academicJournals

Vol. 15(18), pp. 759-767, 4 May, 2016
DOI: 10.5897/AJB2015.14666
Article Number: 0DFE43A58325
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Lipase-producing fungi for potential wastewater treatment and bioenergy production

Celson Rodrigues[1]*, Sérvio Túlio Alves Cassini[1], Paulo Wagner Pereira Antunes[1], Laura Marina Pinotti[2], Regina de Pinho Keller[1] and Ricardo Franci Gonçalves[1]

[1]Technological Center, Department of Environmental Engineering, Federal University of Espírito Santo-UFES, Campus Goiabeiras, Avenue Fernando Ferrari 514, CEP 29075-910, Vitória - ES, Brazil.
[2]Department of Engineering and Technology, University Center North of Espírito Santo, Federal University of Espírito Santo - UFES, Campus of São Mateus, Highway BR 101 North Km 60, CEP 29932-540, São Mateus - ES, Brazil.

Received 23 April, 2015; Accepted 8 April, 2016

The use of fungal biomass as a lipase biocatalyst represents an attractive approach for the treatments of oil wastewater as well as for the production of biodiesel from oil and residual grease, due to its greater stability, possibility of reuse, and lower cost. In this work, 20 filamentous fungi were isolated from the grease trap scum of a restaurant at the Federal University of Espírito Santo, Brazil. The fungi included those belonging to the genera *Aspergillus*, *Beauveria*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium*, *Rhizomucor*, and *Verticillium*. Fungal lipase activity and biomass production were quantified. Lipase activity ranged from 0.13 U mg^{-1} protein of *Rhizomucor* sp. ECGF18 to 18.06 U mg^{-1} protein of *Penicillium* sp. ECGF02, and the biomass production ranged from 7.61 mg mL^{-1} for *Cladosporium* sp. ECGF19 to 12.68 mg mL^{-1} for *Rhizomucor* sp. ECGF18. In the sequence, *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, were previously select and, further evaluated in solid-state fermentation. Results confirmed the high extracellular lipase-activity of *Penicillium* sp. ECG02 and the high intracellular lipase activity of *Rhizomucor* sp. ECG18. *Rhizomucor* sp. ECG18 showed potential for use in future research, in the form of whole-cell lipases, wastewater treatment, and as a biocatalyst in the production of biodiesel from oil residues.

Key words: Lipase-producing fungi, wastewater treatment, bioenergy.

INTRODUCTION

Lipases (such as triacylglycerol acyl hydrolase, E.C. 3.1.1.3) work at the aqueous-organic interface where thay catalyse hhydrolytic reactions of triglycerides as well as esterification, transesterification, or interesterification in low-water environments (Nagarajan, 2012). New applied lipase technology, including using it in genetic engineering methods and diverse applications in the food, chemical, and pharmaceutical industry means the current global market for lipase is expanding (Salihu et al., 2012). In addition, there is great interest in lipase use for the treatment of high lipid-content effluents for the production of bioenergy (Cammarota and Freire, 2006;

*Corresponding author. E-mail: celsonrodrigues@yahoo.com.br. Tel: +55 27 99984-5207.

Alberton et al., 2010; Singh and Mukhopadhyay, 2012; Hama and Kondo, 2013; Guldhe et al., 2015).

An increasing number of publications and patent applications reflect a worldwide interest in the use of lipases for the synthesis of biodiesel. An extensive review was published by Christopher et al. (2014) covering many aspects of these studies, including the nature and properties of the enzyme catalyst. However, the use of commercial lipases for the transesterification of biodiesel, as well as for the hydrolysis of oil effluents, is not currently competitive with other processes. Although advantageous under the environmental point of view, at the moment, the main drawback is the production cost of these enzymes (Guldhe et al., 2015).

Commercial lipases are produced, primarily, by microorganisms such bacteria, yeasts, and filamentous fungi, due to their short generation time, ease of genetic manipulation, easily increased scalability, purification, specificity, and stability (Nagarajan, 2012).

Filamentous fungi are preferred sources of lipase production because they are easily extracted from fermentation processes, are considered safe and easy to hand, and can potentially be used as whole cells (Alberton et al., 2010; Singh and Mukhopadhyay, 2012; Andrade et al., 2014). Filamentous fungi have proven to be the most convenient biosystem for industrial applications because of their strong cell walls. *Rhizopus oryzae*, *Rhizopus chinensis*, *Aspergillus niger*, and *Mucor circinelloides* have all been studied as whole cell biocatalysts by several research groups (Fukuda et al., 2009; Andrade et al., 2014).

Filamentous fungi show favorable characteristics for the growth and lipase production in solid-state fermentation systems (Singh and Mukhopadhyay, 2012). Solid substrates are effective for the growth of filamentous fungi, due to the environmental similarity with their natural habitat, which result in higher enzyme production, lower demand for water and, energy, and easy aeration. This is a low-cost alternative, can potentially be used to clean modern oil residues or can be used by the agroindustry for the production of enzymes (Colen et al., 2006; Griebeler et al., 2011). In addition, they may be used in the form of whole-cells lipase, directly used in solid fermentation conditions (Fukuda et al., 2009; Li and Zong, 2010; Athalye et al., 2013; Talukder et al., 2013; Ferrarezi et al., 2014; Guldhe et al., 2015). The advantage of such an approach that it does not require an enzyme purification step, and the whole-cells and solid substrates can act as immobilizing agents for enzyme support (Kumar and Kanwar, 2012; Salihu et al., 2012; Farias et al., 2015; Carvalho et al. 2015a; Carvalho et al., 2015b).

Fungi are capable of producing several enzymes for their survival within a wide range of substrates. In view of the interesting applications of lipases, it could be of tremendous value to screen and identify fungi with the highest potential for the biodegradation of oils and fats.

There are two basic types of fungal lipases, the extracellular lipase, which is secreted into the liquid medium, and the intracellular lipase, which is inside the cell or linked to its membrane or cell wall, intracellular lipase, also called whole-cell lipase, is less expensive to use in full-scale. Therefore, the isolation and selection of new fungal strains with high levels of intracellular lipolytic activity for use as whole-cell lipase in wastewater treatment and biodiesel production is of great interest (Fukuda et al., 2009; Carvalho et al., 2015a).

Although, different screening strategies have been proposed for the determination of lipase extracellular activity, assays using agar plates are highly recommended, because it is an easier method with lower cost. However, agar plates are often insufficient or clear for quantifying intracellular lipase activity of various strains, and the process results in losses to the isolation and selection processes; therefore, these must be complemented with other methods (Gopinath et al., 2014).

The aim of this work was to isolate filamentous fungi from grease trap scum and to select favorable isolates based on their high lipase production and fungal biomass, production while in submerged fermentation. This work also assessed, the potential characteristics of the selected isolates for use in future research, in the form of whole-cell lipases, wastewater treatment, and biocatalysis of biodiesel from oil residues found in environmental sanitation.

MATERIALS AND METHODS

The aim of this study was to isolate and select efficient lipase-producing fungi for potential whole-cell lipase use in the hydrolysis of oily wastewater and synthesis of fatty acid esters from oil residues. Figure 1 shows the experimental steps of the work.

We proposed the concomitant use of submerged fermentation methods and solid-state fermentation to produce strains of filamentous fungi with high potential of lipase intracellular. Table 1 shows shows the main methods used, in accordance with the purposes of the research.

Isolation and identification of lipase-producing fungi

Collection and characterization of grease trap scum

The source of filamentous fungi lipase producers was scum that had for a period of 3 months, accumulated on the surface of, a grease trap in a restaurant at the Federal University of Espírito Santo, Brazil. Five 200 mL samples were collected from different surface points of the trap and stored in previously sterilized 500 mL Erlenmeyer flasks. The scum was quantified using the following parameters: volatile solids (VS), chemical oxygen demand (COD), content of oils and greases (O&G), and pH, in accordance with the "Standard Methods for the Examination of Water and Wastewater" (APHA, 2005). The values of the parameters were VS (mg L^{-1}) = 922110.45, COD (mg L^{-1}) = 1429001.83, O&G (mg L^{-1}) = 921936.67, and pH = 6.08, while those relating to ECG-RU were VS (mg L^{-1}) = 756472.75, COD (mg L^{-1}) = 922991.29, O&G (mg L^{-1}) = 595478.25, and pH = 5.60.

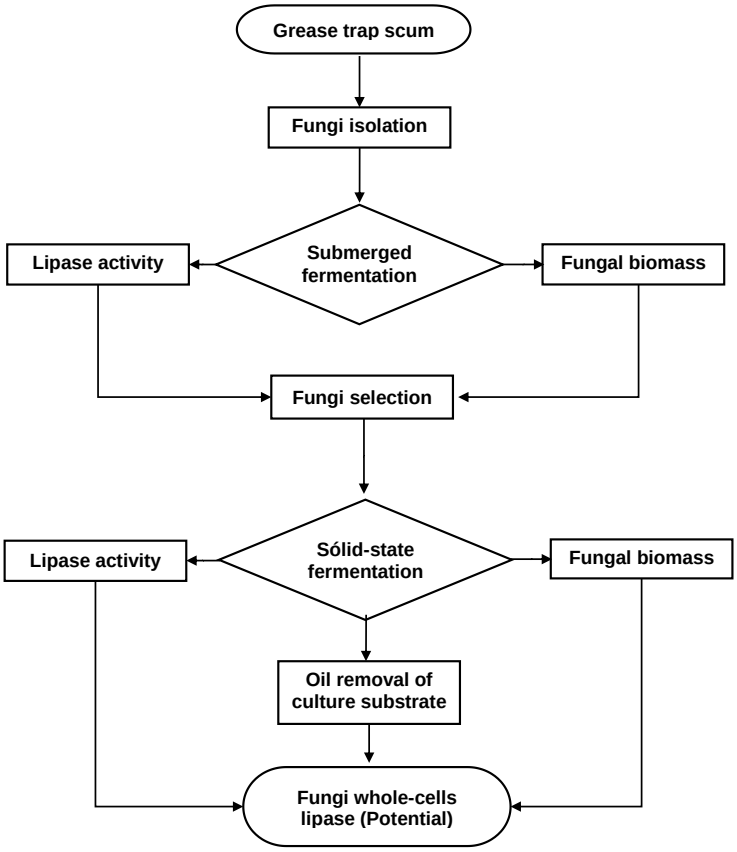

Figure 1. A flowchart of the main steps of this work, for the isolation and selection of lipase-producing fungi for potential whole-cell lipase use.

Table 1. Main parameters, units, methods and correspondent references in this work, for the isolation and selection of lipase-producing fungi for potential whole-cell lipase use.

Parameter	Unit	Method	References
pH	-	Potentiometric	APHA (2005)
COD	mg L^{-1}	Closed reflux colorimetric	APHA (2005)
O&G/SO removal	mg L^{-1}	Soxhlet extraction	APHA (2005)
Volatile solids	mg L^{-1}	Gravimetric	APHA (2005)
Fungal genus	-	Microculture method	Barnett and Hunter (1998)
Protein	mg	Bradford method	Bradford (1976)
Lipase activity	U mg^{-1}	Colorimetric	Winkler and Stuckmann (1979); Rowe and Howard (2002)
Ergosterol	µg	HPLC	Montgomery et al. (2000)
Fungal biomass	mg L^{-1}	Gravimetric	Colen et al. (2006)
	mg g^{-1}	HPLC	Montgomery et al. (2000)

pH, hydrogen ionic potential; COD, chemical oxygen demand; O&G, oils and greases; HPLC, high performance liquid chromatography; APHA, American Public Health Association.

Isolation of lipase-producing fungi

For the proliferation of microorganisms, 40 mL of each each sample was poured into a 250 mL Erlenmeyer, along with 50 mL of minimum mineral culture medium (MM) and 10 mL of soybean oil (Liza), emulsified with 0.1% Tween 80 (MMSO). The MM consisted of (g L^{-1}): $(NH_4)_2 SO_4$, 5.0 g; KH_2PO_4, 0.9 g; NaCl, 1.0 g; $MgSO_4$. $7H_2O$, 0.3; Na_2HPO_4, 6.2 g; soybean oil 10% and 1 mL micronutrients solution ($FeCl_3$. $6H_2O$, 2000 mg; $ZnCl_2$, 50 mg; $CuCl_2$. $2H_2O$, 30 mg; $MnCl_2$. $2H_2O$, 500 mg; $(NH_4)_6.Mo_7O_{24}$.$4H_2O$,

50 mg; $AlCl_3$, 50 mg; $CoCl_3$. $6H_2O$, 2000 mg).

The flasks with enriched samples stirred at 120 rpm in an orbital shaker (Benchtop Shaker Incubator NT 712, Novatécnica) at 30°C for 96 h. Then, 1 mL aliquots of enriched medium were mixed with MM plus 10% commercial soybean oil (Liza) and 0.1% Tween 80 (MMSO), and 1.7% agar, and were poured in previously autoclaved 90 mm diameter Petri dishes supplemented with 50 ppm of streptomycin and 50 ppm chloramphenicol and incubated for 120 h (DBO-Biotec BT424) at 28°C. After the incubation period, colonies of the filamentous fungi were successively transfered to new Petri dishes, and all strains obtained were successively transferred to new Petri dishes, and all strains obtained were transferred to culture tubes containing BDA (potato-dextrose-agar) and stored at 5°C.

Identification of lipase-producing fungi

The previously isolated fungi were identified by microcultivation technique to distinguish which samples were promising for lipase production. The fungi samples were individually inoculated on a slice of agar laid on a sterile glass slide and covered by a sterile coverslip. The slide was then placed in a Petri dish, and the setup was incubated for 96 h at 25°C. Then, the coverslip with the adhered hyphae was removed and stained with a cotton blue dye. The same procedure was adopted to examine spores and hyphae bound to the slide. The identification of different fungi genera was based on the macroscopic morphology of the colonies and the fructification structures of the strains, following the key of investigation of genera proposed by Barnett and Hunter (1998).

Selection of lipase-producing fungi in submerged fermentation

The filamentous fungi, obtained in the previous step, were evaluated for lipase activity and fungal biomass production on submerged fermentation. The submerged fermentation was performed with a completely randomized design with 20 fungi strains and three replications, and the means were compared by the Tukey test at 5% probability. This step of the work permitted the selection of two fungal isolates for subsequent work step, one of them by highest value displayed of the lipase activity and the other by the highest value of fungal biomass production.

Lipase activity determination

The experiments were conducted using 125 mL Erlenmeyer flasks, containing 50 mL MM (sterile) with an additional 10% soybean oil and 0.1% Tween 80 (v/v) as sole carbon sources. In each flask, the medium was inoculated with 1 mL of an aqueous suspension of fungus at a concentration of 10^7 spores mL^{-1} followed by an incubation of 120 h on an orbital shaker at 30°C and 150 rpm. After incubation, the content of each Erlenmeyer flask was vacuum-filtered and three 1 mL aliquots of liquid fraction were transferred to sterile Eppendorf tubes and centrifuged at 15000 rpm for 20 min at 4°C (Hettich Mikro 22R, Andreas Hettich GmbH Co. KG &, D-78532 Tuttlingen). The resulting supernatant was evaluated for lipase activity using p-nitrophenyl palmitate (pNPP, Sigma) as a substrate, as stated by Winkler and Stuckmann (1979) and Rowe and Howard (2002).

Samples of 0.1 mL of the supernatant were mixed with 0.9 mL substrate solution of the following composition: 3 mg of pNPP dissolved in 1 mL propanol diluted in 9 mL of tris-HCl pH 8.0 and containing 40 mg of Triton X-100 and 10 mg of arabic gum. After 30 min of incubation at 37°C, absorbance at 410 nm was measured by spectrophotometry (Ultrospec 1000, Pharmacia Biotech), against a control without enzyme. A lipase activity unit (U) was defined as the amount of enzyme to release one mol of p-nitrophenol min^{-1}. The protein measurements were conducted by the method of Bradford (Bradford, 1976).

Fungal biomass determination

For quantification by gravimetry, the fungal biomass was separated from the supernatant by filtration with Whatman filter paper nº 1, followed by acetone cleaning and successive washes with milli-Q water. The biomass was then transferred to filter papers previously weighed and dried at 80°C, and then the biomass was dried until the weight remained constant (meaning that all the water evaporated). The dry biomass was calculated by determining the difference between the measurements at the beginning and end of the previous step, with and without the presence of biomasses as studied by Colen et al. (2006).

Lipase production in solid-state fermentation

Fungal biomass production and removal of the soybean oil of cultive substrate by *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 (selected in the previous step, respectively, by lipase activity and fungal biomass values), in intervals of 24 h, and ≥ 120 h, in solid-state fermentation conditions were assessed for lipase activity. The cultive substrate used was prepared from at mixture of 50% sand and 50% vermiculite (v/v) previously washed and completely dried at 100°C. The mixture (40 g) was added to 250 mL Erlenmeyer flasks capped with hydrophobic cotton. Mineral medium minimum (MM) was added to the sand-vermiculite mixture in a sufficient volume to achieve 50% of field capacity. This mixture was autoclaved at 121°C for 20 min, the pH was adjusted to 4.5 with H_2SO_4 1.5 mol L^{-1}, and 10% soybean oil (Liza) was added as the exclusive carbon source and inducer for lipase production. Each Erlenmeyer was inoculated with 1 mL of suspension of 10^7 spores mL^{-1} and incubated at 30°C for 120 h. Three replications were used for each parameter evaluated and for each assessment time as follows: 0 (initial time), 24, 48, 72, 96, and 120 h of incubation.

Lipase activity determination

The method for the lipase extraction of fermented solids was as the same as that used by Alberton et al. (2010), except agitation time, which was reduced from 1 h to 30 min. Each fermented solid (10 g) was transferred to a 250 mL Erlenmeyer, 100 mL of a 2% NaCl solution was added, and then the mixture was agitated on a rotary shaker for 30 min at 200 rpm and 30°C. The resulting suspension was filtered through cheesecloth, and the excess liquid was manually squeezed out. The extract was centrifuged for 10 min at 12500 rpm. The quantification of lipase activity proceeded as described in the previous method.

Oil removal of culture substrate

Substrate soybean oil content was quantified using the method of continuous extraction apparatus Soxhlet type, according to the "Standard Methods for the Examination of Water and Wastewater" (APHA, 2005), which is based on the gravimetric quantification of material extracted with hexane. One (1) g samples analysed were collected at 0 (date of installation of the experiment), 24, 48, 72, 96 and 120 h, and on occasions, were modified with HCl to reach a pH of 2.0. The samples were stored in a freezer at - 25°C, until used in analyses, when the sample concentrations were expressed in mg g^{-1} substrate dry weight (dried at 80°C).

Fungal biomass determination

Fungal biomass was indirectly quantified, through the determination from ergosterol content, via high performance liquid chromatography (HPLC), as observed in Montgomery et al. (2000), with some modifications. For the extraction of sample ergosterol, 1 g of substrate was put in a test tube, 5 mL of methanol was added and the sample was vortexed for 1 min. After resting for 10 min, the supernatant was transferred to a 1.5 mL Eppendorf and centrifuged for 10 min at 10000 rpm at 22°C. Then, the supernatants were filtered using 0.22 µ filter paper and were stored at - 25°C for later use.

The binary solvent system HPLC equipment (Shimadzu, model LC-20 AD/T, Japan) had a 100 × 2.1 mm C18 column, attached to a filter with a porosity of 0.5 × 0.004 µm. The mobile phase T was isocractic with methanol (HPLC grade) and water (Milli-Q) 95:5 (v/v), filtered and degassed for 15 min, with a flow rate of 0.5 mL min^{-1}. The race lasted approximately 7 min and was identified a peak integration of ergosterol through the comparison of retention times of the standard and the sample, was verified by the purity of the absorbance spectra obtained at the beginning, peak, and end of peak, at 282 nm. For the construction of the linear standard curve (outside standardization), the point at which it crossed the origin and covered the range of concentration of the samples, ergosterol was used with the minimum purity of 95% and 7 points for reading, whose concentrations ranged from 5.0 to 500 ug mL^{-1}.

To measure the content of ergosterol in a pure biomass sample of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, the fungi were grown in 250 mL Erlenmeyer flasks with MMOS, for 30 days in a 150 rpm shaker at 30°C. At 10, 20 and 30 days, their biomass was collected through filtration, followed by acetone cleaning and successive washes with milli-Q water. These samples were then transferred to filter papers previously weighed and dried at 80°C, and then the biomass was dried until the wight remained constant (meaning that all the water evaporated). The dry biomass was calculated by determining the difference between the measurements at the beginning and end of the previous steps, with and without the presence of biomasses as studied by Colen et al. (2006).

Samples of 1 g of dry biomass were frozen using liquid nitrogen, ground in a mortar, mixed with 5 mL methanol, and then the material was collected and transferred to culture tubes. We then followed he previously decribed preparation of material for chromatographic analysis. Based on the reasons, ergosterol-dry biomass obtained for both isolated in the collection dates, arrived to the fungal biomass values. After conversion, the average (n = 3), values of ergosterol content in mg g^{-1} fungal biomass in *Penicillium* sp. ECGF02 were 1.28 (10 days), 1.57 (20 days), and 1.49 (30 days), and in *Rhizomucor* sp. ECGF18 they were 1.55 (10 days), 1.52 (20 days), and 1.54 (30 days). The finals results of fungal biomass were expressed as mg g^{-1} cultive substrate that had been subjected to a temperature of 80°C until they reached a constant weight.

Statistical analysis

The analysis of variance followed by the Tukey test was applied for the statistical analyses of the obtained data. The data were analyzed with the aid of Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA). All analyses were performed considering a level of 95% confidence ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation and identification of lipase-producing fungi

In this work, it was possible to obtain 20 filamentous fungi isolates from grease trap scum, belonging to the genera *Aspergillus*, *Beauveria*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium*, *Rhizomucor* and *Verticillium*, after successively growing them on MMSO plus bacterial antibiotics. Their taxonomic identification was based on macroscopic structures and as the colonies grew in BDA on Petri dishes, but was also based on microscopic structures and comparisons to specific literature descriptions (Barnett and Hunter, 1998). The 20 isolates were stored in BDA at 4°C, and allowed to multiply until it was time to evaluate their lipase activity and fungal biomass production, which occurred after incubation for 120 h in MMSO at 30°C, while shaking at 150 rpm, on submerged fermentation (Table 2).

Selection of lipase-producing fungi in submerged fermentation

The enzymatic activity and biomass production of the 20 previously filamentous fungi isolated and placed in submerdeg fermentation can be seen in Table 2. The results indicate that the fungi significantly influence ($p \leq 0.05$) the enzymatic degradation reaction of the p-nitrophenyl palmitate.

The lipase activity evaluated on supernatant (extracellular activity) ranged from 0.13 ± 0.03 U mg^{-1} protein of *Rhizomucor* sp. ECGF18 to 18.06 ± 0.36 U mg^{-1} protein of *Penicillium* sp. ECGF02. The dry weight biomass ranged from 7.82 ± 0.13 mg mL^{-1} of culture liquid medium for *Cladosporium* sp. ECGF19 to 12.68 ± 0.15 mg mL^{-1} culture liquid medium for *Rhizomucor* sp. ECGF18. Overall, statistically significant differences for lipase activity and fungal biomass were observed between the values obtained for various isolates belonging to different taxonomic genera and between isolates of the same genus (Table 2).

Using a similar methodology to quantify but a longer incubation time, Baron et al. (2005) observed a lipase activity of 11.82 ± 1.35 U mg^{-1} protein for *P. coryophilum* IOC 4211, after 144 h of incubation at 29°C and 120 rpm. Using a distinct method of quantification, Carvalho et al. (2005) obtained a lipase activity of 13.0 U mL^{-1} and a biomass of 14.2 mg mL^{-1} for *P.restrictum*, and a lipase activity of 10.5 U mL^{-1} and biomass of 6.56 mg mL^{-1} for *P. solitum*.

The following species of the *Penicillium* genus were previously reported as good producers of extracellular lipase activity: *P. citrinum, P. cyclopium, P. simplicissimum, P. caseicolum, P. restrictum, P. expansum, P. corylophilum, P. chrysogenum, P. roqueforti, P. camembertii, P. crustosum* and *P. abeanum*, among others (Baron et al., 2005; Carvalho et al., 2005; Li and Zong, 2010; Rigo et al., 2012). However, it is necessary to consider that the experimental results of lipase activity and growth of biomass will likely be different due to natural, diverse, baseline activities

Table 2. Taxonomic classification, lipase activity (LA) and fungal biomass production (FB) on mineral minimal culture media and 10% soybean oil (MMSO) in submerged fermentation (SF).

Isolate	Taxonomic classification	LA* (U mg^{-1} protein)	FB* (mg mL^{-1} MMSO)
ECGF02	*Penicillium*	18.06 ± 0.36^{a}	10.19 ± 0.13^{cdef}
ECGF09	*Geotrichum*	14.25 ± 0.31^{b}	9.23 ± 0.18^{h}
ECGF01	*Beauveria*	9.13 ± 0.23^{c}	9.62 ± 0.14^{g}
ECGF19	*Cladosporium*	6.10 ± 0.22^{d}	7.61 ± 0.15^{j}
ECGF15	*Cladosporium*	4.85 ± 0.16^{e}	7.82 ± 0.13^{j}
ECGF03	*Colletotrichum*	3.79 ± 0.07^{f}	9.71 ± 0.16^{g}
ECGF20	*Penicillium*	2.74 ± 0.17^{g}	10.12 ± 0.16^{ef}
ECGF13	*Penicillium*	2.56 ± 0.05^{g}	8.77 ± 0.12^{i}
ECGF12	*Colletotrichum*	2.49 ± 0.25^{g}	9.05 ± 0.18^{hi}
ECGF16	*Colletotrichum*	2.36 ± 0.39^{g}	9.02 ± 0.13^{hi}
ECGF11	*Aspergillus*	1.53 ± 0.25^{h}	10.45 ± 0.11^{cde}
ECGF14	*Botrytis*	1.44 ± 0.11^{h}	11.89 ± 0.10^{b}
ECGF17	*Geotrichum*	0.51 ± 0.10^{i}	9.07 ± 0.15^{hi}
ECGF05	*Beauveria*	0.47 ± 0.01^{i}	10.74 ± 0.19^{c}
ECGF04	*Fusarium*	0.39 ± 0.04^{i}	10.27 ± 0.12^{cde}
ECGF10	*Fusarium*	0.28 ± 0.08^{i}	10.25 ± 0.80^{cde}
ECGF06	*Rhizomucor*	0.25 ± 0.01^{i}	9.91 ± 0.12^{fg}
ECGF07	*Verticillium*	0.18 ± 0.01^{i}	10.50 ± 0.15^{cd}
ECGF08	*Aspergillus*	0.18 ± 0.05^{i}	9.87 ± 0.21f^{g}
ECGF18	*Rhizomucor*	0.13 ± 0.03^{i}	12.68 ± 0.15^{a}

* = Means (n = 3) followed by same letters, do not differ statistically by Tukey test (p = 0.05).

between species and within the same species, but there is more likely an influence of factors such as the composition of the culture media, temperature, pH, agitation and forms of quantification, as reviewed by Turky (2013) and reported by Talukder et al. (2013) and Bueno et al. (2014).

The genus *Rhizomucor*, mainly represented by the species *R. mihei*, stands out as a top producer of lipase, and as such, it is currently one of the most marketed extracellular lipase producers; it was the first for whose lipase structure was reported, having its activation interface well elucidated and resulting in its basic use for enzyme modeling studies. An extensive review was recently published stating the main uses of LRM (*Rhizomucor mihei* lipase) and featuring some of the most relevant aspects in its use for the processing of oils and fats, including its use in the hydrolysis of glycerides, transesterification, esterification, acidolyses, and intersterification (Rodriguez and Fernando-Lafuente, 2010).

In this results suggest that the more effective lipase activity of *Penicillium* sp. ECGF02 (18.06 ± 0.36 U mg^{-1} protein) was mainly intercellular. In contrast, the value of the extracellular lipase activity of *Rhizomucor* sp. ECGF18 (0.13 ± 0.03 U mg^{-1} protein) did not express its lipolytic potential, because this isolate showed greater biomass production (12.68 ± 0.15 mg mL^{-1}) in the liquid medium (Table 2). For *Rhizomucor* sp. ECGF18, the results suggest that the greater lipolytic potential was intracellular (membrane-bound or cell-wall lipase). Thus, *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 were selected, in solid-state fermentation, for evaluation of lipase activity, fungal biomass production, and removal of soybean oil in culture substrate, to confirm their lipolytic profile and their potential use as whole-cell lipases in solid culture medium.

Lipase production in solid-state fermentation

Figure 2 shows the mean results from solid-state fermentation (SSF) for *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, for lipase activity, fungal biomass production, and soybean oil removal of culture substrate (120 h growth).

Culture substrate soybean oil removal was quantified through the widely used method in the area of environmental sanitation for the determination of the content of oil and grease in wastewater, solid waste, and sludge (APHA, 2005). This methodological tool was used to indirectly estimate the hydrolytic capacity of two fungal isolates evaluated in solid-state fermentation. The activity and the fungal biomass (120 h) for *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 were better than in submerged fermentation. These results are similar to those reported by Alberton et al. (2010) and Rigo et al.

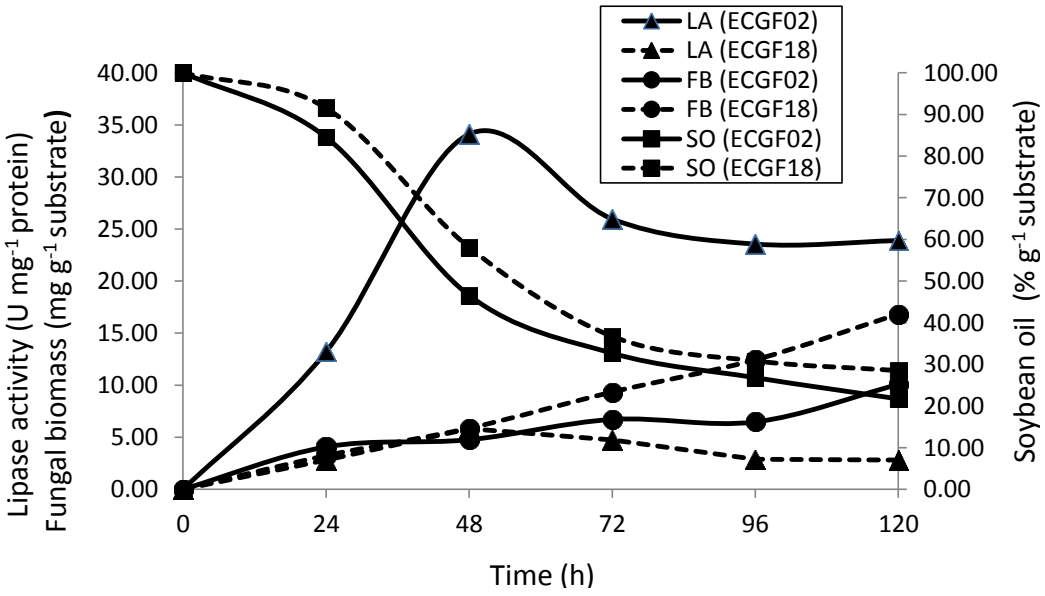

Figure 2. Lipase activity (LA), fungal biomass (FB) and soybean oil content (SO) in the culture substrate of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 in solid-state fermentation.

Table 3. Correlation and linear regression coefficients alongside values obtained from lipase activity (LA), fungal biomass (FB), and reduction of soybean oil (rOS) of substrate cultivation of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 in solid-state fermentation (FES).

Interactions	Correlations and linear regression coefficients	
	ECGF02	**ECGF18**
LA × FB	Correlation = 0.1313 Y = 0.04109X + 5.4279 R^2 = 0.01725 p Value = 0.64083	Correlation = 0.3351 Y = - 1.27831X + 14.4042 R^2 = 0.11228 p Value = 0.22213
LA × rSO	Correlation = 0.542807 Y = 1.85664X + 12.6804 R^2 = 0.29464 p Value = 0.03654	Correlation = 0.08288 Y = - 1.6991X + 60.241 R^2 = 0.00687 p Value = 0.76897
FB × rSO	Correlation = 0.81253 Y = 8.8829X + 0.48021 R^2 = 0.66021 p Value = 0.00023	Correlation = 0.901646 Y = 4.84411X + 7.5517 R^2 = 0.812967 p Value = 0.0000044

(2012) and are the physical conditions closest to those of the natural habitat of the filamentous fungi.

Because the soybean oil was the oily component of the substrate as well as the exclusive source of carbon for fungal growth, we inferred the potential lipase activity through its removal (consumption), as implicated in its transformation to biomass production by the growing fungi isolates. Soybean oil removal of 79.30 ± 0.43% by *Penicillium* sp. ECGF02 and of 71.50 ± 0.32% by *Rhizomucor* sp. ECGF18, allows a similar interpretation to that of submerged fermentation that assigned the production of intracellular lipase, indirectly estimated, the significant responsibility for lipolytic profile presented by *Rhizomucor* sp. ECGF18.

The results of the analyses of correlation and linear regression between lipase activity, fungal biomass production and soybean oil removal, by *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, in conditions of solid-state fermentation, were presented in Table 3. The obtained statistical values were attributed to variations in the lipase activity (growing and decreasing) throughout the 120 h, with peaks (48 h) of 34.11 ± 0.62 U mg^{-1}

protein for *Penicillium* sp. ECGF02 and 5.81 ± 0.25 U mg^{-1} protein for *Rhizomucor* sp. ECGF18 (Figure 2). The fact that the values obtained for lipase activity depended on the method used to quantify it, expressed that extracellular lipase activity could be directly measured, but intracellular activity could not. Satisfactory linearity was verified with a correlation between fungal biomass and soybean oil removal parameters, for *Penicillium* sp. ECGF02 (R = 0.81) and *Rhizomucor* sp. ECGF18 (R = 0.90), these were strong and positive for both, with a greater statistical significance of the latter (Table 3).

Emphasis has been given to studies on the production and application of intracellular lipase produced by fungi as *Rhizopus oryzae* (Athalye et al., 2013), *Rhizopus microsporus* (Alberton et al., 2010), *Mucor circinelloides* (Andrade et al., 2014), *Thermomucor indicae* (Ferrarezi et al., 2014) among others, on which extensive reviews have been published, in regards to its applications as biocatalysts, whether, in the form of whole cells or lipolytic biomass, with the potential for use in industrial processes, sanitation and bioenergy production (Fukuda et al., 2009). As an application the aimed to hydrolyze sanitary sewage of dairy industry oil, with level of oils and greases above 1300 mg L^{-1}, Alberton et al. (2010) tested a solid-fermented lipase of *Rhizopus microsporus*, and found that reduced oil and grease levels to below 300 mg L^{-1}. Rigo et al. (2012) have proposed that noncommercial lipase preparation is more suitable for the hydrolysis of meat industry effluents due to its higher hydrolytic rates. Furthermore, its cost is low because it can be crudely produced from enzymatic preparations of agro-industrial residues. On the other hand, aiming at the great interest in generating biodiesel from used oil, n,n-bis(2-hydroxyethyl) - ethanol, Andrade et al. (2014) selected the fungus *Mucor circinelloides* URM 4182 as an integral cell biocatalyst with the greatest potential to be used in the process, resulting in an 83.22% yield in the conversion process.

Fungal lipases are mostly extracellular, and their production is greatly influenced by nutritional and physicochemical factors such as temperature, pH, nitrogen and carbon sources, the presence of lipids, inorganic salts, the intensity and extend of agitation, and dissolved oxygen concentration. Because the major factor for the expression of lipase activity usually studied as the carbon source, these enzymes are generally produced in the presence of a lipid such as oil or any inducer, such as fatty acids, triglycerols, hydrolysable esters, bile salts, tweens, and glycerol, though few authors have produced good yields in the absence of fats and oils (Singh and Mukhopadhyay, 2012; Turki, 2013).

In this work, the soybean oil, at a concentration of 10%, served as a sole source of carbon in the culture substrate as well as a lipase inducer for the 20 strains of filamentous fungi in submerged fermentation, and for *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18, in sequence, in solid-state fermentation. Such a concentration allows a greater selective pressure on lipase-producing strains and differs from that reported by most authors, who use oil concentrations of or lower than the 2%, they considered sufficient for enzyme-induction. Usually, the carbon concentration must be greater than that of nitrogen to favor the pathways for lipase biosynthesis in these microorganisms (Bueno et al., 2014, Ramos-Sánchez et al., 2015).

The use of the mixture of sand and vermiculite to the natural soil simulation, proved convenient as a substrate for the solid-state fermentation conditions adopted in this study. These inert minerals were not considered a source of nutrients to the fungi evaluated. Therefore, the growth and lipolytic activity of *Penicillium* sp. ECGF02 and *Rhizomucor* sp. ECGF18 were associated with simple factors, best defined as consistent, with simple nutritional composition and low cost. Thus, we believe that these factors contribute to the ease in reproducing these experiments and reliability of the results obtained.

Conclusion

Here it was possible to obtain 20 filamentous fungi isolates from grease trap scum found in a restaurant at Federal University of Espírito Santo, Brazil. The 20 fungi belong to the genera *Aspergillus*, *Beauveria*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Penicillium*, *Rhizomucor*, and *Verticillium*. In submerged fermentation, *Penicillium* sp. ECGF02 showed greater lipase extracellular activity, while *Rhizomucor* sp. ECGF18 showed less, despite having the highest biomass production among all isolates evaluated. In solid-state fermentation, lipase production, biomass production, and soybean oil removal from culture substrate, confirmed the high extracellular lipase activity of *Penicillium* sp. ECG02 and the high intracellular activity by *Rhizomucor* sp. ECGF18. The methodology used in this work proved to be efficient for the characterization of the lipolytic activity of the isolates evaluated. This was an important factor to define before determining its potential use in the production and use of fungal lipases, because the main idea was to lessen the technical and economic burdens of lipase production. This work takes an initial step by isolating new intracellular lipase-producers for commercial interest a whole-cells lipase use. Thus, because of their high lipase intracellular activity, *Rhizomucor* sp. ECG18, showed the potential for use in future research, as whole-cell lipases, are potentially useful in wastewater treatment and biocatalysts in the production of biodiesel from oily residues found in the modern human environment.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors express their acknowledgments to the Pro-Rectorate for Research and Póst-Graduate of the Federal University of Espírito Santo (Brazil) and to the Sanitation Laboratory (LABSAN-UFES) for financial and material support for this work.

REFERENCES

Alberton D, Mitchell DA, Cordova J, Peralta-Zamora P, Krieger N. (2010). Production of a fermented solid containing lipases of *Rhizopus microsporus* and its application in the pre-hydrolysis of a high-fat dairy wastewater. Food Technol. Biotechnol. 48:28-35.

American Public Health Association - APHA (2005). Standard Methods for the Examination of Water and Wastewater, 21th edition, Washington: Port City Press, USA.

Andrade GSS, Carvalho AKF, Romero CM, Oliveira PC, Castro HF (2014). *Mucor circinelloides* whole-cells as a biocatalyst for the production of ethyl esters based on babassu oil. Bioprocess Biosyst. Eng. 37:2539-2548.

Athalye S, Sharma-Shivappa R, Peretti S, Kolar P, Davis JP (2013). Producing biodiesel from cottonseed oil using *Rhizopus oryzae* ATCC #34612 whole cell biocatalysts: culture media and cultivation period optimization. Energy Sustain. Dev. 17:331-336.

Barnett HC, Hunter BB (1998). 4th edition. Illustrated genera of imperfect fungi. New York: Burgess Publisher.

Baron AM, Inez M, Sarquis M, Baigori M, Mitchell DA, Krieger N (2005). Comparative study of the synthesis of *n*-butyl-oleate using a crude lipolytic extract of *Penicillium coryophilum* in water-restricted environments. J. Mol. Catal. B Enzym. 34:25-32.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Bueno PRM, Oliveira TF, Caliari M, Castiglioni GL, Soares Junior MS (2014) Selection and optimization of extracelular lipase production using agro-industrial waste. Afr. J. Biotechnol. 13:566-573.

Cammarota MC, Freire DMG (2006). A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. Bioresour. Technol. 97:2195-2210

Carvalho AKF, Faria ELP, Rivaldi JD, Andrade GSS, Oliveira PC, Castro HF (2015a). Performance of whole-cells lipase derived from *Mucor circinelloides* as a catalyst in the ethanolysis of non-edible vegetable oils under batch and continuous run conditions. Ind. Crops Prod. 67:287-294.

Carvalho AKF, Rivaldi JD, Barbosa JC, Castro HF (2015b). Biosynthesis, characterization and enzymatic transesterification of single cell oil of *Mucor circinelloides* - A sustainable pathway for biofuel production. Bioresour. Technol. 181:47-53.

Carvalho PO, Calafatti SA, Marassi MM, Silva DM, Contesini FJ, Bizaco R (2005). Potential of enantioselective biocatalysis of microbial lipases. New Chem. 28:614-621.

Colen G, Junqueira RG, Moraes-Santos T (2006). Isolation and screening of alkaline lipase-producing fungi from Brazilian savanna soil. World J. Microbiol. Biotechnol. 22:881-885.

Farias CM, Souza OC, Souza MA, Cruz R, Magalhães OMC, Medeiros EV, Moreira KA, Souza-Motta CM (2015). High-level production by *Aspergillus candidus* URM 5611 under solid state fermentation (SSF) using waste from *Siagrus coronata* (Martius) Becari. Afr. J. Biotechnol. 14:820-828.

Ferrarezi AL, Ohe THK, Borges JP, Brito RR, Siqueira MR, Vendramini PH, Quilles Jr. JC, Nunes CCC, Bonilla-Rodriguez GO, Boscolo M, Da-Silva R, Gomes E (2014). Production and characterization of lipases and immobilization of whole cell of the thermophilic *Thermomucor indicae seudaticae* N31 for transesterification reaction. J. Mol. Catal. B Enzym. 107:106-113.

Fukuda H, Kondo A, Tamalampudi S (2009). Bioenergy: sustainable fuel from biomass by yeast and fungal whole-cell biocatalysts. Biochem. Eng. J. 44:2-12.

Gopinath SCB, Anbu P, Lakshmipriya T, Hilda A (2014). Strategies to characterize fungal lipases for applications in medicine and dairy industry. BioMed Res. Int. Vol. 2013, Article ID 154549. 10 p.

Guldhe A, Singh B, Mutanda T, Permaul K, Bux F (2015). Advances in synthesis of biodiesel via enzyme catalysis: Novel and sustainable approaches. Renew. Sustain. Energy Rev. 41:1447-1464.

Hama S, Kondo A (2013). Enzymatic biodiesel production: An overview of potential feedstocks. Bioresour. Technol. 135:386-395.

Kumar A, Kanwar SS (2012). Lipase production in solid-state fermentation (SSF): recent developments and biotechnological application. Dyn. Biochem. Process Biotechnol. Mol. Biol. 6:13-27.

Li N, Zong M (2010). Lipases from the genus *Penicillium*, production, purification, characterization and applications. J. Mol. Catal. B Enzym. 66:43-54.

Montgomery HJ, Monreal CM, Young JC, Seifert KA (2000). Determinination of soil fungal biomass from soil ergosterol analyses. Soil Biol. Biochem. 32:1207-1217.

Nagarajan S (2012). New tools for exploring “old friends microbial lipases”. Appl. Biochem. Biotechnol.168:1163-1196.

Ramos-Sánchez LB, Cujilema-Quitio MC, Julian-Ricardo MC, Cordova J, Fichers P. (2015). Fungal lipase production by solid-state fermentation. J. Bioprocess. Biotechnol. 5:203-212.

Rigo E, Ninow JL, Tsai SM, Durrer A, Foltran LL, Remonatto D, Sychoschy M, Vardanega R, Oliveira D, Treichel H, Di Luccio M (2012). Preliminary characterization of novel extra-cellular lipase from *Penicillium crustosum* under solid-state fermentation and its potential application for triglycerides hydrolysis. Food Bioprocess Technol. 5: 1592-1600.

Rodriguez RC, Fernandez-Lafuente R (2010). Lipase from *Rhizomucor miehei* as a biocatalyst in fats and oils modification. J. Mol. Catal. B Enzym. 66:15-32.

Rowe L, Howard GT (2002). Growth of *Bacillus subtilis* on polyurethane and the purification and characterization of a polyurethanase-lipase enzyme. Int. Biodeterior. Biodegrad. 50:33-40.

Salihu A, Zahangir MA, Abdul Karim MI, Salle HM (2012). Lipase production: an insight in the utilization of renewable agricultural residues. Resour. Conserv. Recycl. 58:36-44.

Singh AK, Mukhopadhyay M. (2012). Overview of fungal lipase: a review. Appl. Biochem. Biotechnol. 166:486-520.

Talukder MR, Lee HZS, Low RF, Pei-Lyn LC, Warzecha D, Wu, J (2013). Potential use of whole cell lipase from a newly isolated *Aspergillus nomius* for methanolysis of palm oil to biodiesel. J. Mol. Catal. B Enzym. 89:108-113.

Turky S (2013). Towards the development of systems for high-yield production of microbial lipases. Biotechnol. Lett. 35:1551-1560.

Winkler UK, Stuckmann M (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol. 138:663-670.