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# Decalactone production by *Yarrowia lipolytica* under increased $O_2$ transfer rates

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**Abstract**

*Yarrowia lipolytica* converts methyl ricinoleate to γ-decalactone, a high-value fruity aroma compound. The highest amount of 3-hydroxy-γ-decalactone produced by the yeast (263 mg $l^{-1}$) occurred by increasing the $k_La$ up to 120 $h^{-1}$ at atmospheric pressure; above it, its concentration decreased, suggesting a predominance of the activity of 3-hydroxyacyl-CoA dehydrogenase. Cultures were grown under high-pressure, i.e., under increased $O_2$ solubility, but, although growth was accelerated, γ-decalactone production decreased. However, by applying 0.5 MPa during growth and biotransformation gave increased concentrations of dec−2-en-4-olide and dec-3-en-4-olide (70 mg $l^{-1}$).

## Introduction

γ-Decalactone is an aroma compound of industrial interest that can be produced biotechnologically by some microorganisms (Aguedo *et al.* 2004, Schrader *et al.* 2004). The strictly aerobic yeast, *Yarrowia lipolytica*, can transform methyl ricinoleate, using peroxisomal β-oxidation (Waché *et al.* 2001), into γ-decalactone, and is used industrially (Barth & Gaillardin 1996).

The accumulation of γ-decalactone in the medium depends on the production and degradation rates of the compound by the cells. In both cases the β-oxidative pathway is implied 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-hydroxydecanoate) can be detected in the medium (Gatfield *et al.* 1993, Waché *et al.* 2003). The accumulation of these different decalactones in the medium gives an indication of the various activities of the enzymes of the pathway. For example, γ-decalactone accumulation increases when acyl-CoA oxidase activity decreases (Waché *et al.* 2001). However, for the most part, the regulation of this pathway in yeast remains misunderstood.

The concentration of dissolved $O_2$ and the oxidative state of the medium influence metabolic pathways of *Y. lipolytica*, as the production of organic acids (Kamzolova *et al.* 2003) or the cytochrome-dependent, alkane oxidation (Fickers *et al.* 2005).

The activities of the peroxisomal β-oxidation enzymes, acyl-CoA oxidase and 3-hydroxyacyl-CoA dehydrogenase, may be influenced by $O_2$ which is necessary for the regeneration of the cofactors $FAD^+$ and, more indirectly $NAD^+$ (Figure 1) (Bakker *et al.* 2001). Thus, the $O_2$ influence on β-oxidation may be important in the process of γ-decalactone production and, more generally, a better

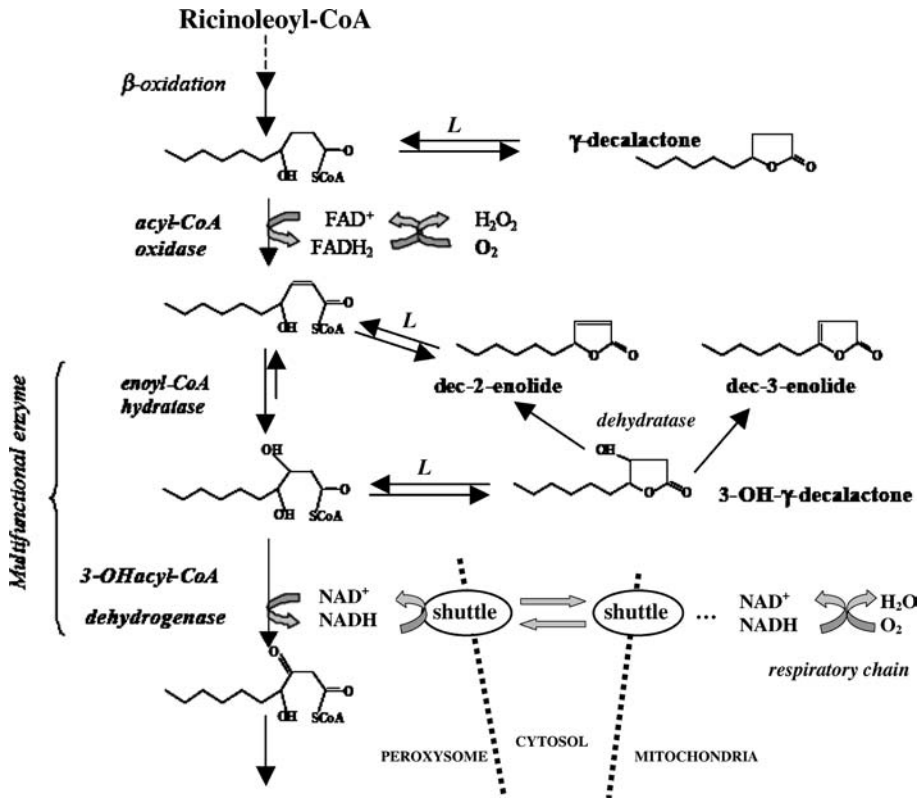


*Fig. 1.* β-Oxidation cycle from 4-hydroxydecanoate (the direct precursor of γ-decalactone) during the degradation of ricinoleoyl-CoA. *L*, lactonase: it is not known whether CoA esters or fatty acids are subject to lactonization. Shuttle mechanisms leading to oxidation of NADH and the link with the mitochondrial respiratory chain have been simplified (Bakker *et al.* 2001).

understanding of this peroxisomal pathway is of interest from a fundamental point of view.

The $O_2$ transfer rate (OTR) from the gas to the liquid medium can be improved by increasing the aeration and stirring rates at atmospheric pressure (enhances $k_La$) and also by raising air pressure in the reactor (increases $O_2$ solubility) (Yang & Wang 1992, Belo *et al.* 2003). The effect of pressure on the growth of *Y. lipolytica* in glucose medium has now been evaluated and the production of γ-decalactone and of three other lactones, followed in the biotransformation medium at different oxygenation conditions, in an attempt to investigate the influence of $O_2$ on of the pathway.

## Materials and methods

### *Microorganism and media*

*Yarrowia lipolytica* W29 (ATCC 20460) was grown in glucose medium as previously described (Aguedo *et al.* 2005).

The biotransformation medium was composed of 10 g methyl ricinoleate $l^{-1}$, 6.7 g yeast nitrogen base $l^{-1}$, 2.5 g $NH_4Cl$ $l^{-1}$ and 1 g Tween 80 $l^{-1}$.

### *Batch cultures*

A 2 l-bioreactor (Biolab, B. Braun, Germany) and a 600 ml stainless steel high-pressure bioreactor (model 4563, Parr, USA) were used, with 1.7 l and 400 ml of biotransformation medium respectively. Cells were harvested (6000 *g*, 5 min) from the initial medium, washed twice, resuspended in the biotransformation medium and fed into the bioreactor. Experiments in the Biolab bioreactor were carried out at agitation rates of 300 and 600 rpm and at aeration rates of 0.3–1.8 vvm. In the pressurized reactor an agitation rate of 400 rpm and an aeration rate of 0.9 vvm were used.

### *Lactone quantification*

Lactones were extracted from 2 ml broth with diethyl ether. The organic phase was analysed by

GC-MS (Varian 3400 GC with a CPWAX-52-CB column, coupled to Varian Saturn II MS).

## Results and discussion

The production of decalactones from 4-hydroxydecanoate was determined under different conditions of operation in the two bioreactors. Figure 2 shows the time course of lactone concentration obtained in the bioreactor at atmospheric pressure.

In all cases, the production of $\gamma$-decalactone was between 80 and 110 mg $l^{-1}$. Increasing the $k_La$ from 26 to 123 $h^{-1}$ (by the increase of stirring and aeration rates), prevented the depletion of $O_2$ from the medium during the phase of $\gamma$-decalactone production and the concentration of 3-hydroxy-$\gamma$-decalactone increased from 122 to 263 mg $l^{-1}$, with a smaller increase of 2- and 3-decenolides (Figure 2a and b). A further increase of $k_La$ to 162 $h^{-1}$ by an aeration rate of 1.8 vvm, maintained the dissolved $O_2$ at its maximum level but caused a substantial decrease in the concentrations of the three decalactones (Figure 2c). Increasing oxygenation first led to an increase in the concentration of 3-hydroxy-$\gamma$-decalactone and of both decenolides (Figure 2b), which suggests that the activity of acyl-CoA oxidase was being stimulated, as it requires $FAD^+$ and utilizes $O_2$ for its regeneration. However, a further increase in oxygenation drastically reduced the concentrations of these three compounds (Figure 2c).

It seems here that acyl-CoA oxidase controls the exit of the compounds from the pathway up to an $O_2$ level corresponding to a $k_La$ around 120 $h^{-1}$ and for higher values, the regulation by 3-hydroxy-acyl-CoA dehydrogenase prevails (Figure 1).

In all the cases, the concentration of dec-2-en-4-olide was higher than that of dec-3-en-4-olide.

The application of 0.5 MPa stimulated cell growth compared to the atmospheric conditions (Figure 3). Such a pressure increases the $O_2$ mass transfer to the medium and to the cells, leading to a positive effect on cell metabolism. When the cells were grown under a pressure of 1 MPa, growth was inhibited after 10 h exposure, suggesting hyperbaric stress on the cells.

In order to evaluate the effect of pressure on the metabolic pathway, as a first step the concentration of lactones in the supernatant was followed in the Parr bioreactor under atmospheric pressure. Under these experimental conditions, the production of decalactones was slower and a maximal concentration of $\gamma$-decalactone around

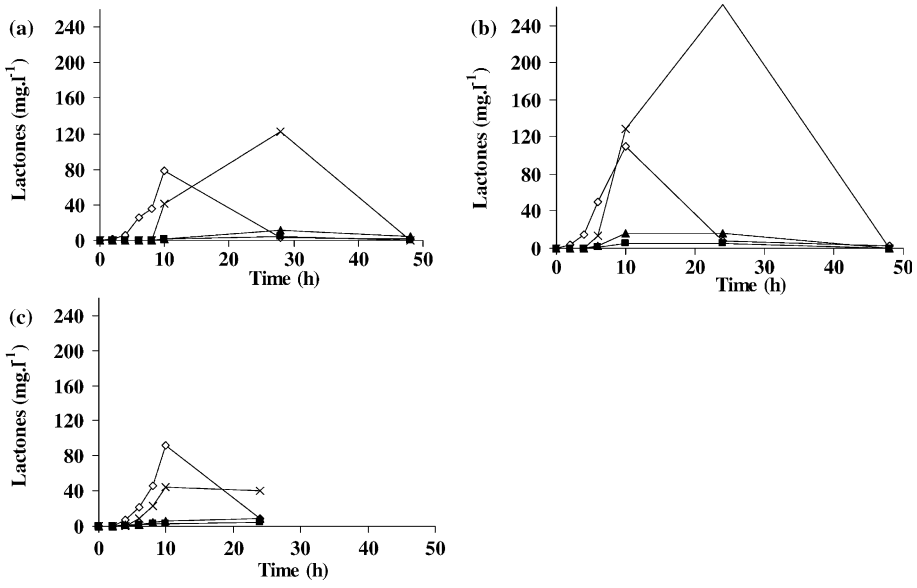


*Fig. 2.* $\gamma$-Decalactone (◇), 3-hydroxy-$\gamma$-decalactone (×), dec-2 (▲) and 3-en-4-olide (■) production profiles by *Y. lipolytica*, under atmospheric conditions, with different operation conditions: (a) agitation of 300 rpm and an air flow rate of 0.3 vvm; (b) 600 rpm and 0.9 vvm; (c) 600 rpm and 1.8 vvm.

300 mg $l^{-1}$ was reached after 25–30 h, but that of 3-hydroxy-$\gamma$-decalactone was in the same concentration range as previously (Figure 4a). When a higher pressure was applied, the excretion of 3-hydroxy-$\gamma$-decalactone was stimulated and the concentration of both decenolides was high (Figure 4b and c). Thus, the use of pressure improved the production of $\gamma$-decalactone, but increased the concentration of its oxidation compounds, suggesting that acyl-CoA oxidase was more active than 3-hydroxy-acyl-CoA dehydrogenase.

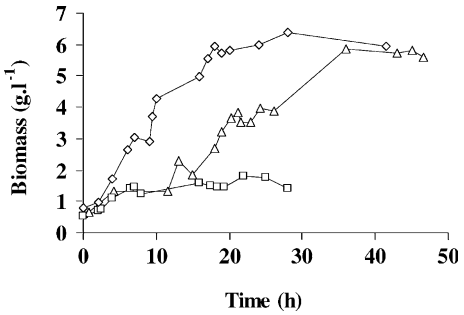


*Fig. 3*. Growth of *Y. lipolytica* in the high-pressure reactor with an agitation of 400 rpm and an air flow rate of 0.9 vvm, under atmospheric conditions (△), under pressures of 0.5 MPa (◇) and 1 MPa (□).

The effect of pre-adaptation of cells to pressure in the glucose medium before their transfer to the biotransformation medium was also investigated, but no influence on decalactone production profiles was detected (not shown). However, when both growth and then the biotransformation were performed at 0.5 MPa, the concentration of 3-hydroxy-$\gamma$-decalactone was equal to 120 mg $l^{-1}$ and that of both decenolides reached 70 mg $l^{-1}$ (Figure 4d). An explanation for this result is lacking: the occurrence of an induction of the dehydratase activity that converts 3-hydroxy-$\gamma$-decalactone into both decenolides would explain it. It is worth noting that dec-3-en-4-olide exhibits a peach note

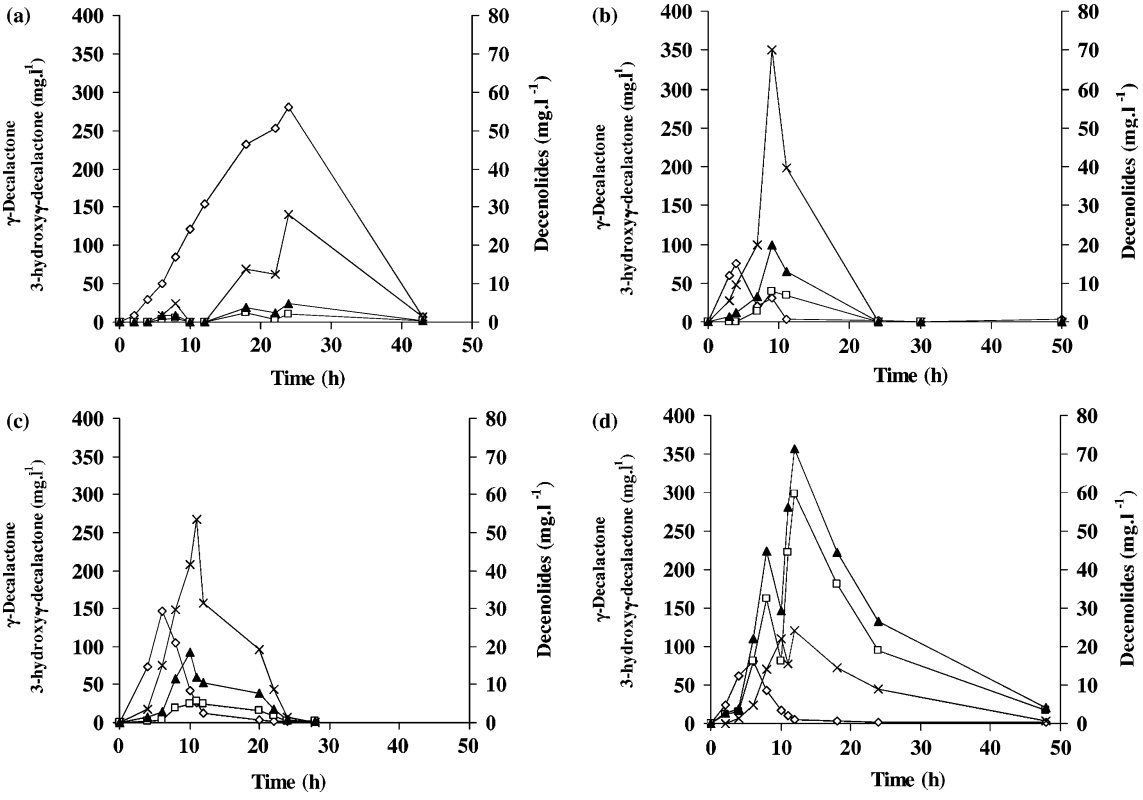


*Fig. 4*. $\gamma$-Decalactone (◇), 3-hydroxy-$\gamma$-decalactone (×), dec-2 (▲) and 3-enolide (■) production profiles by *Y. lipolytica*, in the high-pressure bioreactor with an agitation of 400 rpm and an air flow rate of 0.9 vvm, at different pressures: (a) 0.1 MPa; (b) 0.5 MPa; (c), 1 MPa and (d) 0.5 MPa during growth and 0.5 MPa during biotransformation.

more powerful than that of γ-decalactone, thus its production would be an open possibility but it would need to be separated from its isomer, dec-2-en-4-olide, which gives a mushroom taste (Gatfield *et al*. 1993). Another possible valorization would be the conversion of dec-3-en-4-olide to γ-decalactone, as patented with *S. cerevisiae* (Gatfield 1995).

## Conclusions

β-Oxidation in *Y. lipolytica* is sensitive to the oxidative state of the medium: from a single substrate, the pathway can give rise to different amounts of compounds with ten carbons. The control of the pathway by dehydrogenase seems to prevail when $O_2$ exceeds a threshold (corresponding to a $k_La$ value around 120 $h^{-1}$). The non-improvement of γ-decalactone production indicates that $O_2$ is not a limiting factor for the process; however, it is an important factor in the breakdown of decalactones: for example it may avoid the formation of γ-decalactone from 4-hydroxydecanoate when this is further oxidized.

In order to obtain higher γ-decalactone yields, $O_2$ in the medium can be decreased when the concentration of γ-decalactone is high (Figure 4a).

An important production of decenolides was achieved under a pressure of 0.5 MPa (Figure 4d), i.e. by using conditions that decrease the energy costs and the shear stress, when compared to the mechanical agitation necessary to reach the same OTR under atmospheric pressure (Knoll *et al*. 2005).

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