Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 34, No. 01, pp. 29 - 39, January - March, 2017
dx.doi.org/10.1590/0104-6632.20170341s20140227

SYNTHESIS, CHARACTERIZATION AND APPLICATION OF A POLYURETHANE-BASED SUPPORT FOR IMMOBILIZING MEMBRANE-BOUND LIPASE

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(Submitted: December 15, 2014 ; Revised: October 10, 2015 ; Accepted: November 17, 2015)

Abstract - This study conducted an assessment of polyurethane foams that were synthesized by one-shot process and used as a low-cost support to immobilize *Mucor circinelloides* URM 4182 whole-cells presenting high lipolytic activity. Polyols with different molecular weights (1100 to 6000 g mol^{-1}) were applied to synthesize the polymer matrix, and the agitation speed effect was used for controlling the average pore size of the investigated polyurethane foams. The physical and mechanical properties of the polymers were evaluated by standard test methods, and their morphology was identified by Scanning Electron Microscopy. The immobilization procedure efficiency was assessed by quantifying the capability of the matrices to attach the cells and the catalytic activity of the biocatalysts in both aqueous (olive oil hydrolysis) and non-aqueous media (ethanolysis of babassu oil) under single and consecutive batch runs. Although all synthesized matrices were suitable to immobilize the whole cells with high catalytic performance, a better set of parameters was attained when the polyol ether with molecular weight of 6000 g mol^{-1} and 1100 g mol^{-1} was used. Both matrices yielded immobilized biocatalysts with high hydrolysis and transesterification activities, and exhibited a satisfactory operational stability with 96% and 81% retention of their initial hydrolytic and transesterification activities after three consecutive batch runs.
Keywords: Polyurethane; Lipase; *Mucor circinelloides*.

INTRODUCTION

Hydrolytic enzymes such as lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) have been extensively used in biocatalysis. Lipases make up a versatile group of enzymes that are able to hydrolyze triglycerides at a lipid-water interface, and have a number of potential applications due to their capability of catalyzing a wide range of reactions with broad substrate specificity (Talukder *et al*., 2013). However, in several applications, the use of such enzymes is limited by economic considerations which are mainly associated with complex purification procedures and poor stability. Although an immobilization of lipases can constitute improvements in the enzyme's features, e.g., stability, activity, selectivity or specificity, such a procedure is costly and, in some cases, it promotes difficulties in the implementation of processes (Adachi *et al*., 2013). In this context, the use of intracellular lipase (whole-cell biocatalyst) is a

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major breakthrough in cost-effectiveness and sustainable biodiesel production, as it substantially reduces cost by avoiding complex isolation, purification, and extracellular lipase immobilization processes (Tamalanpudi *et al*., 2008).

Among the established whole-cell biocatalyst systems, filamentous fungi are regarded as the most robust biocatalysts for industrial applications (Romero *et al*., 2012). Utilizing fungal cells immobilized with porous BSPs (Biomass Support Particles) is an attractive technology for lowering lipase production costs, since complex purification processes are not necessary (Ban *et al*., 2002). Therefore, this technology is effective for bulk production commodities, such as biodiesel (Sun *et al*., 2011).

Several works have been reported concerning biodiesel production using whole-cells derived from *Rhizopus oryzae* (Hama *et al*., 2004; Ban *et al*., 2008), *Pseudomonas fragi* (Schuepp *et al*., 1997), *Penicillium cyclopium* (Legier and Comeau, 1992), *Aspergillus niger* (Xiao *et al*., 2010; Romero *et al*., 2012) and *Mucor circinelloides* (Antczak *et al*., 2002; Szczesna-Antczak *et al*., 2004; Andrade *et al*., 2012).

Commercial polyurethane foams – PUFs, are the most commonly used matrices for immobilizing whole-cells in almost all these studies, due to their availability at low cost and properties such as great mechanical resistance and elasticity. This polymer also presents a high porosity level (near 97%) and a large adsorption surface that reduces oxygen diffusion limitations, which is a particularly relevant factor for aerobic microorganisms (De Ory *et al*., 2006). Nevertheless, other important properties are not suitable to immobilize all kinds of microorganisms, particularly fungi that have different growth morphology.

Hence, the preparation of polyurethane foams is reported herein to be used as an affordable support to immobilize *Mucor circinelloides* URM 4182 whole-cells presenting high lipolytic activity. This filamentous fungus was selected based on earlier studies in which it was considered to be effective membrane-bound lipase producer that grows well in triacylglycerol-containing culture media with high transesterification yield (Andrade *et al*., 2012; Andrade *et al*., 2014). All PUFs were synthesized aiming at evaluating the influence of rotation speed and type of polyol to be used in each formulation. The physical-mechanical properties of the matrix were used as a parameter to select the best support for immobilizing the whole-cells. The catalytic performance of the biocatalyst was assessed in both hydrolysis and transesterification reactions. Babassu oil was chosen as feedstock in the transesterification reactions so as to enable a comparison with previous works (Andrade *et al*., 2012; 2014).

MATERIALS AND METHODS

Reagents

All reagents used in the PUFs synthesis were obtained from commercial sources. Lupranat T80A® - 80% isomer mixture 2,4- and 20% 2,6-toluene diisocyanate (TDI), Lupranol 1100® (MW = 1100 g mol^{-1}, OH value = 104 mg KOH g^{-1}), Lupranol B50® (MW = 1100 g mol^{-1}, OH value = 104 mg KOH g^{-1}), Lupranol 2090® (MW = 6000 g mol^{-1}, OH value = 28 mg KOH g^{-1}), Lupraphen 8107® (MW = 2400 g mol^{-1}, OH value = 61 mg KOH g^{-1}) and Toluene-2,4-diisocyanate (TDI) were supplied by BASF Ltd. (Guaratingueta, SP, Brazil). The catalysts tin octanoate, tertiary amine and surfactant polysiloxane copolymer were supplied by EVONIK (Americana, SP, Brazil). Distilled water was used as foaming agent and ethanol as cleaning solvent. Ethanol 99.8% and *tert*-butanol were purchased from Cromoline (Diadema, SP, Brazil). Refined, bleached and deodorized babassu oil was provided by Basf Ltd. (Jacarei, SP-Brazil) with the following properties: acid number: 0.65 mg KOH g^{-1}; peroxide value: 1.82 mEq kg^{-1}; iodine number: 25 g I_2 g^{-1}; saponification number: 238 mg KOH g^{-1} and the following composition of fatty acids (% wt): capric (3.5), caprilic (4.5), lauric (44.7), myristic (17.5), palmitic (9.7), stearic (3.1), oleic (15.2) and linoleic (1.8) and average molecular weight of TAGs: 709.9 g mol^{-1}. Commercial virgin olive oil (0.3% acidity), which was purchased in a local market, was used to determine the hydrolytic activity of the biocatalysts. All other reagents were of analytical grade.

Microorganism

All experiments were carried out using cells of the *Mucor circinelloides* URM 4182 fungus from the culture collection of URM (University of Recife Mycology) at the Federal University of Pernambuco (Recife, PE, Brazil), as previously selected by Andrade *et al*. (2012). PDA (Potato Dextrose Agar – Difco) was used as solid culture medium for fungi propagation. The liquid basal medium used for cell growth contained: soy peptone (Himedia) 70 g L^{-1}; $NaNO_3$ (Vetec) 1.0 g L^{-1}; KH_2PO_4 (Synth) 1.0 g L^{-1}; $MgSO_4.7H_2O$ (Vetec) 0.5 g L^{-1} and olive oil (Carbonell) 30 g L^{-1}.

PUFs Preparation

PUFs were prepared by a one-shot process. At first, each polyol was mixed with the tin catalyst and stirred for 20 seconds at 1000 rpm (first agitation) to ensure a homogeneous mixture, followed by the addition of the amine catalyst, surfactant and water (ASW), under the same agitation for 15 s. TDI was then added into the premixed components and stirred for 15s (second agitation) at different speeds (500 rpm, 1500 rpm and 2500 rpm). Finally, the mixture was poured into an open rectangular mold (250 mm×200 mm×250 mm) to produce free-rise foams at 70 °C for 30 min. The obtained PUFs were left at room temperature for 24 h for a complete cure before being cut to specific dimensions for the assays to be carried out. All formulations are shown in Table 1, which were selected in agreement with the technical expertise of Evonik® aiming at preparing flexible foams with similar properties to those used in previous works (Andrade *et al*., 2012; Andrade *et al*., 2014).

Immobilized Whole-Cell Biocatalyst Preparation

Polyurethane-immobilized whole-cells were prepared by inoculating fungal cells ($1x10^6$ spores/mL medium) in 250 mL conical flasks containing 100 mL of basal medium and 0.4 g of each cuboidal PUF (100 cubes of 6 mm) that were subjected to prior sterilization (121 °C/ 15 min) and incubated for 72 h at 28 °C on a reciprocal shaker (170 rpm), according to the methodology described by Andrade *et al.* (2012). The PUF-immobilized whole cell system was separated from the liquid medium by filtration, washed twice with distilled water and acetone and dried under vacuum for 24 h to attain a water content lower than 10%.

Biodiesel Synthesis

The transesterification reactions were performed in closed Erlenmeyer flasks (250 mL) containing 60 g of substrate consisting of babassu oil and anhydrous ethanol at a molar ratio of 1:6, and *tert*-butanol as solvent at 1:1 proportion. The mixtures were incubated with immobilized whole-cells at a fixed proportion of 20% wt. in relation to the total weight of reactants involved in the reaction medium (Urioste *et al*., 2008). Reactions were carried out at 35 ºC for a maximum period of 120 h in a reciprocal shaker (170 rpm).

Batch Operational Stability Tests

The biocatalyst's operational stability was assayed using the immobilized whole-cells (20% wt. of reaction medium) and substrate containing babassu oil and ethanol (molar ratio = 6) in successive batch runs (35 °C/ 120 h). At the end of each batch, the recovered immobilized whole-cells were rinsed with *tert*-butanol in order to remove any substrate or product that was eventually retained in the matrix. The recovered immobilized whole-cells were filtered under vacuum and dried to attain the required water level (lower than 10%). Then, a fresh medium was introduced in an Erlenmeyer flask containing the biocatalyst, and a new run was conducted keeping the same proportion between the reactants and the biocatalyst. Hydrolytic and transesterification activities were estimated at the end of each cycle, and expressed as $\mu mol.g^{-1}.min^{-1}$ (Kaushiva *et al*., 2000; Andrade *et al*., 2012).

Table 1: Formulations for the synthesis of PUFs.

Material	Amount (grams)	Polymer Designation						
		PL1100	PL1101	PL3000	PL3001	PL3002	PL6000	Polyester
Polyol	200	Lupranol 1100®	Lupranol 1100®	Lupranol B50®	Lupranol B50®	Lupranol B50®	Lupranol 2090®	Lupraphen 8107®
TDI	95-121	TDI 80/20® (121g)	TDI 80/20® (121g)	TDI 80/20® (104g)	TDI 80/20® (104g)	TDI 80/20® (104g)	TDI 80/20® (95g)	TDI 80/20® (116g)
Surfactant	1.6	Tegostab 8228®	Tegostab 8228®	Tegostab 8228®	Tegostab 8228®	Tegostab 8228®	Tegostab 8228®	Tegostab B8300®
Amine catalyst	0.5	Tegoamin 33®	Tegoamin 33®	Tegoamin 33®	Tegoamin 33®	Tegoamin 33®	Tegoamin 33®	Tegoamin E12®
Tin catalyst	0.3	Kosmos 29®	Kosmos 29®	Kosmos 29®	Kosmos 29®	Kosmos 29®	Kosmos 29®	Kosmos 29®
Solvent	8.0	Water	Water	Water	Water	Water	Water	Water
1st agitation (rpm)		1000	1000	1000	1000	1000	1000	1000
2nd agitation (rpm)		2500	500	2500	500	1500	2500	2500

Downstream Procedure

At the end of each batch run, the immobilized whole-cells were recovered from the reaction medium, and the organic phase was washed three times with tap water to remove the free glycerol that had been formed as by-product. Residual ethanol and *tert*-butanol were evaporated in a rotary evaporator at 100 °C for 20 min under atmospheric pressure. The remaining water was removed by adding sodium sulfate salt.

PUFs Characterization

The PUFs morphologies were surveyed using a LEO1450VP scanning electron microscope (SEM, Schott Zeiss, Brazil). The material was cut, attached in a suitable support and then sputter-coated with gold before observation. SEM micrographs of the foams were treated using the *Image J* software (National Institutes of Health, NIH) to obtain the average pore diameter.

The air permeability test was assessed according to ASTM D3574-02, and it expresses the amount of closed pore in the foam. Then, the PUFs were submitted to an air flow of 14 L min^{-1} and their flow resistance was expressed on the basis of line pressure gain, which is directly proportional to the amount of closed pores.

The apparent densities of the foams were measured according to ASTM D3574-02. The dried weight divided by the calculated volume gave the density in g cm^{-3}. Five cubic foam samples for each PUF were measured and averaged.

The water sorption and ethanol and *tert*-butanol absorption capacities were determined by modified ASTM F 716/726. The PUFs were cut into pieces of 30x30x10 mm, then weighed and immersed in the solvent for up to 72 hours in closed flasks. Periodically, each piece was taken from the flask and weighed. Both sorption and absorption capacities were calculated according to Equation (1):

$$\text{Water sorption (g / g)} = \frac{S_w - S_0}{S_0} \quad (1)$$

where S_0 is the initial dry weight of the sorbent and S_w is the wet weight of the sorbent (in the wake of water sorption). The same test was conducted with ethanol and *tert*-butanol. All analyses were performed in triplicate and the results shown are mean values with less than 5% error.

Hydrolytic Activity

The intracellular lipase activity was measured by hydrolysis using an olive oil emulsion according to the modification proposed by Andrade *et al*. (2012). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of free fatty acid per min under the assay conditions (30 °C and pH 7).

Ethyl Esters Quantification

The ethyl esters (FAEE) formed in the transesterification reaction were analyzed by FID gas chromatography (Varian CG 3800, Inc. Corporate Headquarters, 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 previously established conditions (Urioste *et al*., 2008). The theoretical ester concentrations were calculated by taking into account the fatty acid composition and initial weight mass in the reaction medium, and the FAEE yield (%) was defined as the ratio between the product and the theoretical esters concentrations (Urioste *et al*., 2008).

Purified FAAE Characterization

The absolute viscosity of FAEE was determined with a LVDV-II cone and plate spindle Brookfield viscometer (Brookfield Viscometers Ltd., U.K.) 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. Ethyl esters samples of 0.5 mL were used and measurements were replicated three times (Urioste *et al*., 2008). The density of FAEE was found with a DMA 35N EX digital densimeter (Anton Paar). The temperature was maintained at 20 °C during the assays. Ethyl esters samples of 2.0 mL were used and measurements were replicated three times.

RESULTS AND DISCUSSION

PUFs Synthesis and Characterization

The morphologies and pore sizes were affected by agitation speed, as can be observed in Figure 1 (a-g) and Table 2. For the same formulation, lower agitation speed increased the average pore size. Figure 1 a-b shows that PL1100 prepared with a second agita-

tion speed at 2500 rpm had an average pore size (0.30 ± 0.12 mm) that was smaller than that of the same formulation, renamed PL1101, with 0.57 ± 0.16 mm of average pore size at an agitation speed of 500 rpm. The same behavior was observed in the PL3000 series. Figure 1c-e shows that, for PL 3000 at 2500 rpm, an average pore size of 0.24 ± 0.14 mm was achieved. PL 3001 was prepared with an agitation speed of 500 rpm and the average pore size was 0.85 ± 0.14 mm, while PL3002 was prepared at 1500 rpm and the average pore size was 0.52 ± 0.14 mm. PL 6000 and Polyester, both prepared with an agitation speed of 2500 rpm, had an average pore size of 0.25 ± 0.10 mm and 0.32 ± 0.09 mm, respectively.

These results indicate that, keeping the same formulation, the PUF pore size can be influenced only by changing the second agitation speed. A higher agitation speed increased the nucleation of air microbubbles inside the polymer for a particular formulation. It should also be considered that as the volume of carbon dioxide (CO_2) formed is the same at all agitation speeds, the amount of CO_2 in each microbubble will be lower in the foam with a greater number of nucleated microbubbles, resulting in the formation of small cells with a greater amount of pores per area (Kaushiva *et al*., 2000).

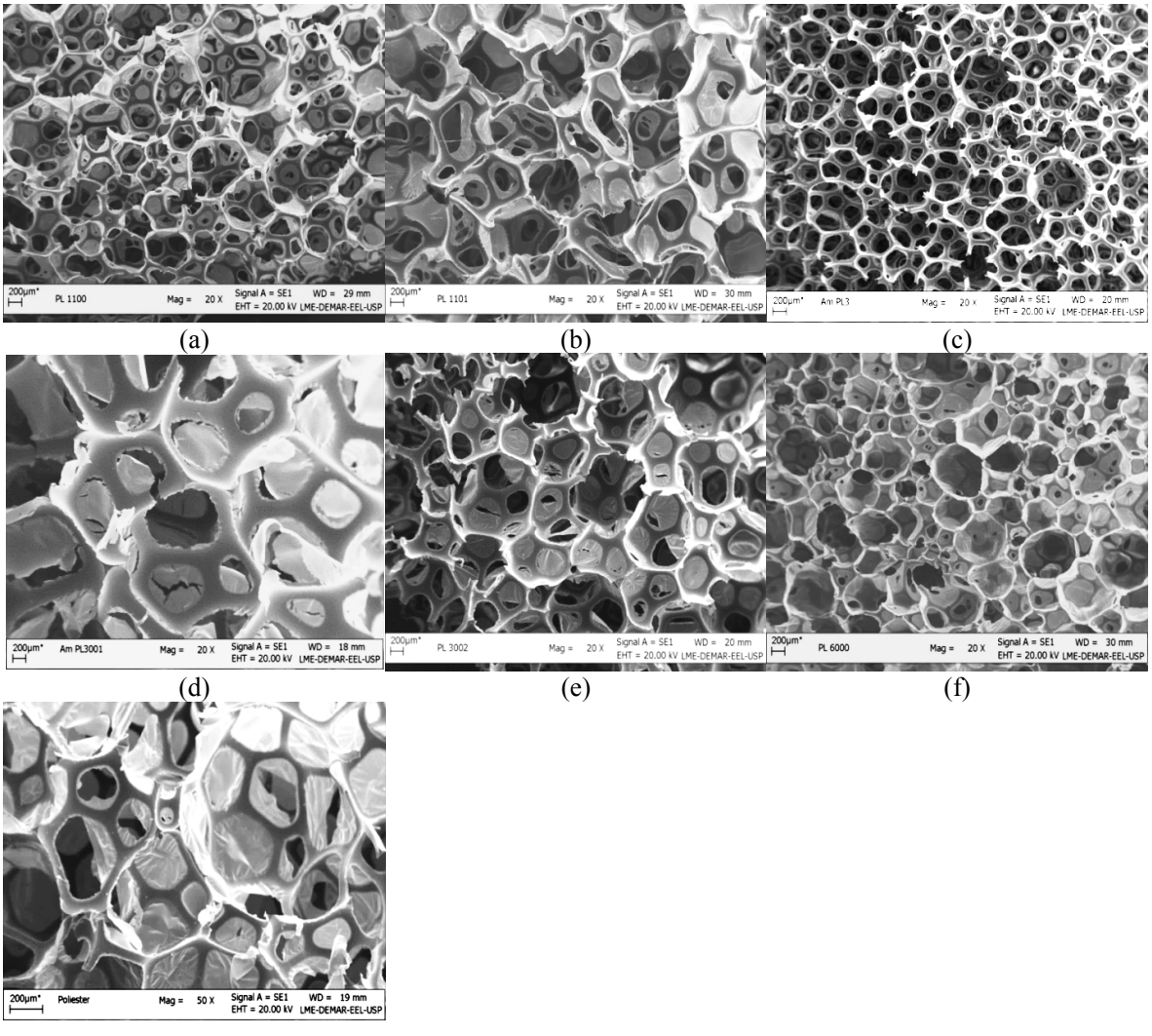

Figure 1: SEM images of PUFs made with (a) PL1100 and 2500 rpm, (b) PL1101 and 500 rpm**,** (c) PL 3000 and 2500 rpm, (d) PL3001 and 500 rpm, (e) PL3002 and 1500 rpm, (f) PL6000 and 2500 rpm, (g) Polyester and 2500 rpm.

Table 2: PUF characterization.

PUF designation	Second agitation (rpm)	Pore size (mm)	Apparent density (kg m^{-3})	Air permeability – pressure drop (mm H_2O)
PL1100	2500	0.30 ± 0.12	22.0	13 – 14
PL1101	500	0.57 ± 0.16	24.0	25 – 26
PL3000	2500	0.24 ± 0.14	33.0	6 – 7
PL3001	500	0.85 ± 0.21	25.0	3 – 4
PL3002	1500	0.52 ± 0.14	27.0	4 – 5
PL6000	2500	0.25 ± 0.10	23.0	20 – 21
Polyester	2500	0.32 ± 0.14	23.0	100 – 110

The physical and mechanical properties of the polymers were evaluated by standard test methods (Table 2). The apparent density did not show a linear relationship with, either the agitation speed or with the pore size, attaining values in the range of 22–25 kg m^{-3} (Table 2). The porosity of the PUFs was measured by an air permeability test, since there is a relation between the air flow resistance (pressure drop) and the amount of closed pores. The PUF, named "Polyester", showed the highest pressure drop, 100-110 mm H_2O, indicating a significant number of closed pores. The PL3000 series synthesized with Lupranol B50® had the greater number of open pores and the lowest pressure drop (Table 2).

The results were also analyzed statistically by ANOVA for the major effects (PUF type and second agitation). These factors revealed only a significant effect ($p < 0.05$) on the pore size. The results also demonstrate that the second agitation substantially affected the pore size of PUF.

The water diffusion within the polymeric matrixes, as shown in Figure 2, could be related to the polyol type, air permeability and pore size. Among the PUFs, Polyester showed the lowest water sorption capacity (14.4 g g^{-1}) after 72 h, probably due to the large amount of closed pores and hydrophobic character. However, both PL1100 and PL3000 showed the highest values of water sorption after the same period, 27.7 g g^{-1}. As the water sorption is related to polarity, an increase in the amount of ethylene oxide pushes the hydrophilic character of the matrix up, and the larger the amount of propylene oxide, the more evident the hydrophobic character of the matrix is going to be (Thomson, 2005). As can also be seen in Figure 2, after 48 hours of immersion, all PUFs had reached equilibrium at maximum water retention and no further sorption was observed.

Polymer matrices with large surface area, uniform porosity and good stability are usually permeable to fluids, mainly organic solvents. The solvent sorption capacities of PUFs indicated that all foams were instantly swollen with *tert*-butanol and ethanol (Figure 3), and they exhibited resistance to water diffusion. Gopakuma and Nair (2005) used polyurethane and a natural rubber block copolymer for studying the solvent diffusion at different polyol to isocyanate molar ratios. According to these authors, solvents having solubility parameter values higher or lower than those of the foam interact with only one polymeric segment of the copolymer. The authors also concluded that decreasing the polyol to isocyanate molar ratio helps increase the swelling degree of the matrices due to decreasing alofanate bonds, which are those responsible for cross-linking the polymer. A high solubility value promotes the best interaction between the flexible (polyol chains) and rigid segments of the matrix, allowing better solvent diffusion within the polymeric matrix. Considering that all foams have no diffusion problems to adsorb ethanol and *tert*-butanol (reagents used in the biodiesel synthesis), a decision was made to carry out immobilization assays using all synthesized supports, although the PL1100 and PL1101 matrices showed better efficiency in adsorbing both solvents (Figure 3).

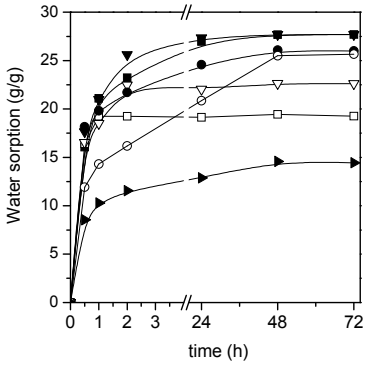

Figure 2: Water sorption capacity of PUFs made with different formulations as displayed in Table 1. Symbols: (■) PL1100, (□) PL1101, (▼) PL3000, (▽) PL3001, (●) PL3002, (○) PL6000, (►) Polyester.

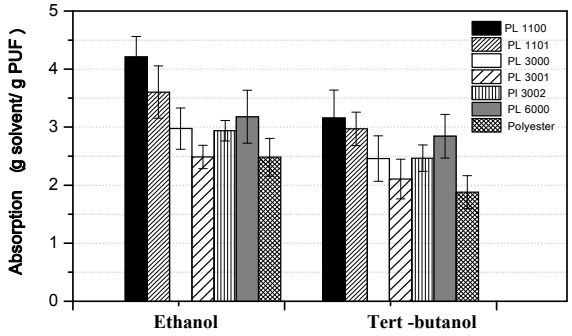

Figure 3: Solvent absorption capacity of PUFs made with different formulations as displayed in Table 1.

Immobilization of Whole Cells from *M. circinelloides* on Different PUFs

M. circinelloides cells were immobilized *in situ* on all synthesized foams according to the methodology previously described by Andrade *et al*. (2012). The immobilization with PUF was a natural consequence of cell growth during cultivation and is the most common procedure to obtain immobilized whole-cells. As can be seen in Figure 4 a-g (SEM images), strong cellular adhesions were verified on all matrices, which mean that the cells would not be easily released from the PUF particles, even under vigorous agitation. *M. circinelloides* whole-cells are fully entangled filaments that facilitate the formation of a strong biofilm inside and around PUF particles.

Intracellular lipase activities and immobilization ratios (proportional to the fixed biomass per gram of foam) are shown in Figure 5. Although all formulations were found to be suitable to immobilize *M. circinelloides* cells, the PUF formulation affected both the attachment of cells to the support and the lipolytic activities, attaining values ranging from 1.4 to 2.4 $g_{biomass}/g_{PUF}$ and lipase activities from 32 to 52 U g^{-1}, respectively.

Immobilized whole-cells on PL1100 showed the highest immobilization ratio, which is probably due to the hydrophobic character of the polyol used in this formulation, thus increasing the interaction between the cells and the matrix, as can be seen in the SEM image (Figure 4a). This can provide the immobilized biomass with additional protection against shear forces. On the other hand, the immobilization ratio could also be related to the PUF pore size, since PL3001 and PL1101 which have the highest values of average pore size showed the lowest amount of immobilized biomass.

Among the polymers, PL6000 gave the maximum intracellular lipase production (52 U g^{-1}), which was slightly superior to that of previously reported results (49 U g^{-1}) using the same fungal strain and commercial foam particles as support (Scotch-Brite^{MR})

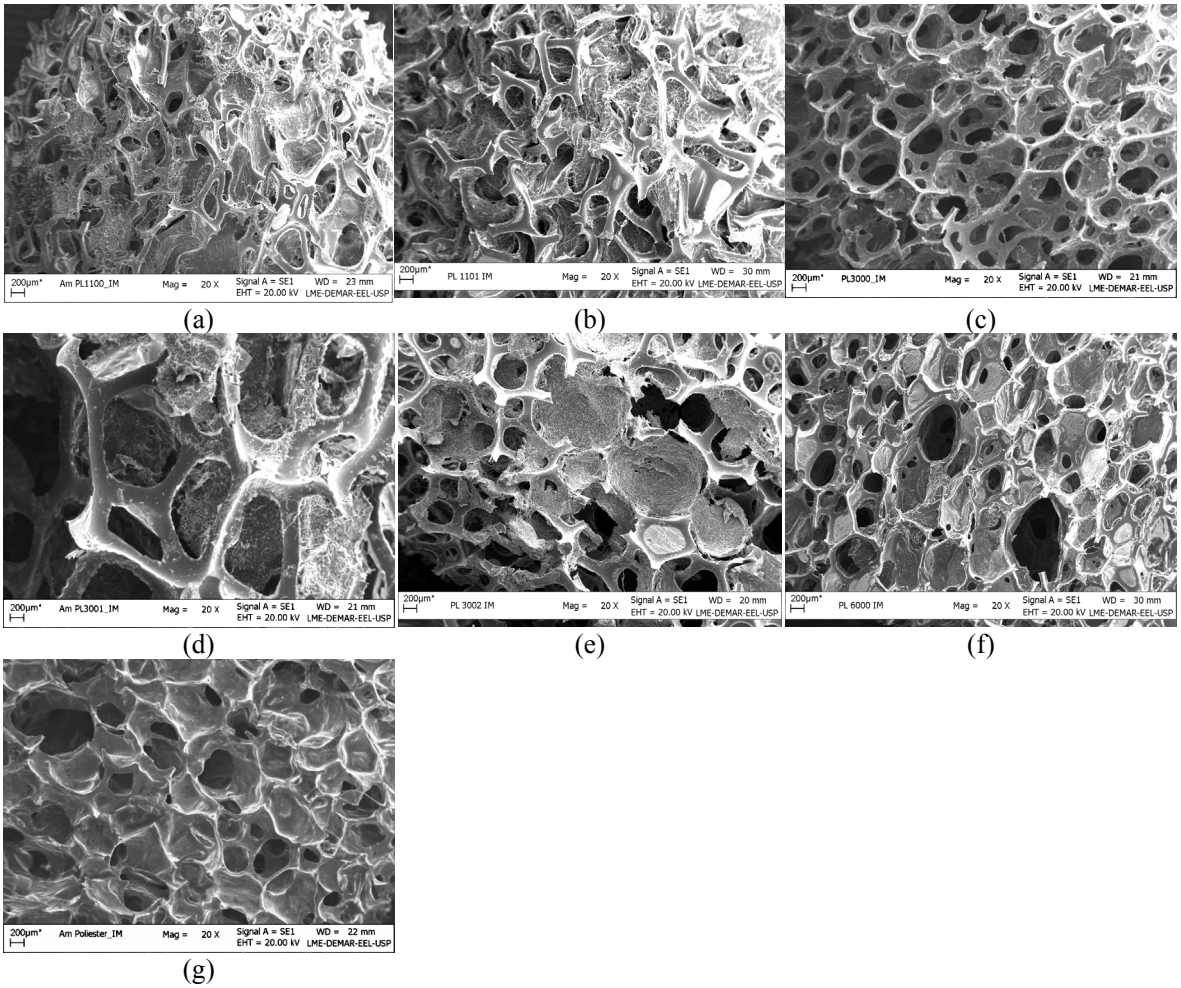

Figure 4: SEM images of *M. circinelloides* immobilized on PUFs (a) PL1100, (b) PL1101**,** (c) PL3000, (d) PL3001, (e) PL3002, (f) PL6000, (g) Polyester.

(Andrade *et al*., 2012). A remarkable cell growth (high immobilization ratios) was also achieved for PL1100 and PL 3002 matrices, although slightly lower hydrolytic activities were found (respectively 42 and 47 U g^{-1}). Such differences can be attributed to the different properties of each PUF. The matrices PL1101, PL3001 and Polyester, which have low water sorption capacity, produced immobilized catalysts with low hydrolytic activities (< 40 U g^{-1}), probably due to the mass transfer limitations of the substrate to the mycelia within the matrix pores.

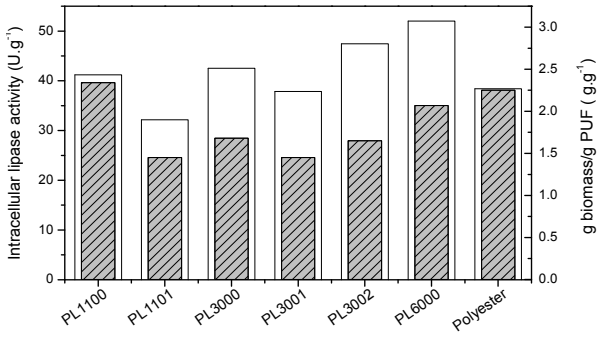

Figure 5: Intracellular lipase activity (open bar) and ratio biomass/PUF (closed bar) obtained from *M. circinelloides* immobilized on PUFs cultivated with olive oil (3%) at 30 °C and 170 rpm for 72 h.

Catalytic Performance of the Immobilized Whole-Cells

The biocatalysts catalytic performance was assessed in the transesterification reaction of babassu oil with ethanol, and the results are shown in Figure 6 and Table 3.

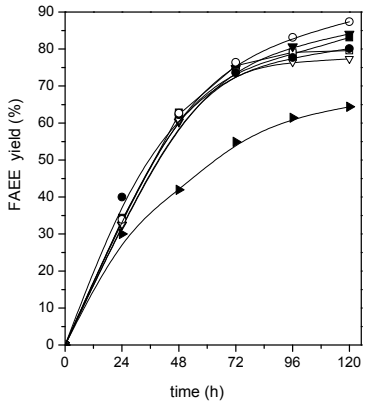

Figure 6: FAEE yields attained as function of time in the ethanolysis of babassu oil using *M. circinelloides* whole cells immobilized on (■) PL1100, (□) PL1101, (▼) PL3000, (▽) PL3001, (●) PL3002, (○) PL6000, (►) Polyester.

Table 3: Properties of the purified product obtained in the transesterification reaction of babassu oil and ethanol catalyzed by *M. circinelloides* immobilized on different PFUs

Biocatalyst immobilized in PUF	Property		
	FAEE (%)	Viscosity (mm^2 s^{-1})	Density (kg m^{-3})
PL1100	83.1 ± 6.3	5.8 ± 1.9	883
PL1101	79.5 ± 1.4	6.1 ± 0.4	887
PL3000	84.1 ± 2.7	5.6 ± 0.2	886
PL3001	77.3 ± 0.3	6.6 ± 0.9	888
PL3002	80.1 ± 4.4	6.1 ± 0.1	886
PL6000	87.3 ± 1.1	5.3 ± 0.4	884
Polyester	64.4 ± 2.3	10.4 ± 0.4	891

The reaction progress in terms of FAEE yield is displayed in Figure 6. These values indicate that both the reaction rate and ester yield are dependent on the immobilized cells activity, which is directly related to the matrix used. The highest FAEE yield (87.3%) was achieved using cells immobilized on PL6000. Slightly lower FAEE yields (83.1 and 84.1%) were achieved in the transesterification reactions mediated by cells immobilized on PL1100 and PL3000. Similar results were produced in earlier studies (Andrade *et al*., 2012), when whole-cells of *M. circinelloides* were immobilized on commercial PUF, and reached 83.2% yield in the ethanolysis of babassu oil.

Low performances were attained with the other biocatalyst, and the lowest yield (64.4%) was achieved for the cells immobilized on Polyester. This result can be explained by the physical properties of the matrix (Polyester), which has a large number of closed pores that influenced the insertion and adhesion of mycelia inside the pores. In addition, this matrix exhibited the lowest water sorption capacity, as shown in Figure 2, which indicates a hydrophobic character.

These results are favorable compared to those described in literature. Chen and Lin (2010) evaluated a circulating packed-bed bioreactor system using fibrous nonwoven fabric as matrix for immobilizing *Rhizopus oryzae* cells, and used the resulting immobilized derivative to mediate the methanolysis of soybean oil, attaining, under optimized conditions, a maximum yield of 70.8%. Athalye *et al.* (2013) immobilized *Rhizopus oryzae* cells on novel rigid polyethylene biomass supports and employed the biocatalyst in the methanolysis of cottonseed oil, reporting yields as low as 27.9%. These studies suggested that polyurethane foam is still the finest support to immobilize whole-cells for biodiesel production due to

its properties such as a very large surface area and suitable porosity to promote the cells growth.

Whole-Cells Operational Stability

The operational stability of the cells immobilized on PL1100, PL3000 and PL6000, which achieved the highest FAEE yields, were assayed under repeated batch runs. At the end of each batch run (120 h), the immobilized biocatalyst was removed from the reaction medium and treated by the previously described methodology. Hydrolytic and transesterification activities were estimated at the end of each batch and the first batch was taken as 100% active.

Figures 7 and 8 show the relative activities (hydrolysis and transesterification) as function of operational time. After three consecutive batches, a decrease in both activities for the three immobilized whole-cells was observed as a consequence of the cell weight loss during the repeated batches. For all assays, the initial weight of the immobilized cells markedly decreased (average value was estimated to be at least 30%) after three repeat cycles. Therefore, the hydrolytic activity decrease can be related to the cell exfoliation caused by agitation, resulting in a significant loss of cells during the biocatalyst recovery by filtration.

Regarding the transesterification activity, the strategy of balancing the biocatalyst loading as a means of decreasing the substrate volume proportionally enabled an assessment of the actual activity loss due to biocatalyst inhibition by the glycerol formed as byproduct in the transesterification reaction.

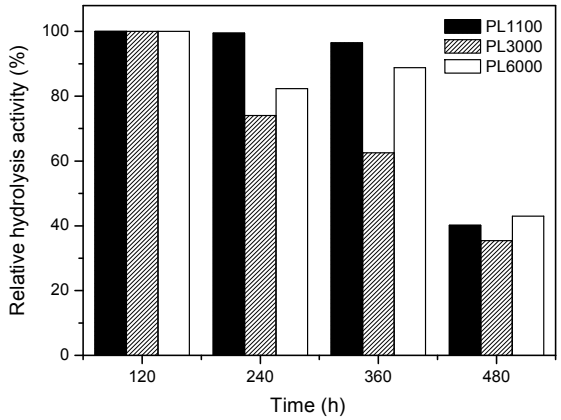

Figure 7: Relative activity of immobilized biocatalyst during consecutive batch hydrolysis runs, taking as 100% active the original activity of the whole cells immobilized on PL 1101 (42 U g^{-1}); PL 3000 (42 U g^{-1}) and PL 6000 (52 U g^{-1}).

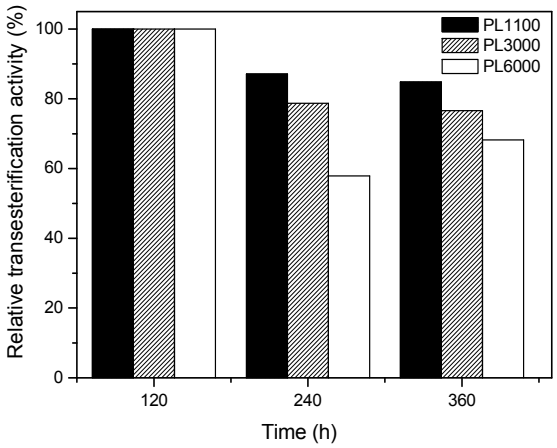

Figure 8: Relative activity of immobilized biocatalyst during consecutive batch transesterification runs, taking as 100% active the original activity of the whole cells immobilized on PL 1101 (210 μM.g^{-1}.min^{-1}); PL 3000 (225 μM g^{-1} min^{-1}); PL 6000 (240 μM g^{-1} min^{-1}).

An alternative approach to avoid the biocatalyst weight loss is to perform the assay in a basket batch reactor (Baltaru *et al*., 2009) or under a continuous run (Yoshida *et al*., 2012).

CONCLUSIONS

Polyurethane foams were synthesized, characterized, and used as support for the immobilization of *Mucor circinelloides* whole-cells and assessed as biocatalyst in the transesterification reaction of babassu oil with ethanol. PL1100 and PL1101 showed optimum interactions with ethanol and *tert*-butanol. PL6000 was the best foam in the ethanolysis and PL1100 was that with the highest operational stability. A smaller microbubble diameter resulted in good dispersion, permeability, and water sorption of the biofilm to the porous matrix. These were the most important properties for suitable nutrient diffusion, allowing whole-cell growth and intracellular lipase production. The immobilization was influenced by the pore diameter of the polymer matrix, water sorption and solvent absorption, as well as the type of polyol used in the synthesis. Furthermore, the hydrophobic and hydrophilic character of the matrix influenced the attachment of the microorganism, providing better compatibility with the reaction medium, aiding the nutrient and substrate transfers.

ACKNOWLEDGMENTS

The authors are grateful for the financial support provided by CAPES and CNPq (*Conselho Nacional*

de Desenvolvimento Científico e Tecnológico), Brazil. They would also like to acknowledge and demonstrate their appreciation for the remarkable language revision provided by FAV Language Services.

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