Appl Biochem Biotechnol (2011) 164:755–766
DOI 10.1007/s12010-011-9171-9

Concentration, Partial Characterization, and Immobilization of Lipase Extract from *P. brevicompactum* by Solid-State Fermentation of Babassu Cake and Castor Bean Cake

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Received: 7 June 2010 / Accepted: 9 January 2011 /
Published online: 22 January 2011

Abstract One relevant limitation hindering the industrial application of microbial lipases has been attributed to their production cost, which is determined by the production yield, enzyme stability among other. The objective of this work was to evaluate the concentration and immobilization of lipase extracts from *Penicillium brevicompactum* obtained by solid-state fermentation of babassu cake and castor bean cake. The precipitation with ammonium sulfate 60% of saturation of crude extract obtained with babassu cake as raw material showed an enhancement in hydrolytic and esterification activities from 31.82 to 227.57 U/g and from 170.92 to 207.40 U/g, respectively. Concentrated lipase extracts showed preference to medium-chain triglycerides and fatty acids. It is shown that the enzyme activity is maintained during storage at low temperatures (4 and −10°C) for up to 30 days. Higher esterification activities were achieved when the lipase extract was immobilized in sodium alginate and activated coal.

Keywords Lipase · Concentration · Partial characterization · Immobilization · Babassu cake · Castor bean cake

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Introduction

Lipases (E.C. 3.1.1.3) and esterases (E.C. 3.1.3.1) constitute an important group of enzymes associated to the metabolism and hydrolysis of lipids. This class of biocatalyst is found in animal and vegetal organisms and in microorganisms [1]. The catalytic properties of lipases, such as selectivity and estereoespecificity, can be controlled through manipulation of reaction conditions. The influence of the reaction medium composition on lipases properties is associated to a complex mechanism of enzymes action, which involves conformational changes in their structures [2].

One relevant limitation to industrial application of microbial lipases has been related to their production cost, which is determined by the production yield, experimental conditions of the process and enzyme stability [3]. Therefore, it is interesting to develop strategies to enhance the process productivity by employing newly isolated high-enzyme producer microorganisms as well as to perform the process optimization through manipulation of the main variables that affect the process. Besides, the application of lipases in large-scale industrial processes requires careful investigation of potential techniques suitable for the reuse and increase the enzyme stability, such as concentration/purification and immobilization strategies. The use of agroindustrial residues as raw materials can contribute to reduce the total production cost of these enzymes [4]. Additionally, lipases should be selected for each industrial application based on their substrate specificity and with regard to stability, pH, and temperature [5].

Based on the aspects mentioned above, the main objective of this work was to evaluate the concentration and immobilization of lipase extracts obtained by solid-state fermentation of *Penicillium brevicompactum* using babassu cake and castor bean cake as raw materials and then to perform a partial characterization of the concentrated lipase extracts obtained.

Materials and Methods

Microorganism

The fungus *P. brevicompactum* was previously isolated by Freire et al. from wastes from the babassu oil industry [6]. This microorganism was identified as good lipase-producer using methodologies of screening in solid and liquid media [6]. The strain was maintained in agar slants at 4°C and also at −80°C in glycerol.

Solid-State Fermentation

The substrates used for lipases production by solid-state fermentation were castor bean cake (kindly donated by Petrobras) and babassu cake (provided by Tobasa Bioindustrial de Babaçu S.A, Palmas, TO, Brazil). Both substrates were stored at −15°C and used without further treatment. The experiments for lipase production were carried out by adding aqueous solutions containing soybean oil as supplement to the raw materials (about 10 g of dry matter) and the resulting medium was then sterilized at 121°C for 20 min in conical reactors covered with hydrophobic fabric. The sterile media were then inoculated with 10^7 spores/g dry substrate, and the moisture content was adjusted according to an experimental design (Table 1). Cultivation was carried out in an incubator with humidified air injection at a fixed temperature of 30°C.

Table 1 Matrix of the full experimental design (real and coded values) with responses in terms of esterification and hydrolytic activities of the concentrated lipase extracts obtained by solid-state fermentation of babassu cake after 72 and 96 h of fermentation and *P. brevicompactum* as microorganism

Experimental conditions			Hydrolytic activity (U/g) Fermentation time (h)		Esterification activity (U/g) Fermentation time (h)	
Run	Moisture content (%)	Soybean oil (%)	72	96	72	96
1	60 (−1)	1 (−1)	0.00	52.66	28.23	77.28
2	80 (1)	1 (−1)	0.00	0.00	0.00	0.00
3	60 (−1)	3 (1)	18.10	13.39	79.95	22.98
4	80 (1)	3 (1)	33.53	5.25	207.40	106.56
5	56 (−1.41)	2 (0)	95.51	120.45	0.00	0.00
6	84 (1.41)	2 (0)	138.70	76.21	0.00	120.45
7	70 (0)	0.6 (−1.41)	227.57	19.74	38.42	60.13
8	70 (0)	3.4 (1.41)	15.00	40.56	35.50	31.73
9	70 (0)	2 (0)	45.84	30.72	39.08	40.90
10	70 (0)	2 (0)	42.97	35.17	41.09	41.20
11	70 (0)	2 (0)	48.71	39.63	42.06	39.70

Enzyme extraction at the end of the fermentation was carried out using phosphate buffer (100 mmol/L, pH 7.0) at a solid–liquid ratio of 1:5 in an orbital shaker (Marconi, MA 410, Brazil) at 200 rpm, 35°C for 30 min. After the solids' filtration, the supernatant was used for analytical assays.

Determination of Hydrolytic Activity

Hydrolytic activity of lipases was assayed using an emulsion of olive oil (5 wt.%) in Arabic gum (5 wt.%) in different buffers to evaluate the enzyme activity. After incubation for 15 min at 37°C and 160 rpm, the reaction was stopped by addition of a solution of acetone/ethanol (1:1). The fatty acids produced due to the hydrolysis were titrated with NaOH 0.05 mol/L in a pH stat (Mettler-Toledo DL50). Control assays (blanks) were carried out adding the acetone/ethanol solution right after enzyme addition. One unit of lipase activity was defined as the amount of enzyme preparation necessary to produce 1 μmol free acid/min under the assay conditions. The results are expressed in terms of units per gram of dry substrate [2].

Determination of Esterification Activity

The esterification activity of the lyophilized crude lipase extract was quantified by the synthesis reaction using oleic acid and ethanol (molar ratio 1:1) [7]. The reaction was carried out at 40°C, 160 rpm, and 40 min. Firstly, 0.1 g of the crude extract was added to the substrates and kept by the pre-determined reaction time in an orbital shaker. Aliquots of 500 μL were withdrawn from the reaction medium at the beginning and the end of the reaction. To each sample, 20 mL of acetone–ethanol solution (1:1; *v*/*v*) was added. The amount of acid consumed was determined by titrametric method using NaOH 0.035 mol/L. One unit of lipase activity was defined as the amount of dry enzyme preparation to the consumption of 1 μmol acid/min under the assay conditions. The results are expressed in terms of units per gram of dry substrate.

Concentration of the Crude Enzymatic Extracts

The concentration of crude lipase extracts was performed by addition of ammonium sulfate to 150 mL of extract until 60% of saturation. The mixture was kept at 4°C in an ice bath with constant agitation, under pH controlled to 7.0 by addition of NaOH 20%. The mixture was then transferred to centrifuge tubes and kept at −10°C for 5 h. After this time, the medium was centrifuged at 4°C and 5,400×*g* for 30 min. The supernatant was discarded and the precipitate removed by adding the minimum amount of sodium phosphate buffer 100 mM pH 7.0 necessary to resuspend the pellets [8, 9]. Samples were frozen at −80°C and lyophilized for 48 h.

Specificity of the Concentrated Enzymatic Extracts

The concentrated lyophilized lipase extracts produced by solid-state fermentation under optimized experimental conditions were characterized regarding the esterification activity under different alcohols (methanol, ethanol, *n*-propanol, and *n*-butanol) and fatty acids (lauric (12:0), oleic (18:0), and butiric (4:0)). The same extracts were also evaluated in terms of hydrolytic activity under different triglycerides using castor bean (*Ricinnus communis* L.), palm, *Jatropha curcas*, coconut, olive, and soybean oils as substrates [10–12].

Stability of the Concentrated Lipase Extracts at Low Temperatures

The stability at low temperatures was carried out by storage of lyophilized concentrated lipase extracts at 4 and −10°C. The measurement of hydrolytic and esterification activities were performed at each 10 days of storage, during a total period of 86 days.

Immobilization of the Concentrated Enzymatic Extracts

Accurel EP 100 as Support

The lyophilized preparations were solubilized in phosphate buffer (0.05 mol/L pH 7.0) and submitted to preferential immobilization by physical adsorption on hydrophobic support (Accurel® MP 1000, Nortec). Carrier preparation was performed adding 10 mL of ethanol to 1,000 mg of the carrier. After 30 min, supernatant was poured out and the carrier was washed repeatedly with distilled water until ethanol was completely removed. To the prepared support was added 50 mL of enzyme solution and kept under magnetic agitation at 5°C for 2 h. Immobilization was performed with magnetic stirring in ice cooler with aliquots sampled periodically (0, 1, 5, 10, 15, 20, 30, 60, 90, 120 min) for protein content assay. Supernatant and carrier with enzyme were also assayed for enzyme activity, as described previously.

Sodium Alginate and Activated Coal as Support

Firstly, a gel solution was prepared by adding 16.5 g of distilled water to 0.75 g of sodium alginate, followed by heating for alginate dissolution and addition of 12.5 g of sucrose. After gel cooling, 5 mL of enzymatic solution, 3.5 mL of glutaraldehyde, and 0.75 g of activated coal were added to gel solution [13]. A peristaltic pump was used for gel pumping to acetate buffer (0.1 mol/L, pH 4.8) containing sodium chloride 0.2 mol/L and 3.5% of glutaraldehyde, at 10°C under agitation.

Immobilized enzymes were kept under refrigeration for 24 h. Then, they were washed with distilled water and a last wash was carried out using acetate buffer. After this procedure, enzymes were immersed in calcium chloride solution 0.05 mol/L. Samples were taken periodically for protein content assay, following the methodology of Bradford [14]. Both the supernatant and the carrier with enzyme were also assayed for enzyme activity, as described previously.

Characterization of the Immobilized Extracts

The textural analysis of immobilized catalysts was carried out from isotherms of adsorption/desorption of N_2. Specific superficial area, average porous volume, and porous diameter were determined by AUTOSORB-1 (Quantachrome, Nova-2200e model) equipment. Before analysis, samples were treated under vacuum at 100°C for complete drying. Measurements were carried out at using liquid N_2 (at −196°C). The textural parameters were determined from the obtained isotherms. The superficial specific area was determined by the BET method and the average porous diameter by Barret, Joynere, and Halenda (BJH) method in the adsorption limit.

Results and Discussion

Concentration of Crude Enzymatic Extracts

Tables 1 and 2 present the results obtained from the execution of the full experimental design (real and coded values) with responses given in terms of esterification and hydrolytic activities of the concentrated lipase extracts obtained by solid-state fermentation of babassu cake and castor bean cake using *P. brevicompactum* as microorganism, respectively.

Table 2 Matrix of the full experimental design (real and coded values) with responses in terms of esterification and hydrolytic activities of the concentrated lipase extracts obtained by solid-state fermentation of castor bean cake after 72 and 96 h of fermentation and *P. brevicompactum* as microorganism

Experimental conditions			Hydrolytic activity (U/g) Fermentation time (h)		Esterification activity (U/g) Fermentation time (h)	
Run	Moisture content (%)	Soybean oil (%)	72	96	72	96
1	60 (−1)	1 (−1)	0.00	63.36	20.94	98.79
2	80 (1)	1 (−1)	0.00	128.26	0.00	0.00
3	60 (−1)	3 (1)	7.86	2.86	70.44	0.00
4	80 (1)	3 (1)	0.00	0.00	74.68	72.01
5	56 (−1.41)	2 (0)	28.66	115.18	98.00	44.76
6	84 (1.41)	2 (0)	0.00	0.00	0.00	77.01
7	70 (0)	0.6 (−1.41)	57.12	49.71	72.75	78.90
8	70 (0)	3.4 (1.41)	106.35	0.00	71.83	0.00
9	70 (0)	2 (0)	13.11	33.89	119.75	110.37
10	70 (0)	2 (0)	17.53	29.55	105.31	115.99
11	70 (0)	2 (0)	12.46	33.18	115.65	107.53

The results shown in Tables 1 and 2 correspond to the concentrated crude enzymatic extracts, after precipitation with ammonium sulfate (60% of saturation) during 5 h which is a condition that was established previously by Menoncin et al. [9]. A direct comparison between the results obtained for non-concentrated (results not shown) and concentrated extracts indicates that, in several experimental conditions, an enhancement in lipase activity was achieved by the precipitation step. As an example, the hydrolytic activities obtained in the experimental condition 7 for babassu cake were 31.82 and 227.57 U/g for the crude and concentrated extracts, respectively, whereas for the crude and concentrated extracts produced using castor bean cake were 0.10 and 57.12 U/g, respectively. In terms of esterification activities, the same behavior was observed: the values obtained in the experimental condition 4 of Tables 1 and 2 using babassu cake as substrate, esterification activities of 170.92 and 207.40 U/g were obtained for crude and precipitated enzymatic extract, respectively. In the same experimental condition for castor meal as substrate, activities were enhanced from 48.08 to 74.68 U/g after the precipitation step.

The concentration of saturation (60–70%) has already been described in the literature as adequate for precipitation of enzymes produced by different microorganisms and substrates [8, 15–18]. Lipases produced by *Pseudomonas aeruginosa* [19], *Pseudomonas fluorescens* [20], *Aspergillus niger* [21], *Antrodia cinnamomea* [8], and *Bacillus* sp. RSJ-1 [17] were concentrated using ammonium sulfate at 80% of saturation. Extracts obtained from *Bacillus coagulans* MTCC-6375 [16] and *A. cinnamomea* [8] could be concentrated at 30% of saturation, whereas 40% of saturation was used for lipases from *Clostridium tetanomorphum* [22] and 20% for that obtained from *Favobacterium odoratum* [23]. The concentration of crude lipase extract of *Pseudomonas* sp. (MSI057) by precipitation with ammonium sulfate at 80% of saturation resulted in a purification of 6.62-fold.

The statistical analysis (Statistica 6.0, Statsoft, USA) of the experimental data obtained for each system permitted to obtain the effect of each studied variables on the activities of precipitated enzymatic extracts. Results are presented in Figs. 1 and 2, for babassu cake and castor bean cake as substrates, respectively.

From Fig. 1(a) one can observe that for both 72 and 96 h of fermentation, the variables soybean oil concentration and moisture content presented a significant negative and positive effect on hydrolytic activities, respectively. Figure 1(b) shows that considering a 95% confidence interval (p=0.05), after 72 h of fermentation, only the quadratic term of moisture content was significant for esterification activity. For 96 h of fermentation, all process variables presented a positive significant effect on esterification activity.

Figure 2 presents the Pareto charts of the significance of process variables in the hydrolytic (A) and esterification (B) activities of the concentrated lipase extract obtained by solid-state fermentation of *P. brevicompactum* in castor bean cake, after 72 (a) and 96 (b) hours of fermentation. Inspection of this figure at 72 h of fermentation reveals that soybean oil concentration presented a positive significant effect on the hydrolytic activity of precipitated extract. For esterification activity, a positive effect of soybean oil concentration, moisture content, and also the interaction between the variables was observed for 72 h of fermentation, considering a confidence level equal to 95%.

Table 3 presents the specific esterification activities of lipase extracts obtained from solid-state fermentation of *P. brevicompactum* using babassu cake and castor bean cake as substrates, where the most promising results in terms of esterification activities obtained from the experimental design are presented. Higher specific activities were achieved for precipitated lipase extract from babassu cake.

Lower specific activities were obtained by Shu et al. (12.7 U/mg of protein) in precipitated lipase extract of *A. cinnamomea* using ammonium sulfate by 70% of saturation

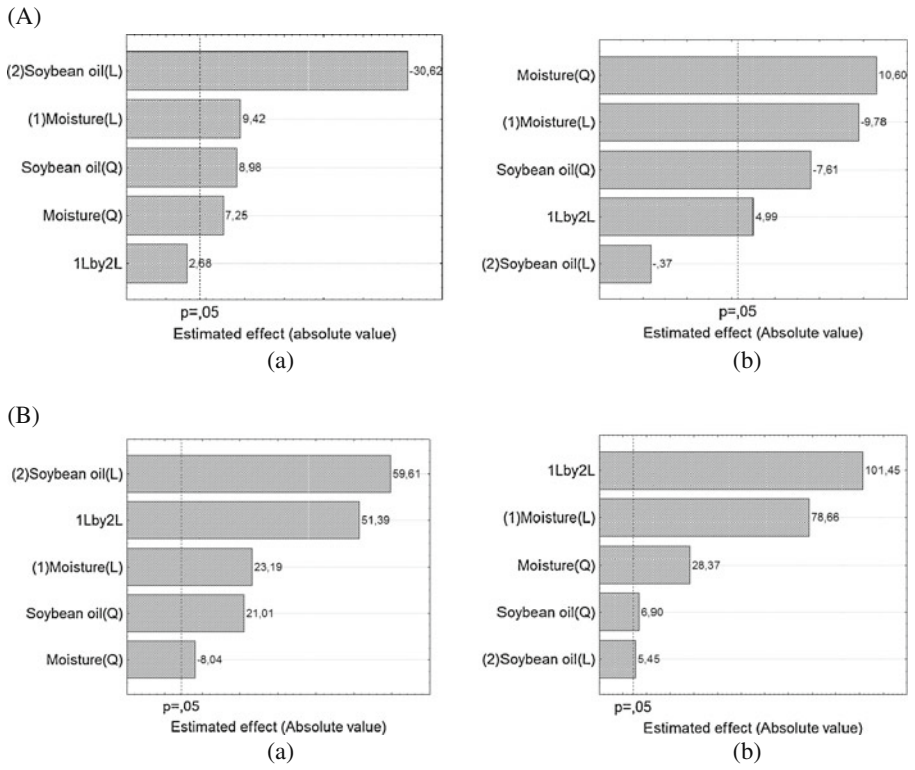

Fig. 1 Pareto charts of the effects of process variables on the hydrolytic (**a**) and esterification (**b**) activities of the concentrated lipase extract obtained by solid-state fermentation of *P. brevicompactum* in babassu cake, after 72 (**a**) and 96 (**b**) hours of fermentation

[8]. Jesus et al. achieved 14.1 U/mg of protein using ammonium sulfate 80% of saturation for precipitation of lipases from *Penicillium restrictum* [24]. Kanwar et al., in the concentration of lipase from *Pseudomonas* with ammonium sulfate 60% of saturation, reached a maximum activity of 19.46 U/mg of protein [16]. Sharma et al. obtained 44.82 U/mg of protein for precipitation of lipases from *Bacillus* sp. RSJ-1 using 30% to 70% of saturation [17]. Pastore et al., performing the concentration of lipases from *Rhizopus* sp., with ammonium sulfate at 70% of saturation, obtained activities in the order of 103 U/mg of protein [25]. Abbas et al. concentrated lipases from *Mucor* sp. using ammonium sulfate at 75% of saturation, reaching activities of 129 U/mg of protein [26]. The precipitation of cod liver lipases at 60% of saturation led to activities of 521 U/mg of protein [27].

In a general way, one may conclude that significant differences are found in the literature, showing that enzyme activities can vary as a function mainly of experimental conditions and microorganism.

Specificity of the Concentrated Lipase Extract in Terms of Esterification Activity

The influence of different alcohols on esterification activity of the lipase extract was first evaluated using oleic acid as substrate. The extract obtained from babassu cake showed higher affinity to ethanol compared to other alcohols. This result was observed for all tested

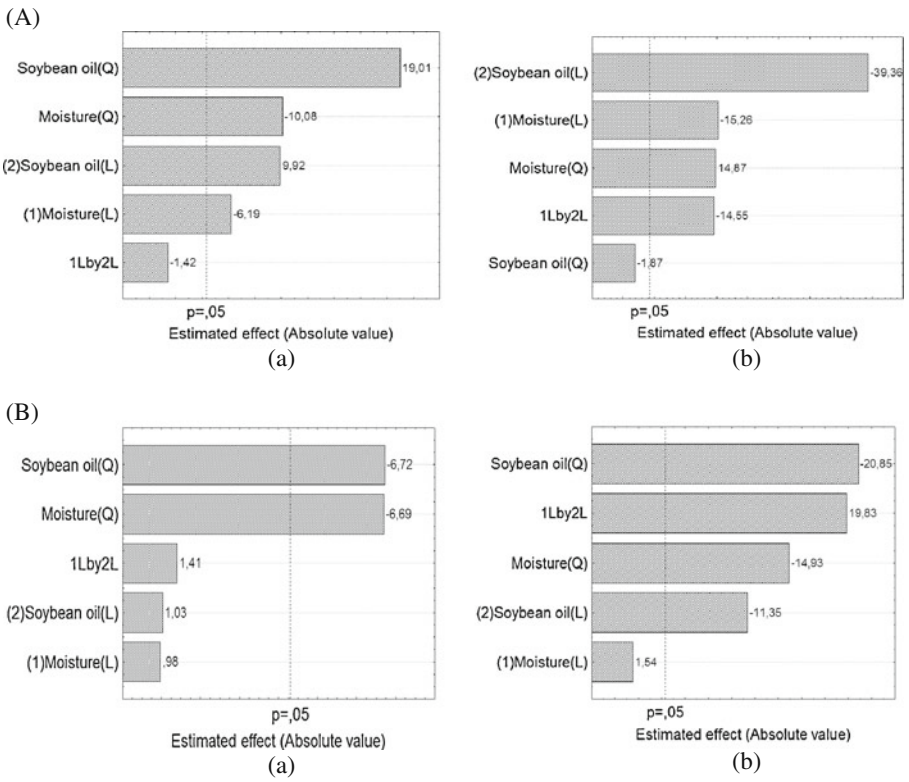

Fig. 2 Pareto charts of the effects of process variables on the hydrolytic (**a**) and esterification (**b**) activities of the concentrated lipase extract obtained by solid-state fermentation of *P. brevicompactum* in castor bean cake, after 72 (**a**) and 96 (**b**) hours of fermentation

samples. When butyric acid was used as substrate, higher affinity was observed for alcohols of long chain.

In a general way, it was possible to verify that the enzymatic concentrated extract presented preference for fatty acids of similar length chain of that from the substrate of fermentation and alcohols of long chain. Similar behavior was observed by Rigo et al. for lipases produced by solid-state fermentation of a newly isolated strain of *Penicillium* sp.

Table 3 Specific esterification activities of lipase extracts obtained from solid-state fermentation of *P. brevicompactum* using babassu cake and castor bean cake as substrate

Substrate		Esterification activity (U/g)	Protein content (mg/g)	Specific esterification activity (U/mg)
Babassu cake	Crude extract	170	4.99	34.07
	Concentrated extract	207	1.39	149.90
Castor bean cake	Crude extract	138	3.75	36.80
	Concentrated extract	110	15.28	7.20

and soybean meal as substrate. The substitution of ethanol by methanol led to a reduction of esterification activity from 104.97 to 1.57 U/g [28].

Dörmo et al. studied the influence of the alcohol chain length on the esterification activity of oleic acid using a commercial lipase (Novozym 435) [29]. These authors investigated the use of ethanol, *n*-propanol, tert-butanol, and iso-pentanol and verified higher yields when alcohols of higher chain lengths were used [29].

Rua and Ballesteros emphasized that different enzyme activities from same lipases in different reaction media can be related to the number of enzyme molecules present in the medium [30]. Besides, Bertollini et al. described that lipases can have different mechanisms able to promote their adaptation in different reaction media [31].

Specificity of the Concentrated Lipase Extract in Terms of Hydrolytic Activity

Recent studies [32] comparing the activities of 32 commercial lipases in aqueous and organic media demonstrated that there is not a fixed relation between hydrolytic and esterification activities. These results emphasize the relevance of evaluating the catalytic ability of lipases directly in organic medium. In the present work (data not shown), lipases demonstrated preference for triglycerides of medium unsaturated chain.

Taking into account some works from the literature, one can see that lipases show different preference as a function of substrate and microorganism used for enzyme production. Lipase from *Bacillus* sp. A30-1 presented higher specificity to medium chain triglycerides, as trilaurin (C12:0) and tricaprilin (C10:0) [12]. The same behavior was obtained for extracellular lipases produced by *Rhizopus oryzae* [33]. Lipases from *Aspergillus carneus* presented good activities in a wide type of triglycerides (C4 to C18) [34].

Stability of the Concentrated Lipase Extract at Low Temperatures

The stability of the concentrated lipase extract was evaluated keeping it at 4 and −10°C by measuring the hydrolytic and esterification activities each 10 up to 86 days of storage. Here, four samples were used for each storage temperature: crude and concentrated extracts of *P. brevicompactum* and babassu cake and crude and concentrated extracts of *P. brevicompactum* and castor bean cake. The extracts kept their initial hydrolytic and esterification activities until 30 and 86 days at 4°C, respectively. After these periods, the lipases lost more than 90% of their initial activities. Similar behavior was observed when the extracts were stored at −10°C.

Immobilization of Concentrated Lipase Extracts

Table 4 presents the results obtained in the immobilization of concentrated lipase extract using Accurel EP100 and sodium alginate and activated coal as supports. For this step, the experimental conditions that led to higher esterification activities in production and precipitation of lipase extracts were tested.

Higher esterification activities were achieved when the lipase extract was immobilized in sodium alginate and activated coal. This result may be of relevance due to the low cost of this support and also the lack found in the literature related to the use of activated coal for lipases immobilization [35, 36].

The immobilization yield obtained for both tested supports can be considered low compared to some results reported in the literature. Kaewthong et al. achieved immobilization yields for lipase Amano PS of 37.16 and 31.10% using Accurel EP100

Table 4 Immobilization of concentrated lipase extracts produced by SSF of agroindustrial cakes, using Accurel EP100 and sodium alginate and activated coal as supports

Experimental condition	Yield (%)	Hydrolytic activity (U/g)		Esterification activity (U/g)	
		Accurel EP100	Sodium alginate + Activated coal	Accurel EP100	Sodium alginate + Activated coal
Precipitated extract (babassu cake 72 h of fermentation 80% of moisture content 3% of soybean oil)	20.34	0.00	2.14	22.15	26.05
Crude extract (babassu cake 72 h of fermentation 70% of moisture content 3% of soybean oil)	19.89	9.13	10.46	15.09	42.79
Crude extract (castor bean cake 96 h of fermentation 70% of moisture content 2% of soybean oil)	22.56	12.45	16.89	30.12	39.67
Precipitated extract (castor bean cake 96 h of fermentation 70% of moisture content 2% of soybean oil)	27.30	8.12	5.67	35.18	45.67

(<200 μm) Accurel EP100 (200–400 μm) as supports, respectively [35]. For the same enzyme, yields of 0.79%, 3.56%, 6.42%, and 0.36% were achieved using calcium carbonate, Celite, silica gel, and activated coal as supports, respectively [35].

The lipase extract obtained in run 4 of the experimental design (Table 1), concentrated with ammonium sulfate 60% of saturation and immobilized using sodium alginate and activated coal as support was characterized in this work in terms of superficial area (A_{BET}) determined by the BET isotherm, porous volume (V_{BJH}) determined by BJH model and average porous diameter, also determined by BJH model. Structural characterization of a commercial immobilized lipase (Novozym 435) was also carried out so as to compare the results obtained for the non-commercial immobilized biocatalyst. The average porous diameter of concentrated lipase extract was lower (4.24 nm) compared to the commercial preparation (15.0 nm). It is known that small pore diameters may hinder or even avoiding the access of reactants to the internal structure of the catalyst, i.e. to the active sites of the enzyme, making difficult the diffusion, thus limiting mass transfer process and accordingly decreasing reaction yield, interfering in possible future applications of the enzyme.

Conclusions

The precipitation with ammonium sulfate 60% of crude lipase extract obtained by solid-state fermentation of *P. brevicompactum* and babassu cake as raw materials showed an enhancement on hydrolytic and esterification activities of 31.82 to 227.57 and 170.92 to 207.40 U/g, respectively. Lipase concentrated extracts showed preference to both medium chain length triglycerides and fatty acids. The enzyme storage at low temperatures permitted the observation that the extract kept its activity both at 4 and −10°C until 30 days, at least. Higher esterification activities were achieved when the lipase extract was immobilized in sodium alginate and activated coal.

Acknowledgements The authors thank PETROBRAS for the financial support of this work and scholarship.

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