Extraction of Lipase from *Burkholderia cepacia* by PEG/Phosphate ATPS and Its Biochemical Characterization

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ABSTRACT

This work aimed to study the partitioning of a lipase produced by Burkholderia cepacia *in PEG/Phosphate aqueous two phase system (ATPS) and its characterization. Lipase was produced by* B. cepacia *strains in a fermenter. Enzyme partitioning occurred at pH 6.0 and 8.0, using PEG 1500 and 6000 on two tie lines. Metal ions, pH and temperature effects on enzyme activity were evaluated. Five milliliter of 7.5% olive oil emulsion with 2.5% gum-arabic in 0.1M sodium phosphate buffer at pH 8.0 and 37ºC were used for the activity determinations. Results showed that crude stratum from* B. cepacia *was partitioned by PEG1500/phosphate ATPS at pH 6.0 or 8.0 for, which the partitioning coefficients were 108- and 209-folds. Lipase presented optimal activity conditions at 37ºC and pH 8.0; it showed pH-stability for 4 h of incubation at different pH values at 37ºC. Metal ions such as Mn^{2+}, Co^{2+}, I^- and Ca^{2+} sustained enzymatic activities; however, it was inhibited by the presence of Fe^{2+}, Hg^{2+} and Al^{3+}. K_m and V_{max} values were 0.258 U/mg and 43.90 g/L, respectively. A molecular weight of 33 kDa and an isoelectric point at pH 5.0 were determined by SDS-PAGE and IFS electrophoresis, respectively.*

Key words: lipase, *Burkholderia cepacia*, aqueous two phase system, partitioning, characterization

INTRODUCTION

Lipases (triglycerol acyl-hydrolases E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of water insoluble triglycerides to di and mono-acylglycerides, free fatty and glycerol. Lipases have been utilized for the esterification, transesterification and resolutions of chiral substrates. Usually, the highest catalytic activity of lipases occurs in water/lipid interface, and they are activated in the presence of emulsified esters. This definition disregards the enzymes which perform their hydrolytic functions under water-soluble substrates (esterases) or which hydrolyze other lipids as acylhydrolases, cholesterol esterase, thiol-esterases, among others (Borgström and Brockman 1984; Balcão 1996; Paiva et al. 2000).

Hydrolytic enzymes constitute the main class of enzymes employed in the industry. Lipases catalyze lipid hydrolysis, which make them attractive for the applications in diversified industrial sectors. Lipases usage as additives in detergents still represents the main industrial application of these enzymes (Kirk et al. 2002; Ranganathan et al. 2008). The hydrolytic capability of lipases has been explored in paper and cellulose industry to remove hydrophobic material from the wood, undesired to paper

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manufacture (Jaerger and Reetz 1998). This capability is also employed to treat the effluents that may be used to remove the oil from the residual waters of factories, restaurants, residences, or even of oil refineries that pollute soil and water, as well as to remove fat deposits formed in tubing systems for hot water, beverages or liquid food. Lipases have also been responsible for the development of aromas in biodiesel, cheese and derivatives, alcoholic drinks, chocolates and desserts, by the selective hydrolysis of triacylglycerols and release of fatty acids, which act as flavorings or as their precursors (Borgström and Brockman 1984; Cammarota et al. 2001; Pandey et al. 1999; Pencreac'h and Barrati 1996; Ranganathan et al. 2008; Sharma et al. 2002; Singh and Banerjee 2007).

Lipases can be found in different organisms, including animals, plants, fungi and bacteria. From Claude Bernard discovery, lipases were initially obtained from the animal pancreas, but owing to the difficulty of obtaining these sources, there was an increase in the interest for microbial lipases which, due to the easy production and greater source of microorganisms capable of synthesizing them, are the most commonly used. The main procedure of producing the lipases by fermentative processes demonstrates control facility and productive capability, as well as reduced obtainment cost (Borgström and Brockman 1984; Hasan et al. 2006). Among the microorganisms that produce lipase, *Rhizopus*, *Aspergillus and Mucor* fungi, *Pseudomonas* bacteria and *Candida* yeasts are outstanding. Fast cellular growth is one of the advantages of bacterial sources as enzyme producers, besides being considered of high biotechnological potential due to its stability at high temperatures and organic solvents. Depending on the source, lipases may have molecular weight ranging from 20 to 75 kDa, optimal pH activity between 6.0 and 8.0 and at temperatures between 30 and 40°C (Borgström and Brockman 1984; Beisson et al. 2000; Kordel et al. 1991).

Approximately 20% of biotransformation derives from the reactions with lipases; this is why they comprise one of the most important groups of biotechnological value. Therefore, lipase biochemical characterization is extremely important to know and establish its application conditions. Literature describes the studies in which this enzyme performance ranges vary according to the utilized microorganism. In this study, *Burkholderia cepacia* lipase partitioning and characterization was carried out so as to know its performance.

MATERIALS

Microorganism
A*Burkholderia cepacia* strain was utilized, which was obtained from André Tosello Foundation and kept in test tubes at 4°C in nutrient agar.

Equipment
Sterilization of growth and production medium and glass wares was performed at 121°C for 15 minutes. The shaker utilized for lipolytic analysis and enzymatic characterization was TE-421 from Tecnal, and BIOFLO III reactor from New Brunswick Scientific was used in the production of the lipases.

Reagents
Acetone, ethyl alcohol, monobasic sodium phosphate, dibasic sodium phosphate, sodium hydroxide, and other salts, both with PA degree, were used to determine the characterization and fermentation stage, were obtained from Synth (Diadema-SP); Arabic-gum, agar-agar, yeast extract and peptone were obtained from Oxoid (England, London); Liza soybean oil and Gallo olive oil were bought from a retail store locally. Polyethylene glycol (PEG) with molecular weight of 6000 was obtained from Sigma Chemical (St. Louis, USA), as well as a mixture of salts for stock solution: monobasic potassium phosphate and dibasic potassium phosphate, both from Synth (Diadema-SP). Commercial lipase used in the electrophoresis was obtained from Sigma Chemical (St. Louis, USA).

METHODS

Inoculums preparation and fermentative system
Inoculums was transferred from test tubes to Erlenmeyer flasks of 125mL, containing yeast extract (3g/L), peptone (3g/L), KH_2PO_4 (4g/L), $MgSO_4$ (0.2 g/L) and 3% of soybean oil. Microorganism adaptation was performed during 48h at 30°C and 150 rpm. After this, inoculums was transferred to a bioreactor Bioflo III (with

useful volume of 5000mL, containing a medium of 3000mL). Air was provided by the compressor, sterilized by glass wool filter and with flow controlled by rotameters. The culture medium had the same concentrations as those of the reagents prepared for the inoculums, but with 6% (v/v) of soybean oil to induce lipase production. Fermentation was performed at 30ºC, pH 7.0, 1.5 vvm of aeration and stirring of 150 rpm for 96 h (Macedo et al. 1997; Padilha et al. 2009a and 2009b; Pastore et al. 2003).

Making of enzymatic extract
During intervals of 24 h, 15 mL of the fermented medium were collected and centrifuged for 10 min at 2016g. Supernatants were used as crude enzymatic extract (Macedo et al. 1997; Padilha et al. 2009a and 2009b; Pastore et al. 2003).

Determination of the lipolytic activity
Lipolytic activity was determined according to the methodology by Kamimura et al. (1999), Macedo (1997) and Pastore et al. (2003). Lipase assay was performed with olive oil emulsion, which was prepared by mixing 25 mL of olive oil and 75 mL Arabic-gum 7% solution in a homogenizer for 4 min. The reaction mixture containing 5.0 mL of olive oil emulsion, 2.0 mL of 100 mM phosphate buffer (pH 8.0) and 1.0 mL of the culture was incubated at 37 °C for 30 min with orbital shaking at 150 rpm. The reaction was immediately stopped after incubation by the addition of 15 mL acetone:ethanol mixture (1:1, v/v), and the liberated free fatty acids were titrated with 50 mM NaOH. One unit (U) of lipase activity was defined as the amount which liberated 1 μmol of fatty acid per min.

Enzyme characterization
The optimum pH of the enzyme was determined by measuring its activity for 30 min in the pH range of 3.0-11.0 at 37°C±2°C, using 5.0 mL of olive oil emulsion at 100mM: citrate buffer (pH from 3.0 to 5.0); phosphate buffer (pH from 6.0 to 8.0); carbonate-bicarbonate buffer (pHs from 9.0 to 11.0). The optimum temperature was obtained measuring its activity, for 30 min at temperatures between 25 and 50°C±2°C, using the olive oil emulsion acetate, at pH 8.0. The activation energy was determined by Arrhenius method. Initial rates of hydrolysis of the olive oil were determined at various substrate concentrations (0.5-50%, p/p) at pH 8.0 and 37°C. The kinetic constants K_m and

V_{max} were estimated by Lineweaver-Burk method. Ions effect also determined to observe the activation and inhibition of ions such as Co^{2+}, Hg^{2+}, Cu^{2+}, Ba^{2+}, Fe^{2+} and others (Aguilar et al. 2000; Biazus et al. 2009; Padilha et al. 2009a; Toledo et al. 2007; Wanderley et al. 2004). Proteins content was measured by Bradford method (Bradford, 1976).

Binodal curves and tie lines
Solutions of PEG 6000 and 1500 (50%, w/w) and phosphate buffer (20%, w/w) of known concentrations were prepared at pH 6.0 and 8.0. A given volume of PEG solution was added to the buffer solution until became turbid. The phase compositions were determined to obtain the binodal curve and tie-line length. PEG 6000 (or PEG 1500) and phosphate buffer solutions were mixed together until equal volumes of the phases (Albertsson 1986; Biazus et al. 2006 and 2007; Diamond and Hsu 1992; Ferreira et al. 2009 and 2011; Ferreira et al. 2007; Fileti et al. 2009 and 2010; Padilha et al. 2011).

Purification stage
In this study, PEG and phosphate salt at pH 6.0 were used for ATPS with two tie lines. Iit was put into graduated centrifuge tubes, with a total weight of 8g, by weighing the appropriate amounts of PEG 6000 and 1500 at 50% and phosphate salt at 20%. A 0.2mL from crude stratum was added into the tubes, stirred in vortex and, after reaching the diffusive balance (between 12 and 24 h), 1.0 mL of sample for each stage was collected (Albertsson 1986; Biazus et al. 2006 and 2007; Diamond and Hsu 1992; Ferreira et al. 2011; Fileti et al. 2009 and 2010; Ferreira et al. 2009). Determination of total protein concentration in inferior and superior stages was measured by the method of Bradford (1976). Enzymatic activity was determined by the methodology described by Macedo et al. (1997) and Pastore et al. (2003). The partition coefficient was obtained with equation 1:

$$K = \frac{C_{top}}{C_{bottom}} \qquad (1)$$

SDS-PAGE Electrophoresis and isoelectric focusing (IEF)
Before and after the purification in aqueous two-phase system (ATPS) PEG/phosphate salt, lipase samples were analyzed by SDS-PAGE electrophoresis, performed with Mini Protean III

(BioRad, USA) and utilizing polyacrylamide gel according to protocol introduced by Laemmli (1970), in a concentration of 7.5%. Samples were treated with the buffers containing SDS in denaturant and reducing conditions (with the presence of β-mercaptoethanol). Denaturation of sample proteins was performed by heating at 100 ºC for 10 min. Aliquots of 15 μL of each sample and 5 μL of low molecular weight indicator were applied on the gels of 7.5%. These gels were submitted to a voltage of 180 V, in a vertical bowl. Low molecular weight indicator contained the following proteins: phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa), and both kits from GE Healthcare (USA). For the electrophoresis of isoelectric focusing (IEF), the equipment PhastSystem was utilized (Pharmacia,

Sweden) at 410Vh and acrylamide gradient gels in 5% concentration ranging between pH 3.0 and 9.0 from GE Healthcare (USA), in accordance with the method described by the manufacturer. In both the techniques, gel coloration was performed with silver nitrate (Biazus et al. 2007 and 2009; Fileti et al. 2009; Morrissey 1981).

RESULTS AND DISCUSSION

Binodal curves and tie lines
Figures 1 and 2 show the pH effect on phase diagrams of PEG/phosphate ATPS. There was an influence of pH, which dislocated the binodal curves of these ATPS. For the reduced pH, there was a large need of PEG concentration for two-phase formation (Albertsson 1986; Ferreira et al. 2007; Silva and Franco 2000).
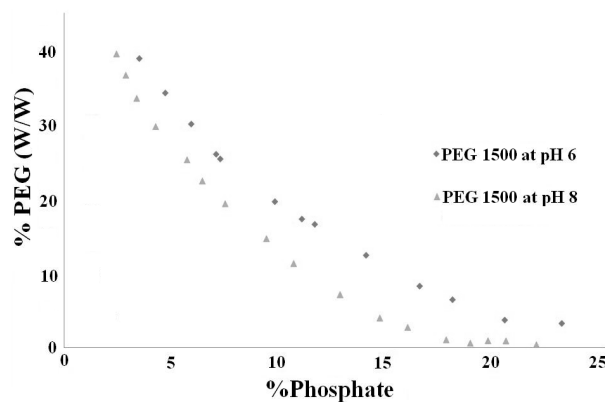

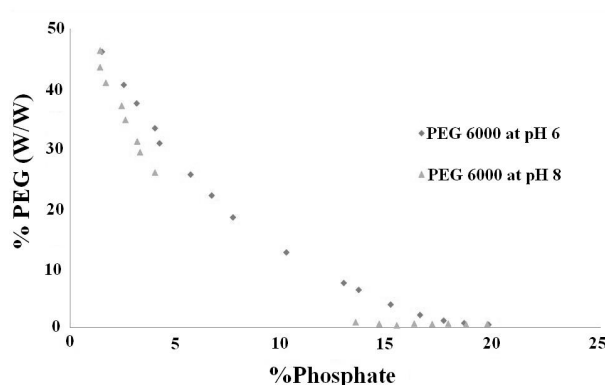**Figure 1 -** Phase diagrams of PEG 1500/Phosphate ATPS on pH effect, at 25°C.

Figure 2 - Phase diagrams of PEG 6000/Phosphate ATPS on pH effect, at 25°C.

Figures 3 and 4 show the effect of the PEG molar weight on phase diagrams of PEG /phosphate ATPS. The lower molar mass needed of a large PEG concentration for two-phase formation (Albertsson, 1986; Silva and Franco, 2000). However, according to Diamond and Hsu (1992), the pH and PEG molar weight effects on PEG-salt systems was not fully elucidated.

Tables 1 and 2 show the tie-line compositions for PEG 1500 and 6000/ phosphate at pH 6.0 and 8.0, respectively. There were significant differences among the composition systems.
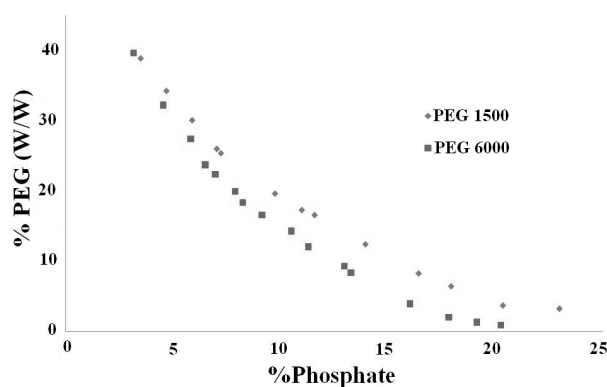

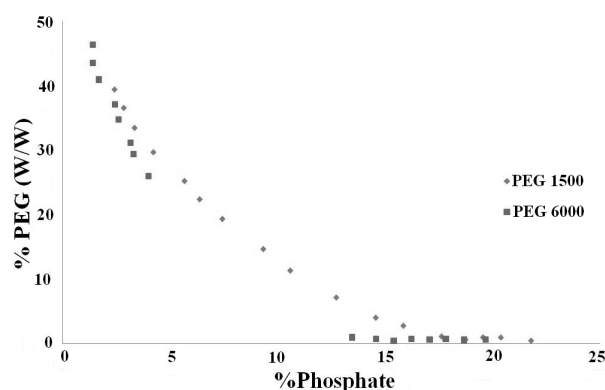**Figure 3 -** Phase diagrams of PEG/Phosphate ATPS at pH 6 and 25°C.

Figure 4 - Phase diagrams of PEG/Phosphate ATPS at pH 8 and 25°C.

Table 1 - Phase compositions of PEG1500/Phosphate ATPS.

pH	Tie Line	Total System Composition (w/w)			Top Phase Composition (w/w)			Bottom Phase Composition (w/w)		
		PEG	Salt	Water	PEG	Salt	Water	PEG	Salt	Water
6	1	17.8	12.9	69.3	31.0	5.6	63.4	5.0	19.5	75.5
	2	14.8	12.6	72.6	21.0	9.3	69.7	9.0	16.0	75.0
8	1	17.9	12.0	70.1	35.0	3.2	64.8	0.7	20.5	78.8
	2	16.8	11.0	72.2	33.0	3.6	63.4	0.6	18.7	80.7

Table 2 - Phase compositions of PEG 6000/Phosphate ATPS.

pH	Tie Line	Total System Composition (w/w)			Top Phase Composition (w/w)			Bottom Phase Composition (w/w)		
		PEG	Salt	Water	PEG	Salt	Water	PEG	Salt	Water
6	1	19.0	12.3	68.7	36.0	3.4	60.6	1.7	21.0	77.3
	2	9.7	12.3	78.0	17.0	8.5	74.5	2.2	16.0	81.8
8	1	23.7	10.5	65.8	43.6	1.4	55.0	3.0	20.0	77.0
	2	18.3	9.9	73.5	35.0	2.8	62.2	0.5	18.0	81.5

Lipase partitioning by PEG/Phosphate ATPS
Tables 3 and 4 show the assays, with the varying of PEG molar weight pH and tie line (TL). The tables, also showed the activity, protein and specific activity (SA) measured after the equilibrium of phases in the bottom and top phases from PEG1500 and 6000/Phosphate ATPS. These results were used for the calculation of partitioning coefficient (K) of lipase from *B. cepacia*, as shown in Table 5. The best results to lipase purification were at PEG 1500/phosphate ATPS for, the pH 6.0 or 8.0 and the tie lines 2 (high PEG content), which the partitioning coefficient of the specific activity were amongst 108 and 209 times, respectively.
Lipase purification method applied in this work

was very efficiently. These results were very significant, principally, while compared to methods showed by other authors.
Saxena et al. (2003) purified the lipases from *C. Viscosum* in the first process by AOT/iso-octane reversed micelles with an activity recovery of 91% and purification factor of 4.3; in a second purification, Amberlite CG and Shefadex chromatography was used, where an activity recovery of 2.8% and a purification factor of 23 was found; and in a third purification, a lipase from *B. Streamthermofilus* by CM-Sepharose and DEAE-Sepharose chromatography was purified, where an activity recovery of 62.2% and a purification factor of 11.6 were found.

Table 3 - Biomolecules partitioning on top phase by PEG/Phosphate ATPS.

Assay	PEG	pH	TL	Activity (U/mL)	Protein (mg/L)	SA (U/mg)
1	6000	8	2	6.01	122.33	49.15
2	6000	8	1	2.40	121.22	19.84
3	6000	6	2	18.72	222.10	84.29
4	6000	6	1	9.44	181.89	51.92
5	1500	8	2	9.52	73.77	129.03
6	1500	8	1	3.34	35.80	93.29
7	1500	6	2	1.44	214.87	6.68
8	1500	6	1	17.66	99.08	178.24

TL = Tie Line; SA = specific activity

Table 4 - Biomolecules partitioning on bottom phase by PEG/Phosphate ATPS.

Assay	PEG	pH	TL	Activity (U/mL)	Proteins (mg/L)	SA (U/mg)
1	6000	8	2	7.16	1031.89	6.94
2	6000	8	1	11.98	1411.56	8.49
3	6000	6	2	20.12	1415.23	14.22
4	6000	6	1	24.26	2718.23	8.92
5	1500	8	2	2.56	2134.34	1.20
6	1500	8	1	3.84	206.64	18.59
7	1500	6	2	0.02	625.25	0.03
8	1500	6	1	14.98	337.92	44.33

TL = Tie Line; SA = specific activity

Table 5 - Lipase partitioning coefficients by PEG1500 and 6000/Phosphate ATPS.

Assay	PEG	pH	TL	Partitioning coefficient
1	6000	8	2	7.079
2	6000	8	1	2.337
3	6000	6	2	5.928
4	6000	6	1	5.818
5	**1500**	**8**	**2**	**107.8**
6	1500	8	1	5.019
7	**1500**	**6**	**2**	**209.0**
8	1500	6	1	4.021

A lipase *P. aeruginosa* was purified by acetone precipitation and Q-sepharose and Q-sephacril S200 chromatographic steps, and after full purification a purification factor of 21.5-fold was found (Singh and Banerjee 2007). The procedure included phenyl-toyopearl fractionation, DEAE-Sepharose chromatography, and Saphadex300HR chromatography was done to the purification of lipases from *P. fluorecens* HU380. The enzyme was purified 24.3-fold (Kojima and Shimizu 2003). Makhzoum et al (1996) purified a lipases from *P. fluorecens* 2D by hydrophobic interaction chromatography and a purification factor of 25-fold was found.

Lipases from *Rizopus sp.* were purified by the chromatography using the SEPHADEX resin and a purification factor of 5-times was found by Pastore et al. (2003). Bradoo et al. (1999) purified the lipases from *B. stearothermophilus* SB-1 by PEG/phosphate aqueous two-phase systems (ATPS) and a purification factor of 15.27 fold was found.

Temperature effects on lipase activity

Enzyme's catalytic activity is very dependent on the temperature; however, as temperature increases, two effects occur: reaction rate increases and decreases enzyme stability due to thermal disablement; thus, temperature is one of the critical agents on enzymatic activities. In this study, assays from 25 to 50ºC were performed to determine the optimal performance temperature of lipase enzyme. In Figure 5, lipolytic activity results for these conditions are presented. Based on the results, optimal enzyme temperature was 37ºC, at which 100% of relative activity was observed, presenting an activity of 1.85 U/mL and specific activity of 29.30 U/mg. The largest variation found for the values of activity was 17% (between 20 and 37°C).

Some enzymes can lose their activity with small variations in the temperature, since the change in conformation causes lose in its active site specificity to react with the substrate (Biazus et al. 2009; Curvelo Santana et al. 2010). The optimum temperature for the reaction of lipase from *B. cepacia* found in this study was the same found by Pencreac'h and Barrati (1996). Similar values (40ºC) were found by Makhzoum et al. (1996) for *P. fluerescens* lipase, and by Pastore et al. (2003), utilizing *Rhizopus* sp lipases. However, Kojima and Shimizu (2003) characterized *P. fluorecens* HU380 lipases and obtained 45ºC as the optimal temperature whereas Nawani et al. (2006) have found an optimal temperature ranging from 60-65°C for the immobilized lipases and 60°C for free lipases of *Bacillus* sp.

Activation energy of olive oil hydrolysis by the lipases was obtained by linear regression of natural logarithm of the reaction velocity (mol/min.g of enzyme) versus temperature inverse (K), according to Arrhenius equation (Aguilar et al. 2000; Biazus et al. 2009; Toledo et al. 2007) as shown in Figure 6. Considering that the ideal gases constant value was equal to 8.31451 J/mol.K, the activation energy value of this reaction was considered close to 75.8 kJ/mol. There are no reports in literature on this aspect.
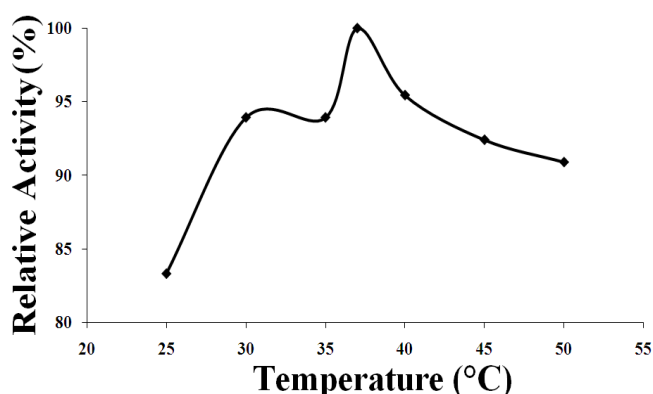

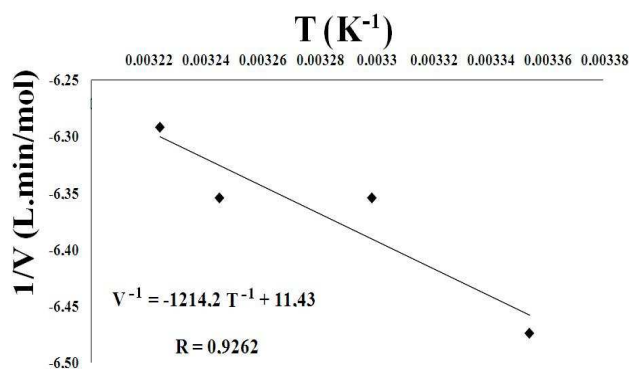**Figure 5** - Temperature effect on lipolytic activity of crude stratum of *B. cepacia.*

Figure 6 - Determination of activation energy of olive oil hydrolysis reaction by *B. cepacia* lipase.

pH effects on lipase activity

pH effects on olive oil hydrolysis by *B. cepacia* lipase were examined in the pH range between 3.0 and 11.0. According to Figure 7, the maximum of relative activity (100%) was observed at pH 8.0 (2.38 U/mL and specific activity of 25.35 U/mg). Also, it was observed that in the range of pH 5.0 and 7.0 80% of the maximum activity was maintained. Reasonable activities were obtained even in more acid pH values, and lower activities in more basic pH values (9.0 and 10.0). At pH 11.0, a decrease in the activity was observed. *B. cepacia* lipase appeared as alkaline and analog to *P. fluorescens* lipase studied by Makhzoum et al. (1996). Similar results were found by Sharma et al. (2002) for *Bacillus* sp at pH 8.0 and 9.0; Bradoo et al. (1999) found optimal values between 3.0 and 6.0 as *B. stearothermophilus* lipase. *P. fluorescens* lipases showed maximum activity at pH 7.0. These values demonstrated that lipase could perform at different ranges of pH. The pH effects on enzymes is due to variations in the ionization state of the system components as their values vary. Thus, pH value is important for the various applications lipase.

Stability of crude enzyme enzymatic activity of *B. cepacia* at different buffers and pH values of 5.0, 8.0 and 11.0 was also examined. The results are shown in Figure 8. Maximum activity (100%) was observed at pH 8.0 in phosphate buffer, after a 4-hour incubation at 37°C, while at pH 5.0 the activity was 35% lower in comparison to the activity at the end of 4 h. At pH 11, activity decreased considerably, although in this alkaline condition and with activity relatively lower, enzyme maintained itself stable along the 4-h incubation. This showed that the enzyme might be applied in different industrial sectors.
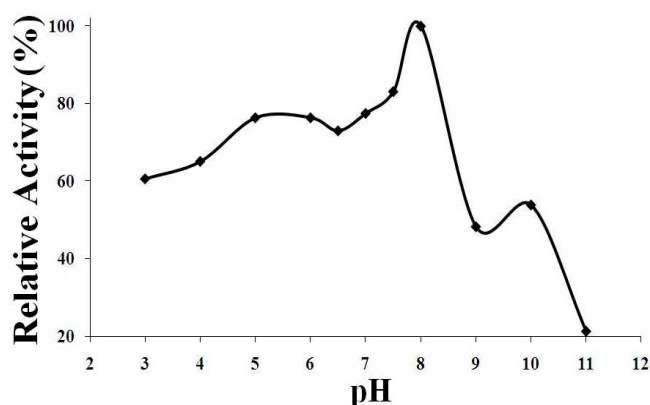

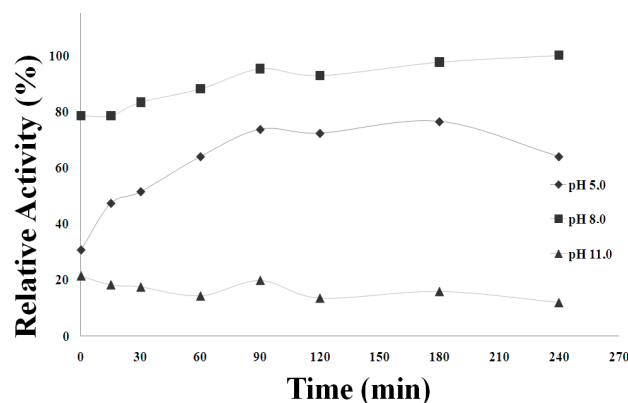**Figure 7 -** pH effects on lipolytic activity from *B. cepacia*.

Figure 8 - Effects on pH stability of crude stratum of *B. cepacia.*

Salt effect on lipase activity

A study to verify for how many days the crude enzymatic extract would maintain its activity with the addition of different ions was carried out (Fig. 9). To analyze the activity and stability in the different ions, tubes containing a certain quantity of crude enzyme were separated. To each tube, 0.3 mL of saline solution was added. One of the tubes, containing only crude enzyme, was kept as a control. These tubes were stored in the refrigerators and the analysis of the activity was performed. This study demonstrated that the salts of Fe^{2+} and Hg^{2+} significantly decreased the lipolytic activity, leading to a disablement in less than 10 days of storage. On the other hand, the addition of the salts of Ba^{2+}, Ca^{2+} and Mn^{2+} managed the store of enzymatic activity for 30 days, with significant activity decreasing as from the 15th day. These ions still maintained a great

percentage of enzymatic activity on the 30th day in comparison to the first day. Salt of I^- led to a significant loss of hydrolytic activity in the first days of storage, although after this period, 23% of enzymatic activity was maintained up to the 30th day. The salts of Al^{3+} Co^{2+}, Cu^{2+} and K^+ led to a decrease in the lipolytic activity of 75, 40, 45 and 50% up to the 10th day, respectively. A study using the crude enzyme was also carried out, and a gradual decrease in the activity along the storage was observed, with a decrease in activity of approximately 80% by the end of the 30th day in comparison to initial storage. Similar studies on *P. fluorescens* lipases were carried out by Makhzoum et al. (1996), in which Co^{2+} and Hg^{2+} proved to be strong inhibitors of this enzyme, whereas Cu^{2+} and Ba^{2+} led to a moderate inhibition, and Fe^{2+} did not have any effects on lipases.
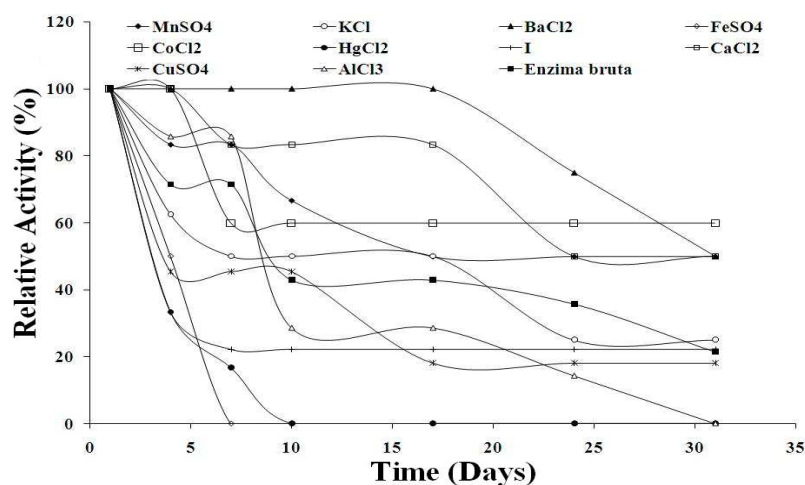

Figure 9 - Ion effects on hydrolytic activity of *B. cepacia* lipase.

Lipolytic activity had a considerable activation (a 40% increase in the activity) by the salt of I⁻. The salts of ions Mn^{2+}, Co^{2+} and Ca^{2+} maintained the enzyme stability along this period and the salts of Fe^{2+}, Hg^{2+} and Al^{3+} performed as inhibitors, leading to a significant enzymatic disablement. The addition of salts to the solution contained *Bacillus* sp RSJ-1 lipases, studied by Sharma et al. (2002), showed enzyme stability in the presence of Ba^{2+}. On the other hand, ions as K^{+1} and Co^{2+} were great inhibitors of lipolytic enzyme. However, Ca^{2+} maintained the enzyme very active (an increase of 125% in the activity in comparison to control sample). In the studies carried out by Makhzoum et al. (1996), Co^{2+} and Ba^{2+} corroborated these results in *P. fluorescens* lipases, and Lima et al. (2004) found, for *P. aurantiogriseum* lipase, some results which were similar to the ones obtained in this study, where the salts of Ba^+, Ca^{2+}, Co^{2+}, Cu^{2+} and Hg^{2+} maintained 70, 79, 105, 69 and 0% of residual activity, respectively.

Kinetics of olive oil hydrolysis

Kinetic parameters determination was performed at 37ºC and pH 8.0. Data experimentally obtained allowed for the obtainment of kinetic curve, which demonstrated the influence of oil and water emulsion containing different proportions of olive oil (0.1 to 50% v/v) on the hydrolysis velocity by *B. cepacia* lipases. It was observed that the activity increased according to the increase in the substrate concentration, (Fig. 10).

As an attempt to describe the kinetic behavior of *B. cepacia* lipase, data were analyzed based on Michaelis-Menten equation ($V_0 = V_{max} *[S] / K_M + [S]$). This equation is graphically represented by plotting V_0 (initial activity) against S (substrate concentration) and describes a rectangular hyperbola. Figure 11 is the linearization of kinetic data according to Lineweaver-Burk methodology. From the equation, it was possible to determine the values of *Km* 0.0258 U/mg and V_{max} 43.90 g/L.
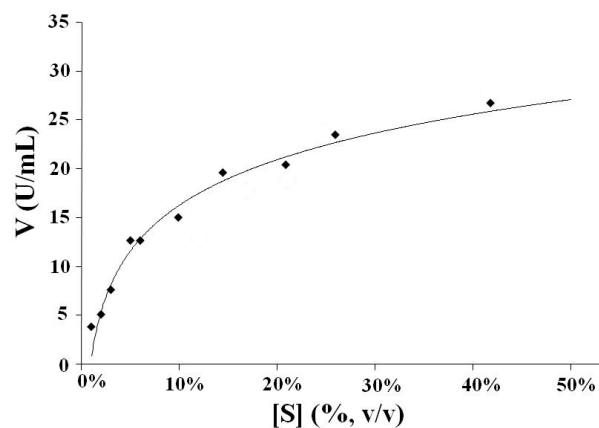

Figure 10 - Kinetic curve of olive oil hydrolysis by *B. cepacia* lipases.
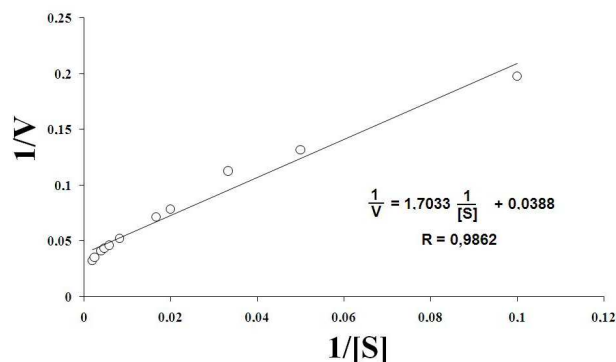

Figure 11 - Lineaweaver-Burk to *B. cepacia* lipase.

Molar weight and isoelectrical point

After the batch ATPS process, the top phase sample of the best system was analyzed by the SDS–PAGE for the determination of enzyme molecular weights and degree of purification. Protein fractions obtained from the purified material by ATPS PEG/phosphate salt at pH 6.0 and from crude enzymatic stratum were analyzed by the electrophoresis by the SDS-PAGE. Figure 12 showed that the extracted enzyme was pure and the molecular weights was 33 kDa. It was noted that the purified lipase (band 1) by ATPS/Phosphate (in this work) was more pure than commercial lipase (band 2).

Comparing the obtained values to literature results, 32 kDa was obtained by Lin et al. (1996) for the purified *P. pseudoalcaligenes* lipase F-111 and Baron (2008), who after lipase purification of *B. cepacia* in Octyl Sepharose CL-4B hydrophobic interaction column, also found the molecular

weight to be 32 kDa. Similar results were obtained by Kordel et al. (1991), where molecular weight obtained for *Pseudomonas* sp lipase was 35 kDa. Lima et al. (2004) found 40 kDa for *B. megaterium* lipase. Lipase molecular weight values from different sources are usually between 19 and 65 kDa (Bandmann et al. 2000), but they can vary from 8 kDa to 180 kDa. In this study, molecular weight value demonstrated that ATPS technique to purify *B. cepacia* lipases was efficient in comparison to the fraction before and after purification with commercial *B. cepacia* lipase.

Figure 13 shows the electrophoresis of isoelectric focusing (IEF) of lipase purified at ATPS pH 6.0. IEF revealed with silver nitrate displayed pI of 5.0. Kordel et al. (1991) found from 4.5 to 4.6 pI for *Pseudonomas* sp lipase; pI of 7.3 was found by Lin et al. (1996) for *P. pseudoalcaligenes* lipase; pI results for *B. cepacia* lipase were not found in the literature up to the present moment.
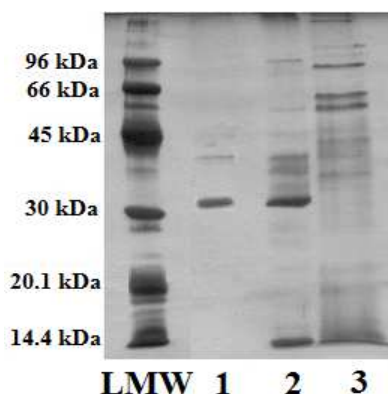

Figure 12 - SDS-PAGE electrophoresis in 12.5% gel, sample in denaturant and reducing conditions. (LMW) low molecular weight indicator; (1) sample of purified lipase in PEG/phosphate salt aqueous two-phase system at pH 6; (2) commercial lipase of *B. cepacia;* (3) crude enzymatic stratum.
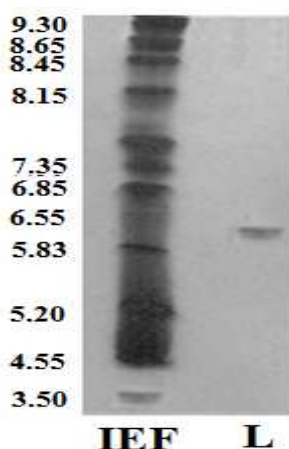

Figure 13 - Electrophoresis of isoelectric focusing at pHs from 3 to 9. (IEF): indicator of isoelectric point; (L): sample of purified lipase in PEG/phosphate salt aqueous two-phase system at pH 6.

CONCLUSIONS

The best condition of partitioning of crude stratum from *Burkholderia cepacia* by PEG/phosphate aqueous two phase system was obtained at pH 6.0 or 8.0, for which the partitioning coefficient were amongst 108 and 209. Lipase enzyme presented optimal activity conditions at $37^{o}C$ and pH 8.0 and showed pH-stability for 4 h at $37^{o}C$. The enzyme activity was positively influenced by the Mn^{2+}, Co^{2+}, I^{-} and Ca^{2+} and was inhibited by the presence of Fe^{2+}, Hg^{2+} and Al^{3+}. Its K_m and V_{max} values were 0.258 U/mg and 43.90 g/L, respectively, and the molecular weight and isoelectric point were 33 kDa and pH 5.0, respectively.

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