Bioprocess Biosyst Eng (2014) 37:2539–2548
DOI 10.1007/s00449-014-1231-4

ORIGINAL PAPER

Mucor circinelloides whole-cells as a biocatalyst for the production of ethyl esters based on babassu oil

Grazielle S. S. Andrade · Ana K. F. Carvalho · Cintia M. Romero · Pedro C. Oliveira · Heizir F. de Castro

Received: 10 March 2014 / Accepted: 28 May 2014 / Published online: 24 June 2014

Abstract The intracellular lipase production by *Mucor circinelloides* URM 4182 was investigated through a step-by-step strategy to attain immobilized whole-cells with high lipase activity. Physicochemical parameters, such as carbon and nitrogen sources, inoculum size and aeration, were studied to determine the optimum conditions for both lipase production and immobilization in polyurethane support. Olive oil and soybean peptone were found to be the best carbon and nitrogen sources, respectively, to enhance the intracellular lipase activity. Low inoculum level and poor aeration rate also provided suitable conditions to attain high lipase activity (64.8 ± 0.8 U g^{-1}). The transesterification activity of the immobilized whole- cells was assayed and optimal reaction conditions for the ethanolysis of babassu oil were determined by experimental design. Statistical analysis showed that *M. circinelloides* whole-cells were able to produce ethyl esters at all tested conditions, with the highest yield attained (98.1 %) at 35 °C using an 1:6 oil-to-ethanol molar ratio. The biocatalyst operational stability was also assayed in a continuous packed bed reactor (PBR) charged with glutaraldehyde (GA) and Aliquat-treated cells revealing half-life of 43.0 ± 0.5 and 20.0 ± 0.8 days, respectively. These results indicate the potential of immobilized *M. circinelloides* URM 4182 whole-cells as a low-cost alternative to conventional biocatalysts in the production of ethyl esters from babassu oil.

Keywords Filamentous fungi · Immobilized whole-cells · Polyurethane · Transesterification · Ethyl esters

G. S. S. Andrade (✉)
Science and Technology Institute, Federal University of Alfenas, Poços de Caldas, Minas Gerais, Brazil
e-mail: grazielle.andrade@unifal-mg.edu.br

A. K. F. Carvalho · P. C. Oliveira · H. F. de Castro (✉)
Engineering School of Lorena, University of São Paulo, 12602-810 Lorena, São Paulo, Brazil
e-mail: heizir@dequi.eel.usp.br

C. M. Romero
Planta Piloto de Procesos Industriales Microbiológicos, Consejo Nacional de Investigaciones Científicas y Técnicas, Av. Belgrano y Pasaje Caseros CP, San Miguel de Tucumán, Tucumán, Argentina
e-mail: cinromero78@hotmail.com

Introduction

Biodiesel produced by alcoholysis of vegetables oils or animal fats is a promising renewable fuel. It is compatible with commercial diesel engines and has clear benefits in comparison to diesel fuel, including enhanced biodegradation, reduced toxicity and a lower emission profile [1, 2].

There has been considerable research on biodiesel production from edible or non-edible oils, particularly through biological alternatives [3, 4]. Biocatalysts exhibit advantages over alkali or acid catalysts: the overall transesterification process is less energy intensive and a complex process of catalyst removal and waste treatment is not required. Enzymatic catalysis using lipases has become more attractive for biodiesel production, since it can overcome the mentioned drawbacks. In particular, it should be noted that the by-product (glycerol) can be easily recovered without complex processing, and also that free fatty acids contained in waste oils and fats can be completely converted to methyl esters [4].

However, in this application lipases are limited by economic considerations that are mainly associated with complex purification procedures and enzyme instability

[5]. Fungal cells with an intrinsically high lipase production in conjunction with porous biomass support particles for use as whole-cell biocatalysts represent an attractive technology for enhancing reaction efficiency and reducing costs [6]. As the whole-cell lipase requires no purification, the technology can be effective for the bulk production of commodities [5].

Whole-cell biocatalysts are simply prepared by cultivation. The enzymes trapped inside the cells are regarded as immobilized and can be separated easily [6]. The cell structure may act as a natural matrix able to protect the enzymes from the possible negative action of external agents, providing an effective analogous to that exerted by common matrix used for enzyme immobilization [7].

Rhizopus oryzae, as a whole-cell catalyst producing intracellular lipase, has been extensively studied for biodiesel production. The reaction can be carried out either in solvent-free or organic solvent systems [8, 9]. Several other intracellular lipases derived from *Rhizopus arrhizus* [10], *Pseudomonas fragi* [11], *Rhizopus chinesis* [12], *Aspergillus niger* [13] and *Mucor circinelloides* [14] have been reported to date. However, these strains have been poorly explored as whole-cell biocatalysts for biodiesel production.

Mucor circinelloides URM 4182 was found to be an effective intracellular membrane-bound lipase producer that grows well in triacylglycerol-containing culture media and shows transesterification activity [15]. Although systematic studies have been performed to address questions relating to the immobilization of the fungus whole-cells [15], no studies have been developed to investigate the effects of cultivation parameters on the *M. circinelloides* membrane-bound lipase, as well as to determine the optimized conditions for ethyl esters (FAEE) synthesis.

Here, additional data on the performance of *M. circinelloides* URM 4182 immobilized whole-cells are provided by first studying the effect of nutritional and physical cultivation parameters on the intracellular lipase synthesis. Then, the potential of immobilized whole-cells attained at the best cultivation conditions was assessed in the ethanolysis of babassu oil for FAEE production using experimental design. The operational stability of the biocatalyst was also evaluated in the transesterification reaction in a continuous packed bed reactor (PBR) charged with glutaraldehyde (GA) and Aliquat-treated cells.

Materials and methods

Microorganism

All experiments were carried out with *M. circinelloides* URM 4182 which has documented lipase activity and was purchased from culture collection URM (University Recife Mycologia) at Federal University of Pernambuco (Pernambuco, Brazil). PDA (Potato Dextrose Agar—Difco) was used as solid culture medium for fungi propagation.

Materials

Polyurethane foams (PUF—Scotch-Brite^{MR}) were purchased from local market and cut down in cubic pieces (6-mm) having pore size of 0.76 ± 0.11 mm and density of 0.02 ± 0.01 g cm^{-3}. Olive, sunflower, Brazil nut and linseed oils were purchased locally having fatty acid composition as described in Table 1. Beef and soy peptone were acquired from Himedia (Mumbai, India). Aliquat ® 336 and corn steep liquor (50 %) were bought from Sigma-Aldrich (St Louis, MO, United States). Glutaraldehyde (25 %), ethanol (99.8 %) and *tert*-butanol were purchased from Cromoline (Diadema, SP, Brazil). Babassu oil was a kind gift from Pulcra (Jacarei, SP, Brazil) having the following composition in fatty acids (wt): (3.5 %) capric, (4.5 %) caprylic, (44.7 %) lauric, (17.5 %) mirystic, (9.7 %) palmitic, (3.1 %) stearic, (15.2 %) oleic and (1.8 %) linoleic, with 709.4 g mol^{-1} average molecular weight. Other characteristics included acid index (0.65 %), saponification index (238 mg KOH g^{-1}), iodine index (25 g I_2 g^{-1}), specific mass (0.85 g cm^{-3}), free fatty acid (0.33 %), oxidation index (1.82 m Eq kg^{-1}) and viscosity (29.5 mm^2 s^{-1}). All other reagents were of analytical grade.

Effect of nutritional and physical parameters on the intracellular lipase production

The fungus strain was grown in aseptically basal medium containing per litre: $NaNO_3$ (1 g), KH_2PO_4 (1 g) and $MgSO_4.7H_2O$ (0.5 g). Various parameters namely carbon source (olive, sunflower, Brazil nut, linseed oils), nitrogen source (beef and soy peptone, corn steep liquor), inoculum level and volume ratio were examined to determine their effect on the lipase production. Erlenmeyer flasks (250 and 500 mL) containing 100 mL of the culture medium together with 100 cubes (6 mm × 6 mm × 6 mm) (about 0.4 g) of

Table 1 Fatty acid composition of the tested vegetable oils [13]

Fatty acid	Amount (wt%)				
	Olive oil	Sunflower oil	Brazil nut oil	Linseed oil	Soybean oil
C16:0—palmitic	11.8	6.8	13.8	5.5	11.4
C18:0—stearic	2.6	4.7	10.2	6.0	4.4
C18:1—oleic	74.2	18.6	29.4	21.0	20.8
C18:2—linoleic	8.5	68.2	45.5	14.0	53.8
C18:3—linolenic	0.7	0.5	0.1	53.5	9.3
Others	2.2	1.2	1.0	–	0.3

polyurethane foams (PUF) subjected to prior sterilization (121 °C/15 min) were inoculated by aseptically transferring number of spores (10^6 and 10^7 spores mL^{-1}) from agar slants and incubated for 72 h at 30 °C on a reciprocal shaker (170 rpm). Immobilization of *M. circinelloides* cells was carried out as a natural consequence of their growth during the cultivation. Samples were taken after 72 h and biomass was separated from the culture broth by filtration, washed with water and acetone and dried under vacuum for 24 h. Dry biomass and the filtrated from culture broth were submitted to analysis of lipase activity [15].

Cross-link treatment of immobilized whole- cells

Cross-link treatment was carried out by adding solution of 0.1 vol.% of GA and 0.1 vol.% of Aliquat ® 336 (both in 0.1 M phosphate buffer, pH 7.0) to the immobilized whole-cells, which were separated from the culture broth, and incubating them at 25 °C for 1 h. Treated whole-cells were recovered by filtration, then washed with tap water and phosphate buffer pH 7.0, and dried for approximately 24 h at room temperature. Water content of treated whole-cells was maintained <10 % to avoid reaction reversibility.

Ethyl esters synthesis and data treatment

The ethanolysis reactions were carried out in closed Erlenmeyer flasks (250 mL) containing 30 g of substrate consisting of babassu oil and anhydrous ethanol at different molar ratio using *tert*-butanol as a solvent [15]. Experiments were performed according to a full 2^2 factorial design. The molar ratio (X_1) of ethanol and babassu oil and the temperature (X_2) were chosen as independent process in the range to cover the intervals commonly used for other lipase preparations (molar ratio from 1:6 to 1:12 and temperature from 35 to 45 °C). The mixtures were incubated with immobilized whole-cells at fixed proportion of 20 % wt in relationship to the total weight of reactants involved in the reaction medium. All runs were performed at random for 96 h under reciprocal shaker (170 rpm). Three runs were carried out at the center point level, for experimental error estimation. The transesterification yield (Y %), defined as ratio of the concentrations of the transformed babassu oil to the initial oil multiplied by 100 was taken as response variable.

Results were analyzed with help of *Statistica* software version 8.0 (StatSoft Co.) to verify the independent variables effect on the response assuming levels of $p < 0.05$ as statistical significance criterion. The statistical significance of the individual regression coefficients and the mathematical model was determined by Fischer test and the proportion of variance explained by the model obtained, was given by the multiple coefficient of determination, R^2.

Operational biocatalyst stability of treated whole-cells

The operational biocatalyst stability was assayed under continuous run using a packed bed reactor (PBR) charged with immobilized treated whole-cells. Ethanolysis runs were performed at 35 °C, for a 14 days running on substrate consisting of babassu oil and anhydrous ethanol molar ratio oil to alcohol (1:6) and *tert*-butanol as solvent. PBR was a jacketed glass column (internal diameter = 45 mm; height = 190 mm, and total volume = 310 cm^3). The temperature was kept by circulating water in the jacket. The substrate was continuously pumped (Sj-1211-Hatto) from a reservoir at 35 °C, through silicone tubing, to the bottom end of the bioreactor at flow rate of 7.8 mL h^{-1}. An amount of 27 g biocatalyst was used, which corresponds to a bulk volume of 29.2 cm^3. Density of the immobilized cells (dry weight) was about 1.08 ± 0.06 g/cm^3. The space time was calculated according to Levenspiel [16]. Aliquots were taken every day to quantify ethyl esters formed by gas chromatography (GC).

The experimental results were plotted and normalized following the Arrhenius equation (Eq. 1) and fitted to a deactivation model of first order:

$$-\ln\frac{A}{A_o} = K_d \times t \quad (1)$$

Half-life of the biocatalyst ($t_{1/2}$) was estimated by Eq. (2), in which $t_{1/2}$ is the half-life, and K_d is the deactivation coefficient.

$$t_{1/2} = \frac{0.693}{K_d} \quad (2)$$

Downstream procedure

When the reaction was completed the lipase was separated from the medium and the organic phase was washed twice with water to remove both the remaining ethanol and the free glycerol as a by-product. Residual water and *tert*-butanol were removed with a rotary evaporator to attain the final fatty acid ethyl ester product.

Analysis

Lipase activity

The intracellular lipase activity was measured by the hydrolysis method using olive oil emulsion according to the modification proposed by Andrade et al. [15]. One unit (U) of enzyme activity was defined as the amount of

enzyme that liberates 1 μmol of free fatty acid per min under the assay conditions (37 °C and pH 7.0).

Monitoring ethyl esters formation

Fatty acid ethyl esters (FAEE) were analyzed in FID gas chromatography Varian GC 3800 model (Varian Inc., Palo Alto, CA, USA) using a 5 % DEGS CHR-WHP 80/100 mesh 6 ft 2.0 mm ID and 1/8 OD column (Restek, Frankel Commerce of Analytic Instruments LTD, SP, Brazil) following previous established conditions [17]. Data was collected using Galaxie Chromatography Data System software version 1.9. Theoretical ester concentrations were calculated by taking into consideration the babassu oil fatty acid composition and its initial weight mass in the reaction medium [17, 18].

Mono, di and triglyceride analysis

Glycerides analysis was performed in an Agilent 1200 Series liquid chromatograph (Agilent Technologies, USA) equipped with an evaporative light scattering detector and Gemini C-18 (5 μm, 150 × 4.6 mm, 110 Å) column at 40 °C, using as mobile phase containing a mixture of acetonitrile (80 %) and methanol (20 %) in a flow rate of 1 mL/min for 6 min, 1.5 mL/min until 30 min and 3.0 mL/min up to 35 min. All the samples were previously dissolved in ethyl acetate-hexane (1:1 v/v), filtered through 0.22 μm membrane filters (Millipore) and injected in a volume of 10 μL. All solvents were of HPLC grade and the assays were done at least in duplicate.

Purified fatty acid ethyl esters (FAEE) analysis

Purified samples were dissolved in deuterated chloroform and spectrum was registered on Varian spectrometer, model Mercury-300 MHz. Conversion of triglycerides was determined by taking the data base generated by ^{1}H nuclear magnetic resonance (^{1}H NMR) using the equation validated by Paiva et al. [19].

Thermal gravimetric analysis (TGA) was employed to verify the influence of the heating rate on the FAEE profile. A Shimadzu TGA 50 thermogravimetric analyzer was used to carry out the analysis of samples by adopting the following analysis conditions: nitrogen flow rate of 50 mL min^{-1}, heating rate of 10 °C min^{-1} in the range of 25–1,000 °C [18].

Fourier transform infrared spectroscopy (FTIR) was used to investigate the compounds formed by the transesterification reaction. The IR spectra were recorded in a Perkin Elmer Spectrum GX, spectrometer, using KBr pellets, in the range of 4,000–400 cm^{-1}.

The absolute viscosity was determined with LVDV-II cone and plate spindle Brookfield viscosimeter (Brookfield Viscometers Ltd., UK) using a CP 42 cone. A circulating water bath was used to maintain the temperature at 40 °C during the assays. The shear stress measurements were taken as a function of shear rate, and the dynamic viscosity was determined as a slope constant. FAEE samples of 0.5 mL were used and measurements were replicated three times. The density was determined with DMA 35 N EX digital densimeter (Anton Paar). The temperature was maintaining at 20 °C during the assays. FAEE samples of 2.0 mL were used and measurements were replicated three times [18].

Results and discussion

Effect of nutritional sources and physical parameters on the lipase production

Five vegetable oils having different profile of fatty acids (Table 1) were tested for their ability to support lipase production by *M. circinelloides* URM 4182. Although all tested oils were able to enhance cell growth and lipase activity, maximum values were achieved when olive oil was used as a carbon source (Table 2).

These results can be related to the high proportion of oleic acid in olive oil, which accounts for more than 70 %. Soybean, sunflower and Brazil nut oils have higher percentages of linoleic acid (54, 68.2 and 45 %, respectively) and lower contents of oleic acid. It has been proposed that fatty acids (including those present in oils) have important effects on the lipase production [12, 13]. Therefore, the hypothesis was that the different effects of oils in the lipase production were caused by fatty acids from the metabolism of oils, with the best lipase production being correlated to the highest oleic acid content.

There are several reports suggesting that the synthesis of intracellular lipases from some microorganisms can be enhanced using either oleic acid or olive oil as inducers. Yang et al. [10] investigated the influence of different fatty acids as a carbon source on the intracellular lipase in *R. arrhizus* whole-cells and found that oleic acid was the best inducer. Shimada et al. [20] indicated that long chain fatty acids participate in the expression of *Geotrichum candidum* lipase genes, and long-chain unsaturated fatty acid esters were the most effective for the lipase synthesis.

The concentration of olive oil in the culture medium was evaluated in the range of 10–100 g L^{-1} taking into consideration that high levels of carbon may inhibited cell growth and poor supply of nutrients may decrease cell growth [12], consequently reducing the lipase production. The concentration of olive oil had significant effect on both

Table 2 Biomass and intracellular lipase activity obtained during cultivation of *M. circinelloides* 4182 whole-cells using different nutritional sources and physical parameters

	Condition	Biomass ($g\ L^{-1}$)	Lipase activity ($U\ g^{-1}$)
Carbon source	Olive oil	33.8 ± 0.7	66.6 ± 0.9
	Soybean oil	33.7 ± 0.8	48.8 ± 1.2
	Sunflower oil	32.1 ± 0.5	47.4 ± 0.8
	Linseed oil	26.4 ± 0.4	46.5 ± 0.9
	BRAZIL nut oil	27.7 ± 0.6	40.4 ± 1.1
Olive oil content ($g\ L^{-1}$)	10	30.4 ± 0.6	58.1 ± 0.7
	30	30.5 ± 0.8	66.8 ± 0.6
	50	41.5 ± 0.5	55.2 ± 1.1
	100	100.2 ± 2.0	55.2 ± 0.8
Nitrogen source	Soybean peptone	31.6 ± 0.7	67.0 ± 0.6
	Beef peptone	30.5 ± 0.8	62.1 ± 1.3
	Corn steep liquor	28.9 ± 0.6	32.2 ± 0.8
Inoculum (spores mL^{-1})	10^6	32.5 ± 0.9	65.7 ± 0.8
	10^7	30.9 ± 0.5	52.6 ± 0.7
Volume ratio	1:2.5	33.5 ± 0.8	66.2 ± 0.6
	1:5.0	37.8 ± 0.7	43.7 ± 0.5

lipase activity and biomass growth, although in different ways (Table 2). The biomass growth was proportional to the concentration of olive oil in the culture medium, and the highest value of biomass (100 $g\ L^{-1}$) was attained when the highest olive oil concentration was used. However, concentrations higher than 30 $g\ L^{-1}$ was found to inhibit the lipase production, presumably due to the free fatty acids formed by the excess of hydrolyzed olive oil, which may change the medium pH, thus repressing the lipase production. Thus, the maximum lipase activity (66.8 $U\ g^{-1}$) was achieved when 30 $g\ L^{-1}$ of olive oil was used. These results are in agreement with those established for *Rhizopus chinensis* cultivation [12], in which an optimum olive oil concentration was found to be 20 $g\ L^{-1}$.

The influence of different nitrogen sources (beef peptone, soybean peptone and corn steep liquor) on the production of intracellular lipase from *M. circinelloides* URM 4182 was assessed at fixed concentration (70 $g\ L^{-1}$). Corn steep liquor negatively affected the intracellular lipase activity reducing it to levels not greater than 32.2 $U\ g^{-1}$. No accentuated differences were found using peptone from beef or soybean, though a slightly higher lipolytic activity (67.0 $U\ g^{-1}$) was achieved when soybean peptone was added to the culture medium. Yang et al. [10] reported that soybean flour and protein provided the highest lipase activity in the cultivation of *R. arrhizus* immobilized cells. These results were attributed to macromolecular protein and oils contained in soybean flour, which were beneficial for the synthesis of lipase.

The spore concentration of the inoculum appears to be a critical factor for the process outcome and also in the immobilized cells and lipase activity [21–24]. Table 2 shows that there was no expressive difference between cells growth, with values of 32.5 and 30.9 $g\ L^{-1}$ for inoculum levels of 10^6 and 10^7 spores mL^{-1}, respectively. Nevertheless, the highest intracellular lipase activity (65.7 $U\ g^{-1}$) was attained using the lowest inoculum level. This behavior could be related to the influence of inoculum in fungal morphology. Teng et al. [25] investigated the relation of *R. chinensis* whole-cell morphology and lipase production employing different inoculum levels. These authors found an increase in lipase production when mycelia grew as fully entangled filaments, which were obtained using lower inoculum levels. It was also reported that inoculum levels higher than 10^7 spores mL^{-1} tend to form dispersed mycelia, leading to reduced lipase production [25].

Aeration rate was also a relevant variable that influenced both lipase production and biomass growth. The lipase production was affected by changes in the volume ratio and the highest productivity was attained at volume ratio of 1:2.5. Lipase activity decreased when higher volume ratio was used (1:5). It was evident from the results that aeration influenced lipase production since a larger volume ratio increases the area growth, improving aeration with the medium components or giving better dispersion of the carbon source. However, high cell growth caused by elevated aeration rate does not necessarily imply a high lipase production (Table 2). Biomass concentration was increased when a 1:5 volume ratio was used (37.8 $g\ L^{-1}$), and the intracellular lipase activity decreased (43.7 $U\ g^{-1}$). A volume ratio of 1:2.5 was the best for intracellular lipase production, with 66.2 $U\ g^{-1}$ lipase activity suggesting that a higher volume ratio induced the lipase secretion to the fermentation broth.

Experimental design

An experimental design was used to study the combined effect of the oil-to-ethanol molar ratio and temperature in the transesterification yield. For each run, the strain was growth under the conditions previously established attaining an average intracellular lipase activity of 64.8 ± 0.8 $U\ g^{-1}$. The experimental matrix together with the transesterification yields (%) is shown in Table 3.

The results showed that transesterification yields were strongly affected by both variables, ranging between 50.2 and 88.8 %. The highest value was achieved when lower molar ratio (1:6) and temperature (35 °C) were used.

Table 3 Experimental design and results according to a full 2^2 factorial design to evaluate the influence of the variables oil-to-ethanol molar ratio and temperature on the transesterification yield

Run	X_1	X_2	X_1 oil-to-ethanol molar ratio	X_2 temperature (°C)	Transesterification yield* (%)
1	−1	−1	1:6	35	88.8
2	+1	−1	1:12	35	68.5
3	−1	+1	1:6	45	79.3
4	+1	+1	1:12	45	50.2
5	0	0	1:9	40	72.7
6	0	0	1:9	40	68.6
7	0	0	1:9	40	69.8

* Reaction time = 96 h

Conversion was markedly decreased for higher molar ratios (>1:9).

The experiment results showed in Table 3 were used to estimate the main variable effects and its interaction. According to the Student's *t* test, both variables had negative and significant main effects for the formation of ethyl esters within the experimental range studied at a 95 % confidence level (Table 4). However its interaction was not statistically significant at the same confidence level.

The most significant effect was the molar ratio between reactants and large excess of ethanol decreased the transesterification yields (runs 2, 4, 5 to 7). Although an excess of alcohol in relation to the stoichiometric amount is usually needed to shift the reaction equilibrium towards the esters formation [3, 8], a large excess of alcohol may inhibit the activity of some lipases [8]. In the present study, ethanol in excess was used to guarantee the medium homogeneity during the process. However, further increases in this excess caused a yield decrease.

The influence of temperature on the transesterification yield was less important than that for oil-to-ethanol molar ratio and its negative effect is in agreement with the thermal stability of *M. circinelloides* URM 4182, which is a mesophilic strain. In addition, according to the literature, fungi that grow and synthesize lipolytic enzymes at a temperature range of 20–30 °C should be less stable to heat treatment [26]. In this study, *M. circinelloides* was grown at 30 °C and, therefore, it is expected that intracellular lipase could be less stable at temperatures higher than 40 °C.

The main effects were fitted by multiple regression analysis (Table 4) to a linear model as no significant value was given by checking the curvature ($p = 0.499$) and the best fitting response function can be written by Eq. (3).

$$\hat{y} = 71.13 - 12.35x_1 - 6.95x_2 \quad (3)$$

where $\hat{y}$ is transesterification yield (%) and x_1 and x_2 are molar ratio and temperature, respectively.

Table 4 Estimated effects, standard errors and Student's t test for transesterification yield in biodiesel production using 2^2 full factorial design

Variable	Effect	Standard error	*p*
Mean	71.13	±0.79	0.000*
X_1	−24.70	±2.10	0.007*
X_2	−13.90	±2.10	0.022*
$X_1 \cdot X_2$	−4.40	±2.10	0.172

X_1 and X_2 represent the variables oil-to-ethanol molar ratio and temperature, respectively

* Significant at 95 % confidence level

Table 5 Analysis of variance (ANOVA) for the regression of the model that represents transesterification yield using the 2^2 full factorial design

Source	Sum of squares	Degree of freedom	Mean square	*F*	
				Value	Prob > *F*
Model	822.66	3	274.22	61.71	0.0160
X_1	610.10	1	610.09	137.30	0.0072
X_2	193.20	1	193.21	43.48	0.0222
Lack of fit	3.05	1	3.05	0.69	0.4947
Pure error	8.90	2	4.44		
Cor total	834.59	6			

$R^2 = 0.9857$

The statistical significance of this model was evaluated by the *F* test (Table 5), which revealed that this regression is statistically significant at a 95 % probability level. The model did not show lack of fit and the determination coefficient ($R^2 = 0.9893$) indicates that the model can explain 98.93 % of the variability.

According to this study, the maximum transesterification yield can be obtained, working at the lowest level for both variables, that is, molar ratio of 1:6 (oil to ethanol) and temperature of 35 °C. In order to validate the model (Eq. 3), ethanolysis runs were performed under the optimal conditions predicted by the model. The time-course of the reactions is presented in Fig. 1 in which the concentration values for ethyl esters showed a typical kinetic profile attaining 73.5 wt% in 96 h (yield = 90.5 ± 1.7 %). This result is in good agreement with the predicted value made by the model. Extending the reaction time for an additional 24 h the value for ethyl ester concentration achieved its maximum 78.6 wt% corresponding to an yield of 98.1 ± 1.2 %.

The main ester produced was ethyl laurate (C12), followed by ethyl myristate (C14) and ethyl oleate (C18:1). Other esters were produced at lower amounts (Table 6). This profile was expected, considering the babassu oil fatty acid composition. Although high FAEE yield (98.1 ± 1.2 %) was achieved considerable

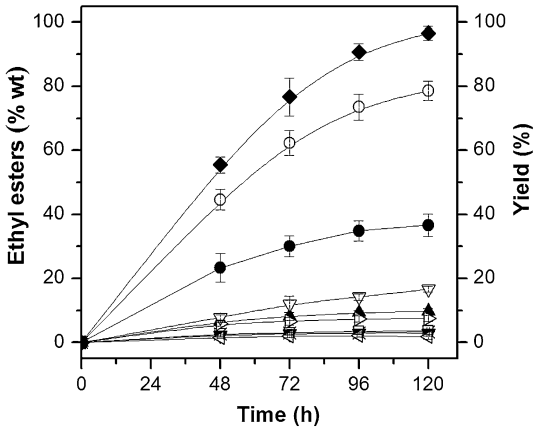

Fig. 1 Profile for alkyl esters formation in the ethanolysis of babassu oil catalyzed by immobilized *M. circinelloides* 4182 whole-cells. *Asterisk* C8; *open square* C10; *filled circle* C12; *filled triangle* C14; *right sided triangle* C16; *filled inverted triangle* C18; *open inverted triangle* C18:1; *left sided triangle* C18:2; *open circle* total ethyl esters; *filled diamond* yield

Table 6 Properties of the purified sample obtained from the ethanolysis of babassu oil catalyzed by immobilized *M. circinelloides* URM 4182 whole-cells

Property	Value
Density 20 °C (kg m^{-3})	877
Kinematic viscosity at 40 °C (mm^2 s^{-1})	4.3
Water content (%)	0.2
Ethyl esters composition (%wt)	
Ethyl caprylate (C8)	3.5
Ethyl capronate (C10)	4.5
Ethyl laurate (C12)	44.8
Ethyl myristate (C14)	16.8
Ethyl palmitate (C16)	9.8
Ethyl stearate (C18)	3.7
Ethyl oleate (C18:1)	13.9
Ethyl linoleate (C18:2)	1.1
FAEE (%)	98.1
Monoglyceride (wt%)	1.5
Diglyceride (wt%)	0.4
Triglyceride	0

amounts of monoglycerides (1.5 ± 0.3 wt%) and diglycerides (0.4 ± 0.1 wt%) remained after the transesterification. This can be associated to the specific of the *M. circinelloides* which is classified as 1(3)-regiospecific lipase.

Properties of fatty acid ethyl esters (FAEE)

To confirm these results, the purified product was analyzed by ^{1}H NMR (Fig. 2), TGA (Fig. 3) and FTIR (Fig. 4). Specific gravity, water content and viscosity were also assessed.

The purified FAEE had a viscosity of 4.3 mm^2 s^{-1} at 40 °C and density of 877 kg m^{-3} at 20 °C (Table 6). Viscosity value confirmed the occurrence of the transesterification reaction due to the consistent viscosity reduction when compared to the initial value estimated for the feedstock (29.5 mm^2 s^{-1}). Viscosity and density values are in accordance with effective technical standards which indicated values, respectively from 1.9 to 6.0 mm^2 s^{-1} and from 850 to 900 kg m^{-3} and similar with data reported for the FAEE based on babassu oil obtained by immobilized extracellular lipase [18].

Quality and purity of the FAEE was assessed by ^{1}H NMR analysis based on monitoring the variations of ester ethoxy and glycerol methylene hydrogens in the region of 4.05–4.40 ppm. Spectra of ^{1}H NMR from FAEE are showed in Fig. 2.

It can be noted that the spectrum attributed to the protons on the 1 and 3 carbon atoms of the glyceril group (CH_2) at 4.2 ppm is totally absent, indicating the conversion of triacylglycerol into 98.5 % of FAEE. This result is reinforced by the appearance of a quartet signal at 4.1 ppm concerning to FAEE. The yield (98.5 %) calculated by this technique is well-correlated with GC and HPLC analysis of the same sample.

The satisfactory performance of the biocatalyst was also confirmed by thermogravimetry analysis (TGA). The TGA curve (Fig. 3) shows the thermal behavior of FAEE from babassu oil and mineral diesel, used as a parameter control. FAEE showed only one step of thermal decomposition in temperature ranging from 23 to 402 °C with 98 % weight loss, which is associated with the evaporation and/or combustion of the ethyl esters. Carvalho et al. [18] investigated the enzymatic transesterification of different feedstocks using *Burkholderia cepacia* lipase immobilized on silica-PVA as catalyst and results evaluated by ^{1}H NMR, as well as GC and TGA techniques showed similar values as those obtained here.

Fourier Transform Infrared spectroscopy (FTIR) was also used in this study to evaluate FAEE quality by identifying functional groups and bands corresponding to stretching and vibrations in biodiesel and feedstock samples. No attempt was made to quantify the transesterification yields using this methodology. Instead, the ethyl esters quality was assessed by FTIR and correlated with other analytical data obtained, in order to crosscheck the results.

Figure 4 showed absorption bands in the regions of 1,750 cm^{-1} corresponding to carbonyl axial deformation (–C=O) axial, 1,170 cm^{-1} related to the ester axial deformation (C–O–C) and 722 cm^{-1} attributed to the group –$(CH_2)_n$. This behavior is similar to that reported for biodiesel samples obtained from different feedstocks,

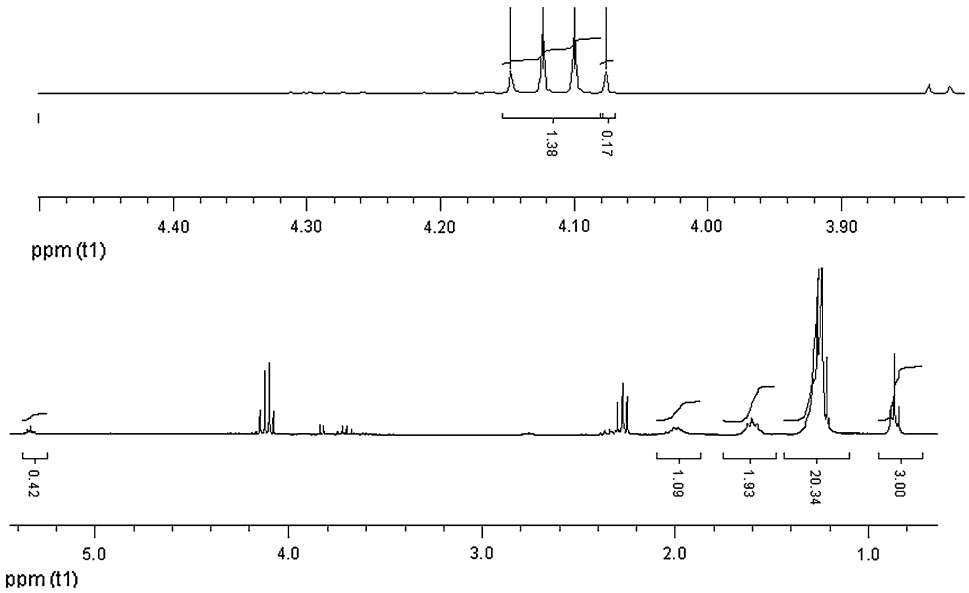

Fig. 2 Integrated ^{1}H NMR spectrum of ethyl esters from ethanolysis of babassu oil catalyzed by immobilized *M. circinelloides* URM 4182 whole-cells

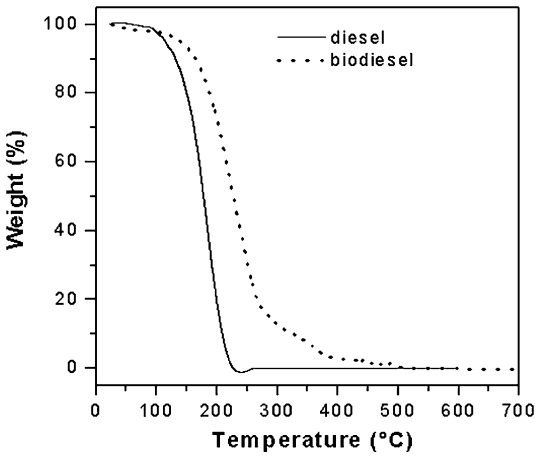

Fig. 3 TGA curves of mineral diesel and ethyl esters from ethanolysis of babassu oil catalyzed by immobilized *M. circinelloides* URM 4182 whole -cells

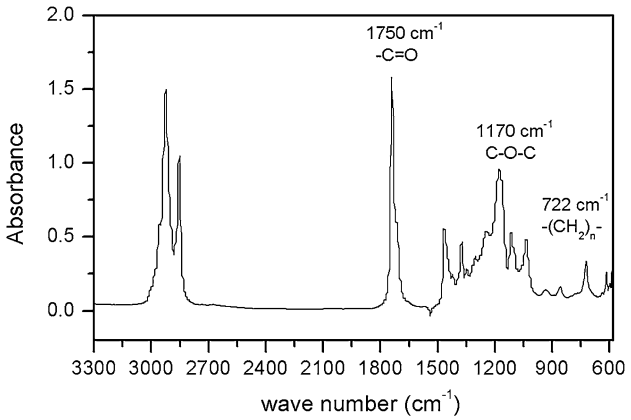

Fig. 4 IR spectra for ethyl esters from ethanolysis of babassu oil catalyzed by immobilized *M. circinelloides* URM 4182 whole-cells

according to Soares et al. [27]. The absence of a broad band in the 3,100–3,500 cm^{-1} region related to the axial deformation characteristic of OH groups suggests that the ethyl esters sample had low water content (0.2 %), as can also be verified by thermogravimetric analysis, discussed previously.

Effect of treatment M. circinelloides URM 4182 whole-cells with GA and Aliquat® 336 in the biocatalyst operational stability

Catalytic activities of immobilized whole-cells may be decreased during repeated batch uses, due to cell exfoliation caused by vigorous agitation in a shaker and other critical parameters that should be taken into consideration following the treatment (e.g. rinsing) of the biocatalyst after recovery. Alternatively, the biocatalyst stability can be assessed in a continuous run.

In a previous study using immobilized whole-cells [15], the stability of the untreated biocatalyst was evaluated in the ethanolysis of babassu oil in a continuous PBR. After 14 days at 35 °C, FAEE concentration varied between 55.0 ± 0.7 and 52.1 ± 0.6 wt% and a half-life of 26 days and a deactivation coefficient of 2.7 × 10^{-2} day^{-1} were estimated.

In the present study, the immobilized whole-cells were submitted to a cross-link treatment with GA, widely reported as a cross-link agent, and Aliquat® 336, an ionic liquid successfully tested as additive in lipase immobilization by encapsulation, in order to achieve a higher FAEE content and improve the half-life of the biocatalyst [28].

GA treatment did not affect the lipase activity, since similar immobilized whole cells without treatment

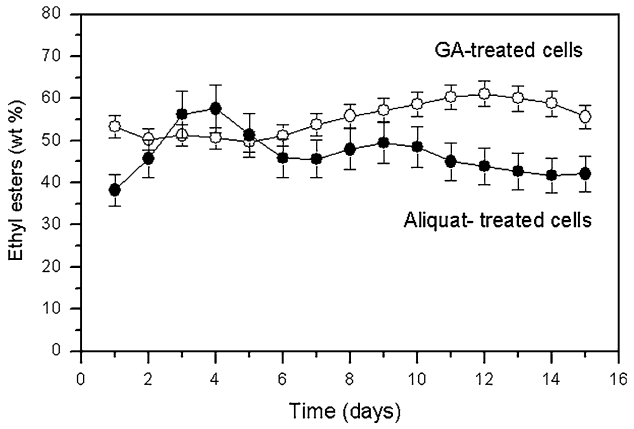

Fig. 5 Continuous ethanolysis running on babassu oil catalyzed by GA (*filled circle*) and Aliquat® 336 (*open circle*) treated immobilized *M. circinelloides* URM 4182 whole-cells. Reaction conditions: PBR charged with immobilized cells, feed medium consisting of molar ratio oil-to babbasu oil at 1:6, space time 36 h and 35 °C

(control) showed lipase activity of 65.7 U g^{-1} and after GA treatment, a similar value (63.2 U g^{-1}) was found. In contrast, the Aliquat® 336 treatment decreased the lipase activity to 39.2 U g^{-1}, indicating strong denaturation of the intracellular lipase by this hydrophobic agent. This behavior was not observed in extracellular lipase immobilization, as reported by Souza et al. [28].

Figure 5 indicate different performances according to the treatment whole cells, in the ethanolysis of babassu oil under continuous flow. Although GA-treated whole cells promoted a slight turbulence at the beginning of the reaction, FAEE concentration was increased during the transesterification, reaching a maximum value of 61.4 % wt. which corresponds to 75.5 % of FAEE yield and a product with 8.4 mm^2 s^{-1} of viscosity. On the other hand, the use of whole-cells treated with Aliquat® 336 led to a sharp oscillation in FAEE concentration throughout the period of operation of the reactor. FAEE concentration ranged from 35.2 to 63.8 wt% yields of 43.3 to 78.5 %, with viscosities of 13.5 and 8.7 mm^2 s^{-1}, respectively.

GA-treated whole cells was the most efficiently system tested, since improved the stability of biocatalyst revealing a half-life of 43 days and a deactivation coefficient of 1.6×10^{-2} day^{-1}. Comparing to our previous work with non-treated cells [15], the half-life of immobilized whole cells had a 1.7-fold increase, suggesting the potential of GA cross-link treatment to extend the usability of whole-cells under continuous operation. The improved stability of GA-treated cells can be attributed to the dense film at the particle surface resulting in reduced lipase leakage from the whole-cells [29]. Otherwise, lower values were obtained in reaction with whole-cells treated with Aliquat® 336 that reached only a half-life of 20 days and a deactivation coefficient of 3.4×10^{-2} day^{-1}. This low stability of Aliquat-treated whole-cells can be related to the denaturation of intracellular lipase caused by this ionic liquid.

Conclusions

Suitable cultivation conditions for *M. circinelloides* URM 4182 whole-cells immobilized in polyurethane foams were determined. The high lipase activity was confirmed by transesterification reactions with babassu oil and ethanol, resulting in a suitable product to be used as biofuel. Results also demonstrated good potential for running the transesterification reaction on a continuous basis, due to the high operational stability of the biocatalyst measured in the presence of substrate and products. Many challenges still remain and investigations should try to make the system more efficient, particularly by enhancing the reaction rate. Cost reduction is the major advantage of using whole-cells as biocatalysts in the FAEE synthesis.

Acknowledgments The authors are grateful for the financial support provided by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil.

References

1. Balat M, Balat HA (2010) Progress in biodiesel processing. Appl Energ 87:1815–1835
2. Helwani Z, Othman MR, Aziz N, Fernando WJN, Kim J (2009) Technologies for production of biodiesel focusing on green catalytic techniques: a review. Fuel Process Technol 90:1502–1514
3. Stamenkovic OS, Velickovic AV, Veljkovic VB (2011) The production of biodiesel from vegetable oils by ethanolysis: current state and perspectives. Fuel 90:3141–3155
4. Adamczak M, Bornscheuer UT, Bednarski W (2009) The application of biotechnological methods for the synthesis of biodiesel. Eur J Lipid Sci Technol 111:808–813
5. Christopher LP, Kumar H, Zambare VP (2014) Enzymatic biodiesel: challenges and opportunities. Appl Energy 119:497–520
6. Parawira W (2009) Biotechnological production of biodiesel fuel using biocatalysed transesterification: a review. Crit Rev Biotechnol 29:82–93
7. Fukuda H, Kondo A, Tamalampudi S (2009) Bioenergy: sustainable fuels from biomass by yeast and fungal whole-cell. Biocatal Biochem Eng J 44:2–12
8. Arumugam A, Ponnusami V (2014) Biodiesel production from *Calophyllum inophyllum* oil using lipase producing *Rhizopus oryzae* cells immobilized within reticulated foams. Renew Energy 64:276–282
9. Li W, Du W, Liu D (2008) *Rhizopus oryzae* whole-cell catalyzed biodiesel production from oleic acid in *tert*-butanol medium. Energy Fuels 22:155–158
10. Yang X, Wang B, Cui F, Tan T (2005) Production of lipase by repeated batch fermentation with immobilized *Rhizopus arrhizus*. Process Biochem 40:2095–2103

11. Schuepp C, Kermasha S, Michalski MC, Morin A (1997) Production, partial purification and characterisation of lipases from *Pseudomonas fragi* CRDA 037. Process Biochem 32:225–232
12. Wang D, Xu Y, Shan T (2008) Effects of oils and oil-related substrates on the synthetic activity of membrane-bound lipase from *Rhizopus chinensis* and optimization of the lipase fermentation media. Biochem Eng J 41:30–37
13. Firestone D (2006) Physical and chemical characteristics of oils, fats and waxes, 2nd edn. AOCS Press, Champaign, p 238
14. Szczesna-Antczak M, Antczak T, Rzyska M, Modrzejewska Z, Patura J, Kalinowska H, Bielecki S (2004) Stabilization of an intracellular *Mucor circinelloides* lipase for application in non-aqueous media. J Mol Catal B Enzym 29:163–171
15. Andrade GSS, Freitas L, Oliveira PC, De Castro HF (2012) Screening, immobilization and utilization of whole cell biocatalysts to mediate the ethanolysis of babassu oil. J Mol Catal B Enzym 84:183–188
16. Levenspiel O (1972) Chemical reaction engineering, 2nd edn. Wiley, New York
17. Urioste D, Castro MBA, Biaggio FC, De Castro HF (2008) Synthesis of chromatographic standards and establishment of a method for the quantification of the fatty ester composition of biodiesel from babassu oil. Quim Nova 31:407–412
18. Carvalho AKF, Da Ros PCM, Teixeira LF, Andrade GSS, Zanin GM, De Castro HF (2013) Assessing the potential of non-edible oils and residual fat to be used as a feedstock source in the enzymatic ethanolysis reaction. Ind Crops Prod 50:485–493
19. Paiva EJM, Silva MLCP, Barboza JCS, Oliveira PC, De Castro HF, Giordani DS (2013) Non-edible babassu oil as a new source for energy production—a feasibility transesterification survey assisted by ultrasound. Ultrason Sonochem 20:833–838
20. Shimada Y, Sugihara A, Nagao T, Tominaga Y (1992) Induction of *Geotrichum candidum* lipase by long-chain fatty acids. J Ferment Bioeng 74:77–80
21. Papagianni M, Mattey M (2006) Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterization of mycelial morphology. Microb Cell Fact. doi:10.1186/1475-2859-5-3
22. Papagianni M, Moo-Young M (2002) Protease secretion in glucoamylase producer *Aspergillus niger c*ultures: fungal morphology and inoculum effects. Process Biochem 37:1271–1278
23. Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv 22:189–259
24. Teng Y, Xu Y (2008) Culture condition improvement for whole-cell lipase production in submerged fermentation by *Rhizopus chinensis* using statistical method. Bioresour Technol 99:3900–3907
25. Teng Y, Xu Y, Wang D (2009) Changes in morphology of *Rhizopus chinensis* in submerged fermentation and their effect on production of mycelium-bound lipase. Bioprocess Biosyst Eng 33:397–405
26. Fernandez-Lahore HM, Auday RM, Fraile ER, Bonino MBJ, Pirpignani L, Machalinski C, Cascone O (1999) Purification and characterization of an acid proteinase from mesophilic *Mucor* sp. solid-state cultures. J Pept Res 53:599–605
27. Soares IP, Rezende TF, Pereira RCC, Santos CG, Fortes ICP (2011) Determination of biodiesel adulteration with raw vegetable oil from ATR-FTIR data using chemometric tools. J Brazil Chem Soc 22:1229–1235
28. Souza RL, Resende WC, Barao CE, Zanin GM, De Castro HF, Santos OAA, Fricks AT, Figueiredo RT, Lima AS, Soares CMF (2012) Influence of the use of Aliquat 336 in the immobilization procedure in sol-gel of lipase from *Bacillus* sp. ITP-001. J Mol Catal B Enzym 84:152–159
29. Sun T, Du W, Liu D, Dai L (2010) Improved catalytic performance of GA cross-linking treated *Rhizopus oryzae* IFO 4697 whole cell for biodiesel production. Process Biochem 45:1192–1195