

## Development of bioprocesses based in biphasic media involving the yeast *Yarrowia lipolytica*

Nelma Gomes, Cristiana Gonçalves, Marlene Lopes, Mário Aguedo, José A.  
Teixeira, Manuel Mota, Isabel Belo\*

IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering,  
University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal.

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### Abstract

*Yarrowia lipolytica* has been intensively used in our research group to develop different bioprocesses based in the ability of this yeast to use hydrophobic substrates. Work has been focused in the optimization of the production of  $\gamma$ -decalactone and lipase, and to degrade and detoxify olive oil wastewater producing simultaneously high-value compounds, such as enzymes. Studies of the oxygen mass transfer phenomenon in biphasic media was also conducted and applied to select the most adequate oxygenation conditions for bioprocesses. Application of increased air pressures has also been proven to be an effective mean to improve oxygen transfer rate into *Y. lipolytica* cultures.

### 1 Introduction

The ascomycetous yeast *Yarrowia lipolytica* is a natural dimorphic fungus, which forms yeast cells, pseudohyphae and septate hyphae. The morphology is determined both by growth conditions (aeration, carbon and nitrogen sources, pH, etc) and by the genetic background of the strain (Barth and Gaillardin, 1997).

*Y. lipolytica* is one of the more intensively studied non-conventional yeast species. This microorganism is not only of interest for fundamental research, but also for biotechnological applications (Barth and Gaillardin, 1997). As it is considered as non-pathogenic and as GRAS by the American Food and Drug Administration, this yeast has been used in several industrial applications like production of single cell protein, peach flavour and citric acid (Beckerich et al., 1998). The range of substrates used by *Yarrowia lipolytica* include alkanes, fatty acids, organic acids, proteins and some sugars, which also contributes to the interest of this yeast (Fickers et al., 2005).

In our laboratory we have been exploring the abilities of this yeast in three main different fields: optimization of a peach-like aroma compound production; optimization of lipase production using hyperbaric conditions; and valorization of olive mill wastewater (OMW) by producing high-value compounds while degrading this waste.

### 2 Aroma production

$\gamma$ -Decalactone is a peach-like aroma compound well known in several food and beverages, reason why the food industry has a high interest in its biotechnological production. One of the better known applications is the biotransformation of ricinoleic acid catalysed by yeasts with GRAS status, conferring a natural label to the aroma, which is very important, considering the increasing health- and nutrition-conscious lifestyles.

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\* Corresponding author. Tel + 351-253604413. E-mail:ibelo@deb.uminho.pt

Ricinoleic acid (12-hydroxy-octadec-9-enoic acid) is a hydroxylated C<sub>18</sub> fatty acid that in its esterified form is the major constituent (about 86%) of castor oil, making it an abundant compound.

The yeast *Yarrowia lipolytica* is able to carry out the biotransformation of ricinoleic acid into  $\gamma$ -decalactone. This process involves the substrate degradation through the peroxisomal  $\beta$ -oxidation (Figure 1), leading to the formation of 4-hydroxydecanoic acid, which cyclises into  $\gamma$ -decalactone (Blin-Perrin et al., 2000).

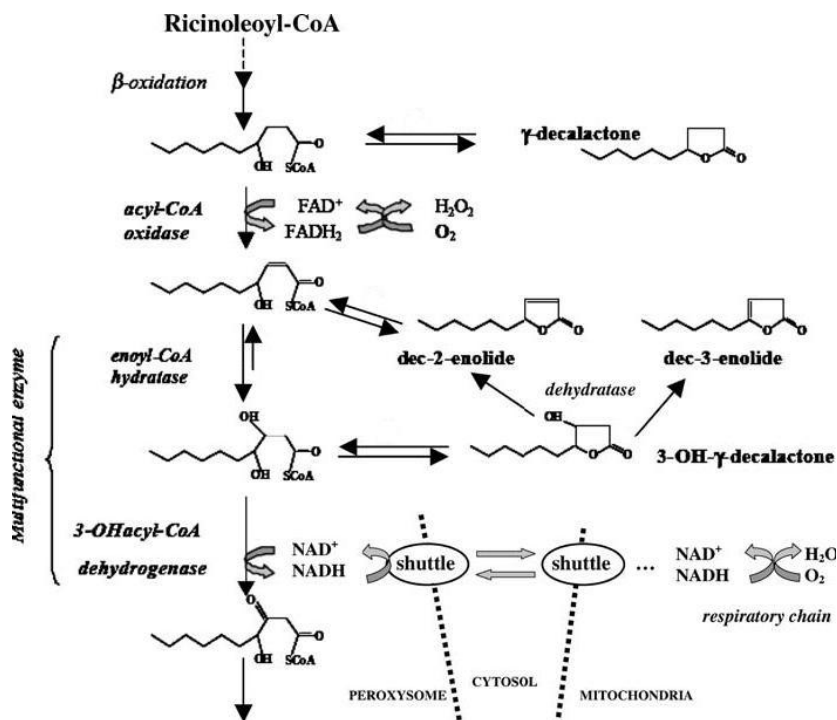


Figure 1 -  $\beta$ -Oxidation cycle from 4-hydroxydecanoic acid (the direct precursor of  $\gamma$ -decalactone) during the degradation of ricinoleoyl-CoA.

The production of  $\gamma$ -decalactone has been intensively investigated in order to better understand the all process and optimize it (Aguedo, 2002; Waché et al., 2001). Oxygen availability in the medium is a determining factor in the process, intervening in the control of the peroxisomal  $\beta$ -oxidation pathway. Thus, work has focused in the optimization and modelization of oxygen mass transfer in this complex multi-phase system composed by an aqueous and an organic phase as well as a gaseous and a solid phase (Gomes et al., 2007).

#### Oxygen mass transfer in the biphasic system

Oxygen transfer rate (OTR) in a system is a function of the oxygen volumetric mass transfer coefficient ( $k_L a$ ) and the oxygen solubility in the medium. For a specific bioreactor and medium, it is possible to increase  $k_L a$  and, consequently, OTR, using high agitation and aeration rates. However, this causes high power consumption, significantly increasing operation costs. The addition of a second, water-immiscible phase, in which oxygen has a higher solubility, has been proposed by several authors as an alternative mean of OTR improvement. Usually, experiments described in the literature use a hydrocarbon or a perfluorocarbon (PFC) as an inert dispersed phase in the aqueous medium. In the system herein described, the organic phase is the substrate used by the cells and not an inert compound used with the only purpose of increasing OTR. The effects of adding a second liquid organic phase on  $k_L a$  may change ( $k_L a$  values may increase, decrease, or remain constant) depending on the nature of the organic compound and the operating conditions (Dumont and Delmas, 2003). So, in order to take into account, not only the effect of the

organic phase, but also the effect of surfactant and operating conditions (agitation and aeration rates) on  $k_La$ , an empirical correlation was proposed, assuming that the two liquid phases behave as a single homogeneous phase (Eq. 1).

$$k_La = 243 \cdot \left( \frac{P_g}{V} \right)^{0.6} \cdot (v_s)^{0.6} \cdot (1 - X_{RM})^3 \cdot (1 - X_{Tween})^{-141} \quad \text{Eq. (1)}$$

Where  $P_g$  represents the power input of the aerated bioreactor;  $V$ , the bioreactor working volume;  $v_s$ , the superficial gas velocity through the bioreactor;  $X_{ORG}$ , the fraction of the organic phase;  $X_{Tween}$ , the fraction of surfactant; and  $\delta$ ,  $\alpha$ ,  $\beta$  and  $\gamma$  are numerical constants.

This correlation enables the prediction of  $k_La$  as a function of operating conditions and organic phase composition for the biotransformation system (Gomes et al., 2007).

An interaction effect between the organic phase and the surfactant was found: the organic phase increased  $k_La$  in the absence of the surfactant but had an opposite effect when Tween 80 was available in the medium. This is due to a change in the surfactant 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. The presence of surfactant inhibits the mass transfer because its accumulation at the gas-liquid interface causes a decrease in the liquid surface elements renewal (turbulence near the interface) and it implies that oxygen concentration in the liquid phase zone, 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 (Goméz-Díaz et al., 2008).

#### Influence of oxygen transfer rate on the aroma production

The influence of  $k_La$  on  $\gamma$ -decalactone production by the biotransformation of methyl ricinoleate (ricinoleic acid source) was proven through the performance of several experiments at different operating conditions, in a 2-L stirred bioreactor. A maximal aroma compound production ( $141 \pm 21 \text{ mg L}^{-1}$ ) was achieved for a  $k_La$  of  $70 \text{ h}^{-1}$ , obtained at  $27^\circ\text{C}$  with agitation and aeration rates of 400 rpm and 0.6 vvm, respectively, 1% (v/v) methyl ricinoleate and a cellular density of  $1.4 \times 10^8 \text{ cells mL}^{-1}$ .

Previous works in the group have demonstrated that hyperbaric air can be successfully applied to yeast cultivation as a way of improving the OTR to aerobic cultures (Belo et al., 2003). Based on this, the effect of increased air pressures on the oxygen mass transfer improvement for the production of  $\gamma$ -decalactone was studied (Aguedo et al., 2005). Essays were carried out in a 600-mL stainless steel high-pressure bioreactor. Air pressures of 1 bar (atmospheric pressure), 5 bar and 10 bar were applied on the cell growth and on the biotransformation of ricinoleic acid. The application of 5 bar accelerated cell growth, compared to the atmospheric conditions. However, when 10 bar was used, growth was inhibited after 10 h exposure, suggesting hyperbaric stress on the cells.

Besides  $\gamma$ -decalactone, also other compounds (3-hydroxy- $\gamma$ -decalactone, dec-2-en-4-olide and dec-3-en-4-olide) proceeding from the direct precursor of  $\gamma$ -decalactone (4-hydroxydecanoic acid) were analyzed (Figure 1).

Results revealed that oxygen plays an important role on the enzymes that control the  $\beta$ -oxidation metabolic pathway. Low oxygen concentrations in the medium induced the control of the  $\beta$ -oxydation pathway by acyl-CoA oxidase and therefore, an accumulation of  $\gamma$ -decalactone occurred. On the contrary, when higher oxygen levels were used, the control of the pathway was carried out by 3-hydroxyacyl-CoA dehydrogenase, resulting in an accumulation of 3-hydroxy- $\gamma$ -decalactone.

#### Effect of two different substrates and its concentrations on the aroma production

Since oxygen is more soluble in the organic phase than in the aqueous phase, increasing oil concentration leads to an oxygen mass transfer improvement.

We investigated the influence of two different ricinoleic acid sources (castor oil and methyl ricinoleate) at several concentrations, on the production of  $\gamma$ -decalactone by *Y. lipolytica* (Gomes et al., 2008). Experiments were carried out in 500-mL flasks. The highest amounts of  $\gamma$ -decalactone (around  $2 \text{ g L}^{-1}$ ) were achieved with oil concentrations of 3% and 5% (v/v) for each substrate, but as the process was quite long, the productivity of the process was small ( $10$  to  $14.5 \text{ mg L}^{-1} \text{ h}^{-1}$ ). In order to improve this, experiments with 3% (v/v) methyl ricinoleate were carried out in a 2-L stirred bioreactor, under different agitation and aeration rates. The highest concentration and productivity achieved for the aroma production was approximately  $1 \text{ g L}^{-1}$  and  $87.6 \text{ mg L}^{-1} \text{ h}^{-1}$ , respectively, with an agitation rate of 600 rpm and  $5 \text{ L min}^{-1}$  of aeration (Figure 2). However, contrary to the essays in flask, in bioreactor, high concentrations of 3-hydroxy- $\gamma$ -decalactone were produced for all conditions tested, reaching a maximal value of  $8 \text{ g L}^{-1}$ , using 600 rpm and  $5 \text{ L min}^{-1}$  of agitation and aeration rates, respectively. The accumulation of this compound and of  $\gamma$ -decalactone were directly proportional to the increase of OTR in the medium. Work now proceeds in the optimization of the OTR conditions to improve the overall process.

### 3 Lipase production

Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes finding vast applications in food, dairy, detergent and pharmaceutical industries.

Due to the facility of single cell use and to the high secretion ability, *Yarrowia lipolytica* has revealed to be an interesting and alternative organism to be used in lipase production (Pignede, 1998).

The increase of lipase productivity during the fermentation process is of great importance since lower costs of production could promote new industrial applications. The extracellular lipase production is affected by different environmental factors such as pH, temperature, medium composition, aeration, agitation and proteases present in the medium. Moreover, the presence of some compounds (i.e. fatty acids, triglycerides and surfactants) has often shown to induce lipase secretion (Dalmau et al., 2000).

#### Effect of medium composition and pH on lipase production

Lipase production by the strain *Y. lipolytica* W29 was previously studied through a experimental  $2^{4-1}$  fractionated factorial design (Lopes et al., 2007). A culture medium composed by YNB without aminoacids, at pH 7.2, with an arabic gum concentration of 0.5% (w/v) promoted the highest values of lipase activity ( $247 \text{ U L}^{-1}$ ) and productivity ( $10.7 \text{ U L}^{-1} \text{ h}^{-1}$ ). The presence of Tween 80 revealed to have a negative effect on lipase activity. Contrary to other studies (Dalmau et al., 2000; Fickers et al., 2003), the increase of olive oil concentration did not improve lipase activity and the presence of glucose did not inhibit the enzyme production.

#### Effect of increased air pressures on cell growth and morphology and on lipase production

The amount of available oxygen to *Y. lipolytica* is an important parameter since this strain is strictly aerobic. In order to obtain a further insight on the influence of moderate total air pressure both on yeast growth and on lipase production, *Y. lipolytica* was grown in 300-mL cylindrical stainless steel pressurized bioreactor where the variation of OTR with pressure could be modeled (Lopes et al., 2008). Despite the OTR increase of 2.3-fold when the pressure varied from 4 bar up to 8 bar, no differences were observed in the cellular growth.

An increase of the lipase activity at 8 bar ( $550 \text{ U L}^{-1}$ ) of 5.5-fold and 2.3-fold was obtained compared with the experiments under atmospheric pressure and 4 bar, respectively. The rise of the total air pressure from 1 bar up to 8 bar, led to a 4.8-fold improvement in the lipase productivity ( $11 \text{ U L}^{-1} \text{ h}^{-1}$ ). Besides lipase production, protease secretion into the medium was also observed. Its activity was inhibited by air pressure of 8 bar, indicating that pressure has different effect on lipase and protease activities.

Simultaneously, the effect of hyperbaric air in the morphology of this dimorphic yeast has been investigated using an automatic image analysis technique. It was observed that cell exposure to increased air pressure did not induced hyphae formation but led to a cell size decrease for the 8 bar cultures (Lopes et al., 2008).

#### Lipase induction by castor oil and methyl ricinoleate

Besides olive oil, other compounds can be used to induce lipase activity in *Y. lipolytica*. This is the case of the oils used in the biotransformation process of aroma production mentioned above. Lipase activity was detected in experiments with different concentrations of both ricinoleic acid sources, castor oil (CO) and methyl ricinoleate (MR) (Table 1).

**Table 1 – Maximum lipolytic activity obtained for different operating conditions.**

Experiments	[Substrate] (% v/v)	Maximum lipolytic activity (U L <sup>-1</sup> )
w/ washed cells	[MR] = 3	19 ± 3.9 (169.5 h)
w/o washed cells	[MR] = 3	105 ± 4.8 (271.5 h)
	[CO] = 3	42 ± 7.1 (192 h)

Methyl ricinoleate seemed to be more adequate than castor oil to induce lipase production, since the lipolytic activity was 2.5-fold higher with MR than with CO, for the conditions tested. Moreover, the enzyme production seems to be significantly affected by the hydrophobic characteristics of the cell, since a higher lipase activity was found when the biotransformation was carried out with cells that did not suffer the washing pre-treatment.

#### **4 Olive mill wastewater**

The ability of hydrophobic substrates use by *Yarrowia lipolytica* strains has been used not only to develop industrial biprocesses but also to solve environmental problems, such as bioremediation and lipidic wastewaters treatment (Fickers et al., 2005). Olive mill wastewater (OMW) is an example of those environmental problems resulting from the olive mill industry. OMW is a dark-colored stable emulsion effluent, characterized by a high polluting load.

The organic fraction of OMW includes sugars, tannins, polyphenols, polyalcohols, pectins and lipids, resulting in high values of chemical oxygen demand (COD). Thus, OMW can represent a possible renewal resource that might be a basis for fermentation processes.

Yeast species such as *Yarrowia lipolytica* can grow well in OMW media, consume the organic material and, simultaneously, produce biomass and other valuable products like enzymes and organic acids (D'Annibale et al., 2006), contributing for the valorization of OMW, which is the aim of the current investigation in our laboratory. Thus, in a first stage, work focused on the potential use of OMW as a culture medium for different *Yarrowia lipolytica* strains; i.e. their ability to grow and to produce high-value compounds, such as enzymes (e.g.: lipases), while degrading and detoxifying this waste was evaluated. For this purpose, different OMW from different regions of Portugal have been collected and characterized. Although all strains were able to grow in OMW based media without dilution, to consume almost all of the sugars present in the media and to significantly reduce COD values, the most adapted strains were selected.

Investigation now proceeds on the optimization of OMW fermentation process on a lab-scale bioreactor.

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