A Solid-State Bioprocess for Selecting Lipase-Producing Filamentous Fungi

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Z. Naturforsch. **64 c**, 131–137 (2009); received March 25/May 23, 2008

A solid-state bioprocess with wheat bran and rice husk as substrate was used to isolate filamentous fungi with lipase activity from dairy effluent and soil contaminated with diesel oil. The lipase activity was measured in units, with one unit (U) being defined as the amount of enzyme required to liberate 1 μmol of fatty acids per minute per gram of bran substrate (1 U = 1 μmol min^{-1} g^{-1}). We obtained 24 isolates of filamentous fungi with lipase activity, 17 from the dairy effluent and 7 from the diesel oil-contaminated soil. The best lipase producers were the dairy effluent isolate *Aspergillus* E-6, with a maximum lipase activity of 49.81 U, and *Aspergillus* isolate O-4 recovered from the diesel oil-contaminated soil, with a maximum lipase activity of 45.49 U. Both isolates produced their maximum lipase activity eight days after the start of the bioprocess.

Key words: Filamentous Fungi, Lipase, Wheat Bran

Introduction

Solid-state bioprocesses involve the growth of microorganisms on the exterior and interior of substrates or matrices consisting of humid porous particles with a liquid phase, which provides sufficient water activity (A_w) to ensure growth and metabolism of the target microorganism but does not exceed the carrying capacity of the matrix. Filamentous fungi have received the greatest attention as candidate organisms for low A_w solid-state bioprocesses because under such conditions they show better growth than other classes of microorganisms (Pandey, 2003; Pandey *et al*., 2000; Couto and Sanromán, 2006). The ability of filamentous fungi to grow in solid-state bioprocesses reflects the fact that these microorganisms grow naturally on fruits, grains and agricultural and forestry residues under conditions very similar to those present in solid-state bioprocesses, which typically have A_w values in the order of 0.7 to 0.8, generally the optimal levels for fungal growth. A further advantage of solid-state bioprocesses is that they can utilize corn, rice, soya and wheat bran and straw, cassava residues, sugar-cane bagasse and other agro-industrial residues and thus alleviate the environmental pollution caused by the large amounts of such residues, while they also reduce the costs of bioprocesses (Raimbault, 1998).

Lipases (triacylglycerol-acyl-hydrolases, E.C. 3.1.1.3.) are industrially important biochemicals. Filamentous fungi are known to be good lipase producers; examples are *Aspergillus niger* (Mahadik *et al*., 2002), *Fusarium solani* (Maia *et al*., 2001), *Rhizopus oligosporus* (Ul-Haq *et al*., 2002) and members of the genera *Geotrichum*, *Mucor* and *Penicillium* (Sharma *et al*., 2001).

Lipases represent a group of cheap, biocatalysts with low specificity, which can hydrolyze the ester bonds of water-insoluble substrates at the water/substrate interface and can also catalyze the partial or total hydrolysis of triacylglycerol (TAG) to produce diacylglycerol (DAG), monoacylglycerol (MAG), glycerol and free fatty acids (Sharma *et al*., 2001). Lipases have many uses, including acceleration of cheese curing and others in the food industry (Sharma *et al*., 2001; Hasan *et al*., 2006), the separation of racemic mixtures (Rao *et al*.,

0939–5075/2009/0100–0131 $ 06.00

1993), detergent production (Hasan *et al.*, 2006), effluent treatment (Castro *et al.*, 2004; Mendes and Castro, 2005; Rosa *et al.*, 2006), and the production of cosmetics and pharmaceuticals (Elibol and Ozer, 2000).

The object of the research described in this paper was to isolate filamentous fungi for the production of lipases using a solid-state bioprocess.

Material and Methods

Sample collection, fungal isolation, identification and maintenance

Soil samples were collected at depths of 50 cm to 100 cm from an area contaminated with diesel oil, and dairy effluents rich in lipids and fatty acids were collected from the settling tanks and activated sludge bioreactors of a dairy, both in the city of Passo Fundo, in the southern Brazilian state of Rio Grande do Sul. Filamentous fungi were isolated from the soil samples by serial dilution in peptone water [0.1 % (w/v) peptone; Acumedium Manufacturers, Baltimore, USA], 0.1 mL of the 10^{-5} dilution being spread onto plates of potato dextrose agar (PDA; Acumedium Manufacturers) acidified to pH 3.5 with a 10 % solution of tartaric acid. Isolation from the dairy effluents was accomplished by direct inoculation of 0.3 mL of undiluted effluent onto the surface of acidified PDA. In all cases the plates were incubated at 30 °C for 5 d, and individual colonies were transferred to test tubes containing 0.1 % peptone water. The fungal isolates were replicated on acidified PDA until consistant colonies were obtained; representative material from these colonies was maintained at 4 °C on PDA slopes. The fungal isolates were identified to the genus level using the slide microcultivation technique (Ribeiro and Soares, 2000).

Selection of lipase-producing filamentous fungi

The fungal isolates were plated onto 30 mL of PDA and incubated at 30 °C for 5 d until sporulation. After incubation, 20 mL of distilled water containing 0.1 % (v/v) Tween 80 (Sigma) were added to each plate, and the fungal spores were scraped from the mycelium using a sterilized loop. The spore and mycelium suspension were filtered through sterilized gauze to remove the hyphae.

The medium was prepared using 15 g rice husk, 90 g of wheat bran and 75 mL of a salt solution containing 2 g L^{-1} KH_2PO_4, 1 g L^{-1} $MgSO_4$, 10 mL L^{-1} of trace-element solution [containing (mg L^{-1}) $FeSO_4 \cdot 7H_2O$ (0.63); $MnSO_4$ (0.01); and $ZnSO_4$ (0.62)] and distilled water to give 1 L of medium. The medium was autoclaved at 103 kPa for 20 min. Thereafter the pH value was corrected to 6.0 by the addition of a 1.5 mol L^{-1} sulfuric acid and the humidity was adjusted to 60 % by adding sterile distilled water. Olive oil was added at a content of 2 % (v/v) to induce lipases.

For each fungal strain, 50 g of medium were added to each of two 300 mL conical flasks and inoculated with 2.5 mL of spore suspension (corresponding to $2 \cdot 10^6$ spores g^{-1}). The flasks were incubated at 30 °C for 10 d and 10 g samples removed every 48 h for lipase analysis.

Determination of lipase activity

Lipases were extracted from the samples by adding 1 g of medium to 10 mL of phosphate buffer (0.1 M; pH 7.0) in a 250 mL conical flask which was shaken at 160 revs min^{-1} for 30 min at 37 °C; thereafter the liquid was decanted and filtered through a Whatman number 4 filter paper to remove any suspended solids.

Lipase activity was determined, in duplicate, using the method of Burkert *et al.* (2004) where lipases in the culture medium extract are quantified by alkaline titration of the fatty acids released when aliquots of the filtrate are mixed with a water emulsion of olive oil using gum Arabic as the suspending agent. For each sample, 2 mL of phosphate buffer (0.1 M; pH 7.0) and 5 mL of olive oil emulsion [25 % (v/v) olive oil plus 75 % (v/v) of a 7 % (w/v) aqueous solution of gum Arabic] were placed in an 125 mL conical flask, and 1 mL of culture medium extract was added. The flasks were shaken at 160 revs min^{-1} for 30 min at 37 °C, and the reaction was stopped by the addition of 15 mL of 1:1:1 (v/v) acetone/ethanol/water. The fatty acids liberated were titrated with 0.05 M aqueous NaOH using phenolphthalein as indicator. The lipase activity was measured in units, with one unit (U) being defined as the amount of enzyme required to liberate 1 μmol of fatty acids per minute per gram of bran substrate (1 U = 1 μmol min^{-1} g^{-1}) under the conditions specified.

Statistical analysis

Lipase activity as a function of time was tabulated and plotted. The highest lipase activities were subjected to analysis of variance (ANOVA)

Table I. Analysis of variance of the maximum lipase activities of fungal isolates recovered from dairy effluent (n = 17) and soil contaminated with diesel oil (n = 7). The blocks represent the replicate bioprocesses.

Experiment	Sum of squares	Degrees of freedom (n – 1)	Mean square	F value	p
Dairy effluent					
Mean	101516.9	1	101516.9	105680.4	< 0.001
Blocks	1.5	1	1.5	1.6	0.2147
Fungi (n = 17)	2005.1	16	125.3	130.5	< 0.001
Error	48.0	50	1.0		
Soil + diesel oil					
Mean	35402.38	1	35402.38	31887.91	< 0.001
Blocks	0.46	1	0.46	0.41	0.5278
Fungi (n = 7)	974.76	6	162.46	146.33	< 0.001
Error	22.20	20	1.11		

Table II. Maximum lipase activity ± standard deviation (SD) of the fungal isolates recovered from dairy effluent (E) and soil contaminated with diesel oil (O). Lipase activity was measured in units, with one unit (U) being defined as the amount of enzyme required to liberate 1 μmol of fatty acids per minute per gram of bran substrate (1 U = 1 μmol min^{-1} g^{-1} of bran).

Maximum lipase activity [U] ± SD of filamentous fungi isolated from different sources

Dairy effluent		Soil contaminated with diesel oil	
Isolate and genus	Lipase activity*	Isolate and genus	Lipase activity*
E-6 *Aspergillus*	49.81 ± 0.75^{a}	O-4 *Aspergillus*	45.49 ± 0.69^{a}
E-17 *Aspergillus*	45.92 ± 1.14^{b}	O-7 *Trichoderma*	40.09 ± 0.34^{b}
E-1 *Fusarium*	43.56 ± 0.60^{bc}	O-1 *Aspergillus*	36.88 ± 0.67^{c}
E-8 *Aspergillus*	43.26 ± 0.92^{c}	O-5 *Aspergillus*	35.37 ± 2.48^{cd}
E-20 *Penicillium*	43.25 ± 1.55^{c}	O-8 *Aspergillus*	33.37 ± 0.67^{de}
E-19 *Trichoderma*	41.76 ± 0.11^{cd}	O-6 *Penicillium*	32.85 ± 0.34^{e}
E-18 *Trichoderma*	39.60 ± 0.68^{de}	O-3 *Penicillium*	24.96 ± 0.69^{f}
E-13 *Trichoderma*	39.52 ± 0.89^{de}		
E-7 *Aspergillus*	39.12 ± 0.10^{ef}		
E-15 *Aspergillus*	36.84 ± 0.55^{fg}		
E-14 *Fusarium*	36.37 ± 0.12^{gh}		
E-5 *Fusarium*	36.32 ± 0.66^{gh}		
E-10 *Aspergillus*	35.69 ± 0.93^{gh}		
E-9 *Aspergillus*	34.23 ± 0.52^{hi}		
E-16 unidentified	32.25 ± 0.03^{ij}		
E-12 *Penicillium*	30.51 ± 1.88^{jl}		
E-3 *Penicillium*	28.84 ± 0.94^{l}		

* Within each column means with the same superscript were not significantly different by the Tukey test at p = 0.05. Numbers are rounded to two significant decimal places.

and the means compared using the Tukey honest difference (HSD) test (Box *et al.*, 1978).

Results and Discussion

Fungal isolation, identification and maximum lipase production

We recovered 17 filamentous fungi (isolates E-1, E-3, E-5 to E-10 and E-12 to E-20) from the dairy effluent and seven filamentous fungi from the soil contaminated with diesel oil (isolates O-1 and O-3 to O-8), all subsequently shown to have lipase activity. Analysis of variance (Table I) of the maximum lipase activities (Table II) of the isolates from both sources indicated that there were significant differences ($p \leq 0.01$) between the isolates in each group. The F-value for the blocks, representing the number of replicate bioprocesses, was not

significant (Table I) at the probability level stated, indicating acceptable repeatability for the duplicate bioprocessor runs.

For the group of isolates recovered from dairy effluent, the Tukey test showed significant differences ($p = 0.05$) in the maximum lipase activity, with the *Aspergillus* isolate E-6 presenting a significantly higher ($p < 0.05$) maximum lipase activity [(49.81 ± 0.75) U] than the other isolates in this group (Table II). The *Aspergillus* isolate E-17 and the *Fusarium* isolate E-1 produced the second highest maximum lipase activity in this group and were not statistically different ($p > 0.05$) in terms of their maximum lipase activity, the maximum lipase activity of these two isolates being 45.92 U and 43.56 U, respectively. Isolates E-8, E-19 and E-20 produced statistically similar ($p > 0.05$) lipase activities, the maximum lipase activity for these isolates ranking third at 42.95 U. The remaining dairy effluent isolates presented maximum lipase activities of less than 40 U, with the lowest maximum activity (28.84 U) being produced by the *Penicillium* isolate E-3 (Table II). For the group of isolates recovered from soil contaminated with diesel oil the Tukey test also showed significant differences ($p = 0.05$) in the maximum lipase activity, with the *Aspergillus* isolate O-4 producing a significantly higher ($p < 0.05$) the maximum lipase activity [(45.49 ± 0.69) U] than the other isolates in this group. The *Trichoderma* isolate O-7 produced the second highest maximum lipase activity [(40.09 ± 0.34) U] in this group and was a significantly better ($p < 0.05$) lipase producer than isolates O-1, O-5, O-8 and O-6, which presented maximum lipase activities ranging from 32.85 U to 36.88 U (Table II). The lowest maximum activity (24.96 U) in this group was produced by *Penicillium* isolate O-3 (Table II).

Lipase production as a function of time

The maximum lipase activity as a function of time is shown in Fig. 1 for isolates E-1, E-3, E-5 to E-10 and E-12 to E-20 and in Fig. 2 for isolates O-1 and O-3 to O-8.

For the dairy effluent isolates the maximum lipase activities occurred eight days after inoculation and were 49.81 U for *Aspergillus* isolate E-6 (Fig. 1a) and 45.92 U for *Aspergillus* isolate E-17 (Fig. 1d).

The isolates recovered from soil contaminated with diesel oil also produced their maximum lipase activities eight days after inoculation; *Aspergillus* isolate O-4 produced 45.49 U (Fig. 2a) and *Trichoderma* isolate O-7 produced 40.09 U (Fig. 2b).

Most studies of lipase production by bacteria, filamentous fungi and yeasts used submerged culture, with solid-state processes having received relatively little attention. Recently, however, increasing emphasis has been given to the conversion of agroindustrial residues to lipases using solid-state bioprocesses (Couto *et al*., 2006), and the importance of using solid substrates to isolate lipolytic microorganisms has been recognized, with filamentous fungi being considered to be the microorganisms best adapted to such substrates (Raimbault, 1998; Pandey *et al*., 2000; Pandey, 2003). Filamentous fungi are known to be good enzyme producers (Maia *et al*., 2001) and fungal lipases are preferred for industrial applications, especially in the food industry (Mahadik *et al*., 2002). Sharma *et al*. (2001) cite many fungal strains as being good lipase producers, including members of the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Mucor*, *Penicillium*, *Rhizomucor* and *Rhizopus*. D'Annibale *et al*. (2006) grew various fungi and yeasts (the fungi *Aspergillus*, *Geotrichum*, *Penicillium*, *Rhizopus* and the yeast *Candida*) in submerged culture, using effluents from the olive oil industry as substrate, and found that the highest lipase activities were produced by *Candida cylindracea* strain NRRL Y-17506 [0.6 international U (IU) mL^{-1}] and *Geotrichum candidum* NRRL Y-553 (0.52 IU mL^{-1}). The highest lipase activities per unit volume were produced by *Penicillium citrinum* strains ISRIM 118 (5.42 IU L^{-1} h^{-1}) and NRRL 3754 (4.58 IU L^{-1} h^{-1}).

In our study we found that the best lipase producers were members of the genus *Aspergillus* isolated from dairy effluent (isolate E-6, 49.81 U) and soil contaminated with diesel oil (isolate O-4, 45.49 U). Many authors have reported *Aspergillus* strains to be good producers of lipases. For example, Mahadik *et al*. (2002) investigated the ability of *Aspergillus*, *Geotrichum*, *Mucor*, *Penicillium* and *Rhizopus* to form butyl esters from butter fat and found that *Rhizopus arrhizus* and *Aspergillus niger* produced the best results, with *Aspergillus niger* presenting the highest production of extracellular lipases and subsequently being used by these authors for lipases production using a solid-state bioprocess with wheat bran as the main component of the culture medium. Falony *et al.* (2006) and Damaso *et al*. (2005) used *A. niger* for lipase

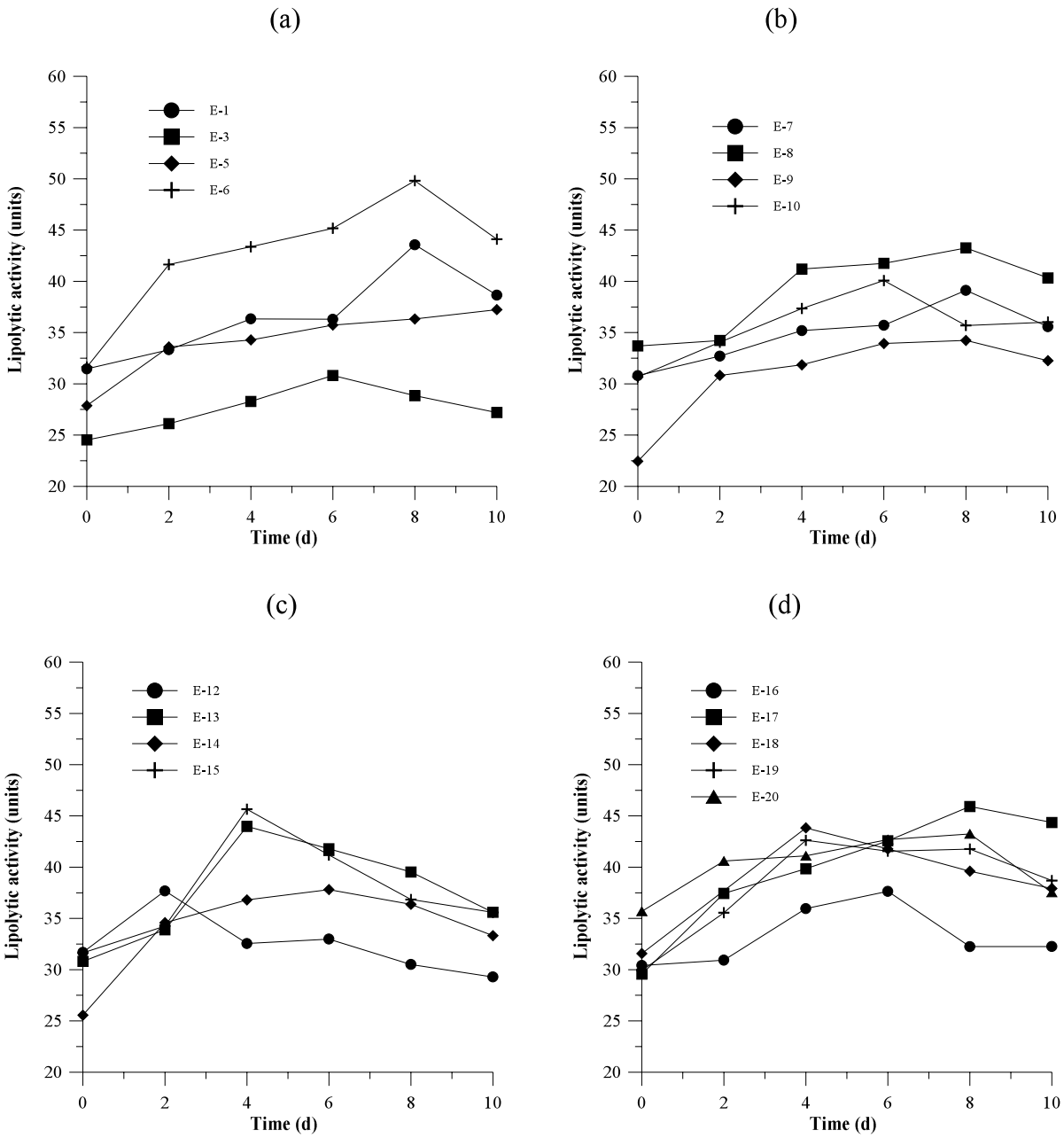

Fig. 1. Maximum lipase activity as a function of time for isolates (a) E-1, E-3, E-5 and E-6, (b) E-7 to E-10, (c) E-12 to E-15, and (d) E-16 to E-20. Lipase activity was measured in units, with one unit (U) being defined as the amount of enzyme required to liberate 1 μmol of fatty acids per minute per gram of bran substrate (μmol min^{-1} g^{-1}).

production in solid-state bioprocesses with wheat bran as the principal component of the cultivation medium, while Montesino *et al.* (2005) used *A. niger* for the degradation of liquid residues rich in fat. Ul-Haq *et al.* (2002) screened *A. niger*, *A. wentii*, *Mucor lipolytica*, *Mucor* sp., *Penicillium* sp. and various *Rhizopus* species (*oryzae, oligosporus, nigricans* and *arrhizus*) for lipase production in solid-state medium and found that, although *A. wentii* produced lipases, the highest lipase activi-

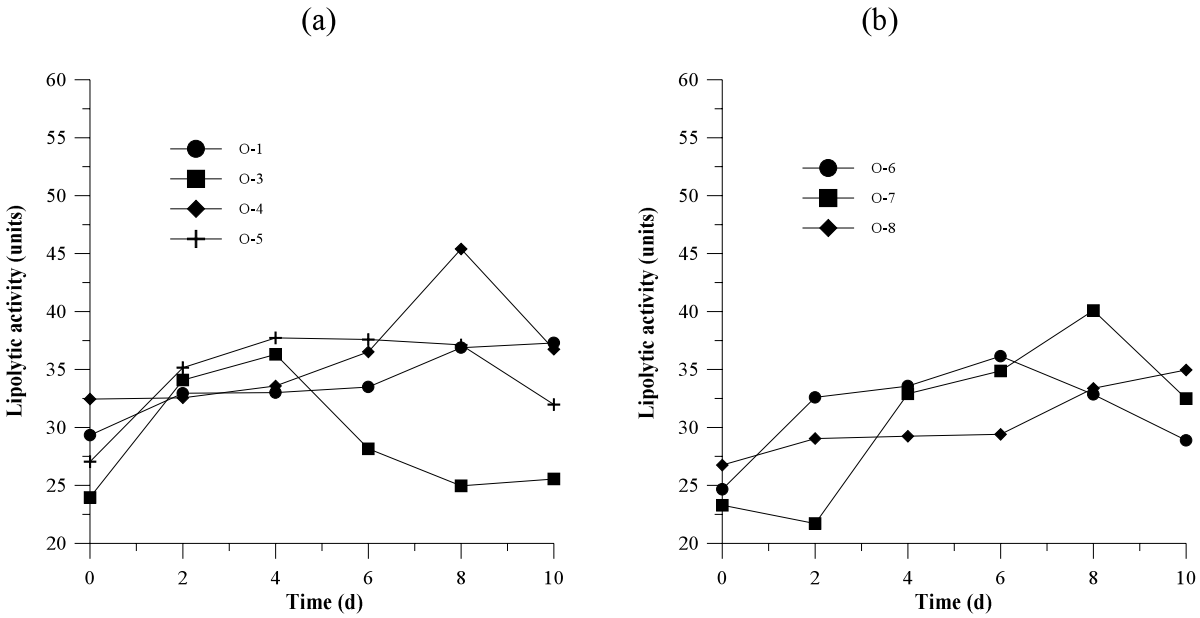

Fig. 2. Maximum lipase activity as a function of time for isolates (a) O-1, O-3, O-4 and O-5, and (b) O-6, O-7 and O-8. Lipase activity was measured in units, with one unit (U) being defined as the amount of enzyme required to liberate 1 μmol of fatty acids per minute per gram of bran substrate (μmol min^{-1} g^{-1}).

ties were obtained with *R. oligosporus* growing in wheat bran at 30 °C.

There have been many reports on lipase production by *Penicillium* isolates; for example, Gombert *et al.* (1999) reported that *Penicillium restrictum* was a good lipase producer in submerged culture, and D'Annibale *et al.* (2006) reported that when growing on olive oil effluent the highest lipase activities per unit volume were produced by *P. citrinum* strains ISRIM 118 (5.42 IU L^{-1} h^{-1}) and NRRL 3754 (4.58 IU L^{-1} h^{-1}). However, in our study only *Penicillium* isolate E-20 produced relatively high lipase activities while the other *Penicillium* isolates produced low lipase activities compared to the isolates belonging to other fungal genera, irrespective of the source of the isolate (Table II).

Leal *et al.* (2002) used *P. restrictum* and a solid-state bioprocess based on babaçu palm seed cake (a residue of babaçu oil production) as substrate to produce an enzymatic extract with high lipase activity that was used to hydrolyze a dairy effluent with a fat content of 180 mg L^{-1} to 1.200 mg L^{-1} which was subsequently submitted to anaerobic treatment, the efficiency of which was increased by pre-treatment with fungal lipase. Rosa *et al.* (2006) also used *P. restrictum* and a solid-state bioprocess in which the waste of babaçu oil industry served to produce lipases which were used to pretreat a semi-synthetic effluent containing milk fat which was subsequently treated by a continuously activated sludge process; the results showed that pre-treatment with lipase facilitated the subsequent processing of the effluent. These reports suggest that the strains isolated by us could be used to produce bran containing lipases for the efficient treatment of dairy effluent; this would be especially useful for treating the effluent from which they had been isolated and to which they were, therefore, adapted.

Two of the *Trichoderma* isolates recovered by us from the dairy effluents and the single isolates obtained from the contaminated soil showed a high maximum lipase activity of about 40 U, although this was not as high as for the best *Aspergillus* isolates which showed maximum activities ranging from 45 to 49 U. It is interesting to note that we found no reports in the literature regarding lipase production by *Trichoderma*. Furthermore, the *Fusarium* isolates obtained in our study were good, or reasonable, lipase producers, supporting the work of Maia *et al.* (2001) who found that *Fusarium solani* produced good levels of lipase activity.

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