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

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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 ± 0.2) % were achieved.

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 (± 1) °C and atmospheric pressure. The impact of
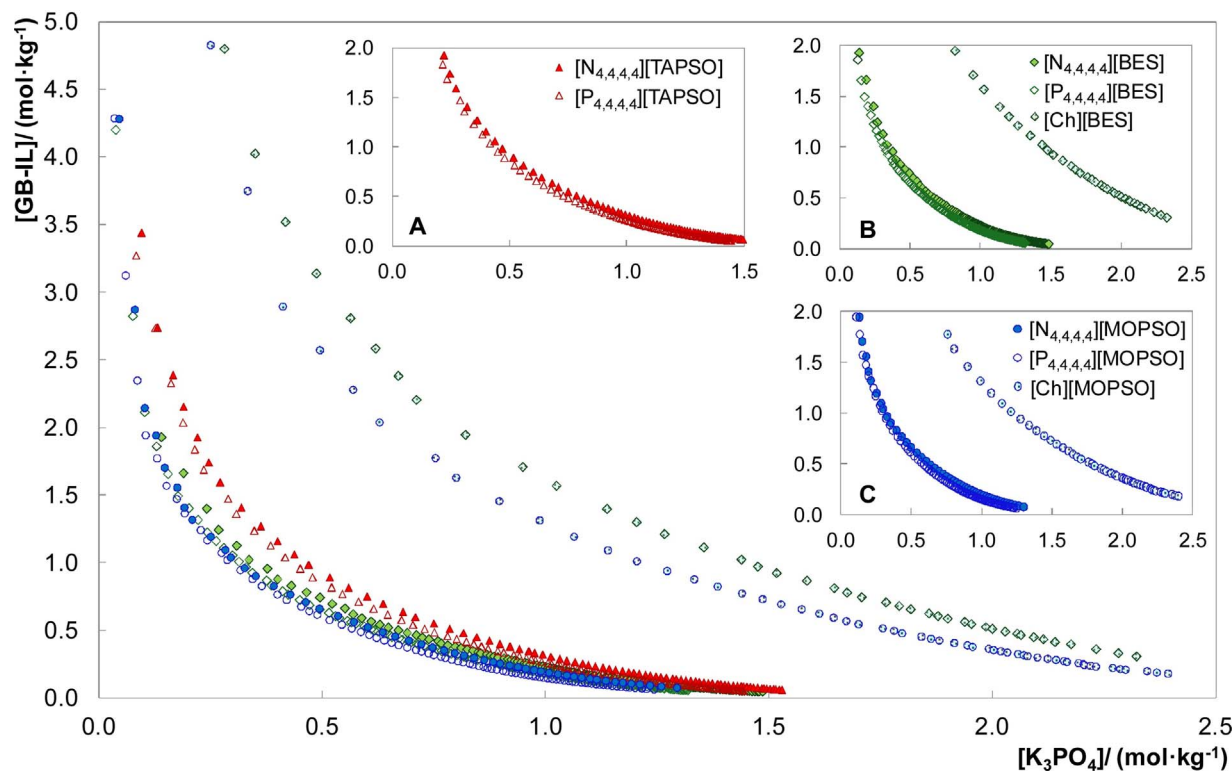
Fig. 1. Phase diagrams for the ternary systems composed of GB-IL + K₃PO₄ + H₂O at T = 25 (± 1) °C. To study the cation effect, the comparison between the binodal curves obtained for GB-ILs based on [TAPSO]⁻ (**A**), [BES]⁻ (**B**) and [MOPSO]⁻ (**C**) is highlighted in the insets included in this figure.
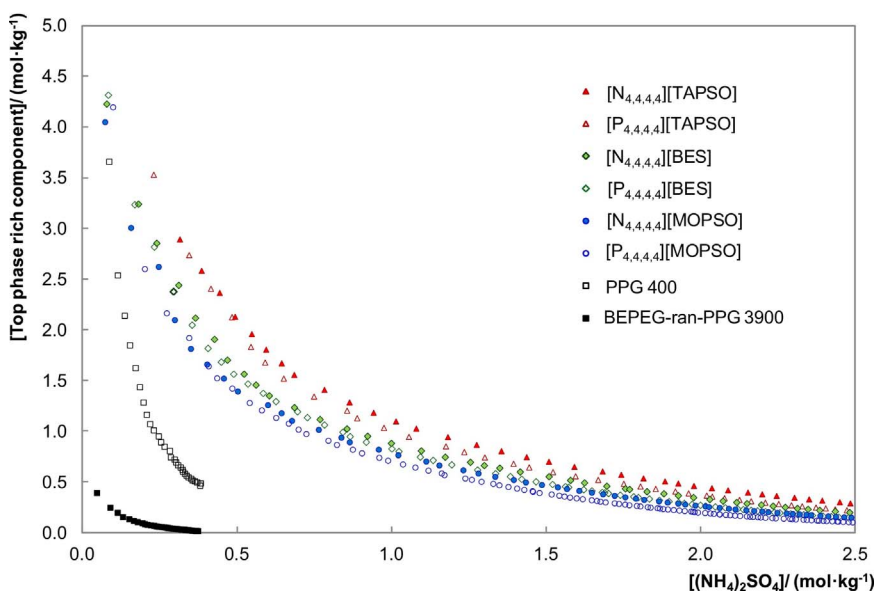

Fig. 2. Phase diagrams for the ternary systems composed of GB-IL/PPG 400/BEPEG-ran-PPG 3900 + (NH₄)₂SO₄ + H₂O at T = 25 (± 1) °C.

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 ≥99%. The [N₄,₄,₄,₄]OH (40 wt% in H₂O), [P₄,₄,₄,₄]OH (40 wt% in H₂O), [Ch]OH (46 wt% in H₂O) solutions, potassium phosphate tribasic (K₃PO₄, purity ≥ 98%), potassium carbonate (K₂CO₃, purity ≥ 99%), potassium phosphate monobasic (KH₂PO₄, purity ≥ 99.5%), potassium phosphate dibasic (K₂HPO₄, purity ≥ 99%), PEG with a molar mass of 400 g mol⁻¹ (PEG 400) and 1000 g mol⁻¹ (PEG 1000), PPG of 400 g mol⁻¹ (PPG 400), PEG-ran-

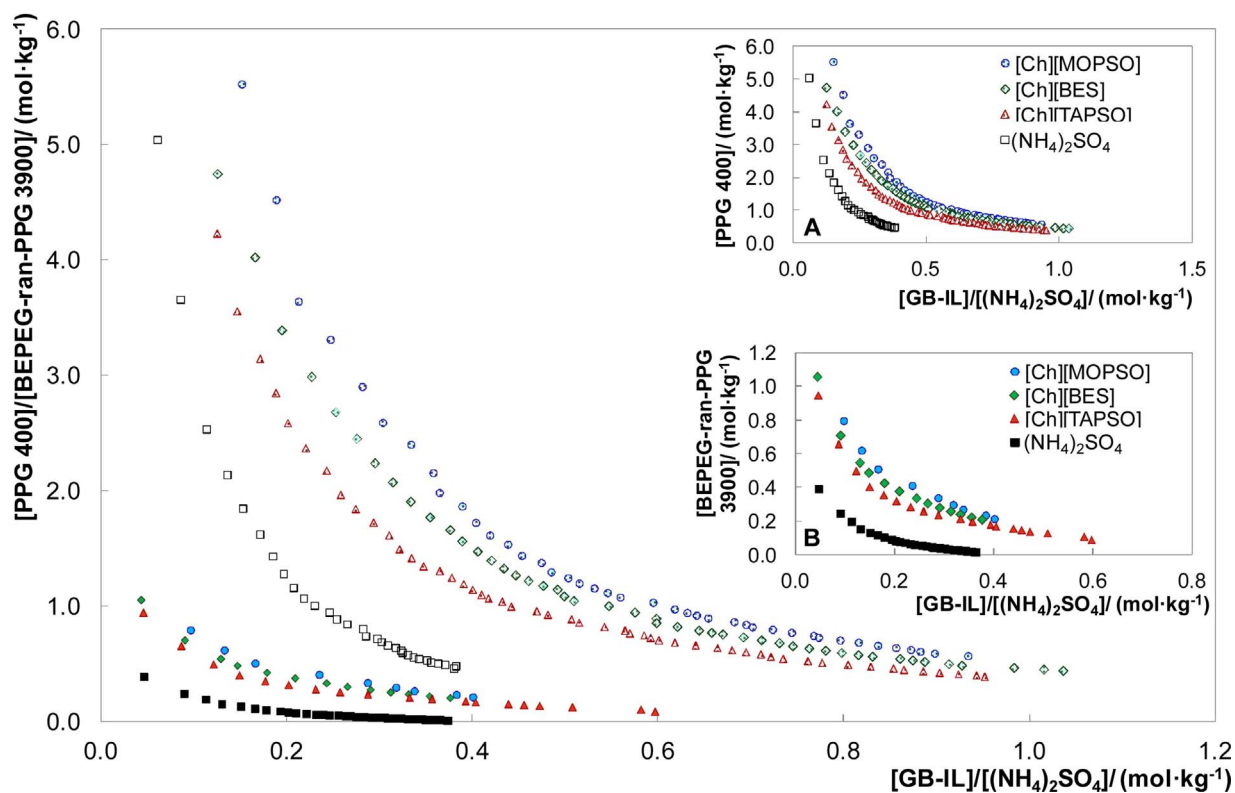**Fig. 3.** Phase diagrams for the ternary systems composed of PPG 400 (inlet **A**)/BEPEG-ran-PPG 3900 (inlet **B**) + GB-IL/(NH$_4$)$_2$SO$_4$ + H$_2$O at T = 25 (± 1) °C.

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$_E$ ± σ	R$_T^E$ ± σ (%)	R$_B^E$ ± 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$_4$; 15.53	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 ± 0.1
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 ± 0.4
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 ± 0.2	93.5 ± 0.1	–

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

[a] The enzyme recovery data were calculated for the ABS phase in which the lipase is more concentrated.

PPG of 2500 g mol^{-1} (PEG-ran-PPG 2500) and BEPEG-ran-PPG of 3900 g·mol^{-1} (BEPEG-ran-PPG 3900), 4-nitrophenyl laurate (*p*-NPL, purity ≥ 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 ((NH$_4$)$_2$SO$_4$, purity ≥ 99.5%) was acquired from Merck. Methanol (purity ≥ 99.9%), acetonitrile (purity ≥ 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 Fig. 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
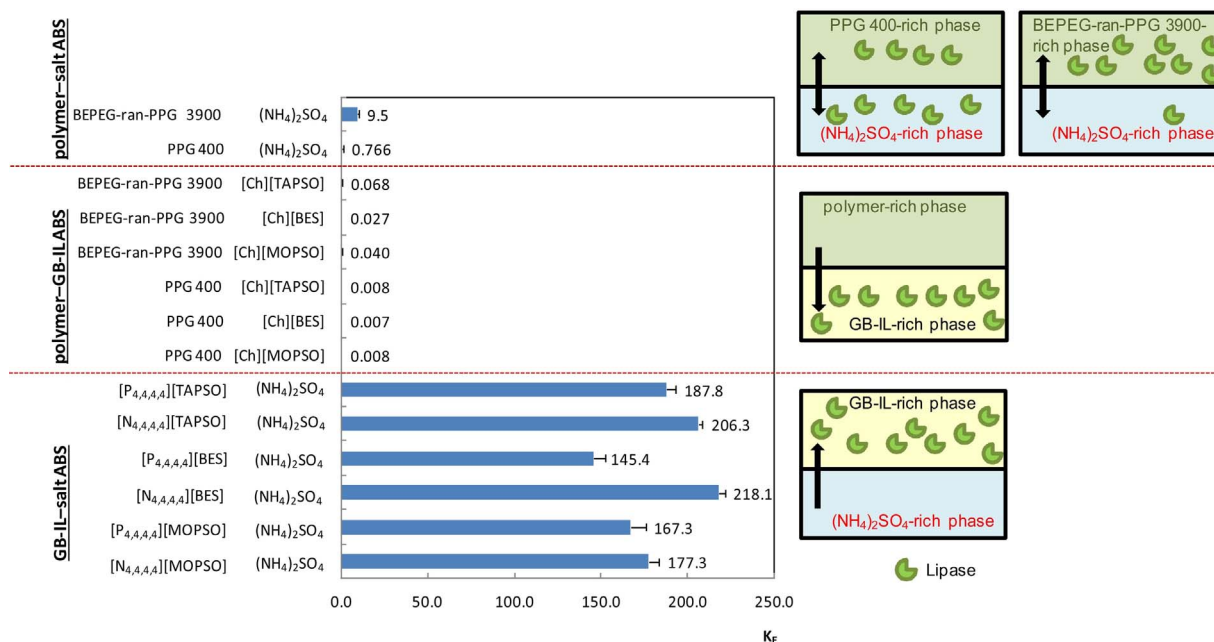
Fig. 4. Partition coefficients of the commercial *P. cepacia* lipase in the ABS studied.

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 $\pm$ σ
Production	Submerged fermentation	223.55	–
Pre-purification	Centrifugation at 4 °C, 10 000 $\times$ g, 15 min	975.43	4.4 $\pm$ 0.1
	Ammonium sulfate precipitation (35% saturation level at 0 °C)	1707.52	7.6 $\pm$ 0.2

Note: SA $-$ enzyme specific activity, PF $-$ purification factor.

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 1000*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)–(3) [27,33].

$$K_E = \frac{EA_T}{EA_B} \tag{1}$$

$$R_T^E = \frac{100}{1 + \frac{1}{R_V K_E}} \tag{2}$$

$$R_B^E = \frac{100}{1 + R_V K_E} \tag{3}$$

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$_2$PO$_4$/K$_2$HPO$_4$ 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 (ΔAbs min^{-1}) was analyzed. One unit (U) of enzyme activity is defined as the amount of enzyme that produces 1 µmol of *p*-NP *per* minute, being this parameter expressed in U 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].
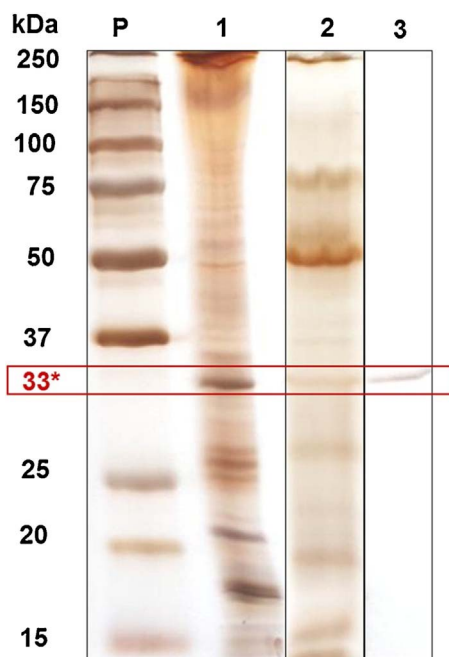
Table 3
Enzyme partition and recovery, purification fold, partition coefficient of the total proteins and selectivity parameters at the end of the purification scheme using GB-IL-based ABS for the extracellular lipase from *B. cepacia* ST8 produced *via* submerged fermentation.

[N$_{4,4,4,4}$][BES] (wt%)	(NH$_4$)$_2$SO$_4$ (wt%)	R$_V$	K$_E$ $\pm$ σ	R$_T^E$ $\pm$ σ (%)	PF $\pm$ σ	K$_P$ $\pm$ σ	S$_{E/P}$ $\pm$ σ
34.87	10.04	8.0	9.12 $\pm$ 0.04	98.65 $\pm$ 0.01	8.9 $\pm$ 0.4	4.2 $\pm$ 0.4	2.2 $\pm$ 0.2
25.03	15.02	1.6	10.7 $\pm$ 0.6	94.4 $\pm$ 0.3	9.8 $\pm$ 0.6	3.2 $\pm$ 0.5	3.5 $\pm$ 0.6
25.00	15.98	1.3	12.8 $\pm$ 0.8	94.1 $\pm$ 0.3	11 $\pm$ 1	2.9 $\pm$ 0.3	4.5 $\pm$ 0.7
16.08	20.01	0.4	39 $\pm$ 2	93.7 $\pm$ 0.3	15.8 $\pm$ 0.4	1.8 $\pm$ 0.1	22 $\pm$ 2
9.99	24.98	0.2	93 $\pm$ 3	94.0 $\pm$ 0.2	22.4 $\pm$ 0.7	1.6 $\pm$ 0.1	57 $\pm$ 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.

Fig. 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 faction 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 $(NH_4)_2SO_4$ 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].

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 centre 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 (Memmert, 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 to 35% and then in a second step, from 35 to 70%. The protein pellets collected by centrifugation at 10000*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 $S_{E/P}$) parameters were determined, as described by Eqs. (4)–(7) [27].

$$K_P = \frac{C_T}{C_B} \tag{4}$$

$$SA = \frac{EA}{C} \tag{5}$$

$$PF = \frac{SA}{SA_i} \tag{6}$$
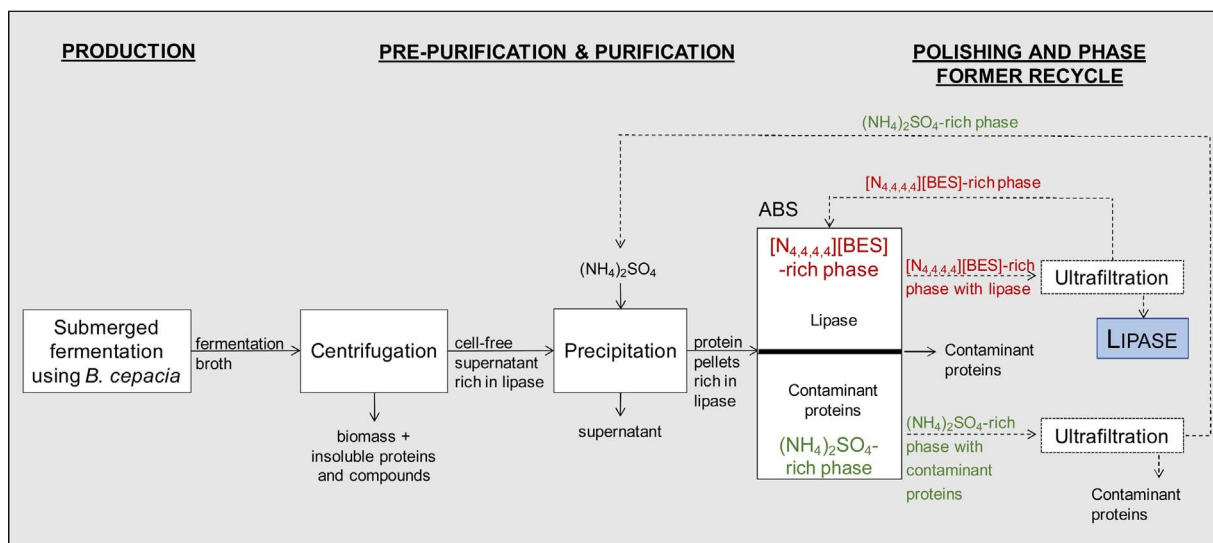
$$S_{E/P} = \frac{K_E}{K_P} \tag{7}$$

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

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 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 h, 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 (Fig. S2), which are closely related to the hydrophilic/hydrophobic nature of their cations (Fig. 1) and anions (Fig. 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 $(NH_4)_2SO_4$ (Fig. 2), are easily inducing the ABS formation with the hydrophobic polymers, namely PPG 400 and BEPEG-ran-PPG 3900 (Fig. 3). Additionally, the trend of the salt effect complies with the well-known Hofmeister series [42] (Fig. 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 Fig. 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 Fig. 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 ± 7–218 ± 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-IL (K_E = 0.007 ± 0.002–0.068 ± 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 ± 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 ± 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 behaviour 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 (Figs. S6 and 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 (Fig. S8), were studied. For that purpose, the fermentation broth was collected after the lipase production and subjected to a pre-treatment with an ammonium sulfate 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 ± 0.7) was verified, since the lipase (K_E = 93 ± 3) and the contaminant proteins are concentrated in opposite phases (K_P = 1.6 ± 0.1), in addition to the contaminants being physically

separated by precipitation at the two-phase interface (Fig. 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 (Fig. 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 (Fig. 6).

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 obtaining a lipase with high purity (PF = 22.4 ± 0.7) and recovery yield (R_T^E = 94.0 ± 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.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2017.08.020.

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