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Isolation and screening of alkaline lipase-producing fungi from Brazilian savanna soil

Gecernir Colen*, Roberto Gonçalves Junqueira and Tasso Moraes-Santos
Departamento de Alimentos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627. Pampulha. Campus UFMG, 31270-901, Belo Horizonte, MG, Brazil
**Author for correspondence: Tel.: +31-3499-6912, Fax: +31-3499-6988, E-mail: gcolen@farmacia.ufmg.br*

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Summary

Fifty-nine lipase-producing fungal strains were isolated from Brazilian savanna soil by employing enrichment culture tecniques. An agar plate medium containing bile salts and olive oil emulsion was employed for isolating and growing fungi in primary screening assay. Twenty-one strains were selected by the ratio of the lipolytic halo radius and the colonies radius. Eleven strains were considered good producers under conditions of submerged liquid fermentation (shaken cultures) and solid-state fermentation. The most productive strain, identified as *Colletotrichum gloesporioides*, produced 27,700 U/l of lipase under optimized conditions and the crude lipase preparation was capable of hydrolysing a broad range of substrates including lard, natural oils and tributyrin.

Introduction

Some of the commercially important lipase-producing fungi are of the genera *Rhizopus, Geotrichum, Rhizomucor, Aspergillus, Candida* and *Penicillium* (Tan *et al*. 2003; Ellaiah *et al*. 2004; Larios *et al*. 2004). The industrial demand for new sources of lipases with different enzymatic characteristics stimulate the isolation and selection of new strains of lipolytic microorganisms (Vargas *et al*. 2004). However, lipase production by filamentous fungi varies according to the strain, the composition of the growth medium and cultivation conditions such as the carbon and nitrogen sources, pH and temperature (Sharma *et al*. 2001; Cihangir & Sarikaya 2004).

In general, the selection begins with microbial growth on agar plates. It is known that the success or failure in selecting microorganisms depends on the primary selection. The difficulties in selecting lipolytic fungi on agar plates are related to the exuberant growth, low lipolytic activity and interference of dyes with fungal metabolites (Jensen 1983). After the primary selection procedures it is usual to submit the strains to a submerged culture for evaluation of the enzyme production. In nature, soil habitat fungi grow attached to solid substrates, but with different characteristics from those growing in *in vitro* conditions. The distinct *in vitro* experimental conditions of submerged liquid fermentation (SLF) and solid-state fermentation (SSF) can alter gene expression, changing phenotypes such as growth, production of secondary metabolites and enzymes (Iwashita 2002; Hölker *et al*. 2004); therefore, the strains could be selected under both SLF and SSF conditions, simultaneously.

The objective of this study was to screen fungi for significant lipase production. This report includes: (a) a description of the techniques used to isolate and to select lipase-producing fungi from samples of Brazilian savanna soil; (b) the screening of alkaline lipase-producing strains; (c) the evaluation of some fermentation parameters related to the best lipase-producing fungus.

Materials and methods

Isolation of filamentous fungi

Savanna soil samples (500 g) from the north of the Minas Gerais state, Brazil, were collected under the 'pequizeiro' tree (*Caryocar brasiliense* Camb) and under the avocado tree (*Persea americana* Miller), both producing oleaginous fruits. Shavings (10 g) of the oleaginous seeds of the barú (*Dipteryx alata* Vog.), a native Brazilian savanna tree, were added to the soil samples, and they were kept in a humid chamber at 25–30 °C for 7 days. Ten grams of each sample were resuspended in 90 ml of a 0.1% (v/v) solution of Tween 80 in distilled water. The suspensions were used to inoculate a selective agar medium that contained (per litre): 0.5 g yeast

extract, 5.0 g $(NH_4)_2SO_4$, 2.0 g $(NH_2)_2CO$, 1.0 g $MgSO_4{\cdot}7H_2O$, 1.0 g NaCl, 2.0 g bile salts, 10.0 ml olive oil, 20.0 g bacteriological agar, pH 7.0. Before adding the oil, the mixture was heated to melt the agar and then emulsified using a Waring blender.

From the 59 isolated strains numbers 41 and 42 were identified as *Colletotrichum gloesporioides* by the Andre Tosello Foundation, Campinas, São Paulo, Brazil, based on conidial morphology. The conidia of *C. gloesporioides* were hyaline and cylindrical and obclavate in shape (Baxter *et al.* 1985; Bernstein *et al.* 1995). All stock cultures were cultivated in potato dextrose agar (PDA) at 30 °C and stored at 4 °C.

Screening of lipolytic fungi

The first screening was carried out on the same plate medium as was used for isolation, as described above. Culture plates inoculated with the 59 fungal strains were incubated at 30 °C for 72 h and the radius (r) of the colonies and the radius (R) of the clear hydrolytic halos around them were measured. Twenty-five strains with the ratio R/r equal to or higher than 1.2 were selected and grown both in SLF and in SSF conditions. The filtrates as described below were assayed in ATV or AEPV plates (see description below) and the strains (11) with a halo diameter equal to or greater than 2 mm were titrimetically analysed.

Submerged liquid fermentation

A five-milliliter spores suspension (10^6 spores/ml), prepared by washing 7–10 day-old PDA slants with sterile distilled water, was used to provide an inoculum for each 250-ml shake-flask containing 50 ml of the liquid growth medium (per litre): 20.0 g bacteriological peptone, 8.0 ml olive oil, 0.6 g $MgSO_4{\cdot}7H_2O$, 1.0 g KH_2PO_4, 1.0 g NH_4NO_3. The initial pH was adjusted to 7.0. After 72 h of incubation, at 30 °C in a rotatory shaker (Marconi, Piracicaba, SP, Brazil) operating at 160 rev/min, the biomass was separated by filtration through a Whatman filter paper, and the culture filtrates were used as the enzyme sources. The results were expressed as the mean of at least three independent measurements.

Solid-state fermentation

The basal fermentation medium contained: 30.0 g bacteriological peptone, 250 ml olive oil emulsion (25.0 ml olive oil in 100 ml of solution containing 1.25 g of arabic gum), 100 ml saline solution, 650 ml distilled water. The initial pH was adjusted to 7.0. The saline solution in distilled water contained (per litre): 100.0 g $(NH_4)_2HPO_4$, 2.0 g $MgSO_4{\cdot}7H_2O$, 2.0 g KCl. A sample (15.0 g) of washed and dried rice hulls was added to a series of 250 ml flasks, moistened with 10.0 ml of the fermentation medium and sterilized at 121 °C for 20 min. After cooling, three flasks were inoculated with each strain using 2.5 ml of the spore suspension (as prepared above) and incubated at 30 °C for 4 days. Enzyme extraction was performed by adding 100 ml of distilled water to the solid mouldy medium and shaking the mixture in a rotatory shaker (100 rev/min) for 60 min. The extracts were then squeezed through a cloth and clarified by centrifugation at $10{,}000\times g$ for 5 min. The supernatants were assayed for lipase activity.

Lipase production

Lipase production by strain 41 of *C. gloesporioides* was carried out at 30 °C in 250-ml shake flasks with 50 ml of the medium using a rotatory shaker (150 rev/min) for 96 h. The flasks were inoculated with 1 ml of a spore suspension or 5 ml of a vegetative inoculum (see explanation in Table 2 footnote). The following parameters were determined: biomass, lipolytic activity, yield, productivity and specific growth rate.

Assay of lipolytic activity

Plate assay

The culture filtrates (from SLF and SSF) were first submitted to diffusion into agar gel that contained (per litre of 0.1 M Tris–HCl, pH 8.9): 15.0 g bacteriological agar, 80.0 mg Victoria blue B, 6.0 ml tributyrin (ATV) or 15.0 ml of a emulsion prepared by dispersion of 25.0 ml of olive oil in 100.0 ml of 2% (v/v) polyvinyl alcohol solution (AEPV). After agar solidification, wells of 7.0 mm diameter were prepared and 50 μl samples of the filtrate were added to each well in triplicate and incubated at 30 °C for 18 h. Lipolytic activity was identified by a deep blue color halo and the diameter was measured. The use of olive oil and tributyrin follows Jensen (1983) and Smith & Hass (1992) who recomend the use of two different substrates for screening of lipase-producing microorganisms.

Titrimetric assay

Lipolytic activity was measured according to Watanabe *et al.* (1977). The reaction mixture contained: 2.5 ml of 0.1 M Tris–HCl buffer pH 8.0; 2.5 ml of 25% (v/v) olive oil emulsion in 2% (v/v) polyvinyl alcohol solution; 1.0 ml of the enzyme filtrate. The olive oil emulsion was formed using an ultra-turrax dispersor T25 (Ika, Wilmington, NC, USA). The reaction mixture was incubated at 30 °C on a reciprocal shaker (Fanem, SP, Brazil) at 45 cycles/min. After 10 min, 10 ml of an acetone/ethanol (1:1, v/v) solution was added to stop the reaction and the free fatty acid released was determined by titration with 0.05 N NaOH using thymolphthalein as indicator. Culture filtrate boiled for 10 min was used as control. One unit of lipase was defined as the amount of enzyme required to release one μmol of fatty acid per minute under the specified conditions.

Biomass dry weight

The mycelia were separated by filtration, washed twice with a butanol–ethanol mixture (1:1, v/v) and then

washed with deionized water. The biomass was dried to constant weight at 80 °C.

Hydrolysis of lipidic substrates by C. gloesporioides crude lipase

The hydrolysis of various natural oils, lard and tributyrin by the fermentation broth (crude enzyme) was carried out using the same procedure for titrimetric assay, but adding double the volume of the reaction mixture and incubated for 30 min.

Results and discussion

The oleaginous seed shavings from baru were essential to isolate fungi since they create the necessary enrichment conditions for fungal isolation. The fungal mycelia attached to the oleaginous seed shavings added to the soil samples were noted after three days and 59 fungus strains were isolated. From these 59 strains, lipolytic halos around colonies were observed in 25 of them (21 with R/r ratio equal to or higher than 1.2). The opacity of the agar plate medium developed in the present work, in which the bile salts present partial solubility (pH 7.0), created the conditions for obtaining isolated colonies of adequate size with better diffusion and lipase action.

Table 1 shows the lipolytic activity results from 11 alkaline lipase-producing fungi cultured in submerged and SSF conditions. In both conditions strain 41, identified as *C. gloesporioides*, showed the highest lipolytic activity, therefore it was chosen for further studies on lipase production.

The fermentation parameters related to lipase production by the *C. gloesporioides* cultivated using spores and vegetative inocula are shown in Table 2. It may be noted that lipase activity is dependent on fermentation time. However, the biomass production decreased from 72 to 96 h with lipase activity practically the same. As a consequence the yield was higher at 96 h of fermentation.

Table 1. Screening of alkaline lipase producing fungi growing under conditions of submerged liquid fermentation and solid-state fermentation.

Strain number	Submerged fermentation[a]			Solid-state fermentation[b]		
	Halo diameter in agar gel plates (mm)[c]		Lipolytic activity (U/ml)[d]	Halo diameter in agar gel plates (mm)		Lipolytic activity (U/ml)[d]
	Tributirin	Olive oil emulsion		Tributirin	Olive oil emulsion	
41	14.0	6.0	18.8	7.0	2.0	3.5
42	12.5	5.0	14.0	1.0	1.0	2.5
44	6.0	0.0	1.0	4.0	1.0	1.5
45	7.0	2.0	11.0	0.0	0.0	Nr
47	0.0	5.0	3.0	2.0	1.0	2.0
58	3.0	6.0	5.0	1.0	1.0	1.0
62	2.0	6.0	3.5	0.0	0.0	Nr
66	12.0	4.0	4.0	0.0	0.0	Nr
67	1.0	2.0	2.0	3.0	1.0	1.0
68	0.0	3.0	0.0	0.0	1.0	1.5
73	9.0	8.0	7.0	1.0	1.0	4.0

[a]72 h of incubation; [b]96 h of incubation; [c]50 μl of each broth per well and incubation at 30 °C for 18 h; [d]The activity was measured by titrimetric assay, in alkaline conditions, using olive oil emulsion as substrate; means of triplicate runs, two repetitions. Strains that produced at least 1.5 U/ml on solid-state fermentation or 2.5 U/ml on submerged fermentation were considered good producers.
Nr – not realised.

Table 2. Fermentation parameters of a batch culture of *C. gloesporioides* growing in shaken liquid medium in Erlenmeyer flasks[a].

Inoculum	Fermentation time (h)	Final pH	Lipase[b] (U/l)	Biomass (mg/ml)	Yield $Y_{p/x}$ (U/mg)	Productivity (U/l/h)	Specific growth μ (h^{-1})
Spores	24	6.7	3500	9.2	0.4	145.8	0.09
	48	7.4	18,800	14.9	1.2	391.7	0.06
	72	8.2	25,700	12.5	2.1	356.9	0.03
	96	8.0	24,300	8.1	3.0	253.1	0.02
Vegetative[c]	24	6.2	18,300	11.1	1.6	762.5	0.10
	48	7.4	27,700	13.0	2.1	577.1	0.05
	72	8.1	26,800	11.0	2.5	372.2	0.03
	96	8.4	27,400	10.3	2.7	285.4	0.02

[a]The culture medium contained (g/l): 20.0 peptone; 8.0 ml olive oil; 0.6 $MgSO_4 \cdot 7H_2O$; 1.0 KH_2PO_4; 1.0 $NaNO_3$; pH 6.5. The results are means of four replicates repeated twice; [b]The lipolytic activity was measured by titrimetric assay. The reaction was carried out in 0.1 M Tris–HCl pH 8.9 buffer; [c]The mycelial mass obtained after 24 h of incubation in the same culture medium for fermentation was used as vegetative inoculum.

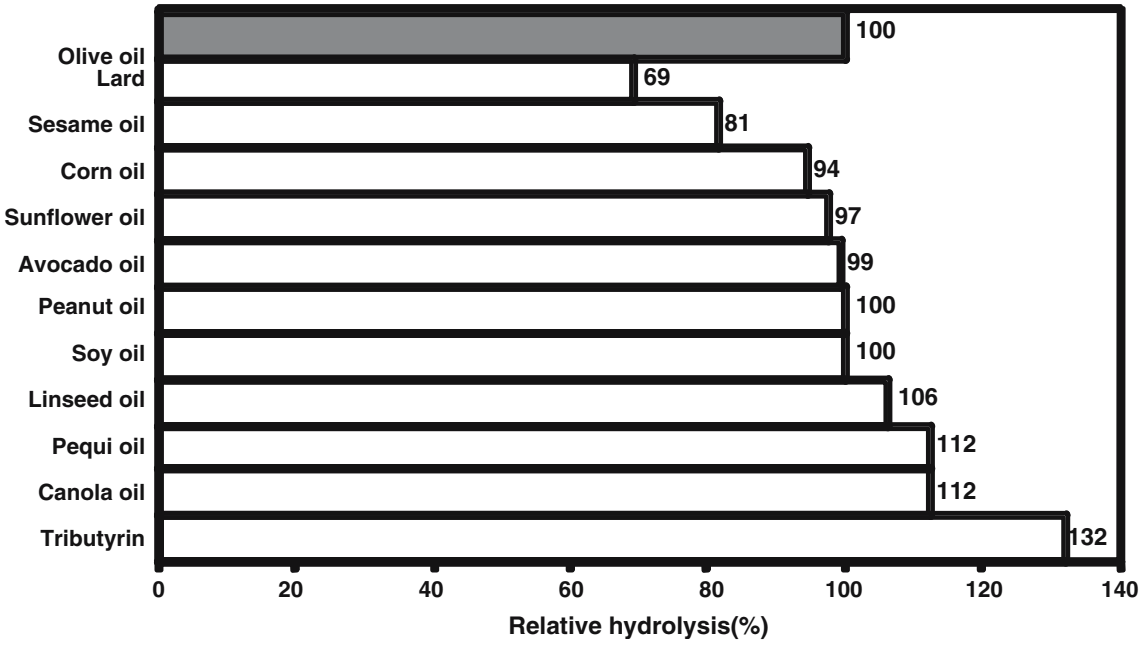

Figure 1. Relative hydrolysis of different lipidic substrate by *C. gloesporioides* crude lipase. The results are mean values of duplicate assays carried out with 1 ml of fermentation broth containing 16 U/ml of lipase. Pequi oil: *Caryocar brasiliense* Camb fruit pulp oil.

This could be explained by the lost biomass, without affecting the production of lipase if it is assumed that the remaining mycelia are more efficient in producing the enzyme. This explanation does not mean that the speed of enzyme production should be kept the same, since the time spent for enzyme production. Therefore the decreased productivity was around 29% and the time difference was 25%, which can explain why the only 5% lipase activity was lost. This reasoning does not contradict the specific growth rate that was shown to decrease with time. However, using vegetative inoculum the highest activity was reached at 48 h without practically losing biomass and preserving the yield. The productivity decreased from 48 to 96 h incubation time, and the above explanation fits to this case as well the specific growth rate. The highest lipase activity obtained with both inocula is comparable of that (26,000 U/l) of Tan *et al.* (2004) using peptone as organic N source for *Penicillium camemberti.*

Figure 1 shows the hydrolytic activity of the *C. gloesporioides* crude lipase on several oils, lard, and tributyrin. Taking olive oil as a 100%, the lowest value was found for lard and the highest for tributyrin. It can be concluded therefore that the isolated lipase-producing fungus is effective for hydrolysing a broad spectrum of substrates, which agrees with other researcher (Tan *et al*. 2004).

Conclusions

Lipase-producing fungi were successfully isolated from soil. Enrichment of the soil with oleaginous seed shavings (baru seed) was essential for fungi isolation. Bile salts and a dispersed form of olive oil were essential for isolation and evaluation of lipolytic activity.

A strain of *C. gloesporioides* was the best in producing alkaline lipase, which was able to hydrolyse a broad range of oils and lard.

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