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ORIGINAL PAPER

Adding value to a toxic residue from the biodiesel industry: production of two distinct pool of lipases from *Penicillium simplicissimum* in castor bean waste

Mateus G. Godoy · Melissa L. E. Gutarra · Aline M. Castro · Olga L. T. Machado · Denise M. G. Freire

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Abstract In countries with a strong agricultural base, such as Brazil, the generation of solid residues is very high. In some cases, these wastes present no utility due to their toxic and allergenic compounds, and so are an environmental concern. The castor bean (*Ricinus communis*) is a promising candidate for biodiesel production. From the biodiesel production process developed in the Petrobras Research Center using castor bean seeds, a toxic and alkaline waste is produced. The use of agroindustrial wastes in solid-state fermentation (SSF) is a very interesting alternative for obtaining enzymes at low cost. Therefore, in this work, castor bean waste was used, without any treatment, as a culture medium for fungal growth and lipase production. The fungus *Penicillium simplicissimum* was able to grow and produce an enzyme in this waste. In order to maximize the enzyme production, two sequential designs–Plackett-Burman (variable screening) followed by central composite rotatable design (CCRD)—were carried out, attaining a considerable increase in lipase production, reaching an activity of 155.0 U/g after 96 h of fermentation. The use of experimental design strategy was efficient, leading to an increase of 340% in the lipase production. Zymography showed the presence of different lipases in the crude extract. The partial characterization of such extract showed the occurrence of two lipase pools with distinct characteristics of pH and temperature of action: one group with optimal action at pH 6.5 and 45°C and another one at pH 9.0 and 25°C. These results demonstrate how to add value to a toxic and worthless residue through the production of lipases with distinct characteristics. This pool of enzymes, produced through a low cost methodology, can be applied in different areas of biotechnology.

Keywords Lipase · Solid-state fermentation · Castor bean · Optimization

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M. G. Godoy (✉) · M. L. E. Gutarra · D. M. G. Freire (✉)
Departamento de Bioquímica, Laboratório de Biotecnologia Microbiana - 549-1 e 2, Instituto de Química, Centro de Tecnologia, Universidade Federal do Rio de Janeiro, Av. Athos da Silveira Ramos, 149, Bloco A, Rio de Janeiro, RJ CEP 21941-909, Brazil
e-mail: godoy@iq.ufrj.br

D. M. G. Freire
e-mail: freire@iq.ufrj.br

A. M. Castro
Cenpes—PETROBRAS, Rio de Janeiro, RJ, Brazil

O. L. T. Machado
Universidade Estadual do Norte Fluminense, Centro de Biociências e Biotecnologia, Campos dos Goytacazes, RJ, Brazil

Introduction

Lipases are glycerol ester hydrolases (EC 3.1.1.3) defined as enzymes that catalyze the hydrolysis of carboxylic ester bonds present in triacylglycerols, releasing diacylglycerols, monoacylglycerols, glycerol and fatty acids [34]. However, these enzymes are also able to catalyze synthesis reactions such as esterification, acidolysis, alcoholysis, interesterification and aminolysis in non-aqueous environments [18]. Since the lipases are a product of industrial interest, their production must be combined with cost reduction, which can be achieved through the use of low cost culture media (residues) from agro-industry. In some applications of this enzyme, e.g. in wastewater treatment and production of

biodiesel, the production cost of the biocatalyst is a limiting factor in the viability of these industrial processes [6].

One way to obtain low cost lipases is through a process named solid-state fermentation (SSF) [6]. The SSF process is basically the use of a solid culture medium as a nutrient source and as a support to microorganism growth. The amount of water in this solid matrix must be enough for the growth and metabolism of the cells, but must not exceed the maximum water retention capacity of its matrix, i.e., this process must be carried out in the absence of or with a minimum of free water [26, 31].

Another way to reduce the cost of enzyme production processes is to obtain high enzymatic activity, which can be reached through the optimization of production. Experimental design techniques are widely used as tools in the optimization process of enzyme production [4, 13, 19, 22, 36, 38]. Such techniques allow researchers to determine the optimum conditions for reaching high activity levels and for verifying possible interaction effects between the studied variables.

Several studies have been carried out to obtain lipases by SSF using different agro-industrial residues, such as wheat bran [35], babassu cake [16, 17], *Jatropha* cake [25], sugarcane bagasse [8, 32], soybean meal [38], olive cake [8], and so on. There are several residues from biodiesel production, arising from the oil extraction of soybean, castor bean and *Jatropha curcas* seeds, among others [39]. The PETROBRAS Research Center (Cenpes) developed a process of biodiesel production from castor bean seeds [21] in which a residue with no utility is generated, due its toxicity and alkalinity, called castor bean waste. Godoy et al. [11] used this residue as a solid culture medium by SSF, eliminating its toxicity and reducing its allergenicity. In the present study, the castor bean waste was used as a solid matrix for *Penicillium simplicissimum* lipase production. A screening experimental design (Plackett-Burman) was initially used to determine significant factors, followed by a Central Composite Rotatable Design to optimize the culture conditions. In addition, the crude extract was partially characterized respecting the specificity of lipase and its optimum pH and temperature of reaction.

Materials and methods

Microorganism and propagation

The *Penicillium simplicissimum* strain was selected by Gutarra et al. [14] as a promising lipase producer in solid-state fermentation. To obtain spores, the fungal strain was propagated at 30°C for 7 days in a medium with the following composition (% w/v): soluble starch 2.0; $MgSO_4 \cdot 7H_2O$ 0.025; KH_2PO_4 0.05; $CaCO_3$ 0.5; yeast extract 0.1; olive oil 1.0; and agar 1.0. The spores were suspended in phosphate buffer (100 mM, pH 7) and counted in a cell counting chamber.

Solid-state fermentation

Castor bean waste, a solid residue from biodiesel production [21], was used as the solid-state fermentation culture medium. The waste was ground in a laboratory knife mill and separated in a sieve shaker. Final waste particle sizes ranged from 0.42–0.60, 0.60–0.85 and 0.85–1.18 mm of diameter. The castor bean medium was supplemented with sugar cane molasses of different concentrations and moisturized to different percentages (w/w). The fermentations were carried out in lab-scale, tray-type bioreactors containing 20 g of waste. The medium was inoculated and incubated at 30°C in a climatic chamber with relative air humidity set to 95%. Fermentation samples (whole trays) were removed at 24-h intervals for up to 120 h.

Glucosamine determination

Biomass quantification was done indirectly through the determination of N-acetylglucosamine in the fungal biomass. The experimental procedure to determine N-acetylglucosamine followed the method described by Aidoo et al. [1]. Thus, biomass concentration was indirectly expressed as mg of N-acetylglucosamine per gram of castor bean waste (mg/g).

Enzyme extraction

After fermentation, phosphate buffer (100 mM, pH 7.0, 5 mL/g) was added to each tray containing the fermented solids. The enzyme extraction was carried out in a rotary shaker at 35°C and 200 rpm for 20 min. Afterwards solid-liquid separation was done by pressing followed by centrifugation at 2000 *g* for 5 min [12]. The supernatant was used for lipase activity determination and then stored at −20°C for further analysis.

Protein determination

The protein content of the crude enzyme extract was determined by Bradford protein assay [2] and was expressed as mg of total proteins per gram of castor bean waste.

Protease activity

Protease activity was determined by the method described by Charney and Tomarelli [7], based on the formation of stained proteins derived from the digestion of azocasein

solution precipitated with trichloroacetic acid (TCA). The absorbance of this solution was then determined in a spectrophotometer at 428 nm. One unit of protease activity (U) was defined as the amount of enzyme causing an absorbance unit difference between the sample and its corresponding control per minute under the described assay conditions. Enzyme activity was expressed as units per gram of the initial dry solid medium.

Lipase activity determination

Lipase activity was measured by two different ways: spectrophotometric and titrimetric methods. The first one is a rapid and sensitive methodology and it was used throughout the enzyme production optimization assays. However, as the substrates used in the spectrophotometric method (*p*-NP esters) are unstable at high temperatures and present low absorbance at alkaline pH values, the titrimetric method was used to study the effects of temperature and pH on the lipase activity.

Spectrophotometric method

Lipase activity was measured using *p*-nitrophenyl laurate (*p*NP-laurate) as substrate. The enzyme activity was determined by the addition of 0.05 mL of the crude enzyme to a solution of 2.2 mL of 25 mM phosphate buffer (pH 7.0) and 0.25 mL of 2.5 mM *p*NP-laurate. The hydrolysis reaction was carried out at 30°C and measured over time up to 10 min at 412 nm. One unit of lipase activity is defined as the amount of enzyme which releases 1 μmole of *p*-nitrophenol under assay conditions. Enzyme activity was expressed as units per gram of castor bean waste.

The specific activity was calculated as the ratio of lipase activity (U/g) and protein content (mg/g), being expressed as units per mg of total proteins.

Titrimetric method

The enzyme extract (1 mL) was added to an emulsion (19 mL) of 5% (w/v) olive oil and 5% (w/v) arabic gum in 120 mM Universal Buffer [3] at different pH values, and incubated at 35°C and 200 rpm for 15 min. The reaction time was fixed after initial rates were determined. The reaction was interrupted by the addition of an acetone-ethanol mixture (1:1 v/v), which also promoted the extraction of free fatty acids. These fatty acids were titrated with a pH-stat (Mettler Toledo) using 0.04 N NaOH up to a final pH of 11. Reaction blanks were carried out adding the acetone-ethanol mixture prior to the enzyme extract [10]. One lipase unit was defined as the enzyme amount that causes the release of 1 μmole of fatty acids per minute, under assay conditions. Enzyme activity was expressed as units per gram of castor bean waste.

Temperature and pH effects on lipase activity

A Central Composite Rotatable Design (CCRD) was used to optimize the reaction conditions (temperature and pH). The tested temperatures were 25, 28, 35, 42 and 45°C and pH of 6.50, 6.86, 7.75, 8.64 and 9.0. In this case, the lipase activity was determined by the titrimetric method, as previously described, due to the low stability of *p*-NP esters (spectrophotometric method) towards alkaline pH and high temperatures. The statistical analysis of the results was done with the software Statistica 7.0 (Statsoft, USA).

Zymography

In situ lipase activity was detected by zymography following non-denaturing PAGE [23]. After electrophoresis, the lipase activity bands were identified by a zymogram using α-naphthyl acetate 0.02 % and Fast Blue RR salt 0.05% in 0.1 M sodium phosphate buffer, pH 6.2 [20].

Fatty-acid chain length preference of *P. simplicissimum* lipase

Lipase activity was determined by the spectrophotometric method, as described before, using different *p*NP-esters (*p*NP-butyrate, C4:0; *p*NP-caprilate, C8:0; *p*NP-laurate, C12:0; *p*NP-palmitate, C16:0, all from Sigma-Aldrich) at 30°C and pH 7.0.

Results and discussion

Optimization of lipase production in SSF

In previous work, Godoy et al. [11] used washed castor bean waste to produce lipases, reaching good activity levels (44.8 U/g). In the present work, the castor bean waste was used without any pretreatment, avoiding one more stage in the process. For this, a Plackett-Burman experimental design with 12 assays (PB12) was carried out, in order to evaluate the effects on the lipase production of the variables inoculum concentration (IC), molasses concentration (MC), initial moisture (IM) and particle size (PS) of the solids. Through this experimental design technique, it is possible to identify the variables that had the most influence on lipase activity and determine their best ranges.

The fungus *Penicillium simplicissimum* was able to grow and to produce lipase using *in natura* castor bean waste as nutrient (Table 1). The real and coded experimental conditions and values of maximum lipase activity

Table 1 Experimental values of maximum lipase activity for the different experimental conditions (real variable values and coded levels in parenthesis) of the PB12

Assay	IC	MC	IM	PS	Maximum activity (U/g)
1	10^8 (1)	2 (−1)	42 (1)	425–600 (−1)	7.00
2	10^8 (1)	10 (1)	34 (−1)	850–1180 (1)	1.56
3	10^7 (−1)	10 (1)	42 (1)	425–600 (−1)	1.24
4	10^8 (1)	2 (−1)	42 (1)	850–1180 (1)	22.97
5	10^8 (1)	10 (1)	34 (−1)	850–1180 (1)	1.56
6	10^8 (1)	10 (1)	42 (1)	425–600 (−1)	5.08
7	10^7 (−1)	10 (1)	42 (1)	850–1180 (1)	9.49
8	10^7 (−1)	2 (−1)	42 (1)	850–1180 (1)	10.86
9	10^7 (−1)	2 (−1)	34 (−1)	850–1180 (1)	0.88
10	10^8 (1)	2 (−1)	34 (−1)	425–600 (−1)	1.01
11	10^7 (−1)	10 (1)	34 (−1)	425–600 (−1)	0.47
12	10^7 (−1)	2 (−1)	34 (−1)	425–600 (−1)	0.15
13 (C)	5.5×10^7 (0)	6 (0)	38 (0)	600–850 (0)	4.85
14 (C)	5.5×10^7 (0)	6 (0)	38 (0)	600–850 (0)	3.58
15 (C)	5.5×10^7 (0)	6 (0)	38 (0)	600–850 (0)	5.82

IC inoculum concentration (spores/g), *MC* molasses concentration (%), *IM* initial moisture (%), *PS* particle size (μm)

(U/g) obtained under different assay conditions are shown in Table 1. The highest lipase activity found in this first experimental design was about 23 U/g (assay 4).

After statistical analysis of the responses obtained, it was possible to identify the variables that had effects on lipase production by the *P. simplicissimum* in the *in natura* castor bean waste. Using a *P*-value less than 0.1, the analysis indicated that only the inoculum concentration showed no statistically significant effect on the maximum lipase activity. Therefore, it would be possible to use any concentration within the studied range. The use of a high concentration of spores could be a problem in the scaling-up process. So, the use of lower concentration (10^7 spores/g) is more interesting. Particle size showed a positive effect on lipase production, indicating better results with particles between 850 and 1180 μm. This result is possibly related to the compaction of the fermentation bed which occurred with smaller particles, whereas larger particles avoid compaction, thus allowing greater aeration. However, particle sizes above 1180 μm represent only remnants of seed husks, besides being a small portion compared to all the waste (about 5%). In further experiments, it was found that there are no statistically significant differences in the lipase production when particles less than or equal to 1180 μm are used (data not shown). So, a wider range of particle size (0–1180 mm) was employed in the following experimental design, resulting in an almost complete recovery of waste (about 95%). The initial moisture showed the greatest and a positive statistically significant effect on the lipase production. Moisture and, consequently, water activity are the parameters that affect microbial growth in SSF. Using the range of particle sizes less than or equal to 1180 μm, it was possible to increase moisture content range to a maximum of 55% in the next experimental design. The molasses concentration showed a negative effect on the studied response, indicating that 2%, the shift to a shorter range, or even no use of molasses would lead to higher lipase production.

After this first step for variable screening through PB12, a Central Composite Rotatable Design (CCRD) was used to optimize the lipase production. In CCRD, the concentration of molasses and initial moisture content ranged from 0–4% and from 42–55%, respectively (Table 2). As above mentioned, the particle size and inoculum concentration were fixed at 0–1180 μm and 10^7 spores/g, respectively.

The maximum lipase activity obtained in each assay and the experimental conditions of the CCRD are presented in

Table 2 Experimental values of lipase activity for the different experimental conditions (real variable values and coded levels in parenthesis) of the Central Composite Rotatable Design (CCRD)

Assay	MC	IM	Maximum activity (U/g)
1	0.58 (−1)	43.9 (−1)	99.7
2	3.42 (1)	43.9 (−1)	106.9
3	0.58 (−1)	53.1 (1)	86.5
4	3.42 (1)	53.1 (1)	110.4
5	0.00 (−1.41)	48.5 (0)	107.2
6	4.00 (1.41)	48.5 (0)	106.5
7	2.00 (0)	42.0 (−1.41)	25.4
8	2.00 (0)	55.0 (1.41)	8.1
9 (C)	2.00 (0)	48.5 (0)	127.9
10 (C)	2.00 (0)	48.5 (0)	117.4
11 (C)	2.00 (0)	48.5 (0)	105.2

MC molasses concentration (%), *IM* initial moisture (%)

Table 2. The best results were obtained in the central points. Lipase activities above 100 U/g were reached under different experiment conditions. Statistical analysis of experimental data allowed us to estimate the effects of both variables and the interactions between them. To analyze the significance of the variables' effects, the *t*-test and *P*-value were used. Using a 10% level of significance ($P < 0.1$), only the moisture (quadratic term) showed a significant effect on the lipase activity. Using regression analysis, a second-order model equation (Eq. 1) was obtained for the maximum lipase activity (A) as a function of the statistically significant parameters ($P < 0.1$), which was the initial moisture (quadratic term).

$$A(\text{U/g}) = 121.32 - 41.79\,\text{IM}^2 \quad (1)$$

The analysis of variance (ANOVA) indicated that the generated model (Eq. 1) was statistically significant. A coefficient of determination equal to 0.73 is considered reasonable for this type of process and, taking into account the inherent variability of bioprocesses, does not invalidate the generated model. The *F*-test was highly significant for the model, showing the calculated critical value 7.2 times higher than the tabulated F ($F_{0.1;1;9} = 3.36$). Thus, it is possible to construct a response surface (Fig. 1) from the model.

Higher lipase activities are achieved when the moisture is around 48.5% (Aw = 0.999). Moisture levels above 52% and below 45% begin to affect the fermentation process (Fig. 1). Although there is no visible water in the process below 55% moisture, excessive moisture seems to interfere in lipase production by the fungus. An excess of moisture can lead to compression of the solid matrix, hindering the dispersion of oxygen in the medium [24, 30]. Likewise, very low moisture (below 45%) also leads to a decrease in lipase production by the fungus in the castor bean waste. The moisture, and consequently the water activity, are parameters that affect the microbial growth in SSF. Bacteria usually grow at high values of water activity, while some filamentous fungi and yeasts can grow in lower values (0.6–0.7) [28]. Although the group of fungi grows well under low moisture conditions, low moisture levels affect the production of several metabolites [9].

The use of molasses as a supplement in the fermentation was a non-significant variable in this process. Thus, the CCRD indicates that it is possible to use any concentration value of molasses within the studied range (0–4%), without significantly affecting the lipase production. So, it is possible to eliminate supplementation of the culture medium with molasses, thus decreasing the cost and complexity of the process.

Through the employment of the experimental design tool, it was possible to optimize lipase production by the fungus *P. simplicissimum* in the non-treated castor bean waste, reaching activities above 100 U/g. In order to validate the model generated for the production of lipase, a kinetics of lipase production was carried out in the optimum conditions found in the PB12 (IC: 10^7 spores/g, PS: 0–1180 μm) and CCRD (IM: 48.5% and without the addition of molasses) (Fig. 2). Some parameters, such as protease production and fungal growth (through the dosage of N-acetylglucosamine), were also evaluated up to 120 h of fermentation (Fig. 2).

After the optimization process, the fungus *P. simplicissimum* showed maximum lipase activity of about 155.0 U/g (Fig. 2) and specific activity of 9.0 U/mg of total protein after 96 h of fermentation. The maximum achieved yield was 2.1 U/g h at 72 h of fermentation. The lipase production by the fungus was more efficient in the non-treated castor bean waste than in the washed castor bean waste [11], with a maximum activity about 346%

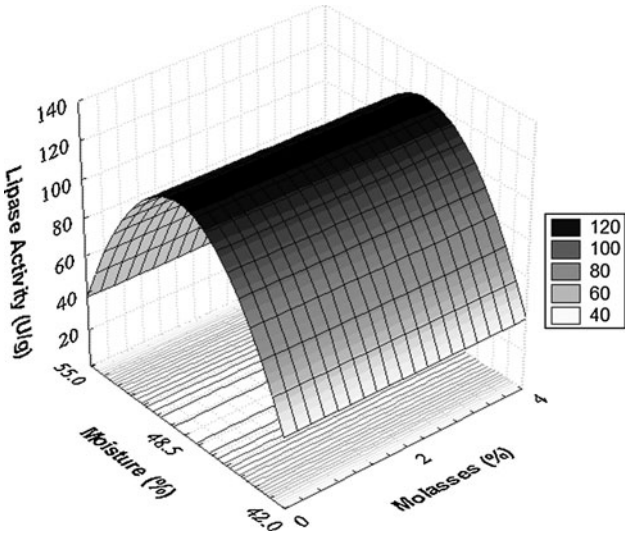

Fig. 1 Response surface diagram for lipase activity as a function of initial moisture and molasses concentration

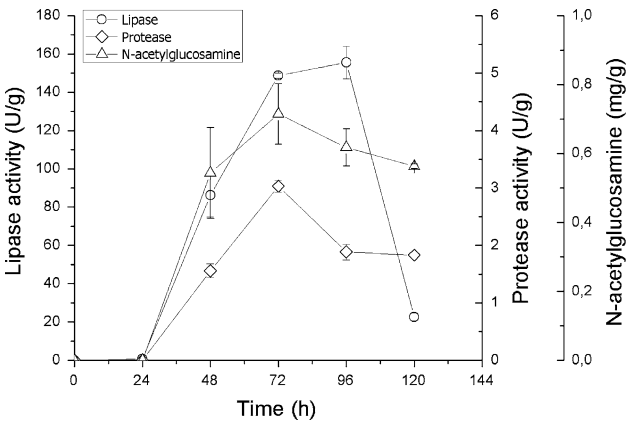

Fig. 2 Profile of growth, lipase and protease production by *P. simplicissimum* in solid-state fermentation of castor bean waste

higher. This increased production may be associated with omission of the treatment process, in which part of the nutrients present in the waste are carried away by the water used in the washing step prior to fermentation. Thus, the non-treated waste probably has a greater variety and concentration of nutrients than the washed waste, furthering the *P. simplicissimum* growth. In babassu cake, lipase production by the same fungus was found to be associated with the growth [15]. In the present work, the lipase production using castor bean waste also appears to be associated with cell growth (Fig. 2).

Solid state fermentation uses agro-industrial solid residues, which present a very complex composition. Microorganisms that grow in this type of process need to produce several enzymes able to degrade and make available the nutrients present in the culture medium, among them proteases [12, 27]. Depending on the application, this enzyme pool may be desirable, as in wastewater treatment [5, 33, 37] or in the leather industry [29]. On the other hand, the presence of proteases may be undesirable because they can catalyze the hydrolysis of the produced lipases, generating an unstable product. Thus, protease production was also assessed throughout the fermentation (Fig. 2). The highest protease production was obtained at 72 h of fermentation, with an activity of 3.0 U/g. This is a low protease production when compared to others in SSF, and about three times lower than that found, for instance, by Rosa et al. [33] with *Penicillium restrictum* in babassu cake. Between 72 and 96 h of fermentation, the amount of produced lipase was stable at around 150.0 U/g, with a strong decrease at 120 h. This decrease in the amount of lipase could be explained by the protease production by the fungus in the culture medium (Fig. 2), which could be acting on the lipases after a shortage of nutrients in the later time of fermentation [12], although this effect is much less frequent in SSF [40]. Rosa et al. [33], using babassu cake as culture medium, obtained a maximum production of lipase and protease by *Penicillium restrictum* in 20 and 24 h of fermentation, respectively, with a large decrease of lipase after that period, whereas the protease remained constant. The authors attribute the decrease of lipase amount to proteolysis or pH increase, as previously observed by Freire et al. [10] and Gombert et al. [12].

Temperature and pH effects on lipase activity

After optimizing lipase production, a CCRD was carried out to evaluate the optimum conditions of temperature and pH for lipases present in the lyophilized extract (Table 3). The studied pH and temperature ranges were from 6.5 to 9.0 and 25 to 45°C, respectively.

A statistical analysis was carried out to estimate the variable effects and the interactions between them. The effects of standardized variables (*t*-values) and significance probability test (*P*-value) were used to assess the effects of pH and temperature (*T*) on the lipase activity. Using a 10% level of significance ($P < 0.1$), it was observed that the pH (quadratic term—*Q*), temperature (linear term—*L*) and the interaction pH-temperature had significant effects on the lipase activity.

Table 3 Experimental values of lipase activity for the different experimental conditions (real variable values and coded levels in parenthesis) of the Central Composite Rotatable Design (CCRD) for partial enzyme characterization

Assay	pH	Temperture (°C)	Lipase activity (U/g)[a]
1	6.86 (−1)	28 (−1)	18.3
2	8.64 (1)	28 (−1)	24.7
3	6.86 (−1)	42 (1)	36.8
4	8.64 (1)	42 (1)	20.8
5	6.50 (−1.41)	35 (0)	31.9
6	9.00 (1.41)	35 (0)	31.5
7	7.75 (0)	25 (−1.41)	18.6
8	7.75 (0)	45 (1.41)	20.4
9 (C)	7.75 (0)	35 (0)	16.3
10 (C)	7.75 (0)	35 (0)	15.5
11 (C)	7.75 (0)	35 (0)	18.0

[a] Lipase activity was measured by the titrimetric method and expressed in units per gram of lyophilized mass

Based on the results, it was possible to construct an empirical quadratic model (Eq. 2) to study the response using the significant coded variables, where *LA* is the lipase activity (expressed in units per gram of lyophilized extract) and *T* is the temperature.

$$LA(\mathrm{U/g}) = 16.6 + 7.5\,\mathrm{pH}^2 + 2.2\,T - 5.6\,\mathrm{pH}\ \mathrm{T} \qquad (2)$$

The generated model was considered predictive by analysis of variance (ANOVA), since it showed a good coefficient of determination ($R^2 = 0.896$) and the *F* test value (20.13) was 6.5 times higher than the critical value (3.07). Based on these observations, the model was used to generate the response surface (Fig. 3) for lipase activity.

The response surface (Fig. 3) shows the trend of two optimum conditions of temperature and pH: one at pH 6.5 (−1.41) and temperature 45°C (+1.41) and another at pH 9.0 (+1.41) and temperature 25°C (−1.41). This behavior indicates the presence of two lipases or two pools of lipases with distinct characteristics of optimum temperature and pH. In order to validate the generated model and to verify the tendency of two optimum reaction conditions, two assays in conditions closer to the optimum were carried out (Table 4). A good correlation between the predicted values and the real results was observed, confirming the good fit of the model.

In order to verify the presence of more than one lipase in the crude enzyme extract, a zymogram using α-naphthyl

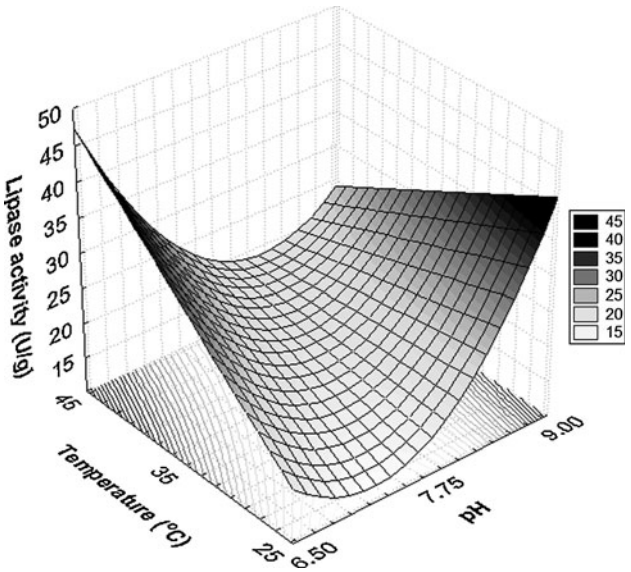

Fig. 3 Response surface diagram for lipase activity as a function of temperature and pH of reaction medium

Table 4 Model validation

Temperature	pH	Experimental activity (U/g)[a]	Predicted activity (U/g)
25°C (−1.41)	9.0 (+1.41)	44.0	39.5
45°C (+1.41)	6.5 (−1.41)	50.3	45.7

[a] Lipase activity was measured by titrimetric method and expressed in units per gram of lyophilized mass

acetate as substrate was carried out (Fig. 4). This substrate is used to identify the presence of enzymes with lipase/esterase activity. Nine bands with lipase/esterase activity were observed in the zymogram (Fig. 4), indicating, indeed, the presence of a pool of enzyme.

It is interesting to note how extracts of the same fungus grown on different substrates differ from one another. The enzymes present in the crude extract from the same fungus strain grown in babassu cake showed different characteristics than the one obtained in the castor bean waste [16]. In babassu cake, the fungus *P. simplicissimum* produced lipases with optimum activity at 50°C and pH between 4.0 and 5.0. When grown in castor bean waste, at least two lipase groups with distinct characteristics of temperature and pH were observed in the present study. These distinct characteristics allow the crude enzyme extract to be used in different fields of biotechnology.

Substrate chain length preference of *P. simplicissimum* lipase

Different p-NP esters were used to study the specificity of crude lipases from *P. simplicissimum*. The crude extract

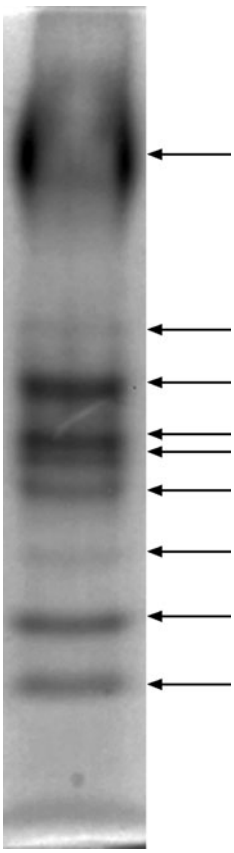

Fig. 4 Zymogram of the crude extract of *P. simplicis*simum using α-naphtyl acetate as substrate. The *arrow* indicates the bands revealed by the zymogram

showed no activity for p-NP butyrate, but showed high activity for p-NP caprylate, laurate and palmitate (Fig. 5). The *P. simplicissimum* crude lipase produced using babassu cake as substrate shows activity with pNP-butyrate [16], in opposition to the present work, in which this activity was not detected.

The null activity with short chain substrate (C4:0) and the high activity with medium and long chain substrates (C8:0, C12:0 and C16:0) are indicative that the enzymes present in crude extract are true lipases and not esterases.

Conclusions

The use of the experimental design technique was successful. The fungus *P. simplicissimum* reached a high production of lipase in non-treated castor bean waste. The optimum culture conditions determined by the Plackett-Burmam and CCRD experimental designs were inoculum concentration: 10^7 spores/g; particle size: 0–1180 μm; initial moisture: 48.5% and without molasses supplementation. In the validation of the generated model, the maximum activity achieved was 155.0 U/g with a high specific activity (9.0 U/mg of protein). The use of the experimental design strategy was efficient, leading to an increase of 340% in lipase production.

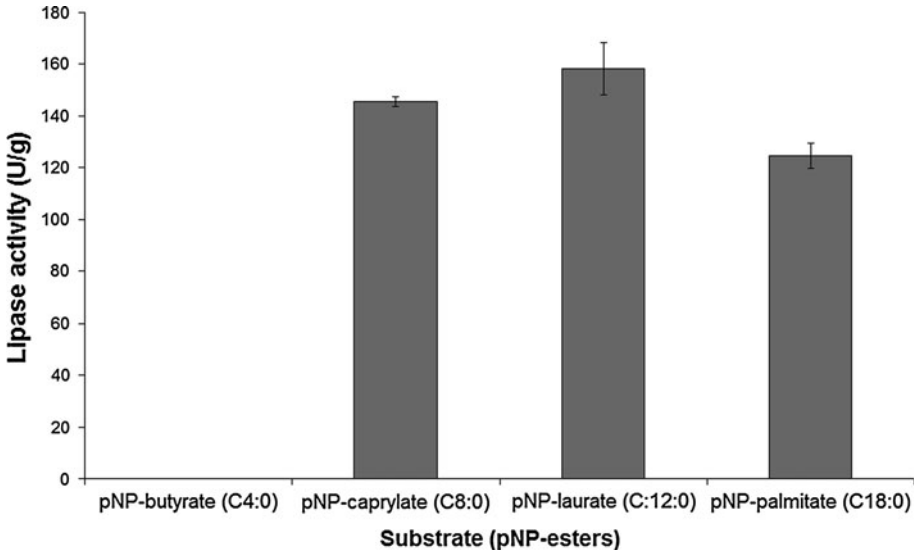

Fig. 5 Substrate chain length preference of *P. simplicissimum* lipase produced by solid-state fermentation in castor bean waste

The assays carried out with the crude enzyme preparation from the *P. simplicissimum* grown in castor bean waste indicate the presence of at least two lipases (or pools of lipases) with different characteristics of pH and temperature. One group, with optimal action at pH 6.5 and 45°C, and another at pH 9.0 and 25°C. The hypothesis of the presence of more than one lipase is even more evident when we observe the appearance of nine bands in the zymography.

The enzymes present in the crude extract were able to hydrolyze substrates with medium-chain fatty esters. This feature indicates that this enzyme preparation could be useful for transesterification reactions for oils with high content of medium-chain fatty acids, for example.

Thus, it was possible add value to an undesirable and worthless waste through the production of two pools of lipase with distinct characteristics. This fact extends the range of use of these low-cost lipases in different biotechnology fields.

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