Short communication

Effect of *POX* genotype and *Lip2p* overexpression on lactone production and reconsumption by *Yarrowia lipolytica* using castor oil as substrate

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ABSTRACT

γ-Decalactone production from ricinoleic acid biotransformation by *Yarrowia lipolytica* has drawn the attention of many authors and, in the last years, molecular and physiological developments were made through the use of engineered strains in novel culture conditions.

The purpose of this work was to monitor the performance of modified *Y. lipolytica* strains in their lipid metabolism specifically at the β-oxidation pathway (MTLY40-2P strain, disrupted in the native genes *POX2-5* and expressing an ectopic *Aox2p*-encoding gene) or at the triglyceride hydrolysis (JMY3010 strain, *Lip2p* overexpressed) using castor oil as substrate, in a lab-scale bioreactor. Using step-wise fed-batch cultures of MTLY40-2P strain, a γ-decalactone concentration of 7 g L⁻¹ was reached. γ-Decalactone reconsumption was prevented and hydroxylactone production was reduced by *Y. lipolytica* MTLY40-2P strain. Moreover, substantial increase in the initial rate of aroma production was obtained with strain overexpressing *LIP2* gene due to the fast hydrolysis of castor oil.

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1. Introduction

Biotechnological production of γ-decalactone has great interest in the food industry due to the higher acceptability by consumers in comparison with similar products obtained by chemical synthesis. This yeast is able to produce γ-decalactone by means of hydroxylated C₁₈ fatty acid (ricinoleic acid) transformation. After γ-decalactone production pathway unravelled by Okui et al. [1] many other authors explored this topic, as is the case of Endrizzi-Joran et al. [2] who described the β-oxidation as the pathway for γ-decalactone production, further explored by Gatfield et al. [3], Haffner and Tressl [4] and Spinnler et al. [5]. Feron et al. [6] and Feron et al. [7] focused on the toxicity levels of this lactone, while Endrizzi-Joran [8] detailed the degradation pathway of γ-decalactone. In *Yarrowia lipolytica* the evidence of the involvement of β-oxidation and specifically of the acyl-CoA oxidases (Aox) in γ-decalactone production was obtained by Waché et al. [9]. The identification of the Aox encoding genes [10–12] and the characterization of these isoenzymes [13,14] paved the way to intensive metabolic engineering to better understand and control

γ-decalactone production [10,15–20]. Environmental conditions also revealed to be very important in aroma production [21,22] and particularly the aspects of oxygen mass transfer from air into the culture medium [23,24].

The yeast *Y. lipolytica* possesses a family of six acyl-CoA oxidases (Aox1 to 6 encoded by *POX1* to *POX6* [12]). The pathway first enzyme is generally considered as the limiting step in the catalysis [16]. The *POX1* disruption resulted in an increased β-oxidation activity but a decrease on the γ-decalactone production [25]. From the *POX* cluster, only two of the coded proteins exhibit high activity and chain-length specificity, one being long-chain-specific, Aox2 [14] and the other short-chain-specific, Aox3 [13]. The role of the other Aox's is less evident. Aox4, Aox5 exhibited a weak activity in the whole spectrum of straight-chain acyl-CoA (from C₄ to C₁₈) and, Aox1 and Aox6 did not exhibit any detectable activity. Latest studies to improve γ-decalactone production concerns β-oxidation pathway engineering [9,19,25,26]. Deleting Aox3 leads to slow γ-decalactone reconsumption [10] and others lactones production [19]. Short-chain β-oxidation was blocked by *POX3* disruption and resulted in 10-fold yield increase [10,16]. In addition, modification of β-oxidation fluxes at the multifunctional enzyme can trigger the switch between the production of γ-decalactone and the production of hydroxy-related lactones [21]. The yeast *Y. lipolytica* seems to have a low activity at the multifunctional enzyme level leading

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to the accumulation of three hydroxy-related lactones. The production of hydroxylactone with the *Y. lipolytica* Δ pox2pox3 is close to zero whereas γ -decalactone production continues [16]. These results are supported by Titorenko and coworkers [27] who demonstrated that Aox2p and Aox3p enzymes are required to assemble the global acyl-CoA oxidase complex and target it into peroxisomes.

Besides metabolic engineering, strategies to improve aroma production aim into culture conditions experimentation. The effect of medium composition on γ -decalactone production has been studied by Braga et al. [28] who investigated the influence of different castor oil concentrations in batch cultures and concluded that the best substrate concentration was 30 g L⁻¹ of castor oil, obtaining 1.8 g L⁻¹ of γ -decalactone. The fed-batch operation is usually applied to obtain high yields and productivities, comparing to batch mode, by controlling the nutrient feeding. Gomes et al. [22] obtained a γ -decalactone productivity of 0.043 g L⁻¹ h⁻¹ for a step-wise fed-batch operation applied to *Y. lipolytica* cultures, when 30 g L⁻¹ methyl ricinoleate was fed twice to the bioreactor.

In this study we report the characterization of γ -decalactone production in a lab-scale bioreactor using two *Y. lipolytica* mutant strains and castor oil as substrate: one with modifications in the lipid metabolism at the β -oxidation pathway (acyl-CoA oxidases); and another with improved triglyceride hydrolysis ability due to the *LIP2* overexpression.

2. Experimental procedures

2.1. Microorganism, media and culture conditions

The strains used in this work, which are listed in Table 1, were *Y. lipolytica* W29 (ATCC 20460) and its derived mutants with modifications in the lipid metabolism at the peroxisomal β -oxidation pathway (deleted for acyl-CoA oxidases encoding genes and *POX2* overexpression) – MTLY40-2P strain or at extracellular lipase expression (*LIP2* overexpression) – JMY3010 strain.

Cells were cultured for 48 h on YPDA medium (agar 30 g L⁻¹, glucose 20 g L⁻¹, peptone 20 g L⁻¹, yeast extract 10 g L⁻¹) at 27 °C. The cell colonies previously prepared were used to inoculate (cell density of 0.5 g L⁻¹) a 500 mL baffled Erlenmeyer flask containing 200 mL of glucose medium (YPD medium: glucose 20 g L⁻¹, peptone 20 g L⁻¹, yeast extract 10 g L⁻¹). Cultures were incubated at 140 rpm, at 27 °C for 19 h, until the total glucose consumption, and that subculture was used to inoculate 1.7 L of YPD medium in the bioreactor, to give an initial cell concentration of 0.5 g L⁻¹. After the cell growth phase of 19 h (final cell concentration of 30 g L⁻¹), the components of the biotransformation medium were added to the culture to initiate the biotransformation phase. The composition of the biotransformation medium was 6.7 g L⁻¹ YNB (yeast nitrogen base) with amino acids, 2.5 g L⁻¹ NH₄Cl, 30 g L⁻¹ or 60 g L⁻¹ Castor oil (CO) and 3 g L⁻¹ or 6 g L⁻¹ Tween 80, respectively.

Table 1
Yarrowia lipolytica strains used in this study.

Strain	Relevant genotype	Reference
W29 (ATCC 20460)	Wild type (WT)	[29]
MTLY40-2P	Δ pox2 Δ pox3 Δ pox4 Δ pox5 + pPOX2 ^a -POX2	[16]
JMY3010	W29 derivative containing an additional copy of <i>LIP2</i> , pTEF- <i>LIP2</i>	Unpublished

^a The pPOX2 promoter contained a deletion that results in a decrease catabolic repression [30].

2.2. Biotransformations

Experiments were carried out in a 3.7 L operating volume bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland). Cellular growth occurred at 27 °C, with 500 rpm and 3 L min⁻¹ of aeration rate for 19 h until a final cell density of 30 g L⁻¹ and total glucose consumption. After the cell growth phase biotransformations were performed at 650 rpm with an aeration rate of 5.1 L min⁻¹, and pH 6, with two different castor oil concentrations, 30 g L⁻¹ according with previous works [28,31] and the double of this concentration (60 g L⁻¹). Afterwards, a step-wise fed-batch strategy was performed, based on two additions of 60 g L⁻¹ castor oil.

2.3. Sampling and analytical methods

Samples were collected through time for analysis of extracellular lipase activity, lactone quantification and cell concentration. Extracellular lipase activity was measured in the supernatant by a spectrophotometric method using *p*-nitrophenyl butyrate as substrate [32]. Lactones were extracted from 2 mL samples with 2 mL diethyl ether and the organic phase was analyzed by GC [33]. Cell concentration was estimated using a Neubauer-improved counting chamber [34].

3. Results and discussion

3.1. Batch cultures

The MTLY40-2P was proven to produce high levels of γ -decalactone [16] from methyl ricinoleate due to severely decreased short-chain fatty acid degradation and enhanced long-chain fatty-acid β -oxidation through the overexpression of Aox2p [10]. To our knowledge this is the first time that a study aims to analyze the performance of this strain in CO medium. Braga et al. [28] and Braga et al. [33] pointed out the importance of extracellular lipases for the fast release of ricinoleic acid from castor oil. Thus, the JMY3010 that has an additional copy of *LIP2* coding for the main extracellular lipase, highly expressed under the strong and constitutive promoter of the TEF gene (translation elongation factor 1), was also studied using CO as substrate.

Fig. 1 shows γ -decalactone production in batch cultures of the three strains with 30 g L⁻¹ and 60 g L⁻¹ of CO. Briefly, it was observed that for all strains the increase of CO concentration from 30 g L⁻¹ to 60 g L⁻¹ increased the aroma production. This increase was higher for WT and MTLY40-2P reaching the value of γ -decalactone of 4.1 ± 0.4 g L⁻¹. This value is the highest ever reported with MTLY40-2P. Groguenin et al. [16] using methyl ricinoleate as substrate with this strain in flasks assays obtained a γ -decalactone maximum concentration of 300 mg L⁻¹. The two mutant strains produced higher aroma concentration for 30 g L⁻¹ CO than the WT. The influence of different substrate concentrations on γ -dodecalactone production by permeabilized *Waltomyces lipofer* cells was also studied by An et al. [35]. They observed a proportional increase in the production of γ -dodecalactone with a substrate concentration increase. Nevertheless, for substrate concentrations higher than 60 g L⁻¹ the production reached a plateau. On the other hand, Alchihab et al. [36] observed that γ -decalactone concentration obtained with the strain *Rhodotorula aurantiaca* was not proportional to the concentration of castor oil added.

The kinetic profile of γ -decalactone production for the WT shows a maximum of aroma production followed by a rapid aroma reconsumption, typical for this strain in batch cultures on several substrates [21–24,31,33]. The mutants contrasting profile exhibit no significant decrease at the end of the experiment, except for the MTLY40-2P with 60 g L⁻¹ CO where a 30% decrease in γ -decalactone concentration occurred.

Fig. 1. Effect of substrate concentration on γ -decalactone (filled symbols) and 3-hydroxy- γ -decalactone (open symbols) production: (a) 30 g L⁻¹ of castor oil; (b) 60 g L⁻¹ of castor oil. Data are presented as mean with the standard deviation of three independent experiments.

The MTL40-2P exhibited higher γ -decalactone production than the W29. Indeed the disruption of *POX3* coding the short-chain specific enzyme (Aox3) avoided degradation of the aroma or its precursor *via* continuation of β -oxidation after C₁₀ level or lactone reconsumption. Strains lacking the short-chain Aox (Aox3) consumed more slowly γ -decalactone [10,19,37]. The slight reconsumption observed at 60 g L⁻¹ CO is probably a result of the basal transcription levels of *Aox1* and *Aox6*. However, the lack of γ -decalactone reconsumption in JMY3010 was unexpected as this strain was not modified for its β -oxidation and possibly one of the hydrolysis products of castor oil, rapidly generated by overexpressed of *LIP2*, could be involved in the regulation of the β -oxidation pathway or other flux responsible for lactone reconsumption. We hypothesize that rapid release of glycerol upon CO hydrolysis could result in repression of *POX*, *MFE1* and *POT1* genes in this strain. Indeed, these genes were shown to be repressed by glucose and glycerol in a previous transcriptomic analysis (Morin and Nicaud, unpublished results). For example, *POX2* and *POX3* expression is about 10-fold lower in glucose

and glycerol compared to oil or fatty acid media. Even *POX3* appeared to be more repressed than *POX2* by glycerol than by glucose. This may explain the slowdown production after 10 h in JMY3010 compared to the WT and the absence of γ -decalactone reconsumption.

Since β -oxidation pathway may lead to the accumulation of other compounds derived from 4-hydroxydecanoic acid (precursor of γ -decalactone), the production of 3-hydroxy- γ -decalactone was analyzed (Fig. 1). As expected higher hydroxy-lactone concentration was achieved for strains that were not manipulated at β -oxidation level (W29 and JMY3010). Moreover, 3-hydroxy- γ -decalactone production increases as the CO concentration increases from 30 g L⁻¹ to 60 g L⁻¹ for these two strains. In this case, when more substrate is present the production of hydroxy-related lactones increases due to the substrate availability, since the pathway is not blocked after C₁₀. Also, for W29 3-hydroxy- γ -decalactone increases as γ -decalactone is consumed, by opposition with MTL40-2P (*POX3* disrupted gene) for which hydroxylactone production is reduced.

Table 2

Maximum γ -decalactone and 3-hydroxy- γ -decalactone productivity in batch cultures. Data are presented as the mean with the standard deviation of three independent experiments.

Strain	[CO] (g L ⁻¹)	γ -Decalactone productivity _{maximum} (g L ⁻¹ h ⁻¹)	3-Hydroxy- γ -decalactone productivity _{maximum} (g L ⁻¹ h ⁻¹)
W29	30	0.08 ± 0.01	0.02 ± 0.01
	60	0.21 ± 0.03	0.07 ± 0.01
MTLY40-2P	30	0.08 ± 0.01	0.01 ± 0.004
	60	0.14 ± 0.02	0.02 ± 0.002
JMY3010	30	0.13 ± 0.02	0.03 ± 0.01
	60	0.08 ± 0.02	0.12 ± 0.02

Table 3

Maximum γ -decalactone and 3-hydroxy- γ -decalactone productivity in step-wise fed-batch cultures. Data are presented as the mean with the standard deviation of three independent experiments.

Strain	γ -Decalactone productivity step-wise fed-batch (g L ⁻¹ h ⁻¹)	3-Hydroxy- γ -decalactone productivity step-wise fed-batch (g L ⁻¹ h ⁻¹)
W29	0.21 ± 0.03	0.05 ± 0.01
MTLY40-2P	0.10 ± 0.02	0.03 ± 0.01
JMY3010	0.05 ± 0.01	0.02 ± 0.01

Besides the differences observed in maximal aroma concentration obtained with the increase of CO concentration, the production rate was also changed. For W29, the increase in CO concentration leads to an increase in aroma production rate (a 4-fold increase in maximum value, 444 mg L⁻¹ h⁻¹, after 10 and 24 h), however a lag phase delay is observed at 60 g L⁻¹ CO when compared to 30 g L⁻¹ (Fig. 1). Also, a 2-fold increase in the γ -decalactone consumption rate was observed with the CO increase (−104 mg L⁻¹ h⁻¹ vs −211 mg L⁻¹ h⁻¹). For MTLY40-2P a lower initial (first 7 h) production rate was observed compared to W29. However, at 30 g L⁻¹ higher values of production rate were reached than for W29 (c.a. 2.5-fold). The increase of CO concentration for MTLY40-2P did not affect the maximum production rate attained but γ -decalactone consumption was observed. As JMY3010 has an additional copy of *LIP2* gene, thus producing more lipase than the W29 and MTLY40-2P, a fast initial aroma production occurred particularly for 30 g L⁻¹ CO. For W29 and JMY3010 the increase of CO leads to an increase in the maximum production rate.

The lipase production was analyzed and higher activity was obtained with JMY3010 (2000 U L⁻¹ to 3500 U L⁻¹) as expected. For W29 and MTLY40-2P lower activity was observed (1000 U L⁻¹ and 840 U L⁻¹, respectively). These differences in extracellular lipase explain the delay observed in the γ -decalactone production for MTLY40-2P that was eliminated in JMY3010, overexpressing *LIP2* gene. The existence of a lag phase particularly in MTLY40-2P in γ -decalactone production can be attributed to the time necessary for the synthesis of lipases, responsible for castor oil hydrolysis, and increasing ricinoleic acid availability (which is the substrate for the aroma synthesis) to the cells. This fact was also reported by Braga et al. [33] in which the addition of an extracellular enzyme during aroma production by *Y. lipolytica* W29 resulted in a reduction of the lag phase observed for γ -decalactone, since in these conditions the oil is hydrolyzed into ricinoleic acid earlier. In an industrial point of view, this process is not the most adequate since it is cost- and time-consuming. Thus, overexpressing *Lip2* enzyme would bridge the gap of this problem by improving γ -decalactone production rate with no extra costs.

From Table 2 is clear that the highest aroma productivity was obtained for WT, since the highest aroma concentrations were achieved with this strain and MTLY40-2P, but due to the lag phase

Fig. 2. γ -Decalactone (■) and 3-hydroxy- γ -decalactone (□) production in a step-wise fed-batch, after a batch phase with 60 g L⁻¹ castor oil. The solid arrows indicate 60 g L⁻¹ castor oil addition to the medium. Data are presented as mean with the standard deviation of three independent experiments.

of this last, more time was needed to achieve the maximum concentration, leading to a lower global productivity. For JMY3010 a higher production rate was observed but the maximal aroma concentration obtained was lower, leading to lower productivities.

Concerning 3-hydroxy- γ -decalactone, the highest productivity was obtained with JMY3010 at higher CO concentration, contrarily to γ -decalactone, which showed lower productivity under those conditions.

3.2. Step-wise fed-batch culture

As a prospect to improve γ -decalactone productivity, a step-wise fed-batch strategy was attempted in which 60 g L⁻¹ of castor oil was added in two pulses to the bioreactor (Fig. 2).

For W29 and JMY3010 no greater improvement in γ -decalactone production was achieved by using step-wise fed-batch culture, where only a 1.2-fold increase in aroma concentration was observed. On the other hand, a greater increase in aroma production was obtained for MTLY40-2P; in fact a 2-fold increase in γ -decalactone production was achieved leading to a maximum aroma concentration of 7 g L⁻¹. Ambid et al. [38] studied the γ -decalactone production with *Sporobolomyces odoratus* and methyl ricinoleate additions with 24 h gaps, and a 3-fold improvement in aroma fed-batch production was obtained compared with batch mode, but the maximum aroma concentration reached was only 142.7 mg L⁻¹.

Concerning the production of 3-hydroxy- γ -decalactone, in general, the concentrations obtained in fed-batch were similar with those obtained in batch for higher CO concentration (Fig. 1).

Tables 2 and 3 summarize the productivity values of γ -decalactone obtained with step-wise fed-batch and batch.

The highest γ -decalactone concentration was achieved with MTLY40-2P and around 2-fold improvement of maximum aroma concentration is observed in step-wise fed-batch mode compared to the batch culture, which have important impact in downstream processing cost savings.

Regarding 3-hydroxy- γ -decalactone productivity, higher values were obtained in batch mode since more time was needed to achieve the maximum hydroxyl-lactone concentration in fed-batch, leading to a lower global productivity.

4. Conclusions

Nowadays, industrial downstream process is one of the highest expensive step in any biotechnological setting. Therefore, is of the upmost importance the increase of studies aiming to achieve higher catalytic and capable strains from metabolic engineering pathways modification that would lead to an increase in accumulation of a desired product as well as reduction of other by products. In this study the effect of *POX* genotype and *LIP2* overexpression on γ -decalactone production and reconsumption during castor oil use by *Y. lipolytica* was analyzed.

Our results show that using CO as substrate, mutant MTLY40-2P (disrupted in the genes *POX2-5* and overexpressing *Aox2p*) prevented decrease of aroma at the end of the experiment and the production of hydroxylactone was minimized. Moreover, the overexpression of *Lip2p* gene increased the γ -decalactone production rate.

The γ -decalactone production by *Y. lipolytica* strains of different genotypes has been analyzed and castor oil concentration of 60 g L⁻¹ were shown to be the most adequate condition for γ -decalactone production in batch cultures. Although productivities in batch and step-wise fed-batch approaches were quite similar, a 2-fold improvement of maximum aroma concentration was obtained with MTLY40-2P in step-wise fed-batch mode compared to batch culture.

Considering the impact of castor oil hydrolysis to improve γ -decalactone production rate preliminary results highlight the importance to direct metabolic engineering in combining the *POX* level and *LIP2* overexpression. We hypothesize that the glycerol repression is a key point, and that fine tuning of lipase expression and β -oxidation genes expression would probably lead to further improve γ -decalactone production.

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