Applied Biochemistry and Biotechnology (2019) 189:1108–1126
https://doi.org/10.1007/s12010-019-03041-x

Economic Method for Extraction/Purification of a *Burkholderia cepacia* Lipase with Potential Biotechnology Application

G. S. Padilha [1] · W. R. Osório [1,2]

Received: 11 February 2019 / Accepted: 10 May 2019 /
Published online: 6 June 2019

Abstract
In order to recover biomolecules, a single downstream processing step is carried out. In this sense, an aqueous two-phase system (ATPS) containing polyethylene glycol (PEG) and potassium phosphate salts is used. Intending the purification of *Burkholderia cepacia* (Bc) lipase, the effects of the molecular masses of 1500 (PEG 1500), 4000 (PEG 4000), and 6000 (PEG 6000), pH (6, 7, and 8) and distinct tie line lengths are perfomed. Although this is reasonable reported in literature, a study covering an economical production aspect considering the Bc is scarce. This characterizes a novelty proposed in this investigation. Lipase is recovered in a polymer phase at lower pH value. PEG 1500/phosphate salt ATPS at pH 6 is considered a good method with ~ 98% of the extraction efficiency. Another contribution of this proposed investigation concerns to a biotechnological material synthesis, which is applied in several advanced and revolutionize engineering practices. Additionally, an economic analysis of the proposed method indicates a minimal sale price (~ US$410/L) inducing to a future and potential commercial application.

Keywords Aqueous two-phase systems · *Burkholderia cepacia* lipase · Profit · Purification · Market price

Introduction

As a valuable tool for the purification and separation of biomolecules, the aqueous two-phase systems (ATPS) have been applied. This mild technique and its applications have widely been

✉ G. S. Padilha
giovana.padilha@fca.unicamp.br

[1] School of Applied Sciences/FCA, Research Group in Manufacturing of Advanced Materials, University of Campinas, UNICAMP, Campus Limeira, Limeira, SP, Brazil

[2] School of Technology, University of Campinas, UNICAMP - Campus I, Limeira, SP 13484-332, Brazil

reported in several separation processeses [1–4]. However, in order to separate and to purify the biomolecules, two immiscible polymer phases can be used. A mixture between a polymer and a high ionic strength salt can be utilized [4]. Besides, a mixture between two distinct polymers with different molecular mass and salt [5] by using ionic liquids and organic solvents can also be considered [3]. Under these circumstances, the biomolecules are separated according to their surface properties. The partition can be influenced by size, electrical charge, hydrophobicity, and biospecific recognition [6, 7]. Biotechnological applications at low cost can be considered since this mentioned technique promotes high selectivity, and the preservation of the enzyme activity can be easily scaled up. The considerable aspect of these ATPS systems is that both the examined phases are aqueous (containing significant water content), due to the dissolution in water [3, 8–11].

Among the enzymes, the microbial lipases (triacylglycerol–hydrolase E.C. 3.1.1.3) produced in the fermentation broths, in which secondary or intermediate products are generated, a purification step is required. Using the ATPS technique and appropriate conditions, in one of the phases and the target protein content in other phase, the cell fragments can be confined [12, 13].

Lipases are enzymes used in industrial applications such as detergent [14], food [15, 16], pulp and paper [17], and pharmaceutical industries [18]. Additionally, it can also be applied in the novel biotechnological applications, e.g., bioremediation [19], synthesis of biofuels [20], hydraulic fluids [21], and flavor compounds [22]. In some cases, the use of a more purified lipase is required. The commonly utilized products for the lipase purification are ammonium sulfate precipitation [23], reverse micelle [24], and chromatography methods [9, 14, 25] Commonly, only one-step purification method is not sufficient to provide a determined level or purity level required. With this, the combination of more than one purification technique to improve purification and to reach higher yields is required. Consequently, the lipase obtained using the conventional methods becoming more time-consuming and difficult to use for industrial applications due to its corresponding costs increased [26].

Lopes et al. [27, 28] have demonstrated an economic viability of a pharmaceutical product from a purified pineapple juice by membrane separation process. This has indicated a cost lower between 9 and 13 times lower than a common commercial product. Although polyethylene glycol (PEG)/phosphate system is commonly investigated, an economical viability investigation using *Burkholderia cepacia* lipase is scarce. This indicates a novelty corroborated in this present investigation, which can be potentially applied in a sustainable system into biofuel production. Although is complicate to provide a technical detail concerning to specific biofuel yield using lipase, it is remarked that independently of the adopted extraction/purification process, a more purified lipase/enzyme provides a higher product yield. Based on this, if a cheaper purification/extraction lipase is attained, an economic and environmentally friendly product can potentially be commercialized.

An experimental planning in order to evaluate the effects of molecular mass of PEG (1500, 4000 and 6000), pH (6, 7 and 8), and tie lines (PEG and salt equilibrium concentrations) on the partition coefficient is carried out.

Material and Methods

Materials

From Sigma-Aldrich® [29], three distinctive polyethylene glycols with different molecular weights, i.e., 1500 (PEG 1500), 4000 (PEG 4000), and 6000 (PEG 6000), were utilized.

Acetone, ethyl alcohol, monobasic sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, magnesium sulfate, calcium chloride, sodium alginate, and sodium hydroxide are also utilized. These were purchased from Synth (Diadema, Brazil). These aforementioned products are analytical grades and used without further purification. Arabic gum, agar–agar, yeast extract, and bacteriological peptone were obtained from Oxoid (London, UK). From the local market, both the soybean and olive oils (Liza and Gallo trademarks, respectively) were purchased. Their corresponding maximal acidities are 0.3% and 0.5%, respectively.

Burkholderia cepacia strains, inoculum preparation, and lipase production

Burkholderia cepacia strains supplied from André Tosello Foundation (Campinas, Brazil) were kept at 4 °C on agar–agar medium. In flasks of 125 mL containing salts (KH_2PO_4—4 g/L and $MgSO_4$—0.2 g/L), yeast extract (3 g/L), bacteriological peptone (3 g/L), and soybean oil (3% *v*/*v*), inoculum for 48 h at 30 °C and under 200 rpm was cultivated. The lipase production was carried out into a 5-L bioreactor type Bioflow at 30 °C in the same conditions of inoculum. It is remarked that 60% of the total production capacity of the aforementioned bioreactor was used. However, a higher soybean oil volume (6% *v*/*v*) than one is considered. From the cells by centrifugation (2000×*g* per 10 min) after 96 h, the supernatant was separated. This was used as enzymatic extract rich in lipase, since *Burkholderia cepacia* induces to an extracellular lipase [30].

Lipase Assays

Based on the olive oil emulsion method previously proposed by Souza et al. [3] and Padilha et al. [9, 22], the enzymatic extract and purified extract of *Burkholderia cepacia* lipase with few modifications were produced. By mixing 75 mL of olive oil and 25 mL of a 7% Arabic gum solution (*w*/*v*), 5 mL was added in 2 mL of 100 mM sodium phosphate buffered at pH 8 and 1 mL of enzyme extract. After 15 min at 37 °C and 200 rpm, the reaction was stopped with 15 mL of acetone:ethyl alcohol (1:1 *v*/*v*). The liberated fatty acids were titrated with a 500-mM sodium hydroxide solution. Phenolphthalein as an acid–base indicator was also used. One unit (U) of the lipase activity was defined as the amount of the enzyme that liberated 1 μmol of free fatty acid per min (μmol/min) under the assay conditions (37 °C, pH 8, 200 rpm).

According to the method described by Bradford [31] using bovine serum albumin as standard and by measuring the absorbance at 595 nm, the protein concentration was determined. The specific activity was calculated as activity for each mass of protein ($U/mg_{protein}$) content into the enzymatic extract or the purified extract.

Aqueous Two-Phase System

Into 15 mL of the graduated glass tubes, the aqueous biphasic systems were attained. Stock solutions of the polymer (50% *w*/*w*) and the potassium phosphate salt (22.5% *w*/*w*) were used. Inside a tube, different mixtures of the polyethylene glycols solution with distinct molecular masses (1500, 4000, and 6000) considering three distinctive buffered pH values (i.e., 6, 7, and 8) were added. Each one of the examined mixtures was prepared gravimetrically ($\pm 10^{-3}$ g). A given volume of the polymer solution was added to the buffer solution until it turned turbid. This indicates the beginning of the two-phase system. The binodal curve was

previously obtained [9]. The enzymatic extract (200 μL) was added into the tubes with different ATPS compositions, and stirred vortex during 2 min was produced. After the phase separation and the formation of the well-defined interface between the top and the bottom phases (24 h at 25 °C under atmospheric pressure), the samples of the top and the bottom were carefully withdrawn by using a Pasteur pipette, as previously reported [2]. It is worth noting that although a relative long-term period seems to be reached, a diffusive balance considering this mentioned period should be attained. Besides, it is remarkable that none enzyme activity tending to drop was observed. The total amount of the system for 8 g of the lipase extraction is adjusted. The aliquots from each phase were analyzed in order to determine the enzyme activity and protein concentration, as previously described. Triplicate experimentation was adopted for all experiments. The results are represented by the average of three independent experiments.

Analytical Methods

A thermodynamic parameter, commonly used to measure the difference of the intensive properties between the equilibrium phases, is the tie line length. This parameter is calculated based on the differences in the concentrations in the top and bottom phases, according to Eq. 1. For this purpose, the concentrations of PEG and salt in the two-phases are considered.

$$\mathrm{TLL} = \sqrt{\left[(\mathrm{PEG})_t - (\mathrm{PEG})_b\right]^2 + \left[(S)_t - (S)_b\right]^2} \tag{1}$$

where ΔPEG and Δ*S* are the differences in concentration between the top and bottom phases of both the polymer and salt. The subindexes "T" and "B" correspond with top and bottom phases, respectively.

The tie lines are isothermal lines connecting with the binodal curves. Based on this parameter tie line length (TLL), it can be described the composition of the top and bottom phases and to indicate that the phase which is prevalent to *Burkholderia cepacia* lipase be located or formed. Table 1 shows the tie line compositions and tie line lengths for the average molecular masses of the examined PEG (1500, 4000, and 6000) and the potassium phosphate salt at pH 6, 7, and 8, as also previously reported [9].

The partition coefficient for the lipase activity (K_L) in the ATPS was calculated using Eq. 2.

$$K_\mathrm{L}^\mathrm{T} = \frac{\mathrm{L}_T}{\mathrm{L}_B} \text{ or } K_\mathrm{L}^\mathrm{B} = \frac{L_\mathrm{B}}{L_\mathrm{T}} \tag{2}$$

where K_L^T and K_L^B are the enzyme partition coefficients for the top and the bottom phases, respectively. L_T and L_B are the lipase activities in the top and bottom phases, respectively.

The protein partition coefficient is determined by using Eq. 3.

$$K^\mathrm{T} = \frac{C_\mathrm{T}}{C_\mathrm{B}} \text{ or } K^\mathrm{B} = \frac{C_\mathrm{B}}{C_\mathrm{T}} \tag{3}$$

where K^T and K^B are the protein partition coefficients for the top and bottom phases, respectively. C_T and C_B are protein concentrations in the PEG (top phase) and salt (bottom

Table 1 Compositions of PEG and potassium phosphate attained at top and bottom phases

		Top phase composition (%, *w*/*w*)			Bottom phase composition (%, *w*/*w*)			TLL
	pH	PEG	Salt	Water	PEG	Salt	Water	–
PEG 1500	6	21.0	9.3	69.7	9.0	16.0	75.0	13.8
		31.0	5.6	63.4	5.0	19.5	75.5	29.5
	7	25.0	6.6	68.4	4.5	16.5	79.0	22.8
		37.5	3.1	59.4	1.5	21.0	77.5	40.2
	8	29.0	4.4	66.6	1.9	16.8	81.3	29.8
		35.0	3.2	64.8	0.7	20.5	78.8	38.4
PEG 4000	6	12.5	9.0	78.5	2.2	17.9	80.1	13.6
		32.0	4.6	63.4	1.4	21.7	76.9	35.1
	7	18.5	2.1	79.4	8.3	14.5	77.2	16.1
		26.0	5.3	68.7	6.4	22.0	71.6	25.8
	8	28.5	4.0	67.5	1.0	14.0	85.0	29.3
		19.5	2.2	78.3	2.4	37.0	60.6	38.8
PEG 6000	6	17.0	8.5	74.5	2.2	16.0	81.8	16.6
		36.0	3.4	60.6	1.7	21.0	77.3	38.6
	7	29.0	4.2	66.8	1.5	17.5	82.2	30.6
		38.5	2.3	59.2	4.2	21.0	74.8	39.1
	8	35.0	2.8	62.2	18	0.5	81.5	17.2
		43.6	1.4	55.0	20	3.0	77.0	23.7

phase). The lipase purification factor (LPF) and the extraction efficiency of the lipase (EE_L) in the top and bottom phases are determined by using Eqs. 4 and 5.

$$\mathrm{LPF}^{\mathrm{T}} = \frac{\mathrm{SA_T}}{\mathrm{SA_{crude}}} \text{ or } \mathrm{LPF}^{\mathrm{B}} = \frac{\mathrm{SA_B}}{\mathrm{SA_{crude}}} \tag{4}$$

$$\mathrm{EE_L}^{\mathrm{T}} = \frac{K_{\mathrm{L}}^{\mathrm{T}} R_{\mathrm{V}}^{\mathrm{T}}.100}{1 + \left(K_{\mathrm{L}}^{\mathrm{T}} R_{\mathrm{V}}^{\mathrm{T}}\right)} \text{ or } \mathrm{EE_L}^{\mathrm{TB}} = \frac{K_{\mathrm{L}}^{\mathrm{B}} R_{\mathrm{V}}^{\mathrm{B}} \cdot 100}{1 + \left(K_{\mathrm{L}}^{\mathrm{B}} R_{\mathrm{V}}^{\mathrm{B}}\right)} \tag{5}$$

The specific activity partition coefficient after purification is determined by using Eq. 6.

$$\mathrm{SA}^{\mathrm{T}} = \frac{\mathrm{L_T}}{\mathrm{C_T}} \text{ or } \mathrm{SA}^{\mathrm{B}} = \frac{\mathrm{L_B}}{\mathrm{C_B}} \tag{6}$$

where SA = L/C, determining the ratio of the enzyme activity to the protein concentration in the top or bottom phase, respectively. The phase volume ratio (R_V) is determined using Eq. 7.

$$R_{\mathrm{V}}^{\mathrm{T}} = \frac{V_{\mathrm{T}}}{V_{\mathrm{B}}} \text{ or } R_{\mathrm{V}}^{\mathrm{B}} = \frac{V_{\mathrm{B}}}{V_{\mathrm{T}}} \tag{7}$$

As reference (control specimen), the phases of the same two-phase system without lipase were used.

SDS–PAGE Electrophoresis

Before and after the lipase partitions in the PEG/potassium phosphate salt ATPS, the SDS–PAGE electrophoresis analyses were carried. For this purpose, a Mini Protean III (Bio-Rad®, USA) and utilizing a polyacrylamide gel were also utilized, as prescribed in specific protocol [32]. The samples were treated with the buffers containing SDS in the denaturant and reduced conditions (with the presence of β-mercaptoethanol). The denaturation of the protein samples was performed by heating at 100 °C for 10 min. The aliquots of 15 μL of each sample and 5 μL of low molecular weight indicator into the gels of 7.5% were applied. Into a vertical bowl under a voltage of 180 V, the mentioned gels were subjected. Low molecular weight indicator contains the following proteins, phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) from GE Healthcare (USA).

Production Cost Determination

A PEG 1500/potassium phosphate salt ATPS at pH 6 was considered as an optimum condition for the lipase partition. Fixed and variable costs are used and the average total and variable, and marginal costs are also determined. The minimum price (MP) was calculated based on the sum of the fixed and variable costs, monthly production and involved material, as shown in Eq. 8 [33, 34]. For this purpose, a range of the fixed and variable costs are taken in account, as will forwardly be discussed. In order to propose the sale prices or the selling prices (SP) with distinctive markup values, Eq. 9 is used, as previously reported [33]. The mentioned distinctive markups (i.e., 25, 37.5, and 50%) concatenated with a range of the two commonly used distinct financial taxes are also considered.

$$\mathrm{MP}\ (\mathrm{US\$/L}) = \frac{\Sigma(\text{Monthly cost})}{\text{Monthly production}} + \text{Unit variable cost} \tag{8}$$

$$\mathrm{SP}\ (\mathrm{US\$}) = \frac{\mathrm{MP}}{1-(\text{tax fraction} + \text{markup})} \tag{9}$$

A work month with 22 days and 8 h per day is also taken in account. Based on the fact that a 5-L bioreactor, as previously described, and of about 60% of its total capacity was utilized, it seems that a small-scale production is dominant. From the economic and business aspects, the small- and the large-scale productions have their disadvantages and advantages, which are not focused on this investigation. For example, a small-scale production can be a higher overhead charge (e.g., cost of rent) and cost of credit (rate of interest) than a large-scale ones. However, the costs and the sales prices are determined considering a monthly production of about 30 L correlated with those fixed and variable costs, as will also forwardly be discussed.

Considering these limitations, between the lipase production and the purification stages after 8 days of production, a volume of about 21 L is obtained. From this volume, approximately 50% are effectively used since only the top phase is successfully used. This indicates a production of about 30 L/month when 60% of the reactor production capacity is used. It is worth noting that if a large-scale production is considered, for instance, using a bioreactor

with volume capacity of about 1200 L, considerable variations related mainly with applied material and equipment are attained. For this reason, the groups of the involved costs are considered using distinct cost ranges. This seems that the market or commercialization in both the emerging and developing countries can be reasonably comprised.

Results and Discussion

Binodal Curves for Different Molecular Masses of PEG and Potassium Phosphate Salt

According to that reported by Padilha et al. [9, 22], polyethylene glycols of the following average molecular masses and potassium phosphate salt to induce ATPS formation were evaluated. Besides, the titration method for the determination of the binodal curves is used. The binodal curve separates the single-phase system from the biphasic system, i.e., below the binodal curve, the system is homogeneous and above it, a heterogeneous condition is predominant. Zhou et al. [26] have reported that the partition behavior of the biomolecules in ATPS is very sensitive with variation in the PEG molecular mass. With this, the effects of both the PEG molecular mass and pH are evaluated. These factors seem to affect the charge, stability, structure, and characteristics of the biomolecules in the system. Besides, their partition behavior can also be influenced [11]. Figure 1 shows the binodal curves of PEG/potassium phosphate salt at distinctive three pH values, i.e., 6, 7, and 8. Since these values are close to maximal activity (pH 8) and isoeletric point (pH 6) of *Burkholderia cepacia* lipase, they were considered [9, 11]. Additionally, it was observed that the increase of the PEG molecular weight, the concentration, and salt solution necessary for the ATPS formations has been decreased. In this present investigation, the aforementioned mentioned behavior was also similarly characterized.

The binodal curve is shifted to the right when the same pH value is considered and when the PEG 1500, 4000, and 6000 are also compared. This effect can be explained due to the increase of the $H_2PO_4^-/HPO_4^{-2}$ ratio, while the pH values are decreased.

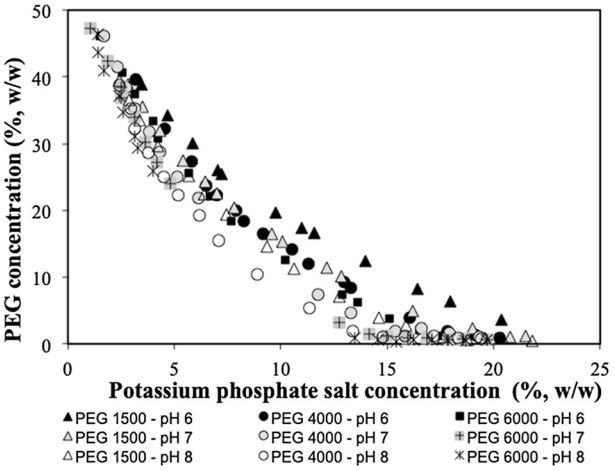

Fig. 1 Phase diagrams for binary systems composed of PEG and potassium phosphate salt, at 25 ± (0.1) °C and atmospheric pressure

Considering a same range of the examined PEG molecular mass (i.e., PEG 1500, 4000, and 6000), a lower pH value induces to a higher PEG/potassium phosphate salt concentration ratio than other examined pH values, as shown in Fig. 1. On the other hand, in a general way, when the PEG values in same pH values are compared, it seems that the highest PEG/phosphate salt concentration ratio is that of the PEG 1500. An intermediate value is that of the PEG 4000, and the lowest is that of the PEG 6000.

TLLs

From the experimental results, no substantial variations of the TLL values as a function of the examined pH values are observed. However, it is worth noting that at pH 6, for both the PEG 1500 and 4000 values, a noticeable difference is observed. Considering the maximum–minimum values of the TLL at pH 6, it can be seen that for the PEG 1500, 4000, and 6000, their corresponding values are 15.7 (e.g., 29.5–13.8), 21.5, and 22.0, respectively, as shown in Table 1.

Additionally, the highest TLL is attained when a PEG 1500 at pH 7 is considered. It is remarkable that only in this reaction the mentioned behavior was observed. The effect of the TLL observed by [35] showed that the increase of the tie line length has generally induced to the increase of the partition coefficient for the total protein and ribonucleic acid examined. However, the enzyme partition in the ATPS depends on the molecular mass and concentration of polymers, surface properties, molecular load, as well as the ionic composition between the phases. Some of these parameters, in this paper, will be forwardly analyzed and discussed.

Concerning to pH, the proteins show partition behavior that is broadly related to their net positive or negative charges considering its isoelectric points [35]. In the pH value close to the isoelectric point, only the electrostatic effects are considered due to the sum proteins to be practically 0. *Burkholderia cepacia* lipase had an isoelectric of 6.0 [9] or really close of 6.0 [36]. It is known that lipase enzymes have isoelectric pH in the range 6–7, which induces to a positive charge lipase surface in pH = 6. Also, it is reported that some proteins are negatively charged and at the top phase, PEG-salt systems are preferred. On the other hand, a positively charged is commonly partitioning at the bottom phase, as previously reported by Kavakcıoglu et al. [37–39]. Additionally, it is also known that pH equal to isoelectric point of the protein, the positive and negative charges are equilibrated, and the enzyme is neutrally charged [39]. Based on this, it is supposed that a neutral charge is prevalent in both top and bottom phases. Besides, according to the systems, the polymer and salt concentrations in the top and bottom phases are lowest when shorter TLLs are attained.

Burkholderia cepacia Lipase Partition in ATPS

In order to test the lipase partition in PEG/salt and systems, initially, the lipase activity and the protein concentration in the top and bottom phases for all the examined TLL were measured, as demonstrated in Table 2. From these results, it is observed that the transfer of the protein from the top to the bottom phase for all molecular masses of PEG and pH has clearly occurred. This means that the bottom phase shows a majority of protein when compared to the top phase. This amount of proteins seems to be associated with the impurities content into the enzymatic extract observed in all conditions examined. For the tie line lengths at PEG 1500 and PEG 6000, it is possible to note that a higher lipase affinity for the top phase is prevalent. However, for a PEG 4000, a distinctive behavior was verified.

Table 2 Experimental results of the lipase activities and protein concentrations at the top and bottom phases in different TLLs

TLL	Top phase			Bottom phase		
	Lipase activity (U/mL)	Protein (mg/mL)	Volume (mL)	Lipase activity (U/mL)	Protein (mg/mL)	Volume (mL)
Crude enzymatic extract						
–	3.30	0.786				
13.6	2.44	0.198	3.7	9.68	0.523	3.5
13.8	14.44	0.214	3.4	0.20	0.575	3.8
16.1	4.99	0.196	3.9	18.65	0.510	3.3
16.6	18.72	0.222	3.3	2.12	0.415	3.7
17.2	10.01	0.122	3.5	7.16	0.531	3.6
22.8	9.52	0.243	3.2	2.56	0.576	4.0
23.7	12.40	0.191	3.3	1.98	0.511	4.0
25.8	3.55	0.163	4.3	19.66	0.568	2.9
29.3	5.63	0.103	3.7	11.98	0.604	3.5
29.5	17.66	0.199	3.4	1.98	0.538	3.8
29.8	5.65	0.121	3.4	2.54	0.686	3.8
30.6	17.87	0.202	3.1	6.65	0.523	4.1
35.1	7.87	0.187	4.0	20.12	0.606	3.2
38.4	4.84	0.136	2.6	3.34	0.606	4.6
38.6	19.44	0.182	3.6	4.26	0.598	3.8
38.8	3.46	0.156	3.9	18.20	0.545	3.3
39.1	14.14	0.203	3.4	2.60	0.543	3.8
40.2	8.65	0.054	3.1	2.14	0.786	4.1

A higher affinity of the lipase at the bottom phase was observed. Additionally, when a PEG 1500 at pH 6 is considered, the top phase concentrated almost all the lipase activities in relation to the bottom phase are prevalent. This means that the PEG 1500 at pH 6 indicates a better condition for the lipase purification since the lipase activity was lower than at the bottom phase. Although in Table 2, the PEG 6000 with pH 6 (TLL = 38.6) shows an activity 19.44 of the top and 4.26 for the bottom, a top-to-bottom ratio evidences that the lipase activity at bottom is remained (~ 20%). Similarly, when both PEG 6000, pH = 6 TLL = 16.6 and PEG 6000, pH = 7 TLL = 30.6 are analyzed, the top–to-bottom ratios of the lipase activities indicate are also remained, i.e., of about ~ 40% and ~ 30% of the remained activities in the bottom. This indicates lower activity or ratio when compared with that of ~ 98% considering PEG 1500 at pH 6, as aforementioned.

With the decrease of the pH, the enzyme can be more positively charged since the system pH affects the ionizable protein groups and the protein surface charges are modified [40]. The protein concentration (K_{SA}) in this process condition is increased, as shown in Table 3. From these results, the best result is correlated with the lowest TLL value.

Partition of *Burkholderia cepacia* Lipase in ATPS

In order to evaluate the extraction of the enzymatic crude rich in the lipase, an aqueous two-phase system is used. In this process, the lipase is partitioned in one of the phases, while the cell fragments in another phase are retained [11]. As discussed above, the charge, the size, and the hydrophobicity of the biomolecules are complex parameters and depend of the success of the ATPS partitioning. The partition process is very sensible to these mentioned parameters.

Table 3 Partition of *Burkholderia cepacia* lipase by aqueous two phase system (PEG/phosphate)

	pH	TLL	K_L	SA (U/mg)	R_V
PEG 1500	6	13.8^{T}_{B}	72.2	67.5	0.89
			0.01	0.35	1.12
		29.5^{T}_{B}	8.9	88.7	0.89
			0.1	3.7	1.12
	7	22.8^{T}_{B}	3.7	39.2	0.80
			0.3	4.4	1.25
		40.2^{T}_{B}	4.0	160.1	0.76
			0.3	2.8	1.32
	8	29.8^{T}_{B}	2.2	46.7	0.89
			0.5	3.7	1.12
		38.4^{T}_{B}	1.5	35.6	0.57
			0.7	5.5	1.8
PEG 4000	6	13.6^{T}_{B}	0.25	12.3	1.06
			3.8	18.5	0.95
		35.1^{T}_{B}	0.4	42.1	1.25
			2.6	33.2	0.80
	7	16.1^{T}_{B}	0.3	25.5	1.18
			3.7	36.6	0.85
		25.8^{T}_{B}	0.2	22.8	1.48
			5.5	34.6	0.67
	8	29.3^{T}_{B}	0.5	54.7	1.06
			2.1	19.8	0.95
		38.8^{T}_{B}	0.2	22.2	1.18
			5.3	33.4	0.85
PEG 6000	6	16.6^{T}_{B}	8.8	84.3	0.89
			0.1	5.1	1.12
		38.6^{T}_{B}	4.6	106.8	0.95
			0.2	7.1	1.06
	7	30.6^{T}_{B}	2.7	88.5	0.76
			0.4	12.7	1.32
		39.1^{T}_{B}	5.4	69.7	0.89
			0.2	4.8	1.12
	8	17.2^{T}_{B}	1.4	82.1	0.97
			0.7	13.5	1.03
		23.7^{T}_{B}	6.3	64.9	0.83
			0.2	3.9	1.21

T and B subscript designate top and bottom phase, respectively

K_L lipase partition coefficient, *SA* the ratio of the enzyme activity to the protein concentration in a sample, R_V phase volume ratio, *LPF* lipase purification factor, EE_L extraction efficient of lipase

Thus, some factors such as pH, volume ratio, component contents, type of aqueous two-phase system, molecular mass, phase volume ratio, and other ones have important roles to change the biomolecules characteristics. With this, the tendency of the biomolecules to interact with the phase-forming components, to increase or to decrease the yields, is significantly affected [12, 41].

In order to determine the TLL values for each molecular mass of PEG and pH, Eq. 9 was used. In order to facilitate the interpretation of those values shown in Table 2, for each examined pH and TLL, the parameters K_L, SA and R_V were determined. For this purpose, K_L is determined by using lipase activity from the top per bottom when the top phase is aimed and the bottom per top when the bottom is aimed. We consider TLL = 13.8 at pH 6, PEG 1500, from Table 2, 14.44/0.2 and 0.2/14.44, respectively. SA is calculated using the lipase activity divided by protein concentration. For instance, when TLL = 13.8 at pH 6, PEG 1500 is

considered, from Table 2, the lipase activity (14.44) divided by protein concentration (0.2) determines SA = 67.5 for the top (T) phase. Also, these values are used to determine for the bottom (B) phase and ~ 0.35 is reached (0.2/0.575). Finally, R_V is determined using Eq. 7 and 3.4 and 3.8 values from Table 2.

It is remembered that the significance of each one of the aforementioned parameters was previously described. LPF and EE_L correspond to the lipase purification factor and the extraction efficient of the lipase, respectively. These parameters associated with TLL (at top and bottom phases) with the three distinct pHs (6, 7, and 8) and PEG molecular masses (1500, 4000, and 6000) are shown in Fig. 2a, b, and c, respectively. In order to calculate the LPF, in each one of the examined sample, 1 mL of the crude lipase with activity and a protein amount of 3.3 ± 0.5 U/mL and 0.786 ± 0.08 mg/mL are utilized respectively.

It can also be observed that the PEG 1500 has a lower molecular mass than other examined PEGs, which induces to higher fluidity related with lower viscosity in contact with potassium phosphate. This corroborates with a higher mass transfer to the top phase when the bottom phase is compared. This seems to be intrinsically associated with the characteristic demonstrated by an aqueous two-phase system (ATPS) with PEG 1500 content, as shown in Fig. 2a.

Two first columns are associated with the results of TTL at the top (symbol T) and other two columns with TTL at the bottom (symbol B) for each examined pH, as shown in Fig. 2. Based on the experimental results, a first analysis indicates that the three distinctive PEGs evidence different trends between both LPF and EE_L with TTL at top and bottom and with distinctive pH values.

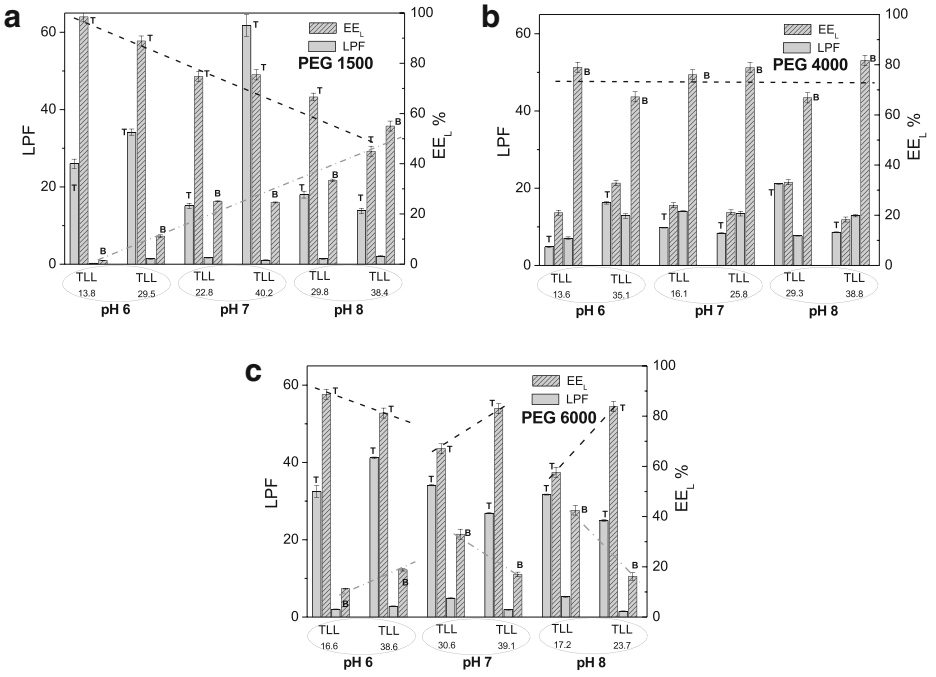

Fig. 2 Correlation between LPF (lipase purification factor) and EE_L (extraction efficient of lipase) with TLLs (tie line lengths) for three distinct pH values considering PEGs, **a** 1500, **b** 4000, and **c** 6000 at 25 ± (0.1) °C and under atmospheric pressure

Considering the PEG 1500, indistinctively of the examined pH, the top phase defines the highest values of the EE_L. Besides, with increasing pH, a decreasing trend of the EE_L is clearly characterized. It is worth noting that an inverse trend of the EE_L values correlated with the bottom phase is exhibited, as shown in Fig. 2a. When other PEGs are analyzed, this same trend is not characterized. When the PEG 4000 is analyzed and indistinctively of the pH examined, the highest results of the EE_L are those corresponding with the bottom phases, as shown in Fig. 2b. On the other hand, when the PEG 6000 is analyzed, a distinct behavior between LPF and EE_L as a function of both the TLL and pH examined is characterized. At pH 6, the lower TLL has higher EE_L than other ones, while at pH 6 and 8, the higher TLLs also attain the high EE_L values. Evidently, the bottom phase behavior depicts an opposite trend with respect to the top phase, as shown in Fig. 2c.

A general comparison among the examined PEGs, the highest EE_L is that of the PEG 1500 at pH 6 in the top phase (98% and $K_L = 72.2$). It is remarked that the resulting TLL also is maximized in terms of the extraction (89.9%), as observed in TLL of 29.5 in the top phase. However, the K_L value attains of about 8 times lower than other examined samples. The same behavior was observed for the PEG 6000 at pH 6 and TLL 16.6 in the top phase. The majority of the tests indicate that the lipase preferred a more hydrophobic phase (top), which is represented when the value of $K_L > 1$ is attained. For the assays with PEG 4000, the values were smaller than 1 in the top phase, and the lipase at the bottom phase (potassium phosphate) has preferentially occurred. Jiang et al. [37] have also demonstrated a similar behavior, and it was evidenced that the PEG exclusion from the protein domain is more important than the polymer protein interaction. This was considered as the main effect driving the protein partition to the salt phase when a weightier PEG molecular mass is used. In this result, for a lighter PEG molecular, the results of the partition with K_L is more significant in lighter PEG masses with an isoelectric point (pH 6) using low TLL value.

It is remembered that the phase volume ratio (R_V) was used to attain the optimization due to its direct correlation with the EE_L determination. The R_V effect has been investigated by Chew et al. [41]. It was reported that R_V was not a parameter that alone displayed an exert influence to the partitioning behavior, as confirmed by Ramakrishnan et al. [42]. They have demonstrated this behavior when analyzing the partition coefficient of a total protein and *Enterococcus faecium* lipase that followed a decreasing trend with the decrease of the R_V. It was reported by Chow et al. [43] that a same behavior in the partition of a bovine serum albumin is observed. In this paper, the R_V values showed the same behavior and they are dependent of the PEG molecular mass and pH in both the top and bottom phases.

The highest purification factor of the lipase from an extract crude was that of the ATPS. The purity of the partitioned lipase was assessed with the SDS–PAGE, as depicted in Fig. 3. The crude enzymatic extract (line 1) has demonstrated multiple bands due to the impurity contents in both the reaction medium and the culture. When a low molecular weight indicator (LWM) with a commercial *Burkholderia cepacia* lipase is exhibited, at line 3, a target lipase with molecular mass of the microbial lipase from *Burkholderia cepacia* is characterized, as also shown in Fig. 3. In this aforementioned situation, the other bands are not revealed, which indicates a possible separation of the lipase from the contaminant compounds. On the other hand, into line 4 for the bottom phase, this behavior was expected due to a higher protein content and lower lipase amount is prevalent, as shown in Table 3. All SDS–PAGE images were obtained at pH 6, indicating a better EE_L results than other examined. This is intimately associated with the isoelectric point of *Burkholderia cepacia*.

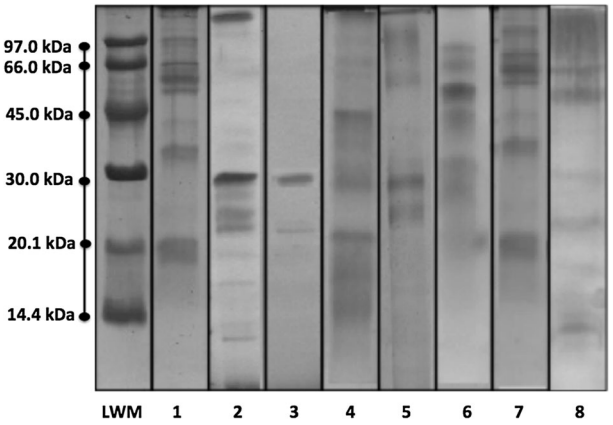

Fig. 3 Typical SDS–PAGE electrophoresis results using a 12.5% gel; samples in denaturant and reducing conditions. (LWM) low molecular weight indicator: (1) crude enzymatic extract; (2) commercial lipase from *Burkholderia cepacia*; (3) purified *Burkholderia cepacia* lipase in the PEG 1500, at pH 6 and TLL 13.8 at top phase; (4) purified *Burkholderia cepacia* lipase in the PEG 1500, at pH 6 and TLL 13.8 at bottom phase; (5) purified *Burkholderia cepacia* lipase in the PEG 6000, at pH 6 and TLL 16.6 at top phase; (6) purified *Burkholderia cepacia* lipase in the PEG 6000, at pH 6 and TLL 13.8 at bottom phase; (7) purified *Burkholderia cepacia* lipase in the PEG 4000, at pH 6 and TLL 13.6 at top phase; and (8) purified *Burkholderia cepacia* lipase in the PEG 4000, at pH 6 and TLL 13.6 at bottom phase

Lines 5 and 6 show the target lipase with other impurities in distinct TLL for the PEG 6000 and pH 6 and top and bottom phases. For the PEG 4000, the better lipase activity and values in the bottom phase are characterized, as also evidenced in Fig. 2b. This indicates that the target is more expressive in the bottom phase due to a higher lipase activity than the EE_L value.

The Enzyme Production Costs

In order to determine both the minimum price and sale price (breakeven point) [33, 34], the variable costs were constituted, as shown in Table 4. Additionally, the fixed costs were also determined, as shown in Table 5. The variable costs were defined in two distinctive groups (i.e., material cost and employees wages and/or salaries and its finance charges). Based on the fact that there is a reasonable difficult to fix a group costs considering distinctive economic conditions and countries, the ranges for these costs are considered, as aforementioned. This consideration was adopted in order to attain the possible variations of each examined item when different countries or economic factors and conditions are considered.

The material cost shown in Table 4 was deduced based on the condition for the PEG 1500, pH 6 and TLL 13.8, as shown in Tables 1, 2, and 3. The considered range prices are those

Table 4 The variable costs considering the cost of materials involved for 1 L of lipase produced using PEG 1500 and the estimated costs related with employees and finance charges applied

Item	Range costs (US$/unit)
Lipase and purification materials (including PEG 1500)	15 to 25
Fermentation broth materials	100 to 150
Subtotal unity cost (materials)	115 to 175
Employees (selling commission) + financial rates	4000 to 9000
Total variable costs	4115 to 9175

Table 5 The total fixed costs for the enzyme production involving area industrial plant, equipment group, and its energy consumption and maintenance

Item	Cost (US$) × 1000	Life cycle (years)	Monthly cost (US$)
Hangar (production area)	80 to 120	10	8 to 12
Equipment group	12.5 to 22.5	10	105 to 190
Energy (KWh)	–	–	300 to 400
Maintenance	–	–	150 to 280
Employees (production)+ financial rates	–	–	5 to 8×10^3
Total fixed costs			~ 13.5 to 21×10^3

currently practiced at Brazil market, which can be different when another country is considered. It can be seen that the all involved material cost attains a range between US$115 and 175 per liter of the extracted lipase, PEG, and the fermentation materials. A range for the total variable costs between US$4115 and 9175 is considered. This involves the employees' salaries and wages and its finance charges (rates), as well as the material used. It is recognized that both working restriction laws associated with different economic conditions in each different country define distinctive number of employees (technicians or chemical engineers). It seems that adopting a range for the wages/salaries, the distinct and differentiates employee careers and working/occupation places at emerging or developing markets can be considered.

The fixed cost for the lipase production based on 100 m^2 of a considered small industrial area production (located at São Paulo state, Brazil) and the correlated equipment and their maintenance and the energy consumption are considered. A total fixed cost between US$13,500 and 21,000 per month is attained. A daily energy consumption of about 100 kWh during 8 h of working day is considered, as shown in Table 5.

Based on a CVP (cost–volume–profit) analysis considering a trend to long-run cost curve (LRC), with 10 years of the life cycle (Table 5), the typical average total cost (ATC), the average variable cost (AVC), and the marginal cost (MC), as defined by Cafferky and Wentworth [34], Gaither and Faizer [44], and Shepherd [45], as a function of the distinct production capacities (L per month), are shown in Fig. 4.

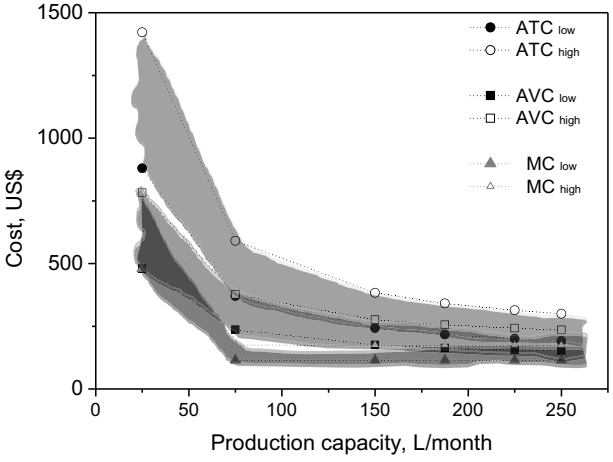

Fig. 4 Calculated ranges for the average total cost (ATC), average variable cost (AVC), and marginal cost (MC) as a function of the production capacities for the extracted of lipase using a PEG 1500/potassium phosphate ATPS at pH 6

The costs ATC, AVC, and MC are defined by each one of the corresponding costs divided the quantity (total production in L/month), as reported by Cafferky and Wentworth [34]. A total production of 150 L/month using 5 bioreactors corresponds to 60% of the production capacity. From Tables 4 and 5, the ranges of each one of the examined costs are characterized, as shown in Fig. 4. From the economical point of view, when a 100% total production capacity using 5 bioreactors is considered, the MC is lower than the ATC. This means that the marginal revenue will also be higher than the MC. Considering the limitations for a 100% production, the revenue and profit seem to be reasonably attained. Forwardly, a market price will be compared, in order to analyze the selling price and corresponding markup and profit.

By using Eq. 7 and those costs shown in Tables 4 and 5, the minimum price varying with the production capacity is determined, as shown in Fig. 5. It can be seen that the three distinct profits levels (markup values) are considered, i.e., 25% (0.25), 37.5% (0.375), and 50% (0.50). The high and low values of the fixed costs (i.e., US$10,000 and 16,000), the variables cost (i.e., US$9115 and 15,175), and the tax fractions (i.e., ~0.3 and 0.4) generate distinctive sale prices as a function of the production capacity levels, as shown in Fig. 5a and b, respectively.

The proposed sale prices are determined using Eq. 8 considering the three distinctive markup values, and a range of the tax fraction between ~0.3 and 0.4 is also taken in account. The production capacity levels correspond with those capacities in liters per month shown in Fig. 4, i.e., 25, 75, 150, 187.5, 225, and 250 L/month, which correspond with those production capacity percentages of 10, 30, 60, 75, 90, and 100%. It is remembered that these proposed sale prices are determined using a PEG 1500/potassium phosphate ATPS at pH 6. Although two different cost conditions are used, the range between 30 and 75% of the production capacity (i.e., levels 3 to 5) is considered as a region or range, which has a reasonable price stability. This is based on the fact that a slight standard deviation is possible when average prices are considered.

Considering the limitations related to a small-scale production capacity, it seems that the proposed selling prices are reasonably competitive and promised when compared with a commercial product (~US$150 per 50 g) [29]. It is remarked that a market price comparison between two products with a similar technologies constitutes a complicate task and a weakness in this comparison is associated. For instance, significant differences are intrinsically related with the production method (freeze drying/lyophilization, spray drying, or other), the lipase stability, and purity. A drawback characteristic concerning to the proposed lipase product is related with its aqueous condition. On the other hand, due to the fact that no other proteins are prevalent in the lipase, as observed in a commercial product, an advantage favoring this proposed product seems to be reasonably established, as shown in Fig. 3.

Although the aforementioned limitations comprise for a market price comparison, based on the commercialized products, a selling price of about US$1000 per liter is predictable. Adopting this speculate commercial price associated with 25% of markup concatenated with low value conditions for all involved costs and taxes (as previously commented), the production capacities from the level 3 (60%) up to 100% (250 L/month) indicate sale prices lower than speculate/predictable selling price (i.e., US$1000/L), as shown in Fig. 5b. The proposed sale price are ~39% and ~47% lower than the predictable US$1000 per liter, when 60% and 90% of the production capacities are considered, respectively.

Additionally, it can also be seen that the sale price is competitive when a 37.5% markup is taken in account. When the resulting costs and taxes are not favorable, i.e., a higher

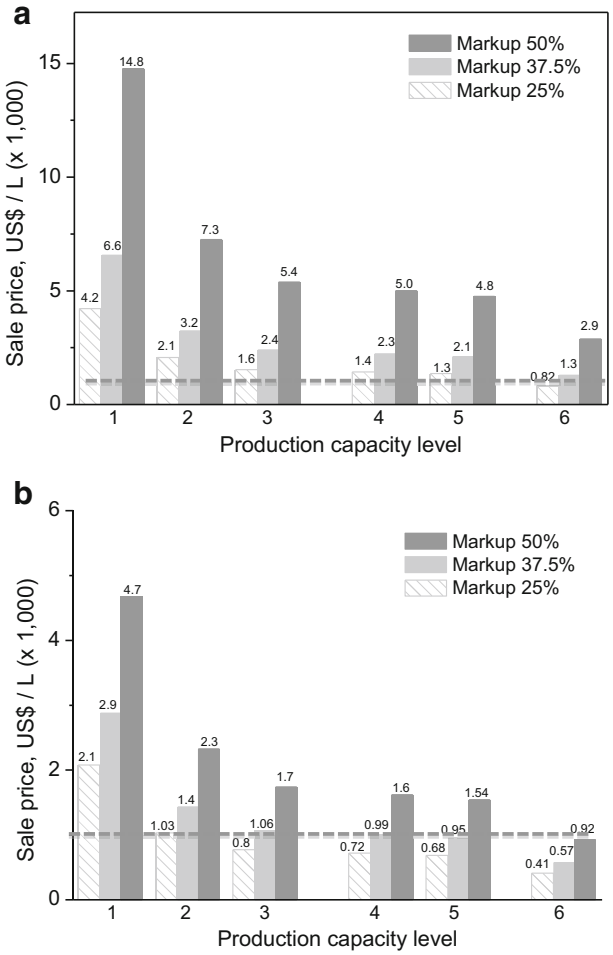

Fig. 5 Calculated sale prices considering three distinctive markups (profits), i.e., 25%, 37.5%, and 50%, and high (**a**) and low (**b**) values for ATC and AVC and tax fractions for an extracted lipase using a PEG 1500/ potassium phosphate ATPS at pH 6

range of the costs are involved, a selling price lower than the estimated US$1000/L is provided only when the total production capacity associated with a markup ~25% is considered.

From the aforementioned discussion, it is induced that a small-scale production considering a 25% markup and a proposed sale price lower (~40%) than speculate/predictable US$1000/L are attained. This suggests a promise and reasonable competitive selling condition. It is worth noting that the transportation (FOB), exportation taxes, and its storage/delivering cost conditions are not involved. Since an aqueous nature related with proposed lipase associated with a small-scale production, it seems that a pull system production trend to just-in-time is prevalent and more adequate. Based on this and those resulting costs involved, when an internal and particular market, the proposed small-scale production and their resulting final cost involved seem to be a promise, economical, and environmentally-friendly *Burkholderia cepacia* lipase extraction and production.

Concluding Remarks

The modeling of the partitioning shows a square dependence of the partition to the molecular mass of the polymer and the hyperbolic to the pH and tie line length (TLL). The optimization shows that the optimal condition is low pH and molar masses of polymer and a large TLL or the third TLL from PEG 1500/potassium phosphate ATPS at pH 6. In this condition, a partition coefficient over 200 folds is found. It is also found that the three distinct PEGs examined have induced to different trends between LPF and EE_L with TLL at top and bottom for the examined pH values. Independent of the examined pH, the highest value for EE_L is that of the PEG 1500. Based on this, an optimum condition is characterized and a PEG 1500/phosphate salt ATPS at pH 6 is economically attained. It is concluded that the proposed sale prices are between ~40 and ~50% lower (cheaper) than a speculate market price ~US$1000 per liter, when the production capacity levels of 60% and 90% associated with a markup of 25% are considered.

Funding Information This work was financially supported by the FAEPEX-UNICAMP (Grant 2194/16).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Gadade, P. R., Sardare, M. D., & Chavan, A. R. (2013). Study of extraction of methylene blue from synthetic waste water using liquid emulsion membrane technology. *Canadian Journal of Chemical Engineering, 91*, 80–89.
2. Khayati, G., & Alizadeh, S. (2013). Extraction of lipase from Rhodotorula glutinis fermentation culture by aqueous two-phase partitioning. *Fluid Phase Equilibria, 353*(15), 132–134.
3. Souza, R. L., Lima, R. A., Coutinho, J. A. P., Soares, C. M. F., & Lima, A. S. (2015). Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification. *Separation and Purification Technology, 155*, 118–126.
4. Carvalho, T., Finotelli, P. V., Bonomo, R. C. F., Franco, M., & Amaral, P. F. F. (2017). Evaluating aqueous two-phase systems for *Yarrowia lipolytica* extracellular lipase purification. *Process Biochemistry, 53*, 259–266.
5. Bassani, G., Farruggia, B., Nerli, B., Romanini, D., & Picó, G. (2007). Porcine pancreatic lipase partition in potassium phosphate–polyethylene glycol aqueous two-phase systems. *Journal of Chromatography, 859A*, 222–228.
6. Albertsson, P. A. (1986). *Partition of cell particles and macromolecules*. New York: John Willey.
7. Diamond, A. D., & Hsu, J. T. (1992). Aqueous two-phase systems for biomolecule separation. *Advances in Biochemical Engineering, 47*, 89–135.
8. Zaslasvsky, B. Y. (1994). *Aqueous two-phase partitioning - physical chemistry and bioanalytical applications*. New York: CRC press.
9. Padilha, G. S., Santana, J. C. C., Alegre, R. M., & Tambourgi, E. B. (2012). Extraction of lipase from *Burkholderia cepacia* by PEG/phosphate ATPS and its biochemical characterization. *Brazilian Archives of Biology and Technology, 55*(1), 7–19.
10. Duarte, A. W. F., Lopes, A. M., Molino, J. V. D., Pessoa, A., & Sette, L. D. (2015). Liquid–liquid extraction of lipase produced by psychrotrophic yeast *Leucosporidium scottii* L117 using aqueous two-phase systems. *Separation and Purification Technology, 156*, 215–225.
11. Tan, C. H., Show, P. L., Ooi, C. W., Ng, E. P., Lan, J. C. W., & Ling, T. C. (2015). Novel lipase purification methods – a review of the latest developments. *Biotechnol., 10*(1), 31–44.

12. Raja, S., Murty, V. R., Thivaharan, V., Vinayagam, R., & Ramesh, V. (2011). Aqueous two phase systems for the recovery of biomolecules—a review. *Science and Technology, 1*(1), 7–16.
13. Ventura, S. P. M., Sousa, S. G., Freire, M. G., Serafim, L. S., & Coutinho, J. A. P. (2011). Design of ionic liquids for lipase purification. *Journal of Chromatography, 879B*(26), 2679–2687.
14. Sharma, P., Sharma, N., Sharma, P., Pathania, S., & Handa, S. (2017). Purification and characterization of a halotolerant and thermotolerant lipase produced from a novel bacteria *Brevibacterium halotolerans* PS4 IKX671556I and its application in detergent formulations. *Proceedings of the Indian National Science Academy, 22*(3), 681–687. https://doi.org/10.16943/ptinsa/2017/49025.
15. Sun, J., Yu, B., Curran, P., & Liu, S. Q. (2012). Optimisation of flavour ester biosynthesis in an aqueous system of coconut cream and fusel oil catalysed by lipase. *Food Chemistry, 135*(4), 2714–2720.
16. Ivić, J. T., Veličković, D., Dimitrijević, A., Bezbradica, D., Dragačević, V., Jankulović, M. G., & Milosavić, N. (2016). Design of biocompatible immobilized *Candida rugosa* lipase with potential application in food industry. *Journal of Science and Food Agriculture, 96*(12), 4281–4287.
17. Gutiérrez, A., Del Rio, J. C., & Martínez, A. T. (2009). Microbial and enzymatic control of pitch in the pulp and paper industry. *Applied Microbiology and Biotechnology, 82*(6), 1005–1018.
18. Ünlü, A., Tanriseven, A., Sezen, I. Y., & Çelik, A. (2015). A new lipase as a pharmaceutical target for battling infections caused by *Staphylococcus aureus*, gene cloning and biochemical characterization. *Biotechnology and Applied Biochemistry, 62*(5), 642–651.
19. Kumar, S., Mathur, A., Singh, V., Nandy, S., Khare, S. K., & Negi, S. (2012). Bioremediation of waste cooking oil using a novel lipase produced by *Penicillium chrysogenum* SNP5 grown in solid medium containing waste grease. *Bioresource Technology, 120*, 300–304.
20. Foukis, A., Gkini, O. A., Stergiou, P. Y., Sakkas, V. A., Dima, A., Boura, K., Koutinas, A., & Papamichael, E. M. (2017). Sustainable production of a new generation biofuel by lipase-catalyzed esterification of fatty acids from liquid industrial waste biomass. *Bioresource Technology, 238*, 122–128.
21. Åkerman, C. O., Hagström, A. E. V., Mollaahmad, M. A., Karlsson, S., & Hatti-Kaul, R. (2011). Biolubricant synthesis using immobilised lipase: process optimisation of trimethylolpropane oleate production. *Process Biochemistry, 46*(12), 2225–2231.
22. Padilha, G. S., Tambourgi, E. B., & Monte Alegre, R. (2018). Evaluation of lipase from *Burkholderia cepacia* immobilized in alginate beads and application in the synthesis of banana flavor (isoamyl acetate). *Chemical Engineering Communications, 205*(1), 23–33.
23. Mhetras, N. C., Bastawde, K. B., & Gokdale, D. V. (2009). Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207. *Bioresource Technology, 100*(3), 1486–1490.
24. Gaikaiwari, R. P., Wagh, S. A., & Kulkarni, B. D. (2012). Efficient lipase purification using reverse micellar extraction. *Bioresource Technology, 108*, 224–230.
25. Trodler, P., Nieveler, J., Rusnak, M., Schmid, R. D., & Pleiss, J. (2008). Rational design of a new one-step purification strategy for *Candida antarctica* lipase B by ion-exchange chromatography. *Journal of Chromatography, 1179A*(2), 161–167.
26. Zhou, Y. J., Hu, C. L., Wang, N., Zhang, W. W., & Yu, X. Q. (2013). Purification of porcine pancreatic lipase by aqueous two-phase systems of polyethylene glycol and potassium phosphate. *Journal of Chromatography, 926*, 77–82.
27. Lopes, F. L. G., Severo, J. J. B., Souza, R. R., Ehrhardt, D. D., Santana, J. C. C., & Tambourgi, E. B. (2009). Concentration by membrane separation processes of a medicinal product obtained from pineapple pulp. *Brazilian Archives of Biology and Technology, 52*(2), 457–464.
28. Lopes, F. L. G., Sbruzzi, D., Barros, K. V. G., Ferreira, J. F., Santana, J. C. C., Souza, R. R., & Tambourgi, E. B. (2012). Viability in the production of a drug extracted from *Ananas comosus* by a flat membrane system. *Brazilian Archives of Biology and Technology, 55*(3), 465–470.
29. Sigma, Chemical Company 2018. Accessed from www.sigmaaldrich.com/Sigma-Aldrich
30. Kordel, M., Hofmann, B., Schomburg, D., & Schmid, R. (1991). Extracellular lipase of *Pseudomonas sp* strain ATCC 21808, purification, characterization, crystallization and preliminary X-ray diffraction data. *Journal of Bacteriology, 177*, 4836–4841.
31. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein. *Analytical Biochemistry, 72*(1-2), 248–254.
32. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature., 227*(5259), 680–685.
33. Almeida, P. F., Silva, J. R., Lannes, S. C. L., Farias, T. M. B., & Santana, J. C. C. (2013). Quality assurance and economic feasibility of an innovative product obtained from a byproduct of the meat industry in Brazil. *African Journal of Business Management, 7*(27), 2745–2756.
34. Cafferky, M. E., & Wentworth, J. (2014). *Breakeven analysis, the definitive guide to cost-volume-profit analysis* (Second ed.). Business Expert Press.

35. Huddleston, J. G., Ottomar, K. W., Ngonyani, D. M., & Lyddiatt, A. (1991). Influence of system and molecular parameters upon fractionation of intracellular proteins from Saccharomyces by aqueous two-phase partition. *Enzyme and Microbial Technology, 13*(1), 24–32.
36. Show, P. L., Tan, C. P., Anuar, M. S., Ariff, A., Yusof, Y. A., Chen, S. K., & Ling, T. C. (2012). Extractive fermentation for improved production and recovery of lipase derived from *Burkholderia cepacia* using a thermoseparating polymer in aqueous two-phase systems. *Bioresource Technology, 116*, 226–233.
37. Kavakçıoglu, B., & Tarhan, L. (2013). Initial purification of catalase from Phanerochaete chrysosporium by partitioning in poly(ethylene glycol)/salt aqueous two phase systems. *Separation and Purification Technology, 105*, 8–14.
38. Kavakçıoglu, B., Tongul, B., & Tarhan, L. (2016). Aqueous two-phase system purification for superoxide dismutase induced by menadione from Phanerochaete chrysosporium. *Artificial Cells, Nanomedicine, and Biotechnology, 45*(2), 380–388.
39. Rabe, M., Verdes, D., & Seeger, S. (2011). Understanding protein adsorption phenomena at solid surfaces. *Advances in Colloid and Interface Science, 162*(1-2), 87–106.
40. Jiang, Z. G., Zhang, H. D., & Wang, W. T. (2015). Purification of papain by metal affinity partitioning in aqueous two-phase polyethylene glycol/sodium sulfate systems. *Journal of Separation Science, 38*(8), 1426–1432.
41. Chew, L. C., Annuar, M. S. M., Show, P. L., & Ling, T. C. (2015). Extractive bioconversion of polycaprolactone by *Burkholderia cepacia* lipase in an aqueous two-phase system. *Biochemical Engineering Journal, 101*, 9–17.
42. Ramakrishnan, V., Goveas, L. C., Suralikerimath, N., Jampani, C., Halami, P. M., & Narayan, B. (2016). Extraction and purification of lipase from *Enterococcus faecium* MTCC5695 by PEG/phosphate aqueous-two phase system (ATPS) and its biochemical characterization. *Biocatalysis and Agricultural Biotechnology, 6*, 19–27.
43. Chow, Y. H., Yap, Y. J., Tan, C. P., Anuar, M. S., Tejo, B. A., Show, P. L., Ariff, A. B., Ng, E. P., & Ling, T. C. (2015). Characterization of bovine serum albumin partitioning behaviors in polymer-salt aqueous two-phase systems. *Journal of Bioscience and Bioengineering, 120*(1), 85–90.
44. Gaither N, Faizer G (1999) Production and operations management. Octave Edition, South-Western Publishing Co.
45. Shepherd R (1981). Theory of cost and production functions. Springer.