Oxygen effect in γ-Decalactone production through biotransformation of ricinoleic acid

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Abstract: γ-Decalactone can be produced biotechnologically from the degradation of ricinoleic acid by yeasts, namely *Yarrowia lipolytica*. Preliminary studies using two different ricinoleic acid sources (methyl ricinoleate and castor oil) at different concentrations were tested as substrates, in flask experiments. Although high amounts of γ-decalactone (up to 2 $g \cdot L^{-1}$) were achieved with oil concentrations of 3% and 5% (v/v) for each substrate, the productivity of the process was small (10 to 14.5 $mg \cdot L^{-1} \cdot h^{-1}$). In order to increase these values, essays were conducted in a 2-L bioreactor, with 3% (v/v) methyl ricinoleate at different aeration and agitation rates, in the herein presented work. Under these conditions, the highest amount of γ-decalactone achieved was lower (approximately 1 $g \cdot L^{-1}$). However, the productivity of the process was much higher: 87 $mg \cdot L^{-1} \cdot h^{-1}$. Furthermore, another compound derived from the direct precursor of γ-decalactone, 3-hydroxy-γ-decalactone, was detected in large amounts (up to 8 $g \cdot L^{-1}$), which implies a change in the metabolic pathway control.

Keywords: γ-Decalactone, *Yarrowia lipolytica,* biotransformation, methyl ricinoleate, oxygen, 3-hydroxy-γ-decalactone

1. INTRODUCTION

γ-Decalactone is a peach-like aroma compound of industrial interest that can be produced biotechnologically through the biotransformation of ricinoleic acid (12-hydroxy-octadec-9-enoic acid), catalyzed by some yeasts. One of the yeasts able to perform this biotransformation is *Yarrowia lipolytica* (Aguedo et al., 2004), a strictly aerobic microorganism and one of the most intensively studied non-conventional yeast species (Barth and Gaillardin, 1997). The process involves the substrate biodegradation through the peroxisomal β-oxidation, leading to the formation of 4-hydroxydecanoic acid, which cyclizes into γ-decalatone (Blin-Perrin et al., 2000).

Ricinoleic acid is a hydroxylated C_{18} fatty acid that in its esterified form is the major constituent (about 86%) of castor oil, which makes it an abundant compound, being the precursor most usually used in the production of γ-decalactone. In some cases, substrates of the process are castor oil hydrolysates, fatty acids or esters of these compounds (Page and Eilerman, 1996).

The accumulation of γ-decalactone in the medium depends on the rates of production and degradation by the cells. In both cases, the peroxisomal β-oxidation pathway is involved and several compounds (3-hydroxy-γ-decalactone, dec-2-en-4-olide and dec-3-en-4-olide), proceeding from the direct precursor of γ-decalactone (4-hydroxydecanoic acid) can be detected in the medium (Waché et al., 2003). The accumulation of these compounds in the medium 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^+ (Bakker et al., 2001) and therefore, influence the production of γ-decalactone (Figure 1).

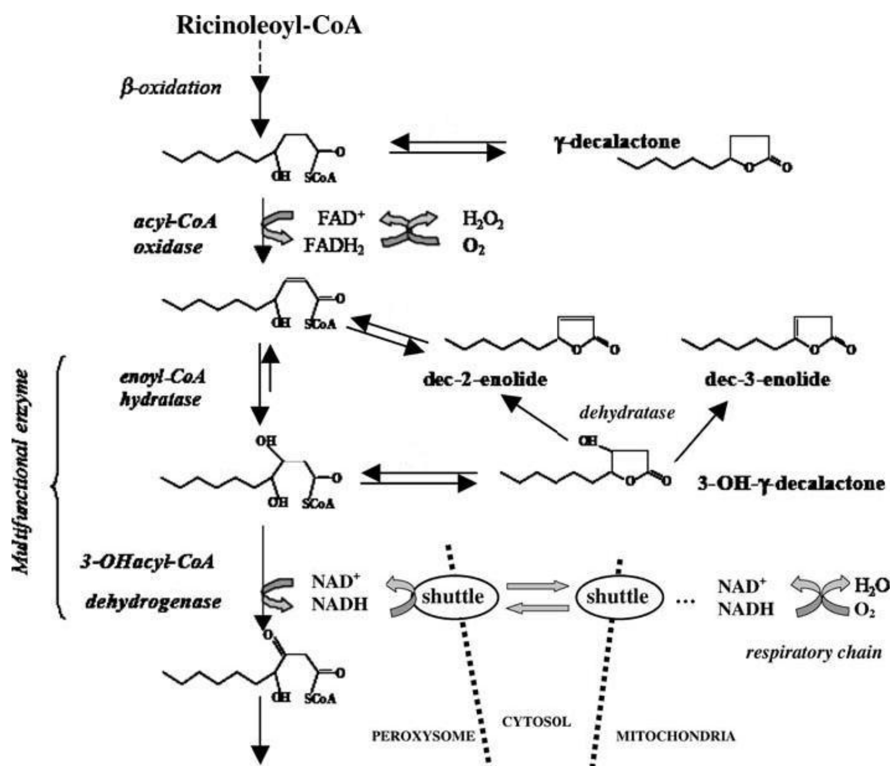

Fig. 1. β-Oxidation cycle from the direct precursor of γ-decalactone (4-hydroxydecanoic acid) during the degradation of ricinoleoyl-CoA. Shuttle mechanisms leading to oxidation of NADH and the link with the mitochondrial respiratory chain have been simplified (Waché et al., 2003).

Previous studies have shown the importance of oxygen mass transfer in the overall process (Aguedo et al., 2005; Gomes et al., 2007). Since oxygen is more soluble in the organic phase (methyl ricinoleate or castor oil) than in the aqueous phase, increasing oil concentration leads to the oxygen mass transfer improvement, which is important for the process efficiency. Oxygen transfer rate from the gas to the liquid medium can also be improved by increasing the aeration and stirring rates.

The influence of several concentrations (1%, 3% and 5% (v/v)) of two different ricinoleic acid sources (castor oil and methyl ricinoleate) on the production of γ-decalactone by *Y. lipolytica*, was previously studied in flask experiments. High amounts of γ-decalactone (up to 2 $g \cdot L^{-1}$) were achieved with oil concentrations of 3% and 5% (v/v) for each substrate, however the productivity of the process was small (10 to 14.5 $mg \cdot L^{-1} \cdot h^{-1}$) (data not shown).

In the present work, after selecting the best conditions in flask experiments, the production of γ-decalactone was analyzed in a 2-L stirred bioreactor under different oxygenation conditions, in order to analyze the role of oxygen in the system.

2. MATERIALS AND METHODS

1.1 Microorganism, media and culture conditions

Yarrowia lipolytica W29 (ATCC20460) was cultured for 48 h on YPDA medium (30 $g \cdot L^{-1}$ agar, 20 $g \cdot L^{-1}$ glucose, 20 $g \cdot L^{-1}$ peptone, 10 $g \cdot L^{-1}$ yeast extract) at 27 ºC and used to inoculate (to an optical density at 600 nm (OD_{600}) of 1) the bioreactor containing 1.7 L of YPD medium. When the culture reached the late logarithmic growth phase (OD_{600} of 4) and the whole glucose was consumed, the biotransformation medium components (6.7 $g \cdot L^{-1}$ Yeast Nitrogen

Base (YNB) with amino acids, 2.5 g·L^{-1} NH_4Cl, 30 g·L^{-1} methyl ricinoleate (MR) and 3 g·L^{-1} Tween 80), were added in order to start the biotransformation phase.

1.2 Bioreactor

Experiments were carried out in a 2-L bioreactor (Biolab, B. BRAUN, Germany) using 3% (v/v) MR as substrate. Agitation rates of 400 and 600 rpm were used, and the aeration rates tested were 1, 3 and 5 L·min^{-1}.

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

1.3 γ-Decalactone extraction and quantification

For the quantification of lactones, 1.5-mL medium samples were collected at appropriate intervals and extracted with 1.5 mL of diethyl ether by 10 gentle shakings after addition of γ-undecalactone as internal standard. After 5 min, the ether phase was separated and analyzed by gas chromatography (Varian 3800 GC) with a TR-WAX capillary column (30m×0.32mm×0.25µm) with Helium as a carrier gas. The temperatures of the split injector and the detector were set to 250 ºC and 300 ºC, respectively. The oven temperature was programmed to increase from 60 ºC to 145 ºC at a rate of 5 ºC min^{-1} and then to 180 ºC at a rate of 2 ºC min^{-1}.

3. RESULTS AND DISCUSSION

Since similar amounts of γ-decalactone (up to 2 g·L^{-1}) were achieved, in flask essays, with oil concentrations of 3% and 5% (v/v) for both substrates, methyl ricinoleate and castor oil (data not shown), further investigation proceeded with 3% (v/v) of MR. In order to increase the productivities obtained in flasks, experiments were carried out in a 2-L bioreactor, varying the oxygenation of the medium, through the manipulation of agitation and aeration rates.

The time course of dissolved oxygen concentration during the growth and the biotransformation phases is depicted in Figure 2. A complete depletion of dissolved oxygen occurred in the medium, at the end of the growth phase, using aeration rates of 1 and 3 L·min^{-1} with an agitation of 400 rpm.

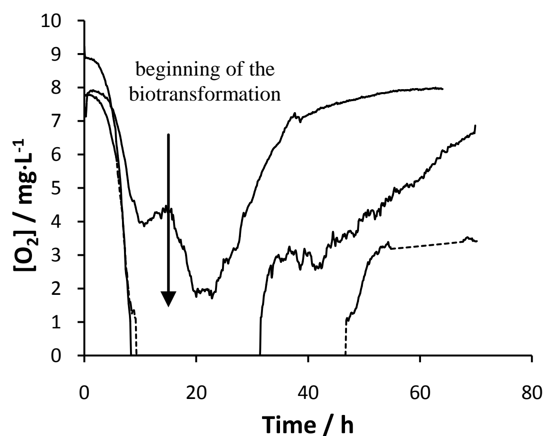

Fig. 2. Time course of dissolved oxygen concentration during the growth and the biotransformation phases in the bioreactor, under distinct aeration and agitation rates: (—) 400 rpm and 1 L·min^{-1}; (…) 400 rpm and 3 L·min^{-1}; (– – –) 600 rpm and 5 L·min^{-1}.

The maximum concentration of γ-decalactone obtained was around 1 $g{\cdot}L^{-1}$, when the agitation and aeration rates used were of 600 rpm and 5 $L{\cdot}min^{-1}$, respectively (Figure 3). For the lowest and the highest oxygenation conditions tested in this work (400 rpm, 1 $L{\cdot}min^{-1}$; and 600 rpm, 5 $L{\cdot}min^{-1}$, respectively), after the maximum value has been reached, at 12 hours of biotransformation, the aroma concentration started to decrease. A complete disappearance from the medium was observed after 30 hours of biotransformation, for the lowest oxygenation conditions. For the highest ones, after 48 hours, there was still some aroma left in the medium. However, for the intermediate oxygenation conditions, the maximum γ-decalactone concentration was achieved at 15 hours of biotransformation and this value remained constant until the end of the experiment (at 54 hours of biotransformation). The γ-decalactone disappearance is due to the ability of yeasts to consume the aroma as a carbon source (Aguedo, 2002).

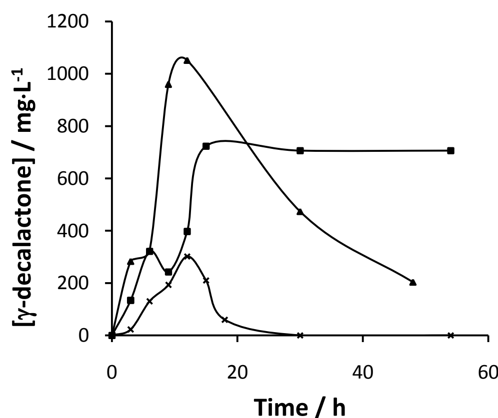

Fig. 3. Accumulation of γ-decalactone in the biotransformation medium under different operating conditions in the bioreactor: (×) 400 rpm and 1 $L{\cdot}min^{-1}$; (■) 400 rpm and 3 $L{\cdot}min^{-1}$; (▲) 600 rpm and 5 $L{\cdot}min^{-1}$.

It is notorious that, for the conditions tested, the accumulation of γ-decalactone is directly proportional to the enhancement of oxygen transfer to the medium. This result is in disagreement with the results obtained by Aguedo *et al*. (2005) and García *et al*. (2007). Both of these works concluded that low oxygen concentrations in the medium induce the control of the β-oxidation pathway by acyl-CoA oxidase and therefore, an accumulation of γ-decalactone occurs.

Despite the lower concentrations, the aroma production was much faster in the bioreactor and, therefore, the productivities achieved were greatly improved (Table 1).

Table 1 Productivity of γ-decalactone production in the bioreactor.

Operating conditions	Productivity ($mg{\cdot}L^{-1}{\cdot}h^{-1}$)
400 rpm; 1 $L{\cdot}min^{-1}$	25.2
400 rpm; 3 $L{\cdot}min^{-1}$	48.2
600 rpm; 5 $L{\cdot}min^{-1}$	87.6

Since it is known that oxygen may influence the activities of the enzymes of the peroxisomal β-oxidation pathway (by allowing the accumulation of other compounds derived from the 4-hydroxydecanoic acid), the production of another compound, 3-hydroxy-γ-decalactone, was also analyzed and surprising concentrations of this compound were detected (Figure 4).

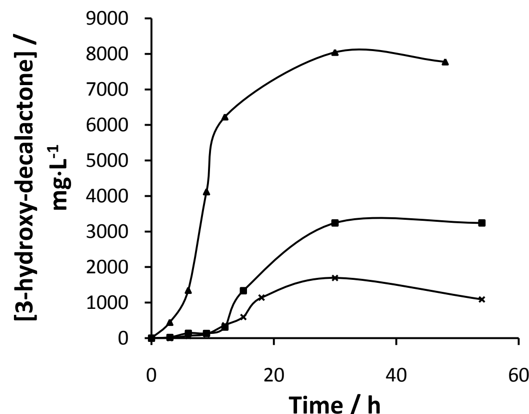

Fig. 4. Accumulation of 3-hydroxy-γ-decalactone in the biotransformation medium under different operating conditions in the bioreactor: (×) 400 rpm and 1 L·min^{-1}; (■) 400 rpm and 3 L·min^{-1}; (▲) 600 rpm and 5 L·min^{-1}.

The increase of 3-hydroxy-γ-decalactone concentration in the medium was also directly proportional to the enhancement of oxygen transfer. This result is in accordance to the results obtained previously by Aguedo *et al*. (2005) where it was observed that 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-γ-decalactone. Also García *et al*. (2007) have reported that this compound was promoted using high aeration conditions.

This study adds some more data on the effect of oxygen on the degradation of an oily substrate by yeast cells, notably on the control of the metabolic pathway involved. In the present case, it contributes also to a better knowledge of the industrial process leading to the bio-production of natural aroma compounds. However, further work is necessary and it now proceeds with experiments under controlled dissolved oxygen concentrations, in order to better understand the role of oxygen in the overall process and to improve the productivity of γ-decalactone production. Furthermore, it is known that *Yarrowia lipolytica* is also a producer of lipase (Lopes et al., 2008), enzyme able to catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. Therefore, lipases present in the medium may improve the availability of the substrate to the microorganism. However, its activity may be influenced by the activity of proteases since these enzymes may cause changes in lipase characteristics and also to degrade them (Kulkarni and Gadre, 1999). Thus, the role of these two extracellular enzymes is also being studied and these results will be presented.

REFERENCES

Aguedo M. (2002). Biotransformation de lipides en arômes par *Yarrowia lipolytica*: interactions avec le substrat, excrétion et toxicité des métabolites produits. PhD thesis, Université de Bourgogne, France.

Aguedo, M., Ly, M. H., Belo, I., Teixeira, J. A., Belin, J.-M., Waché, Y. (2004). The use of enzymes and microorganisms for the production of aroma compounds from lipids. Food Technol. Biotechnol.. 42, 327-336.

Aguedo, M., Gomes, N., Escamilla-Garcia, E., Waché, Y., Mota, M., Teixeira, J.A., Belo, I. (2005). Decalactone production by *Yarrowia lipolytica* under increased O_2 transfer rates. Biotech. Lett., 27, 1617-1621.

Bakker, B. M., Overkamp, K. M., van Maris, A. J. A., Kötter, P., Luttik, M. A. H., van Dijken, J. P., Pronk, J.T. (2001). Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev., 25, 15-37.

Barth. G., Gaillardin, C. (1997). Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. FEMS Microbiol. Rev., 19, 219-237.

Blin-Perrin, C., Molle, D., Dufosse, L., Le-Quere, J.-L., Viel, C., Mauvais, G., Feron, G. (2000). Metabolism of ricinoleic acid into γ-decalactone: β-oxidation and long chain acyl intermediates of ricinoleic acid in the genus *Sporidiobolus* sp. FEMS Microbiol. Lett., 188, 69-74.

Garcia, E. E., Belin, J.-M., Waché, Y. (2007). Use of a Doehlert factorial design to investigate the effects of pH and aeration on the accumulation of lactones by *Yarrowia lipolytica*. J. Appl. Microbiol., 103, 1508-1515.

Gomes, N., Aguedo, M., Teixeira, J.A., Belo, I. (2007). Oxygen mass transfer in a biphasic medium: Influence on the biotransformation of methyl ricinoleate into γ-decalactone by the yeast *Yarrowia lipolytica*. Biochem. Eng. J., 35, 380-386.

Kulkarni, N., Gadre, R. V. (1999). A novel alkaline, thermostable, protease-free lipase from *Pseudomonas* sp. Biotechnol. Lett., 21, 897-899.

Lopes, M., Gomes, N., Gonçalves, C., Coelho, M. A. Z., Mota, M., Belo, I. (2008). *Yarrowia lipolytica* lipase production enhanced by increased air pressure. Lett. Appl. Microbiol., 46, 255-260.

Page, G. V., Eilerman, R. G. (1996). Process for the Preparation of Gamma and Delta Lactones. European Patent EP0417203.

Waché, Y., Aguedo, M., Nicaud, J.-M., Belin, J.-M. (2003). Catabolism of hydroxyacids and biotechnological production of lactones by *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol., 61, 393-404.