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ORIGINAL ARTICLE

Overproduction and properties of lipase by a wild strain of *Burkholderia lata* LBBIO-BL02 using chicken fat

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Abstract In this work, we report the overproduction of lipases by a new wild strain of *Burkholderia lata* (LBBIO-BL02) in submerged fermentation to seek an economically attractive bioprocess. The best fermentation medium composition was containing chicken fat (12.5 mL/L) and ammonium phosphate (15 g/L) at 35ºC and pH 7.0, resulting in a lipase titer of 1137.82 U/mL and 2146.83 U/mg. The lipase characterization exhibited maximum activity at 55 °C and pH 8.0. The lipase retained 100, 93 and 85 % of its maximum activity at 45, 50 and 60 °C, respectively, and 78, 82 and 100 % at pH 2.2, 3 and 10, respectively. The enzyme was successfully immobilized on Celite by adsorption and showed a promising future for various organic syntheses because of its stability in organic solvents. All the results shows that *Burkholderia lata* LBBIO-BL02 is a superior lipase-producing bacterium and its enzyme showed good potential for industrial and biotechnology application.

Keywords Chicken fat · Lipase · Overproduction · Bioprocess design · Immobilization · Celite

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Introduction

The *Burkholderia cepacia* complex (Bcc) comprises a group of closely related organisms that occupies diverse ecological niches such as soil, water, animals, plants, and humans. The Bcc strains can be used for multiple applications as bio control, bioprocess, bioremediation, and plant growth promotion purposes, but their capacity as opportunistic bacteria to cause human infections hampers their use in these biotechnological applications (Vanlaere et al. 2009; Lipuma 2010). The *Burkholderia lata* species was differentiated from Bcc in 2009 (Vanlaere et al. 2009). According to a recent research describing Bcc species that are involved in respiratory tract infection in cystic fibrosis, the *B. lata* strains was not cited as a pathogen (Lipuma 2010). There are no records in the literature using this bacteria in biotechnology studies.

Bcc is an important group in the current research on biotechnology. Lipases from the Bcc strains shows biocatalysis capability in organic solvents media and are used in biofuel production, chiral reactions for pharmaceutical products, and racemic resolutions (Kawakami et al. 2011; Li et al. 2013), which make them attractive for the applications in diversified industrial sectors. Despite this, the industrial use of lipases is limited by the high cost of its production and the necessary purity grade according to its use. Because of this, finding a new strain with an overproducing lipase and the potential of using low cost nutrients such as agro-industrial residues and inorganic salts in microbial fermentation for enzyme production are extremely important in dictating future uses of lipases.

Medium optimization for overproduction of the enzyme is an important step for its commercial usage and involves a number of physico-chemical parameters such as the composition of production medium, the carbon and nitrogen sources, minerals and trace metals, pH, temperature, aeration, and inoculum age (Jaeger et al. 1994; Gupta et al. 2004). One of the most critical factors in producing microbial lipases is the choice of carbon sources as enzyme inducers. Compounds such as plant seed oils (triacylglycerols), free fatty acids, surfactants, bile salts and glycerol have been included in the nutrient medium to increase levels of lipase activity (Gupta et al. 2004). Olive oil has frequently been cited as an inducer of Bcc lipases (Boekema et al. 2007); however, vegetable oils are regarded as expensive fermentation substrates and are mainly used as a food stock.

Animal fat has the advantages of wide availability and low cost, and waste meat processing. Chicken fat is disposed by the poultry processing industry (Arnaud et al. 2004), which has been used to produce biodiesel; however, it can also be used for obtaining bioproducts with high added value such as enzymes. The average cost of the disposal is of US$0.55/L, against US$3.15/L of olive oil (2014).

The use of pure enzymes in biotechnology can be costly and its disposal after reaction is uneconomical. Furthermore, recovery of the reaction medium may be difficult. The development of immobilization techniques have been important for providing reuse of enzymes to facilitate separation of products and increase stability in organic solvents (Chang et al. 2007). As an inert support material, Celite® 545 consists of highly porous diatomaceous earth beads composed of more than 90 % silica (SiO_2) with a superficial area of 2.19 m^2/g (SIGMA). Because of its chemical inertness and interconnected pore structure (Chang et al. 2007), Celite® 545 is very suitable for enzyme immobilization by physical adsorption. There is a growing interest in the use of Celite beads as immobilization support material to enhance reaction rates by providing a better distribution of the catalyst (Mateo et al. 2007; Chang et al. 2007), besides the economic advantages of enzyme reutilization.

The present study was started with the aim of developing an efficient and economic fermentation media for overproduction of lipase by a new Bcc specie *Burkholderia lata* LBBIO-BL02. Besides the production, this work evaluated the enzyme properties of free and immobilized Celite® 545.

Material and methods

Strain identification

The bacterial strain LBBIO-BL02 was isolated in our laboratory in a fungal culture contamination and identified by morphological and molecular methods (16S RNA) at Fundação André Tosello (FAT), Campinas - SP - Brasil.

Production of lipase in submerged fermentation (SmF)

The bacteria cultivation was started with a pre-inoculum in 250 mL Erlenmeyer flasks containing 50 mL of Luria-Bertani (LB) medium with an incubation time of about 48 h (until the order of 10^8 cells/mL) at 30ºC and with 180 rpm orbital shaking. After this, 1 mL of the culture was passed to 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium maintained under the same conditions. The standard fermentation medium was composed of K_2HPO_4 (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), NaCl (0.38 g/L) and $FeSO_4 \cdot 7H_2O$ (0.01 g/L). The C and N sources varied according to the assay. Bacterial growth was monitored during the cultivation by counting cells in a Neubauer chamber.

The effect of different C and N sources

For lipase production by *B. lata* in SmF was evaluated with chicken fat or different oil sources: olive, corn, soybean, crambe, palm, and linseed (10 mL/L).

To study the influence of N sources in the lipase production by *B. lata*, we studied six different organic and inorganic N sources using a 2^{6-2} fractional design method (Table 2). The N sources evaluated were yeast extract, urea, ammonium sulfate, sodium nitrate, ammonium nitrate, and ammonium phosphate. The chicken fat (10.0 mL/L) was used as a carbon source. Table 2 shows the independent factors (Xi), their levels, and experimental design in terms of coded (−1, 0, and +1) and non-coded (actual value) variables.

The influence of the concentrations of the C (X_1) and N (X_2) source were investigated by a 2-level, 2-factor factorial design requiring 12 experiments with four central points and four axial points. The variables and their levels selected for lipase production were C (0.0–25.0 mL/L) as N source (0.0–12.0 g/L). Table 4 shows the independent factors (X_i), their levels, and the experimental design in terms of the coded ($-\alpha$, −1, 0, 1, and $+\alpha$) and the non-coded (actual value) variables. The analyses were performed using STATISTICA 8.0 software (Statsoft Inc.) to calculate the main effects of the variables, their interactions, and to perform the analysis of variance (ANOVA).

The response of variables, Y (lipase activity, U/mL), may be approximated by the polynomial equations:

CCD 2^2:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where: Y is the predicted response, β_0 is the offset term, β_i the linear effect, β_{ii} the squared effect, and βij the interaction effect.

Effect of the temperature and initial pH on lipase production

The effect of initial culture pH on lipase production was studied using univariate study in shake flasks at different initial pH values (pH 3 to 12). The pH of the medium was adjusted to the desired value by using either 1 mol/L NaOH or 1 mol/L HCl prior to the sterilization process.

Temperatures ranging from 30 to 45 °C were tested for their effect on lipase production by *B. lata* with 48 and 72 h of fermentation.

Enzyme biochemical characterization

To determine the pH effect on the lipase activity of the crude extract, assays were performed varying the reaction medium pH between pH 2.3 and 10. The method had to be adapted as described below, because *p*-nitrophenol (*p*NP) doesn't absorb color below pH 6.0. The reaction was carried out in tubes with 4.5 mL of reaction medium pre-equilibrated at 37 °C. After adding 0.5 mL of enzyme solution, 0.1 mL aliquots were removed, added to 0.9 mL of phosphate buffer 0.05 mol/L pH 8.0 and transferred to an ice bath. The activities were calculated based on the concentration of product in 1 min using the molar extinction coefficient determined at pH 8.0.

The pH stability was determined by incubating crude extract in buffers with pH values from 2.3 to 10 for 1 h at room temperature. After incubation, residual activity was determined using phosphate buffer 0.05 mol/L pH 8.0 by the standard method.

To determine the effect of temperature on the enzyme activity, the enzymatic reaction was carried out at temperatures between 20 and 90 °C, in phosphate buffer at 0.05 mol/L pH 8.0.

The temperature stability was determined by incubating the enzyme extract at temperatures between 20 and 70 °C in the absence of substrate. Residual lipase activity was determined at 37 °C by the standard method.

Immobilization by adsorption on Celite® 545 and stability in organic solvents

The immobilized enzyme on Celite® 545 was prepared as described by Chang et al. (2007) with modifications. In 5 g of support was added 5 mL of enzyme followed by addition of 50 mL of isopropanol at −10 °C. The mixture was homogenized under gentle agitation and immobilized lipase was collected by filtration and washed twice with n-hexane to remove non-adsorbed impurities and enzyme.

The FT-IR spectra of Celite-545, free lipase, and Celite-545 adsorbed lipase were recorded on a Bruker Tensor 37 FTIR spectrometer using the potassium bromide pellet method. The pellets were prepared from a mixture of 200 mg potassium bromide, 3 mg of the samples, and 1.5 mg potassium ferricyanide as an internal standard. The acquisition conditions were: spectral width 4000 to 500 cm^{-1}, 32 accumulations, and 4 cm^{-1} resolution.

To determine the stability of lipolytic activity of free and immobilized enzyme in polar organic solvents, methanol, ethanol, isopropanol, and acetone were used in different concentrations (25, 50, 75, and 100 %, v/v) in incubation for 1 h at room temperature. The residual activity was determined according to the standard method.

Analytical methods

Lipase activity determination

The hydrolysis of *p*NPP was used to follow the trials of fermentation and biochemical characterization. The lipase activity was measured by the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) as first described by Winkler and Stuckmann (Winkler and Stuckmann 1979). One unit of lipase activity is defined as the release of 1 mmol/min of *p*-nitrophenol (*p*NP). The molar extinction coefficient of *p*NP (1.5× $s10^4$ mol/L x cm) was used to correlate the concentration of product from the absorbance readings.

The lipase activity determination by titration was used for characterization of enzymatic activity against natural substrates and triacylglycerols of different chain sizes. The reaction medium consisted of 0.2 mol/L of substrate, 6 % (w/v) of Triton X-100, and 74 % (v/v) citrate-phosphate buffer 0.05 mol/L pH 7.0. To this was added 1 mL of lipase to 5 mL of enzyme reaction medium and with incubation for 30 min at 55 °C under strong agitation (300 rpm). After incubation, 16 mL of a 1:1 (v/v) mixture of acetone and ethanol was added and this solution was titrated with NaOH 0.1 mol/L. One unit of enzyme activity was equivalent to 1 mmol of fatty acid released per minute.

Protein concentration assay

Protein concentration was determined according to the procedure described by Bradford (Bradford 1976) using BSA as a standard protein.

Gas chromatography (GC) analysis of fatty acids

The fatty acid derived from chicken fat were identified at Technology Laboratory of Animal Products (UNESP – Jaboticabal/SP) by GC using a Shimadzu 14B Gas Chromatograph connected to a OMEGAWAX250 capillary column

(30 m x 0.25 mm x 0.25 μm). The injector and detector temperatures were set at 250 and 280 °C, respectively. The column temperature was initially maintained at 100 °C for 2 min, increased to 220 °C at 4 °C/min, and finally held at 300 °C for 25 min. A mix of fatty acids (SIGMA) was used as standard.

Results

Strain identification

Morphologically, the strain introduced in the form of large Gram-negative bacilli and the sequencing of the 16S RNA, allowed the identification of the strain as *Burkholderia lata*. The strain was deposited at the culture collection of the Faculdade de Ciências e Letras de Assis – UNESP/Assis under LBBIO-BL02 code. This species was differentiated from *Burkholderia cepacia* complex (Bcc) in 2009 (Vanlaere et al. 2009), and there are no records in the literature of lipase production by this strain.

Production of lipase in submerged fermentation

The effect of different carbon sources

Throughout the study, the cultures with our *B. lata* LBBIO-BL02 strain had markedly high lipolytic activity, volumetric and specific, presenting unusual values in comparison with the literature. With the exception of fermentation with linseed oil, all other carbon sources produced lipase above 150 U/mL. Chicken fat showed the highest lipase activity (323 U/mL) and the highest specific activity (1619 U/mg) compared to other sources (Table 1). Specific activity above 1000 U/mg was also obtained with olive and corn oils.

Although chicken fat is an animal fat, the analysis of its fatty acids composition found a high percentage of oleic, linoleic, and linolenic acids (57 %), similar to vegetable oils traditionally used in lipase production by microorganisms. The contents of these fatty acids has been linked to induction of enzyme production, which may explain the good results obtained with chicken fat.

Beyond inducing the increased production of the enzyme, chicken fat provided the best Production cost/Lipase activity rate, defined as the best carbon source for stimulating lipase production by *Burkholderia lata* strain with high enzyme production at low cost (Table 1). Moreover, it is interesting to note that Production cost/Lipase activity rate is even competitive with other carbon sources, such as corn, soybean, crambe, and palm oils.

The effect of different N sources

To study the effect of different N sources, organic and inorganic, a fractional design 2^{6-2} was performed. In this study, the highest enzyme activities was found in experimental run 9 using sodium nitrate (X_4) and ammonium phosphate (X_6), with lipase activity of 373.0 U/mL and 4696.29 U/mg (Table 2). The specific activity obtained in this experiment has no precedent in the literature. Inorganic nitrogen sources were found to increase lipase synthesis by *B. lata* grown in the presence of chicken fat. From the analysis of the effects, it was possible to observe that only X_6 was significant to a confidence level of $p<0.05$ with a positive influence (Table 3). Therefore, ammonium phosphate was chosen for the sequence of experiments. Besides being the N source to induce increased production of the enzyme, the salt ammonium phosphate is at a lower cost than organic sources, and its presence hinders subsequent stages of protein purification.

Carbon and nitrogen concentrations

The results show the importance of chicken fat and ammonium phosphate to produce lipase by *B. lata*. However, the concentration of these nutrients requires evaluation, and there is the possibility of interaction between them. Since the conventional method (one factor at a time) used for optimization does not indicate the interactions between the significant components, a statistical approach for optimizing the significant media components has been employed.

In the experimental design performed, there were obtained lipolytic activities ranging from 0.06 to 425.00 U/mL. The highest activity was observed in Experiment 8 (425.00 U/mL), which included the largest concentration of the nitrogen source (0, α+). Table 4 describes the planning matrix, with its coded variables, and the predicted and observed results.

Considering the volumetric activity obtained in the experiments, based on the analysis of the effects, the interaction between X_1 (oil chicken) and X_2 (ammonium phosphate) was not significant at a confidence level of $p<0.05$ (Table 5). However, the isolated variables had significant effects. Thus, the concentrations of C and N sources have positive influences on the response, that is, increasing these variables will increase the value of enzyme activity causing an average activity increase of 115.5 and 304.8 U/mL when passing level (−) to level (+), respectively.

Considering the specific activity obtained in the experiments, the concentration of ammonium phosphate has a significant influence, providing an average increase of 1064 U/mg when passing from level (−) to the level (+). The concentration of the carbon source had no significant effect on specific activity.

The F test demonstrated that for the variables studied, $F_{calc}>F_{tab}$ (Table 5) was meaningful, and the model was valid.

Table 1 Influence of different oils and fat as carbon sources on lipase production by *B. lata* in SmF

Substrate	Lipase activity (U/mL)	Production cost/Lipase activity* ($/million units)	Specific activity (U/mg)	Fatty acids content of C sources				
				Oleic ($^{\Delta 9}$18:1)	Linoleic ($^{\Delta 9,12}$18:2)	Linolenic ($^{\Delta 9,12,15}$18:3)	Erucic ($^{\Delta 13}$22:1)	Palmitic (16:0)
Chicken fat	323.81±43.17	0.02	1619.05	43	14	0.7	–	25
Corn oil	258.44±19.21	0.05	1292.20	35	45	1	–	10
Soybean oil	207.66±23.75	0.05	988.86	21	54	6	0.3	11
Olive oil	207.12±34.08	0.14	1218.35	70	10	0.2	–	16
Palm oil (dendê)	202.35±7.21	0.06	249.81	40	17	–	–	34
Crambe oil	187.10±20.33	0.04	668.21	18	9	6	56	2
Linseed	8.23±2.92	1.52	30.48	11	15	40	–	2

*Commercial value of the carbon sources was obtained from the Agricultural Marketing Resource Center (USDA Economic Research Service 2014)

Table 2 Fractional design matrix (factorial 2^{6-2}) containing four repetitions at the central point for the production of lipase by *B. lata* in SmF

Run	Variables in coded levels						Response	
	X_1	X_2	X_3	X_4	X_5	X_6	*Y* (U/mL)	Specific Activity (U/mg)
1	−1	−1	−1	−1	−1	−1	0.47	13.74
2	1	−1	−1	−1	1	−1	6.08	138.34
3	−1	1	−1	−1	1	1	22.47	355.64
4	1	1	−1	−1	−1	1	59.33	933.29
5	−1	−1	1	−1	1	1	11.03	282.94
6	1	−1	1	−1	−1	1	28.83	190.35
7	−1	1	1	−1	−1	−1	5.92	50.74
8	1	1	1	−1	1	−1	5.08	629.73
9	−1	−1	−1	1	−1	1	373.00	4696.29
10	1	−1	−1	1	1	1	76.33	497.33
11	−1	1	−1	1	1	−1	2.91	582.00
12	1	1	−1	1	−1	−1	2.03	203.33
13	−1	−1	1	1	1	−1	2.54	100.36
14	1	−1	1	1	−1	−1	1.58	102.15
15	−1	1	1	1	−1	1	75.80	334.75
16	1	1	1	1	1	1	0.24	23.67
17	0	0	0	0	0	0	9.91	154.40
18	0	0	0	0	0	0	19.72	503.88
19	0	0	0	0	0	0	7.23	196.27
2								
0	0	0	0	0	0	0	14.62	224.99

Factors	Real levels		
	−1	0	+1
X_1 Yeast extract (g/L)	0.0	1.0	2.0
X_2 Urea (g/L)	0.0	2.15	4.3
X_3 Ammonium sulfate (g/L)	0.0	4.7	9.4
X_4 Sodium nitrate (g/L)	0.0	6.0	12.0
X_5 Ammonium nitrate (g/L)	0	2,85	5,7
X_6 Ammonium phosphate (g/L)	0	3	6

Table 3 Analysis of variance obtained for the 2^{6-2} fractional design with different N sources. Chicken fat was used as the carbon source at 30ºC and 180 rpm

Factor	Ss	Df	Ms	Fcalc	*p*-Value
X_1	6187.2	1	6187.19	1.297699	0.275190
X_2	6645.6	1	6645.56	1.393840	0.258909
X_3	10588.1	1	10588.14	2.220754	0.160025
X_4	9762.0	1	9762.03	2.047487	0.176055
X_5	11039.8	1	11039.77	2.315481	0.152038
X_6	24057.5	1	24057.46	5.045808	0.042699
Residue	61981.5	13	4767.81		
Total	130261.7	19			

Ss, sum of squares; *df*, degrees of freedom; *Ms*, mean square; R^2 =0.5242; R=0.7240; F_{tab} (1; 13; 0.05)=4.67

Thus, an empirical mathematical model for lipase is possible (Equation 1).

$$Y(\mathrm{U/mL}) = 236.21 + 57.78.(X_1) - 55.61.(X_1)^2 + 152.40.(X_2) - 4,70.(X_2)^2 \quad (1)$$

The 3-D response surface and contour plots obtained for the model are shown in Fig. 1a and b, respectively. As previously mentioned, when the concentrations of N source (X_2) increased, the enzymatic activity also increased; thus, the highest lipase activities were obtained at the highest concentrations of X_2 (ammonium phosphate).

It is possible observe that this model is not conclusive, since the response surface does not have a region where the enzymatic activity is maximal but has a growing response. To variable X_1 (chicken fat), it is possible to indicate the most suitable concentration to induce enzyme production (12.5 mL/L). However, for the variable X_2, the results indicate that better production of the enzyme would occur at concentrations above 20 g/L. Since the concentration of C and N sources showed no interaction and the concentration of the most suitable carbon source was determined, then a univariate experiment was conducted to find the best concentration of ammonium phosphate.

Although the factorial design indicates that the lipase production would be favored by adding more than 20 g/L of ammonium phosphate to the culture medium, in the univariate study there was no significant increase in activity between 15 and 25 g/L, including a marked decreased activity with 30 g/L

Table 5 Analysis of variance obtained for the 2^2 CCD with chicken fat (X_1) and ammonium sulfate (X_2)

Factor	Ss	df	Ms	Fcalc	*p*-Value
X_1(L)	26635.6	1	26635.6	6.29071	0.046020
X_1(Q)	19634.1	1	19634.1	4.63711	0.074755
X_2(L)	185259.0	1	185259.0	43.75384	0.000575
X_2(Q)	140.6	1	140.6	0.03321	0.861407
X_1 x X_2	671.5	1	671.5	0.15859	0.704239
Residue	25404.7	6	4234.1		
Total	257863.4	11			

Ss, sum of squares; df: degrees of freedom; *Ms*, mean square; R^2 = 0.9015; R=0.9495; F_{tab} (1; 6; 0.06)=5.99

Table 4 CCD matrix (factorial 2^2) containing four repetitions at the central point for the production of lipase by *Burkholderia lata* LBBIO-BL2 in SmF

Run	Variables in coded levels		Response		
	X_1	X_2	*Y* (U/mL) - Predicted	*Y* (U/mL) -Observed	Specific activity (U/mg)
1	−1	−1	0.00	5.46	90.15
2	−1	+1	257.54	297.00	1439.00
3	+1	−1	68.31	42.96	196.03
4	+1	+1	399.02	386.33	618.07
5	-α	0	44.15	0.06	4.31
6	+α	0	207.11	237.00	632.19
7	0	-α	11.96	13.83	26.52
8	0	+α	441.73	425.66	1781.97
9	0	0	236.20	179.33	666.53
10	0	0	236.20	169.00	908.93
11	0	0	236.20	255.00	869.27
12	0	0	236.20	341.67	1708.33
Factors	Actual levels				
	-α	−1	0	+1	+α
X_1 (Chicken fat, mL/L)	0.00	0.35	1.25	2.15	2.50
X_2 (Ammonium phosphate, g/L)	0.00	1.75	6.00	10.15	12.00

α=1.41

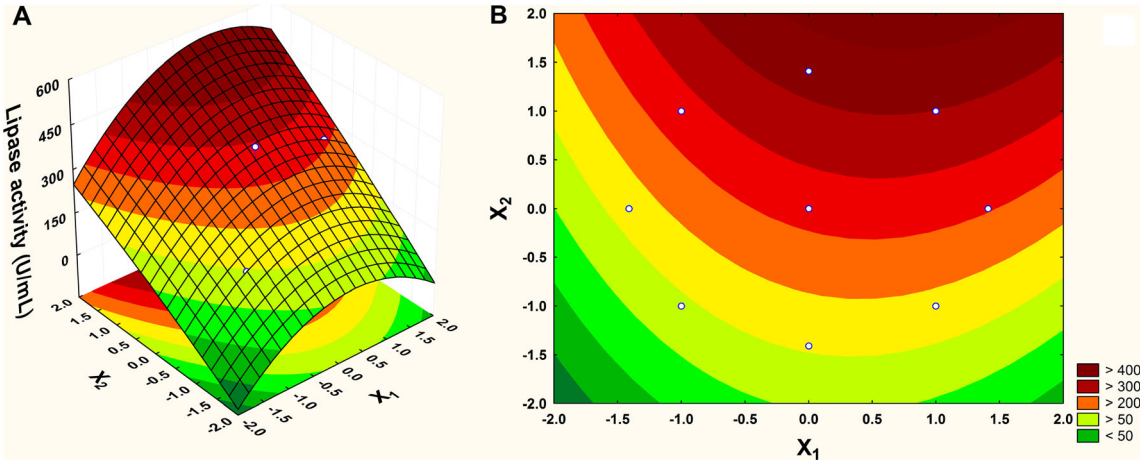

Fig. 1 Three-dimensional response surface (**a**) and contour (**b**) plots of lipase activity (U/mL) obtained by submerged fermentation of *Burkholderia lata* LBBIO-BL2 on medium with various concentrations of chicken fat (X_1) and ammonium phosphate (X_2)

of salt. Increasing the N source up to 15 g/L, the lipase production was maximum (183.77 U/mL and 1647.26 U/mg) (Fig. 2).

Effect of the initial pH and the temperature on lipase production

To evaluate the effect of the initial pH of the culture medium and the incubation temperature on lipase production by *B. lata*, fermentation was carried out at 12.5 mL/L of chicken fat and 15 g/L of ammonium phosphate. The enzymatic activity was followed specially in two different conditions: pH 8 and 37 °C (standard conditions in fermentation experiments) and pH 7 and 55 °C (a condition established after the characterization of the enzyme, described below).

The initial culture pH had prominent effects on the lipase production by *B. lata*. Initial pH of culture medium ranging from 7 to 9 promoted good lipase production with the initial pH of 8 showing the best lipase activity (Fig. 3). In contrast, pH below 6 and above 9 on the pH of the initial culture has drastic negative effects.

The effect of initial pH of the culture medium is directly related to the microorganism's growth. The highest enzyme activity was obtained exactly in fermentations where higher cell growth occurred. As a consequence of cell growth, beside enzymatic activity, acidification of the medium was also seen in all experiments. Cultures started at pH 7 had a final pH of 5.06, while the pH experiments initiated in 8 and 9, finished with a pH around 6.5. It is interesting to notice how small differences in pH can also provide important effects on the metabolism of the microorganism. Comparing the cultures at pH 6 and the control (without correction, pH 6.43), there was double the activity in the control flask and lower acidification after 72 h of cultivation (pH 5.26 and 3.6 in the control flasks and adjusted at pH 6, respectively), although approximately with the same cell growth.

At a different temperature fermentation, considering the incubation time of 72 h, it was observed that the culture temperature had an effect on the lipase production, with increased activity between 30 and 35 °C (Table 6). However, the experiment was also accompanied with 48 h of culture, and in this period it is evident the highest activity was at 35 °C. The cell growth did not change between 48 and 72 h, suggesting that even at 48 h, the bacteria was already in the stationary phase of cell growth. Thus, the different activities obtained are probably due to metabolic changes caused by temperature.

Kinetic characterization

For kinetic characterization studies, the lipase was produced in basal culture media containing chicken fat (12.5 mL/L) and ammonium phosphate (15 g/L) at 30 °C, 180 rpm, pH 6.6 for 72 h resulting in an enzyme extract, 250 U/mL (standard method assay).

The effect of temperature on the enzyme activity in a range between 20 and 90 °C was evaluated (Fig. 4b). The highest activities were observed in the range of 55 °C (542.76 U/mL) at 65 °C (510.26 U/mL), but considerable activity was also observed at high temperatures with 138.92 U/mL at 85 °C (25.60 % of the activity measured at 55 °C).

To determine the thermal stability of *B. lata* lipase, the enzyme extract was incubated at temperatures between 20 and 70 °C (Fig. 4a). It was observed that *B. lata* lipase, even after 60 min of incubation at 50 and 60 °C, held 93.3 and 85.4 % of the initial activity. However, when the temperature increased to 70 °C, there was total loss of enzyme stability.

The effect of pH on *B. lata* lipase was investigated at pH values between 2.2 and 10. Higher activities were observed in the pH range between 4.0 and 9.0, with maximum activity at pH 7.0. Reasonable activities were obtained even at more

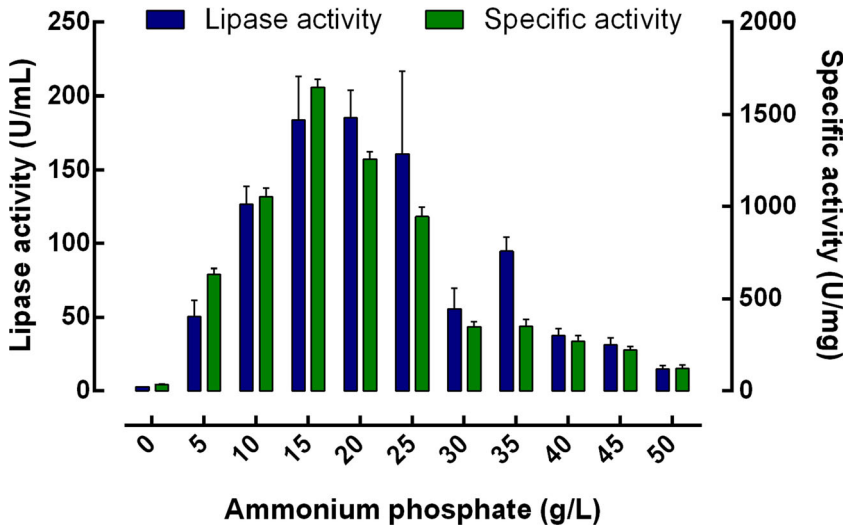

Fig. 2 Influence of ammonium phosphate concentration as the N source on the production of lipase by *Burkholderia lata* in SmF. Culture conditions: 12.5 mL/L of chicken fat; 180 rpm, 30 °C, 72 h

acidic pH values, at pH 3.0 and 4.0 we observed 65.14 and 89.56 % of relative activity, respectively (Fig. 4d).

The stability of the enzymatic activity of *B. lata* lipase at different pH values was investigated. The enzyme was stable at pH values between 3.0 and 10.0, after incubation for 1 h at 25 °C (Fig. 4c). Even at pH 2.2, the enzyme showed 78.20 % residual activity. When in contact with the phosphate-citrate buffer pH 3.5 to 6.0 and KH_2PO_4-NaOH buffer pH 6.0 and 8.0, there was an activation of the lipase in relation to the activity of the enzyme not incubated. This effect has been reported for other lipases, but no mechanism has been proposed to explain this phenomenon.

Activity on different substrates

The *B. lata* lipase showed high hydrolysis activity against triacylglycerol of different chain sizes, synthetic and natural (Table 7). The triolein is the typical substrate for lipases, and it is not hydrolyzed by esterases, thus, the ability to hydrolyze this triacylglycerols indicates the presence of true lipases in the enzyme extract. This definition applies even when considering that the rate of hydrolysis of triolein was lower than that of other short chain triglycerides such as tributyrin (4:0).

Using different natural substrates, the *B. lata* lipase shows the most hydrolysis activity on linseed and soy oils, and chicken fat for which the predominant fatty acids are linolenic, linoleic, and oleic acid, respectively, as already shown in Table 1. Despite variations in degree of saturation in the fatty acids, they have the same size chain (18 carbons).

Lipase immobilization by adsorption on Celite® 545

After characterization of the enzyme, the crude extract was used in adsorption tests with immobilization on Celite® 545. This support consists mainly of silica SiO_2 (91.5 %) with an average particle size of 0.002 mm^2 (range 0.02-0.1 mm) measured in our

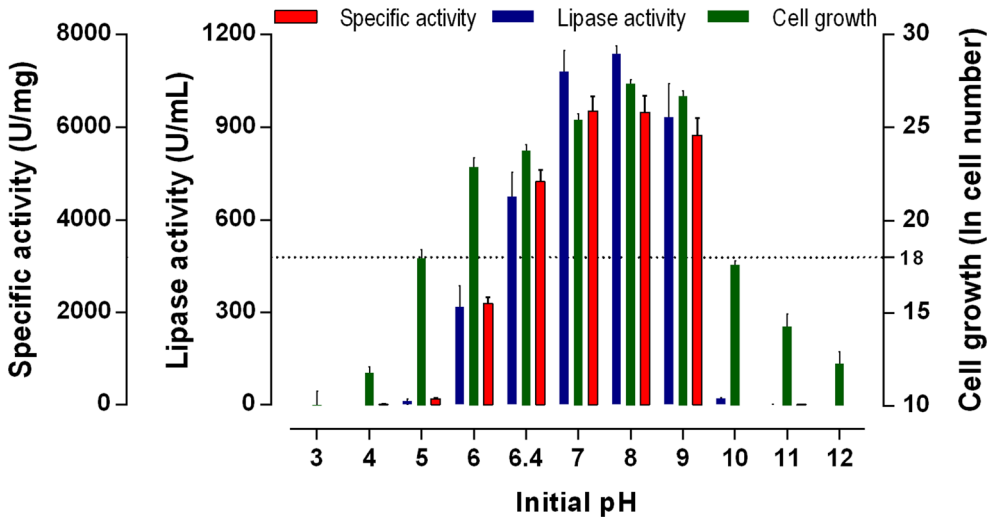

Fig. 3 Lipase production by *B. lata* and cell growth in culture medium with different initial pHs. The initial ln cell number added to fermentation was 18. Culture medium contained 1.25 mL/L of chicken fat and 15 g/L of ammonium phosphate. Control fermentation without pH adjustment was at pH 6.4. Culture conditions: 180 rpm, 30 °C and 72 h. Test conditions: citrate-phosphate buffer 0.05 mol/L pH 7.0, 55 °C. Assays carried out in triplicate

Table 6 Lipase production by *B. lata* and cell growth in fermentations performed at different temperatures

Time (h)	Temperature (°C)	Activity (U/mL; pH 7; 55 °C)	Activity (U/mL; pH 8; 37 °C)	Cell growth (ln)
48	30	630.38±37.72	192.77±14.62	24.73
	35	1124.91±128.23	359.11±32.26	26.13
	40	471.94±178.03	240.74±4.85	25.96
	45	0.00±0.00	0.00±0.00	8.87
72	30	1137.83±25.15	323.65±17.27	25.43
	35	997.81±96.01	319.11±± 57.56	26.41
	40	587.90±24.01	171.56±34.69	26.07
	45	0.00±0.00	0.00±0.00	0.00

The initial ln cell number added to fermentation was 18. Culture medium containing 1.25 mL/L of chicken fat and 15 g/L of ammonium phosphate. Culture conditions: 180 rpm, pH 8.00

laboratory. Celite also shows a permeability of 4.8 Darcies, a pore size of 17 microns and a superficial area of 2.19 m^2/g (manufacturer's data).

For immobilization tests we used enzyme extract with an activity of 135.45 U/mL. To 5 g of the support was added an equivalent to 1354.50 U of enzyme activity. After

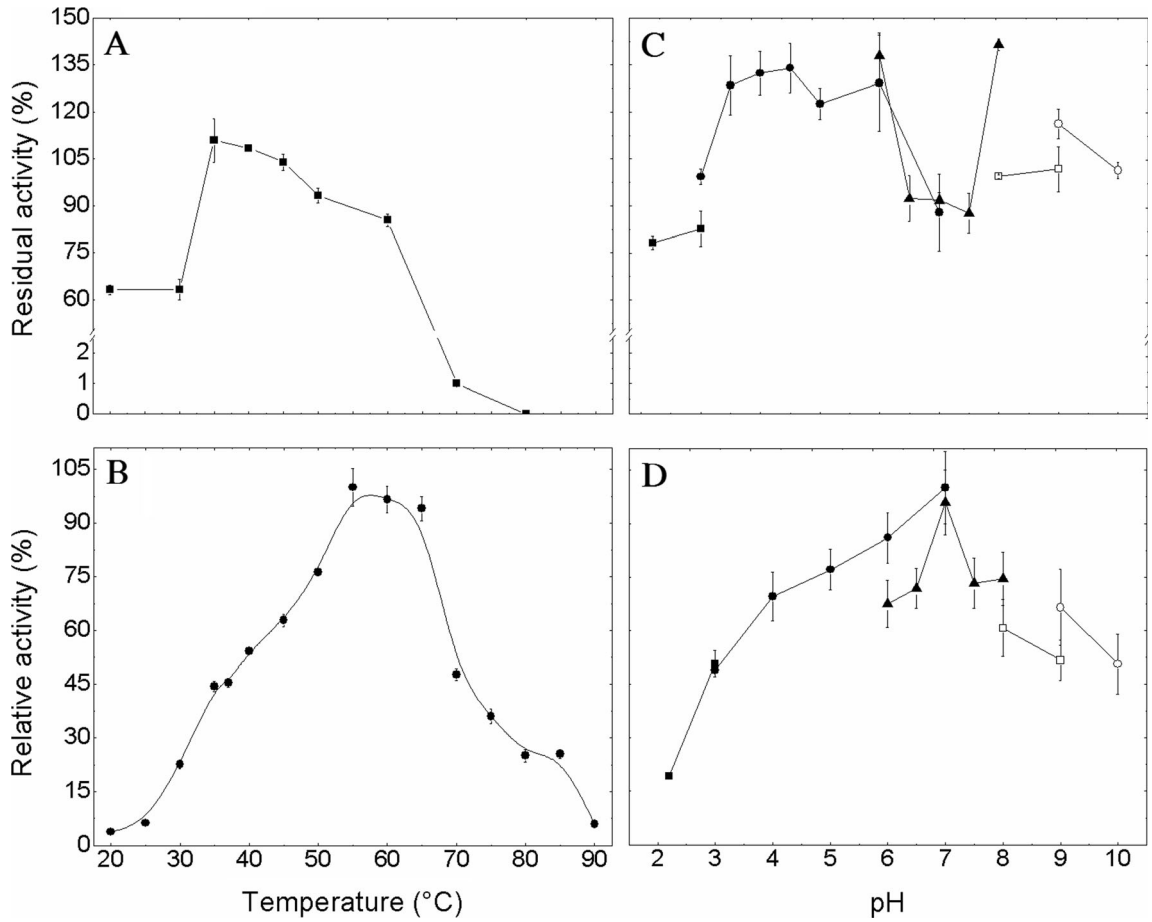

Fig. 4 Effect of temperature on the stability (**a**) and activity (**b**) of *B. lata* lipase. Test conditions: temperature between 20 and 90 °C, phosphate buffer 0.05 mol/L pH 8.0. Assays were carried out in triplicate. All activities was calculated relative to the activity measured at the standard temperature of 55 °C (542.76 U/mL, 100 %). Effect of pH on the stability (C) and activity (D) of *B. lata* lipase at different pHs. Test conditions: 37 °C. Residual activity in the stability tests after 1 h of incubation at 25 °C. Buffers used in the two assays (0.05 mol/L): Glycine-HCl pH 2.2 to 3.0 (■), citrate-phosphate pH 3.0 to 7, 0 (●), KH_2PO_4-NaOH pH 6.0 to 8.0 (▲), Tris–HCl pH 8.0 and 9.0 (□) and Glycine-NaOH pH 9 and 10 (○). The activities were compared with the activity determined in phosphate buffer 0.05 mol/L pH 8.0 without prior incubation (100 %, 250 U/mL). Assays carried out in triplicate

Table 7 *Burkholderia lata* lipase activity on different substrates

Substrate	Lipase activity (U/mL)
p-Nitrophenyl palmitate (*p*NPP,16:0)	445.18±13.5
Triolein ($^{\Delta 9}$18:1)	405.06±11.3
Tributyrin (4:0)	581.46±5.7
Linseed oil	470.19±26.1
Chicken fat	378.92±15.0
Soybean oil	346.26±20.4
Corn oil	195.99±25.9
Olive oil	120.85±31.5
Crambe oil	117.59±25.9

Test conditions: 55 °C, pH 7.0. determinations in triplicates

immobilization, we obtained the activity of the immobilized enzyme with 1041.47 U/g support, with an exceptional yield of 768.89 %, superior to those found in the literature (Chang et al. 2007).

The immobilized enzyme, the Celite, and the free lipase were analyzed by FT-IR to ascertain the nature of the enzyme/carrier interaction. The spectra of immobilized enzyme showed amide bands typical of lipases, but with less noise than free lipase, which can be explained by a possible selective attachment of the protein to the solid support providing a crude purification. The band at 1088 cm^{-1} (siloxane group -Si-O-Si-) of the support remains predominant. No additional band in the spectrum for the immobilized derivative was observed indicating that no covalent bond was between the enzyme and support, but that there was physical adsorption.

The lipolytic activity of the immobilized enzyme using *p*NPP as substrate was followed by five subsequent cycles with washing the support with isopropanol between cycles. In this aqueous reaction environment, rapid desorption of the enzyme was observed. Decreases were observed in the activity of 34.5, 64.2, 84.3, and 95.4 % between the second and fifth recycles.

However, for the enzyme application of the catalysis, the reaction medium is generally composed of organic solvents. We lack additional evaluation of free and immobilized enzyme stability in this environment. Here can be observed great stability for both polar and nonpolar solvents. For the nonpolar, there is emphasis on the n-hexane (Table 8). For free enzyme better results were obtained in 50 % ethanol, n-hexane, and heptane with residual activities of 178.38, 256.96, and 261.54 %, respectively. For the immobilized enzyme, n-hexane showed the highest activity when compared to the initial (115.40 %).

Discussion

Most lipases are inducible enzymes and addition of oleic, linoleic, and linolenic acids proved to enhance lipase production. Natural substrates of lipase are triglycerides of long chains, so triolein is used in most cases as a standard substrate (Gupta et al. 2004). The selection of a carbon source that is also an inducer of enzyme production for the content of long-chain fatty acids, but has also affordable cost, is one of the most common concerns in studies of lipase production by microorganisms.

In this sense, the results presented here have great interest for enzyme industries. In cultures of *B. lata* LBBIO-BL02 in this work there was obtained high activity in comparison with the literature, using as a carbon source chicken fat from disposals of the poultry industry. Although of an animal source, chicken fat has a high content of unsaturated long chain fatty acids (>57 %), 43 % of it is oleic acid; justifying it as good source for lipase production, as observed in other studies conducted by our group for lipase production by *Fusarium* sp (GFC) (Oliveira and Lima 2014). Besides our work there is no report in the literature of lipase production using chicken oil. Olive oil is currently used as an inducer of lipases (Boekema et al. 2007); however, this increases the production cost by seven times compared to chicken fat.

Chicken fat is a disposal of the poultry industry together with the viscera, feathers, and blood (Arnaud et al. 2004), contributing to the increase of waste released in nature. Use of this fatty material for enzyme production of high added value appears as an excellent alternative for the industry, with great appeal related to the preservation of the environment.

Apart from chicken fat, the present production cost/lipase production rate is quite competitive the crambe and palm oils. The crambe (*Crambe abyssinica*) is a non-food oilseed, its oil is toxic for human or animal consumption and has exclusively industrial application. The *B. lata* lipase production in the EU, where the culture is already deployed, can be favorable.

Palm oil is extracted from palm (*Elaeais guineensis*), cultivated in the Brazilian northeast and the western coast of Africa. It has a food use, but is limited to local customs. Considering that the oil is extracted in poor regions, their use for lipase production could represent, in addition to economic viability for the bioprocess, increased income for the population.

Besides being a carbon source, the type of N source in the medium has great influence in lipase production by microorganisms. Generally, organic N is preferred, such as tryptone, peptone, and yeast extract (Gupta et al. 2004), which have been used as a nitrogen source for lipase production by various *Burkholderia* sp. (Dalal et al. 2008; Lau et al. 2011; Lo et al. 2012). The cost of those sources is high compared to that of inorganic N salts (Oliveira et al. 2013), and its presence hinders subsequent stages of protein purification.

Table 8 Study of stability *Burkholderia lata* LBBIO-BL02 lipase from different organic solvents and concentrations

	Log P	Concentration (%)	Free enzyme Residual activity (%)	Immobilized enzyme Residual activity (%)
Control			100.0±8.2	100.0±2.5
Methanol	−0.73	25	86.5±21.5	33.0±5.3
		50	119.7±5.3	28.2±4.6
		75	50.7±6.4	22.8±5.8
		100	0.0±0.0	36.9±3.0
Ethanol	−0.24	25	105.2±12.8	37.7±3.1
		50	178.4±7.8	16.7±7.0
		75	98.8±8.6	19.5±6.3
		100	28.2±8.4	48.5±10.9
Iso-propanol	−0.28	25	162.7±7.2	21.7±0.6
		50	82.4±39.5	19.9±0.6
		75	91.5±12.0	17.0±0.5
		100	72.0±7.9	10.3±0.6
Acetone	−0.23	25	72.5±9.8	36.7±3.0
		50	70.5±5.7	31.4±0.7
		75	61.0±3.2	26.8±3.1
		100	72.3±4.2	33.1±1.8
Butanol	0.8	100	6.1±3.3	21.4±2.5
Iso-amyl	1.42	100	41.0±0.0	28.4±0.9
Octanol	2.8	100	41.4±11.2	21.3±2.4
Toluene	2.5	100	115.7±45.7	42.1±2.2
Hexane	3.5	100	257.0±5.4	115.4±3.0
Heptane	4	100	261.5±8.2	59.5±6.0
Octane	4.51	100	44.5±1.6	65.6±4.9

[a] The percentages values accompanying polar organic solvents correspond to the amount of solvent in phosphate buffer 0.05 mol/L pH 8.0. Tests in triplicate. Log P: Solvent hydrophobicity measurement computed by the logarithm of partition coefficient of a given solvent in a octanol/water system standard. Hydrophilic solvents: $-2.5<\log P<0$

In this work the best N source was an inorganic salt (ammonium phosphate).Reports in the literature show that the lipase production could be improved by inorganic nitrogen sources while the cell growth was influenced by organic ones (Liu et al. 2012). The results indicated that the expensive organic N sources can be replaced by lower cost inorganic N salts with a gain of lipase activity. They are significant, considering the cost advantage and the ease of enzyme purification. Rathi et al. (2001) reported that ammonium phosphate did not affect the lipase production by a *B. cepacia* strain significantly; however, higher specific activity was observed.

The best pH of a culture medium to have the maximum lipase production by *Burkholderia* isolates is reported to be around 7–9. Rathi et al. (2001) reported the *B. cepacia* preferred pH around 7.0 and 9.0, respectively, for optimal growth and lipase production, Lau et al. (2011) found the best pH for lipase production by *B. cenocepacia* ST8 was 9.0. Others microorganism, such as *Bacillus coagulans* BTS-3 (Kumar et al. 2005) and *Pseudomonas* sp. MSI057 (Kiran et al. 2008) showed the best lipase production at pH 8.5 and 9.0, respectively. Hence, initial culture pH is an important factor which affects the lipase production, and it varies with the types of microorganisms.

The *B. lata* LBBIO-BL02 lipase activity and stability in the range of temperature of 37 to 65 °C may make it interesting for use in biocatalysis, as these characteristics are not common to lipases from mesophilic bacterial strains such as *B. lata*. Even after 1 h incubation at pH extreme acid 2.2 and alkaline10.0, *B. lata* lipase remained active showing residual activity of 78.20 and 101.36, respectively. The activity and stability shown by the *B. lata* lipase at acid pH are not common among lipases produced by bacteria, which are generally more stable and active at neutral or alkaline pH values.

Wang et al. (2009), studying *B. cepacia* lipase, reported maximum activity of the enzyme on *p*NPP at 30 °C and pH 9.0, while keeping only 44 % residual activity after 60 min at 50 °C, has activity decrease of 50 % at pH 5. To the other hand, Park et al. (2007) reported that lipase from *Burkholderia* sp. HY-10 exhibited the highest *p*NPP hydrolysis at 60 °C and pH 8.5, but low stability at higher temperature

(25 % at 60 °C). Yang et al. (2007), using a titrimetric assay, found the optimal temperature for *B. cepacia* G63 at 70 °C and pH 8.0, and kept stable at a temperature range of 40–70 °C. Liu et al. (2006) reported that the optimal reaction conditions of lipase from *Burkholderia* sp. C20 were pH 9.0 and 55 °C (titrimetric assay).

The results of activity and stability in a wide pH range (3.0 to 10.0), are also desirable in catalysis and are added to the good characteristics in terms of temperature by the *B. lata* lipase.

After pH and composition of the fermentation media studies, the enzymatic activity of the fermentation broth was 1137.82 U/mL with 0.53 mg/mL of protein, resulting in a specific activity of 2146.83 U/mg. In the literature, strains of *Burkholderia cepacia* (Rathi et al. 2001), *Burkholderia cepacia* ATCC 25609, *Burkholderia multivorans* V2 (Dandavate et al. 2009), and other lipase-producing bacteria, such as *Pseudomonas aeruginosa* (Joshi and Khare 2013) and *Bacillus sphaericus* MTCC 7526 (Joseph and Ramteke 2012), produced, on average, 2 to 4 mg of protein per mL of fermentation extract, resulting in a specific activity of 51.0, 0.23, 1.76, 0.28, and 182.8 U/mg, respectively.

The overproduction of lipase by *Yarrowia lipolytica* have been well-studied during the last 10–15 years. The optimization of conditions for overproduction of lipase was done (Fickers et al. 2011). Pignède et al. (2000) using a genetically modified *Y. lipolytica* was able to produce 0.2 U/mL (titrimetric assay using olive oil). Our *B.lata* LBBIO-BL02 strain, using the same assay method was able to show activity of 120 U/mL. That demonstrated the advantage of *B. lata* LBBIO-BL02 wild strain in comparison to a traditional yeast producer.

Thus, the *B. lata* strain studied shows lipase production with higher volumetric and specific activity than reported in the literature for both synthetic substrates (*p*NPP), triolein, and natural oils, justifying the interest in this enzyme study.

The *B. lata* lipase was easily immobilized on Celite. Celite (diatomaceous earth) is one of the most popular carriers in immobilization of lipases due, mostly, for its affordable price, and it can be utilized both in adsorption and covalent attachment. Celite is hydrophilic, and its structure and properties can vary significantly as a function of production process. Several Celite types are commercially available, and the pore size of Celite can vary from μm to mm, and the shape of the particles can be rods or beads. The shape and porosity greatly affects the adsorption of enzymes as well as the ability to retain water inside the pores. Many commercial lipase preparations are based on Celite powder, e.g., Amano lipase PS-D from *Burkholderia cepacia* (Hara 2011).

With *B. lata* immobilized lipase, it was possible to use it for up to three cycles of hydrolysis while maintaining 35 % of the initial activity (1041.47 U/g). In cases of immobilization on Celite, the yields of hydrolysis recycles cited in the literature are low, with a loss of up to 70 % in the first recycle, due to the existing weak hydrophobic interaction between the enzyme/support on the adsorption (Chang et al. 2007). The desorption may be increased in the presence of a surfactant (Triton X-100) normally used in the preparation of emulsified substrates.

The use of lipases in biocatalysis, however, occurs especially in organic solvent medium due to the high solubility of the substrates and products, to hydrolytic reverse reaction, and modification of the enzyme specificity. In principle, the ability to catalyze reactions in organic solvent medium was been mainly described as characteristic of lipases (Jaeger et al. 1994). Furthermore, the enzyme activity in these systems is typically much smaller than in aqueous solutions (Pencreac'h and Baratti 2001). In an apparent paradox, the stability of the protein is lower in water miscible solvents ($-2.5<\text{Log } P<0$) than in hydrophobic solvents ($\log P>0$). The poor stability in hydrophilic solvents represent an issue for the use of lipases involving, for example, the esterification of sugars in the production of biosurfactants, since these reactions consists of polar solvents such as 2-methyl-2-butanol (Soultani et al. 2001). Accordingly, the *B. lata* lipase presents unusual and very desirable characteristics, since its stability remained above 50 % for all miscible solvents in concentrations of up to 75 %. These results are similar to those obtained for *Burkholderia cepacia* G63, confirming the high stability in water miscible organic solvents of lipases produced by the *Burkholderia* genus (Yang et al. 2007).

Conclusion

The findings of this work demonstrated overproducing lipase by a wild strain of *Burkholderia lata* LBBIO-BL02. The bacterial isolate was found to be a producer of lipase with market potential, with high lipase activity obtained on a low cost nutrient medium. The lipase activity obtained was 1137.82 U/mL and 2146.83 U/mg with desirable characteristics. Moreover, the use of chicken fat as an inducer for lipase production appears as an excellent alternative, with great appeal related to the environment. Lipase was successfully immobilized on Celite by adsorption and showed a promising future as it allows easy immobilization with a simple and inexpensive process.

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