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Lipase Production by Solid-State Fermentation

Cultivation Conditions and Operation of Tray and Packed-Bed Bioreactors

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Abstract

The production of lipase by *Penicillium simplicissimum* in solid-state fermentation was studied using babassu cake as the basal medium. Tray-type and packed-bed bioreactors were employed. In the former, the influence of temperature; content of the medium, and medium supplementation with olive oil, sugarcane molasses, corn steep liquor, and yeast hydrolysate was studied. For all combinations of supplements, a temperature of 30°C, a moisture content of 70%, and a concentration of carbon source of 6.25% (m/m, dry basis) provided optimum conditions for lipase production. When used as single supplements olive oil and molasses also were able to provide high lipase activities (20 U/g). Using packed-bed bioreactors and molasses-supplemented medium, optimum conditions for enzyme production were air superficial velocities above 55 cm/min and temperatures below 28°C. The lower temperature optimum found for these reactors is probably related to radial heat gradient formation inside the packed bed. Maximum lipase activities obtained in these bioreactors (26.4 U/g) were 30% higher than in tray-type reactors.

Index Entries: Lipase; packed-bed bioreactor; tray-type bioreactor; *Penicillium simplicissimum*; solid-state fermentation.

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Introduction

Lipases (glycerol ester hydrolases, EC 3.1.1.3) form a group of enzymes responsible for the hydrolysis of ester bonds of triacylglycerols to form glycerol and fatty acids. However, in water-restricted environments, most lipases can act in the reverse way, catalyzing esterification and transesterification reactions. Thus, owing to the different types of reactions, they are able to catalyze, and owing to their regio- and enantioselectivities, lipases have been increasingly employed in a broad range of applications, such as in the manufacture of detergents, in the food, leather, pulp and paper, and pharmaceutical industries, for the production of fine chemicals; and for oily wastewater treatment *(1)*.

Industrial enzymes can be produced both by submerged fermentation and solid-state fermentation. Although most commercial processes are currently based on submerged fermentation, a growing interest in solid-state fermentation for the production of biomolecules has been observed in recent years, mainly owing to the availability of low-cost carbon sources, such as agricultural residues, which can be used as raw materials *(2)*. However, the low moisture levels and the poor mixing characteristics usually result in a highly heterogeneous environment, in overheating inside the solid medium, and in oxygen transfer limitations *(3,4)*. Therefore, the availability of adequate bioreactor configurations for solid-state fermentation is still limited *(5)*, and further studies are required to enable successful application of solid-state fermentation at large scales *(3)*.

The aim of the present work was to investigate the production of lipases in solid-state fermentation by a strain of the fungus *Penicillium simplicissimum*. Tray-type bioreactors were used to study the influence of temperature, moisture content, and medium supplementation on enzyme production. Employing the conditions determined as the best, further process optimization was carried out using packed-bed bioreactors. A statistical experimental design was used throughout this work.

Materials and Methods

Microrganism and Culture Media

A *P. simplicissimum* strain isolated by Freire *(6)* and selected by Gutarra *(7)* as an excellent lipase producer in solid-state fermentation was employed. For inoculum preparation, the fungus was propagated in culture tubes at 30°C for 7 d in the following medium: 2.0% (m/v) soluble starch, 0.025% (m/v) $MgSO_4 \cdot 7H_2O$, 0.05% (m/v) KH_2PO_4, 0.5% (m/v) $CaCO_3$, 0.1% (m/v) yeast extract, 1.0% (m/v) olive oil, 1.0% (m/v) agar. Spores were then recovered from the tubes, suspended in 0.05 *M* phosphate buffer (pH 7.0) and quantified using a Neubauer chamber. For solid-state fermentation runs, babassu cake, a solid residue of the industrial production of babassu oil, kindly donated by Tobasa S.A. (Tocantinópolis, Brazil), was used as basal culture medium. Before use, the cake was cominuted and sieved, and particles in the range of 0.21–0.42 mm were used. Inocula of 10^7 spores/g of cake were employed.

Table 1
Elemental Analysis
of Components of Culture Medium

	Composition (% m/m)	
Material	C	N
Babassu cake	44.56	3.24
OO	75.77	2.68
MO	31.92	0.44
YH	9.65	3.00
CS	18.75	3.40

Table 2
Variables Studied in Tray-Type Bioreactors[a]

	Levels				
Variable	–2	–1	0	1	2
T (°C)	25	28.80	32.5	36.30	40
M (%)	40	50.00	60.0	70.00	80
C (%)	1	2.75	4.5	6.25	8
N (%)	1	2.75	4.5	6.25	8

[a] *C*, carbon source concentration; *M*, moisture content; *N*, nitrogen source concentration; and *T*, temperature. The absolute and normalized levels are given.

Solid-State Fermentation in Tray Bioreactors

Tray bioreactors containing 10 g of babassu cake with a working height of 1 cm were incubated for 48 h in chambers with temperature control and humidified air injection at 95% saturation. Medium supplementation was studied through the addition of substances rich in either carbon or nitrogen. Supplements used were olive oil (OO) and sugarcane molasses (MO) (carbon sources), and corn steep liquor (CS) and yeast hydrolysate (YH) (nitrogen sources). The elemental composition of these materials, as determined by carbon, hydrogen, and nitrogen (CHN) analysis using a Perkin-Elmer 2400-CHN elemental analyzer, is given in Table 1. Response surface experimental design was used as a tool to investigate the influence of cultivation temperature (*T*), initial moisture content (*M*), and concentration of the carbon (*C*) and nitrogen (*N*) sources. Four groups of experiments were carried out, by combining the two C and the two N sources. The variable ranges tested are presented in Table 2, as both absolute and normalized values. To evaluate lipase production in babassu cake enriched with each single supplement, further experiments were conducted at 28.8°C and 70% moisture, with the supplement added at a 6.25% (m/m, dry basis) concentration.

Table 3
Variables Studied in Packed-Bed Bioreactors [a]

	Levels				
Variables	–1.41	–1	0	1	1.41
T (°C)	25.8	27.0	30.0	33.0	34.2
V (cm min^{-1})	6.4	15.9	39.8	63.7	73.2

[a] T, temperature; V, air superficial velocity. The absolute and normalized levels are given.

Solid-State Fermentation in Packed-Bed Bioreactors

Water-jacketed packed-bed bioreactors with an id of 4 cm and a working height of 14 cm containing 30 g of cake were aerated with water-saturated air. Basal medium (babassu cake) was supplemented with 6.25% (m/m, dry basis) mo and moistened to a level of 70%. Fermentations were carried out for 48 h. A response surface experimental design was employed to investigate the effects of air superficial velocity (V) and temperature (T) on lipase production. The variable ranges tested are presented in Table 3, as both absolute and normalized values.

Statistical Experimental Design

Experimental design and statistical analysis of experimental data were performed using the software *Statistica* 5.1. Quadratic models were used to describe lipase activities as a function of the normalized variables, whereby only terms presenting a high statistical significance ($p < 0.1$) were included.

Enzyme Extraction

After fermentation, enzyme extraction was carried out by adding 0.1 *M* phosphate buffer (pH 7.0) to the fermented solids at a ratio of 5 mL/g and by shaking the resulting suspension at 200 rpm and 35°C for 20 min. Subsequently, solid-liquid separation was achieved through pressing followed by centrifugation at 1000*g* for 2 min. The supernatant was used for determination of lipase activity *(8)*.

Lipase Assay

Lipase activity was determined as described previously *(9,10)*. One lipase activity unit is defined as the amount of enzyme that produces 1 µmol of fatty acids/min, under the assay conditions.

Results and Discussion

Cultivation Conditions for Lipase Production by Solid-State Fermentation in Tray Bioreactors

The influence of *T*, *M*, and *C* and *N* sources on the production of lipase by *P. simplicissimum* was investigated. OO and MO were the carbon

Table 4
Response Surface Experimental Design Carried Out in Tray-Type Bioreactors [a]

				Lipase activity (U/g)			
T (°C)	M (%)	C (%)	N (%)	OO-CS	OO-YH	MO-CS	MO-YH
28.8	50	2.75	2.75	10.0	2.6	5.7	1.2
36.3	50	2.75	2.75	7.3	4.0	6.2	6.3
28.8	70	2.75	2.75	12.8	2.4	0.0	0.0
36.3	70	2.75	2.75	7.9	12.6	9.1	5.5
28.8	50	6.25	2.75	8.3	3.3	3.0	2.2
36.3	50	6.25	2.75	0.8	0.0	0.0	0.0
28.8	70	6.25	2.75	21.5	17.0	18.7	16.2
36.3	70	6.25	2.75	13.3	13.1	10.0	11.8
28.8	50	2.75	6.25	5.0	0.0	0.0	0.0
36.3	50	2.75	6.25	3.7	1.3	0.7	0.0
28.8	70	2.75	6.25	7.4	4.8	3.4	0.0
36.3	70	2.75	6.25	9.0	5.8	7.7	3.0
28.8	50	6.25	6.25	5.9	2.7	0.0	0.7
36.3	50	6.25	6.25	0.0	0.0	0.0	0.0
28.8	70	6.25	6.25	19.1	14.2	13.7	9.1
36.3	70	6.25	6.25	11.4	9.3	6.0	4.9
25.0	60	4.5	4.5	14.1	12.6	12.5	10.8
40.0	60	4.5	4.5	0.8	0.0	0.0	1.1
32.5	40	4.5	4.5	1.5	4.7	0.7	2.0
32.5	80	4.5	4.5	3.5	1.2	3.9	0.0
32.5	60	1	4.5	13.1	10.4	8.6	2.4
32.5	60	8	4.5	17.7	12.6	6.0	8.0
32.5	60	4.5	1	11.6	12.7	13.6	8.7
32.5	60	4.5	8	16.6	12.7	16.8	14.5
32.5	60	4.5	4.5	16.6	15.5	13.6	10.6
32.5	60	4.5	4.5	16.4	15.6	13.9	10.8
32.5	60	4.5	4.5	16.7	15.7	13.2	10.3

[a] Lipase activities obtained for the different supplement combinations (OO-CS, OO-YH, MO-CS, MO-YH) are given.

sources studied, because OO is usually reported as an inducer of lipase production *(11)*, whereas MO is an abundant, lower-cost carbon source. CS and YH were investigated as nitrogen sources, Because these substances are organic sources available at relatively low costs.

Four groups of experiments were conducted, by combining each of the carbon sources with each of the nitrogen sources. Fermentation time was set at 48 h, because at this time *P. simplicissimum* presents maximum lipase production *(7)*.

Table 4 gives the response surface experimental design that was employed, and the lipase activities that were obtained for the different experimental conditions. Note that for all supplement combinations the best conditions among those tested were a temperature of 28.8°C, a mois-

ture level of 70%, a carbon source concentration of 6.25%, and a nitrogen source concentration of 2.75%. Under these conditions, the highest lipase activity (21.5 U/g) was obtained for OO and CS supplementation.

Quadratic models were obtained for the supplement combinations OO-CS (Eq. 1), OO-YH (Eq. 2), MO-CS (Eq. 3) and MO-YH (Eq. 4) through a statistical analysis of the data shown in Table 4. These models describe lipase activity ([LA], expressed in U/g) as a function of the normalized statistically significant variables.

$$LA = 14.95 \pm 2.64 \cdot T \pm 2.12 \cdot T^2 + 2.72 \cdot M \pm 3.38 \cdot M^2 + 1.10 \cdot C \pm 1.38 \cdot T \cdot C + 2.44 \cdot M \cdot C \quad (1)$$

$$LA = 13.55 \pm 2.36 \cdot T^2 + 2.42 \cdot M \pm 3.20 \cdot M^2 \pm 1.07 \cdot C^2 \pm 1.79 \cdot T \cdot C + 1.86 \cdot M \cdot C \quad (2)$$

$$LA = 13.55 \pm 2.24 \cdot T^2 + 2.47 \cdot M \pm 3.24 \cdot M^2 \pm 1.98 \cdot C^2 \pm 2.13 \cdot T \cdot C + 2.37 \cdot M \cdot C \quad (3)$$

$$LA = 10.38 \pm 1.48 \cdot T^2 + 1.50 \cdot M \pm 2.72 \cdot M^2 + 1.65 \cdot C \pm 1.66 \cdot C^2 + 2.38 \cdot M \cdot C \quad (4)$$

For all supplement combinations, the concentration of *N* did not exert any statistically significant effect on lipase production in the concentration range tested (1–8% m/m), whereas *T*, *M*, *C*, as well as interactions of these variables presented a statistically significant influence on lipase activity.

To analyze the optimum variable ranges, response surface charts were plotted and are presented in Fig. 1. They show lipase activity as a function of carbon source concentration and temperature, for a nitrogen source concentration of 2.75%, and a moisture content of 70%. It can be observed in Fig. 1 that although the effect of the concentration of the nitrogen source was shown to be insignificant, the type of nitrogen source added to the medium exerted a considerable influence on lipase activity, especially when the carbon source used was OO. It has also been observed for *P. restrictum* that the type of nitrogen source has a greater effect on lipase production than its concentration *(9)*.

For temperature, the optimum range was approx 30°C for all supplement combinations (Fig. 1). Temperature levels below the optimum caused rather slight decreases in lipase activity, whereas temperatures above the optimum levels resulted in steep drops in enzyme production, with lipase activities below 1 U/g being obtained at 40°C for all supplement combinations (Table 4). Other researchers have reported similar behavior for the production of lipase by *Rhizopus oligosporus* in submerged fermentation *(12)*.

According to Fig. 1, a positive effect of the carbon source concentration was observed for all supplement concentrations, with optimum levels obtained at about 6.25%, except for OO-CS, for which higher OO concentrations (8%) were required. This is in agreement with the findings of Mahadik et al. *(11)*, who observed that lipase production was mostly influenced by the carbon content of the medium when the carbon source was a lipid. However, studies on microbial physiology have shown that the effect of a given carbon source on lipase production seems to be dependent on the regulation mechanisms of each microorganism *(13)*. Kamini et al. *(14)*, e.g., observed that lipase production by *Aspergillus niger* in solid-state fermen-

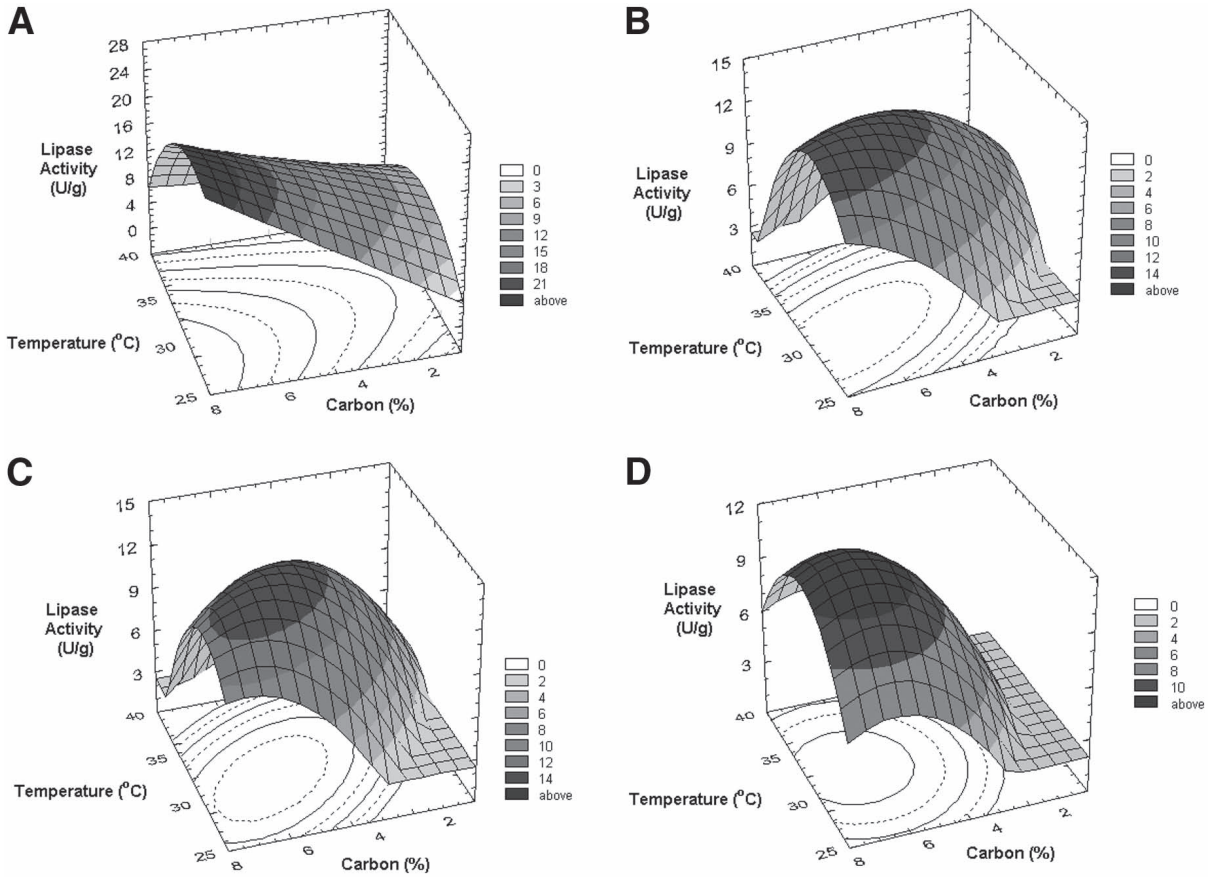

Fig. 1. Response surfaces obtained for **(A)** OO-CS, **(B)** OO-YH, **(C)** MO-CS, and **(D)** MO-YH. Moisture content: 70%; nitrogen source concentration: 2.75%.

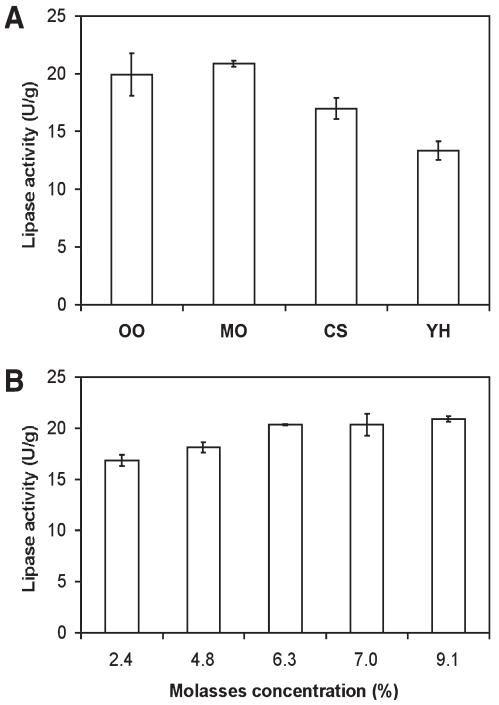

Fig. 2. **(A)** Effect of supplementation of basal medium with either OO, MO, CS or YH on lipase production (supplement concentration: 6.25%); **(B)** effect of MO concentration (dry basis) on lipase production. Temperature: 28.8°C; moisture content: 70%.

tation was not significantly affected by medium supplementation with lipids and carbohydrates.

A positive influence of the moisture content on lipase production was observed until a level of 70%. For runs carried out at 80% moisture content, *P. simplicissimum* presented only superficial growth and lipase activities below 0.5 U/g were obtained for all supplement combinations (Table 4). According to Mitchell et al. *(15)*, moisture contents above an optimum level result in great decreases in the production of microbial metabolites in solid-state fermentation, because high moisture levels can cause agglomeration of medium particles and lead to oxygen transfer limitations.

Because it was shown that the nitrogen source concentration did not present a significant effect on lipase production when used in combination with OO and MO, further tests were carried out to investigate lipase production in babassu cake enriched with each single supplement (OO, MO, CS, or YH). As shown in Fig. 2A, the use of only MO or OO as supplement yielded lipase activities as high as 20 U/g to be obtained, while corn steep liquor and YH yielded lower enzyme levels (17.0 and 13.4 U/g, respectively). Lipase levels obtained through medium supplementation with only MO or OO were approximately equal to those obtained when these supplements were combined with the nitrogen sources (Table 4).

Table 5
Response Surface Experimental Design Conducted in Packed-Bed Bioreactors [a]

T (°C)	*V* (cm/min)	Lipase activity (U/g)
25.8	39.8	16.7
27.0	15.9	19.6
27.0	63.7	26.4
30.0	6.4	18.8
30.0	39.8	20.6
30.0	39.8	21.5
30.0	39.8	20.3
30.0	73.2	20.9
33.0	15.9	12.1
33.0	63.7	11.2
34.2	39.8	18.7

[a]Lipase activities obtained for the different temperatures (*T*) and air superficial velocities (*V*) are given. Culture medium: babassu cake with 6.25% molasses; moisture content: 70%.

Because MO is a byproduct of the sugar industry, presenting characteristics such as low cost, abundancy, and easy storage at room temperature, this carbon source seems to be a more advantageous supplement for lipase production than OO. Thus, further experiments were carried out to verify the influence of MO concentration when employed as a single supplement. As shown in Fig. 2B, an increase in carbon availability in the culture medium up to an MO concentration of 6.3% caused an increase in lipase activity. Higher MO concentrations did not further increase enzyme production but also did not repress enzyme synthesis. Thus, these data confirm the results of the response surface experimental design, which indicated levels of about 6.25% as optimum carbon source concentrations.

Lipase Production by Solid-State Fermentation in Packed-Bed Bioreactors

Packed-bed bioreactors are mechanically simple devices that allow a better control of oxygen transfer and moisture content through forced aeration of medium bed with humidified air. In our study, the influence of *V* and *T* on lipase production by *P. simplicissimum* in these bioreactors was investigated. A response surface experimental design for testing these variables was employed, and the results are given in Table 5.

Through statistical analysis of the results, a quadratic model (Eq. 5) was obtained to describe lipase activity ([LA], expressed in U/g) as a function of the normalized *T* and *V*. Both variables, including their quadratic

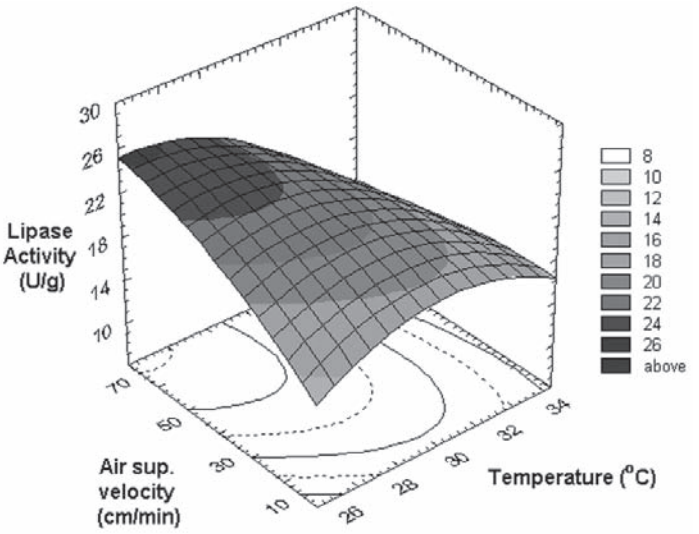

Fig. 3. Effect of T and V on lipase production by *P. simplicissimum* in packed-bed bioreactors.

terms and the interaction among them, were shown to be statistically significant:

$$LA = 20.79 \pm 2.46T \pm 1.91 \cdot T^2 + 1.11 \cdot V \pm 0.85 \cdot V^2 \pm 1.93 \cdot T \cdot V \quad (5)$$

The response surface obtained (Fig. 3) indicates that optimum conditions for lipase production in packed-bed bioreactors occur for a combination of temperatures <28°C and air superficial velocities >55 cm/min.

In this type of bioreactor, a negative effect of the temperature was observed in the whole range tested. By contrasti, in tray-type reactors, temperature levels of about 30°C were determined to be optimum. This different behavior observed in both types of bioreactors is probably related to heat gradient formation inside the packed-bed bioreactor. Employing bioreactors similar to those used in the present work, Saucedo-Castañeda et al. *(16)* observed radial thermal gradients with temperature differences as high as 10°C between the center of the reactor and its wall. Thus, the lower temperature, found as optimum for the packed-bed bioreactor, is probably related to the fact that the real temperature in the inner portion of the medium bed is higher than the temperature set in the water jacket.

Air superficial velocity presented a positive effect on lipase production in the whole range tested, showing that velocities up to 73.2 cm/min did not cause problems of medium drying owing to evaporation of water in the bed, such as discussed by Mitchell et al. *(4)*. The positive effect of the air superficial velocity is most probably related to the capacity of the airstream to remove heat, avoiding temperature levels that would be deleterious to the microorganism, rather than to any improvement in oxygen transfer, because in this type of bioreactor oxygen is usually not limiting *(17)*.

The highest lipase activities obtained in packed-bed bioreactors were 26.4 U/g, which is approx 30% higher than those found in tray-type bioreactors. This indicates the adequacy of packed-bed bioreactors for solid-state fermentation processes.

This lipase activity level obtained in solid-state fermentation in the present work (26.4 U/g) is higher than that (6.9 U/g) obtained for this fungus in submerged fermentation in a 10-L fermentor, as reported by Sztajer et al. *(18)*. When compared to other microorganisms grown in solid-state fermentation, the lipase activities obtained in the present work are in the same range as those observed for *Penicillium restrictum* cultivated in babassu cake (30.3 U/g) *(8)*, *Rhizopus oligosporus* in almond meal (48 U/g) *(12)*, *Candida rugosa* in rice straw (37 U/g) *(19)*, and *Rhizomucor pusillus* in sugarcane bagasse and olive cake, 50% each (20.2 U/g) *(20)*.

Conclusions

In the present work, the production of lipase by *P. simplicissimum* in solid-state fermentation was investigated. In tray-type bioreactors, it was shown that, considering the carbon and nitrogen sources tested, the concentration of the nitrogen source did not exert a significant effect on lipase production, whereas temperature, moisture content, and carbon source concentration did present significant effects on enzyme synthesis. For all combinations of supplements, a temperature of 30°C, a moisture content of 70%, and a concentration of the carbon source of about 6.25% provided optimum conditions for lipase production. Furthermore, it was shown that MO can be used as a single supplement to enrich babassu cake, providing lipase activities as high as when used in combination with CS or YH.

In packed-bed bioreactors, the aeration rate was shown to have a positive effect on lipase production, whereas temperature had a negative effect in the whole range tested. Maximum lipase activities were obtained for air superficial velocities > 55 cm/min combined with temperatures lower than 28°C. When compared with tray-type bioreactors, the temperature optimum obtained for packed-bed bioreactors was lower, suggesting the probable existence of radial temperature gradients inside the bioreactor. Maximum lipase activities in packed-bed bioreactors were approx 30% higher than maxima observed in tray-type bioreactors, indicating the potentiality of employing such bioreactors for the production of enzymes by solid-state fermentation.

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