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Lipase purification using ionic liquids as adjuvants in aqueous two-phase systems†

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Aqueous two-phase systems (ATPS) are efficient, environmentally friendly, and "biocompatible" separation processes, which allow the recovery of enzymes. The most common systems are based on polymers and salts, and recently, to overcome the low polarity difference between the phases of the polymeric systems, ATPS based on ionic liquids (ILs) were proposed and have been successfully applied in this field. This work discusses the use of imidazolium-based ILs not as phase forming compounds but as adjuvants (5 wt%) in ATPS of polyethylene glycol systems (1500, 4000, 6000 and 8000 g mol^{-1}) with potassium phosphate buffer at pH 7, in the extraction and purification of a lipase produced by submerged fermentation by *Bacillus* sp. ITP-001. An initial optimization study was carried out with the commercial lipase B from *Candida antarctica* (CaLB) allowing us to further purify the commercial CaLB (purification factor = 5.2). Using the optimized conditions, a purification factor of 245 for the lipase from *Bacillus* sp. ITP-001 was achieved with 1-hexyl-3-methyl imidazolium chloride. The high purification factor is a consequence of the favorable interactions between the IL and the contaminant proteins that migrate for the PEG-rich phase, where the IL also concentrates preferentially, while the enzyme remains in the salt-rich phase.

Introduction

Lipases, triacylglycerol ester hydrolases (EC 3.1.1.3), especially of microbial origin, occupy a place of prominence among biocatalysts owing to their novel and multifold applications in organic synthesis, detergent formulation, nutrition, use as biosensors, and bioremediation, among others.[1–3] Most commercial applications do not require lipase preparations; a certain degree of purity, however, enables efficient and successful usage. Purified lipase preparations are required in industries employing the enzymes for the biocatalytic production of fine chemicals, pharmaceuticals and cosmetics.[1,4] The main constraints to the production of high purity enzymes are the multiple steps necessary to purify them, which are in general technically difficult, may induce deactivation of the enzyme, or require a high consumption of energy and chemicals.[5] In this sense, significant efforts have been focused on the development of new or adapted technologies to perform the purification of enzymes, with lower costs, but still they should be efficient, sustainable and biocompatible with the enzyme conformational structure.[6] In this context, aqueous two-phase systems (ATPS) have been proposed as alternative extraction methodologies for the extraction/purification of enzymes and other molecules, such as proteins, genetic materials, biopharmaceuticals, cells and organelles.[7–9] The ATPS can become the basis for a liquid–liquid extraction process for protein extraction, even purification, that can prevent their denaturation or loss of biological activity. This characteristic is usually attributed to the high water content and low interfacial tension of the systems which protect the proteins.[10] Despite the well-known advantages offered by these systems, ATPS are typically composed of polymers (such as polyethylene glycol, PEG, or dextran),[11–14] their applicability being restricted due to the limited range of polarities of the coexisting phases. Recently, to overcome this limitation and enlarge the range of polarity differences between the coexisting phases, the use of ionic liquids (ILs) in ATPS was proposed.[15–21] One of the main advantages of the application of ILs in the formation of ATPS is the possibility to manipulate their physicochemical properties[22] by the proper combination/manipulation of the cation/anion/alkyl chain of the ILs.[23]

For enzymes, the capability to retain the catalytic activity is important for the success of the extraction process and it is therefore essential that the hydrophilic ILs and their aqueous solutions possess balanced IL–enzyme interactions, which

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means a strong capacity to dissolve the enzyme but not too strong that would disrupt their structure and/or interact with their active sites, causing the deactivation of the enzyme.[24] Hydrophilic ILs were previously applied in the preparation of ATPS and their use as extraction systems for the separation of four different lipases: *Thermomyces lanuginosus* lipase,[25] CaLB,[26] CaLA,[20] and the *Bacillus* sp. ITP-001 lipase[21] with a purification factor (PF) of 51. In all cases, the imidazolium family presented the best results.[20,21,25,26] However, the application of ILs as phase promoters, when conjugated with different inorganic or organic salts, or even with polymers, to promote the formation of ATPS, normally requires the use of large concentrations of salts or polymers, making the extraction process more expensive and less sustainable. The use of small quantities of ILs as adjuvants[27] in conventional polymer–polymer of polymer–salt ATPS appears as an alternative to overcome this problem. Pereira *et al.*[27] have demonstrated that the incorporation of 5 wt% of an IL in a polymer + salt common ATPS was able to modify the polarity of the aqueous phases, leading to better control and a more efficient separation process. Recently, Souza *et al.*[28] have also described that the presence of a small concentration of different ILs of the type [$C_n$mim]Cl, although presenting a minor effect upon the ATPS formation, had a major impact on the PEG-rich phase characteristics and thus, on the extraction parameters (*i.e.* higher partition coefficients and extraction efficiencies for two dyes). In addition, Almeida *et al.*[29] used ILs as adjuvants in conventional PEG + Na_2SO_4 ATPS providing enhanced extraction efficiencies for the extraction of different antioxidants (vanillic and syringic acids). However, all these studies concern the use of model systems and there is no previous attempt to apply ILs as adjuvants in a polymer + salt applied to the extraction and purification of compounds from real systems such as fermentation broths. In this context, this work concentrates efforts on the application of ATPS based on four polymers (PEG 1500, PEG 4000, PEG 6000 and PEG 8000) with potassium phosphate buffer (K_2HPO_4/KH_2PO_4) at pH 7, using four imidazolium-based ILs as adjuvants (1-alkyl-3-methylimidazolium chloride [$C_n$mim]Cl, n = 2, 4, 6, 8) at 5 wt %. These allowed the study of various conditions, namely the molecular weight of the polymer and the alkyl chain length of the ILs, that were investigated in terms of their effect on the purification of the commercial lipase B from *Candida antarctica* (CaLB) that is here used as a model enzyme to evaluate the purification performance of these new ATPS. Subsequently, representative ATPS using ILs as adjuvants were employed to study the purification performance of *Bacillus* sp. ITP-001 lipase produced by submerged fermentation.

Experimental section

Materials

The present study was carried out using different polyethylene glycol (PEG) polymers of average molecular weights 1500, 4000, 6000 and 8000 g mol^{-1} (abbreviated as PEG 1500, PEG 4000, PEG 6000, and PEG 8000, respectively), supplied by Sigma Aldrich® and used as received. Aqueous solutions of potassium phosphate buffer at pH 7 were used. The salts used in the preparation of the potassium phosphate buffer K_2HPO_4 (purity ≥98 wt%) and KH_2PO_4 (purity ≥99.5 wt%) were purchased from Sigma Aldrich®. All ILs were purchased from Iolitec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 98%, their chemical structures are shown in Fig. 1. The protein bovine serum albumin (BSA, purity ≥97%) was obtained from Merck. The model enzyme used, *Candida antarctica* lipase B (here abbreviated as CaLB), was kindly offered by Novozymes A/S, Bagsværd, Denmark and the enzyme from *Bacillus* sp. ITP-001 was obtained by submerged fermentation.

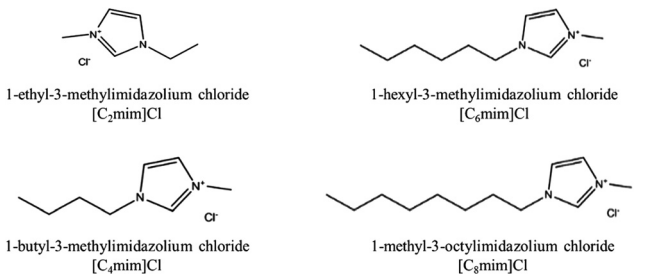

Fig. 1 Chemical structure, full name and abbreviation of all ILs investigated.

Production of lipase by *Bacillus* sp. ITP-001

Fermentation conditions

The lipolytic enzyme was produced by *Bacillus* sp. ITP-001 by submerged fermentation. The microorganisms were isolated from an oil contaminated soil and stored at the Instituto de Tecnologia e Pesquisa – ITP (Aracaju – Sergipe, Brazil). The strain was cultivated in 500 mL Erlenmeyer flasks containing 200 mL of a suitable medium with the following composition (%, w/v): KH_2PO_4 (0.1), $MgSO_4{\cdot}7H_2O$ (0.05), $NaNO_3$ (0.3), yeast extract (0.6), peptone (0.13), and starch (2.0) as the carbon source. The fermentation conditions were as follows: the initial pH around 7, the incubation temperature equal to 37 °C, and the stirring speed at 1700 rpm. After 72 h of cultivation, coconut oil (4%, w/v) and Triton X-100 were added as inductors as described by Feitosa *et al.*[30]

Pre-purification steps

The fermented broth was centrifuged at 10 000 rpm for 30 min, the biomass was discharged and the supernatant was used to determine the enzymatic activity and the total protein concentration, just in the final step of the fermentation process. The protein contaminants in the cell-free fermented broth were precipitated using ammonium sulphate at 80% (w/v) and room temperature, and then the broth was centrifuged at 10 000 rpm for 30 min to separate the aqueous solution from the precipitate (mainly composed of the denatured contaminant proteins). Following the protein denaturation, the aqueous solution obtained was dialyzed using a MD 25 dialysis bag (cut-off: 10 000–12 000 Da) against ultra-pure water

for 24 h at 4 °C. The dialyzed solution (dialysate) containing lipase from *Bacillus* sp. ITP-001 was then used in the preparation of the ATPS under study.

Preparation of the ATPS

The composition of the mixture points selected for the extraction experiments concerning the model lipase CaLB is shown in Table A.1 (ESI†). These were chosen to minimize or avoid the denaturation or unfolding of the target enzyme at the interface and during the experiments. Considering the optimization step, all systems contained approximately 5 wt% of CaLB, and, when present, 5 wt% of IL [$C_n$mim]Cl (n = 2, 4, 6 and 8)]. A potassium phosphate buffer solution (K_2HPO_4/KH_2PO_4) was prepared by the addition of dibasic (K_2HPO_4) and monobasic (KH_2PO_4) potassium phosphate at a ratio of 1.087 (w/w) and pH 7. Considering the study of extraction of a lipase produced *via* fermentation (by the *Bacillus* sp. ITP-001) and purified by applying ATPS, the extraction systems were prepared by adding 15 wt% of PEG (1500, 6000 or 8000), 15 wt% of K_2HPO_4/KH_2PO_4 (potassium phosphate buffer) + 5 wt% of each [$C_n$mim]Cl (n = 2, 4, 6, 8) – Table A.2 in ESI.† In this particular case, potassium phosphate buffer was prepared with a dialysate solution, where the lipolytic lipase from *Bacillus* sp. ITP-001 is concentrated. Then, the buffer was directly used in the preparation of extraction systems in the proportions previously mentioned. The mixture points selected form two immiscible aqueous phases which is confirmed by the quaternary phase diagrams published elsewhere.[28] Each mixture was prepared gravimetrically within $\pm 10^{-4}$ g, vigorously stirred and left to equilibrate for at least 12 h and at 25.0 (±0.1) °C. After this treatment, both phases became clear and transparent, the interface being well defined. The phases were carefully withdrawn using a pipette for the top phase and a syringe with a long needle for the bottom phase. The volumes and weights were determined in graduated test tubes (the total mass of each extraction system prepared was 5 g). The partition coefficients of the main contaminant proteins (K_P) and the enzyme (K_E) are defined by eqn (1) and (2).

$$K_P = \frac{C_T}{C_B} \quad (1)$$

$$K_E = \frac{EA_T}{EA_B} \quad (2)$$

where C_T and C_B are, respectively, the total protein concentration (mg mL^{-1}) in the top and bottom phases, and EA_T and EA_B represent the enzyme activity (U L^{-1}) in the top and bottom phases, respectively. In order to evaluate the purification process, the specific enzyme activity (SA, U mg^{-1} protein) calculated by eqn (3), the volume ratio between the top and bottom phases (R_V), the contaminant protein recovery taking into account the top phase (R_{PT}, %), the enzyme recovery in the bottom phase (R_{EB}, %), and the purification factor (PF – fold) were calculated accordingly to eqn (4)–(6).

$$SA = \frac{EA}{C} \quad (3)$$

$$R_{PT} = \frac{100}{1 + (1/R_V K_P)} \quad (4)$$

$$R_{EB} = \frac{100}{1 + R_V K_E} \quad (5)$$

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

where C is the total protein concentration (mg mL^{-1}). The purification factor (PF) was calculated using the ratio between the SA in the top or bottom phases (which is dependent on the phase in which the enzyme is concentrated) and the specific activity (SA_i) of lipase from *Bacillus* sp. ITP-001 after the centrifugation.

Enzyme assay

The lipolytic activity was measured according to a method proposed by Soares *et al.*[31] The substrate was prepared by mixing 50 mL of olive oil with 50 mL of Arabic gum solution (7%, w/v). The reaction mixture, containing 5 mL of the oil emulsion, 2 mL of sodium phosphate buffer (100 mM and pH 7) and 1 mL of enzyme extract, was incubated in a thermostated bath reactor for 5 min at 37 °C. A blank/control titration was performed on a sample where the enzyme was replaced with distilled water. After 5 min of reaction, an aliquot of ≈0.33 g was taken and added to 2 mL of a solution composed of acetone–ethanol–water (1 : 1 : 1). The exact weight of each aliquot was determined at the end of the addition procedure. The fatty acids produced were titrated with a potassium hydroxide solution (40 mM) in the presence of phenolphthalein as an indicator. One unit (U) of enzyme activity is defined as the amount of enzyme that produces 1 μmol of free fatty acids per minute (μmol min^{-1}) under the assay conditions (37 °C, pH 7 and 100 rpm).

Protein assay

The total protein concentration was determined by the Bradford's method[32] using bovine serum albumin (BSA) as the standard, using a Shimadzu PharmaSpec UV-1700, at 595 nm, and a calibration curve previously established for the standard protein BSA. To eliminate the influence of the IL presence on the protein concentration analysis, one control system for each extraction point but without an enzyme was prepared under the same process conditions.

SDS-PAGE electrophoresis

Electrophoresis of samples using a commercial model enzyme (CaLB) was performed with the Amersham ECL™ Gel from GE Healthcare Life Sciences using polyacrylamide gels (resolving: 20% and stacking: 4%) with a running buffer consisting of 250 mM Tris HCl, 1.92 M glycine, 1% SDS as described by Laemmli.[33] Gels were stained with Coomassie Blue R-250. Full-range molecular weight markers (VWR) were used as protein standards (GE Healthcare Life Sciences). All gels were analyzed using the Image Lab 3.0 (Bio-Rad) analysis tool. For the samples of lipase from fermented broth electrophoresis was

performed with the Mini-PROTEAN II System (BioRad, Brazil) using 12% resolving gels and 5% stacking gels as described by Laemmli.[33] Proteins were visualized by staining with a silver stain. Protein markers used were trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine albumin (66.2 kDa), and phosphorylase (97.4 kDa), all purchased from BioRad (Brazil).

Results and discussion

Optimization approach

This work is on the use of ATPS with ILs as adjuvants for the purification of a lipolytic enzyme from the fermentation broth, a lipase produced by submerged fermentation from *Bacillus* sp. ITP-001. The initial optimization of the ATPS was carried out using the commercial CaLB with a high purity level, aiming at understanding the most important mechanisms behind the partition of the enzyme between the two aqueous phases. The success of the application of ATPS in extraction processes is largely dependent on the ability to manipulate the properties of the aqueous phases to obtain appropriate partition of the (bio)molecules of interest, aiming at the specific selectivity in the partition of both the target enzyme and contaminant proteins. In this work, several ATPS in the form PEG + K_2HPO_4/KH_2PO_4 + water + [$C_n$mim]Cl; n = 2, 4, 6 and 8 (when present) were investigated. The buffer was used in all experiments, because it made possible to keep the pH constant during the entire experiments, allowing retainment of the enzyme structure and charge.[19,34] To control the partition of the biomolecules in ATPS containing ILs, different parameters can be considered: the IL chemical structures, the amount of IL, salt, and polymer, the polymer molecular weight, the temperature, and the pH. In the first section of results, this work discusses the principal effects promoted by the presence/absence of ILs and different polymer molecular weights in the partition of the commercial CaLB on the studied ATPS.

Effect of the ionic liquid and its chemical structure

The partition of CaLB was analyzed considering different ATPS based on PEG 1500 + K_2HPO_4/KH_2PO_4 (pH 7) + 5 wt% [$C_n$mim]Cl; n = 2, 4, 6, 8 (when present) + water + 5 wt% of CaLB aqueous solution. Since this commercial enzyme from Novozymes is already pure, the expectable purification in the optimization section is obviously limited.[26] However, and following the same approach of Ventura *et al.*[26] the purification factor was calculated. Fig. 2 shows the results of the partition coefficients for the enzyme (K_E) and the contaminant proteins (K_P), and the purification factor (PF) for CaLB (details about the recovery of CaLB and the contaminant proteins are described in Table A.3 in ESI†). The results depicted in Fig. 2 show the partition of CaLB and the contaminant proteins for opposite phases (K_E < 1 and K_P > 1). CaLB is thus more concentrated in the salt-rich phase. This can be easily justified by the fact that at pH 7, CaLB is negatively charged, isoelectric point pI = 6,[35,36] resulting in an increase of its affinity for the more

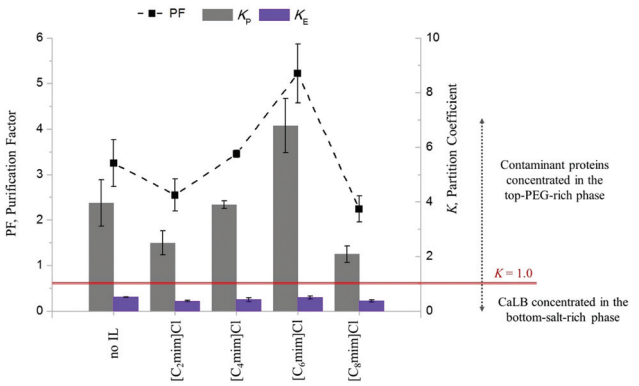

Fig. 2 Results for the purification factors (PFs), and partition coefficients of the contaminant proteins (K_P) and CaLB (K_E) by applying systems based on PEG 1500 + 5 wt% of [$C_n$mim]Cl (n = 2, 4, 6 and 8) + water + K_2HPO_4/KH_2PO_4, at pH 7 and 25 (±0.1) °C and atmospheric pressure.

hydrophilic phase.[12,26] However, the contaminant proteins (as evidenced in a previous study of ours[26] by an electrophoresis analysis performed on the commercial lipase) are preferentially partitioning to the PEG-rich phase (K_P > 1). In fact, the contaminant proteins are partitioned for the PEG layer closely following the partition of the ILs used, as recently described.[28,29] This behavior seems to be justified by the stronger interactions between the contaminant proteins and the polymeric phase (already shown in Souza *et al.*[28]), not only due to the presence of the polymer (identified by the K_P > 1 for the system in the absence of IL), but also due to the presence of the ILs, as can be concluded from the same tendency described by the partition of the contaminant proteins (K_P results here described) and the ILs for the polymer layer (K_{IL} previously described in the literature[28]). The ILs have different affinities for the PEG-rich phase. The results obtained for the K_{IL} show that the partition of the ILs for the PEG-rich phase increases with the IL hydrophobicity up to [C_6mim]Cl, and then decreases again for [C_8mim]Cl due to micelle formation (Fig. A.1†) as discussed by us in a previous study.[28] It seems that the use of these ATPS conjugated with the ILs is actually more efficient in the manipulation of the contaminant proteins partition than in the control of the CaLB partition.

The tendency of the contaminant proteins to migrate to the PEG-rich phase according to the results of Fig. 2 is in the order [C_8mim]Cl < [C_2mim]Cl < [C_4mim]Cl < no IL < [C_6mim]Cl. Based on the K_P data, it is suggested that more than just the hydrophobic interactions are controlling their partition, also electrostatic interactions, van der Waals forces and hydrogen-bonding are playing a significant role, due to the presence of ILs.[27,28] Finally, the decrease observed for [C_8mim]Cl relative to [C_6mim]Cl seems to be related to the possible formation of aggregates by the IL' self-aggregation, thus changing the balance of interactions acting in the ATPS bulk.[37] The negative effect of the presence of IL's aggregates was already shown in other work,[38] but it should be clarified that this behavior only

occurs under certain processing conditions and for specific molecules. The values for the purification factor are also depicted in Fig. 2. The dependence of K_P on PF was more clearly observed when ILs are used as adjuvants. The purification factor is the last parameter to be taken into account, since the main purpose of this work is to identify the best systems to promote the purification of a lipolytic enzyme from the fermentation broth, separating them from the remaining contaminants (mainly other proteins also produced in the submerged fermentation). From a careful analysis of the PF data, it is concluded that the best system used is the ATPS based on [C_6mim]Cl. The presence of [C_6mim]Cl increases the PF from 3.25 ± 0.65 (without IL) to 5.22 ± 0.65 (with IL). These results are mainly justified by the higher partition of the contaminant proteins for the PEG-rich phase in the presence of the IL, while CaLB is still accumulated in the salt-rich phase. Again, we call the attention to the fact that this enzyme is a commercial sample in which the purity level is already high, and even then, it is possible to increase the PF to 5.22 ± 0.65, a clear sign of the capacity of these systems to improve the enzyme purity level. Moreover, they show to perform even better than the common IL-based ATPS composed of 25 wt% [C_8mim]Cl and 30 wt% of K_2HPO_4/KH_2PO_4 (pH 7) that presented a PF = 2.6 ± 0.1.[26]

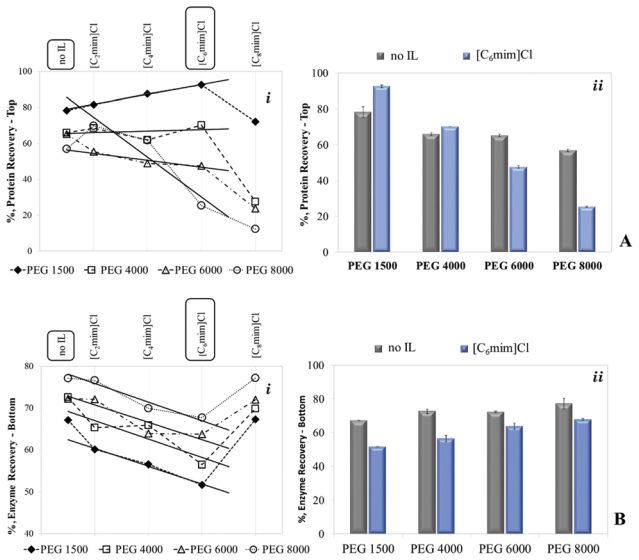

Fig. 3 Effect of different PEG molecular weights on the recovery of the contaminant proteins to the top phase (A) and the enzyme to the bottom phase (B): (i) with the elongation of the IL′ alkyl chain and (ii) by comparing systems with and without the presence of [C_6mim]Cl.

Effect of the PEG molecular weight

Besides the use of ILs as adjuvants for the control of the contaminant protein partition, the polymer molecular weight also plays an important role in the partition phenomenon. The partition of CaLB was analyzed considering different ATPS based on PEG (1500, 4000, 6000 and 8000) + K_2HPO_4/KH_2PO_4 (pH 7) + IL [$C_n$mim]Cl; n = 2, 4, 6, 8 (when present) + water + CaLB aqueous solution (Table A.1†). The principal results considering the partition of CaLB and the contaminant proteins for the bottom and top phases, respectively, are presented in Fig. 3.

The profiles presented in Fig. 3A demonstrate the effect of the polymer molecular weight increase conjugated with the elongation of the ILs' alkyl chain. The trend lines in Fig. 3A(i) suggest an inversion of the partition of the contaminant proteins from the top to the bottom phase, with the simultaneous increase in both the polymer molecular weight and IL alkyl chain length. The results from Fig. 3A(ii) suggest that most contaminant proteins are concentrated in the top phase, and their partition decreases with the increase of the polymer molecular weight, by the following order: PEG 1500 > PEG 4000 > PEG 6000 > PEG 8000. It can be concluded that the presence of ILs is more advantageous regarding the separation of the CaLB and the contaminant proteins when polymers of lower molecular weight are used. As recently reported by Souza *et al.*[28] from the results obtained for the K_{IL} in quaternary ATPS considering both PEG 1500 and PEG 8000, the IL migration for the PEG-rich phase is favored for these polymers (Fig. A.1†). Actually, and in the particular case of [C_6mim]Cl, the extraction of the contaminant proteins decreases considerably when PEG 6000 or PEG 8000 [Fig. 3A(ii)] are used. These results can be justified by the differences regarding the intrinsic viscosity of the two phases, as reported by Kirincic and Klofutar.[39] The authors show that the increase of the molecular weight of PEG increased the viscosity of the aqueous solution of PEG from 4.41 $cm^3\ g^{-1}$ (PEG 400) to 14.15 $cm^3\ g^{-1}$ (PEG 4000) and 42.02 $cm^3\ g^{-1}$ (PEG 2000). The partition of CaLB was also evaluated (Fig. 3B) and in general, the results showed the preferential enzyme accumulation in the salt-rich (bottom) phase, the contaminant proteins being accumulated in the opposite phase. The partition tendency observed for CaLB is not as pronounced as the tendency found for the contaminant proteins. However, it is possible to identify an increase in the enzyme recovery at *circa* 20% with the increase in the molecular weight of PEG [from PEG 1500 to PEG 8000, Fig. 3B(i)]. To confirm the tendencies observed and the selective separation of CaLB from the contaminant proteins, despite the use of a commercial lipase in the optimization step, electrophoresis analysis was carried out and the main results are shown in Fig. 4.

The electrophoresis gel (Fig. 4) is divided into 4 main lanes, lane A represents the molecular mass standard, lane B represents the commercial CaLB and the remaining lanes represent the bottom phases of two distinct ATPS applied in the optimization step. Analyzing carefully lane B, it is clearly seen the presence of the principal enzyme CaLB at *circa* 35 kDa and two other bands less pronounced representing two contaminant proteins (numbers 2 and 3), showing that this commercial enzyme is of limited purity. Lanes C and D represent the bottom phases of ATPS with IL [C_2mim]Cl and [C_6mim]Cl, respectively. Specifically in lane C it can be seen that there is a small additional band (5), which is not found in lane D. In this case, it is evident that the ATPS using IL ([C_6mim]Cl) are more efficient for the isolation of enzymes in the bottom

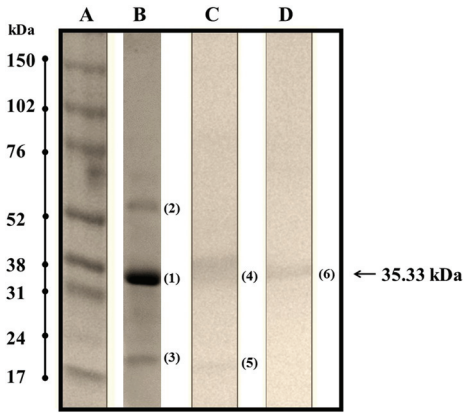

Fig. 4 Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) patterns of CaLB. Lane A: molecular mass standard (17–150 kDa), lane B: commercial CaLB, lanes C and D: CaLB purified from 2 ATPS: PEG 1500 + 5 wt% of $[C_2mim]Cl$ + water + K_2HPO_4/KH_2PO_4 lane C bottom phase; PEG 1500 + 5 wt% of $[C_6mim]Cl$ + water + K_2HPO_4/KH_2PO_4 lane D bottom phase.

phase of the system, since only a band (6) was found in lane D. Thus, the observations by SDS-PAGE support the purification results previously reported.

Production and pre-purification of lipase from *Bacillus* sp. ITP-001

Having evaluated the purification capacities of these new ATPS, they were further employed in order to demonstrate the purification of lipase from *Bacillus* sp. ITP-001, produced by submerged fermentation from a real matrix. The partition of the target enzyme and the main contaminants (normally proteins) present in the fermentation media were considered in the analysis. Moreover, two distinct approaches were applied, namely (i) the use of a pre-purification step before the use of ATPS and (ii) the direct application of ATPS to extract and purify the lipase without a pre-purification step. This process is described in Fig. 5, in which the process diagram is presented from the production to the final step of purification considering the application of ATPS using ILs as adjuvants. Table 1 reports several parameters, namely the enzymatic activity (EA – U mL^{-1}), the total protein concentration (C – mg mL^{-1}), the specific activity (SA – U mg^{-1}) and the purification factor (PF – fold) obtained as the output of each step, in particular the production, in which the fermentation broth is obtained and at the end of the pre-purification step, by obtaining the dialysate (route i). In route i, the salt $(NH_4)_2SO_4$ was used to precipitate some of the contaminant proteins (the precipitation step is described in the process diagram), the lipase is concentrated in the supernatant and a large amount of contaminant proteins was removed. Following the precipitation process, the supernatant was dialyzed aiming at removing the low molecular weight compounds, including inorganic salts from the fermentation and the precipitation process. In this case, the purification factor of the dialysate was around 17.16 fold, in agreement with the results previously reported by us for this pre-purification step.[12,21] In the next section, routes i and ii will be addressed taking into account only the step of purification by ATPS.

Purification of lipase using ATPS

After the optimization studies carried out using the commercial CaLB, the ATPS representing the best extraction conditions (higher PFs) were applied to the purification of an extracellular lipolytic enzyme produced by *Bacillus* sp. ITP-001. The extraction systems used in this task were composed of 15 wt% of PEG (1500, 4000 and 8000) + 15 wt% of K_2HPO_4/KH_2PO_4 + 5 wt% of each IL. Here, phosphate buffer was prepared with the dialysate (where the lipolytic lipase from *Bacillus* sp. ITP-001 is accumulated) and it was directly introduced in the preparation of all ATPS under study. The selected ATPS using ILs as adjuvants have revealed a great performance in the puri-

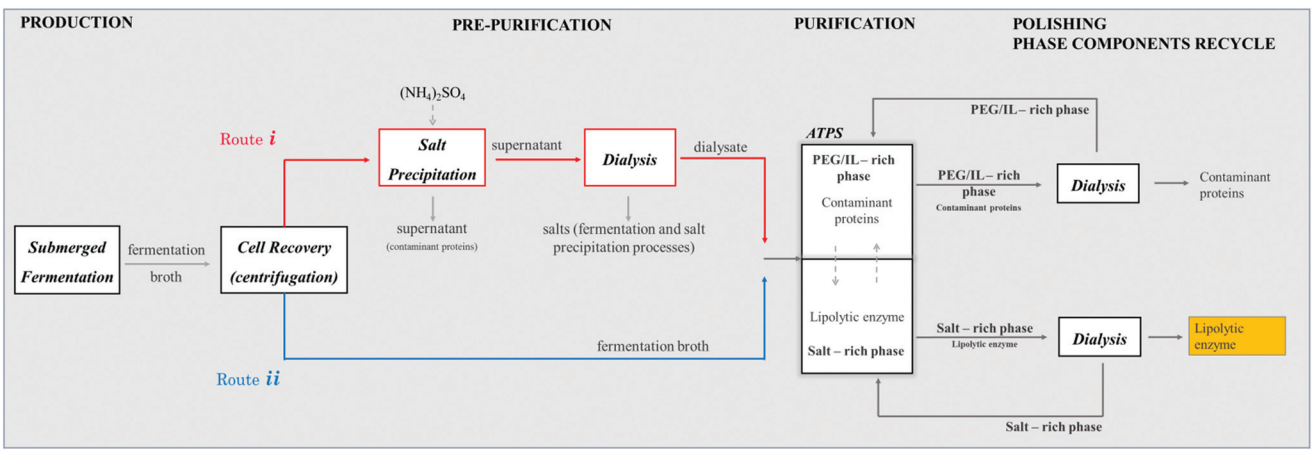

Fig. 5 Flow chart of the two routes for the production and purification of the lipolytic enzyme produced by *Bacillus* sp. ITP-001 *via* submerged fermentation: route i – with pre-purification steps, including precipitation with $(NH_4)_2SO_4$ followed by dialysis; route ii – without any pre-purification step. The polishing of the lipolytic enzyme and recycle of the phase components are also integrated in this process of purification.

Table 1 Purification factor, enzymatic activity, specific activity, and protein concentration at the end of each step of the production and pre-purification of lipase produced by *Bacillus* sp. ITP-001

Steps	Process	EA (U mL^{-1})	C (mg mL^{-1})	SA (U mg^{-1})	PF (fold)
Production	Fermentation	9430.7	1.16	8129.2	—
Pre-purification	Dialysis	9287.8	0.07	139 473.4	17.16

fication of the lipolytic lipase produced from *Bacillus* sp. ITP-001 (Table 2).

Concerning route i, the PF achieved for the lipase from *Bacillus* sp. ITP-001 increased from 175.6 ± 2.4 using the ATPS without any IL to 245.0 ± 9.5 using the ATPS with [C_6mim]Cl. Additional tests were performed for the route i purification approach regarding the variation in the polymer molecular size conjugated with [C_2mim]Cl and [C_6mim]Cl (Table 2).

Regarding the effect of the polymer molecular weight, the results seem to corroborate the data previously found in the optimization step by using the commercial CaLB, despite the fact that the purification results achieved for the lipase produced by *Bacillus* sp. ITP-001 are much more significant due to the completely different level of purity of the lipase samples (the fermentation broth has a higher content of contaminant proteins). The same tendencies of purification were already demonstrated in previous studies from our group, in which the purification of the same lipolytic enzyme (from *Bacillus* sp. ITP-001) was studied by applying simpler ATPS based on ILs and salts as separation agents, namely using 25 wt% of [C_8mim]Cl and 30 wt% of the same buffer (pH 7) used in this work. However, a new result of utmost importance appears in this work: the use of ATPS composed of ILs as adjuvants allowed much higher PFs (route i: PF = 245.0 ± 9.5) when compared with the IL based ATPS for which PF = 51 ± 2[21] and the common polymeric ATPS (PF lower than 30, taking into account the differences under some conditions namely the pH).[12] Table 3 shows a comparison between the PF of all ATPS purification approaches previously reported for the lipase produced by *Bacillus* sp. ITP-001, with the results presented in this work for the purification of lipase from *Bacillus* sp. ITP-001. The improvement in the purification capacity presented by the ATPS using IL as an adjuvant over all the other purification systems previously studied is patented. There seems to be a synergy between the IL and the PEG that maximizes the PF achieved with the advantage of using a much lower IL concentration, enhancing thus the biocompatibility of the system while lowering its cost and environmental impact.

To support the interpretation of the results concerning the ATPS purification capacity of PEG + potassium phosphate buffer + IL as an adjuvant, electrophoresis analysis was performed using samples of the bottom phase (with [C_6mim]Cl,

Table 2 Recovery parameters for the contaminant proteins and lipase from *Bacillus* sp. ITP-001, partition coefficients and purification factor achieved in routes i and ii, using PEG (1500, 4000 and 8000) + K_2HPO_4/KH_2PO_4 (prepared with the fermentation broth containing the target lipase) + IL (when present) + water, at 25 (±0.1) °C and atmospheric pressure

Route	Polymer	ATPS	$R_{EB} \pm \sigma$	$R_{PT} \pm \sigma$	$K_E \pm \sigma$	$K_P \pm \sigma$	PF ± σ
i	PEG 1500	No IL	79.23 ± 0.12	56.69 ± 0.80	0.209 ± 0.004	1.07 ± 0.04	175.61 ± 2.36
		[C_2mim]Cl	77.19 ± 0.26	49.61 ± 0.94	0.269 ± 0.003	0.92 ± 0.03	169.34 ± 0.81
		[C_4mim]Cl	77.48 ± 0.53	54.09 ± 1.18	0.231 ± 0.008	0.95 ± 0.04	220.04 ± 7.68
		[C_6mim]Cl	76.62 ± 2.52	62.81 ± 0.39	0.237 ± 0.026	1.32 ± 0.01	244.99 ± 9.52
		[C_8mim]Cl	90.16 ± 0.53	37.40 ± 0.04	0.109 ± 0.006	0.60 ± 0.003	100.76 ± 2.96
	PEG 4000	[C_2mim]Cl	90.14 ± 0.09	53.16 ± 0.21	0.132 ± 0.002	1.37 ± 0.01	181.32 ± 2.17
		[C_6mim]Cl	79.29 ± 1.59	59.33 ± 0.08	0.233 ± 0.019	1.33 ± 0.02	254.03 ± 1.01
	PEG 8000	[C_2mim]Cl	89.69 ± 0.90	53.31 ± 0.61	0.134 ± 0.014	1.33 ± 0.02	160.58 ± 1.94
		[C_6mim]Cl	80.89 ± 0.84	60.96 ± 0.29	0.266 ± 0.012	1.49 ± 0.01	222.86 ± 2.02
ii	PEG 1500	no IL	83.93 ± 0.41	88.69 ± 0.13	0.175 ± 0.05	7.12 ± 0.08	73.05 ± 2.48
		[C_6mim]Cl	77.60 ± 0.19	94.31 ± 0.28	0.152 ± 0.02	8.90 ± 0.25	103.47 ± 1.23

Table 3 Aqueous two-phase systems applied to purification of lipase from *Bacillus* sp. ITP-001

ATPS			Mass fraction composition (wt%)				
$w1$	$w2$	$w3$	$w1$	$w2$	$w3$	PF	Reference
PEG 1500	K_2HPO_4/KH_2PO_4	[C_6mim]Cl	15	15	5	245.0	This work
PEG 8000	K_2HPO_4/KH_2PO_4	NaCl	20	18	6	201.5	Barbosa *et al.*[12]
[C_8mim]Cl	K_2HPO_4/KH_2PO_4	—	25	30	—	51.0	Ventura *et al.*[21]

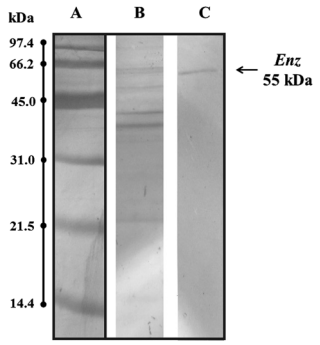

Fig. 6 Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) patterns of the purified lipase from *Bacillus* sp. ITP-001. The purity of the partitioned lipase was assessed using 12% acrylamide gel stained with a silver nitrate solution. Lane A: molecular mass standard (14.4–97.4 kDa), lane B: fermented broth, lane C: bottom phase obtained from the PEG 1500 + 5 wt% of [C_6mim]Cl + water + K_2HPO_4/KH_2PO_4.

here considered the best purification system) and the crude fermentation broth (Fig. 6). The three lanes shown in Fig. 6 represent the molecular mass standard (lane A), the crude fermentation broth (lane B) and the bottom phase obtained from the [C_6mim]Cl-ATPS (lane C). The presence of multiple light bands in lane B confirms the presence of contaminant proteins. In lane C, it is possible to observe the presence of the target enzyme with a molecular weight of around 54 kDa (abbreviated as Enz).

As shown in the diagram of the purification process (Fig. 5), two distinct routes were contemplated, one with a pre-purification step (route i already discussed) and the other described without any pre-purification step, named route ii, these results are also presented in Table 2. These final tests were performed using the system PEG 1500 + K_2HPO_4/KH_2PO_4 + water without any IL, presenting a PF of 175.6 ± 2.4 and 73.1 ± 2.5 for routes ii and i, respectively, and in the presence of [C_6mim]Cl, which presented a PF of 103.5 ± 1.2 and 245.0 ± 9.5 for routes ii and i, respectively. In this sense, the results indicate that the lipase purification is reduced in about 40%, when comparing routes ii and i. Although with values 40% smaller in the purification of lipase, the second approach of purification without the pre-purification step may be considered as an alternative for downstream processes of industrial sectors which do not require a high degree of purification of the lipase, since this will significantly decrease the costs of the purification process.

For the proposed process to be of industrial relevance the isolation of the enzyme from the (top) PEG/IL-rich phase and the recycling of the phase forming components must be considered after the purification step. It is here proposed that the two phases are recycled by the application of dialysis as shown in Fig. 5. In these processes the contaminant proteins will be removed from the (bottom) salt-rich phase and the lipolytic enzyme will be isolated in aqueous solution from the (top) PEG/IL-rich phase. Due to the large difference in the molecular weight of the proteins (including the target enzyme) and the phase forming components of the ATPS it is possible to easily separate them and to recycle the PEG/IL and salt-rich phases in the process to be reused in the preparation of the ATPS.

Conclusion

In this work ATPS using ILs as additives were used for the purification of a lipolytic enzyme from a fermentation broth. ATPS composed of different polyethylene glycols, PEG 1500, 4000, 6000 and 8000, and using various ILs of the type [$C_n$mim]Cl (n = 2, 4, 6, 8) as adjuvants (5 wt%) were studied to analyze the effect of the IL cation alkyl side chain length and the molecular weight of PEGs, upon the purification of lipases. After the initial optimization studies based on a commercial lipase (CaLB), the best systems identified were applied to the purification of a lipolytic enzyme produced in a submerged fermentation by the *Bacillus* sp. ITP-001. The purification factors regarding the extraction of the *Bacillus* sp. ITP-001 enzyme from the fermentation broth here achieved (PF = 245.0 ± 9.5) were much higher than those previously reported for ATPS of polymer + salt[12] and IL + salt.[21] The strategy of using ILs as adjuvants in ATPS, instead of phase forming compounds, as described in this work, seems to be very efficient for the purification process of biomolecules. The wide range of ILs available, and their designer solvent character, reinforces the capacity of this approach by allowing the selection of the best IL that would optimize the extractability and selectivity of a target molecule. The results here reported suggest that a synergetic effect between the IL and the PEG is present on these systems, maximizing the purification factor obtained, with the advantage of using a much lower IL concentration, enhancing thus the biocompatibility of these systems to be applied as a downstream process for other enzymes or biopharmaceuticals, while lowering its cost and environmental impact.

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