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Isolation of Lipase-Producing Yeasts from Industrial Oily Residues in Different Culture Media

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A B S T R A C T

The main goal of this work was to collect industrial oily residues and isolate lipolytic yeasts by selecting the best strain in relation to lipolytic and esterification activities and investigate the increase of production in shake flasks. A total of 78 yeasts were isolated on Yeast Malt agar and 44 strains with lipase-producing potential pre-selected on tributyrin agar. The yeast identified as *Yarrowia lipolytica* showed the highest lipolytic and esterification activities, reaching 2.67 U/mL and 464.65 U/g, respectively, at 48 h. Medium 4 showed major lipolytic (2.23 U/mL) and esterification (324.98 U/g) activity, concomitantly. The components of this medium were used in the factorial design; it was observed that an increase in the concentration of $MgSO_4$ and olive oil decreased lipolytic activity significantly ($p<0.05$), and the other variables did not influence lipase production. The esterification activity and cell concentration were not affected significantly ($p>0.05$) for any of the variables studied. An increase in lipolytic activity was reached in trial 9 (5.12 U/mL) and trial 1 (5.23 U/mL) and the maximum esterification activity was 133.19 U/g (trial 14). Additionally, the trial 15 showed relevant activities (4.32 U/mL and 105.22 U/g) beyond the cell concentration (10.30 g/L), obtained concomitantly.

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes capable of catalysing the hydrolysis of long-chain triglycerides to diglycerides, monoglycerides, glycerol and free fatty acids at the oil-water interface (Treichel *et al.*, 2010; Verma *et al.*, 2012). Besides hydrolysis, these enzymes may also catalyse esterification, trans-esterification, inter-esterification, alcoholysis, acidolysis, aminolysis and lactonization reactions (Paques and Macedo, 2006). Lipases are ubiquitous in nature and are produced by several plants, animals and microorganisms. Microbial lipases have gained special industrial attention due to their ability towards extremes of temperature, pH, organic solvents and chemo-, regio- and enantioselectivity. Many microorganisms are known as potential

producers of extracellular lipases, including bacteria, yeast, and fungi (Messias *et al.,* 2011; Thakur, 2012).

Among microorganisms, yeasts have been used for the production of lipases, due to advantages like wider substrate range, lower sensitivity to low dissolved oxygen concentrations and heavy metals, higher product yield, rapid growth and ease of genetic manipulation (Fickers *et al.,* 2005; Yang, 2011). Finding promising isolated yeasts can open attractive scientific and commercial perspectives. The composition of the culture medium and environmental conditions has an important effect on microorganism metabolism, which is desired to maximize the production of enzymes and reduce costs (Jia *et al.,* 2015). The greatest factor for the expression of lipase activity is the carbon source used in the medium, since these enzymes are produced in the presence of inducers such as oils, triglycerides, fatty acids, hydrolysable esters, glycerol and Tween (Sharma *et al.,* 2001; Gupta *et al.,* 2004). In addition to the carbon source, nitrogen and essential micronutrients should also be carefully evaluated for growth and increased lipase production (Treichel *et al.,* 2010).

Exploration of the biodiversity of lipase-producing microorganisms with properties suitable for application in various sectors justifies the importance of research and ensures the supply of enzymes, allowing the development of new systems not carried out by enzymes of vegetable or animal origin. The isolation of different strains is the key for discovering novel lipases of interest to industry in general, and in particular of interest to the food industry (Romdhane *et al.,* 2010). Studies indicate the isolation of lipolytic yeasts from various samples, for example, flowers, fruits, soil, leaf surface (Bussamara *et al.,* 2010; Goldbeck and Filho, 2013; Ramesh *et al.,* 2014), butter cheese (Souza *et al.,* 2015), soybean meal (Smaniotto *et al.,* 2012), wastewater and water samples (Odeyemi *et al.,* 2013), among other sources.

The aims of the present study were to isolate lipolytic yeasts from oily industrial residues, selecting the best strain in relation to lipolytic and esterification activities and to investigate the increase of production in shake flasks.

Materials and Methods

Isolation and selection of lipase-producing yeasts using tributyrin

Lipase-producing yeasts were isolated from the residues of crude degummed soybean oil and fish industries located in the city of Rio Grande, Rio Grande do Sul (approximate coordinates, 32° 1' S 52° 5' W). The residues were collected at different points from random industrial processes, and transported in coolers to the Bioprocess Engineering Laboratory of the Federal University of Rio Grande (Rio Grande do Sul, Brasil). The residues were codified as: STB - sludge from degumming tanks, CON - condensate obtained in the oil condensation step, DO - degummed oil residue from the tank background, OE - oil residue in tanks exposed to the environment, SM - soybean meal, F - fishmeal, OPF - oil obtained from pressing of fishmeal, RPF - residue from the fishmeal pressing tank and FP - fish processing residue.

Subsequently, in order to exhaust the endogenous nutritional resources of the microorganisms, samples were serially diluted with sterile distilled water at 25 °C for 24 h and spread onto Petri dishes containing Yeast Malt agar (YM; 1 % glucose, 0.5 % peptone, 0.3 % malt extract, 0.3 % yeast extract and 2 % agar) (Kumar *et al.,* 2012). The dishes were incubated at 25 °C for 48 h in order to isolate individual colonies and test the same for their ability to produce lipase enzymes using

tributyrin agar (Landell *et al.*, 2010). The lipase-producing yeasts were selected according to the formation of a transparent halo around the colonies when cultivated in Petri dishes containing 0.5 % peptone, 0.3 % yeast extract, 2 % agar and 0.1 % tributyrin at pH = 6.0 and incubated at a temperature of 30 °C for 48 h (Goldbeck and Filho, 2013). After 48 h of incubation, the colonies with a halo diameter greater than or equal to 20 mm were selected.

The enzymatic activity index (EAI) is the ratio between the halo diameter and the colony diameter, and classifies the size of halos as small (EAI < 2), average (EAI = 2 to 5) or large (EAI > 5) (Anagnostakis and Hankin, 1975). The selected strains were maintained on an agar slope (YM), covered with a layer of sterile mineral oil and kept at 4 °C.

Pre-selection of lipase-producing wild yeasts in liquid medium

The pre-selected yeasts were cultivated in liquid medium containing 5 % peptone, 0.3 % yeast extract, 0.1 % $MgSO_4$, 0.1 % $NaNO_3$ and 1 % olive oil at pH = 6.0. The inocula were prepared on a GYMP agar slope (2 % glucose, 0.5 % yeast extract, 2 % malt extract, 2 % agar and 0.2 % monobasic sodium phosphate) at 30 °C for 96 h.

After 96 h, 10 mL of liquid medium was added to the agar slope, incubated for 24 h and transferred to Erlenmeyers containing 45 mL of medium at a temperature of 30 °C for 48 h, with agitation at 150 rpm on an orbital shaker. Every 12 h of cultivation, samples were removed and centrifuged at 3640 × *g* for 10 min at 4 °C and lipolytic activity was determined in the crude enzymatic extract. Esterification activity determination was carried out in the lyophilized crude enzymatic extract after 48 h of culture (Goldbeck and Filho, 2013).

Different culture media in the production of lipase

The yeast identified with best lipolytic and esterification activity was cultivated in four media with different compositions: Medium 1 (w/v) 5 % peptone, 1 % soybean oil, 0.1 % $MgSO_4$ and 0.1 % $NaNO_3$ at pH=7.0 (Maldonado *et al.*, 2012), Medium 2, 1 % soybean oil, 0.2 % yeast extract, 0.05 % KH_2PO_4, 0.05 % K_2HPO_4, 0.05 % $MgSO_4.7H_2O$, 0.01 % $CaCl_2$ and 0.01 % NaCl at pH = 6.0 (Mafakher *et al.*, 2010), Medium 3, 0.5 % yeast extract, 1 % KH_2PO_4, 0.1 % $MgSO_4.7H_2O$ and 1 % olive oil at pH = 6.8 (Ramesh *et al.*, 2014) and Medium 4, 5 % peptone, 0.3 % yeast extract, 0.1 % $MgSO_4$, 0.1 % $NaNO_3$ and 1 % olive oil at pH = 6.0 (Goldbeck and Filho, 2013). The inocula and cultures were performed as described previously.

The carbon/nitrogen (C/N) ratio of the different media was estimated using the Solver tool (Excel 2010 software, Microsoft).

Fractional factorial design in the production of lipase

The effects of the concentration of medium components on lipase production were investigated using the fractional factorial design 2^{5-1} with three centre points. The variables and levels studied were peptone (2 to 8 %), yeast extract (0.1 to 0.5 %), $MgSO_4$ (0 to 0.2 %), $NaNO_3$ (0 to 0.2 %) and olive oil (0.5 to 1.5 %). The inocula and cultures were performed as described previously.

Determination of lipolytic activity

Lipolytic activity was measured by titrimetric assay using olive oil as the substrate (Freire *et al.*, 1997). The reaction mixture consisted of 19 mL of olive oil/arabic gum emulsion (5 % olive oil and 5 % arabic gum) in 100 mM

sodium phosphate buffer, pH = 7.0. This mixture was homogenized in a blender for 3 min and the enzymatic reaction started by adding 1 mL of the crude enzymatic extract, incubating for 30 min at 37 °C. The reaction was stopped by adding 20 mL of acetone-ethanol 1:1 (v/v) and the released fatty acids were titrated with 0.05 M NaOH solution to pH = 11.0. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol of fatty acid/min under the described conditions, according to the following Equation 1:

$$LA = \frac{(Va - Vb) * M * 100}{t * v} \quad 1$$

Where *LA* is the lipolytic activity (U/mL), *Va* is the volume of NaOH used for the sample (mL), *Vb* is the volume of NaOH used for the blank (mL), *M* is the molarity of the NaOH solution, *t* is the reaction time (min) and *v* is the volume of enzymatic extract (mL).

Determination of esterification activity

The esterification activity of lyophilized crude enzymatic extracts was measured by the consumption of acid in the esterification reaction with alcohols (1:1, equimolar ratio) with 100 mg of the enzymatic extract. In all experimental runs, a control assay (blank) was always carried out. After incubation for 40 min at 40 °C and 160 rpm, the fatty acids remaining in the aliquot were extracted by the addition of 20 mL of an acetone-ethanol solution (1:1, v/v). The amount of fatty acids was then titrated with 0.035 M NaOH solution until pH = 11.0. Reaction blanks were run in the same way, but adding the sample after addition of acetone-ethanol solution. A unit of esterification activity was defined as the amount of enzyme that consumes 1 µmol/min of oleic acid per minute under the assay conditions (Langone *et al.*, 2002; Bernardes *et al.*, 2007), according to the following Equation 2:

$$EA = \frac{(Va - Vb) * N * 1000 * Vf}{t * m * Vc} \quad 2$$

Where *EA* is the esterification activity (U/g), *Va* is the volume of NaOH used for the sample (mL), *Vb* is the volume of NaOH used for the blank (mL), *M* is the molarity of the NaOH solution, *Vf* is the final reaction volume (mL), *t* is the time (min), *m* is the mass of lyophilized enzymatic extract (g) and *Vc* is the volume withdrawn for titration (mL).

Lipolytic productivity

The lipolytic productivity (U/mL/h) was calculated according to the following Equation 3 (Burkert *et al.*, 2005):

$$LP = \frac{Max\ LA}{t} \quad 3$$

Where *LP* is the lipolytic productivity (U/mL/h), *Max LA* is the maximum lipolytic activity (U/mL) and *t* is the corresponding fermentation time.

Determination of cell concentration

Cell concentration was determined through a gravimetric method, after drying the yeast biomass in 105 °C until constant mass was reached (Celinska and Grajek, 2013).

Determination of pH

The pH of samples was monitored using a digital potentiometer (model MB-10; Marte, São Paulo, Brazil) in accordance with AOAC method no. 972.44 (AOAC, 2000).

Molecular identification

The microorganisms that showed the best lipolytic and esterification activity were submitted for molecular identification at the Institute of Biological Sciences (Federal University of Minas Gerais, Brasil).

Firstly, the strain was incubated in malt extract agar at 25 °C for 24 h. The yeast DNA was extracted and quantified in a Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies). The D1/D2 domains of the 26 S subunit were sequenced by using the primers NL1 (5' GCATATCAATAAGC GGAGGAAAAG 3') and NL4 (5' GGTCC GTGTTTCAAGACGG 3'), according to the methodology described by Lachance *et al.*, (1999).

The nucleotide sequence of the amplified D1/D2 fragment was directly sequenced from the purified PCR product. The sequences obtained were compared with sequences deposited at the GenBank database with the BLAST program (GenBank, U.S. National Library of Medicine, Bethesda, MD).

Statistical analysis

Experiments were conducted in triplicate and the data were statistically analysed using analysis of variance and Tukey's test at 95 % confidence level ($p < 0.05$). A statistical analysis related to the estimated effects of each variable was performed at 90% confidence level ($p < 0.1$). All statistical analysis was performed with STATISTICA v. 8.0 (StatSoft Inc., Tulsa, OK, USA) software.

Results and Discussion

Microorganisms isolated in industrial oily residues

Overall, 78 microorganisms were isolated from industrial oily residues in YM agar. The isolated strains were screened for extracellular lipase using tributyrin agar and 44 were pre-selected from the formation of a transparent halo around the colonies. In the residue of crude degummed soybean oil 24 microorganisms were isolated, while in the fish residue 20 lipase-producing microorganisms were found (Table 1). The use of supplementary solid media containing emulsified triglycerides is the standard methodology for the selection of lipase-producing microorganisms, and tributyrin, which indicates the production of lipase by the presence of a transparent zone around the colonies, is often used as the substrate (Cardenas *et al.*, 2001).

After 48 h of incubation, the agar dishes were analysed according to the production of halos. In this study, of the 44 isolated microorganisms about 4 % showed a large halo (2), 80 % medium (35) and 16 % small (7). The difference in size of the halo can probably be explained either by the amount of extracellular lipase excreted by the microorganism, by the enzyme stability, by the enzyme affinity or by a combination of two or more of these factors (Cardenas *et al.*, 2001).

The highest EAI values were obtained for the microorganisms encoded as OPF1 and DO1, reaching 5.6 and 5.2, respectively. According to Anagnostakis and Hankin (1975), colonies with the highest EAI are those with higher extracellular enzyme activity; however, in this study, this correlation was not observed and the EAI was measured with the sole purpose of classifying the strains isolated from industrial oily residues.

These 44 pre-selected microorganisms were screened for lipase production in liquid medium and strains with the best lipolytic and esterification activity were identified and then the most promising used in a fractional factorial design.

Studies investigating the isolation of lipolytic microorganisms are described in the literature. Goldbeck and Filho (2013) studied lipase-producing wild yeasts isolated from samples of flowers, fruits and soils from some Brazil

biomes and obtained 372 strains; 207 showed a hydrolysis halo around the colonies and the EAI of 44 yeasts was classified as large, 86 as medium and 77 as small. Landell *et al.,* (2010) studied the isolation of yeasts from bromeliad leaves and water tank samples and obtained two new strains identified as *Candida aechmeae* sp. nov. and *Candida vrieseae* sp. nov. Roveda *et al.,* (2010) isolated 21 lipase-producing fungi from dairy effluent in submerged fermentation. Thabet *et al.,* (2012) isolated 19 lipolytic yeasts and three bacteria with EAI between 0.4 and 4.5 from an oil waste site collected from a sunflower oil plant.

The initial results of the study and the literature cited suggest that the industrial oily residues studied are good sources for the isolation of lipase-producing microorganisms, and can be found in various habitats, especially those containing lipids.

Cultivation in liquid medium: lipolytic and esterification activity of pre-selected microorganisms

The lipolytic activity of the 44 pre-selected microorganisms varied from 0.35 to 2.88 U/mL while the esterification activity was between 8.08 and 464.65 U/g (data not presented). Six microorganisms (Table 2) from the fish industry residue stood out for their lipolytic and esterification activities, and were sent for molecular identification.

The microorganism encoded as F1 (Table 2) stood out for its lipolytic (2.67 U/mL) and esterification (464.65 U/g) activity, significantly higher than the other means, obtained concomitantly at 48 h. The microorganism OPF4 (2.88 U/mL) showed the maximum lipolytic activity at 12 h of culture, and there was no statistically significant difference ($p > 0.05$) from F1. The lipolytic productivity of OPF 4 (0.24 U/mL/h) was statistically higher than others.

During the cultivations a decrease in the production of the enzyme after obtaining maximum lipolytic activity (Fig. 1) could be observed except for the F1 microorganism, which possibly is associated with the release of proteases produced during the cell growth phase (Dalmau *et al.,* 2000). A similar fact was reported by Bussamara *et al.,* (2010) who evidenced the presence of proteases in the medium resulting in a reduction in lipolytic activity after 19 h of fermentation of lipase from *Pseudozyma hubeiensis*. The reduction of activity of *Candida rugosa* lipase by proteases was also observed in a study Puthli *et al.,* (2006). Several studies investigated lipase production and isolation by microorganisms (Table 3). Thus, the lipolytic activity of this study presents lower values when compared to the literature cited, reinforcing the need for viable alternatives, such as factorial design, in order to increase enzymatic activity.

In relation to esterification activity (Table 2) microorganisms coded as OPF5 (346.76 U/g), FP-9 (172.53 U/g), OPF3 (151.91 U/g) and F2 (110.81 U/g) also showed relevant results. Rigo *et al.,* (2010) obtained lipolytic yeast from soybean meal with catalytic ability for esterification reactions using the same substrate as the study (oleic acid and ethanol). The highest activities were achieved by the yeasts coded 137Y and 111Y, with 107.94 U/g and 101.67 U/g, respectively. Reinehr *et al.,* (2014) studied esterification reactions between oleic acid and ethanol (molar ratio 1:1) of lipase from *Aspergillus niger* and *Aspergillus fumigatus* isolated from soil contaminated with diesel oil and the activities achieved were 395.43 and 451.5 U/g, respectively, while Ferraz *et al.,* (2012) obtained 189.5 U/g lipase from *Sporobolomyces ruberrimus* using the same substrate in the reaction. Thus, the esterification activity of the yeasts used in this study was slightly higher than the results reported in the literature.

Fig.1 Monitoring the lipolytic activity of microorganisms selected and cultivated at 30 °C, 48 h, 150 rpm

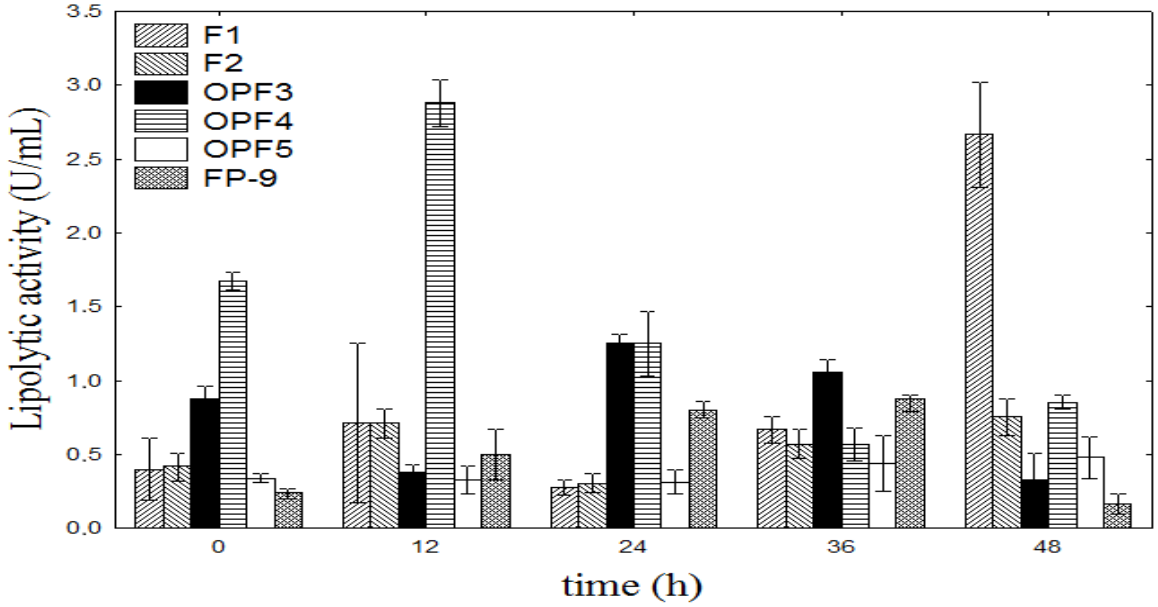

Fig.2 Production of lipase in the four medium culture studied at 48 h cultivation: A) Medium 1, B) Medium 2, C) Medium 3 and D) Medium 4

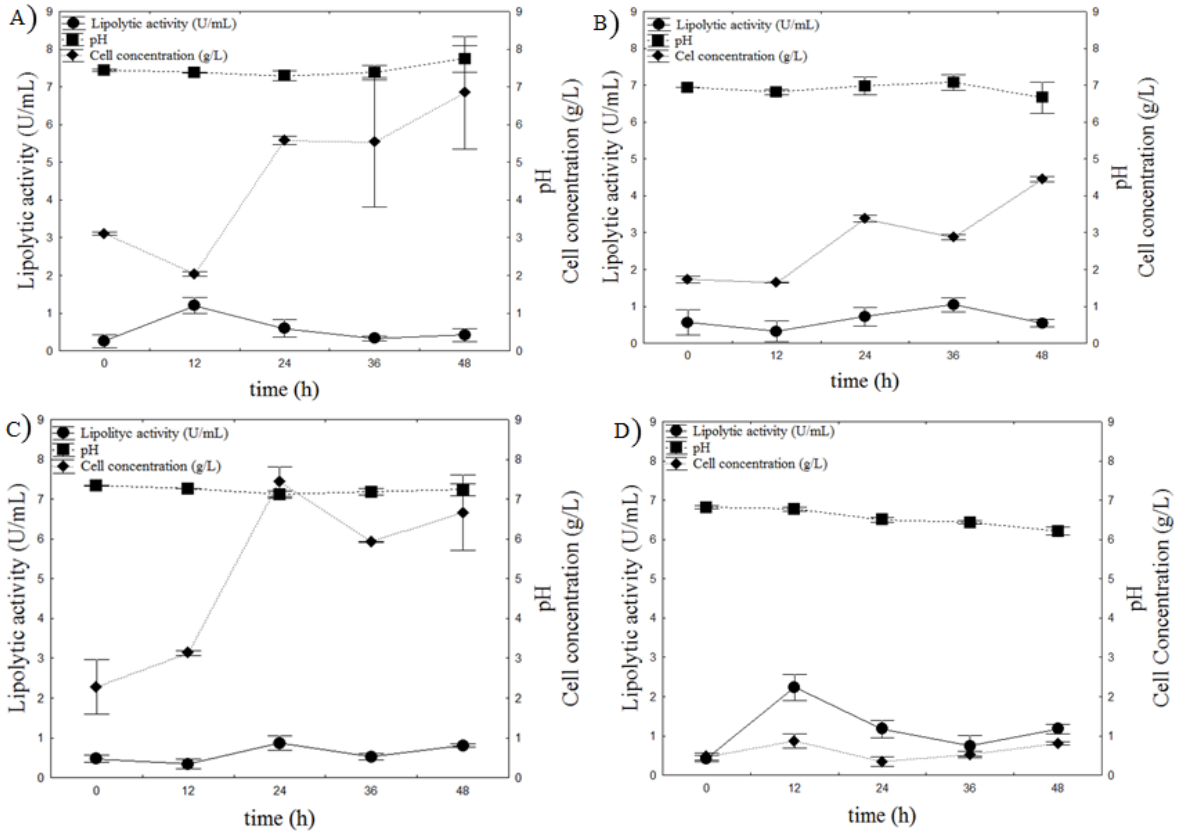

Table.1 Number of microorganisms isolated in YM agar, tributyrin agar and the enzymatic activity index (EAI)

Residue	Total microorganisms	Large halo	Average halo	Small halo
Crude degummed soybean oil	24	1	22	1
Fish	20	1	13	6

Large halo - EAI > 5; average halo - EAI = 2 to 5; and small halo - EAI < 2

Table.2 Maximum lipolytic activity (LA, U/mL), production time (h), lipolytic productivity (Lip. prod., U/mL/h) and esterification activity (EA, U/g) of the six microorganisms selected in residue fish industries

Code	LA	Time	Lip. prod	EA
OPF4	2.88 ± 0.16^{a}	12	0.24 ± 0.01^{a}	59.58 ± 2.79^{e}
OPF5	0.45 ± 0.14^{b}	48	0.01 ± 0.003^{d}	346.76 ± 15.60^{b}
RPF3	2.45 ± 0.30^{a}	12	0.20 ± 0.02^{b}	28.54 ± 4.73^{e}
F1	2.67 ± 0.35^{a}	48	0.06 ± 0.01^{c}	464.65 ± 28.79^{a}
F2	0.76 ± 0.12^{b}	48	0.01 ± 0.003^{d}	110.81 ± 7.45^{d}
FP-9	0.88 ± 0.09^{b}	36	0.02 ± 0.003^{d}	172.53 ± 29.61^{c}

Means±SD (n = 3) with different superscript letters in the same column indicate that there were significant differences ($p < 0.05$)

Table.3 Studies of literature investigating the production of lipase by wild yeasts with lipolytic activity (LA, U/mL) quantified by titrimetric method

Strains	Source	Conditions of production	LA	Ref
Metschnikowia pulcherrima	Flowers, fruit, and soil from Brazil biomes	5 % peptone, 0.3 % yeast extract, 0.1 % $MgSO_4$, 0.1 % $NaNO_3$, 1 % olive oil, 30 °C, 150 rpm and 48 h	4.77	1
Yarrowia lipolytica	Agro-industrial wastewater treatment plants	1 % olive oil, 0.2 % yeast extract, 0.05 % KH_2PO_4, 0.05 % K_2HPO_4, 0.05 % $MgSO_4.7H_2O$, 0.01 % $CaCl_2$, 0.01 % NaCl, 29 °C, 200 rpm and 48 h	11.0	2
Debaryomyces hansenii	Dry-salted olives of Thassos variety	0.5 % yeast extract, 1 % peptone, 0.4 % K_2HPO_4, 0.1 % $MgSO_4.7H_2O$, 1.31 % glucose, 1.9 % olive oil, 0.38 % Tween 80, 30 °C, 150 rpm and 72 h	7.44	3
Candida boidinii	Spent olive from manufacturers of olive oil	1% glucose, 3 % whey powder, 0,8 % $(NH_4)_2SO_4$, 1 % corn steep liquor, 0.5 % olive oil, 30 °C, 120 rpm and 120 h	7.30	4

1 - Goldbeck and Filho (2013); 2 - Mafakher *et al.*, (2010); 3 - Papagora *et al.*, (2013); 4 - Bataiche *et al.*, (2014)

Table.4 Maximum lipolytic activity (LA, U/mL), production time (t, h), lipolytic productivity (Lip. prod., U/mL/h), cell concentration (CC, g/L), biomass productivity (Biom. prod., g/L/h), esterification activity (EA, U/g) and C: N ratio in the four culture mediums lipase from *Y. lipolytica*

Med.	LA	t	Lip. prod.	CC	Biom. prod.	EA	C: N
1	1.20±0.21^{b}	12	0.10±0.02^{b}	2.04±0.06^{b}	0.17±0.005^{c}	47.25±3.79^{b}	3.53
2	1.05±0.19^{b}	36	0.03±0.01^{c}	2.87±0.07^{b}	0.08±0.002^{c}	39.74±1.71^{b}	14.98
3	0.87±0.18^{b}	24	0.04±0.01^{c}	7.45±0.37^{a}	0.31±0.02^{a}	34.90±3.92^{b}	15.94
4	2.23±0.33^{a}	12	0.20±0.19^{a}	0.93±0.09^{c}	0.08±0.02^{c}	324.98 ±62.29^{a}	4.04

Med. - Medium; Means ±SD (n = 3) with different superscript letters in the same column indicate that there were significant differences ($p < 0.05$)

Table.5 Coded levels and real values (in parentheses) for the 2^{5-1} fractional factorial design, lipolytic activity (LA, U/mL), esterification activity (EA, U/g) and cell concentration (CC, g/L)

Trial	Peptone (%)	Yeast extract (%)	$MgSO_4$ (%)	$NaNO_3$ (%)	Olive oil (%)	LA	EA	CC
1	-1 (2)	-1 (0.1)	-1 (0)	-1 (0)	+1 (1.5)	5.23	32.92	6.50
2	+1 (8)	-1 (0.1)	-1 (0)	-1 (0)	-1 (0.5)	3.92	74.65	6.62
3	-1 (2)	+1 (0.5)	-1 (0)	-1 (0)	-1 (0.5)	2.12	18.53	4.73
4	+1 (8)	+1 (0.5)	-1 (0)	-1 (0)	+1 (1.5)	1.06	66.44	1.53
5	-1 (2)	-1 (0.1)	+1 (0.2)	-1 (0)	-1 (0.5)	2.24	51.08	4.92
6	+1 (8)	-1 (0.1)	+1 (0.2)	-1 (0)	+1 (1.5)	1.28	52.58	13.30
7	-1 (2)	+1 (0.5)	+1 (0.2)	-1 (0)	+1 (1.5)	1.52	11.17	3.46
8	+1 (8)	+1 (0.5)	+1 (0.2)	-1 (0)	-1 (0.5)	1.80	11.37	1.76
9	-1 (2)	-1 (0.1)	-1 (0)	+1 (0.2)	-1 (0.5)	5.12	19.74	1.80
10	+1 (8)	-1 (0.1)	-1 (0)	+1 (0.2)	+1 (1.5)	2.61	42.65	2.52
11	-1 (2)	+1 (0.5)	-1 (0)	+1 (0.2)	+1 (1.5)	1.03	24.58	2.40
12	+1 (8)	+1 (0.5)	-1 (0)	+1 (0.2)	-1 (0.5)	2.45	36.04	6.52
13	-1 (2)	-1 (0.1)	+1 (0.2)	+1 (0.2)	+1 (1.5)	0.83	86.22	1.08
14	+1 (8)	-1 (0.1)	+1 (0.2)	+1 (0.2)	-1 (0.5)	1.14	133.19	6.50
15	-1 (2)	+1 (0.5)	+1 (0.2)	+1 (0.2)	-1 (0.5)	4.32	105.22	10.30
16	+1 (8)	+1 (0.5)	+1 (0.2)	+1 (0.2)	+1 (1.5)	0.97	42.24	11.22
17	0 (5)	0 (0.3)	0 (0.1)	0 (0.1)	0 (1)	2.99	67.51	1.66
18	0 (5)	0 (0.3)	0 (0.1)	0 (0.1)	0 (1)	2.35	50.80	1.30
19	0 (5)	0 (0.3)	0 (0.1)	0 (0.1)	0 (1)	2.56	57.08	1.64

Table.6 Main effects of the variables on the lipolytic activity, esterification activity and cell concentration of lipase from *Y. lipolytica*

	Lipolytic activity			Esterification activity			Cell concentration		
Sources of variation	Effects	Std.Err	*t* (13)	Effects	Std.Err	*t* (13)	Effects	Std.Err	*t* (13)
Mean	2.39*	0.26	9.00	51.05	6.84	7.56	4.72	0.91	5.19
Peptone	-0.89	0.58	-1.54	13.71	14.92	0.91	1.84	1.98	0.93
Yeast extract	-0.88	0.58	-1.53	-22.17	14.92	-1.48	-0.16	1.98	-0.08
$MgSO_4$	-1.18*	0.58	-2.03	22.18	14.92	1.48	2.49	1.98	1.25
$NaNO_3$	-0.08	0.58	-0.15	21.39	14.92	1.43	-0.06	1.98	-0.03
Olive oil	-1.07*	0.58	-1.84	-11.39	14.92	-0.76	-0.14	1.98	-0.07

*Significant factors ($p < 0.1$)

Identification of microorganism strains

The molecular identification of the six lipase-producing microorganisms selected revealed that six yeasts were isolated, by sequencing the D1/D2 domains of the subunit (26 S) ribosomal DNA. As an identification tool, the sequencing of this region is relatively simple to apply and yields clear results (Kurtzman and Robnett, 1997; Leaw *et al.*, 2006). Thus, all yeast isolates were identified as *Yarrowia lipolytica* and showed 99 % sequence identity with strain NRRL YB-423.

Selection of culture medium for lipase production by *Y. lipolytica*

Figure 2 shows the lipase production, pH and cell concentration of the four culture media screened for lipase production by *Y. lipolytica*. Medium 4 showed the highest lipolytic and esterification activities, reaching 2.23 U/mL (Fig. 2D and Table 4) and 324.98 U/g, respectively, differing statistically from the other means ($p < 0.05$). In addition to activities, medium 4 also had the highest lipolytic productivity (0.20 U/mL/h) achieved in 12 h of cultivation. The maximum lipolytic activity of each culture medium was differentiated, occurring at times 12, 36, 24 and 12 h for media 1, 2, 3 and 4, respectively. In all culture media, also, there were variations in activity during the cultivations, with a progressive reduction in enzyme activity, which may be due to the secretion of proteases at the end of the logarithmic phase (Dheeman *et al.*, 2010).

The pH was maintained at about 6.0 to 7.0, no pronounced changes being observed from the initial pH. According to Iizumi *et al.*, (1990) lipase has a catalytic activity at pH = 7.0 and is stable in the pH range 5.0 to 8.0, directly influencing the activity, solubility and stability of the enzyme. Benjamin and Pandey (2001) also affirm that neutral pH is generally defined as optimal for lipolytic activity.

Medium 3 showed the highest biomass concentration and, consequently, biomass productivity reached 7.45 g/L and 0.31 g/L/h (Table 4), respectively. There were no significant differences between the mean cell concentration of medium 1 (2.04 g/L) and medium 2 (2.87 g/L) ($p > 0.05$), while medium 4 showed the lowest growth, at 0.93 g/L. Thus, the medium with the highest lipolytic activity (medium 4) was not the medium with the highest cell concentration (medium 3); possibly, this is due to the metabolism of the yeast, since productivity and lipase activity are affected by genetic and

environmental factors (Liu *et al.,* 2015). Another reason for this difference may be related to the growth kinetics of the microorganism and production of the lipase, classifying these processes as non-growth-associated products (Schmidell *et al.,* 2004).

The C:N ratio of the four culture media suggests that medium 4, containing two organic nitrogen sources, peptone and yeast extract, exerted a positive influence on lipolytic (2.23 U/mL) and esterification (324.98 U/g) activity and a negative effect on cell concentration (0.93 g/L). According to Lima *et al.,* (2001) a suitable C:N balance can be critical, especially if the pH is not controlled. Thus, as noted previously also in medium 3, production of the enzyme was not associated with cell growth (Schmidell *et al.,* 2004).

Thus, medium 4 was selected for the continuity of the work, due to higher lipolytic and esterification activity achieved together, aiming to increase the production of extracellular lipase of *Y. lipolytica*.

Effect of culture medium composition on lipase production

The effect of medium composition on the production of lipase by *Y. lipolytica* was evaluated by varying the concentrations of the components in medium 4. The lipolytic activity ranged from 0.83 (trial 13) to 5.23 U/mL (trial 1), esterification activity from 11.17 (trial 7) to 133.19 U/g (trial 14) and cell concentration from 1.08 (trial 13) to 13.30 g/L (trial 6) (Table 5).

The maximum lipolytic activity of trial 1 (5.23 U/mL in 36 h) and trial 9 (5.12 U/mL in 12 h) was obtained in culture media with lower concentrations of peptone, yeast extract and MgSO4. The activities obtained increased about 50 % compared with the activity of medium 4 (2.23 U/mL, Table 4) and also in the central points, varying from 2.35 U/mL (trial 17) to 2.99 U/mL (trial 19). Lower concentrations of constituents contribute to a cost reduction in the production of lipase by *Y. lipolytica*.

Trials 14 (133.19 U/g) and 15 (105.22 U/g) in the fractional factorial design showed the highest esterification activities and the culture medium contained a low concentration of olive oil and high concentrations of $NaNO_3$ and $MgSO_4$. However, comparing these trials with medium 4 (324.98 U/g, Table 4) there was a reduction in esterification activity of approximately 60 %, which may be related to the low concentration of olive oil (0.5 %) as it has proved to be an efficient inducer for lipase production at a concentration of 1 % (w/v). Despite the reduction in esterification activity, trial 15, composed of 2 % peptone, 0.5 % yeast extract, 0.2 % $MgSO_4$, 0.2 % $NaNO_3$ and 0.5 % olive oil, showed relevant lipolytic activity (4.32 U/mL) and cell concentration (10.30 g/L), achieved concomitantly. In relation to cell concentration, trial 6, constituted by 8 % peptone, 0.1 % yeast extract, 0.2 % $MgSO_4$ and 1.5 % olive oil, obtained the highest concentration with 13.30 g/L. This culture medium presented the highest levels of peptone, $MgSO_4$ and olive oil.

The estimated effect for each variable was determined (Table 6) for 48 h of fermentation. An estimate of the main effects can be obtained by evaluating the difference in process performance caused by changing from a low (−1) to high (+1) level of the corresponding factor (Rodrigues and Iemma, 2009). The increase in concentration of $MgSO_4$ and olive oil exerted negative significant effects ($p < 0.1$) on lipolytic activity, decreasing the activity to 1.18 and 1.07 U/mL, respectively. In the fractional factorial design for the five factors studied the

effects were not statistically significant for esterification activity and cell concentration in the range analysed ($p > 0.1$). Thus, the variables that significantly influenced the production of lipase by *Y. lipolytica* were $MgSO_4$ and olive oil. Ramani *et al.*, (2010) studied the production of lipase by *Pseudomonas gessardii* and observed that by adding 0.1 % (w/v) $MgSO_4$ to the medium, there was also a reduction in enzyme activity.

A total of 78 microorganisms were isolated from industrial oily residues and only six strains were selected due to considerable lipolytic and esterification activities in liquid medium. The yeast identified as *Y. lipolytica* was the most promising with higher lipolytic (2.67 U/mL) and esterification (464.65 U/g) activity obtained in conjunction. In the maximization of lipase production, medium 4 showed the best results, indicating in the factorial design that an increase in the concentration of $MgSO_4$ and olive oil variables was significant in lipolytic activity, causing a decrease in its value. The search for new sources for isolating lipase-producing microorganisms, in particular by yeast, is important to find out more stable and selective enzymes applicable in many processes.

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