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Research Article

Culture Conditions and Investigation of Bioreactor Configurations for Lipase Production by *Rhizopus oryzae*

Lipolytic enzymes are the subject of great industrial and academic interest. For this reason, a detailed study of lipolytic enzyme production by the fungus *Rhizopus oryzae* is tackled, and several steps from plate to shake flasks and bioreactor cultures are investigated in order to propose an optimized strategy to perform the biological process. The suitability of several lipidic compounds and surfactants is assessed. Triton X-100 (5 g/L) gives the highest activities with a maximum value of 6320 U/L which is 10-fold the value attained in cultures without addition of lipidic compounds. As there are almost no studies on bench-scale bioreactors, two bioreactor configurations, stirred tank and air-lift, are investigated to determine the most suitable one to carry out the biological reaction. It is demonstrated that the lipolytic activity is strongly enhanced when a stirred-tank bioreactor is used with a maximum value of 3521 U/L within two days which is clearly higher than the values produced by other recently reported species.

Keywords: Bioreactor, Inducers, Lipolytic activity, *Rhizopus oryzae*, Surfactants

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1 Introduction

Lipases (triacylglycerol hydrolase; E.C. 3.1.1.3.) are versatile enzymes that display a broad spectrum of substrate specificity. Their natural substrates are triacylglycerols, but lipases also catalyze the hydrolysis of other water-insoluble carboxyl esters as well as the esterification of primary and secondary alcohols and transesterification reactions. In organic synthesis lies most of the potential of lipases and, in fact, a myriad of reactions of organic synthesis has been conducted with lipases due to their stability in organic solvents. Besides, they are known to exhibit a high degree of enantio- and regioselectivity and they do not require cofactors [1, 2].

These enzymes are widely found throughout the animal and plant kingdoms, as well as in bacteria, fungi, yeasts, and actinomyces, although *Candida*, *Pseudomonas*, *Rhizomucor*, and *Rhizopus* sp. stand out nowadays as sources of the most commercially available enzyme preparations. Of all known enzymes, lipases have attracted the most scientific and industrial attention. The versatility of lipases makes them an obvious choice for potential applications in a variety of sectors ranging from food, detergent, pharmaceutical, leather, textile, cosmetic to paper industries. The most significant industrial applications of lipases are found mainly in the food, detergent, and pharmaceutical sectors. Limitations of the industrial use of these enzymes are owing mainly to their high production costs which may be overcome by molecular technologies and medium optimization, enabling the production of these enzymes at high levels and in a virtually purified form [3–5].

Extensive application of lipases in industry requires the development of adequate large-scale production methods. However, a considerable amount of the research carried out to date has focused on small-scale procedures, while less information is available on the particular characteristics of lipolytic enzyme biosynthesis in bioreactors and the feasibility of diverse culture modes, such as batch, fed-batch, or continuous systems [6–8]. Over the last few months, research concerning lipase production by yeasts and fungi in bioreactors has been employed to obtain high levels of lipase production, thus demonstrating the interest that the scale-up of this kind of biological process is triggering nowadays and then encouraging further research in this field [9–12].

The production of microbial lipases is influenced by several factors, namely the carbon source, temperature, pH, dissolved oxygen concentration, and presence of inducers. These compounds, such as oils and some surfactants, have been described as agents that increase the production of enzymes with lipoly-

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tic activity. Also, in some cases they are essential for lipolytic activity to be detected [4, 12, 13]. Finally, the knowledge of engineering of culture conditions has also been shown to be an effective mode to achieve enzyme preparations enriched in selected isoenzymes which are effective for particular biotechnological applications [14].

Rhizopus oryzae has been previously shown to have potential as an extracellular lipolytic enzyme producer [15–21] and, hence, it can be considered a promising candidate for application in industrial bioprocesses to obtain lipases. As *R. oryzae* presents a particularly attractive lipase production at flask-scale level, in this work several strategies for enhancing lipase biosynthesis at bioreactor scale were explored.

2 Materials and Methods

2.1 Microorganism

R. oryzae (CECT 2340) was grown on peptone-malt extract agar plates. The medium consisted of glucose (10 g/L), peptone (5 g/L), yeast extract (3 g/L), malt extract (3 g/L), and agar (20 g/L) in distilled water. Plates were incubated at 30 °C for ten days and stored at 4 °C between transfers. A cell suspension was obtained by the addition of 10 mL of 0.9 % NaCl solution to a previously prepared agar plate. Unless otherwise stated, all cultures were carried out using a basal medium (pH 6) prepared in distilled water, containing glucose (20 g/L), yeast extract (3 g/L), malt extract (3 g/L), and mycological peptone (5 g/L).

2.2 Tributyrin Agar Plates (Detection of Lipolytic Activity)

The microorganism was grown on peptone-malt extract agar plates to which tributyrin (10 g/L) was added [22]. The medium was emulsified for 7 min at 1000 rpm (Homogeniser IKA T25) before autoclaving. Plates were incubated at 30 °C for one week. Lipolytic activity was identified on the plates as a transparent halo around the colonies after seven days of incubation.

2.3 Shake Flask Cultures

The cultures were carried out at 30 °C in 250-mL Erlenmeyer flasks with 150 mL of culture medium. The flasks were capped with cellulose stoppers, which permit passive aeration and were incubated in an orbital shaker at 150 rpm. Several media with salts and vitamins were tried in order to define the best basal medium (code meanings are indicated in Tab. 1). Moreover, the effect of lipidic compounds and surfactants was evaluated:

- Lipidic compounds: oleic acid (10 mmol/L), olive oil, sunflower oil, corn oil, tributyrin (10 g/L)
- Surfactants: Tween 80 (1 g/L), Triton X-100 (0.5, 1, and 1.5 g/L), CHAPS (0.125, 0.250, 0.5, 1, and 5 g/L)

Samples (1.5 mL) were taken every day, centrifuged, and analyzed. Experiments were conducted twice and samples were analyzed three times. The results in the figures correspond to mean values, the standard deviation being less than 15 %.

2.4 Bioreactor Cultures

A Biostat B air-lift bioreactor (2 L working volume; Braun, Germany) and a stirred-tank bioreactor (3 L working volume) were employed. The temperature was maintained at 30 °C by circulation of temperature-controlled water. Sterile air was supplied to the bioreactor in a continuous way at a rate of 1 L/min. The bioreactor operated in batch mode. Samples (1.5 mL) were collected every day and analyzed in triplicate. The values in the figures correspond to mean values of replicate experiments with a standard deviation of less than 15 %.

2.5 Analytical Methods

2.5.1 Sample Preparation

Cells were harvested by centrifugation (10 min, 5000×*g*) and the supernatant was reserved for extracellular enzyme analysis and determination of reducing sugars.

2.5.2 Lipolytic Activity Assay

The lipolytic activity assay was performed by a spectrophotometric method, using 25 mM *p*-nitrophenyl laurate (*p*-NPL), dissolved in ethanol as substrate in a 50 mM Tris-HCl buffer containing 40 mM $CaCl_2$ (pH 6) [18]. The hydrolysis reaction was allowed to proceed for 20 min at 37 °C and was stopped afterwards by adding 2 M Na_2CO_3. The mixture was kept on ice for at least 15 min before centrifugation (5000×*g* for 10 min). The absorbance of the supernatant was monitored at 400 nm in a Unicam Helios β spectrophotometer. Under these conditions, the molar extinction coefficient was 17 215 ± 0.002 /M/cm$_{\text{e}}$ heck unit One unit (U) of lipolytic activity is defined as the amount of enzyme that produces 1 µmol of product per minute under the above conditions.

2.5.3 Reducing Sugars

Reducing sugars were measured by the dinitrosalicylic acid method using D-glucose as a standard according to Miller [23].

3 Results and Discussion

3.1 Basal Medium Culture

The potential of lipolytic enzymes as industrial biocatalysts has led to a considerable amount of research on this topic. A wide range of aspects has been considered, from the optimization of culture conditions to the study of mechanisms and biochemical properties of the enzymes. The search for new lipase/esterase sources is of interest because it could provide new biocata-

lysts that could either promote novel industrial applications or be used to achieve a better understanding of enzyme mechanisms and structure-function relationships.

First of all, the microorganism was grown on tributyrin agar plates, in order to confirm the occurrence of lipolytic activity. After seven days of culture, transparent halos were observed around the colonies. This method has also successfully been used by a number of authors for screening of lipase/esterase producers [24, 25].

The next step of this research work was to carry out the biological process in shake flasks and to analyze if the conditions are adequate for lipase production. Five different culture media, detailed in Tab. 1, were investigated. As it is observed, the M1 medium is the one which provides higher values of activity as well as of lipolytic production rates.

The addition of salts, vitamins, and the reduction of glucose content in the culture medium involves the reduction of lipase secretion levels. The reason behind this behavior can be the different morphology observed in the several cultures that were carried out. Fungal morphology may play a major role in the metabolite production response, as it has been reported previously by Carlsen et al. [26], Papagianni and Moo-Young [27], and Haack et al. [28] when investigating amylase, protease, and lipase production, respectively. While cultures performed with salts and vitamins showed a clumped-shape mycelium (fully entangled filaments), the M1 and M5 cultures led to a mycelium morphology tending to be dispersed. It seems that salts and vitamins exert some kind of chemical pressure on the microorganism that makes it grow in an aggregated form. This phenomenon has also been observed when the hydrodynamic cultivation conditions leading to shear stress enhanced the extracellular production of metabolites. This fact was reported by Du et al. [29]

Table 1. Culture medium composition, lipolytic activity, and lipolytic enzyme production rate (Q_P) at the stationary phase for the different basal media used in shake flask submerged cultures of *R. oryzae*.

Ref.	Meaning	Compounds	Concentration [g/L]	Lipolytic activity [U/L]	Q_P [U/L·h]
M1	Basal medium	Yeast extract	3	365.5	7.61
		Malt extract	3		
		Glucose	20		
		Mycopeptone	5		
M2	Basal medium + salts	Yeast extract	3	260.0	5.42
		Malt extract	3		
		Glucose	20		
		Mycopeptone	5		
		$NaNO_3$	1		
		KH_2PO_4	0.5		
		K_2HPO_4	0.5		
		$MgSO_4{\cdot}7H_2O$	0.5		
M3	Basal medium + salts + vitamins	Yeast extract	3	240.7	5.01
		Malt extract	3		
		Glucose	20		
		Mycopeptone	5		
		$NaNO_3$	1		
		KH_2PO_4	0.5		
		K_2HPO_4	0.5		
		$MgSO_4{\cdot}7H_2O$	0.5		
		D-Biotine	8 µg/L		
		Thiamine	200 µg/L		
M4	Modified basal medium	$NaNO_3$	1	195.0	4.06
		KH_2PO_4	1		
		$MgSO_4{\cdot}7H_2O$	0.5		
		Yeast extract	70		
M5	Basal medium with 50 % glucose	Yeast extract	3	276.0	5.75
		Malt extract	3		
		Glucose	10		
		Mycopeptone	5		
M6	Basal medium with 50 % glucose and salts	Yeast extract	3	258.8	5.39
		Malt extract	3		
		Glucose	10		
		Mycopeptone	5		
		$NaNO_3$	1		
		KH_2PO_4	0.5		
		K_2HPO_4	0.5		
		$MgSO_4{\cdot}7H_2O$	0.5		

and Teng et al. [30] for other *Rhizopus* strains, as well as by Hama et al. [31] when studying lipolytic enzyme production by *R. oryzae*. Therefore, it can be stated that dispersed mycelium favors extracellular lipase production.

On the other hand, the increase of glucose in the culture medium entails higher levels of lipase synthesis which is in agreement with previous findings in other processes to produce lipases by *Rhizopus* species. Thus, media supplemented with glucose at high levels involved maximum lipase synthesis, as reported by Rajendran and Thangavelu [32] when tackling lipolytic enzyme production by *Rhizopus arrhizus*. In this sense, it is necessary to point out that lipase production by another *Rhizopus* species turned out to reach the optimum when glucose was used as carbon source instead of fructose, sucrose, lactose, maltose, galactose, and mannitol [33]. Therefore, a basal medium containing 20 g/L of glucose was chosen to continue with optimization in order to find the right culture conditions that allow carrying out the biological process at higher scale, maintaining high lipolytic activity levels.

3.2 Addition of Lipidic Compounds

The production of lipase is commonly inducer-dependent. Although there is a variable effect depending on the microorganism and the lipidic substances, in general terms, fatty acids, triglycerides, and some esters have been reported to act as good inducers of lipolytic enzymes [4, 34, 35]. In this case, oleic acid, tributyrin, several oils (from olive, sunflower, and corn), and surfactants (Tween 80, Triton X-100, and CHAPS) were used to check their possible role as lipase inducers. The strategy proposed in this paper is to study if surfactants can exert by themselves an inducer effect in this microorganism. Actually, these substances are usually employed to ameliorate the emulsification degree in biphasic systems, and are added to favor the inducer effect of lipidic substances that are usually immiscible in aqueous media used for biological reactions. The results shown in Fig. 1 reveal that the surfactant Triton X-100 leads to a drastic increase in lipolytic secretion, acting as an inducer of activity. In the same way, CHAPS induced a slight increase in enzyme secretion. On the contrary, oils, tributyrin, and oleic acid possess an inhibitory effect on lipase production. Gulati et al. [36] and Odibo et al. [37] demonstrated that an inducer effect was exerted by Triton X-100 in cultures of *Hendersonula toruloidea* and *Aspergillus terreus*. The lower results obtained in media containing oils are in agreement with the findings of Nahas [38] and Fadiloglu and Erkmen [39] when studying lipase production by *Rhizopus oligosporus* and *R. oryzae*, respectively. They reported that the use of olive oil together with a carbon source was inhibitory for lipolytic production, due to a limited availability of the carbon source to the fungus in media supplemented with oil.

In a second stage of this work, the addition of several concentrations of the inducers which led to the highest values of activity was studied. Thus, 0.5, 1, and 1.5 g/L Triton X-100 and 0.125, 0.250, 0.5, 1, and 5 g/L CHAPS were studied as inducers which increased lipase secretion. As it can be seen in Fig. 2, the trends observed indicate that there is an elevated lipase production when high concentrations of CHAPS and Triton X-100 are used. As it has been previously reported by Deive et al. [40], these two compounds have the ability of solubilizing cell membranes, so this fact could contribute to release intracellular and membrane activity which explains the drastic increase of activity.

3.3 Bioreactor Culture

Up to date, there are no studies concerning lipolytic enzyme production from *R. oryzae* at bioreactor scale. Since the operating conditions existing in a bioreactor are very different from those ruling flask cultures and can strongly affect features, such as the morphology of the fungi, the growth, or the lipolytic synthesis, investigation of the biotechnological process must unavoidably tackle this crucial aspect. In recent years, the need to decrease the time necessary for bioprocess development has

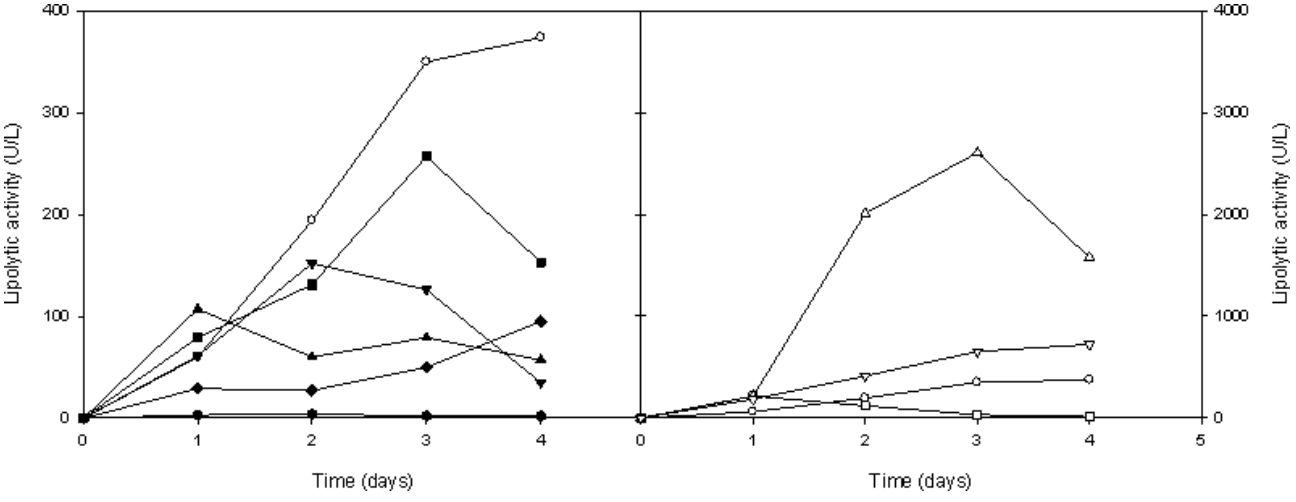

Figure 1. Lipolytic enzyme production in the presence of several oils, fatty acids, and triglycerides (left) and surfactants (right). (■) Control; (□) olive oil; (▲) sunflower oil; (*θ*) corn oil; (▼) oleic acid; (△) tributyrin; (▽) Tween 80; (*ρ*) Triton X-100; (*σ*) CHAPS.

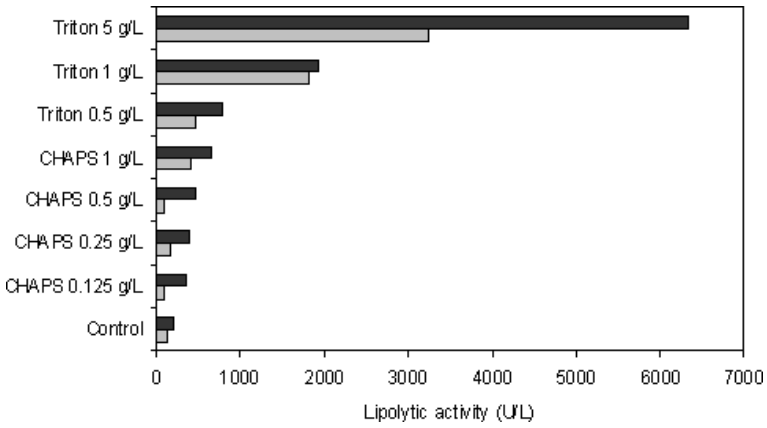

Figure 2. Values of activity at different concentrations of Triton X-100 and CHAPS after two (■) and four (■) days of cultivation.

led to the design and evaluation of a number of small-scale bioreactor systems. These tended to focus on stirred-tank or air-lift designs because of their geometrical similarity with the large-scale bioreactors most likely to be used for industrial manufacture. In this work, these two kinds of bioreactors will be used with the culture medium that turned out to be the optimum at shake flask scale. In all cultures it was determined that the dissolved oxygen concentration of the culture medium became zero in the exponential growth phase, indicating insufficiency of the oxygen supply. However, the maximum lipolytic activity values obtained for a six-days cultivation period are shown in Tab. 2.

It is obvious that high activities can only be achieved by performing the process in a stirred-tank bioreactor (3521.1 U/L). This fact can be related to the different hydrodynamic setting existing inside the bioreactors, in terms of agitation and aeration. On the one hand, an increased transfer area is furthered by forming small-sized bubbles which are dispersed in the culture medium, increasing their residence time on the liquid. On the other hand, a good agitation favors turbulence which leads to a decrease of the thickness of the liquid layer on the gas/liquid interface, reducing the resistance to oxygen mass transfer. This statement is in accordance with the conclusions reported by Lopes et al. [9], who recorded an increase of 96 % in lipase activity values when using 5 bar air pressure instead of 1 bar pressure during the culture of *Yarrowia lipolytica*. In the same way, Deive et al. [41] observed higher lipolytic enzyme levels in a stirred-tank bioreactor compared to an air-lift one.

As it happened in flask cultures, the explanation for this phenomenon can be visually detected from the differences existing between the morphology in both kinds of bioreactor configuration. The culture in the air-lift bioreactor led to round balls mycelium, while in the stirred-tank bioreactor the culture showed a dispersed appearance. This fact is in agreement with that reported by Hama et al. [31] who studied lipase production by *R. oryzae*. López et al. [21] reported a maximum lipolytic enzyme secretion of 500 U/L in an air-lift bioreactor containing immobilized cells of *R. oryzae*. As it can be seen here, the air-lift configuration can only be used when the cells are trapped in a support, otherwise the hydrodynamic conditions could lead to some stress that avoids triggering the extracellular lipolytic enzyme synthesis.

Furthermore, the bioreactor configuration proposed in this paper involves a 7-fold higher increase (about 3500 U/L) than that reported by López et al. [21] when working with the same microorganism. During the last months, several reports were published stressing the need of searching for operating conditions in bioreactors to produce lipolytic enzymes from yeasts [9, 35], bacteria [10], and fungi [21], but maximum activities of 700 U/L, 400 U/L, and 500 U/L, respectively, were obtained via the *p*-nitrophenyl esters method. Therefore, the extremely high levels obtained in this work with the same enzyme determination method point out the suitability of the proposed strategy as well as the bioreactor configuration to be implemented at higher scale for the production of lipolytic enzymes.

Additionally, the values of glucose concentration were analyzed in an attempt to evaluate the metabolic pathways that are followed to synthesize lipolytic enzymes. It has been observed that lipase production is detected only after glucose depletion, a fact that was also concluded previously and that has to do with the energetic demands of the microorganism [32, 38]. Besides, in terms of yield (lipolytic activity with respect to substrate) and lipolytic enzyme production rate (Tab. 2), the use of a stirred-tank bioreactor involves a substantial amelioration with regard to that reported by López et al. [18] when dealing with the production of lipolytic enzymes by immobilized *R. oryzae*.

4 Conclusions

In this work, a successful scale-up of the process to obtain lipases from *R. oryzae* has been approached. The preliminary study with shake flasks rendered possible to determine the most adequate basal medium in terms of lipolytic enzyme secretion. Furthermore, the addition of the surfactants Triton X-100 and CHAPS were the only way to drastically increase the lipolytic activity up to 6000 U/L, maybe due to their ability to modify the characteristics of the cell membrane together with an inducer effect. Therefore, this strategy was carried out in two kinds of bioreactor configuration (air-lift and

Table 2. Lipolytic enzyme production levels and culture yields in batch fermentations operating in an air-lift and stirred-tank bioreactor Biostat B of 2 L and 5 L at 30 °C, 1 L/min air flow, and 100 rpm (in stirred tank).

Bioreactor	Maximum lipolytic activity [U/L]	$Y_{P/S}$ [U/g]	Q_p [U/L·h]	Days required for maximum
Air-lift	83.0	4.17	0.58	6
Stirred tank	3521.1	176.05	73.36	2

stirred tank), thus assessing the suitability of the stirred-tank bioreactor to obtain high levels of lipolytic enzyme when free cells of *Rhizopus oryzae* are used. The results obtained (more than 3500 U/L) permit to conclude that this work poses a significant amelioration of lipase activity levels with regard to other typical values recently reported for other microorganisms.

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The authors have declared no conflict of interest.

Symbols used

Q_p	[U/L·h]	lipolytic enzyme production rate
$Y_{p/s}$	[U/g]	yield of lipolytic activity with respect to substrate

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Research Article: A successful scale-up of the process to obtain high amounts of lipases from *Rhizopus oryzae* has been performed by determination of the most adequate basal medium in terms of lipolytic enzyme secretion, addition of the surfactants Triton X-100 and CHAPS which drastically increased the lipolytic activity, and use of a stirred-tank bioreactor instead of an air-lift reactor.

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