Selection of Lipase-Producing Microorganisms through Submerged Fermentation

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Lipases are enzymes used in various industrial sectors such as food, pharmaceutical and chemical synthesis industries. The selection of microorganisms isolated from soil or wastewater is an alternative to the discovery of new species with high enzymes productivity and with different catalytic activities. In this study, the selection of lipolytic fungi was carried out by submerged fermentation. A total of 27 fungi were used, of which 20 were isolated from dairy effluent and 7 from soil contaminated with diesel oil. The largest producers were the fungi *Penicillium* E-3 with maximum lipolytic activity of 2.81 U, *Trichoderma* E-19 and *Aspergillus* O-8 with maximum activities of 2.34 and 2.03 U where U is the amount of enzyme that releases 1 μmol of fatty acid per min per mL of enzyme extract. The fungi had maximum lipolytic activities on the 4th day of fermentation.

Key words: Filamentous Fungi, Lipase, Screening, Submerged Fermentation

Introduction

Lipases (triacylglycerol-acyl-hydrolases, EC 3.1.1.3.) are enzymes capable of hydrolyzing ester bonds of water-insoluble substrates at the interface between the substrate and water, which catalyze the partial or total hydrolysis of triacylglycerols (TAG) providing diacylglycerols (DAG), monoacylglycerols (MAG), glycerol, and free fatty acids (Sharma *et al.*, 2001). They can be used in manufacturing of detergents (Hasan *et al.*, 2006), in effluent treatment (Mendes and Castro, 2005; Rosa *et al.,* 2006; Castro *et al.*, 2004), for the development of cosmetics, medicines (digestive enzymes) or as clinical reagents (Elibol and Ozer, 2000), and in the resolution of racemic mixtures (Rao *et al.*, 1993). In the food industry they are used in the synthesis of emulsifiers (Kim *et al.*, 2006), to increase the levels of unsaturated fatty acids in lipids (Carvalho *et al.*, 2003), in the production of margarine, in the development of flavours (Larios *et al.*, 2004), and in cheese maturation (Dupuis *et al.*, 1993), among others.

Microorganisms are a commercially advantageous source for enzyme production, particularly lipases. Thus, the selection of new microorganisms that efficiently produce these enzymes and studies on the biosynthetic regulation of these metabolites are very important (Makhsumkhanov *et al.*, 2003).

Fungal lipases are preferred for industrial use, especially in the food industry (Mahadik *et al.*, 2002). The most cited lipase-producing fungi genera are *Aspergillus*, *Rhizopus*, *Penicillium*, *Mucor*, *Geotrichum* and *Fusarium* (D'Annibale *et al.*, 2006; Haq *et al.*, 2002; Mahadik *et al.*, 2002; Maia *et al.*, 2001).

The selection of lipolytic fungi from soils is a new area of interest for researchers because of the potential microflora in this environment (Shukla and Gupta, 2007). Furthermore, environmental conditions in soils, often arising from processes of accumulation of toxic compounds, cause the natural selection of microorganisms, which produce certain metabolites necessary for cellular adaptation and survival.

Another potential source of lipase-producing microorganisms are lipid-rich industrial effluents (Ertugrul *et al.*, 2007; D'Annibale *et al.*, 2006), as they develop the metabolic ability to remain alive

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in the effluent by treating the residues, as reported by Matsumiya *et al.* (2007), who demonstrated the treatment of a lipid-rich effluent through the use of lipase produced by microorganisms isolated from the effluent.

Selection of lipase-producing microorganisms usually starts by their growth on plates containing agar from a lipidic source, as reported by various authors (Roveda *et al.*, 2010; Shukla and Gupta, 2007; Colen, 2006). However, the difficulty in selecting lipolytic fungi using these methods arises from the excessive growth, low lipolytic activities, and the interference of fungal metabolites (Colen, 2006). In addition, the selected microorganisms might not always be good lipase-producers under the conditions of solid-state or submerged fermentation. The objective of the present study was to select lipase-producing fungi using submerged fermentation.

Material and Methods

Microorganisms and maintenance

Fungi were isolated from diesel-contaminated soil and from dairy effluents according to Colla *et al.* (2009), and kept stored at 4 °C in tubes with PDA (potato-dextrose-agar).

Selection of lipase-producing filamentous fungi

The inoculum was prepared by inoculating the fungi in Petri dishes containing 30 mL of solidified PDA medium and incubating at 30 °C for 5 d. After growth, a suspension of spores was prepared by adding 20 mL of a 0.1% Tween (v/v) solution followed by scraping off the spores with a Drigalsky loop and filtration through sterile gauze for retention of hyphae.

The cultivation media were prepared with 10% (w/v) wheat bran, which was boiled at 100 °C for 30 min in 50% of the volume of distilled water. Afterwards, the medium was filtered and the soluble extract added to 10% (v/v) saline, 1% (w/v) sodium nitrate, and 1% (w/v) olive oil as the lipase-production inducer. The saline solution contained 2 g L^{-1} KH_2PO_4, 1 g L^{-1} $MgSO_4$, and 10 mL L^{-1} trace solution, which was composed of 0.63 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 0.01 mg L^{-1} $MnSO_4$, and 0.62 mg L^{-1} $ZnSO_4$ (Bertolin *et al.*, 2001). The liquid medium was autoclaved and its pH value adjusted to 6.0 by using 1.5 M HCl or 1 M NaOH.

For each microorganism used in the selection, two fermentations were carried out by inoculating 5 mL of the suspension of spores to 100 mL of liquid medium in 125-mL Erlenmeyer flasks, with subsequent incubation at 30 °C for 10 d. Samples were withdrawn every 24 h for assessment of lipolytic activity.

Lipolytic activity assessment

The samples were filtered through cotton wool to remove the hyphae, and the filtrates were used for lipolytic activity assessment.

The enzymatic activity was assessed using the method standardized by Burkert *et al.* (2004), which is based on titration with NaOH of the fatty acids released by the lipase action, in the enzymatic extract, over triacylglycerols of olive oil emulsified in arabic gum.

One unit of lipolytic activity was defined as the amount of enzyme that releases 1 μmol of fatty acid per min per mL of enzyme extract (1 U = 1 μmol min^{-1} mL^{-1}) under the test conditions.

Statistical analysis

The results of maximum lipolytic activity were analyzed by analysis of variance (Anova) and Tukey test for comparison of means (Box *et al.*, 1978).

Results and Discussion

The 27 fungi used for lipase production in submerged fermentation and the maximum lipolytic activities reached are presented in Table I. The fungi with the highest mean lipolytic activity among the replicas were E-3, O-8, E-20, E-5, E-16, O-4, and E-19, which belong to the genera *Penicillium, Aspergillus, Fusarium*, and *Trichoderma*, which led to mean lipolytic activities higher than 1.29 U. Out of the isolated fungi, only the strains O-8 and O-4 were isolated from diesel oil-contaminated soil; all other strains were from dairy effluent. According to Sharma *et al.* (2001), among the lipase-producing fungi, the genera *Aspergillus*, *Penicillium*, and *Fusarium* are the best producers, which is in agreement with the findings in our study.

Analysis of variance of the enzyme activity data (Table I) showed significant differences between the fungi ($p < 0.01$) regarding the production of lipases in liquid medium containing wheat bran

Table I. Maximum lipolytic activities produced by the fungi used in submerged fermentation.

Fungus	Genus	Lipolytic activity [U]
E-3	*Penicillium*	2.05 ± 0.21^{a}
O-8	*Aspergillus*	1.89 ± 0.41^{ab}
E-20	*Penicillium*	1.78 ± 1.26^{abc}
E-5	*Fusarium*	1.61 ± 1.68^{abcd}
E-16	Not identified	1.61 ± 0.12^{abcd}
O-4	*Aspergillus*	1.38 ± 0.11^{abcde}
E-19	*Trichoderma*	1.29 ± 0.51^{abcde}
O-2	Not identified	1.23 ± 0.06^{abcdef}
E-6	*Aspergillus*	1.23 ± 0.00^{abcdef}
O-3	*Penicillium*	1.19 ± 0.05^{abcdef}
E-8	*Aspergillus*	1.19 ± 0.06^{abcdef}
E-18	*Trichoderma*	1.16 ± 0.15^{bcdef}
O-6	*Penicillium*	1.14 ± 0.01^{bcdef}
O-1	*Aspergillus*	1.13 ± 0.08^{bcdef}
E-11	Not identified	0.93 ± 0.06^{cdef}
E-10	*Aspergillus*	0.93 ± 0.24^{cdef}
E-1	*Fusarium*	0.85 ± 0.48^{def}
E-13	*Trichoderma*	0.81 ± 0.26^{def}
E-2	Not identified	0.72 ± 0.06^{ef}
E-4	Not identified	0.68 ± 0.12^{ef}
E-9	*Aspergillus*	0.63 ± 0.18^{ef}
E-17	*Aspergillus*	0.61 ± 0.11^{ef}
E-7	*Aspergillus*	0.58 ± 0.06^{ef}
E-21	*Aspergillus*	0.57 ± 0.32^{ef}
O-5	*Aspergillus*	0.54 ± 0.00^{ef}
E-12	*Penicillium*	0.40 ± 0.15^{f}
E-14	*Fusarium*	0.38 ± 0.18^{f}

Mean ± standard deviation. Means followed by different letters are significantly different. Tukey test at a 0.05 significance level.

and olive oil as carbon sources. However, there was a significant difference between the replicas of fermentation ($p < 0.01$). Because of this, the fungi with the highest mean lipolytic activities (E-3, O-8, E-20, E-5, E-16, and E-19) were used in a second stage of selection. Fungus E-19 was chosen rather than the fungus O-4 because it showed higher lipolytic activity in one of the replicas (1.69 U). Moreover, there were no significant differences between E-19 and O-4 (Tukey test, $p > 0.05$).

Fig. 1 shows the lipolytic activity versus time for the fungi E-3 and E-5 (Fig. 1a), E-16 and E-19 (Fig. 1b), and E-20 and O-8 (Fig. 1c). The maximum lipolytic activities were, on average, reached on the 4th day of submerged fermentation. The analysis of variance of data for maximum lipolytic activity in the second stage of selection

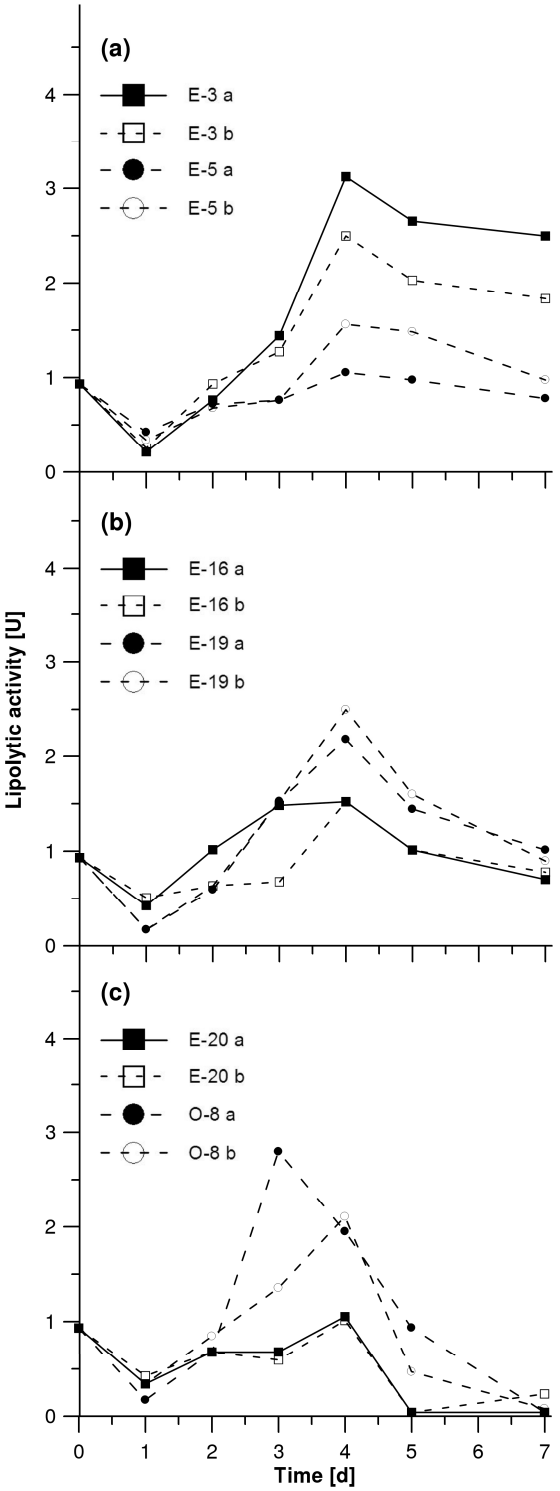

Fig. 1. Lipolytic activities versus time for the fungi (a) E-3 and E-5, (b) E-16 and E-19, (c) E-20 and O-8.

showed significant differences between the six fungi ($p < 0.01$). The F value for the replicas of fermentation was not significant ($p = 0.888$) in the confidence interval tested, indicating repeatability between fermentation replicates in this second stage.

Table II shows the results of lipolytic activity assessment for the six fungi used in the second stage of selection. Fungus E-3 had the highest lipolytic activity, similar to what happened in the first stage. However, the lipolytic activity obtained was equal to that obtained with the fungus E-19 ($p = 0.076$) but higher ($p < 0.05$) than the activities of other fungi. Fungi E-19 and O-8 had the second and third highest lipolytic activities, significantly higher than those of the other fungi ($p < 0.05$) but equal to each other ($p = 0.390$).

The selection of lipase-producing microorganisms has been carried out by several authors (Shukla and Gupta, 2007), who have described a pre-selection stage in Petri dishes containing solid media. These media allow the verification of extracellular production of lipases by forming a transparent halo around the microorganism colonies. In subsequent steps, the microorganisms that present positive results of halo formation are selected via submerged or solid-state fermentation. In the present study, a selection of microorganisms was carried out directly via submerged fermentation, without the pre-selection step in Petri dishes containing semi-solid media, which is advantageous, because the cultivation conditions in semi-solid media are very different from the conditions offered by submerged fermentation, especially in terms of access of the microorganism to micro-nutrients and oxygenation conditions.

Table II. Maximum lipolytic activities reached by fungi used in the second stage of selection of lipase-producing fungi via submerged fermentation.

Fungus and genus	Lipolytic activity [U]
E-3 *Penicillium*	2.81 ± 0.36^{a}
E-19 *Trichoderma*	2.34 ± 0.18^{ab}
O-8 *Aspergillus*	2.03 ± 0.13^{b}
E-16 Not identified	1.52 ± 0.10^{c}
E-5 *Fusarium*	1.31 ± 0.29^{c}
E-20 *Penicillium*	1.03 ± 0.07^{c}

Mean ± standard deviation. Means followed by different letters are significantly different. Tukey test at a 0.05 significance level.

Olive oil was used as inducer for the production of enzymes by fungi because it presented the best results in studies reported by several authors (Teng and Xu, 2008; Wang *et al.*, 2008; Ertugrul *et al.*, 2007; Joshi *et al.*, 2006; Muralidhar *et al.*, 2001; Miranda *et al.*, 1999).

The microorganism with the best lipase production was a strain of the genus *Penicillium*, isolated from dairy industry effluent (strain E-3, 2.81 U). There are several reports regarding the lipase production by strains of *Penicillium*. Gombert *et al.* (1999) reported that *Penicillium restrictum* was a good producer of lipase in submerged fermentation. Also, D'Annibale *et al.* (2006) reported that strains of *P. citrinum* had maximum growth in effluent of the olive oil industry.

The second largest producer of lipase was a strain of *Trichoderma* (E-19, 2.34 U). However, in scientific literature this type of fungus has not been related to the production of lipases, but of other enzymes such as cellulases. When *Trichoderma* strains (E-13, E-18 and E-19) were tested for lipase production in solid-state fermentation by Colla *et al.* (2009), they had a good productivity.

The strain O-8, identified as *Aspergillus*, had a maximum lipolytic activity of 2.03 U, and this genus is recognized as a good producer of lipase, as reported by Kaushik *et al.* (2006), Mahadik *et al.* (2004), Gulati *et al.* (2000), and Pokorny *et al.* (1997).

Roveda *et al.* (2010) used fungi isolated from dairy effluent in PDA added to 5% olive oil for a pre-selection, and they assessed the radial growth of fungi during incubation at 30 °C for 7 d. The best results of fungus radial growth rate were obtained with E-6 (1.77 cm d^{-1}). Fungi E-19 and E-3, which showed high lipolytic activity in our study, had much lower radial growth rates, 0.39 and 0.08 cm d^{-1}. Colla *et al.* (2009) selected these fungi for lipase production via solid-state fermentation, and the best results were obtained with the fungi O-4 and E-6. The fungus E-3, which had the highest lipolytic activity in our study, had the lowest result among the fungi isolated from dairy effluent in solid-state fermentation. These results demonstrate that the ability of growth in PDA added to olive oil showed similar results to those obtained in the selection of these fungi in terms of the production of lipases in solid-state fermentation. However, these fungi did not have

good results in the selection via submerged fermentation.

The method of selection of lipase-producing fungi used in the present study, via growth in submerged processes, allows the selection to be carried out under real conditions of submerged fermentation processes, which does not occur in the selection processes that use growth in Petri dishes.

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