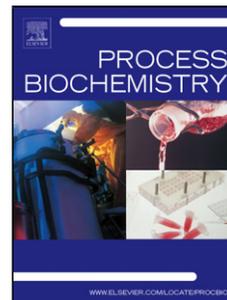


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Lipase production and purification by self-buffering ionic liquid-based aqueous biphasic systems

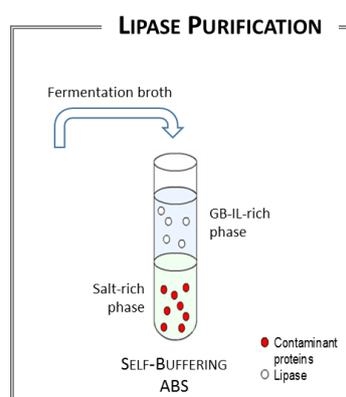
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Graphical abstract



Aqueous biphasic systems composed of self-buffering ionic liquids were used for the purification of an extracellular lipase from its fermentation broth.

Highlights

- Self-buffering aqueous biphasic systems formed with GB-ILs, salts, and (co)polymers
- Lipase preferential partition towards the GB-IL-rich phase
- An extracellular lipase produced *via* submerged fermentation was studied
- An efficient downstream process to recover a microbial lipase was developed

ABSTRACT

In this work, a group of Good's buffer ionic liquids (GB-ILs) comprised of tetrabutylammonium, tetrabutylphosphonium and cholinium cations paired with Good's buffer (GB) anions (MOPSO, BES and TAPSO) was studied. Their distinctive capability to induce the formation of aqueous biphasic systems (ABS) with the salts K_3PO_4 , K_2CO_3 , and $(NH_4)_2SO_4$, and the polymers poly(ethylene glycol) (PEG), poly(propylene glycol) (PPG), and PEG-PPG copolymers was demonstrated. Their application as purification tools to recover a lipase produced *via* submerged fermentation by *Burkholderia cepacia* ST8 was investigated. The lipase was preferentially partitioned towards the GB-IL-rich phase in both the GB-IL + salt and polymer + GB-IL purification systems. Molecular docking studies were performed aiming at to understand the possible interactions between the GB-IL ions and the lipase residues. Furthermore, the selected GB-IL-based ABS was investigated as part of an integrated process developed to successfully recover and purify an extracellular *B. cepacia* ST8 lipase from the fermentation broth, in which a purification factor of 22.4 ± 0.7 and a recovery yield of $(94.0 \pm 0.2) \%$ were achieved.

KEYWORDS: Lipase from *Burkholderia cepacia* ST8, Good's buffer ionic liquids, integrated downstream process, aqueous biphasic systems.

1. Introduction

One of the main problems associated with the production of lipase *via* fermentation is the downstream process [1, 2]. The traditional purification processes, namely the chromatographic methods, membrane separations and liquid-liquid extractions with organic solvents are complex and expensive [3, 4]. In this context, aqueous biphasic systems (ABS) have been focus of much attention during the last years [5, 6]. Despite their high simplicity, increased biocompatibility with different macromolecules, and easiest scale-up [7], they still present some drawbacks to overcome, mainly those found when these systems are applied on enzyme purification. Numerous studies have been devoted to study ABS composed of distinct phase-forming components for the lipase recovery and purification, from polymers (the most commonly studied) like dextran and polyethylene glycol (PEG) [8-16], alcohol [17], thermo-sensitive PEG-polypropylene glycol (PPG) copolymers [18], organic solvents [19] to ionic liquids (ILs) [20-23]. Amongst the phase-forming components, ILs have received great attention, mainly due to their environmentally benign characteristics (non-volatility and non-flammability [24]), but also in terms of their tunable properties assessed by the proper cation/anion combinations [25, 26]. However, in applications dealing with enzymes, IL-based ABS are mostly restricted to the use of buffer solutions, normally based in phosphate or citrate salts to control the pH of the aqueous phases.

Recently, our group has reported the synthesis of a series of water-miscible ILs with buffering capacity, including at pH 7, in which anions derived from the Good's buffers (GBs) were utilized [27]. In this work, the 2-hydroxy-3-morpholinopropanesulfonic acid (MOPSO), 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (BES) and N-[tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropanesulfonic acid (TAPSO), coupled with tetrabutylammonium ($[N_{4,4,4,4}]^+$), tetrabutylphosphonium ($[P_{4,4,4,4}]^+$) and cholinium ($[Ch]^+$) cations [27] were considered. Meanwhile, other works dealt with the use and application of these GB-ILs in the extraction of other macromolecules, namely proteins like the bovine serum albumin (BSA) [28], and immunoglobulin Y [29].

Given the advantageous properties of the GB-ILs, namely their temperature independent buffering capacity, biocompatibility with enzyme, and low toxicity [27], these compounds were further used in this work, on the preparation of ABS composed of GB-ILs with K_3PO_4 , K_2CO_3 and $(NH_4)_2SO_4$, and polymers including PEG, PPG, and

copolymers, namely poly(ethylene glycol-ran-propylene glycol) (PEG-ran-PPG) and poly(ethylene glycol-ran-propylene glycol) monobutyl ether (BEPEG-ran-PPG). The ternary phase diagrams were experimentally determined at $25 (\pm 1) ^\circ\text{C}$ and atmospheric pressure. The impact of different cations and anions of GB-ILs in forming ABS when conjugated with salts or polymers was addressed. The screening of the potential of the ABS formed by GB-IL + salt and polymer + GB-IL was performed by evaluating the partition behaviour of a commercially obtained *Pseudomonas cepacia* lipase, and the results were compared with the experimental data found in this work also for the conventional polymer + salt-based ABS. Additionally, a molecular docking study was conducted, aiming at understanding the interactions between the GB-IL ions with lipase residues. Finally, the most efficient GB-IL-based ABS was further applied in the purification of an extracellular lipase produced by *Burkholderia cepacia* ST8 via submerged fermentation.

2. Experimental section

2.1. Materials

The GBs, including MOPSO, BES and TAPSO were purchased from Sigma-Aldrich with purities $\geq 99\%$. The $[\text{N}_{4,4,4,4}]\text{OH}$ (40 wt% in H_2O), $[\text{P}_{4,4,4,4}]\text{OH}$ (40 wt% in H_2O), $[\text{Ch}]\text{OH}$ (46 wt% in H_2O) solutions, potassium phosphate tribasic (K_3PO_4 , purity $\geq 98\%$), potassium carbonate (K_2CO_3 , purity $\geq 99\%$), potassium phosphate monobasic (KH_2PO_4 , purity $\geq 99.5\%$), potassium phosphate dibasic (K_2HPO_4 , purity $\geq 99\%$), PEG with a molar mass of $400\text{ g}\cdot\text{mol}^{-1}$ (PEG 400) and $1000\text{ g}\cdot\text{mol}^{-1}$ (PEG 1000), PPG of $400\text{ g}\cdot\text{mol}^{-1}$ (PPG 400), PEG-ran-PPG of $2500\text{ g}\cdot\text{mol}^{-1}$ (PEG-ran-PPG 2500) and BEPEG-ran-PPG of $3900\text{ g}\cdot\text{mol}^{-1}$ (BEPEG-ran-PPG 3900), 4-nitrophenyl laurate (*p*-NPL, purity $\geq 98.0\%$), 4-nitrophenol (*p*-NP, spectrophotometric grade), brilliant blue G-250 (microscopy grade), the protein standard of bovine serum albumin (BSA) and lipase from *P. cepacia* were supplied by Sigma-Aldrich. Ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$, purity $\geq 99.5\%$) was acquired from Merck. Methanol (purity $\geq 99.9\%$), acetonitrile (purity $\geq 99.9\%$), ethanol and orthophosphoric acid were purchased from Fisher Scientific. Difco™ nutrient broth containing beef extract and peptone (in a mass ratio of 3:5) was obtained commercially from Becton Dickinson (product code: 234000). All stock solutions and chemicals for polyacrylamide gel electrophoresis and pre-stained protein standard were

obtained from Bio-Rad, and Proteosilver silver stain kit was purchased from Sigma-Aldrich. The water used throughout the work was ultra-pure water treated by a Milli-Q integral water purification system.

2.2. Methods

2.2.1. Synthesis of GB-ILs

All the GB-ILs were synthesized following the same experimental procedures described elsewhere [30], and their chemical structures are presented in Figure S1 (in Supporting Information).

2.2.2. Phase diagrams, tie-lines, and tie-line lengths

The binodal curve of each ternary phase diagram was determined by the cloud point titration method at 25 (± 1) °C and atmospheric pressure, as described in literature [27, 31]. The experimental binodal curves were fitted by least-squares regression to Merchuk equation, as described elsewhere [27, 31, 32]. The tie-lines (TLs) and corresponding tie-line lengths (TLLs) were assessed by the gravimetric method previously described [27]. The consistency of the TLs' compositions was ascertained by using the Othmer–Tobias and Bancroft equations.

2.2.3. Lipase partition in the GB-IL-based ABS

The GB-IL-based ABS were prepared as described in our earlier work [27]. The mixture with a total mass of 5.0 g (included 0.1 wt% of commercial lipase added) was stirred in a vortex and then centrifuged at 1 000 g, at 25 °C for 15 min on an Eppendorf centrifuge (model 5430). The systems were then left for one hour to achieve the thermodynamic equilibrium then, both aqueous phases were separated, their volume measured, and the lipase activity determined. Triplicate independent runs were performed for each test, being the results expressed as their average. The lipase partition behaviour was studied considering the measurement of the partition coefficient of the enzyme (K_E) and the enzyme recovery in the top (R_T^E) and bottom (R_B^E) phases, as defined by Eqs. (1) to (3) [27, 33].

$$K_E = \frac{EA_T}{EA_B} \quad (1)$$

$$R_T^E = \frac{100}{1 + \frac{1}{R_V K_E}} \quad (2)$$

$$R_B^E = \frac{100}{1 + R_V K_E} \quad (3)$$

where EA_T and EA_B are the enzyme activity at the top and bottom phases, and R_V is the volume ratio of both top and bottom phases.

2.2.4. Lipase activity assay

The lipase activity was spectrophotometrically assayed using a UV-Vis spectrophotometer (Shimadzu, model UV-1800) [8, 27, 34]. The substrate solution was prepared by mixing 9.0 % (v/v) of *p*-NPL (20 mM) in ethanol, 89.2 % (v/v) of a KH_2PO_4/K_2HPO_4 solution at 50 mM and pH 7.0 and 1.8 % (v/v) of Triton-X100, and incubated at 37 °C for 10 min prior to addition of sample. The linear range of absorbance variation at 410 nm during 150 s ($\Delta Abs \cdot min^{-1}$) was analyzed. One unit (U) of enzyme activity is defined as the amount of enzyme that produces 1 μ mol of *p*-NP *per* minute, being this parameter expressed in $U \cdot mL^{-1}$.

2.2.5. Protein assay

The Bradford's method was used to quantify the concentration of the total proteins [35], using a UV-Vis spectrophotometer (Shimadzu, UV-1800) at 595 nm. The detailed procedures were described in a previous work [27].

2.2.6. Molecular docking study

Molecular docking was used to simulate the lipase as the receptor and GB-IL, $(NH_4)_2SO_4$, PPG with 5 units of the corresponding monomers and *p*-NPL as the ligands targeting their potential interactions. For this purpose AutoDock Tools Vina 1.5.6 [36] was used. The crystal structure of this lipase from *B. cepacia* (PDB ID: 1YS1) [37] was obtained from the RCSB Protein Data Bank [38]. The centre of *B. cepacia* lipase structure was used as the center of the grid boxes for docking, and the size of each box was assigned as $(60 \times 60 \times 60)$ Å, to cover the whole enzyme. The natural bond orbital (NBO) charges of all ligands were computed using Gaussian 09 [39], based on polarizable continuum model in water at the DFT/B3LYP/6-311++G(d,p) level, and then used in the docking. All the docking calculations were performed using the default procedure implemented in AutoDock Vina [36].

2.2.7. Production and pre-purification of an extracellular lipase from *B. cepacia* ST8

B. cepacia ST8 was cultivated in a 500 mL shake flask consisting of 100 mL of fermentation medium prepared with the Difco™ nutrient broth in the following

composition, 0.325 % (w/v); CaCl₂, 0.1 % (w/v); olive oil, 1.0 % (v/v); and arabic gum, 1.0 % (w/v); and inoculated with a pre-grown culture with the cells growth reaching values around 1.2 of OD_{600nm} in 0.8 % (w/v) of nutrient broth, 5.0 % (v/v), with the initial pH adjusted to 7.0 [34]. The submerged fermentation was carried out in a water bath shaker (Mettler, model WNB22L4) at 37.0 °C for 72 h under constant agitation at 150 rpm. At the end of fermentation, the cells were harvested by centrifugation at 10 000 g at 4 °C for 15 min using a refrigerated centrifuge (Eppendorf, 5810R). The cell-free supernatant (rich in the lipase) obtained was pre-treated by two ammonium sulfate precipitation steps; in a first step from 0-35 % and then in a second step, from 35-70 %. The protein pellets collected by centrifugation at 10 000 × g at 4 °C for 15 min were resuspended in a phosphate buffer solution (50 mM; pH 7.0), for the quantification of the lipase and total proteins.

2.2.8. Lipase purification

The lipase rich fraction (approximately 7.5 wt% in each system) obtained from the ammonium sulfate precipitation step, was further purified using the selected GB-IL-based ABS at different phase compositions and similar TLs. The process was conducted using the same operational conditions previously described in Section 2.2.3. To allow the evaluation of the purification capability of the systems, additional parameters including the partition coefficient of total proteins (K_P), the enzyme specific activity (SA), the purification factor (PF), and the selectivity ($S_{E/P}$) parameters were determined, as described by Eqs. (4 to 7) [27].

$$K_P = \frac{C_T}{C_B} \quad (4)$$

$$SA = \frac{EA}{C} \quad (5)$$

$$PF = \frac{SA}{SA_i} \quad (6)$$

$$S_{E/P} = \frac{K_E}{K_P} \quad (7)$$

where C_T and C_B are the concentration of the total proteins at the top and bottom phases. The enzyme specific activity (SA) can be evaluated based on the ratio of the enzyme

activity (EA) and the protein concentration (C) in the enzyme extract, and (SA_i) is the initial specific enzyme activity before the extraction procedure.

2.2.9. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The SDS-PAGE analysis was performed using an hand cast polyacrylamide gel made of a 12 % revolving gel and a 4 % stacking gel, thus added to a vertical gel electrophoresis unit (Bio-Rad, model mini-PROTEAN tetra cell), running at 160 V for 1 hour, following the protocol described by Laemmli [40]. After the electrophoresis process, the gel was stained through the direct silver staining procedure. The treatment of the protein samples was performed as described elsewhere [17, 41].

3. Results and discussion

The present work aims at investigating the applicability of GB-IL-based ABS to improve the purification of an extracellular lipase produced *via* fermentation by *B. cepacia* ST8. In this case, the possibility of obtaining a lipase with a high purity level is associated with the reduction of the number of steps applied comprising the downstream processing, thus aiming at increasing the sustainability of the process.

3.1. Development of the GB-IL-based ABS on the lipase partition

3.1.1. GB-IL-based ABS with salts and polymers

The preparation of ABS based in the GB-IL + salt and GB-IL + polymer platforms was envisaged by measuring the phase diagrams of nine GB-ILs, comprised of different cations ($[N_{4,4,4,4}]^+$, $[P_{4,4,4,4}]^+$ and $[Ch]^+$) and anions ($[MOPSO]^-$, $[BES]^-$ and $[TAPSO]^-$), and conjugated with aqueous solutions of salts (phosphate, carbonate and sulfate) or polymers (PEG 400, PEG 1000, PPG 400, PEG-ran-PPG 2500 and BEPEG-ran-PPG 3900). The phase diagrams of the GB-IL-based ABS were characterized being further described in Section S1 in Supporting Information.

The GB-ILs studied demonstrated different capacities to form ABS with different types of salts and polymers (Figure S2), which are closely related to the hydrophilic/hydrophobic nature of their cations (Figure 1) and anions (Figure S3), being their effects already addressed in our earliest work [27]. Tetrabutylammonium- and tetrabutylphosphonium-based GB-ILs promoted the two-phase formation in presence of the salts studied, but these do not form ABS with polymers and copolymers, due to their

higher hydrophobicity. The more hydrophilic cholinium-based GB-ILs, which are not able to create ABS with $K_3C_6H_5O_7$ [27] and $(NH_4)_2SO_4$ (Figure 2), are easily inducing the ABS formation with the hydrophobic polymers, namely PPG 400 and BEPEG-ran-PPG 3900 (Figure 3). Additionally, the trend of the salt effect complies with the well-known Hofmeister series [42] (Figure S4). Summing up, the results demonstrated an inverse trend of the GB-ILs phase formation capability when these are conjugated with salts or polymers [25, 43].

3.1.2. Partition of commercial lipase in GB-ILs-based ABS

The GB-IL-based ABS developed were investigated towards the lipase partition by using a commercial *P. cepacia* lipase. Considering their buffering region, the GB-IL-based ABS using $(NH_4)_2SO_4$, PPG 400 and BEPEG-ran-PPG 3900 were selected, and a biphasic mixture point at the shortest TL of each system (Table S16) was chosen in order to maximize the water content in each phase. Additionally, PPG 400 + $(NH_4)_2SO_4$ and BEPEG-ran-PPG 3900 + $(NH_4)_2SO_4$ systems were also included in this study as the conventional systems used to be compared against those based in the GB-ILs, being their pH established to be 5.54 and 4.73, respectively. All the lipase partition systems studied are depicted in Figure S5.

Table 1 presents the systems selected, their compositions, respective TLL applied, and the lipase partition coefficient and recovery results achieved. For all GB-IL-based ABS investigated the complete partition of the lipase towards the GB-IL-rich phase was observed, as shown in Figure 4. For the ABS formed by tetrabutylammonium- or tetrabutylphosphonium-based GB-ILs + $(NH_4)_2SO_4$, the lipase is completely concentrated in the GB-IL-rich phase ($K_E = 145 \pm 7$ to 218 ± 3). However, the lipase partition is not only predominantly driven by hydrophobic interactions, which is proven by the preferential migration of lipase to the bottom hydrophilic GB-IL-rich phase for systems composed of PPG 400/BEPEG-ran-PPG 3900 + cholinium-based GB-ILs ($K_E = 0.007 \pm 0.002$ to 0.068 ± 0.003). When the results found for the conventional (polymer + salt) ABS are analyzed, the main conclusion is that the lipase is similarly distributed between both aqueous phases of PPG 400 and $(NH_4)_2SO_4$ ($K_E = 0.77 \pm 0.03$). Better results were obtained for the system BEPEG-ran-PPG 3900 + $(NH_4)_2SO_4$, in which a preferential partition of the lipase to the top phase is obtained ($K_E = 9.5 \pm 0.2$), nevertheless far below the results obtained for the GB-IL-based ABS. This poor migration of the lipase can be

attributed to the acidic pH conditions of the conventional systems, which affect the charge and the lipase hydrophobic surface properties, consequently changing the preferential interactions of the enzyme with the phase components.

Aiming at to identify the possible GB-IL ions/lipase interactions, a molecular docking study was performed. For the cations $[P_{4,4,4,4}]$, $[N_{4,4,4,4}]^+$ and $[Ch]^+$, the free energies of binding (the negative sign reflects favorable binding) are, respectively, -5.2, -4.9 and -3.2 kcal·mol⁻¹ and for the anions $[MOPSO]^-$, $[BES]^-$ and $[TAPSO]^-$, are respectively, -4.9, -4.6 and -4.5 kcal·mol⁻¹. Besides, lower binding affinities of the $(NH_4)_2SO_4$ ions with lipase were obtained, which are -1.7 and -3.4 kcal·mol⁻¹ for $[NH_4]^+$ and $[SO_4]^{2-}$, and a binding affinity of -5.0 kcal·mol⁻¹ was obtained for PPG 400. The results are consistent with the findings obtained considering the lipase partition behavior study. Actually, the results show the lipase higher (binding) affinity for GB-IL-rich phase than for $(NH_4)_2SO_4$ - or PPG 400-rich phase. The strong binding affinities of GB-IL ions and lipase residues can be attributed to the establishment of hydrogen bond interactions, apart from $[P_{4,4,4,4}]^+$, $[N_{4,4,4,4}]^+$ and PPG 400 that form non polar interactions (Figure S6 and Figure S7). Additionally, most ligands studied are not likely to interact with the active site inside the binding pocket of lipase where the substrate molecules bind for catalytic activity (see details in Section S2).

3.2. Purification of an extracellular lipase produced *via* fermentation

Taking into account the results obtained for the lipase partition, the ABS composed of $[N_{4,4,4,4}][BES] + (NH_4)_2SO_4$ were selected to be applied for the purification of an extracellular lipase produced *via* fermentation by *B. cepacia* ST8. Different phase compositions of the systems with similar short TLL and at fixed pH between 7.00 and 7.30 (Figure S8), were studied. For that purpose, the fermentation broth was collected after the lipase production and subjected to a pre-treatment with an ammonium sulphate solution. The protein fraction with the highest specific enzymatic activity obtained from the precipitation step (Table S18) was directly purified using the ABS based in $[N_{4,4,4,4}][BES] + (NH_4)_2SO_4$, aiming at the adoption of the same salt in both steps of pre-purification and purification.

Table 2 reports the lipase specific enzyme activity at each stage of production and pre-purification, being the purification results presented in Table 3. Again, the same lipase partition behaviour for the GB-IL-rich phase was defined. Its maximum partition

coefficient was obtained for the system composed of 9.99 wt% of $[N_{4,4,4,4}][BES]$ + 24.98 wt% of $(NH_4)_2SO_4$, the system with the lowest volume of GB-IL-rich phase. Moreover, its highest purification performance ($PF = 22.4 \pm 0.7$) was verified, since the lipase ($K_E = 93 \pm 3$) and the contaminant proteins are concentrated in opposite phases ($K_P = 1.6 \pm 0.1$), in addition to the contaminants being physically separated by precipitation at the two-phase interface (Figure S9). The results reveal that when the protein solvation capacity of the GB-IL-rich phase is restricted by its concentration/volume, the respective system demonstrated high selectivity to lipase, thus increasing its purity. This can be attributed to the stronger binding affinities between the GB-IL ions and lipase residues, when compared to the interactions taking place between the contaminant proteins and the GB-IL ions. Besides, the purity of the lipase recovered from the best system was further ascertained through an SDS-PAGE analysis (Figure 5), being the purified lipase found consistent with the molecular weight of 33 kDa previously reported [44].

The proposed scheme showed enhanced purification results when compared with the purification strategy previously reported for *B. cepacia* ST8 lipase ($PF = 14.0$) [44]. Meanwhile, some other works have revealed high purity levels of different bacterial lipases being recovered from the fermentation broth, namely those based in polymers + salt [45], organic solvent + salt [33], and ABS applying ionic liquids as adjuvants [46]. However, it should be remarked the higher complexity (quaternary systems [46] are employed) and the lower biocompatibility (use of organic solvents [33] and low amounts of water [45]) of some of those systems when compared to these reported in this work.

4. Conclusions

In this work an integrated process of production and purification of an extracellular lipase was successfully developed. It was successfully demonstrated the application of the neutral pH-controlled $[N_{4,4,4,4}][BES]$ + $(NH_4)_2SO_4$ -based ABS to improve the purification of an extracellular lipase produced *via* fermentation by *B. cepacia* ST8 not only obtaining a lipase with high purity ($PF = 22.4 \pm 0.7$) and recovery yield ($R_T^E = 94.0 \pm 0.2$ %). It is possible to simplify and merge both the pre-purification and purification steps. Moreover, this adapted downstream process allows the polishing of the target lipase and the recycle of the ABS phase formers, by just applying an ultrafiltration step to each

phase. The proposed strategy not only allows the simplification of lipase downstream process, but also reduces its economic and environmental impact.

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Supporting Information

The supporting information is available free of charge on the website.

References

- [1] E. Dako, A.T. Dadie, A.-M. Bernier, C.K. Jankowski, *The Problems Associated with Enzyme Purification*, INTECH Open Access Publisher 2012.
- [2] G. Carta, A. Jungbauer, *Downstream processing of biotechnology products*, *Protein chromatography: Process development and scale-up* (2010) 1-55.
- [3] R. Gupta, N. Gupta, P. Rathi, *Bacterial lipases: an overview of production, purification and biochemical properties*, *Appl. Microbiol. Biotechnol.* 64(6) (2004) 763–781.
- [4] R. Saxena, A. Sheoran, B. Giri, W.S. Davidson, *Purification strategies for microbial lipases*, *J. Microbiol. Methods* 52(1) (2003) 1-18.
- [5] P.-L. Show, T.-C. Ling, J. C-W Lan, B.-T. Tey, R. N Ramanan, S.-T. Yong, C.-W. Ooi, *Review of microbial lipase purification using aqueous two-phase systems*, *Curr. Org. Chem.* 19(1) (2015) 19-29.
- [6] H. Walter, *Partitioning In Aqueous Two-Phase System: Theory, Methods, Uses, And Applications To Biotechnology*, Elsevier 2012.
- [7] R. Hatti-Kaul, *Aqueous two-phase systems: methods and protocols*, Springer Science & Business Media 2000.
- [8] C.W. Ooi, B.T. Tey, S.L. Hii, A. Ariff, H.S. Wu, J.C.W. Lan, R.S. Juang, S.M.M. Kamal, T.C. Ling, *Direct purification of Burkholderia Pseudomallei lipase from fermentation broth using aqueous two-phase systems*, *Biotechnol. Bioprocess Eng.* 14(6) (2010) 811-818.
- [9] C.W. Ooi, S.L. Hii, S.M.M. Kamal, A. Ariff, T.C. Ling, *Extractive fermentation using aqueous two-phase systems for integrated production and purification of extracellular lipase derived from Burkholderia pseudomallei*, *Process Biochem.* 46(1) (2011) 68-73.
- [10] N. Saisubramanian, S. Sivasubramanian, N. Nandakumar, B. Indirakumar, N.A. Chaudhary, R. Puvanakrishnan, *Two step purification of Acinetobacter sp. lipase and its evaluation as a detergent additive at low temperatures*, *Appl. Biochem. Biotechnol.* 150(2) (2008) 139-156.
- [11] A. Marini, N. Imelio, G. Pico, D. Romanini, B. Farruggia, *Isolation of a Aspergillus niger lipase from a solid culture medium with aqueous two-phase systems*, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 879(22) (2011) 2135-41.

- [12] G.D.S. Padilha, J.C.C. Santana, R.M. Alegre, E.B. Tambourgi, Extraction of lipase from *Burkholderia cepacia* by PEG/Phosphate ATPS and its biochemical characterization, *Braz. Arch. Biol. Technol.* 55(1) (2012) 7–19.
- [13] G. Khayati, S. Alizadeh, Extraction of lipase from *Rhodotorula glutinis* fermentation culture by aqueous two-phase partitioning, *Fluid Phase Equilib.* 353 (2013) 132-134.
- [14] B.-h. Yuan, Y.-j. Cai, X.-r. Liao, L.-h. Yun, F. Zhang, D.-b. Zhang, Isolation and identification of a cold-adapted lipase producing strain from decayed seeds of *Ginkgo biloba* L. and characterization of the lipase, *African Journal of Biotechnology* 9(18) (2010) 2661-2667.
- [15] G. Bassani, P. Fucinos, G. Pico, B. Farruggia, *Candida rugosa* lipase Lip1-polyethyleneglycol interaction and the relation with its partition in aqueous two-phase systems, *Colloids Surf. B. Biointerfaces* 75(2) (2010) 532-7.
- [16] Y.-y. Zhang, J.-h. Liu, Purification and in situ immobilization of lipase from of a mutant of *Trichosporon laibacchii* using aqueous two-phase systems, *J. Chromatogr. B* 878(11) (2010) 909-912.
- [17] C.W. Ooi, B.T. Tey, S.L. Hii, S.M.M. Kamal, J.C.W. Lan, A. Ariff, T.C. Ling, Purification of lipase derived from *Burkholderia pseudomallei* with alcohol/salt-based aqueous two-phase systems, *Process Biochem.* 44(10) (2009) 1083-1087.
- [18] P.L. Show, C.P. Tan, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, T.C. Ling, Primary recovery of lipase derived from *Burkholderia cenocepacia* strain ST8 and recycling of phase components in an aqueous two-phase system, *Biochem. Eng. J.* 60 (2012) 74-80.
- [19] R.L. Souza, R.A. Lima, J.A.P. Coutinho, C.M.F. Soares, Á.S. Lima, Novel aqueous two-phase systems based on tetrahydrofuran and potassium phosphate buffer for purification of lipase, *Process Biochem.* 50(9) (2015) 1459–1467.
- [20] F.J. Deive, A. Rodríguez, A.B. Pereiro, J.M.M. Araújo, M.A. Longo, M.A.Z. Coelho, J.N.C. Lopes, J.M.S.S. Esperança, L.P.N. Rebelo, I.M. Marrucho, Ionic liquid-based aqueous biphasic system for lipase extraction, *Green Chem.* 13(2) (2011) 390–396.
- [21] F.J. Deive, A. Rodríguez, L.P.N. Rebelo, I.M. Marrucho, Extraction of *Candida antarctica* lipase A from aqueous solutions using imidazolium-based ionic liquids, *Sep. Purif. Technol.* 97 (2012) 205–210.

- [22] S.P. Ventura, S.G. Sousa, M.G. Freire, L.S. Serafim, A.S. Lima, J.A. Coutinho, Design of ionic liquids for lipase purification, *J. Chromatogr. B* 879(26) (2011) 2679–2687.
- [23] S.P.M. Ventura, R.L.F. de Barros, J.M. de Pinho Barbosa, C.M.F. Soares, Á.S. Lima, J.A.P. Coutinho, Production and purification of an extracellular lipolytic enzyme using ionic liquid-based aqueous two-phase systems, *Green Chem.* 14(3) (2012) 734–740.
- [24] M. Freemantle, *An introduction to ionic liquids*, Royal Society of chemistry 2010.
- [25] M.G. Freire, A.F. Claudio, J.M. Araujo, J.A. Coutinho, I.M. Marrucho, J.N. Canongia Lopes, L.P. Rebelo, Aqueous biphasic systems: a boost brought about by using ionic liquids, *Chem. Soc. Rev.* 41(14) (2012) 4966–4995.
- [26] S.Y. Lee, I. Khoiroh, C.W. Ooi, T.C. Ling, P.L. Show, Recent Advances in Protein Extraction Using Ionic Liquid-based Aqueous Two-phase Systems, *Sep. Purif. Rev.* (2017) 1-14.
- [27] S.Y. Lee, F.A. Vicente, F.A. e Silva, T.n.E. Sintra, M. Taha, I. Khoiroh, J.o.A. Coutinho, P.L. Show, S.n.P. Ventura, Evaluating self-buffering ionic liquids for biotechnological applications, *ACS Sustain. Chem. Eng.* 3(12) (2015) 3420-3428.
- [28] M. Taha, M.V. Quental, I. Correia, M.G. Freire, J.A.P. Coutinho, Extraction and stability of bovine serum albumin (BSA) using cholinium-based Good's buffers ionic liquids, *Process Biochem.* 50(7) (2015) 1158–1166.
- [29] M. Taha, M.R. Almeida, F.A. Silva, P. Domingues, S.P. Ventura, J.A. Coutinho, M.G. Freire, Novel biocompatible and self-buffering ionic liquids for biopharmaceutical applications, *Chem. Eur. J.* 21(12) (2015) 4781–4788.
- [30] S.Y. Lee, F.A. Vicente, J.A.P. Coutinho, I. Khoiroh, P.L. Show, S.P.M. Ventura, Densities, Viscosities, and Refractive Indexes of Good's Buffer Ionic Liquids, *J. Chem. Eng. Data* 61(7) (2016) 2260-2268.
- [31] C.M. Neves, S. Shahriari, J. Lemus, J.F. Pereira, M.G. Freire, J.A. Coutinho, Aqueous biphasic systems composed of ionic liquids and polypropylene glycol: insights into their liquid–liquid demixing mechanisms, *Phys. Chem. Chem. Phys.* 18(30) (2016) 20571-20582.
- [32] J.C. Merchuk, B.A. Andrews, J.A. Asenjo, Aqueous two-phase systems for protein separation: Studies on phase inversion, *J. Chromatogr. B: Biomed. Sci. Appl.* 711(1) (1998) 285-293.

- [33] R.L. Souza, R.A. Lima, J.A.P. Coutinho, C.M.F. Soares, Á.S. Lima, Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification, *Sep. Purif. Technol.* 155 (2015) 118-126.
- [34] S.Y. Lee, I. Khoiroh, T.C. Ling, P.L. Show, Enhanced recovery of lipase derived from *Burkholderia cepacia* from fermentation broth using recyclable ionic liquid/polymer-based aqueous two-phase systems, *Sep. Purif. Technol.* 179 (2017) 152-160.
- [35] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72(1) (1976) 248–254.
- [36] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, *J. Comput. Chem.* 31(2) (2010) 455-461.
- [37] A. Mezzetti, J.D. Schrag, C.S. Cheong, R.J. Kazlauskas, Mirror-image packing in enantiomer discrimination: molecular basis for the enantioselectivity of *B. cepacia* lipase toward 2-methyl-3-phenyl-1-propanol, *Chem. Biol.* 12(4) (2005) 427-437.
- [38] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, The protein data bank, *Nucleic Acids Res.* 28(1) (2000) 235-242.
- [39] M. Frisch, G. Trucks, H.B. Schlegel, G. Scuseria, M. Robb, J. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. Petersson, Gaussian 09, Revision A. 02, Gaussian, Inc., Wallingford, CT 200 (2009).
- [40] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227(5259) (1970) 680–685.
- [41] P.L. Show, C.P. Tan, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, T.C. Ling, Extractive fermentation for improved production and recovery of lipase derived from *Burkholderia cepacia* using a thermoseparating polymer in aqueous two-phase systems, *Bioresour. Technol.* 116 (2012) 226-233.
- [42] F. Hofmeister, Zur Lehre von der Wirkung der Salze, *Archiv f. experiment. Pathol. u. Pharmakol* 25(1) (1888) 1-30.
- [43] J.F. Pereira, K.A. Kurnia, O.A. Cojocar, G. Gurau, L.P.N. Rebelo, R.D. Rogers, M.G. Freire, J.A. Coutinho, Molecular interactions in aqueous biphasic systems

composed of polyethylene glycol and crystalline vs. liquid cholinium-based salts, *Phys. Chem. Chem. Phys.* 16(12) (2014) 5723-5731.

[44] P.L. Show, C.P. Tan, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, T.C. Ling, Direct recovery of lipase derived from *Burkholderia cepacia* in recycling aqueous two-phase flotation, *Sep. Purif. Technol.* 80(3) (2011) 577-584.

[45] J.M. Barbosa, R.L. Souza, A.T. Fricks, G.M. Zanin, C.M. Soares, A.S. Lima, Purification of lipase produced by a new source of *Bacillus* in submerged fermentation using an aqueous two-phase system, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 879(32) (2011) 3853-3858.

[46] R.L. Souza, S.P.M. Ventura, C.M.F. Soares, J.A.P. Coutinho, Á.S. Lima, Lipase purification using ionic liquids as adjuvants in aqueous two-phase systems, *Green Chem.* 17(5) (2015) 3026-3034.

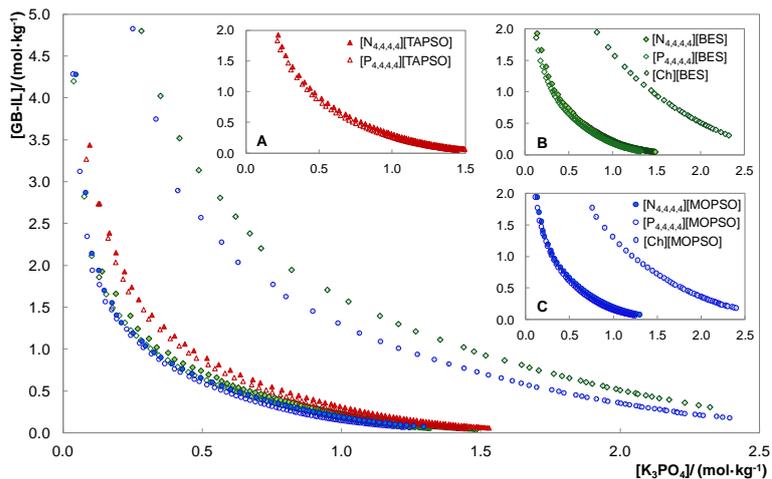


Figure 1. Phase diagrams for the ternary systems composed of GB-IL + K_3PO_4 + H_2O at $T = 25 (\pm 1) ^\circ\text{C}$. To study the cation effect, the comparison between the binodal curves obtained for GB-ILs based on $[\text{TAPSO}]^-$ (A), $[\text{BES}]^-$ (B) and $[\text{MOPSO}]^-$ (C) is highlighted in the insets included in this figure.

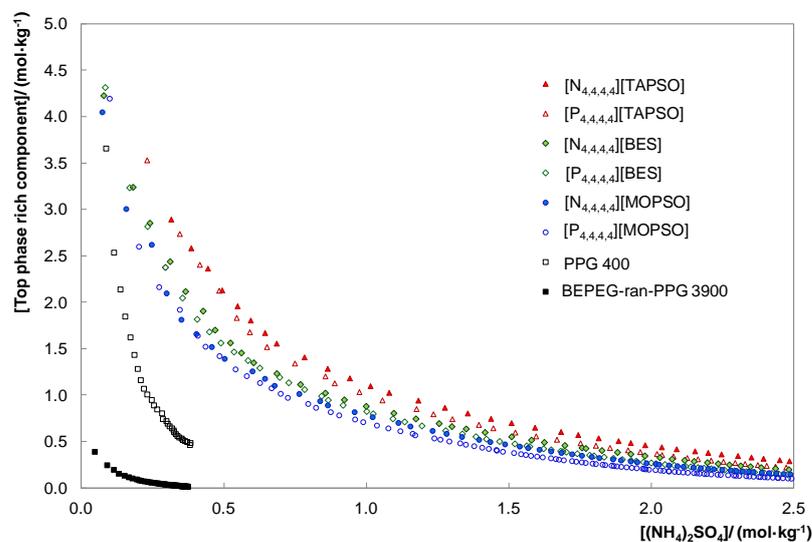


Figure 2. Phase diagrams for the ternary systems composed of GB-IL/PPG 400/BEPEG-ran-PPG 3900 + $(\text{NH}_4)_2\text{SO}_4$ + H_2O at $T = 25 (\pm 1) ^\circ\text{C}$.

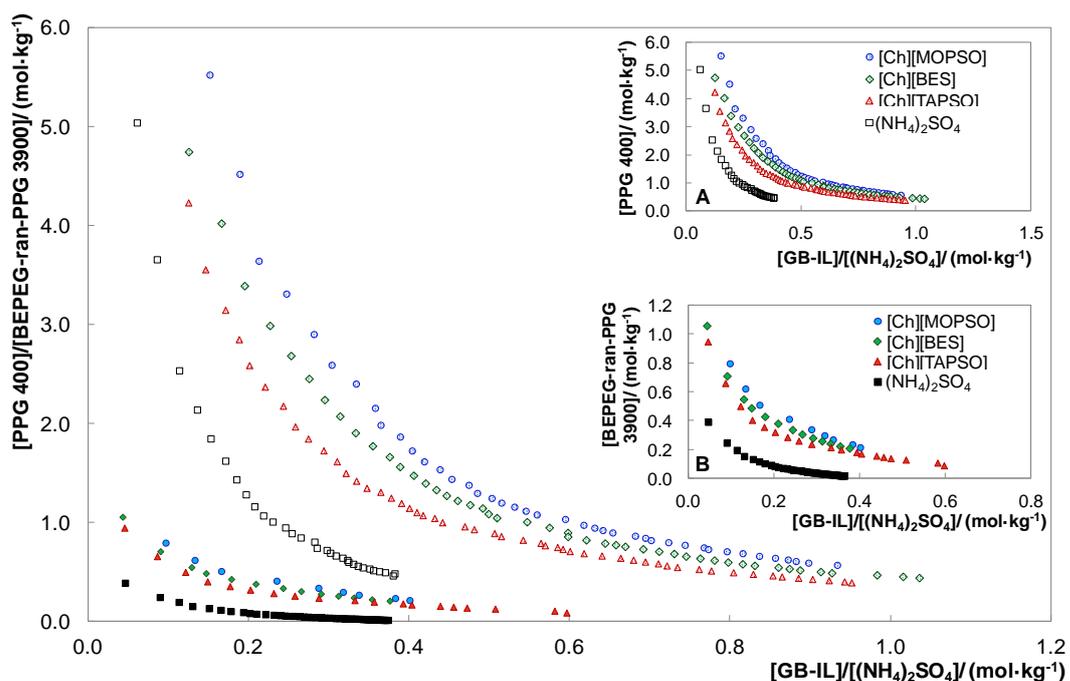
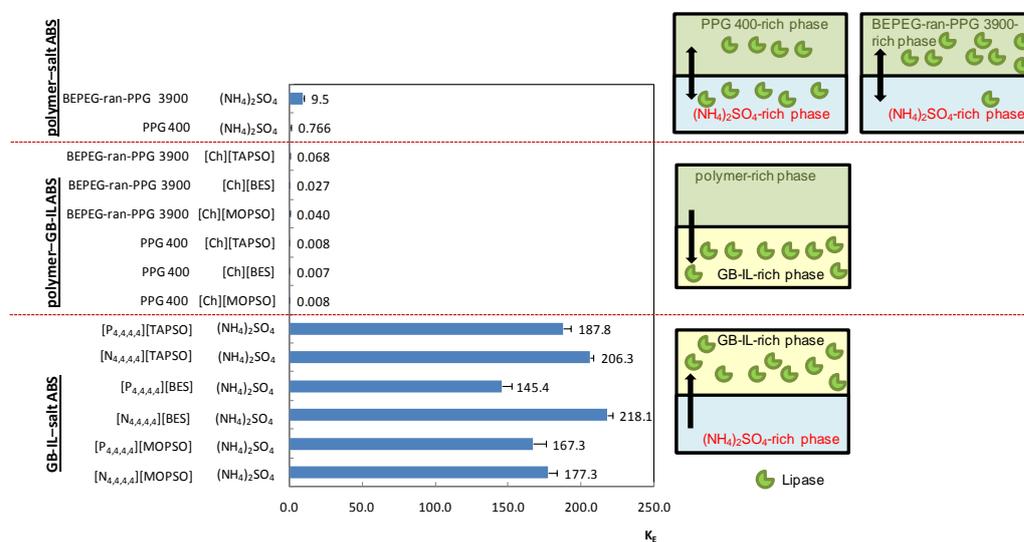


Figure 3. Phase diagrams for the ternary systems composed of PPG 400 (inlet A)/BEPEG-ran-PPG 3900 (inlet B) + GB-IL/ $(\text{NH}_4)_2\text{SO}_4$ + H_2O at $T = 25 (\pm 1) ^\circ\text{C}$.



Figure

4. Partition coefficients of the commercial *P. cepacia* lipase in the ABS studied.

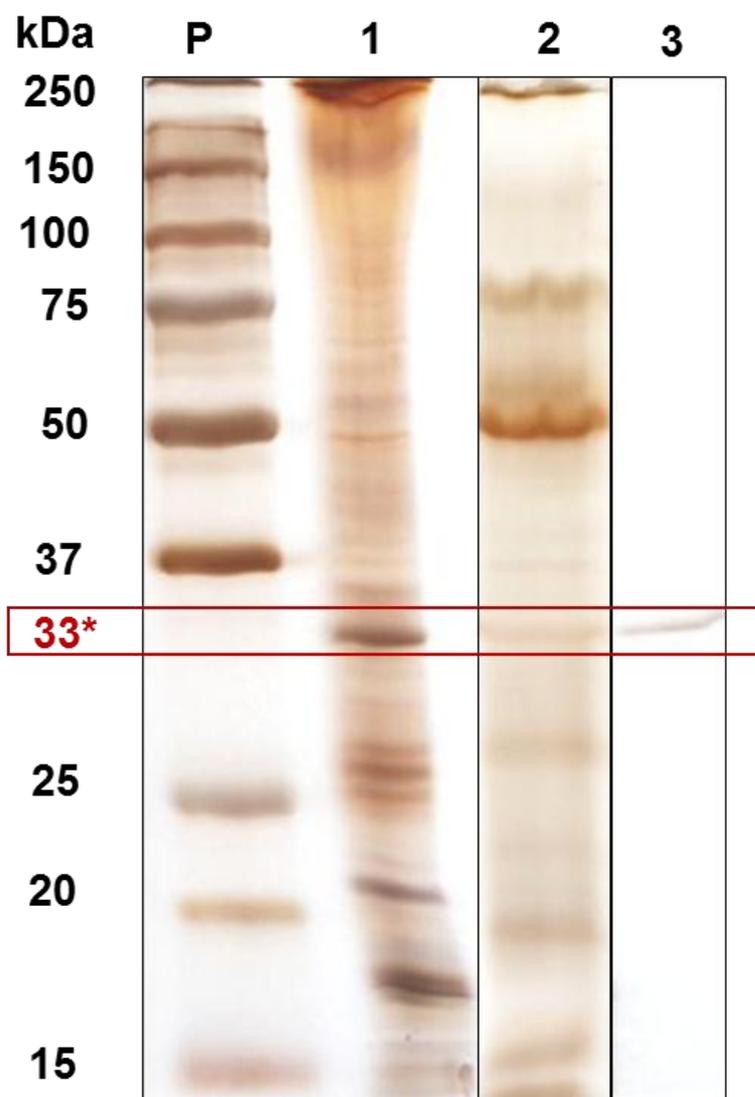


Figure 5. SDS-PAGE analysis of the purified lipase from *B. cepacia* ST8, using 12% of acrylamide gel with silver staining. **Lane P:** protein standard (15-250 kDa), **Lane 1:** harvested fermentation broth; **Lane 2:** protein fraction obtained from the ammonium sulfate precipitation (0-35%); **Lane 3:** protein sample from the GB-IL-rich phase of the system 9.99 wt% of [N_{4,4,4,4}][BES] + 24.98 wt% of (NH₄)₂SO₄ with the pure lipase. *result of molecular weight found for the lipase produced by submerged fermentation using the *B. cepacia* ST8 found in literature [43].

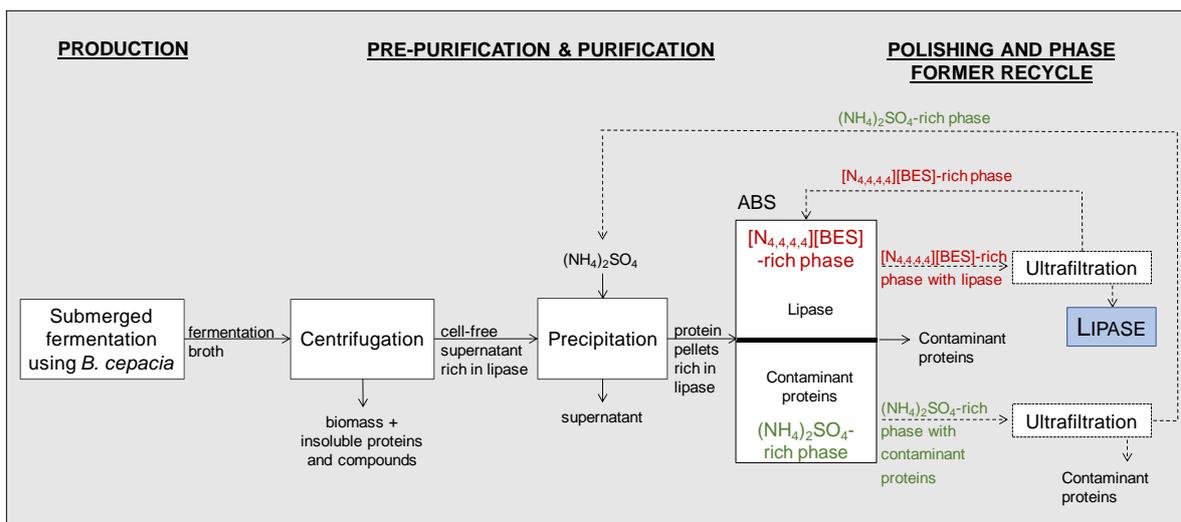


Figure 6. Scheme of the proposed process for the purification of the extracellular lipase produced *via* submerged fermentation.

Table 1. Enzyme partition and recovery^a parameters obtained for the commercial *P. cepacia* lipase in GB-IL-based ABS and respective standard deviations.

Top phase-rich component; (wt%)	Bottom phase-rich component; (wt%)	TLL	R_V	K_E $\pm \sigma$	$R_T^E \pm \sigma$ σ (%)	$R_B^E \pm \sigma$ σ (%)
[N _{4,4,4,4}][MOPSO]; 25.00	(NH ₄) ₂ SO ₄ ; 14.53	38.6	1.5	177 ± 6	99.61 ± 0.03	-
[P _{4,4,4,4}][MOPSO]; 25.02	(NH ₄) ₂ SO ₄ ; 14.50	44.6	0.9	167 ± 9	99.32 ± 0.03	-
[N _{4,4,4,4}][BES]; 24.98	(NH ₄) ₂ SO ₄ ; 15.96	40.6	1.3	218 ± 3	99.63 ± 0.01	-
[P _{4,4,4,4}][BES]; 24.99	(NH ₄) ₂ SO ₄ ; 14.51	34.3	1.3	145 ± 7	99.47 ± 0.05	-
[N _{4,4,4,4}][TAPSO]; 25.01	(NH ₄) ₂ SO ₄ ; 20.06	51.7	0.7	206 ± 2	99.33 ± 0.01	-
[P _{4,4,4,4}][TAPSO]; 24.98	(NH ₄) ₂ SO ₄ ; 20.00	55.3	0.9	188 ± 5	99.43 ± 0.02	-
PPG 400; 45.02	[Ch][MOPSO]; 12.49	49.3	0.8	0.008 ± 0.001	-	99.31 ± 0.09
PPG 400; 44.59	[Ch][BES]; 10.94	44.2	1.0	0.007 ± 0.002	-	99.3 ± 0.2
PPG 400; 45.01	[Ch][TAPSO]; 9.82	39.9	1.0	0.008 ± 0.002	-	99.2 ± 0.2
BEPEG-ran-PPG 3900; 40.09	[Ch][MOPSO]; 15.13	80.2	1.0	0.040 ± 0.009	-	96.2 ± 0.9

BEPEG-ran-PPG 3900; 43.14	[Ch][BES]; 12.67	75.5	1.3	±	-	0.027	0.004	96.8 ± 0.5
BEPEG-ran-PPG 3900; 39.98	[Ch][TAPSO]; 14.03	53.7	1.1	±	-	0.068	0.003	93.0 ± 0.3
PPG 400; 40.08	(NH ₄) ₂ SO ₄ ; 2.52	38.9	0.8	±	-	0.766	0.028	61.5 ± 0.9
BEPEG-ran-PPG 3900; 30.00	(NH ₄) ₂ SO ₄ ; 4.02	41.0	1.5	±	±	9.5 ±	93.5 ±	-
						0.2	0.1	

^aThe enzyme recovery data were calculated for the ABS phase in which the lipase is more concentrated.

Note: TLL – tie line length, R_V – volume ratio, K_E – enzyme partition coefficient, R_T^E – enzyme recovery in the top phase, R_B^E - enzyme recovery in the bottom phase

Table 2. Enzyme specific activity and purification fold at the end of each step of the production and pre-purification of the extracellular lipase produced by *B. cepacia* ST8 via submerged fermentation.

Step	Process	SA (U·mg ⁻¹)	PF ± σ
Production	Submerged fermentation	223.55	-
Pre-purification	Centrifugation at 4 °C, 10 000 × g, 15 min	975.43	4.4 ± 0.1
	Ammonium sulfate precipitation (35 % saturation level at 0 °C)	1707.52	7.6 ± 0.2

Note: SA – enzyme specific activity, PF – purification factor

Table 3. Enzyme partition and recovery, purification fold, partition coefficient of the total proteins and selectivity parameters at the end of the purification scheme using GB-IL-based ABS for the extracellular lipase from *B. cepacia* ST8 produced via submerged fermentation.

[N _{4,4,4,4}][BES] (wt%)	(NH ₄) ₂ SO ₄ (wt%)	R _V	K _E ± σ	R _T ^E ± σ (%)	PF ± σ	K _P ± σ	S _{E/P} ± σ
34.87	10.04	8.0	9.12 ± 0.04	98.65 ± 0.01	8.9 ± 0.4	4.2 ± 0.4	2.2 ± 0.2
25.03	15.02	1.6	10.7 ± 0.6	94.4 ± 0.3	9.8 ± 0.6	3.2 ± 0.5	3.5 ± 0.6
25.00	15.98	1.3	12.8 ± 0.8	94.1 ± 0.3	11 ± 1	2.9 ± 0.3	4.5 ± 0.7
16.08	20.01	0.4	39 ± 2	93.7 ± 0.3	15.8 ± 0.4	1.8 ± 0.1	22 ± 2
9.99	24.98	0.2	93 ± 3	94.0 ± 0.2	22.4 ± 0.7	1.6 ± 0.1	57 ± 5

Note: R_V – volume ratio, K_E – enzyme partition coefficient, R_T^E – enzyme recovery in the top phase, PF – purification factor, K_P – total proteins partition coefficient, S_{E/P} – selectivity