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ORIGINAL PAPER

Lipase Induction in *Yarrowia lipolytica* for Castor Oil Hydrolysis and Its Effect on γ-Decalactone Production

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Abstract γ-Decalactone is an aromatic compound of industrial interest, resulting from the biotransformation of ricinoleic acid, the major constituent of castor oil. In order to increase the availability of the substrate to the cells for the aroma production, castor oil previously hydrolyzed can be used. This hydrolysis may be promoted by enzymatic action, more specifically by lipases. In this work, the influence upon the aroma production of the lipase produced by *Yarrowia lipolytica*, a microorganism able to carry out the biotransformation, was studied. In a first approach, lipase induction conditions were analyzed using different *Y. lipolytica* strains and culture conditions, such as the inoculation mode of the lipase production medium. Lipase production was not affected by the cells centrifugation, so this step was eliminated, reducing the time and phases of the process. Moreover, *Y. lipolytica* W29 was shown to be the most adequate strain for lipase production. To investigate the importance of castor oil hydrolysis, the pre-addition of an inducer of lipase production (olive oil) to the biotransformation medium was tested. Results showed that the highest aroma production (1,600 mg L^{-1}) was obtained without a lipase inducer. However, the pre-induction of lipase decreased the lag phase for γ-decalactone secretion.

Keywords Castor oil · γ-Decalactone · Lipase · *Yarrowia lipolytica*

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Introduction

The production of aromas by means of biotechnological processes has proved to be of great interest, since the acceptability of these products to use in the food industry is greater than similar products obtained by chemical synthesis [1].

γ-Decalactone is an aromatic compound that can be obtained from the biotransformation of ricinoleic acid (the major constituent of castor oil, which is the raw material most commonly used in the biotechnological production of this aroma) by several microorganisms, among which is the aerobe *Yarrowia lipolytica* [2], the yeast used in the present work. Castor oil needs to be hydrolyzed in order to release ricinoleic acid to be used in the process. There are some chemical and physical methods available in the literature to hydrolyze oils. However, enzymatic hydrolysis, contrarily to the other techniques, works at moderate temperatures, allowing the production of an odorless and light-colored ricinoleic acid [3]. Neto et al. [4] studied the influence of castor bean oil previous hydrolyzed, by different microbial lipases, in γ-decalactone production with two fungi strains (*Geotrichum fragrans* and *Geotrichum* sp.) and concluded that the addition of hydrolyzed castor bean oil to the culture medium enhances γ-decalactone production. In γ-decalactone production by *Candida guilliermondii*, Farbood and Willis [5] used a lipase (steapsin) to previously hydrolyze castor oil.

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes comprised from the hydrolases family whose main biological function is catalyzing the hydrolysis of insoluble triglycerides to generate free fatty acids, mono and diglycerides and glycerol. In addition, lipases can catalyze reactions of esterification, interesterification and transesterification in a non-aqueous medium [6]. A wide

range of microorganisms (bacteria, fungi, yeasts) can produce lipases with different enzymological and substrate specificities [7]. *Y. lipolytica* is also known to be a lipase producer [2].

Lipase production can be enhanced by optimization of culture conditions, including pH, temperature, aeration and medium composition. Furthermore, the presence of some compounds (such as fatty acids, triglycerides, among others) has often been shown to induce lipase secretion [8, 9]. However, the activity of the lipase may be influenced by the activity of proteases since these enzymes may cause changes in lipase characteristics and also degrade them [10].

Previous works on lipase production usually involve the centrifugation of cells from the pre-culture, prior to the inoculation of lipase production medium [11]. However, Gomes et al. [2] studied the effect of either washing the cells or not from the pre-culture prior to inoculation of the biotransformation medium, upon the surface hydrophobicity of cells. These authors concluded that washed cells are less hydrophobic than non-washed cells and, consequently, have a lower affinity for the substrate. Considering this, the influence of including the centrifugation step of the cells or not prior to the inoculation of lipase production medium was investigated in the work presented here. After selecting the best conditions and strain to induce lipase production, the impact of this induction before or during the biotransformation of castor oil into γ-decalactone was evaluated. The goal of this work was to improve the production of γ-decalactone accelerating the hydrolysis of castor oil, by the lipase previously produced by *Y. lipolytica* cultures. For this purpose, lipase production was firstly tested using two *Y. lipolytica* strains (W29 ATCC20460 and IMUFRJ 50862) and olive oil as the inducer.

Experimental Procedures

Strains, Media and Culture Conditions

Yarrowia lipolytica strains (W29, which is the ATCC20460 strain, and IMUFRJ 50862 that was isolated from an estuary in the vicinity of Rio de Janeiro [12]) were maintained in YPDA medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 30 g L^{-1} agar) at 4 °C. Cells were used to inoculate (to a cellular concentration of 1.2×10^8 cells mL^{-1}) a 500-mL Erlenmeyer flask with 200 mL of YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose), at 140 rpm and 27 °C, during 24 h, until the cultures reached the late-logarithmic growth phase and the glucose was completely consumed (confirmed by the DNS method [13]).

After the growth phase, the impact of including or not the centrifugation step of the cells prior to the inoculation of the lipase production medium was tested. For this purpose, two different assays were conducted: in the first, the yeast cells were separated from the growth medium by centrifugation (6,000*g*, 5 min) and transferred to the lipase production medium (6.7 g L^{-1} yeast nitrogen base (YNB) without amino acids, 7 g L^{-1} olive oil, 5 g L^{-1} arabic gum and 400 mM Tris–HCl buffer at pH 7.2) contained in baffled Erlenmeyer flasks; in the second experiment, the lipase production medium components (6.7 g L^{-1} YNB without amino acids, 7 g L^{-1} olive oil and 5 g L^{-1} arabic gum) were directly added as a solution to the 200 mL YPD medium containing the cells (without centrifuging them).

The production of lipase was carried out in 500-mL baffled Erlenmeyer flasks containing 200 mL of lipase production medium. Flasks were incubated in an orbital shaker at 140 rpm and 27 °C for 48 h.

For the biotransformation assays, *Y. lipolytica* W29 (ATCC20460) was grown in YPD medium as described above and then, the components of the biotransformation medium (30 g L^{-1} castor oil, 6.7 g L^{-1} YNB with amino acids, 2.5 g L^{-1} NH_4Cl and 3 g L^{-1} Tween 80) were added to the medium, as an emulsion.

After selecting the best conditions and strain for lipase production, the impact of inducing lipase production before or during the biotransformation of castor oil into γ-decalactone, was evaluated. At the end of the exponential growth phase of the inoculum culture, the components of the lipase production medium were added, in order to reduce the steps of the process. Flasks were shaken at 140 rpm and 27 °C for approximately 22 h. After this time, the components of the biotransformation medium were added and the aroma production was followed.

All chemicals were purchased from Sigma-Aldrich (Sintra, Portugal), except for castor oil that was purchased from Interfat, S.A. (Barcelona, Spain).

Analytical Methods

Extracellular and total lipase (extracellular plus lipase linked to the cellular surface) activities were measured in the supernatant and in the samples with cells, by a spectrophotometric method using *p*-nitrophenyl butyrate as substrate, as previously described [14].

Protease was quantified using 0.5% (w/v) azocasein as substrate, in 50 mM acetate buffer at pH 5.0, as described elsewhere [2].

Lactone Extraction and Analyses

For the quantification of γ-decalactone, 2-mL medium samples were removed and their pH was lowered to two

with HCl. The extraction of the aromatic compound was performed with 2 mL diethyl ether by 60 gentle shakes, after addition of γ-undecalactone, as internal standard. After the complete separation of the liquid phases, the ether phase was analyzed by gas chromatography (Varian 3800 instrument, Varian Inc., USA), with a TRWAX capillary column (30 m × 0.32 mm × 0.25 μm, Teknokroma, Spain) with He as the carrier gas. The temperatures of the split injector and the detector were set to 250 and 300 °C, respectively. The oven temperature was programmed to increase from 60 to 145 °C at a rate of 5 °C min^{-1} and then to 180 °C at a rate of 2 °C min^{-1} [2].

Data were analyzed using the acquisition and integration software Star Chromatography Workstation v. 6.30 (Varian Inc., USA) and calibration curves previously obtained for γ-decalactone and γ-undecalactone. The average of various concentration/area ratios was determined, for both compounds, allowing to determine the response coefficient (K), which is obtained from the ratio between the averages corresponding to γ-decalactone and γ-undecalactone. The determination of K allowed quantifying γ-decalactone using Eq. 1.

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

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

Results and Discussion

Influence of Different Inoculation Strategies on Lipase Production

The yeast *Y. lipolytica* has been reported as a microorganism with great potential for lipase production. Nevertheless, the optimization of the culture conditions should be performed for each strain in order to increase lipase production. The presence of some compounds, such as fatty acids, triglycerides and surfactants, induce the secretion of lipase [15], but lipase production can also depend on inoculums' preparation.

Works described in the literature concerning lipase production usually involve the centrifugation of cells from the pre-culture, prior to the inoculation of lipase production medium [9, 11]. However, as previously mentioned [2], washing the cells act negatively on the surface hydrophobicity of cells, reducing their affinity for the substrate. Based on this, two methodologies were tested for the inoculation of the lipase production medium, including a step of harvesting the cells by centrifugation or not, prior to inoculation. Although this is not a cells wash, it removes the majority of the components of the growth culture medium, so we aimed to investigate the influence of centrifuging cells upon lipase production. For these experiments, olive oil, a well-known lipase inducer [16], was used.

Results presented in Table 1 indicate that the production of extracellular lipase, with both strains, is not affected by the cell centrifugation step, since the extracellular lipase activity values are not statistically significant ($p > 6\%$).

The similarity on lipase production with both methodologies tested suggests that the glucose present in the medium, during the cells growth, did not induce lipase production significantly, that only occurred when the medium contained lipid substrates (olive oil).

These results are in accordance with those of Nahas [17] for *Rhizopus oligosporus*, Baillargeon et al. [18] for *Geotrichum candidum*, and Rapp [19] for *Fusarium*, demonstrating that glucose present in the medium did not induced lipase production. Shirazi et al. [20] studied lipase production with *Saccharomyces cerevisiae* DSM 1848 and concluded that when the glucose was present in the medium, no lipase activity was detected. Also, Hegedus and Khachatourians [16] found that lipid sources in the production medium increased the production of lipase by *Beauveria bassiana*.

Table 1 Maximum of lipase and protease activities produced with different strains and conditions, using olive oil as lipase inducer

Yeast strain	Conditions			
	Maximum lipase activity (U L^{-1})		Maximum protease activity (U L^{-1})	
	With cells centrifugation	Without cells centrifugation	With cells centrifugation	Without cells centrifugation
Y. lipolytica W29				
Extracellular activity	449 ± 29	516 ± 27	3,402 ± 286	2,318 ± 102
Total activity	1,070 ± 117	1,009 ± 153	2,785 ± 42	2,475 ± 339
Y. lipolytica IMUFRJ 50862				
Extracellular activity	118 ± 38	205 ± 26	1,507 ± 81	565 ± 14

Data are presented as the mean and standard deviation of three independent experiments

Maximum lipase activity was obtained with around 22 h of incubation

The total lipase activity (extracellular plus the lipase linked to the cells surface) produced by the strain *Y. lipolytica* W29 was measured and the values were statistically similar ($p > 6\%$) for the two methodologies ($1{,}070 \pm 117$ and $1{,}009 \pm 153$, with and without cell centrifugation, respectively).

The differences observed between the values of total and extracellular protease produced by *Y. lipolytica* W29 are not statistically significant ($p > 6\%$). In this case, the results for extracellular and total activity are identical, once the enzyme is not linked to the cells and is secreted to the culture medium.

The extracellular protease activities obtained with the strain *Y. lipolytica* IMUFRJ 50862 are lower in the experiments without the cells centrifugation step, probably due to some residues from the growth medium (such as peptone and yeast extract) that can exert protease inhibition [21]. However, a low activity of these enzymes is beneficial because proteases can degrade proteins present in the medium.

According to the results, the cells centrifugation step does not influence lipase production, so the following lipase production experiments were performed without cells centrifugation, since it reduces the number of steps of the process.

Lipase Production

The highest values of extracellular lipase activity were obtained with the strain *Y. lipolytica* W29. Thus, this strain was selected for the following experiments.

The mechanisms regulating lipase production differ from microorganism to microorganism. Results obtained with *Rhizopus* [22], *Aspergillus* [23] and *Rhodotorula* [24] showed that lipase production is independent of the lipid substrates addition to the culture medium. Moreover, microorganisms such as *G. candidum* [25] need a lipid substrate addition for lipase production. Hegedus and Khachatourians [16] analyzed the effect of adding 2% (w/v) of olive oil to YPD medium and to a medium with glucose and salts (GS medium) and reported a threefold and 1.5-fold increase in lipase production when olive oil was added to the YPD and GS medium, respectively.

Domínguez et al. [15] studied the influence of lipids on lipase production by *Y. lipolytica* CECT 1,240 and observed that the addition of lipidic compounds (olive oil, sunflower oil, tributyrin, oleic acid) to the culture medium increased lipase activity levels two- to four fold with respect to the control culture.

Lipase production by *Y. lipolytica* W29 was therefore analyzed here using olive oil as the inducer.

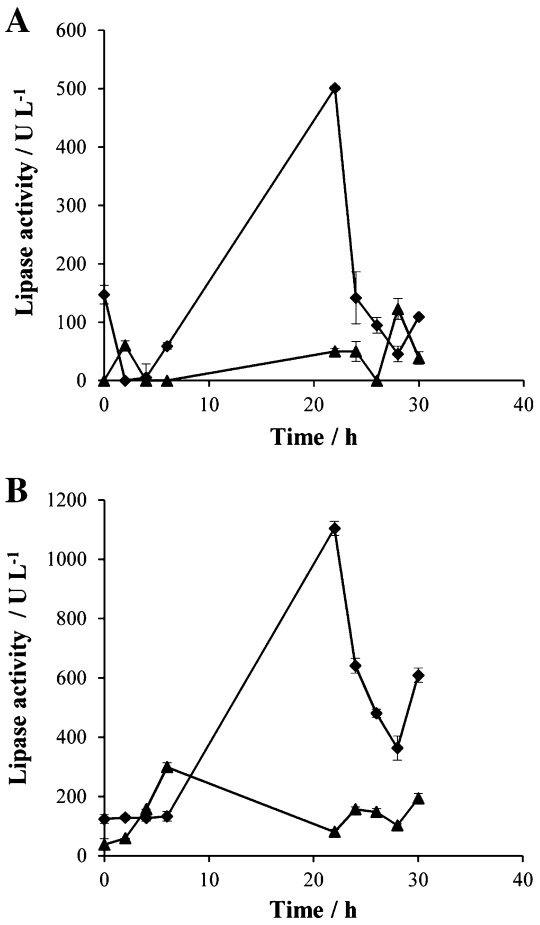

Fig. 1 Lipase activity profiles by *Y. lipolytica* W29 with olive oil (*filled diamonds*) and without inducer (control) (*filled triangles*): **a** extracellular lipase activity, **b** total lipase activity. Data are presented as the mean and standard deviation of three independent experiments

The kinetic profile of extracellular (Fig. 1a) and total lipase activity (Fig. 1b) shows that the lipolytic activity increases up to a maximum, after which it decays. This behavior is in accordance with the results obtained by other authors [26]. In both cases, after 22 h, there is a decrease in lipase production in the medium containing olive oil. In the medium without inducer (control), the extracellular lipase production starts decreasing after 24 h and there is a slight increase in its activity after 26 h, in both cases. After 22 h, the total lipase activity values, in the control medium, show some oscillations (from 80 to 193 U L^{-1}) until the end of the experiment. It is also possible to observe that, in the absence of an inducer, *Y. lipolytica* W29 produces small quantities of lipase.

Besides lipase production, the production of other enzymes, such as proteases, by *Y. lipolytica* strain has been reported [27]. In spite of the fact that the culture medium used favors lipase production, protease activity was detected in the medium. The lipase activity detected in the

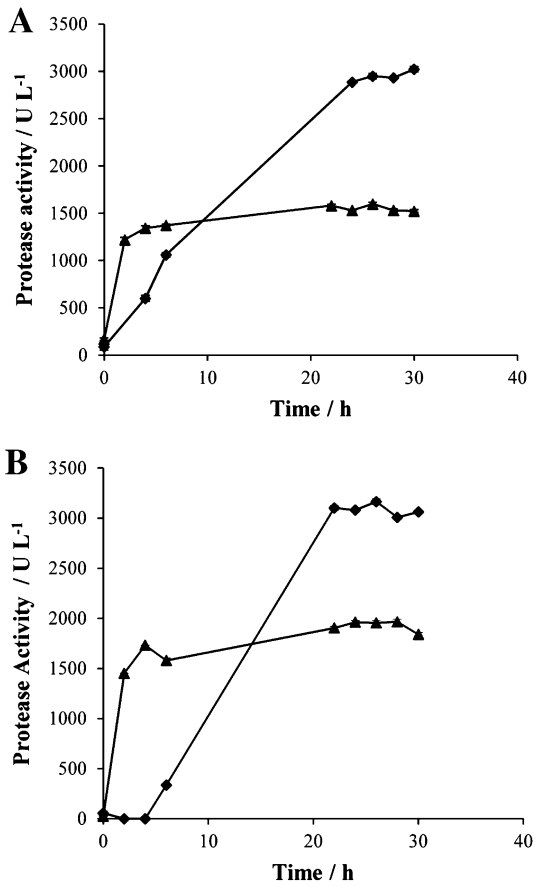

Fig. 2 Protease activity profiles by *Y. lipolytica* W29 with olive oil (*filled diamonds*) and without inducer (control) (*filled triangles*): **a** extracellular protease activity, **b** total protease activity. Data are presented as the mean and standard deviation of three independent experiments

culture medium is the result of a balance between the lipase activity produced and the one degraded by the action of proteolytic enzymes produced by the microorganism during the process [28]. Fig. 2 shows the protease secretion over time.

During the first hours of culture, protease activity was lower in the experiment with olive oil than in the control assay, increasing gradually until the end of the cultivation time.

In almost all experiments, the peak of protease activity occurred after the maximum lipase activity had been reached. Thus, a potential explanation for the lower lipolytic activity is the higher protease activity observed, which would result in lipase degradation. Moreover, the inactivation of lipase by a protease has been observed in a system containing partially purified enzymes produced by *Pseudomonas* sp. no. 33 [29].

The addition of protease inhibitors to the culture medium [like PMSF (phenylmethylsulfonyl fluoride), benzamidine and leupeptin] during the production of lipase may be a way to prevent enzyme degradation and to increase the amount of lipase produced [30].

The results obtained in the production of extracellular and total protease, are quite similar since these enzymes are excreted by the cells to the culture medium and are not linked to the cells.

Biotransformation of Ricinoleic Acid into γ-Decalactone

The aroma production by *Y. lipolytica* W29 was investigated in the presence of different medium compositions. The pre-addition of an inducer of lipase production to the culture medium was tested in order to assess its importance on the hydrolysis of castor oil and consequently in γ-decalactone production. Thus, since the maximum lipase production was attained at 22 h (Fig. 1), the biotransformation medium components were added at this time and the aroma production was followed.

In all experiments, the kinetic profile of γ-decalactone production increases up to a maximum and after that, a decrease in the aroma production is observed, until complete disappearance of the compound (Fig. 3). This decrease in γ-decalactone production is due to the fact that this metabolite is consumed by yeasts as a carbon source [2]. In the case of the pre-induction of lipase by olive oil, the production of γ-decalactone started to be detected after 60 h of fermentation and the maximal concentration was achieved at 140 h of culture. In the experiment without pre-induction of lipase, the aroma production was detected later (approximately at 120 h), and the maximal concentration was obtained at 185 h. Thus, with the addition of a lipase pre-induction step it is possible to obtain a gain around 45 h in the overall process.

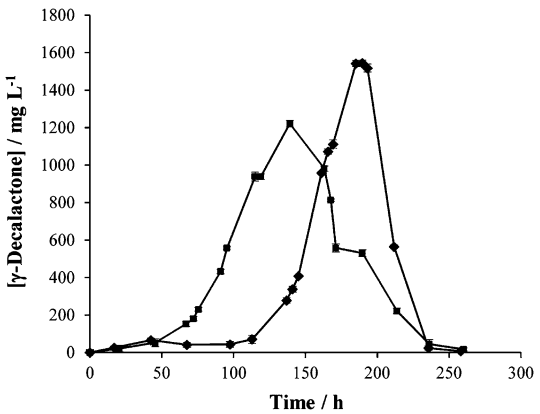

Fig. 3 γ-Decalactone production at different medium compositions: with (*filled squares*) and without olive oil (control) (*filled diamonds*). Data are presented as the mean and standard deviation of three independent experiments

The existence of a lag phase in γ-decalactone production can be attributed to the time necessary for the synthesis of lipases involved in the hydrolysis of castor oil, in order to enable the availability of ricinoleic acid (which is the substrate for the aroma synthesis) to the cells. The addition of a lipase inducer decreases the lag phase, since in these conditions the oil is earlier hydrolyzed into ricinoleic acid.

The lower concentrations of aroma detected in the medium with inducer may be justified by a greater release of castor oil fatty acids [particularly ricinoleic acid (about 90%), linoleic acid (4.2%), oleic acid (3%), stearic acid (1%) and palmitic acid (1%)] which can have a toxic effect on yeasts, leading to a lower γ-decalactone production [31]. Pagot [32] suggested that low γ-decalactone production, in some cases, is due to the fact that ricinoleic acid is the only carbon source present in the biotransformation medium, and part of this acid is used for maintenance of cell growth. Gatfield [33] observed that the degradation of ricinoleic acid can lead to the accumulation of other lactones besides γ-decalactone, such as 3-hydroxy-γ-decalactone, dec-2-enolide and dec-3-enolide, which may also contribute to the low yield of aroma.

The productivities in γ-decalactone (calculated in the biotransformation period) obtained for both media (8.3 ± 1.2 and 8.8 ± 1.3 mg L^{-1} h^{-1}, for media without and with lipase pre-induction, respectively) are not significantly different (which indicates that with the addition of this pre-induction step of lipase production, it is possible to produce γ-decalactone in less time, keeping identical values of aroma productivity).

Conclusions

In order to assess the importance of the lipase preceding induction in *Y. lipolytica* cultures used in the production of γ-decalactone from castor oil, that would enable the faster availability of ricinoleic acid to the cells, the pre-addition of a lipase production inducer (olive oil) to the biotransformation medium was tested, after selecting the best inoculation strategy and strain for lipase production. Of two strains and conditions tested, *Y. lipolytica* W29 revealed itself to be the most adequate strain for lipase production and the cells centrifugation step was eliminated since it was proven not to affect the enzyme production.

Results revealed that with this pre-induction step of lipase production the time needed for maximal production of γ-decalactone is reduced, resulting in similar productivities.

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