinoleic acid source (MR) on the production of γ-decalactone by *Y. lipolytica* is also reported in the present work. After selecting the best conditions in flask experiments, the aroma production was analyzed in a 2-L stirred bioreactor under different aeration conditions.

Thus, the aim of this sub-chapter was to address potentially limiting points of the process which, to our knowledge, have not been tackled yet.

5.1.2 Materials and methods

5.1.2.1 Operating conditions

The source of ricinoleic acid used was MR, at different concentrations: 10, 30 or 50 g L^{-1}. The content in Tween 80 had to be adjusted to maintain the oil/surfactant proportion, i.e., 1, 3 or 5 g L^{-1} Tween 80 were used.

Also, two different methodologies were tested for inoculation of the biotransformation medium: with or without the cell washing step. In the first case, cells from the YPD medium were washed three times in sterile water, centrifuged (6000 g, 5 min) and transferred to the biotransformation medium. In the second case, the components of the biotransformation medium (MR, Tween 80, NH_4Cl and YNB with aminoacids) were directly added to the YPD medium containing cells grown during 24 h, at the late logarithmic phase, with a final OD_{600} of 4.

Samples were collected at appropriate intervals for analysis of cell concentration and viability, aroma compound quantification, lipase and protease activities (section 3.4).

5.1.2.2 Microbial adhesion to hydrocarbons assay

Cells taken from YPD medium (with and without being washed three times in sterile water) were washed twice with phosphate buffer (0.1 M, pH 7), centrifuged (3000 rpm, 5 min) and re-suspended in the same buffer to an OD_{600} of approximately 0.70 (A_0). In a glass tube, one part of this suspension was mixed with six parts of hexadecane. The tube was gently inverted ten times and left until separation of the two phases.

Then, 2 mL of the aqueous phase were removed and the OD_{600} (A) was measured (Aguedo *et al.*, 2003a).

The results are given as percentage of adhesion:

$$\%\,\text{adhesion} = 100 - \left(100 \times \frac{A}{A_0}\right)$$

Eq. 5.1

5.1.3 Results and discussion

5.1.3.1 Flask experiments

5.1.3.1.1 Cell concentration

Two methods to inoculate the biotransformation media were investigated, differing by the performance of a cell washing step after the pre-culture, prior to the inoculation of the biotransformation medium.

Cell growth in the biotransformation medium was approximately 3.6-fold higher when cells were not washed (from 7×10^8 cells mL^{-1} up to 40×10^8 cells mL^{-1}) than in cultures of washed cells (from 7×10^8 cells mL^{-1} up to 11×10^8 cells mL^{-1}). In all cases, cell viability remained practically constant at 100%.

This was probably due to the presence of some residual components of the YPD medium. In addition, the washing procedure may alter the cell surface properties. This hypothesis was tested in the next section.

5.1.3.1.2 Microbial adhesion to hydrocarbons assay

In order to better explain the differences between washed or non-washed cells, the relative surface hydrophobicity of cells was evaluated. Several methods for this have been described (the binding to hydrocarbons, salt aggregation test, adhesion to hydrophobic solid surfaces, partitioning of microorganisms in aqueous polymer two-phase systems, hydrophobic interaction chromatography and measurements of the contact angle of dried cell layers) (Doyle and Rosenberg, 1990). However, the microbial adhesion to hydrocarbons (MATH) assay is the most practical and has a good repeatability (Capizzi and Schwartzbrod, 2001).

The results of the MATH test revealed that 15.3 ± 2.2% of non-washed cells adhered to hexadecane but only 7.9 ± 0.7% of washed cells (results represent an average of three independent experiments ± standard error), clearly showing that washed cells are less hydrophobic than non-washed. As a consequence, cells would possibly have a lower affinity for the substrate.

These differences in cell surface hydrophobicity, and probably in compatibility with the substrate, might explain the observed increase in cell growth in the biotransformation medium when non-washed cells were used, and may also influence the production of γ-decalactone and lipase (discussed in the following sub-chapters).

5.1.3.1.3 γ-Decalactone production

The elimination of the cell washing step had a positive impact not only on cell growth but also on γ-decalactone production (Figure 5.1). While a maximum concentration of 471 ± 71 mg L^{-1} was achieved at 35.5 h with washed cells and 50 g MR L^{-1}, a concentration of 997 ± 150 mg L^{-1} was obtained at 91 h, using the same substrate concentration, without washing the cells.

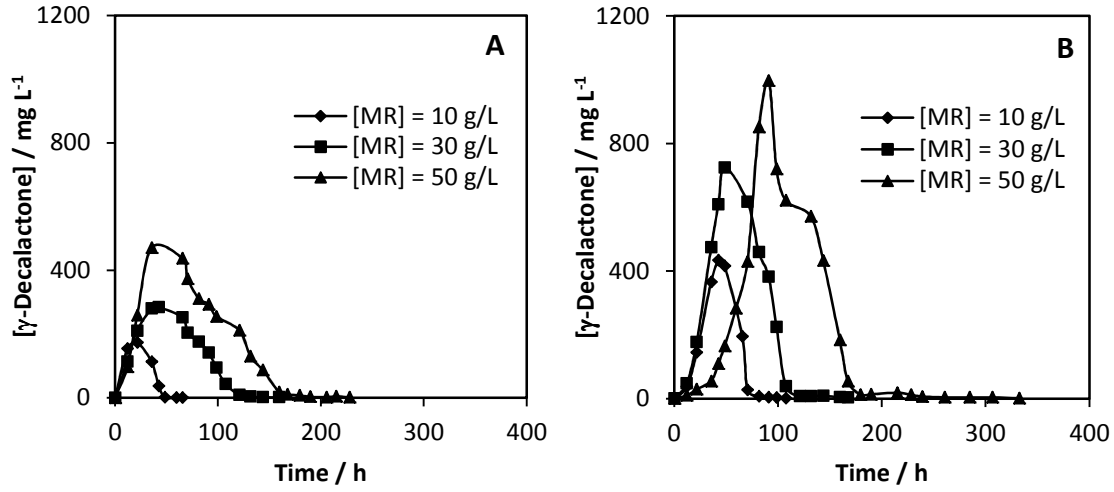

Figure 5.1 - Time course of γ-decalactone concentration in biotransformation medium with different concentrations of methyl ricinoleate (MR) and washed cells (A) or non-washed cells (B) (flask experiments).

The use of more hydrophobic cells increased slightly the affinity for the substrate and consequently its uptake by the cells, improving the overall process. However, another hypothesis is the presence of some residual components from the YPD medium in non-washed cells may account for the differences in the best production time observed with the different approaches. The conversion of ricinoleic acid, through the peroxisomal β-oxidation pathway, will only occur after complete consumption of all the other carbon sources.

Regardless of the start-up procedure adopted, higher concentrations of MR improved the production of γ-decalactone. In all cases, after the maximum content had been reached, the aroma concentration decreased due to the use of γ-decalactone as a carbon source (Aguedo, 2002), until complete disappearing from the medium.

Although the maximum aroma concentrations obtained with washed cells were 2.1- to 2.5-fold lower than those achieved with non-washed cells, the reaction time was faster. However, the volumetric productivities were still lower (Table 5.1). Based purely on volumetric productivity, the highest value (14.9 mg L^{-1} h^{-1}) achieved for 30 g MR L^{-1} with non-washed cells was low compared with that obtained by Aguedo (2002) using 10 g MR L^{-1} and washed cells (27.5 mg L^{-1} h^{-1}), possibly because of the time needed to consume all other carbon sources resulting from the residual YPD medium before the degradation of ricinoleic acid takes place.

Table 5.1 - Maximum productivity of γ-decalactone obtained for different methyl ricinoleate (MR) concentrations and start-up methods in flask experiments.

	[MR] (g L^{-1})	Productivity (mg L^{-1} h^{-1})
With washed cells	10	8.1
	30	6.7
	50	13.3
With non-washed cells	10	10.2
	30	14.9
	50	11.0

Another possible limitation in triglycerides hydrolysis due to poor lipolytic activity in the medium could also contribute to low productivity values, so the activity of extracellular lipase produced in this system was assessed. Proteases potentially decrease lipase activity, so the activity of these enzymes was also determined.

5.1.3.1.4 Enzymatic activities

During the biotransformation of MR into γ-decalactone with washed cells, there was no extracellular lipase production. However, there was also no protease production, indicating that the lack of lipase activity was not due to degradation by proteases, but simply lack of induction under these conditions.

In contrast, lipase and protease induction was observed when non-washed cells were used, possibly because higher substrate uptake by the more hydrophobic cells induced lipase expression and, consequently, protease production, since this enzyme is induced by the presence of extracellular proteins.

For all MR concentrations, there was a simultaneous induction of lipase and protease activities (Figure 5.2). For substrate concentrations of 30 g L^{-1} and 50 g L^{-1}, the activities of both enzymes showed identical profiles, i.e. the maximum activity of one coincided with the maximum of the other. This suggests that without the high proteolytic activity, greater lipase activity levels would probably have been observed. Thus, a potential explanation for the higher lipolytic activity in experiments with 10 g MR L^{-1}, compared with those with 30 g MR L^{-1} and 50 g MR L^{-1}, is the lower protease activity observed, which would result in slower lipase degradation.

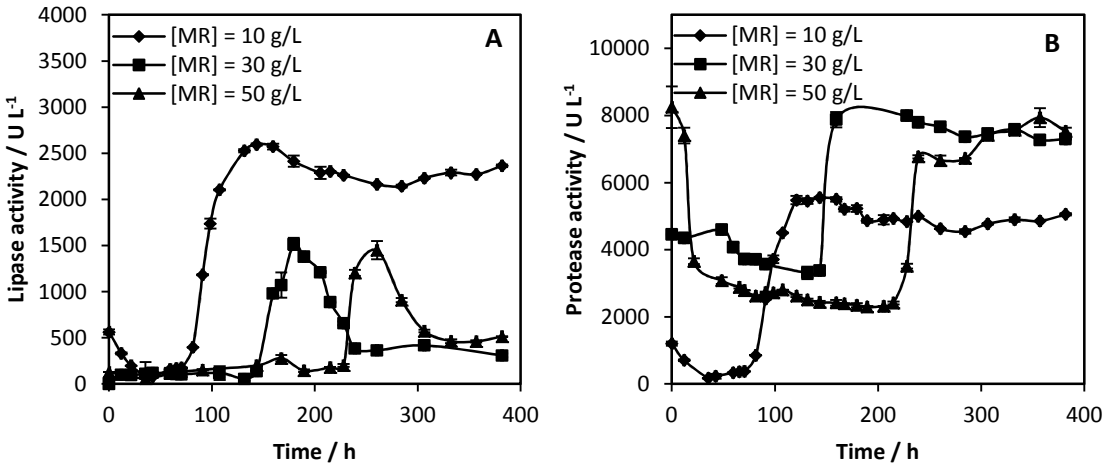

Figure 5.2 - Lipase (A) and protease (B) activity profiles in biotransformation medium with different concentrations of methyl ricinoleate (MR) and non-washed cells (flask experiments). Data are presented as the mean and standard deviation of three independent experiments.

For the range of concentrations tested, increases in oil concentration did not lead to higher lipase activity. In fact, the maximum lipase activity was achieved using the lowest substrate concentration tested, which was 10 g L^{-1}. In literature such a behavior was already reported. For example, Lakshmi *et al.* (1999) examined the effect of different substrates and concentrations on lipase production by *Candida rugosa* and, as in the present work, found that there was an optimum substrate concentration above which no increase in lipolytic activity occurred. In their experiments using castor oil, the maximum lipase activity was obtained using 10 g L^{-1} of substrate; at higher concentrations of oil, lipase activity decreased significantly. Those authors hypothesized that such a behavior could be due to the presence of hydroxy substituted C14 fatty acid esters.

Although the function of lipase is not critical for the production of γ-decalactone from MR since it can be hydrolyzed in the interior of the cell (Romero-Guido *et al.*, 2010), MR was shown to be a lipase inducer. Therefore, besides the aroma production, lipases could also be recovered as from the biotransformation medium as a high value-added by-product, since they have vast applications in food, dairy, detergent and pharmaceutical industries.

5.1.3.2 Bioreactor experiments

Since the highest productivity was achieved with 30 g MR L^{-1}, further investigation proceeded with this substrate concentration, in a 2-L bioreactor.

In order to increase the productivities obtained in flasks, experiments were carried out by varying the oxygen input to the medium, through the manipulation of agitation and aeration rates.

The time course of dissolved oxygen concentration during the growth and biotransformation phases is depicted in Figure 5.3. Complete depletion of dissolved oxygen occurred at the end of the growth phase, using aeration rates of 0.6 and 1.8 vvm, with an agitation rate of 400 rpm. No significant cell growth was observed during the biotransformation phase and cell viability remained practically constant (≈ 100%) during the whole experiment.

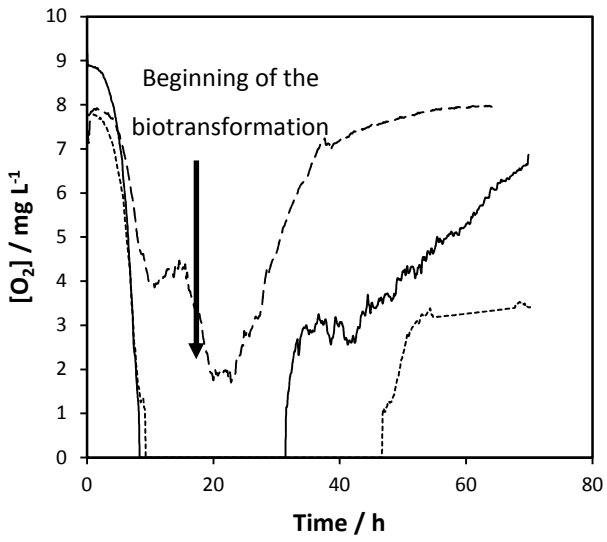

Figure 5.3 - Time course of dissolved oxygen concentration during the growth and biotransformation phases in the bioreactor under different agitation and aeration rates: (____) 400 rpm and 0.6 vvm; (- - -) 400 rpm and 1.8 vvm; (————) 600 rpm and 3 vvm.

The concentrations of γ-decalactone in bioreactor experiments were lower than those achieved in flasks (for the same medium composition) except when the oxygen mass transfer was higher, i.e. when agitation and aeration rates of 600 rpm and 3 vvm, respectively, were used (Figure 5.4). Under these conditions, the maximum concentration obtained was about 1 g L^{-1}, similar to that

obtained in the flask. It is clear that, for the conditions tested, the accumulation of γ-decalactone was directly dependent on the oxygen transfer to the medium. This conflicts with the results of Aguedo *et al.* (2005a), who concluded that low oxygen concentrations in the medium induced the control of the β-oxidation pathway by expression of acyl-CoA oxidase and therefore that an accumulation of γ-decalactone would occur. However, in their work, hyperbaric conditions were used to obtain higher dissolved oxygen concentrations and this may have toxic effects on this aerobic yeast (oxidative stress) (Belo *et al.*, 2005), possibly limiting γ-decalactone production.

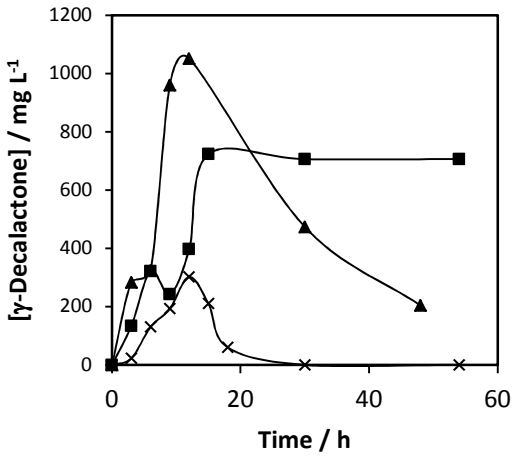

Figure 5.4 - Accumulation of γ-decalactone in the biotransformation medium under different operating conditions in the bioreactor: ×, 400 rpm and 0.6 vvm; ■, 400 rpm and 1.8 vvm; ▲, 600 rpm and 3 vvm.

Aroma production was much faster in the bioreactor and therefore the productivities achieved were greatly improved (Table 5.2).

Table 5.2 - Maximum productivity of γ-decalactone, obtained in bioreactor, under different operating conditions.

Operating conditions	Productivity γ-decalactone (mg L^{-1} h^{-1})
400 rpm; 0.6 vvm	25.2
400 rpm; 1.8 vvm	48.2
600 rpm; 3 vvm	87.6

In previous studies from our research group (Aguedo *et al.*, 2005a), different agitation and aeration rates were also tested in the production of γ-decalactone, using the same bioreactor. The productivities obtained for each pair of conditions were 1 mg L^{-1} h^{-1} (300 rpm and 0.3 vvm), 12.9 mg L^{-1} h^{-1} (600 rpm and 0.9 vvm) and 9 mg L^{-1} h^{-1} (600 rpm and 1.8 vvm). These values are quite low when compared with those achieved in the present more optimized process, probably due to the inclusion of the cell washing procedure prior to inoculation of the biotransformation medium. The present productivities are also much higher than the 14 mg L^{-1} h^{-1} obtained by Waché *et al.* (2001) in experiments carried out in a 2-L bioreactor. However, the oxygen transfer conditions used in their work were lower, since the agitation rate was 300 rpm and the aeration was 0.44 vvm.

Furthermore, the concentration of substrate used was also lower (5 g MR L^{-1}) and the cell washing step was performed. All these differences may explain the discrepancies obtained in the concentration of γ-decalactone.

Since it is known that oxygen may influence the activities of the enzymes of the peroxisomal β-oxidation pathway, allowing the accumulation of other compounds derived from 4-hydroxydecanoic acid (precursor of γ-decalactone), the production of 3-hydroxy-γ-decalactone, dec-2-enolide and dec-3-enolide was also analyzed.

In contrast to flask assays where no production of these compounds was evidenced, surprising concentrations of 3-hydroxy-γ-decalactone (up to 8 g L^{-1}) were detected (Figure 5.5) in the bioreactor.

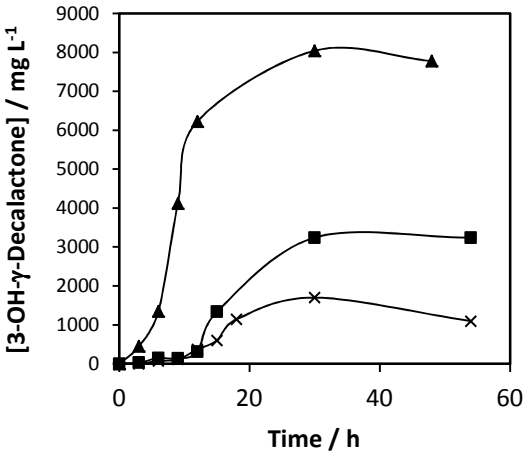

Figure 5.5 - Accumulation of 3-hydroxy-γ-decalactone in the biotransformation medium under different operating conditions in the bioreactor: (×), 400 rpm and 0.6 vvm; (■) 400 rpm and 1.8 vvm; (▲) 600 rpm and 3 vvm.

The increase of 3-hydroxy-γ-decalactone concentration in the medium was also dependent on oxygen transfer in agreement with the results of Aguedo *et al.* (2005a), who observed that 3-hydroxyacyl-CoA dehydrogenase was induced when higher oxygen levels were used, resulting in an accumulation of 3-hydroxy-γ-decalactone. García *et al.* (2007b) have also reported that this compound was formed using high aeration conditions. The productivities obtained for this compound were much higher than those obtained for γ-decalactone (Table 5.3).

Table 5.3 - Maximum productivity of 3-hydroxy-γ-decalactone, obtained in bioreactor, under different operating conditions.

Operating conditions	Productivity 3-OH-γ-decalactone (mg L^{-1} h^{-1})
400 rpm; 0.6 vvm	56.6
400 rpm; 1.8 vvm	108.0
600 rpm; 3 vvm	268.1

The high accumulation of 3-hydroxy-γ-decalactone observed in the present work may be of significant interest, considering that this compound is the precursor of two decenolides with aroma properties: dec-2-enolide (mushroom notes) and dec-3-enolide (fruity notes). These two compounds

result from the dehydration of 3-hydroxy-γ-decalactone. They can accumulate in the medium, but their accumulation is in most of the cases negligible, as found herein. Therefore, 3-hydroxy-γ-decalactone could be used for their formulation by dehydration.

5.1.4 Conclusions

The operating conditions for γ-decalactone production, namely substrate concentration, biotransformation start-up procedure and oxygen transfer, were optimized. The new biotransformation start-up procedure avoids the laborious step of washing cells, slightly increasing their hydrophobicity and leading to a more efficient process, yielding higher product concentrations. The direct influence of oxygen transfer rate on the production of γ-decalactone and 3-hydroxy-γ-decalactone was also demonstrated.

Lipase does not appear to play a limiting role in the production of γ-decalactone from MR. However, high lipase induction by MR was observed so, once the lactones separated from the medium, lipase could be recovered from it, increasing the value of the process.

In the next section of this chapter, the use of a cheaper source of ricinoleic acid than methyl ricinoleate, castor oil, was investigated on γ-decalactone production.

5.2 IMPACT OF CASTOR OIL AND LIPASE-MEDIATED HYDROLYSIS ON γ-DECALACTONE PRODUCTION BY YARROWIA LIPOLYTICA

5.2.1 Introduction

γ-Decalactone can be biotechnologically produced through the biotransformation of ricinoleic acid, an hydroxylated C18 fatty acid that, in its esterified form, is the major constituent of castor oil (CO), a natural and non-toxic oil obtained from the seed of the castor plant, *Ricinus communis* (Neto *et al.*, 2004). This triglyceride is the substrate most usually used in the production of γ-decalactone and it needs to be hydrolyzed in order to release ricinoleic acid. It is a cheaper source of ricinoleic acid than methyl ricinoleate, justifying the study of using this substrate in the aroma production.

There are several methods described to promote oil hydrolysis, yielding high conversion rates. Usually, ricinoleic acid is produced by saponification followed by acidification. Although the reaction conditions are mild (70 °C – 100 °C), the fatty acid obtained has a characteristic unacceptable odor and coloration and there is also a high quantity of the by-product, Na_2SO_4, which is difficult to dispose of (Puthli *et al.*, 2006). The high-temperature and high-pressure processes of manufacturing fatty acids are not suitable for castor oil hydrolysis since they lead to formation of an undesirable product named ricinoleic acid estolide and may cause denaturation of the product (Goswami *et al.*, 2009; Lakshminarayana *et al.*, 1984; Rathod and Pandit, 2009). Considering this, a lipase catalyzed hydrolysis obviates these drawbacks as it operates at moderate temperatures (ca. 35 °C – 40 °C) and produces odorless and light-colored ricinoleic acid (Rathod and Pandit, 2009; Sharon *et al.*, 1998).

Y. lipolytica, besides being one of the yeast species able to carry out the biotransformation of ricinoleic acid into γ-decalactone it is also a lipase producer. Lipases catalyze the hydrolysis of triglycerides into glycerol and free fatty acids. In the previous part of this chapter, MR was shown to induce lipase but this activity was not a limiting factor in the consumption of the substrate. However, using CO, a triglyceride which needs to be hydrolyzed, the presence of lipases in the medium theoretically improves the availability of fatty acid substrates to the microorganism. However, as previously stated, lipase activity may be influenced by the activity of proteases since these enzymes may cause changes in lipase characteristics and also degrade them (Kulkarni and Gadre, 1999).

With the aim of improving the productivity of the aroma, in a first approach, the present work reports this time the influence of different castor oil concentrations on the production of γ-decalactone by *Y. lipolytica*. The production of lipase and protease was monitored during these experiments. The process revealed to be very slow, probably due to an inefficient hydrolysis of the oil. Therefore, as an innovative approach, an enzymatic hydrolysis of the oil was also investigated, using different commercial lipases and operating conditions. Thus, the influence of using in the process castor oil previously hydrolyzed or adding an extracellular lipase to the biotransformation medium was evaluated.

5.2.2 Materials and Methods

5.2.2.1 Operating conditions

In this work the source of ricinoleic acid used was castor oil. In a first approach, different concentrations of CO were tested: 10, 30 or 50 g L^{-1}. The composition in Tween 80 had to be adjusted to maintain the oil/surfactant proportion, as already performed in MR media. Thus, 1, 3 or 5 g L^{-1} Tween 80 were used. For these experiments, the biotransformation medium components (CO, Tween 80, NH_4Cl and YNB with aminoacids) were added to the YPD medium, as an emulsion, in order to start the biotransformation step.

After selecting the optimal substrate concentration, experiments were performed in order to improve the productivity of the process, by using castor oil previously hydrolyzed enzymatically (according to the methodology described in the sequence) or by adding an extracellular lipase to improve the hydrolysis of the oil during the aroma production. In the first case, 10 g of lipase (35 U) were added to the flask containing the biotransformation medium and it was incubated at 140 rpm and 27 °C during 48 h. After the growth phase, the yeast cells were separated from the YPD medium by centrifugation (6000 g, 5 min) and transferred to the biotransformation medium. In the second case, after the growth phase, the cells were transferred (after centrifugation at 6000 g, 5 min) to the biotransformation medium and 10 g of lipase (35 U) were added.

Samples were collected at appropriate intervals for analysis of cell concentration and viability, aroma compound quantification, lipase and protease activities.

5.2.2.2 Enzymatic hydrolysis of castor oil

Several commercial lipases were tested in castor oil hydrolysis: CALB L from *Candida antarctica*, Lipozyme TL IM and Lipolase 100T (enzymes kindly supplied by Novozymes, Bagsvaerd, Denmark).

The reaction mixture consisted of 30 g L^{-1} castor oil, 3 g L^{-1} Tween 80, 3 mL of 10 mM potassium phosphate buffer (pH 6, 7 and 8) and 23.2 U of enzyme. The reaction occurred in an orbital shaker at temperatures of 27 °C or 37 °C, and 200 rpm, during 50 hours. Samples were removed over time and 4 mL acetone/ethanol (1:1 v/v) was added to stop the reaction. Liberated fatty acids were titrated with 0.1 M alcoholic KOH, using phenolphthalein as an indicator.

The percentage of castor oil hydrolysis was calculated as indicated by Eq. 5.2:

$$Hydrolysis\ (\%) = \frac{\frac{M \cdot MM \cdot (V - V_B)}{C_{oil} \cdot V_s}}{SV} \qquad \text{Eq. 5.2}$$

Where M (mol L^{-1}) is the molarity of KOH; MM (g mol^{-1}) is the molar mass of KOH; V (mL) is the KOH volume dispended in the sample titration; V_B (mL) is the KOH volume dispended in the blank titration; C_{oil} (mg mL^{-1}) is the oil concentration in the sample; V_s (mL) is the titrated sample volume; and SV is the saponification value (177 mg g^{-1} KOH, according to the supplier).

5.2.3 Results and Discussion

5.2.3.1 Effect of castor oil concentration on γ-decalactone production

5.2.3.1.1 Cell concentration and viability

Cell concentration and viability were monitored during biotransformation experiments. The cellular concentration appeared to be independent of the substrate concentration, ranging in all cases from

9×10^{8} cells mL^{-1} up to 2×10^{9} cells mL^{-1}. The viability of the cells remained practically constant at 100%, in all cases.

5.2.3.1.2 γ-Decalactone production

The influence of different castor oil concentrations on γ-decalactone production in flask was investigated.

Figure 5.6 indicates a maximum γ-decalactone concentration of 1839 ± 295 mg L^{-1}, obtained at 160 h, with 30 g L^{-1} ofcastor oil. The maximum aroma concentrations obtained with 10 g L^{-1} and 50 g L^{-1} substrate were in both cases approximately 1350 mg γ-decalactone L^{-1}. However, the process was slower when higher oil concentrations were used, with a lag phase of around 100 h before significant amounts of -decalactone were detected in the medium. In contrast, when methyl ricinoleate was used, the maximum of aroma production was attained before or close to 100 h of operation time, depending on the substrate concentration (section 5.1.3.1.3).

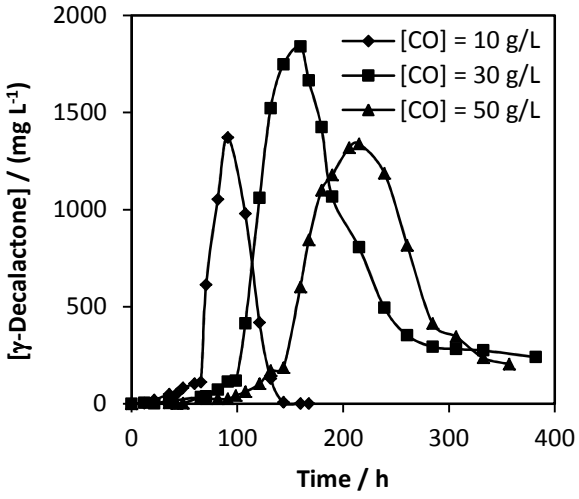

Figure 5.6 - Time course of γ-decalactone accumulation in biotransformation media with different concentrations of castor oil.

After the maximum lactone concentration was reached using 10 g L^{-1} CO, it started to decrease, until complete disappearance from the medium, similarly to what happened using MR media (Figure 5.1). The consumption of the aroma started when no more ricinoleic acid was detected in the medium. The complete disappearance of the aroma from the medium was not observed when 30 g L^{-1} and 50 g L^{-1} CO were used, in which a concentration of oil around 200 mg L^{-1} still remained, suggesting that the metabolic needs of cells decreased.

Despite high concentrations of aroma were obtained, the process was very slow, which may be justified by the use of castor oil as substrate: a limitation by a slow triglyceride hydrolysis may be hypothesized. In order to test this hypothesis, extracellular enzymes, such as lipase and protease, that are known to be produced by *Y. lipolytica*, were analyzed in samples from the biotransformation media.

5.2.3.1.3 Enzymatic activities

Castor oil induced lipase production and, probably protease production as consequence, since this enzyme is induced by the presence of extracellular proteins (Fukushima *et al.*, 1989; Srinivasan and Dhar, 1990). The induction of both enzymes was observed in all cases (Figure 5.7).

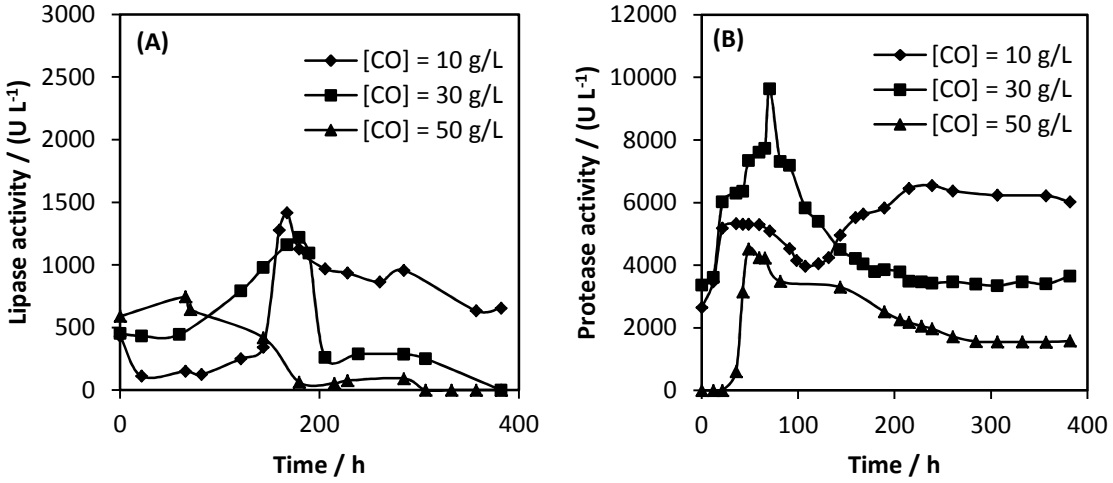

Figure 5.7 - Lipase (A) and protease (B) activity profiles in biotransformation media with different concentrations of castor oil. Data are the mean values of three independent experiments.

For the range of concentrations tested, increases in oil concentration did not lead to higher lipase activity (Figure 5.7A). In fact, the maximum lipase activity (1415 U L^{-1}) was achieved using the lowest castor oil concentration tested, which was 10 g L^{-1}. The same behavior was previously reported (section 5.1.3.1.4; Lakshimi *et al.*, 1999). For example, Lakshimi *et al.* (1999) studied the effect of different substrates and concentrations on lipase production by the yeast *Candida rugosa* and concluded that there was an optimum substrate concentration above which no increase in lipase activity occurred. In their work, the maximum lipolytic activity was achieved using 10 g L^{-1} of all the substrates studied. As previously presented, different concentrations of methyl ricinoleate as substrate were also tested (section 5.1.3.1.4) and the maximum lipase activity was obtained with the lowest oil concentration tested, which was 10 g L^{-1} substrate. This behavior is most likely due to the excess of substrate in the medium. Lipase is probably in the cell wall or membrane and it is not secreted unless the substrate becomes scarce (Pereira-Meirelles *et al.*, 2000).

Nevertheless, the levels of lipase activity would probably be higher without a degradation by proteases, whose activity was high in the biotransformation media (Figure 5.7B).

With exception of the experiment using 50 g L^{-1} CO, where the lipase activity remained lower, the peaks of lipolytic activity were achieved after the maximum accumulation of γ-decalactone occurred (Figure 5.6), meaning that the extracellular lipase was not induced soon enough to affect the oil

utilization by the cells to produce γ-decalactone. This suggests that the biotransformation of ricinoleic acid from castor oil could theoretically be improved by the addition of an extracellular lipase which would accelerate the process and therefore increase its productivity.

5.2.3.2 Enzymatic hydrolysis of castor oil

The enzymatic hydrolysis of oils may be influenced by several external factors, among which pH and temperature. The optimal values of these parameters for castor oil hydrolysis were determined by conducting the same reaction at several pH phosphate buffers (6, 7 and 8) and distinct operating temperatures (27 °C and 37 °C). The efficiency of different commercial lipases (CALB L from *Candida antarctica*, Lipozyme TL IM and Lipolase 100T) on the hydrolysis of the oil was also compared.

Two different temperatures were tested using the immobilized lipases: 37 °C (one of the most widely used temperatures in lipolytic activity determination assays (Lakshimi *et al.*, 1999; Pereira-Meirelles *et al.*, 2000; Pignède *et al.*, 2000; Corzo and Revah, 1999; Amaral *et al.*, 2006b; Reagent Chemical ACS Specifications, 1993) and the optimal temperature given by the supplier (70 °C for Lipozyme TL IM and 55 °C for Lipolase 100T). According to results, the most adequate temperature for both immobilized enzymes, determined with our spectrophotometric method, is 37 °C: 232.2 ± 18.1 U mg^{-1} Lipozyme TL IM and 233.3 ± 41.5 U mg^{-1} Lipolase 100T, in contrast to 87.4 ± 23.2 U mg^{-1} Lipozyme TL IM and 141.3 ± 24.9 U mg^{-1} Lipolase 100T, determined at the optimum temperature given by the supplier (70 °C and 55 °C, respectively). The differences in the enzymatic activities may be justified by the use of different methods and substrates to determine lipolytic activity.

The optimal temperature of CALB L is known to be comprised between 26 °C and 37 °C (Brigida *et al.*, 2007), so no temperature trials were needed. Hence, 37 °C was selected for the following hydrolysis tests.

The ionization of free substrate and free lipase, as well as that of lipase-substrate complex are affected by changes in pH (Tipton and Dixon, 1979). Thus, the resulting effect of varying the phosphate buffer pH (6, 7 and 8) on the rate of castor oil hydrolysis, at 37 °C, was analyzed for each lipase and it is presented on Figure 5.8. From this figure one can observe that the maximum

hydrolysis yield obtained after 30 h (85.3%) with Lipozyme TL IM, is similar for all the pH values studied.

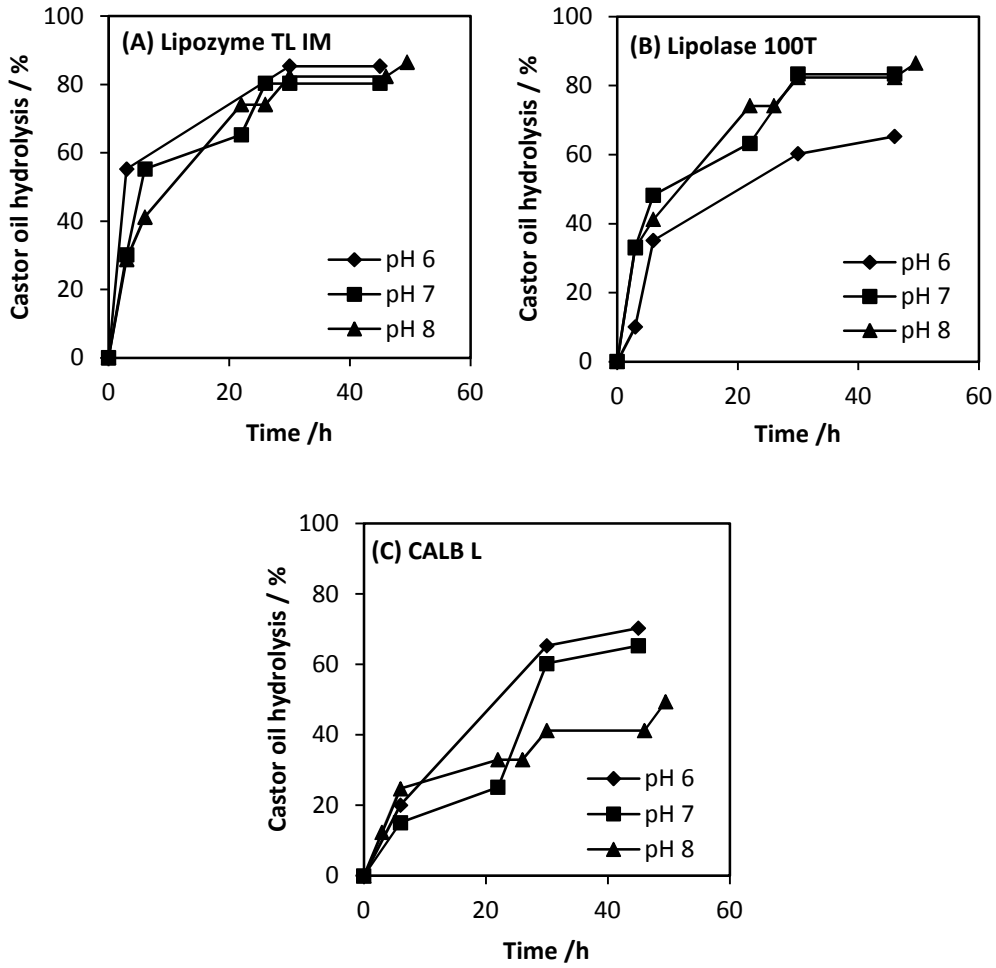

Figure 5.8 - Impact of pH on the rate of castor oil hydrolysis, at 37 °C.

The hydrolysis of castor oil promoted with Lipolase 100T, at pH values of 7 and 8 is quite identical, reaching its highest yield of 83.3 % after 30 h of reaction. pH 6 lead to an oil hydrolysis yield of 65.2 %. Puthli *et al.* (2006) also tested the hydrolysis of castor oil using a conjugated Lipolase enzyme (28 U mL^{-1}) but obtained a maximum hydrolysis percentage of 61 %, at pH 7, after 72 h of reaction.

Among the commercial lipases tested, CALB L was the less efficient in the hydrolysis of castor oil, inducing a maximum hydrolysis yield of 65 %, after 48 h, using pH 7. The lower efficiency of CALB

L may be due to the fact that it is a free enzyme, whereas the others are immobilized. Immobilization can enhance enzyme stability and activity (Nasratun *et al.*, 2009; Sharma *et al.*, 2001), leading to higher oil hydrolysis yields.

In all cases, after 6 h of reaction, the percentage of hydrolysis slowed down, indicating that the enzymatic hydrolysis rate of CO was not linear with time. The reason for this behavior is that in the initial part of the reaction, there are more interactions between oil and enzyme and consequently the hydrolysis rate is faster. Nevertheless, as the reaction time proceeds, higher amounts of fatty acids resulting from the oil hydrolysis accumulate at the interface of the two phases, displacing the enzyme from the interface. This will reduce the contact between lipase and castor oil continually through the hydrolysis process, resulting in a decreased rate of hydrolysis Puthli *et al.* (2006).

Since the best oil hydrolysis yields, achieved with the immobilized lipases, were similar for pH 7 and 8, this last value was chosen because it is near the equivalence point of phenolphthalein, the indicator used in the titrations.

Since the temperature used in the biotransformation process is 27 °C, in a final attempt, it was decided to evaluate the effect of this temperature on the rate of oil hydrolysis and to compare it to the optimum temperature determined for both immobilized enzymes, i.e., 37 °C. Figure 5.9 shows that Lipozyme TL IM leaded to a hydrolysis percentage of 95.4% after 26 h, at 27 °C. The maximum hydrolysis rate (82.3%) obtained using Lipolase 100T was lower, achieved in 30 h, at 37 °C, reason why Lipozyme TL IM was the lipase chosen to be used in the biotransformation experiments.

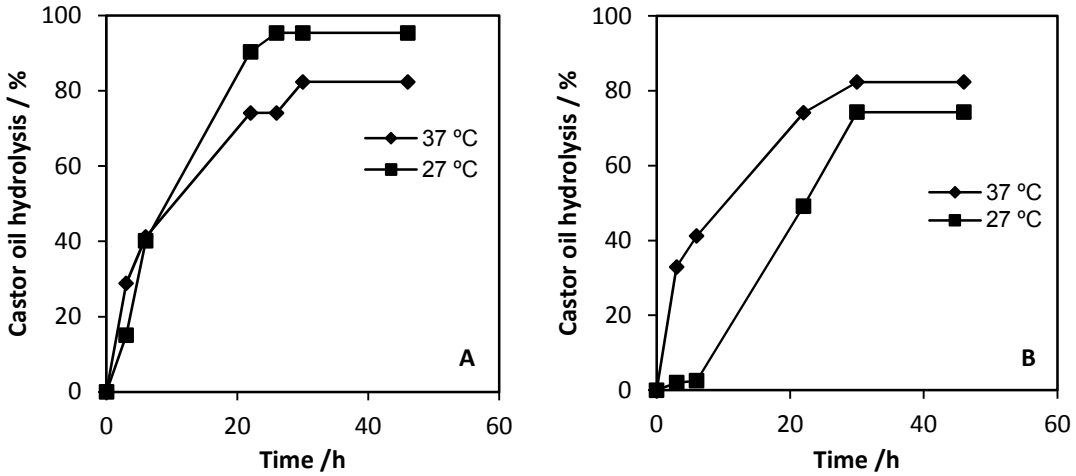

Figure 5.9 - Effect of temperature on castor oil hydrolysis rate, at pH 8, using Lipozyme TL IM (A) and Lipolase 100T (B).

5.2.3.3 Impact of castor oil enzymatic hydrolysis on γ-decalactone production

Two different strategies were investigated and compared with the usual biotransformation process. The first methodology consisted in the addition of an exogenous lipase, Lipozyme TL IM, to the biotransformation medium with the aim of improving the hydrolysis of castor oil; the second strategy consisted in performing a hydrolysis of the oil with the same enzyme, before starting the biotransformation.

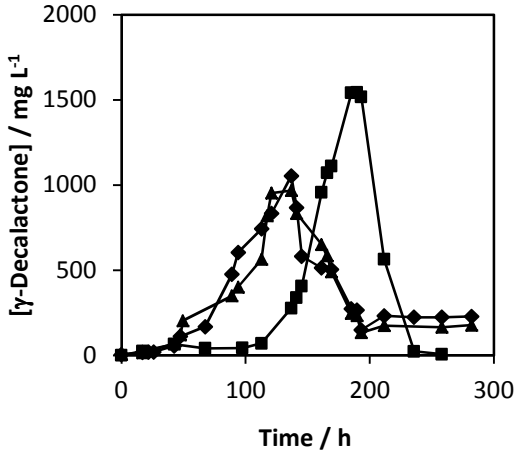

Figure 5.10 - Time course of γ-decalactone accumulation in biotransformation media with 30 g L^{-1} CO as result of different production strategies: (♦) with Lipozyme TL IM added to the medium; (▲) with castor oil previously hydrolyzed by Lipozyme TL IM; (■) without lipase addition (control).

The results presented in Figure 5.10 show that the addition of Lipozyme TL IM during the biotransformation, or the previous hydrolysis of the oil, decreases the lag phase for the production of γ-decalactone from CO, by *Y. lipolytica*. However, although the maximum production of aroma was reached earlier in the experiments with Lipozyme TL IM, the accumulated concentration was lower (ca. 1000 mg L^{-1}, at 137 hours, in both cases) than that obtained in the control test, where no lipase was added (1544 mg L^{-1}, at 190 hours). This is related to the fact that, by adding a lipase to enhance the hydrolysis of castor oil, there is an increased release of fatty acids that can exert a toxic effect in yeasts, leading to a lower production of γ-decalactone (Waché *et al.*, 2001).

Nevertheless, the productivities obtained in the three tested media (7.70 ± 1.15 mg L^{-1} h^{-1}, 7.89 ± 1.18 mg L^{-1} h^{-1} and 8.33 ± 1.24 mg L^{-1} h^{-1}, for medium with Lipozyme TL IM, with CO previously hydrolyzed by Lipozyme TL IM and without lipase addition, respectively) are not significantly different, which indicates that by using an external lipase is possible to produce γ-decalactone in less time keeping identical values of aroma productivity. In fact, a gain of around 50 h in the overall batch process was obtained.

5.2.4 Conclusions

A castor oil concentration of 30 g L^{-1} was shown to be adequate for the production of γ-decalactone in the experimental conditions used.

Three extracellular commercial lipases were investigated for the hydrolysis of CO, at different pH and temperature values. Lipozyme TL IM, at pH 8 and 27 °C gave the most efficient hydrolysis of CO (95%).

The effects, on the aroma production of using CO previously hydrolyzed by the selected enzyme or adding that lipase directly into the biotransformation medium, was evaluated and compared with experiments performed without enzymatic hydrolysis of the oil. As results, the process was faster when lipase was involved and γ-decalactone maximum concentrations were lower, however resulting productivities were similar.

In an innovative way in such a process, it was shown here that with the help of an external lipase, the production of the lactone can be much faster.

After investigating the influence of using several emulsions composed by different concentrations of MR or CO in the production of γ-decalactone, each emulsion was characterized in terms of the oil droplets size, in order to try correlating this information with the aroma production.

5.3 OIL-IN-WATER EMULSIONS CHARACTERIZATION BY LASER GRANULOMETRY AND IMPACT ON γ-DECALACTONE PRODUCTION

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ORIGINAL RESEARCH PAPER

Oil-in-water emulsions characterization by laser granulometry and impact on γ-decalactone production in *Yarrowia lipolytica*

Nelma Gomes · Yves Waché · José A. Teixeira · Isabel Belo

5.3.1 Introduction

Oil-in-water emulsions composed of methyl ricinoleate or castor oil as the organic phase, stabilized by Tween 80, constitute the medium usually used on the biotechnological production of γ-decalactone.

The cells growth in a medium containing lipids occurs directly on droplets as oil solubility in water is limited (Gutierrez and Erickson, 1977) and the growth is, thus, influenced by the size of the oil droplets (Aguedo *et al.*, 2003a).

The interfacial surface of the oil droplets and consequently the surface between the two liquid phases is a determining factor for the degradation of the hydrophobic substrate and, thus, for the cells growth and aroma production (Aguedo *et al.*, 2005a). The lipidic droplets size is influenced by numerous parameters, which may be related to physical and chemical characteristics of the medium (pH, ionic strength, presence of surfactants, etc.) (Wilde, 2000) or to the presence of microorganisms (inoculum concentration, cells surface properties, etc.). During the biotransformation of ricinoleic acid into γ-decalactone, there is a direct contact between the cells surface and the substrate droplets, which may occur by adhesion of cells to the larger droplets surface or by adsorption of micro-droplets at the cells surface (Bakhuis and Bos, 1969).

Since the access of the cells to the substrate depends on the emulsion characteristics, emulsions were characterized by determining the oil droplets size distribution, through laser granulometry, using both oils (MR and CO), at different concentrations and in the presence or absence of cells. Moreover, since the cells surface properties may influence the interaction between the oil and cells, different methodologies were used for inoculum cells preparation, according with work previously described (section 5.1.3.1) This procedure had an impact on cells hydrophobic character and the effect on emulsion behavior was analyzed in the present work. In a last instance, the impact of each emulsion on γ-decalactone production was also investigated.

5.3.2 Materials and methods

5.3.2.1 Operating conditions

MR and CO were used at concentrations from 10 to 50 g L^{-1}. The composition in Tween 80 had to be adjusted to maintain the oil/surfactant proportion. Thus, 1, 3 or 5 g L^{-1} Tween 80 were used.

Two different methodologies were also tested for inoculation of the biotransformation medium: with or without the cell washing step, as described previously in the section 5.1.2.1.

Samples were collected at appropriate intervals for quantification of γ-decalactone.

5.3.2.2 Laser granulometry

The droplet size distribution of MR and CO emulsions was evaluated using a Malvern Mastersizer laser granulometer (Model S2-01, Malvern Instruments, Malvern, UK), based on the Fraunhofer approximation (Agrawal *et al.*, 1991).

Emulsions with MR and CO were analyzed with and without cells precultured during 19 hours on YPD medium. For the essays without cells, the emulsions contained in 500-mL baffled Erlenmeyer flasks were shaken at 140 rpm, 27 °C, during 1.25 h prior to analysis. For the experiments with cells, the emulsions were shaken for 1 h before inoculation and then for more 15 min, with cells, before analysis. A cellular suspension prepared in sterile water was also analyzed, in order to determine the size of the yeast cells. All assays were performed in triplicate.

5.3.3 Results and Discussion

The granulometric analyses of the emulsified MR and CO media without cells, indicated the existence of two distinct droplet populations (Figure 5.11). In the population with smaller dimension, the droplets size remained practically with the same mean value among all MR (1.5 μm) and CO (1.9 μm) concentrations tested. In the bigger size population, the mean droplets diameter increased from 61 μm to 96 μm, with the increase of MR concentration, and from 27 μm to 64 μm, with the increase of CO concentration. Moreover, there was an increase in percentage of larger droplets with the oil concentration increase.

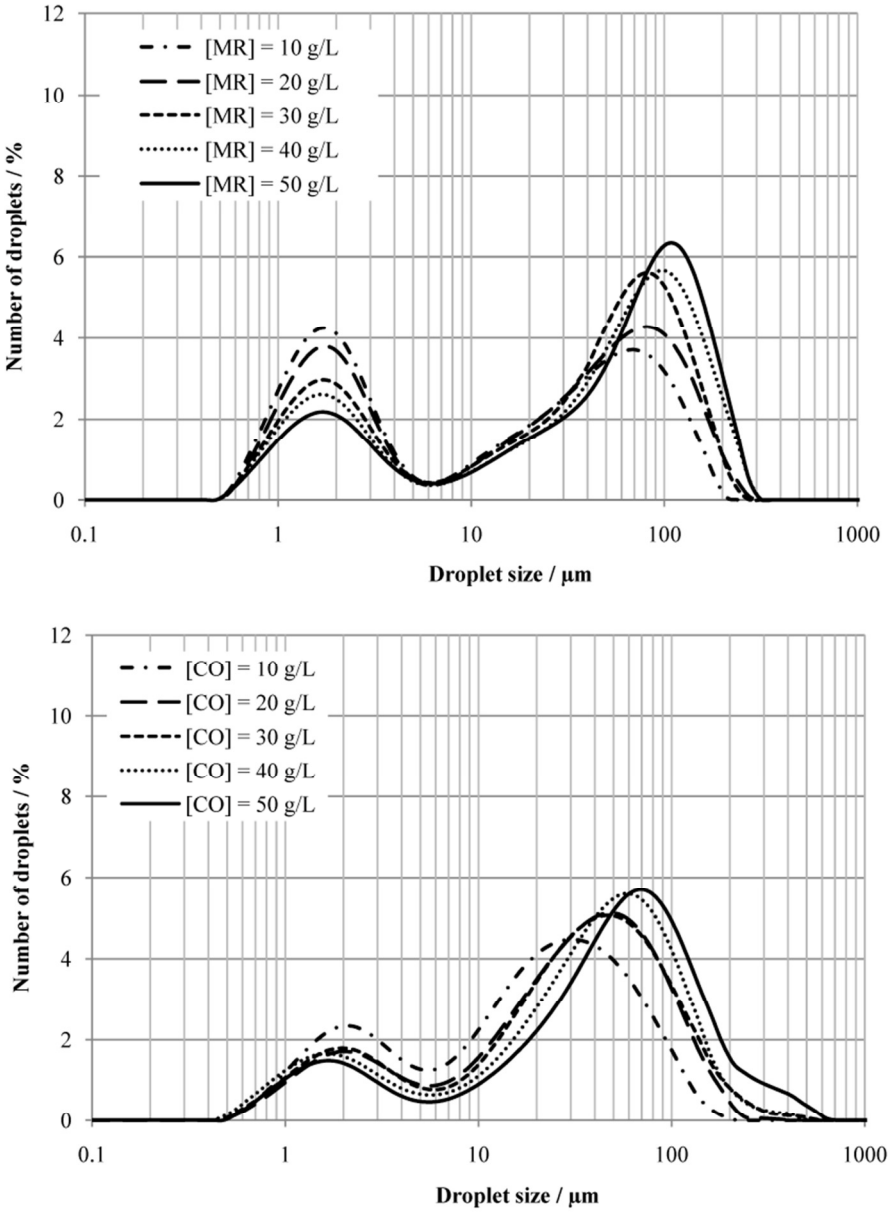

Figure 5.11 - Methyl ricinoleate (MR) and castor oil (CO) droplets size distribution (in µm) in emulsions without cells, related to the number of particles (in %). Data are the mean of three independent experiments.

Aguedo *et al.* (2003a) also characterized an emulsion containing 10 g MR L^{-1}. The results obtained by these authors differ from those presented in this work since they have reported the existence of three distinct droplet populations with mean sizes of 0.1 µm, 3 µm and 43 µm, in the MR emulsion. These divergences may be justified by a variation in the concentration of Tween 80 (0.2 g L^{-1} vs. 1 g L^{-1}, in their work and in the present one, respectively). Thus, it seems that the use of a

higher concentration of surfactant has an impact upon the MR droplets size, since the smaller droplets (0.1 μm) disappeared and a population of larger droplets emerged: 61 μm in this study, compared with 43 μm in their work.

Prior to the determination of droplets size in the presence of cells, the cells size distribution was evaluated by suspending the cells in sterile water (Figure 5.12). A population with mean values of 5.4 μm was detected, corresponding to an oval morphology (Lopes *et al.*, 2008).

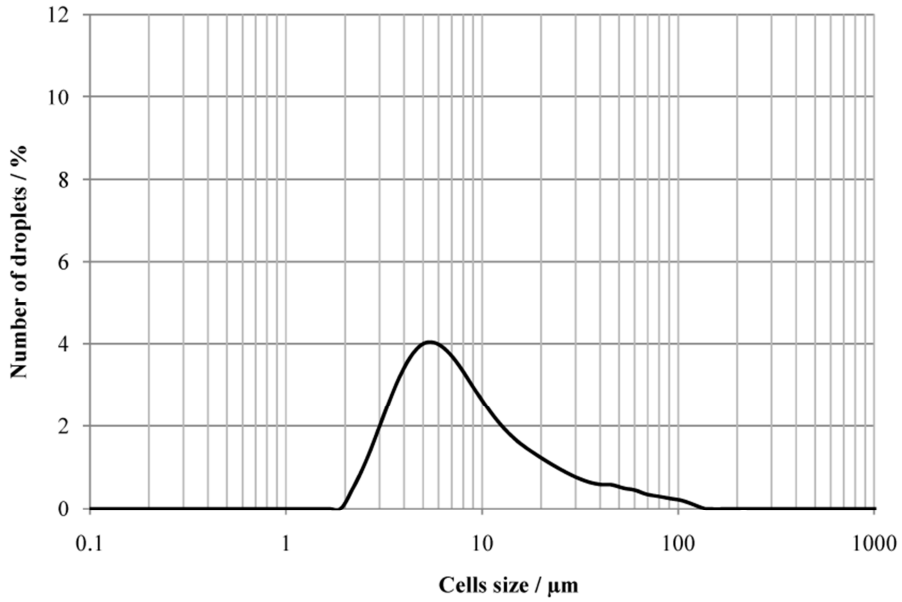

Figure 5.12 - Cells size distribution (in μm) in sterile water, related to the number of particles (in %). Data are the mean of three independent experiments.

When the cells were present at a concentration around 1.5×10^{9} cells mL^{-1} in the MR and CO emulsions, there were still two populations (in some cases, three) with different particle sizes with both washed and non-washed cells (Figure 5.13 and Figure 5.14). However, droplets with 1.5-1.9 μm size disappeared in all emulsions. This disappearance, due to the presence of cells, may be explained by the adhesion of smaller substrate droplets to the cells surface and to the higher number of cell particles that would overshadow the smallest oil droplets (Aguedo *et al.*, 2003a). Nevertheless, the population of cells remained with a mean size of approximately 5.4 μm. The existence of this population was also reported by Aguedo *et al.* (2003a).

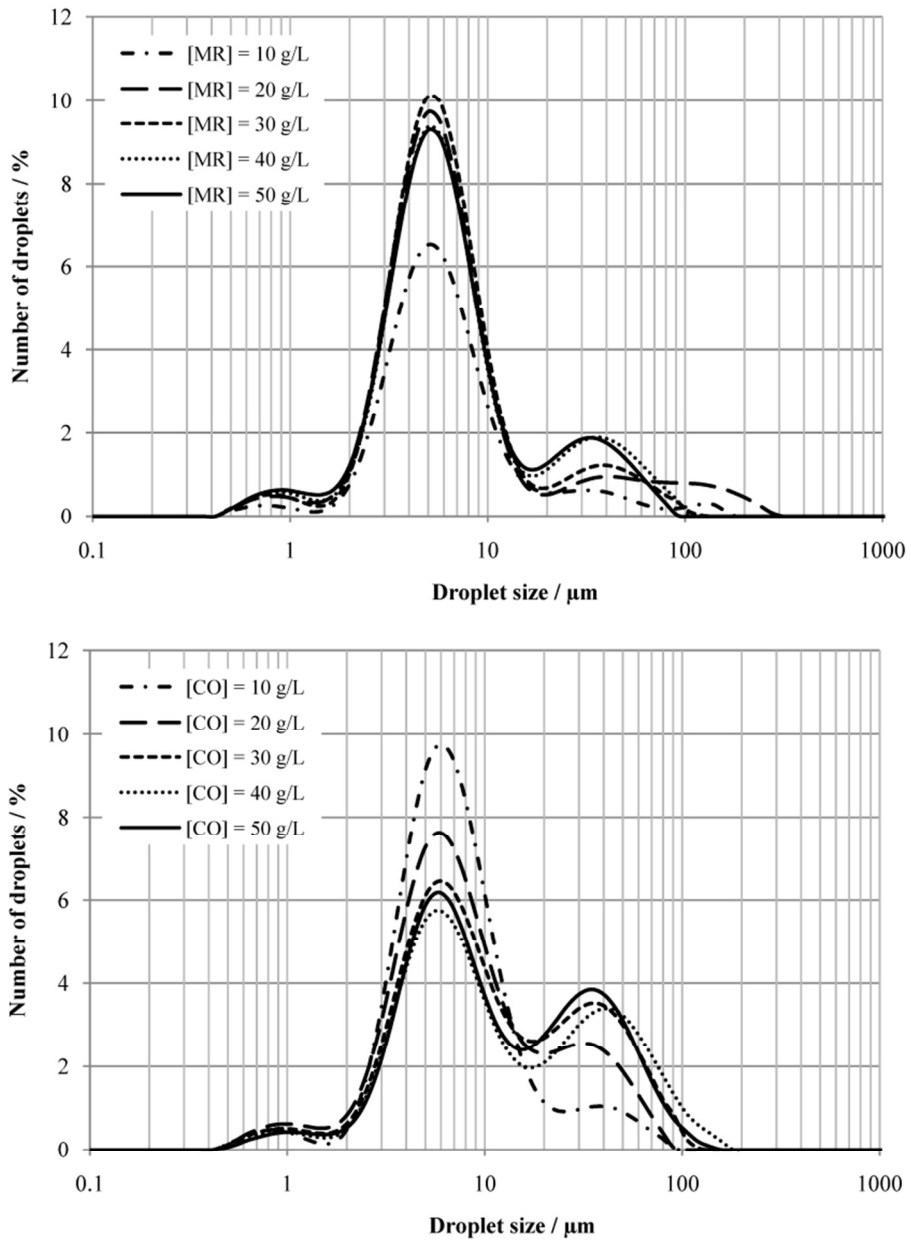

Figure 5.13 - Methyl ricinoleate (MR) and castor oil (CO) droplets size distribution (in µm) in emulsions with washed cells, related to the number of particles (in %). Data are the mean of three independent experiments.

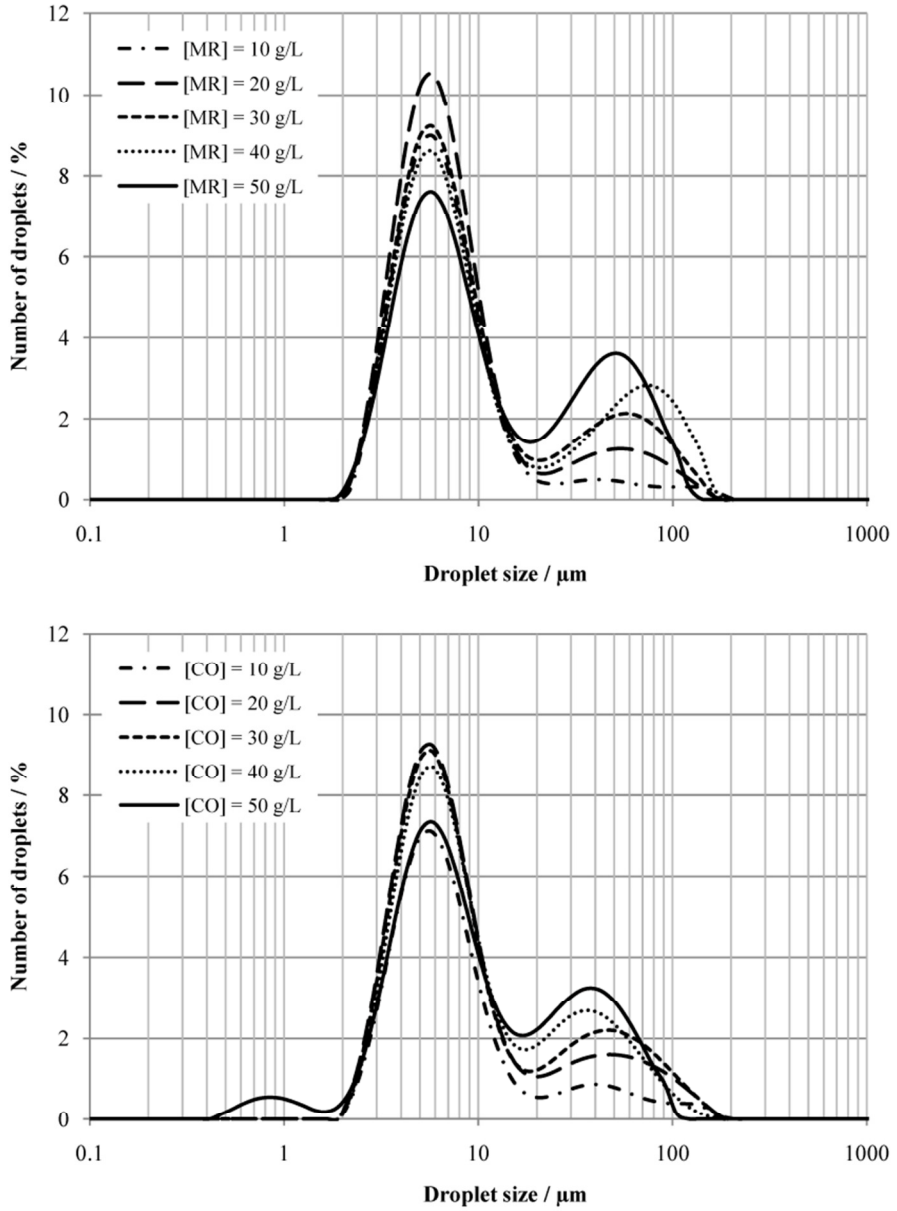

Figure 5.14 - Methyl ricinoleate (MR) and castor oil (CO) droplets size distribution (in µm) in emulsions with non-washed cells, related to the number of particles (in %). Data are the mean of three independent experiments.

When washed cells were used (Figure 5.13), a population with a mean oil droplet size of 0.9 µm was observed, although in a small percentage number, in all experiments and for both oils. This decrease in the oil droplets mean size with the addition of *Y. lipolytica* washed cells was also stated by Waché *et al.* (2000), when using emulsions composed by 5 g MR L^{-1} and 1 g Tween 80 L^{-1}.

Another population, with a mean droplet size comprised between 32 μm and 40 μm was also noticed, in all experiments, and for both substrates, although with a larger number of particles in CO emulsions. Aguedo *et al.* (2003a) also described the existence of a population of MR droplets with a mean size of 42 μm, in emulsions with *Y. lipolytica* washed cells.

In the experiments involving non-washed cells (Figure 5.14), it appears that, as the concentration of MR increased, the size of oil droplets also increased slightly, ranging from 40 μm to 69 μm, except for the experiment with 50 g MR L^{-1}, which presented a mean size of 52 μm.

When comparing the experiments with washed and non-washed cells, using both oils, one can observe that the smallest oil droplets disappeared (except for the case with 50 g CO L^{-1}, where the existence of a small population of oil droplets with a mean size of 0.8 μm was noticed). This disappearance is probably related to the slightly higher hydrophobicity of non-washed cells, as proved previously (section 5.1.3.1.2), allowing droplets to adsorb to their surface, turning its presence not measurable.

Table 5.4 shows that the accumulation of γ-decalactone was directly dependent on the MR concentration, with the greater production of aroma occurring when the substrate concentration was higher. The accumulation of γ-decalactone was also higher when non-washed cells were used. In this situation, the MR droplets size was higher (the smaller population with 0.9 μm disappeared), suggesting that higher particle sizes are more favorable to the production of the aroma, indicating that the adhesion of cells to the substrate occurs by the adsorption of cells around larger oil droplets. In fact, cells possess a much smaller size than the oil droplets and the number of cell particles is far superior to the number of oil particles, which seems to corroborate the hypothesis proposed.

Table 5.4 - Mean droplet size (highest), maximum γ-decalactone accumulation and yield in respect to the substrate ($Y_{P/S}$) achieved in biotransformation media with different MR or CO concentrations and with washed or non-washed cells.

		Washed cells			Non-washed cells		
	[Oil] (g L^{-1})	Maximum mean droplet size (μm)	[γ-Decalactone] (mg L^{-1})	$Y_{P/S}$ (mg g^{-1})	Mean droplet size (μm)	[γ-Decalactone] (mg L^{-1})	$Y_{P/S}$ (mg g^{-1})
MR	10	32.4	173	17.3	5.4; 39.8	434	43.4
	30	39.8	284	9.5	5.4; 60.2	724	24.1
	50	34.7	471	9.4	5.4; 52.5	997	19.9
CO	10	39.8	0	0	5.4; 39.8	1371	137.1
	30	34.7	0	0	5.4; 45.7	1839	61.3
	50	34.7	0	0	5.4; 39.8	1339	26.8

Respecting to the use of CO and non-washed cells, the size of oil droplets varied from 34 μm to 46 μm, for the biggest population (Figure 5.14).

When CO was used as substrate of the process, there was no aroma production when washed cells were used in the biotransformation. When using non-washed cells, the most adequate oil concentration to be employed, in terms of overall productivity, was 30 g L^{-1}, suggesting that the most favorable droplets size corresponds to a diameter of approximately 46 μm, the larger size determined among all CO emulsions. This corroborates the hypothesis previously suggested about larger particle sizes being more favorable to the aroma production.

It is worth noting that the main differences obtained in the aroma production under different operating conditions, cannot be attributed solely to the size of oil droplets, but also to the quantity of available substrate for cells to use in the biotransformation, as well as to the cells surface properties, which determines how the substrate is assimilated. The MATH test showed previously (section 5.1.2.2), that there was a slightly higher adhesion of non-washed than washed cells cells to hexadecane. These differences point out that non-washed cells are more compatible with the substrate, which might also explain the divergences obtained in the production of γ-decalactone, apart from the differences in the oil droplets size.

5.3.4 Conclusions

This study aimed to characterize the oil droplets size distribution on different oil-in-water emulsions, composed by different concentrations of methyl ricinoleate or castor oil and stabilized by a non-ionic surfactant. The impact of the presence of cells on droplets size was shown, as well as the relevance of a washing step of the inoculum cells.

In the experiments without cells, an increase in both oilsconcentrations leaded to an enlargement of the mean oil droplets size. In the presence of cells, there were two or three populations with different particle sizes. However, when cells were washed, a smaller population of both oils droplets was detected.

Moreover, the granulometric characterization of the emulsions was related with γ-decalactone accumulation profiles and allowed to conclude that larger oil droplets favors the aroma production, suggesting that, in this case, the access of cells to the substrate occurs by their adhesion around larger oil droplets.

The obtained results are an important contribution for a better understanding of the factors affecting the assimilation of hydrophobic substrates by *Y. lipolytica*. The impact of substrate concentration and of cells on droplet size distribution and consequently on γ-decalactone production was demonstrated.

After investigating the operating conditions concerning the substrate to be used, its concentration and mode of inoculation, it was concluded that the optimal substrate concentration (independently of the substrate) was 30 g L^{-1}. Between methyl ricinoleate and castor oil, although higher aroma concentrations were achieved using CO, MR was chosen due to the higher productivity obtained and also due to practical questions such as the quantification of substrate by gas chromatography. Indeed, to quantify fatty acids in the case of CO, a hydrolysis and posterior methylation would be necessary; in the case of MR, gas chromatography quantification is simplified. Thus, the following experiments in which different strategies were tested to optimize the production of γ-decalactone were performed using 30 g L^{-1} MR and non-washed cells.

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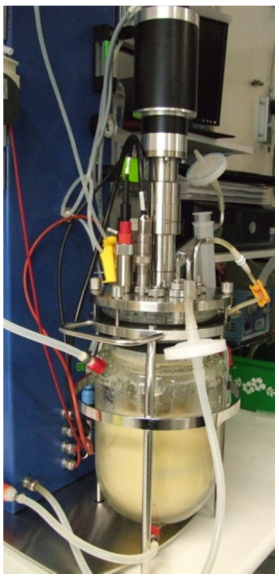

6 DIFFERENT STRATEGIES TO OPTIMIZE THE PRODUCTION OF γ-DECALACTONE

In this chapter, γ-decalactone production was evaluated in a stirred bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland). In a first approach, pH and dissolved oxygen concentration in the biotransformation medium were optimized for γ-decalactone production, using a factorial design. The optimal values of those two factors were used in the subsequent fed-batch experiments performed. The traditional batch mode of operation was compared with two different fed-batch techniques (with constant medium feeding rate and with intermittent feeding), in terms of overall productivity and yield in respect to the substrate. The impact of each operation mode on γ-decalactone production is described.

Finally, the biotransformation of MR was implemented in an airlift bioreactor and the influence of operating conditions upon aroma production was analyzed.

The results presented in this Chapter were published in:

Gomes, N.; Teixeira, J. A.; Belo, I. (2011) Empirical modeling as an experimental approach to optimize lactones production. Catal. Sci. Technol. 1: 86-92.

... and submitted for publication as:

Gomes, N.; Teixeira, J. A.; Belo, I. (2011) Fed-batch versus batch cultures of *Yarrowia lipolytica* for γ-decalactone production from methyl ricinoleate.

6.1 EMPIRICAL MODELLING AS AN EXPERIMENTAL APPROACH TO OPTIMIZE LACTONES PRODUCTION

Adapted from *Catalysis Science & Technology* (2011) 1: 86-92.

Catalysis
Science & Technology

Cite this: *Catal. Sci. Technol.*, 2011, **1**, 86–92

www.rsc.org/catalysis **PAPER**

Empirical modelling as an experimental approach to optimize lactone production

Nelma Gomes, José A. Teixeira and Isabel Belo*

6.1.1 Introduction

It is known that, besides γ-decalactone, other compounds (3-hydroxy-γ-decalactone, dec-2-enolide and dec-3-enolide) derived from its precursor (4-hydroxydecanoic acid) may accumulate in the medium, reducing the yields of γ-decalactone. The accumulation of those compounds gives an indication about the activities of the enzymes of the pathway, namely acyl-CoA oxidase and 3-hydroxyacyl-CoA dehydrogenase. Oxygen may influence their activities since it is necessary for the regeneration of the cofactors FAD and, more indirectly NAD^+ (Figure 2.3) (Bakker *et al.*, 2001) and therefore, influence the production of γ-decalactone and 3-hydroxy-γ-decalactone. For this reason it is imperative to define the most appropriate conditions for lactones production.

Moreover, although *Y. lipolytica* can regulate a relatively steady intracellular pH in different environmental pH (Aguedo, 2002), this parameter could modify some intracellular fluxes and, indirectly, β-oxidation enzyme activities, reason why it is necessary to define the optimal value for operation.

There are several works in literature describing the effect of operating conditions in the production of lactones from the biotransformation of methyl ricinoleate carried out by *Y. lipolytica* (García *et al.*, 2009; Aguedo *et al.*, 2005a; Gomes *et al.*, 2007: García *et al.*, 2007b). However, there are

some discrepancies between the results obtained in those works, especially in what concerns the effect of oxygen, and for that reason the present work was conducted in order to clarify those differences observed. Therefore, a 3^2 full factorial design was employed to determine the influence of pH and dissolved oxygen concentration in the biotransformation medium on the production of lactones from methyl ricinoleate, by the aerobic yeast *Y. lipolytica*, and to optimize the process in bioreactor. This design is an empirical modeling technique used to evaluate, at the same time, the relationship between a set of controllable experimental factors and their observed results.

The main goal of this work was to optimize the operating conditions for γ-decalactone production. However, as high amounts of 3-hydroxy-γ-decalactone were produced simultaneously in the medium, it was also analyzed, in the hope of providing new insights for its production.

6.1.2 Materials and Methods

Biotransformations were carried out as described in the sub-chapter 0.

In order to investigate the effect of important variables such as pH and dissolved oxygen concentration (DO) in the biotransformation medium, on the production of γ-decalactone and 3-hydroxy-γ-decalactone, a full factorial design with two factors at three levels (3^2), with an additional central point to estimate the intrinsic variability (pure error) in the data, was performed. A series of ten experiments was carried out in random order.

The pH and DO values analyzed were: 4.5, 5.6 and 6.7 for pH; and 10%, 30% and 50% for dissolved oxygen concentration in the biotransformation medium.

The setpoint values for pH and DO were automatically controlled by a control unit coupled to the bioreactor. The pH control was performed by addition of 5N KOH or 21% (v/v) orthophosphoric acid, through Peripex peristaltic pumps (Bioengineering, Wald, Switzerland). The DO concentration was controlled by manipulating the agitation and aeration rates, through a cascade control mode.

For statistical calculation, the factors were coded as x_i, at three levels starting from -1, 0 and 1, defined by Eq. 6.1:

$$x_i = \frac{X_i - X_0}{\Delta X_i}$$

Eq. 6.1

With i = 1, 2, 3,... k; and where x_i is the dimensionless coded value of the independent variable X_i; X_0 is the value of X_i at the central point; and ΔX_i is the step change.

A multiple regression analysis of the data was carried out using the Design Expert v.8 software (Stat-Ease, Inc., Minneapolis, MN, USA). The effect of each variable and their interactions on lactones production was studied. The significance of the regression coefficients was tested by a *F*-test. A probability (*p*-value) less than 0.05 for a given factor was considered as significant.

To predict the yield of lactones production, the data were fitted to a second order polynomial equation, which includes all the terms, regardless of their significance (Eq. 6.2):

$$Y = \beta_0 + \beta_1 x_1 + \beta_{11} x_1^{\,2} + \beta_2 x_2 + \beta_{22} x_2^{\,2} + \beta_{12} x_1 x_2 + \beta_{112} x_1^{\,2} x_2 + \beta_{122} x_1 x_2^{\,2} + \beta_{1122} x_1^{\,2} x_2^{\,2}$$

Eq. 6.2

Where, Y is the predicted response (γ-decalactone or 3-hydroxy-γ-decalactone); β_0, is the intercept term; β_1 and β_2, are the linear effects; β_{11}, β_{22}, β_{12}, β_{112}, β_{122} and β_{1122} are model coefficients which were estimated by the factorial design results; x_1 and x_2 are the coded values of the factors (pH and DO, respectively).

The validity of the polynomial models obtained for each lactone was checked by analysis of the residual values, analysis of variance (ANOVA) and by the correlation coefficient, R^2. The significance of the regression coefficients was checked by the *F*-test, where a *p*-value less than 0.05 indicated significance of model terms.

6.1.3 Results and Discussion

The present work is based upon the optimization of operating conditions for lactones production. Biotransformations were carried out in a 3.7-L bioreactor and the optimization was achieved varying pH and dissolved oxygen concentration in the medium.

Table 6.1 shows the experimental matrix for each assay and the respective responses of interest: concentration of γ-decalactone (Y_1) and 3-hydroxy-γ-decalactone (Y_2), both expressed as mg L^{-1}. According to results, the highest γ-decalactone and 3-hydroxy-γ-decalactone concentrations were obtained in the runs no. 5 (pH 5.6; 50% O_2) and 8 (pH 5.6; 30% O_2), respectively.

Table 6.1 - Experimental matrix 3^2: factors levels and experimental results.

			Experimental Responses	
Run no.	X_1 (pH)	X_2 (% O_2)	Y_1, γ-decalactone (mg L^{-1})	Y_2, 3-OH-γ-decalactone (mg L^{-1})
1	-1	1	197	2267
2	-1	-1	164	3127
3	0	-1	424	1198
4	1	1	588	1673
5	0	1	1284	3929
6	-1	0	311	5079
7	1	-1	415	539
8	0	0	326	8016
9	1	0	1192	389
10	0	0	352	8137
level -1	4.5	10		
level 0	5.6	30		
level 1	6.7	50		

The biphasic system used to produce γ-decalactone is very complex, usually presenting low reproducibility, thus becoming a significant problem. However, in this series of experiments, it is possible to conclude that the reproducibility was quite high, since relative standard deviations

(*RSD*) of 5.42% and 1.06% for γ-decalactone and 3-OH-γ-decalactone, respectively, were determined.

Statistical analysis of these data (*p*-values less than 0.05) indicated that pH (x_1) and dissolved oxygen concentration (x_2) have significant effects on the production of both lactones (Table 6.2 and Table 6.3).

Table 6.2 - Results of the 3^2 factorial design: values of coefficient estimates, factor effects and interactions for γ-decalactone concentration.

Source	Estimates	Sum of Squares	Degrees of freedom (*df*)	Mean Square	*F*-value	*p*-value (Prob > *F*)
Model		1.40 × 106	8	1.75 × 105	517.80	0.034
x_1: pH	253.83	4.18 × 105	2	2.09 × 105	618.61	0.028
x_2: O_2	177.67	1.90 × 105	2	9.49 × 104	280.83	0.042
$x_1 \cdot x_2$	35.00	7.90 × 105	4	1.98 × 105	584.25	0.031
Pure Error		338.00	1	338.00		
Cor. Total		1.40 × 106	9			

R^2= 99.98 %; R^2_{adj} (adjusted for *df*) = 99.78 %; *CV* = 3.50 %

Table 6.3 - Results of the 3^2 factorial design: values of coefficient estimates, factor effects and interactions for 3-hydroxy-γ-decalactone concentration.

Source	Estimates	Sum of Squares	Degrees of freedom (*df*)	Mean Square	*F*-value	*p*-value (Prob > *F*)
Model		7.33 × 107	8	9.16 × 106	1251.05	0.022
x_1: pH	-1312.00	2.69 × 107	2	1.35 × 107	1839.79	0.016
x_2: O_2	500.83	2.03 × 107	2	1.02 × 107	1386.78	0.019
$x_1 \cdot x_2$	498.50	1.90 × 107	4	4.74 × 106	647.38	0.030
Pure Error		7320.50	1	7320.50		
Cor. Total		7.33 × 107	9			

R^2= 99.99 %; R^2_{adj} (adjusted for *df*) = 99.91 %; *CV* = 2.49 %

The use of the main response plot allows to determine which of the factors influence the response and to compare the relative strength of the effects. The results show that there is a positive relationship between γ-decalactone production and both factors: pH and DO (Figure 6.1 A and B). The higher the pH and DO values were (in the studied range), the higher the production of γ-decalactone. The higher slope of the line corresponding to pH than the slope of DO indicates that the effect of this factor on γ-decalactone production was stronger than the effect of dissolved oxygen, which is confirmed by the estimate values presented on Table 6.2 (253.83 for pH and 177.67 for dissolved oxygen).

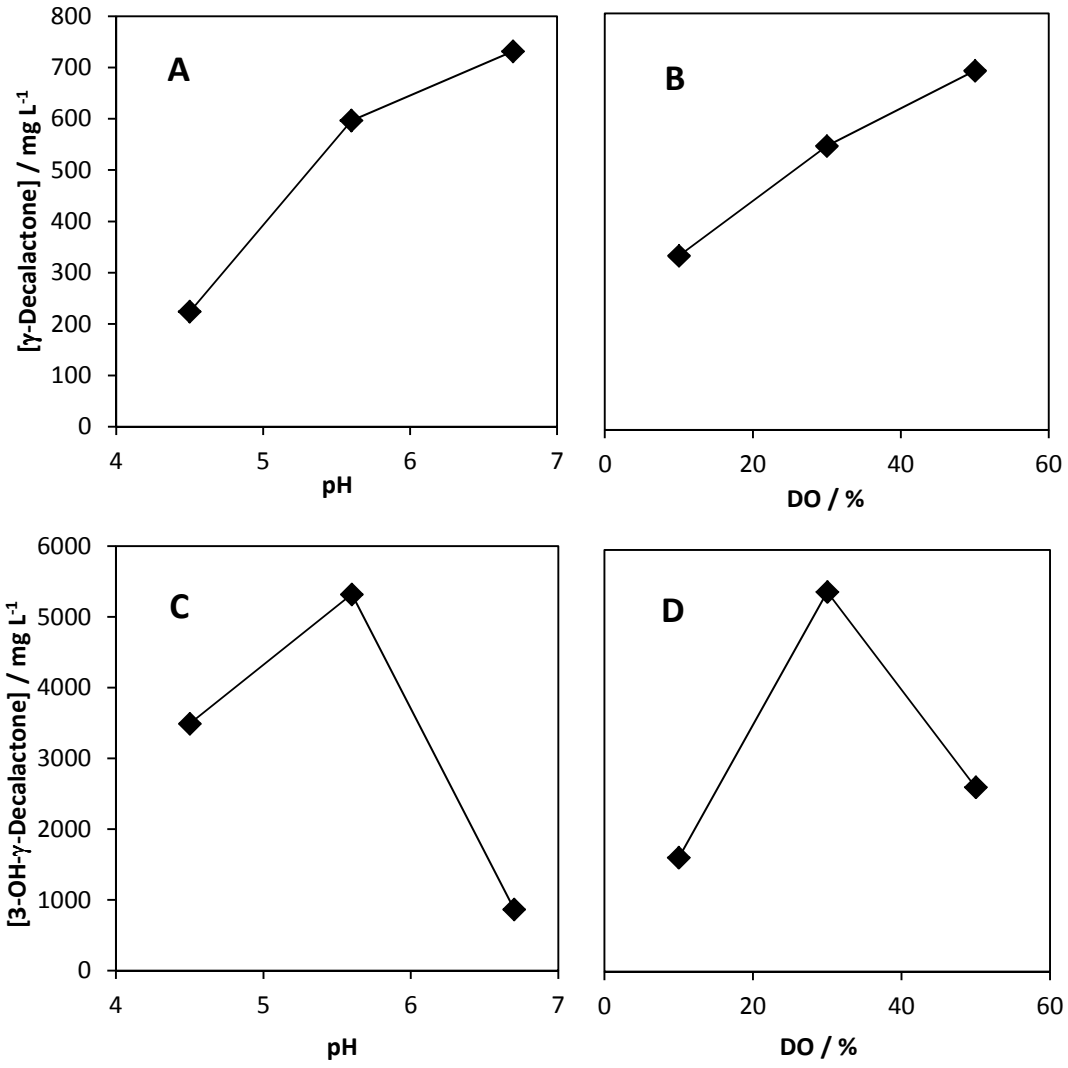

Figure 6.1 – Main response plot of the two factors (pH and dissolved oxygen) on the production of γ-decalactone (A, B) and 3-hydroxy-γ-decalactone (C, D).

Figure 6.1 C and D indicate that, among the experimental conditions tested, the highest 3-hydroxy-γ-decalactone accumulation occurred when intermediate levels of each factor were used: with a pH of 5.6 (5320 mg L^{-1}) and a DO of 30% (5405 mg L^{-1}).

Table 6.3 shows a negative effect of pH on the production of 3-hydroxy-γ-decalactone, an effect which is only valid for pH values higher than 5.6, according to Figure 6.1 C. There was a positive effect of oxygen in the accumulation of 3-hydroxy-γ-decalactone when the concentrations of dissolved oxygen are comprised between 10% and 30%.

Significant interactions ($x_1 \cdot x_2$) between pH and oxygen were observed, so the interaction plots were also constituted (Figure 6.4). The maximum accumulation of γ-decalactone was observed when a pH of 5.6 was used simultaneously with 50% DO (1284 mg L^{-1}) and when a pH of 6.7 was used together with 30% DO (1192 mg L^{-1}). These results are better (in terms of higher aroma concentration obtained) than those observed previously when the individual factor effects were analyzed and the highest γ-decalactone production was obtained using independently the highest levels of each factor (among the range of values tested). This suggests that there is a synergistic effect of pH and dissolved oxygen in the biotransformation medium, meaning that the effect of the two factors on γ-decalactone production is greater than the effect of each factor individually. One factor enhances the effects of the second, leading to a greater γ-decalactone production.

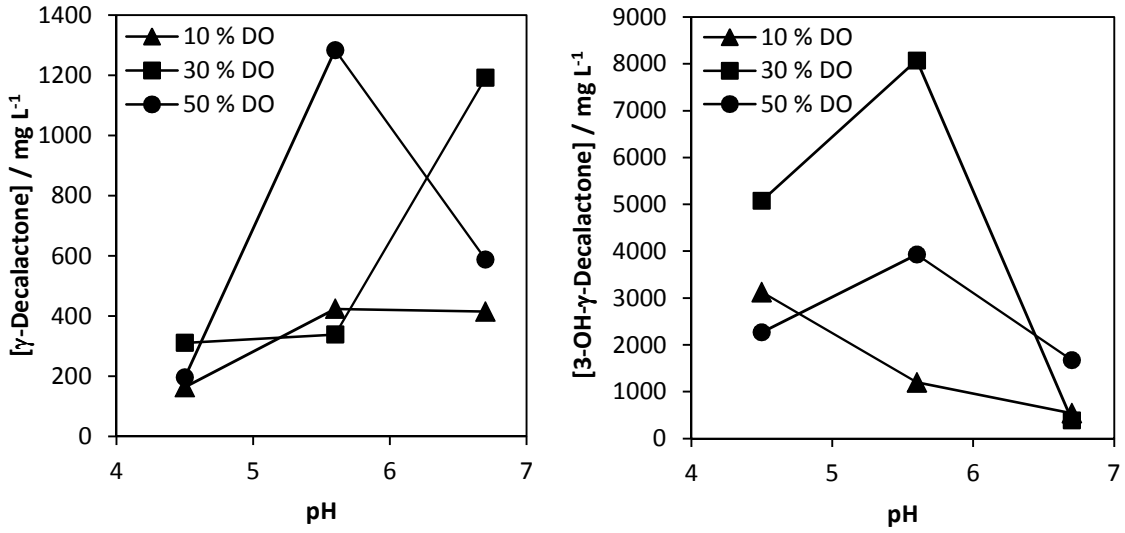

Figure 6.2 – Interaction plot of the two factors (pH and dissolved oxygen) on the production of γ-decalactone (A) and 3-hydroxy-γ-decalactone (B).

The interaction plot displayed on Figure 2.1 B, shows that the maximum 3-hydroxy-γ-decalactone concentration was obtained when a pH of 5.6 was used in the presence of 30% DO (8076 mg L^{-1}). A synergistic effect of both factors was also evidenced in this case.

In order to obtain an empirical relationship between the lactones production and the experimental variables, which will allow predicting the yield of lactones, the data were fitted to Eq. 6.2, based on the calculated regression coefficients and significance of the variables. As result, the following models were obtained, to predict the lactones production, as a function of the coded values of the factors:

$$Y_{\gamma-decalactoв} = 546 + 253.8x_1 - 68.2^2 + 177.7x_2 - 34x_2^{\ 2} + 35x_1x_2 - 126.2x_1^{\ 2}x_2 - 93.3x_1x_2^{\ 2} - 102.8x_1^{\ 2}x_2^{\ 2}$$

Eq. 6.3

$$Y_{3\text{-}OH\text{-}\gamma\text{-}decalactone} = 29197 - 1312x_1 - 7407x_1^2 + 5008x_2 - 7976x_2^2 + 4985x_1x_2 - 4323x_1^2x_2 + 5165x_1x_2^2 + 5201x_1^2x_2^2$$

Eq. 6.4

Where, Y is the predicted response (γ-decalactone or 3-hydroxy-γ-decalactone concentrations); x_1 is the coded value of pH; x_2 is the coded value of dissolved oxygen concentration in the biotransformation medium.

The statistical models proposed for γ-decalactone and 3-hydroxy-γ-decalactone productions are significant since the corresponding *F*-values were 517.80 and 1251.05, respectively (

Table 6.2 and Table 6.3), which mean that there is only a 3.4% and 2.2% chance, respectively, that "Model *F*-Values" that large could occur due to noise.

The calculated coefficient of determination (R^2) for the model corresponding to γ-decalactone production was 0.9998 (

Table 6.2). This high value ensures a good adjustment of the model to the experimental data, indicating that 99.98% of the variability in the response could be explained by the model. The observed low differences (0.002) of the adjusted R^2 value (0.9978) and the R^2 value, further confirm the data accuracy. At the same time, a relatively low value of the coefficient of variation (R^2 = 3.50%) indicated a better precision and reliability of the performed experiments.

Concerning the model proposed for 3-hydroxy-γ-decalactone accumulation, a similar high significance of the model was observed since the R^2 value was 0.9999 (Table 6.3). Therefore, 99.99% of the variability in the response can be explained by the model. Moreover, the low differences observed (0.0008) of the adjusted R^2 value (0.9991) and the R^2 value, also confirm the data accuracy. The small value of the coefficient of variation (R^2 = 2.49%) indicated, as well, a good precision and reliability of the essays.

Three dimensional response surfaces (the graphical representation of the regression equations) were plotted, using MATLAB v. 7.7.0 (The MathWorks, Inc., Natick, MA, USA), to provide a visual interpretation of the interaction between the two factors and facilitate the location of the optimal experimental conditions.

Figure 6.3 illustrates the response surface describing γ-decalactone and 3-hydroxy-γ-decalactone productions, as function of pH and dissolved oxygen concentration.

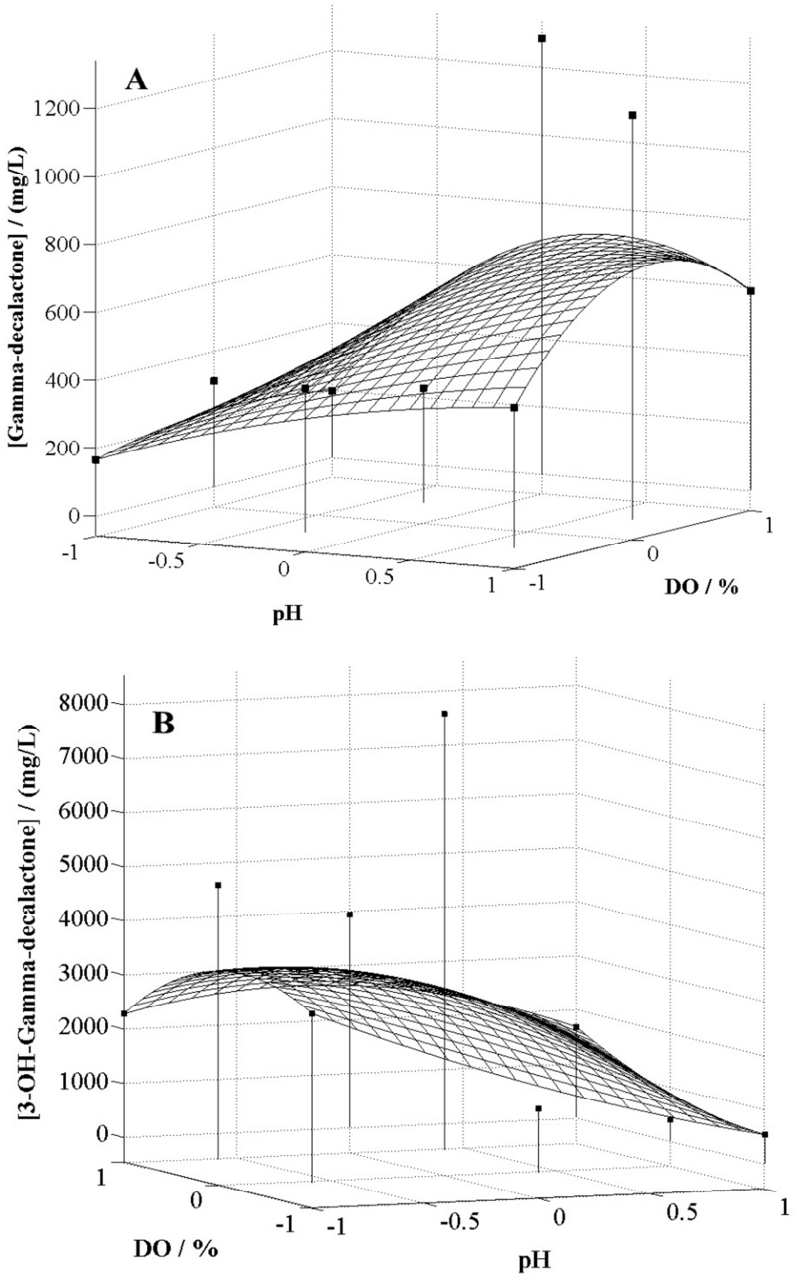

Figure 6.3 – Response surface curve for lactones (γ-decalactone – A; 3-hydroxy-γ-decalactone - B) production by *Y. lipolytica* as a function of pH and dissolved oxygen concentration in the biotransformation medium.

Regarding γ-decalactone accumulation, the trend observed in Figure 6.3A reveals a progressive increase in the response variable with increasing both independent variables up to a threshold value (DO and pH values comprised between 30% - 47% and 5.8 - 6.3, respectively), beyond which it decreased. These values are in accordance with those pointed out by García *et al.* (2009), which referred an optimal pH of 6 for γ-decalactone accumulation and a dissolved oxygen concentration around 40% at the maximum point of production. In a previous section of this work (5.1.3.2) also concluded that the accumulation of γ-decalactone was directly dependent on the oxygen transfer to the medium, in the range of the operating conditions analyzed (between 400 rpm, 0.6 vvm and 600 rpm, 3 vvm), meaning that higher oxygen levels would improve this aroma production.

According to Aguedo *et al.* (2005a) and García *et al.* (2007b), and contrarily to the results obtained in the present work, the maximum γ-decalactone accumulation would occur using low aeration rates. However, Aguedo *et al.* (2005) used higher oxygen concentrations, under hyperbaric conditions, which may had toxic effects on the yeast (oxidative stress), limiting the production of the aroma.

Equations derived by differentiation of the polynomial model given by Eq. 6.3, allowed estimating an optimum γ-decalactone concentration of 718.7 mg L^{-1}, from which it was possible to conclude that the experimental conditions able to simultaneously maximize these parameters would be: pH = 6.17 and DO = 44.4%.

In order to confirm the model adequacy in predicting maximum γ-decalactone concentration, one additional experiment under the optimal conditions was performed. The resulting maximum γ-decalactone accumulation was 680.9 mg L^{-1}, in 7 hours, which was very close to the estimated concentration (718.7 mg γ-decalactone L^{-1}). The good agreement between these two results verified the validity of the model and the existence of an optimal point.

By estimating the 3-hydroxy-γ-decalactone maximum concentration using the equations derived by differentiation of the model represented by Eq. 6.4, all possible solutions obtained corresponded to factors values out of the range tested. Therefore, no optimization was possible for this compound, among the range of operating conditions analyzed. However, Figure 6.3 B suggests that 3-hydroxy-γ-decalactone production would be higher for low pH values and DO around 25%.

The low pH values as the optimum for 3-hydroxy-γ-decalactone accumulation are corroborated by the works of García *et al.* (2007b, 2009). Concerning the effect of oxygen on the production of this lactone, the results herein obtained conflict with the results of Aguedo *et al.* (2005a), García *et al.* (2007b) and those from the section 5.1.3.2, who concluded that high oxygen transfer rates improved the accumulation of 3-hydroxy-γ-decalactone. On the contrary, in another work, García *et al.* (2009) describe a quite low dissolved oxygen concentration in the biotransformation medium (5%) as the optimal oxygen condition for 3-hydroxy-γ-decalactone production. Nevertheless, it is worth highlighting once more that it was not our primary goal to optimize the operating conditions for this lactone. The accumulation of this compound indicates a deviation in the metabolic pathway of γ-decalactone production, decreasing its yields.

6.1.4 Conclusions

This work demonstrated the feasibility of using experimental design tools to screen significant factors with influence in the responses and optimize biotransformation conditions for lactones production.

The factors pH and dissolved oxygen revealed to be significant for the production of both lactones.

The optimized operating conditions for γ-decalactone production were pH 6.17 and dissolved oxygen concentration in the medium of 44.4%. Applying these conditions, γ-decalactone production reached 680.9 mg L^{-1}, which was very similar to the RSM model predicted values of 718.7 mg γ-decalactone L^{-1}.

It was not possible to optimize 3-hydroxy-γ-decalactone production, among the range of operating conditions tested, and there are still some discrepancies among the data available in literature on this matter. Therefore, further studies will be focused on the optimization of the production of this compound, by enlarging the range of operating conditions, namely lowering the pH values.

The operating conditions herein defined as optimal for γ-decalactone production, were used in the experiments described in the next section, in which a comparison between batch and fed-batch cultures was performed.

6.2 FED-BATCH VERSUS BATCH CULTURES OF YARROWIA LIPOLYTICA FOR γ-DECALACTONE PRODUCTION FROM METHYL RICINOLEATE

6.2.1 Introduction

Considering that the primary goal of fermentation research is the cost-effective production of the products, it is important to develop a culture method that allows the production of the desired product at high concentrations and with high productivity and yield.

Fed-batch cultures have been widely employed for the production of various bioproducts including primary and secondary metabolites, proteins and other biopolymers. During fed-batch culture, one or more nutrients are supplied to the bioreactor while cells and products remain in the bioreactor until the end of operation. Fed-batch operation allows achieving higher cell density than batch mode and is often applied to obtain high yields and productivities of the desired product, by controlling the nutrient feeding (Lee *et al.*, 1999b). This feature is especially interesting for our process, considering the potential toxicity of ricinoleic acid to the cells (Feron *et al.*, 1997; Lee *et al.*, 1998; Lee *et al.*, 1999a; Lee *et al.*, 1995; Lin *et al.*, 1996). With this approach, it is possible to supply more substrate to the cells, simultaneously preventing toxic effects.

Several bioprocesses involving the yeast *Y. lipolytica* use the fed-batch operation mode successfully (Fickers *et al.*, 2009; Kim *et al.*, 1999; Kyong and Shin, 2000; Nicaud *et al.*, 2002; Rymowicz *et al.*, 2009; Turki *et al.*, 2010). However, there are very few works using fed-batch fermentation for γ-decalactone production (Kapfer *et al.*, 1989; Kumin and Munch, 1998; Lee *et al.*, 1995) and, to the best of our knowledge, no reports have been published to date on its application for γ-decalactone production from methyl ricinoleate by *Y. lipolytica*. Thus, the main aim of this study was to explore the fed-batch operation strategy to increase the yields of γ-decalactone production, using the optimum operating conditions defined in the previous sub-chapter.

6.2.2 Materials and Methods

Unless otherwise stated, 1.7 L of YPD medium, contained in a 3.7-L bioreactor (type RALF PLUS SOLO, Bioengineering, Wald, Switzerland), was inoculated with *Y. lipolytica* cells until a concentration of 2.4×10^{8} cells mL^{-1}. Cellular growth occurred at 27 °C, 500 rpm and with an initial aeration rate of 3 L min^{-1} for 24 h until the culture reached the late logarithmic growth phase, with a final concentration of 1.0×10^{9} cells mL^{-1}, corresponding to total glucose consumption. The biotransformation medium components were then added as a solution, in order to start the biotransformation stage. The source of ricinoleic acid used was methyl ricinoleate (MR).

Batch and fed-batch cultures were conducted in the bioreactor at the optimal conditions for γ-decalactone production previously determined (pH 6.2 and a dissolved oxygen concentration of 44.4%). The setpoint values of these parameters were automatically controlled by a control unit coupled to the bioreactor.

Two different strategies of fed-batch culture were investigated: constant and intermittent feeding rate. In the first case, the cellular growth occurred in 1 L of glucose medium, at which the biotransformation medium components were added. After the maximum peak of γ-decalactone production was reached, 1.5 L of biotransformation medium began to be fed to the bioreactor at a constant rate of 1 mL min^{-1} (initial dilution rate = 0.06 h^{-1}). In the second approach, the cellular growth occurred in 1.7 L YPD medium, at which the biotransformation medium components were added. When no more MR was detected in the medium, 30 g L^{-1} MR were added to the bioreactor. This addition occurred in two cycles.

Samples were collected at appropriate intervals for analysis of cell concentration and viability (sub-chapter 3.4.1) and for lactones and MR quantification (sub-chapter 3.4.3).

6.2.3 Results and Discussion

The cells of *Y. lipolytica* have the ability to use γ-decalactone as a carbon source, when the substrate is completely consumed (Aguedo, 2002), resulting in the complete disappearance of γ-decalactone from the medium after some hours of batch operation. In order to avoid this, as well as to prevent the inhibitory effects of the substrate (ricinoleic acid) on the cells, two different fed-batch operation strategies were investigated. In a first approach, the biotransformation medium

started to be fed continuously to the bioreactor when γ-decalactone concentration began to decline; secondly, an intermittent fed-batch strategy, in which MR, after its exhaustion, was added in pulses to the bioreactor, was attempted.

The cellular growth was low during the biotransformation in batch and fed-batch modes of operation. The population of *Y. lipolytica* suffered some oscillations that may be justified by the errors associated with the cellular counting method, but, in general, the cellular concentration was comprised between 1.0×10^{9} cells mL^{-1} and 2.5×10^{9} cells mL^{-1}. The viability of the cells remained practically constant at 100% during the whole experiments.

The low cellular growth in lipidic substrates was also reported by Turki *et al.* (2010), being responsible for the cellular mass concentration decrease in fed-batch cultivation, compared to the high-cell mass concentration attainable when glucose is used (Kim *et al.*, 1999).

The production of γ-decalactone and 3-hydroxy-γ-decalactone monitored. The accumulation of the latter is a result of a deviation in the metabolic pathway leading to γ-decalactone, which thus gives indications on the role of the enzymes involved.

The accumulation of both lactones in the biotransformation medium on batch and fed-batch modes is depicted on Figure 6.4.

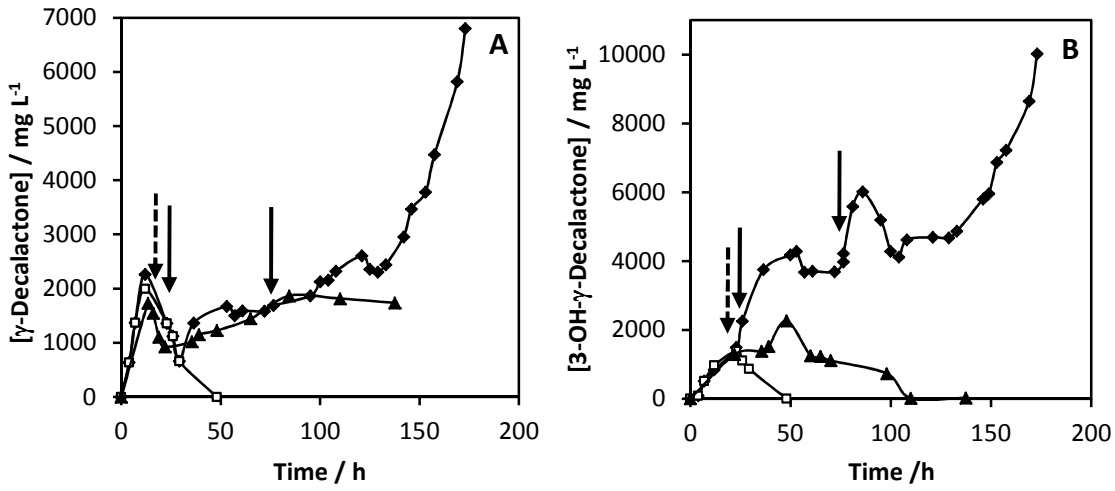

Figure 6.4 – Production of γ-decalactone (A) and 3-hydroxy-γ-decalactone (B) by *Y. lipolytica* cultures in batch (□), fed-batch at a constant medium feeding rate of 1 mL min^{-1} (▲) and with intermittent feeding (◆). The dashed arrow indicates the time at which the biotransformation medium started to be fed to the bioreactor in the fed-batch experiments at constant feeding rate and the solid arrows indicate MR addition to the medium in the intermittent fed-batch strategy.

In the fed-batch cultures, the concentration of both lactones increased after the feeding of additional medium (in the experiments at constant medium feeding rate) and after each MR addition (in the intermittent fed-batch strategy).

In the first fed-batch strategy investigated (conducted at a constant medium feeding rate of 1 mL min^{-1}), the maximum concentration of 1867 mg γ-decalactone L^{-1} obtained after 85 h was slightly inferior to the 1993 mg L^{-1} achieved in the batch culture, after 12 h of biotransformation. In batch culture, after reaching its peak, the aroma concentration decreased progressively until complete disappearance from the medium: this behavior was not observed in this fed-batch strategy. In fact, after the feeding of fresh medium, the aroma concentration increased and it was maintained in the biotransformation medium during the whole experiment. This reinforces the hypothesis of the consumption of lactone by the yeasts only in the absence of oil. Until now, using different culture modes, this was not so evident.

Regarding the accumulation of 3-hydroxy-γ-decalactone, a maximum concentration of 2265 mg L^{-1} was obtained after 48 h of biotransformation in the fed-batch culture, in opposition to 1388 mg L^{-1} achieved in the batch essay, at 23 h. In both cases, after reaching the peak of production, the

concentration of this lactone decreased until complete disappearance from the medium. As far as our knowledge goes, this behavior has never been previously reported. Literature suggests that 3-hydroxy-γ-decalactone is not degraded in the same way as γ-decalactone, since this compound usually remains in the medium (García *et al.*, 2009; Waché *et al.*, 2001). In fact, γ-decalactone started to accumulate at a relatively constant concentration when 3-hydroxy-γ-decalactone concentration began to decrease. Thus, it is possible that, at this point, the β-oxidation pathway is controlled by acyl-CoA oxidase, which implies a higher accumulation of γ-decalactone in detriment of 3-hydroxy-γ-decalactone.

Since this fed-batch approach led to the production of approximately the same amount of γ-decalactone than in batch, but taking more time, the overall productivity of the process was inferior to that obtained in batch. For this reason, and in the expectation of achieving higher γ-decalactone concentrations and productivities, an intermittent fed-batch strategy, based on the intermittent addition of MR was attempted.

Figure 6.4 shows notoriously that each step of MR addition became longer: in the first step (corresponding to the batch mode), 24 h were needed for the total disappearance of MR from the medium; in the second step, 50 h; and in the last step, it took 100 h to completely degrade the substrate. The fact that cells need more time to convert ricinoleic acid into lactones is most likely due to the toxic effect resulting from the exposure of cells to such high lactone concentrations, affecting their performance in the degradation of the substrate. The toxicity that lactones exhibit on microorganisms is potentially a major problem for reaching higher yields (Feron *et al.*, 1996b; Feron *et al.*, 1997). Nevertheless, the concentration of both lactones increased along the whole experiment and high amounts of γ-decalactone and 3-hydroxy-γ-decalactone were achieved with this strategy: 6798 mg L^{-1} and 10018 mg L^{-1}, respectively, after 173 h of biotransformation. The γ-decalactone concentration achieved is slightly higher than the values obtained by Alchihab *et al.* (2010) that used batch cultures of *Rhodotorula aurantiaca* with Macronet resins to recover the γ-decalactone produced, thus reducing the product toxicity effects. Similar behavior describing the product increase until the end of the experiment herein reported was described in other works involving fed-batch cultures (Kim *et al.*, 1999; Kyong and Shin, 2000; Nicaud *et al.*, 2002; Rymowicz *et al.*, 2009).

In the present fed-batch strategy, the control of the pathway seemed mainly carried out by the 3-hydroxyacyl-CoA dehydrogenase, since higher amounts of 3-hydroxy-γ-decalactone were detected in the medium. However, the activity of the acyl-CoA oxidase was high as well, since a high accumulation of γ-decalactone was also noticed.

An increase on γ-decalactone production in fed-batch compared to batch cultures was also reported by Lee *et al.* (1995). These authors attempted two fed-batch strategies to produce γ-decalactone by *Sporobolomyces odorus* from castor oil hydrolysate as source of ricinoleic acid. Initially, the substrate was added intermittently to the bioreactor, to maintain the ricinoleic acid level at approximately 0.06% in the medium. With this strategy, an improvement on γ-decalactone production was achieved: 163.7 mg L^{-1} in opposition to 60.2 mg L^{-1} obtained in batch culture. In accordance with our results, that research group obtained even better results when they used a three-step feeding of castor oil: a maximum of 208 mg γ-decalactone L^{-1} was obtained at 168 h of culture.

The use of fed-batch cultures also allowed Kumin and Munch (1998) to claim in a patent a concentration of 10.5 g γ-decalactone L^{-1} after 60 hours of culture with *Mucor circillenoides* and ethyl decanoate as substrate, which was continuously added at a nutrient broth feeding rate of 2-3 g L^{-1} h^{-1}.

The productivities (P) and the overall lactone yields relative to the substrate ($Y_{L/S}$) for each mode of operation were calculated according to Eq. 6.5 and Eq. 6.6.

$$P = \frac{L_f V_f - L_0 V_0 + \sum_i L_{si} V_{si}}{\left(V_f - V_0\right) t_f}$$

Eq. 6.5

$$Y_{L/S} = \frac{L_f V_f - L_0 V_0 + \sum_i L_{si} V_{si}}{S_0 V_0 - S_f V_f + S_F \int_0^{tf} F(t) dt}$$

Eq. 6.6

Where L is the concentration of lactone; V, the volume of medium; t, the time; S_F, the concentration of substrate in the feeding medium; F, the medium feeding rate; and the subscripts $_f$, $_0$ and $_{si}$ correspond to the final, initial and samples conditions.

The calculated values of productivities and yields were adjusted, taking into account the loss of lactone that occurred due to sampling. For this purpose, the sum of the amount of lactone removed from the reactor in the samples was introduced in the numerator of the equation.

As a summary, the overall productivities and yields of lactones relative to the substrate are displayed on Table 6.4.

Table 6.4 – Productivities (P) and yields in respect to the substrate ($Y_{L/S}$) achieved in each of the biotransformation strategies investigated (batch, fed-batch with constant medium feeding rate and intermittent fed-batch) for both lactones.

	γ-Decalactone		3-OH-γ-Decalactone	
Operation strategies	P (mg L^{-1} h^{-1})	$Y_{L/S}$ (mg g^{-1})	P (mg L^{-1} h^{-1})	$Y_{L/S}$ (mg g^{-1})
Batch	167.5	65.8	60.9	45.6
Fed-batch at constant feeding rate	39.7	38.3	81.2	46.1
Intermittent fed-batch	42.5	72.7	63.8	105.4

Although the productivity of γ-decalactone was considerably higher in the batch mode, the level of substrate conversion to both lactones was higher in the intermittent fed-batch, leading to higher aroma concentrations, which may have an important impact on a subsequent purification process. Nevertheless, the γ-decalactone productivity obtained in the intermittent fed-batch technique was similar to the one obtained by Alchihab *et al.* (2010) which was 45 mg L^{-1} h^{-1} with another yeast strain and CO.

Concerning 3-hydroxy-γ-decalactone, the highest productivity was obtained in the fed-batch with constant feeding rate technique, contrarily to γ-decalactone, which showed the lowest productivity

level, under those conditions. In terms of yields relative to the substrate, the intermittent fed-batch was the strategy more profitable for 3-hydroxy-γ-decalactone production.

6.2.4 Conclusions

γ-Decalactone production by intermittent fed-batch technique using *Y. lipolytica* is a process with high potential for the industrial application since the conversion of ricinoleic acid into γ-decalactone was improved through this process and high values of aroma concentration were reached. However, further studies are required to optimize the process, namely the rate of substrate addition during the biotransformation. Nevertheless, considering that this is the first report on the production of γ-decalactone from methyl ricinoleate by *Y. lipolytica* under fed-batch conditions, the results herein presented are very promising and give new insights for developing an innovative process with a potential for producing the aroma at industrial level.

6.3 LACTONES PRODUCTION IN AN AIRLIFT BIOREACTOR

6.3.1 Introduction

Although stirred tanks are the most common industrial bioreactors used for aerobic fermentations, they are not the best design for microbial cultures, for several reasons: the degree of agitation required to achieve sufficient oxygen mass transfer sometimes cause damage to the microorganisms; the mechanical energy input is high (meaning high costs), resulting in a overheating that has to be controlled. Furthermore, due to their complexity, the stirred reactors are more expensive and less robust than several other types of bioreactors. Based on these disadvantages of the stirred bioreactors, other designs were investigated, among which bubble columns and airlift reactors (Chisty, 1989). The latter also have advantages over the bubble columns: improved mixing and higher mass transfer coefficients, in some cases, due to the higher gas velocities that may be used in the airlifts. The airlift reactors also have a better defined liquid flow than the bubble columns (Chisty, 1989). For all these reasons, the airlift bioreactor was also used to carry out biotransformation experiments at different aeration rates.

This is, to our knowledge, the first work describing the production of γ-decalactone in an airlift bioreactor.

6.3.2 Materials and methods

The yeast cells were grown in three 500 mL-baffled flasks, containing 200 mL of YPD medium initially inoculated with a OD_{600} of 1 (equivalent to 1.2×10^{8} cells mL^{-1}), at 27 °C and 140 rpm. When an OD_{600} of 4 (equivalent to 2×10^{9} cells mL^{-1}) was reached and the glucose was completely consumed, the cellular suspension was centrifuged (6000 g, 5 min), the supernatant rejected and the cells were re-suspended in YNB with aminoacids (section 3.1.4). This solution was used to inoculate 4.5 L of biotransformation medium (without YNB) contained in the airlift.

Methyl ricinoleate at a concentration of 30 g L^{-1} was used as substrate for γ-decalactone production in the airlift.

The aeration rates used in this work were also, generally, higher than those used in the previous studies because, during the biotransformations oxygen depletion occurred, leading to the need of

increasing the gas flow-rate. Thus, gas flow-rates from 1 to 10 L min^{-1} were tested in distinct biotransformations and their impact on lactones production was analyzed.

6.3.3 Results and discussion

The results concerning γ-decalactone production in the airlift reactor, at different aeration rates, are presented on Figure 6.5.

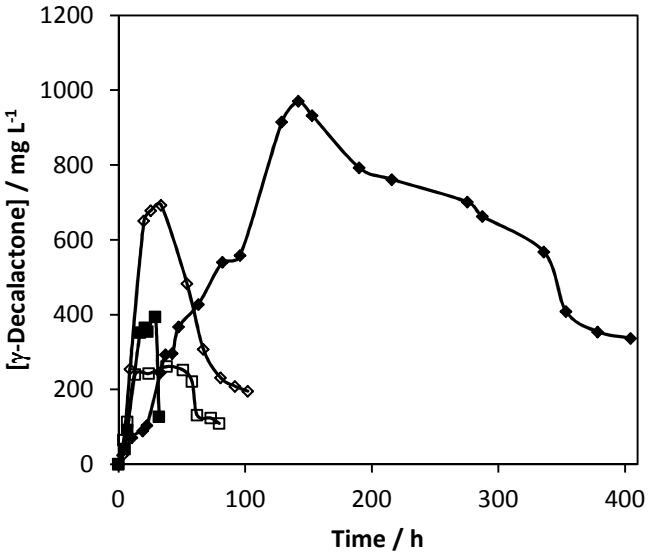

Figure 6.5 – γ-Decalactone production within the airlift bioreactor, with 30 g L^{-1} MR, at distinct gas flow-rates: (♦) 1 L min^{-1}; (◊) 5 L min^{-1}; (■) 7.5 L min^{-1} (□); 10 L min^{-1}.

The highest γ-decalactone production was obtained at an air flow-rate of 1 L min^{-1}. The increase of the aeration rate leaded to inversely proportional aroma concentrations. However, the time needed to reach the peak of γ-decalactone production was also reduced with higher aeration rates, resulting in higher productivities than the obtained using low aeration (Table 6.5).

Table 6.5 - Maximum productivities of γ-decalactone and 3-hydroxy-γ-decalactone, obtained in the airlift reactor, under different operating conditions.

	γ-Decalactone	3-OH-γ-decalactone
Gas flow-rate (L min^{-1})	Productivity (mg L^{-1} h^{-1})	Productivity (mg L^{-1} h^{-1})
1	6.8	1.9
5	20.7	17.6
7.5	17.4	60.6
10	6.9	54.9

It was previously verified (section 4.1.3.3.2) that despite an increase in the gas flow-rate being responsible for an increase in the volumetric mass transfer coefficient, the same does not occur with the liquid side mass transfer coefficient, which decreases with increasing aeration, possibly as a result of the novel distribution of the liquid phase that takes place at increased air flow-rates. This fact may in part justify the results obtained in the aroma production, especially considering that higher aeration rates reduced the time of contact between the gas bubbles and both liquid and solid phase, i.e., the cells.

Moreover, since the increase of liquid velocity does not increase proportionally with the increase of air flow rate (sub-chapter 4.1.2.2) the benefits of high flow-rates in promoting mixing may not be enough to compensate the problems that may arise due to evaporation of products.

However, the effect of oxygen on the enzymes controlling the metabolic pathway that leads to γ-decalactone and 3-hydroxy-γ-decalactone production, cannot be ignored. Previous results (Aguedo *et al.*, 2005a; García *et al.*, 2007b) indicated that the highest γ-decalactone production was attained using low aeration rates, which is in accordance with the results herein obtained.

Furthermore, as previously observed by García (2008) when investigating the production of 3-hydroxy-γ-decalactone in an airlift bioreactor, the use of higher aeration rates (and consequently higher turbulence) led to the formation of a thick layer of cells and lipids adhering to the wall of the reactor. According to this author, the increase of agitation (in this case, due to the raise of aeration rates in the airlift reactor) is responsible for the higher hydrophobic character of the cells surface,

promoting the adhesion to plastic materials, as is the case of *Perspex*, the material from which the airlift reactor is made.

An effect of aeration on cells adhesion has already been reported in yeast (Miki *et al.*, 1982; Millsap *et al.*, 1999). *Candida albicans* was shown to become more hydrophobic at higher aeration, and this property, resulting in cell adhesion to surfaces, was due to the production of hydrophobic proteins whereas the surface was richer in polysaccharides at lower aeration.

As hydrophobic cells tend to adhere to the surfaces, the amount of cells remaining in the biotransformation medium "available" for efficient substrate utilization is lower. Therefore, the great number of cells and oil droplets adhered to the wall surfaces of the airlift bioreactor at higher aeration rates, explains the decrease on γ-decalactone production. Moreover, the formation of biofilms reduces the oxygen transfer, also contributing for a decrease in γ-decalactone production despite the use of higher aeration rates.

In addition to the probable evaporation of the aroma out of the airlift reactor and to the adhesion of the most hydrophobic cells to the bioreactor walls (García, 2008), the low results obtained may also be justified by problems related with scale-up of the process. Negative effects of scale-up were reported before. In the work of Alchihab *et al.* (2010) the production of γ-decalactone by *Rhodotorula aurantiaca* was reduced in 31% by increasing 5 times the volume of the bioreactors used.

3-Hydroxy-γ-decalactone production was also analyzed since, as has been mentioned throughout this work, the accumulation of this compound can provide some information concerning the metabolic pathway control. According to Figure 6.6, the production of 3-hydroxy-γ-decalactone was inverse to that of γ-decalactone: higher aeration rates lead to higher concentrations and productivities (Table 6.5). This could mean that the pathway was controlled by 3-hydroxyacyl-CoA dehydrogenase. These results are corroborated by previous results presented in section 5.1.3.2 and by other works (Aguedo *et al.*, 2005a; García *et al.*, 2007b; García, 2008).

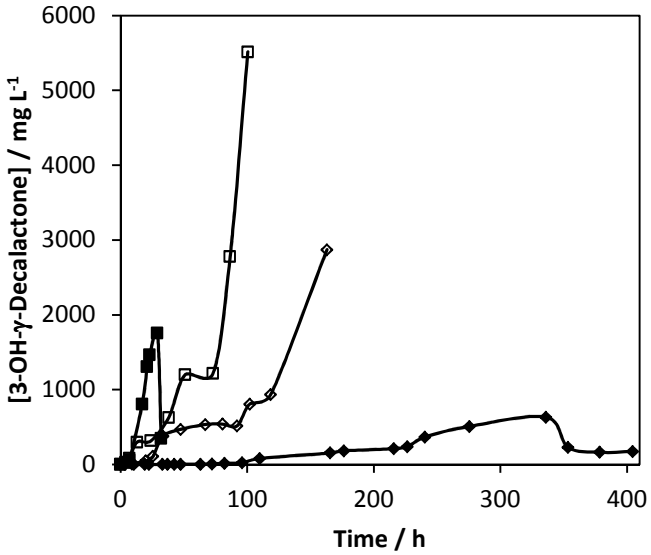

Figure 6.6 – 3-Hydroxy-γ-decalactone production within the airlift bioreactor, with 30 g L^{-1} MR, at distinct gas flow-rates: (♦) 1 L min^{-1}; (◊) 5 L min^{-1}; (■) 7.5 L min^{-1} (□); 10 L min^{-1}.

6.3.4 Conclusions

This work with the airlift reactor was a continuation of a study carried out by García (2008) describing the production of 3-hydroxy-γ-decalactone from methyl ricinoleate by *Y. lipolytica*. However, the present work focused more on the production of γ-decalactone.

The airlift bioreactor revealed to be a cheaper alternative for γ-decalactone production since it avoids the costs associated with agitation needs. However, several problems of the bioreactor design may limit the evaluation of the obtained, namely the impact of the construction material on the cells adhesion and possible evaporation of the aroma compound from the bioreactor. Thus, besides the importance of testing other materials like glass, other alternatives should be considered such as the inclusion of baffles in the different sectors of the reactor to enhance the turbulent mixing and thus preventing cells adhesion.

7 CONCLUSIONS AND FINAL REMARKS

The overall conclusions of the work described are herein presented as well as suggestions for future work related with this field of research.

7.1 FINAL CONCLUSIONS

The biotechnological production of γ-decalactone by biotransformation of ricinoleic acid by microorganisms is an attractive means to produce that aroma with a "natural" label, which is advantageous, considering the preference of consumers. Although there are many works described in the literature about this subject, several factors in the process remain to fully understand and, subsequently, to optimize.

The studies reported in this dissertation were primarily focused on one of those factors: the effect of oxygen in the overall process. Several studies have been performed in order to understand the effect of this compound upon the overall process, however contradictory results were sometimes achieved. In order to contribute to the knowledge on this topic, this work was initially based on the study of the oxygen mass transfer from air to the biotransformation medium. Since it consists of an oil-in-water emulsion stabilized by the non-ionic surfactant Tween 80, the effect of each component of the biotransformation medium in the oxygen absorption process was analyzed in a bubble column and in an airlift bioreactor and is described in CHAPTER 4. The increase of aeration rates is beneficial for both gas-liquid interfacial area and mass transfer due to the increase of turbulence and in the gas hold-up. The surfactant concentration has a positive effect on the gas-liquid interfacial area since it reduces the gas bubbles size and it has a negative effect upon the mass transfer because its molecules are located at the gas-liquid interface, hampering the oxygen mass transfer. Concerning the oil concentration, it has a negative effect upon the gas-liquid interfacial area but it improves mass transfer, since it causes a new distribution of surfactant in the medium, decreasing its concentration in the gas-liquid interface. The overall result is a negative effect of the organic phase upon the global volumetric mass transfer coefficient.

In CHAPTER 5, two different ricinoleic acid sources (methyl ricinoleate and castor oil) were used, in different concentrations, as substrates to produce γ-decalactone. Moreover, different cell inoculation strategies were attempted, differing among each other in the washing or not of the cells. The results revealed that the use of non-washed cells is more beneficial for the aroma production, independently of the substrate used; a concentration of 30 g L^{-1} MR was the most adequate among the range tested, since it was the operating condition that allowed the highest γ-decalactone productivity (14.9 mg $L^{-1} h^{-1}$). This substrate revealed to be a lipase inducer.

In what concerns the use of castor oil as substrate of the process, although high amounts of aroma accumulated (almost 2 g L^{-1}), the process was quite slow, reducing the productivities. An insufficient oil hydrolysis was hypothesized and thus the use of different commercial lipases was attempted as a means to enzymatically hydrolyze castor oil, under different pH and temperature values. Lipozyme TL IM, pH 8 and 27 °C were selected as the most efficient lipase and operating conditions, respectively, to hydrolyze castor oil. The results obtained using castor oil previously hydrolyzed by the selected lipase were compared with the results obtained in experiments in which the enzymatic hydrolysis occurred during the biotransformation and in experiments without adding lipase, indicating that the process was faster when lipase was involved in any form, but the aroma concentrations were lower, resulting in similar productivities.

The characterization of oil droplets size distribution by laser granulometry was performed under different oil (MR and CO) concentrations. The impact of the presence of cells on droplets size was also analyzed as well as the relevance of washing inoculum cells. Furthermore, the granulometric characterization of the emulsions was related with γ-decalactone production and it was observed that, in the presence of non-washed cells, the smaller droplets disappeared, using both oils, which increased γ-decalactone concentration. This result suggested that the access of cells to the substrate occurs by their adhesion around larger oil droplets.

Since best productivity values were achieved with methyl ricinoleate, at a concentration of 30 g L^{-1}, this compound was selected as the substrate to use in the subsequent studies and some experiments were performed in a stirred bioreactor at different aeration and agitation rates, demonstrating the direct influence of oxygen transfer rate on γ-decalactone and 3-hydroxy-γ-decalactone production. In the most parts of the experimental work, 3-hydroxy-γ-decalactone production was also monitored since it can also accumulate in the medium, derived from the same precursor of γ-decalactone (4-hydroxydecanoic acid). The accumulation of this compound indicates a deviation in the metabolic pathway of γ-decalactone production, decreasing its yields.

In CHAPTER 6, different strategies were attempted to optimize the production of γ-decalactone. In a first instance, the response surface methodology indicated a pH of 6.17 and a dissolved oxygen concentration in the medium of 44.4% as the optimized operating conditions for the aroma production. These operating conditions were applied in two fed-batch strategies: with constant medium feeding rate and with intermittent feeding. Both strategies were compared with the

traditional batch mode in terms of overall productivity and yield in respect to the substrate. Although the productivity was considerably higher in the batch mode (167.5 mg L^{-1} h^{-1}), the level of substrate conversion to both lactones (72.7 mg g^{-1} γ-decalactone and 105.4 mg g^{-1} 3-hydroxy-γ-decalactone) was greater in the intermittent fed-batch, allowing the accumulation of high aroma concentrations (6.8 g L^{-1} γ-decalactone and 10.0 g L^{-1} 3-hydroxy-γ-decalactone). The intermittent fed-batch strategy revealed, thus, to be a process with great potential for industrial application. Considering that this was the first report on the production of γ-decalactone from methyl ricinoleate, by *Y. lipolytica*, under fed-batch conditions, the results presented are very promising and give new insights for developing an innovative process with a potential for producing the aroma at industrial level.

Finally, the production of aroma was attempted in an airlift bioreactor due to the advantages of this type of bioreactor, mainly in terms of high power economies, the non-mechanical agitation which avoids damage to cells and the higher mass transfer coefficients attained. The highest γ-decalactone production was obtained at an air flow-rate of 1 L min^{-1}, the lowest aeration rate of the range tested. The aeration rate increase of 5-fold lead to lower aroma concentrations. However, the time needed to reach the peak of γ-decalactone production was also reduced, resulting in higher productivities.

7.2 FUTURE WORK

Although the present work brings new insights on the γ-decalactone biotechnological production, contributing for the optimization of some parameters of relevance for the process, a lot more research is still needed. Therefore, it is necessary to extend the range of operating conditions used in the oxygen mass transfer studies, namely the oil concentration and the aeration rates tested.

Since *Yarrowia lipolytica* is known to be a lipase producer and considering the need of castor oil hydrolysis when used as substrate, inducing lipase production previously to the biotransformation step may reveal to be beneficial for the process.

Considering the toxicity of γ-decalactone to the cells, it would be of interest to use immobilized cells. In this way, cells exhibit a higher tolerance to toxic compounds and thus, better product yields. In this context, the selection of supports for immobilization by adsorption (such as

polypropylene, methyl polymethacrylate, polyurethane foam, among others) and techniques by inclusion (e.g. alginate, chitosan, agarose and *k*-carrageenan) should be performed. Subsequently, the use of immobilized cells in the aroma production must be investigated, as well as the re-usability of the immobilized cells.

An interesting culture strategy to investigate in this bioprocess is the repeated batch culture. This method is known by enhancing the productivity of microbial cultures since the time wasted on the process preparation and on the lag phase is substantially reduced.

This was a preliminary study of the behavior of a biphasic medium in an airlift bioreactor to produce γ-decalactone. Therefore, this production must be optimized and it would be interesting to attempt different culture techniques, such as the continuous culture and repeated batches, recycling the cells. Moreover, operation of the airlift bioreactor should be improved through several modifications including testing other construction materials (like glass) and other design alternatives such as the inclusion of baffles in the different sectors of the reactor to enhance the turbulent mixing and thus preventing cells adhesion.

The method currently used to extract and quantify lactones and lipids involves a liquid-liquid extraction with a solvent (diethyl ether, in this case) and consequent analysis by gas chromatography. This methodology, besides being time consuming, involves several phases, contributing for the increase of experimental errors in the quantification of compounds. Therefore, the development and implementation of methods which would allow a better separation of the biotransformation medium components (lactones, lipids and/or fatty acids resulting from the oil hydrolysis) and subsequent quantification, is of great interest. It would be also of interest to develop recovering techniques of lactones from the medium.

Finally, aiming to turn the process as viable as possible, strategies to turn the process viable is intended to exploit the various bio-products that are produced by the yeast in the biotransformation medium, such as lipase which is induced by MR and 3-hydroxy-γ-decalactone, that was produced in high amounts. This lactone could be used to produce, by dehydration, two decenolides with aroma properties: dec-2-enolide (mushroom notes) and dec-3-enolide (fruity aroma). However, this bioconversion would result in similar concentrations of both decenolides. To overcome the problematic of separating the two enantiomers, an enzymatically bioconversion could be performed.

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