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Expanded bed adsorption of an alkaline lipase from *Pseudomona cepacia*

Giovana da Silva Padilha[a], José Carlos Curvelo-Santana[a,b,*], Ranulfo Monte Alegre[c], Elias Basile Tambourgi[a,**]

[a] *Department of Engineering of Chemical Systems, School of Chemical Engineering, State University of Campinas, University Campus "Zeferino Vaz", Av. Albert Einstein 500, Postal Code: 6066, Barão Geraldo, 13081-970 Campinas, SP, Brazil*
[b] *Department of Exact Sciences, Nine of July University, Vergueiro Street 235/249, Liberdade, 01504-001 São Paulo, SP, Brazil*
[c] *Department of Food Engineering, School of Food Engineering, State University of Campinas, University Campus "Zeferino Vaz", Av. Albert Einstein 500, Postal Code: 6121, Barão Geraldo, 13083-862 Campinas, SP, Brazil*

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ABSTRACT

An extracellular lipase was isolated from *Pseudomona cepacia* by expanded bed adsorption on an Amberlite 410 ion-exchange resin. Enzyme characterization and hydrodynamic study of a chromatography column were done. Enzyme purification was done at three condition of expanded bed height (H): at one and half (6 cm), at two (8 cm) and at three (12 cm) times the fixed bed height (H_0 = 4 cm). The results showed that the experimental data was fitted to the Richardson and Zaki equation, and the comparison between the experimental and calculated terminal velocities showed low relative error. In enzyme purification for better condition, a purification factor of about 80 times was found at 6 cm of expanded bed height, or 1.5 times of expansion degree. Purified lipase had an optimal pH and a temperature of 8 and 37 °C, respectively.

1. Introduction

Lipases (triglycerol acil-hydrolase E.C. 3.1.1.3) are used in many industrial and biotechnological applications, such as detergent formulation, modification of fats and oils, and also used in dairy and pharmaceutical industries [1–5]. Among the diverse classes of microorganisms, the ones most used in the lipase production are: *Achromobacter*, *Alcaligenes*, *Aspergillus*, *Bacillus*, *Candida*, *Humicola*, *Mucor*, *Pseudomona* and *Rizopus*. From the *Pseudomona* species: *P. fragi*, *P. cepacia*, *P. aeruginosa* and *P. fluorescens* have been detached in uses for lipase production [6–13].

They are stable and extremely valuable catalysts for many practical applications, such as, to generate chiral entities from alcohols, carboxylic acid esters, cyanohydrins, chlorohydrins, diols, amines, diamines and amino alcohols, which are used as building blocks for a variety of pharmaceuticals and other fine chemicals. Many lipases are only moderately stable at high temperature and pHs, which can influence their usefulness in some interesting reactions. Using lipases from thermophilic microorganisms, whose resistance to drastic conditions has been developed by nature, can solve this problem [1–5].

Psychrotrophic bacteria grow in refrigerated milk and produce heat-resistant lipases that are implicated in the spoilage of dairy products. Studies on these lipases have concentrated mainly on their unusual heat resistance. Although psychrotroph lipases are considered undesirable in the dairy industry, interesting industrial applications may be found in the future due to their ability to resist heat [1,4]. Biodiesel has become more attractive recently because of its environmental benefits and the fact that it is made from renewable resources. Enzymatic transesterification has attracted much attention for biodiesel production, as it produces a highly pure product and enables easy separation from the by-product, glycerol; therefore, the interest in lipases intensified because of application in transesterification reactions [14,15].

Recently, the purification of microbial lipases have been archived by conventional procedures, including, fractional precipitation, liquid chromatography, gel filtration, aqueous two-phase systems, or a combination of these, usually achieving partial purification and low yields. Hydrophobic interaction chromatography (HIC) has been successfully used due to high surface hydrophobicity of lipases, but it is not industrially compatible, again due to low yields [1,4,6,11,12].

The three principal downstream processes are aqueous two-phase systems extraction, membrane separation, and fluidized bed

* Corresponding author at: Department of Engineering of Chemical Systems, School of Chemical Engineering, State University of Campinas, University Campus "Zeferino Vaz", Av. Albert Einstein 500, Postal Code: 6066, Barão Geraldo, 13081-970 Campinas, SP, Brazil. Tel.: +55 19 32818947.
** Corresponding author.
E-mail addresses: giovana_padilha@yahoo.com.br (G. da Silva Padilha), jccurvelo@yahoo.com.br (J.C. Curvelo-Santana), ranulfo@fea.unicamp.br (R.M. Alegre), eliastam@feq.unicamp.br (E.B. Tambourgi).

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sorption. Expanded bed adsorption (EBA) is a novel purification technique developed from protein chromatography, but different in that it shows the fluidized chromatographic adsorbent bed. It permits crude feeding into the chromatographic column without an initial treatment to eliminate the biological material suspension, and as the bed expands it increases adsorbent surface contact, making interaction with the targeted molecule more effective [16–22]. Presently, several studies on expanded bed adsorption behaviors allow as to better understand the effects of adsorbent type and size [17,18,23–27], bed height, and linear velocity [17,18,26–31] for application in the recovery of important biomolecules.

Dainiak et al. [19] proposed a new technique for treatment of anion exchangers for adsorption of the fermentation product shikimic acid directly from the cell-containing fermentation broth. Amberlite 401 and 458 anionic exchange resins were treated with hydrophilic polymer, poly(acrylic acid) (PAA), to form PAA–Amberlite 401 and PAA–Amberlite 458. The binding capacity of pure shikimic acid was about 81 mg/mL of adsorbent for both cross-linked PAA–Amberlite and native Amberlite in the fluidized mode of column operation. Binding capacity dropped to 17 and 15 mg/mL, respectively, when using filtrate fermentation broth and to about 10 mg/mL for cross-linked PAA–Amberlite when using the fermentation broth containing cells directly. Native Amberlite cannot be used for the direct adsorption of shikimic acid due to the immediate clogging of the column and the collapse of the expanded bed. The cross-linked PAA–Amberlite was used repeatedly for direct adsorption of shikimic acid from industrial fermentation broth.

Human serum albumin (HSA) from very dense *Saccharomyces cerevisiae* suspensions was recovered by expanded bed adsorption in a recent work [28].

In their work, Fernandez-Lahore et al. [25] examined the suitability of ion-selective electrodes (ISE) for a hydrodynamic study in turbid, cell-containing fluids. The enhanced feedstock compatibility of IES is better than that of other tracer sensing devices and allows a better study of bed system hydrodynamics under relevant operating conditions. According to the authors, the data provided a powerful tool for predicting the overall process of adsorption with a defined feedstock type and composition. The best results were obtained using intact yeast cell suspensions at different biomass contents (up to 7.5% wet weight) and buffer conductivities (5–12 mS) in an EBA column filled with the adsorbent Streamline Q XL as the fluidized phase.

With the recent advances in biotechnology, downstream processes are being applied in the purification of biological material, mainly the proteins and enzymes of commercial interest. In view of the development of new technologies, in this present work, the effect of bed expansion and buffer type on the adsorption of lipases from *P. cepaceas* on Amberlite 410 ion-exchange resin was studied. The hydrodynamic behavior of an expanded bed adsorption column on effects of the temperature, biomolecules and salt added to the distilled water were studied to obtain for optimal conditions the lipases recovery on ion-exchange resin.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

An Amberlite IRA 410 ion-exchange resin from VETEC (São Paulo, Brazil) was used. Maize seeds were supplied by EMBRAPA, Aracaju, Sergipe, Brazil. Acetone PA, acetic acid, sodium acetate and, potassium sodium tartrate; were provided by VETEC (São Paulo, Brazil). Potassium hydrogen phosphate, comassie brilliant blue G, di-sodium hydrogen phosphate, agar-agar, sodium hydroxide, yeast extract, peptone, TRIS and chlorine acid were provided by MERCK (Darmstadt, Germany). Olive oil was acquired from a supermarket in Campinas (Brazil).

Table 1
Fluidizers and particles properties.

Temperature (°C)	Properties	H_2O*	Fluidizer PhB	BB
22	ρ_L (kg/m^3)	997.5	1011	1004
	μ (kg/(s m))	9.384×10^{-4}	9.870×10^{-4}	9.872×10^{-4}
28	ρ_L (kg/m^3)	996.8	1007	1023
	μ (kg/(s m))	8.569×10^{-4}	9.106×10^{-4}	9.899×10^{-4}

Particles	Diameter, d_P (m)	ρ_L (kg/m^3)
Amberlite IRA 410**	4.4×10^{-4}	1120

Source: *Streeter [32] and **Sigma [33]. H_2O is the distilled water; PhB is the phosphate buffer; BB is the biomolecule + buffer.

2.1.2. Microorganisms

The *P. cepacia* strains were supplied from André Tosello Foundation, and were maintained at 4 °C on Agar-culture.

2.1.3. Fluidizers and particles

Distilled water (H_2O), 0.05 M phosphate buffer at pH 7 (PhB) and 2% of maize malt in 0.07 M phosphate buffer at pH 7 (BB) were used as fluidizers. In Table 1, the physical properties of fluidizers and adsorbent particles are shown. Water properties were found in the literature [32] and properties of the other fluids were measured by viscosimetry and by the changed weight of 1 mL of fluid volume. These properties are used to calculate U_T by substitution into Eq. (2) and bed voidage by substitution into Eq. (5).

2.1.4. EBA column

In Fig. 1 a scheme of the EBA column used in the present work is shown. The glass column is 1 cm × 30 cm with an adjustable piston and feed flow inlet at the bottom of the column and a product flow outlet at the top. Sixty mesh plates at the feed inlet and at the product outlet were used to avoid loss of adsorbents particles. A ruler was placed at the side of the column for measurement of bed height [17,18,22].

2.2. Methods

2.2.1. Hydrodynamic study

Two grams of Amberlite IR 410 ion-exchange resin was used in all assays; a bed height of 4 cm was obtained with this resin mass. The fluidizer was loaded in the bottom of the column, at laboratorial temperatures of 22 or 27 °C. Linear velocities were between 0.0002 and 0.008 m/s and bed height was measured with the ruler at the side of the column. The Richardson–Zaki index and experimental end velocity ($U_{T,Exp.}$) were obtained with Eq. (4) and the calculated end velocity ($U_{T,Calc.}$) was obtained with Eq. (3) [17,18,24–31]. According to Richardson and Zaki [8], Eq. (1) forms the relationship between fluid velocity (U) and end velocity of the particle (U_t) with

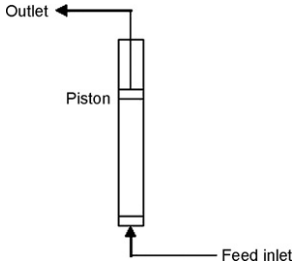

Fig. 1. Scheme of the EBA column.

the bed voidage (ε), which is given as:

$$\frac{U}{U_t} = \varepsilon^n \quad (1)$$

While n is the Richardson–Zaki [8,31] index or expansion index. For the Stokes region, $Re_P < 0.1$, the terminal velocity of an isolated particle (U_T) is given as:

$$U_T = \frac{g\ d_p^2(\rho_P - \rho_L)}{18\mu} \quad (2)$$

And Re_P is given as:

$$Re_P = \frac{d_P \rho_L U}{\mu} \quad (3)$$

With the linearization of Eq. (1), it is possible to obtain n, experimentally with Eq. (5) [17,18,27,30,31]:

$$\ln U = \ln U_T + n \ln \varepsilon \quad (4)$$

2.2.2. Determination of bed voidage (ε)

Bed voidage was obtained by substitution of data on specific mass (ρ_P) and mass (m_P) of the adsorbent particles, area of the cross-section of the column (A_T) and bed height (H), in the following equation:

$$\varepsilon = 1 - \frac{V_P}{V_L} = 1 - \frac{V_P}{A_T H} = 1 - \frac{m_P}{\rho_P A_T H} \quad (5)$$

where V_P is particle volume [17,18,27,30,31].

2.2.3. Enzyme production

P. cepacia strain were pre-grown for 8 h at 30 °C and 150 rpm; in a liquid-culture (125 mL) containing 2 g/L of yeast extract, 5 g/L of peptone and 3% of soy oil. For enzyme production, after being pre-grown, the strains were transferred to a 5 L Bioflow III bioreactor, with 3 L of a liquid-culture containing 2 g/L of yeast extract, 5 g/L of peptone and 6% (v/v) of soy oil. Fermentation occurred at 30 °C, pH 7, and 150 rpm, with 1.5 vvm of filtered-air for 96 h. Extracellular lipases from *P. cepacia* were obtained in supernatant after centrifugation at 7000 rpm every 10 min [7,8,11].

2.2.4. Enzymes assays

Substratum solution was made by mixing 50 mL of olive oil and 50 mL of a 7% (p/v) Arabic-gum emulsion, according to Soares [11,34,35]. An amount of 5 mL of substratum solution was added to 2 mL of 0.1 M phosphate buffer at pH 7 and 1 mL of enzyme solution. A control sample without enzyme was also made. After 10 min at 37 °C the acidity was measured by tittering using 0.02 M of KOH solution. Fenolftaleine was used as an acid–base indicator. Total protein concentration was determined according to the dye binding method of Bradford [36]. One unit of enzyme activity was defined as μmol of acid released per min under the assay conditions. The specific activity was calculated as activity for milligrams protein.

2.2.5. Lipase purification

Expanded bed adsorptions were carried out at 22 °C and pH 7. Adsorbent bed was pre-equilibrated in the working height (6, 8 and 12 cm), with 0.05 M phosphate buffer at pH 7. Five milliliters of enzyme supernatant was loaded in the column bottom for promoting the adsorption of enzymes on Amberlite IRA 410 ion-exchange resin. The elution was profiled with 0.25 M NaCl at 14 mL/min in descendent flux. The activity and the protein content were measured from time to time during the adsorption, washing, and elution periods [17,18,20,22,24,29].

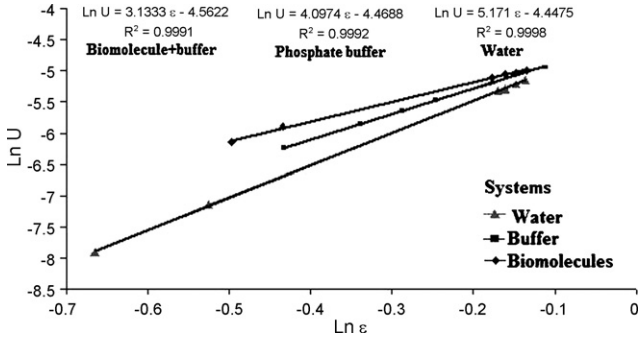

Fig. 2. Richardson–Zaki models, at 22 °C and pH 7.

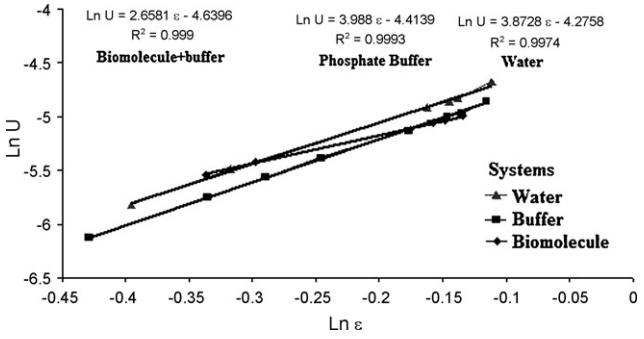

Fig. 3. Richardson–Zaki models, at 27 °C and pH 7.

2.2.6. Enzyme characterization

The optimum pH of the enzyme was determined measuring its activity every 10 min in pH varying from 3.0 to 5.0 at 37 ± 2 °C, using the olive oil substratum solution prepared in the 0.1 M of citrate buffer solutions; 6.0–8.5 at 37 ± 2 °C, using the olive oil substratum solution prepared in the 0.1 M of phosphate buffer solutions and 9–11 at 37 ± 2 °C, using the olive oil substratum solution prepared in the 0.1 M of ammonium buffer solutions. The optimum temperature was obtained measuring its activity every 10 min, at temperatures varying from 25 to 50 ± 2 °C, using the olive oil substratum solution in the 0.1 M phosphate buffer at pH 7.5 [4,9–11,18,37–39]. Activation energy of the olive oil hydrolyses by lipases from *P. cepacia* was calculated by the Arrhenius law [37–39].

3. Results and discussion

In Figs. 2 and 3, the curves of $\ln U$ versus $\ln \varepsilon$ are shown. It can be observed that the multiple correlations are optimal (about 1.0), suggesting that the Richardson–Zaki equation is the best empirical model for predicting particle fluidization. There was a reduction in the value of n when salt (phosphate) and biological material (maize malt) were added to the distilled water systems. It introduces the concept that there is a stronger resistance of resin bed in order water (H_2O) > phosphate buffer (PhB) > biomoleule + buffer (BB). For the fluidizer containing maize malt and salts, there was a large effect of friction on degree of expansion for the particle–particle, particle–liquid and particle–biomolecule interactions, which provoked an increase in the linear velocity of the fluidizer to maintain the bed voidage at the same level as that in the salt system [17,18,24–31]. Analyzing these figures, it can be observed that the values of Richardson–Zaki index decreased in relation to temperature and to the addition of salt and of biomolecules.

Table 2 makes the comparison between the experimental ($U_{T,Exp.}$) and calculated ($U_{T,Calc.}$) particle end velocities for fluidizers and temperatures studied. It can be observed that the errors between the velocities are lower and they are associated with

Table 2
Comparison between the experimental and calculated particles end velocities.

T (°C)	Fluid	U_T (m/s)		Error (%)
		Exp.	*Calc.*	
22	H_2O	0.0134	0.0135	−0.74
	PhB	0.0115	0.0114	+0.88
	BB	0.0104	0.0121	−14.05
28	H_2O	0.0164	0.0147	+11.56
	PhB	0.0125	0.0127	−1.57
	BB	0.0097	0.0099	−2.02

U_T is the end velocity; *Exp.* and *Calc.* are the values of the experimental and calculated end velocities. H_2O is the distilled water; PhB is the phosphate buffer; BB is the biomolecule + buffer.

Table 3
Results of purification of lipases from *Pseudomonas cepacias* supernatant by expanded bed adsorption on Amberlite IRA 410 ion-exchange resin, with the phosphate buffer as fluidizer, at pH 7.

H (cm)	H/H_0	Sample	Protein (mg/L)	Activity (U/mL)	SA (U/mg)	AR (%)	PF
		Crude	65.0	1.715	26.31	100	1
6	1.5	Purified	0.859	1.757	2045	102.45	77.74
8	2	Purified	3.267	1.11	339.8	64.72	12.91
12	3	Purified	1.821	1.349	740.8	78.66	28.16

Where H_0 is the fixed bed height (H = 4 cm); H is fluidized bed height; SA is the specific activity; AR is the activity recovery; PF is the purification factor of lipases.

experimental measurement viscosity and specific mass and the reliability of the data in the table. The low error suggests that the Richardson–Zaki equation is the best empirical model to predict the hydrodynamic behavior of adsorbent particles in expanded bed systems, even when the fluidizer contains a larger amount of biological material in suspension [18,19,23,25,26,28–31]. The fluidized particle end velocity decreased when the salt and biological materials were added. The specific mass (ρ) and the viscosity (μ) of the fluidizers are greater than those of the water fluidizer.

The adsorption of proteins was on mixed-mode fluoride-modified zirconia (FmZr) particles (38–75 μm, surface area of 29 m^2/g and density of 2.8 g/cm^3). Because of the high density of the porous zirconia particles, HSA (4 mg/mL) can be adsorbed in a FmZr bed expanded to three times its height. The expanded bed adsorption of any protein from a suspension containing more than 50 g DCW/L cells had not been previously reported. The FmZr bed expansion characteristics were well represented by the Richardson–Zaki correlation with a particle terminal velocity of 3.1 mm/s and a bed expansion index of 5.4 [28].

Fig. 4 is the chromatographic curves of protein from *P. cepacia* during the expanded bed adsorption processes on the Amberlite IRA 410 ion-exchange resin. By examining the curves, it is possible to identify the adsorption, washing and elution chromatographic stages.

Table 3 shows the results of lipase recovery on Amberlite IRA 410 ion-exchange resin, using phosphate buffer as the fluidizer, at pH 7. From the table, we understand that the phosphate buffer is a promoter of good affinity between amylase and resin, and that at 6 cm of bed height (one and half time of expansion degree) is the best system for lipase recovery, by the expanded bed adsorption on Amberlite IRA 410 ion-exchange resin. There was a decrease of purification factor with expansion degree. The activity recoveries were above 100% of activity from *P. cepacias* supernatant (crude material) showing that Amberlite IRA 410 had a good affinity with this lipase. The original meddle had olive oil and products from olive oil hydrolysis; they are lipase inhibitors and these inhibitors were not captured by resin, because of this the activity recovery was more than 100% and the factor was higher [17,18,20].

Lipases from *Rizopus* sp. were purified by chromatography using the SEPHADEX resin and a purification factor of five times was found [11]. Bradoo et al. [6] purified lipases from *Bacillus stearothermophilus* SB-1 by PEG/phosphate aqueous two-phase systems (ATPS) and a purification factor of 15.27-fold was found [6]. The procedure included phenyl–toyopearl fractionation, DEAE-Sepharose chromatography, and Saphadex 300HR chromatography was done to the purification of lipases from *P. fluorecens* HU380 and the enzyme was purified 24.3-fold [9].

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 [13]. Makhzoum et al. [4] purified lipases from *P. fluorecens* 2D by hydrophobic interaction chromatography and a purification factor of 25-fold was found.

Saxena et al. [40] 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.

Thus, the recovery yield obtained in this work was likely to facilitate the lipase purification by chromatography, and they are more than lipase purified by the following techniques: phenyl–toyopearl and acetone precipitation; Amberlite CG, DEAE-Sepharose, DEA-Sepharose, Sephadex, Sephadex300HR, CM-Sepharose, Q-sepharose and Q-sephacril S200 chromatography process; and hydrophobic interaction chromatographics.

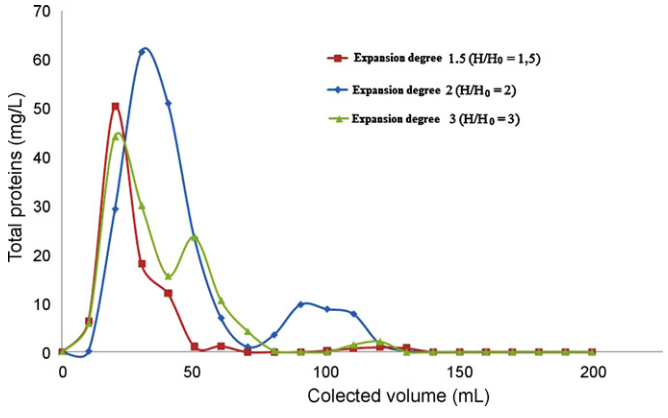

Fig. 4. Protein curves obtained for the passing of *Peseudomonas cepacias* supernatant into the Amberlite IRA 410 bed from chromatographic column.

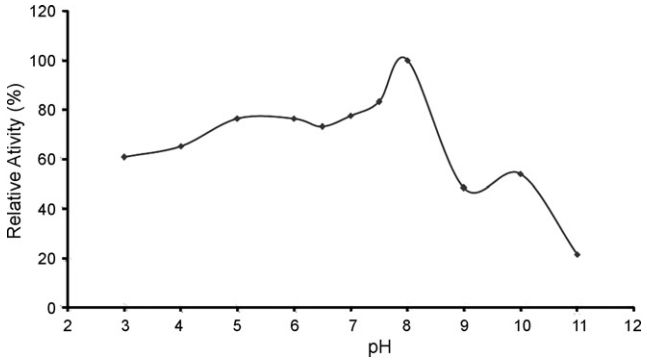

Fig. 5. pH effect on the activity of the lipase from *Pseudomonas cepacia*, at 37 °C.

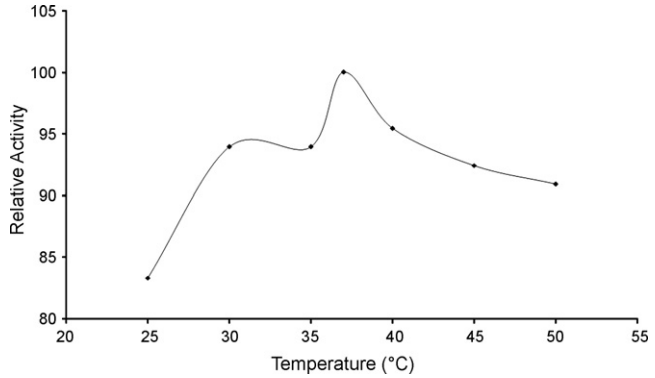

Fig. 6. Temperature effect on the lipase activity from *Pseudomonas cepacia*, at pH 7.5.

The majority of these authors used DEAE-sephacel and DEAE-cellulose resin, which increased the cost of enzyme purification. By using of Amberlite IRA 410 ion-exchange resin and the chromatography on expanded bed condition, it was possible to obtain pure lipase from a crude source at a low cost [11,18,22,24,33]. The high affinity of Amberlite IRA 410 ion-exchange resin for amylases had been perceived by Biazus et al. [17] and Curvelo-Santana [18] from maize malt and for Toledo et al. [22] from maize *Aspergillus niger*, when they purified these enzymes on an expanded bed. Campos [24] also perceived the high affinity of Amberlite IRA410 for purification of bromelain from *Ananas comosus* sp. by expanded bed adsorption. The values of the purification factors only are obtained by affinity chromatography [41].

3.1. Biochemistry characterization

Fig. 5 shows that lipases from *P. cepacia* were kept at 80% of activity in the pH range between pH 5 and 8; this shows that lipase has a large range of working pH. Singh and Banerjee [13] also noted that lipases *P. aeruginosas* are pH stable between the pH 7 and 9. Lipases from *P. fluorecens* HU380 studied by Kojima and Shimizu [9] had pH stability at a pH range between the pH 6 and 7. This figure, it notes that the optimal pH of lipase from *P. cepacia* is pH 8, so this enzyme is an alkaline lipase; this is analogous of the lipase from *P. fluorecens* 2D (pH 8.5), studied by Makhzoum et al. [4]. Sharman [42] obtained a lipase from *Bacillus* sp., with an optimal pH range between pH 8 and 9; however, Bradoo et al. [6] found an optimal pH range for lipase *B. stearothermophilus* SB-1 between the pH 3 and 6. Lipases from *P. fluorecens* HU380 had 7 as an optimal pH [9].

Fig. 6 shows that 80% of lipase activity from *P. cepacia* was kept in the full range of temperatures studied and the optimal temperature

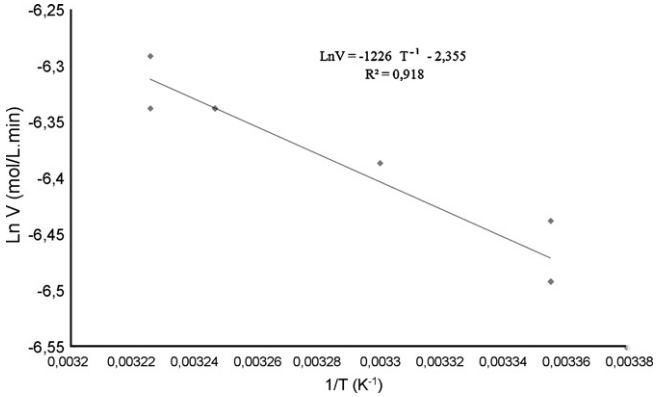

Fig. 7. Determination of activity energy of olive oil hydrolyses by lipases from *Pseudomonas cepacia*, at pH 7.5.

is 37 °C. This introduces that lipases may be used in oil hydrolysis in any temperature among 25–50 °C. Kojima and Shimizu [9] characterized the lipases from *P. fluorecens* HU380 and an optimal temperature was obtained at 45 °C and Makhzoum et al. [4] found an optimal temperature for lipases at 40 °C; however, Nawani et al. [5] obtained the optimal temperatures between 60 and 65 °C for immobilized lipases and 60 °C for free lipases from *Bacillus* sp., and bout were stables at 60 °C/h.

In Fig. 7, the Arrhenius law was used to obtain the activation energy of reaction of oil hydrolysis by lipases from *P. cepacia*, at pH 7.5, according to the method shown for Aguilar et al. [37], Biazus et al. [39] and Curvelo-Santana [38]. Since the gas constant is 8.31451 J/(K mol), the activation energy for the oil hydrolyses is of 10.2 kJ/mol. There is not anything registered on the determination of activation energy for the oil hydrolysis by lipases; however, Aguilar et al. [37] found an activation energy of 32.6 kJ/mol for amylases from *Lactobacillus manihotivorans* LMG 18010^T and Biazus et al. [39] and Curvelo-Santana [38] found a value of 44.5 kJ/mol for the activation energy of starch hydrolyses by amylases from maize malt.

4. Conclusions

The Richardson and Zaki model showed a good fit with the experimental data, showing lower relative error comparison between the experimental and calculated terminal velocities. Phosphate buffer promoted a good affinity between amylase and resin, and that 6 cm of bed height is the best system for lipase purification by the expanded bed adsorption on Amberlite IRA 410 ion-exchange resin, while a purification factor of about 80 times was found at 6 cm of expanded bed height, or a 1.5 times the expansion degree. Purified lipase had an optimal pH and temperature of 8 and 37 °C, respectively, and activation energy of 10.2 kJ/mol was found for the olive oil hydrolyses by lipases from *P. cepacia*. This work showed how to purify the lipases *P. cepacia* by expanded bed adsorption with a high affinity and at low production cost.

Acknowledgment

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