View Online / Journal Homepage / Table of Contents for this issue
Dynamic Article Links

Green Chemistry

Cite this: *Green Chem.*, 2012, **14**, 734

www.rsc.org/greenchem

PAPER

Production and purification of an extracellular lipolytic enzyme using ionic liquid-based aqueous two-phase systems

Sónia Patrícia Marques Ventura,[a] Rafael Lemos Freire de Barros,[b] José Murillo de Pinho Barbosa,[b] Cleide Mara Faria Soares,[b] Álvaro Silva Lima[b] and João Araújo Pereira Coutinho*[a]

Received 9th November 2011, Accepted 16th December 2011
DOI: 10.1039/c2gc16428k

Published on 18 January 2012 on http://pubs.rsc.org | doi:10.1039/C2GC16428K
Downloaded by Universidade de Aveiro (UAveiro) on 05 March 2012

The ability of ionic liquid-based aqueous two-phase systems (ATPS) to purify lipase produced by fermentation is here evaluated and compared against conventional PEG-based ATPS systems. Four ionic liquids, chosen after screening of a larger number of ionic liquids are evaluated, with the maximum purification and higher recovery being obtained for the systems based on $[C_8mim]Cl$. It is shown that IL-based ATPS have a performance superior to PEG-based ATPS for the purification of this enzyme.

1. Introduction

Lipases are glycerol ester hydrolases (E.C. 3.1.1.3), which hydrolyze ester linkages of glycerides at water–oil interface. It is well known that lipases are the most widely used enzymes in organic synthesis and also that more than 20% of the biotransformations are performed with lipases.[1] In addition to their role in synthetic organic chemistry, they also find extensive applications in chemical, pharmaceutical, food and leather industries.[1,2] These enzymes usually display exquisite chemo-selectivity, and stereo-selectivity. Moreover, the crystal structures of many lipases have been solved, facilitating considerably the design of rational engineering strategies. They do not require cofactors nor catalyze side reactions, and are readily available in large scale because most of them are produced by microbial fermentation processes. The traditional methods to purify macromolecules involve several steps, such as ammonium sulfate precipitation, dialysis, ionic and affinity chromatography[3] or electrophoresis,[4] which increases the cost of the production.[5] Liquid–liquid extractive bioconversion processes seem to have, nowadays, a great potential, mainly with the use of aqueous two-phase systems here abbreviated as ATPS. Its advantages lie in the simplicity, low cost and the ease of scale-up of these systems. There is a very large number of works describing the use of ATPS based in polymer/polymer[6] and polymer/salt[7,8] systems which were reported in reviews of this field.[9,10] These systems are usually considered as biocompatible, because of the high water content in both phases, and they possess partition properties favorable to their application in large scale to enzyme separation and purification.[11,12] However, most of polymer-based ATPS display high viscosity[13–15] and normally form opaque aqueous solutions, which could interfere with the quantitative and qualitative analysis of the extracted compounds. During the last decade, ionic liquids (ILs) have emerged as alternative compounds for ATPS formation and with potential to be used in extractive fermentation as solvents. They have unique properties such as negligible vapor pressure, non-flammability, possess a wide electrochemical window, and high thermal and chemical stability.[16–18] Furthermore, they are known for the tunability of their chemical structures and physical properties, and by their strong solvation capability.[18–20] The first approaches to the use of ILs as extractants were carried out applying hydrophobic ILs[21,22] in order to create IL/water biphasic systems. However, compared with the hydrophilic, the hydrophobic species are more expensive,[23] toxic[24,25] and their number is limited.[23] Moreover, the viscosity of the hydrophobic IL phases is characteristically high[23] and the partition coefficients observed are low.[21] Consequently, the attempts to the direct extraction of macromolecules from aqueous medium into an appropriate hydrophobic IL were considered as not practical. In 2003, Rogers and co-workers[26] reported, for the first time, that some hydrophilic ILs could form ATPS in presence of K_3PO_4, and these IL-based ATPS could overcome the limitations of the ATPS based in hydrophobic ILs mentioned above. Since then, significant progress has been made in the use of hydrophilic IL-based ATPS.[27–32] The possibility of forming ATPS with different ILs and inorganic salts[23,33] has been explored and a number of works have been published on the effect of different ATPS conditions[27,30–33] and their use in separation techniques.[23,34,35] In what concerns their application in extraction, ILs were reported as attractive novel separation agents for various small compounds like metals,[36,37] alcohols,[26] organic acids,[38] and biomolecules such as amino-acids.[30,31,39,40] Recently, the possibility to extract some macromolecules, such as proteins and enzymes, was also assessed,[41–48] having in mind that to use ILs as enzyme purification agents it is crucial to obtain both high enzyme activity and stability and also, significant extraction efficiency.[42]

[a]*CICECO, Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal. E-mail: spventura@ua.pt, jcoutinho@ua.pt; Fax: +351-234-370084; Tel: +351-234-370200*
[b]*Programa de Pós-Graduação em Engenharia de Processos, Universidade Tiradentes, Av. Murilo Dantas 300, Farolândia, Aracaju-SE, Brasil. E-mail: engraflemos@gmail.com, murillobarbosa@hotmail.com, cleide_mara@itp.org.br, aslima2001@yahoo.com.br*

This journal is © The Royal Society of Chemistry 2012

Published on 18 January 2012 on http://pubs.rsc.org | doi:10.1039/C2GC16428K
Downloaded by Universidade de Aveiro (UAveiro) on 05 March 2012

The ATPS formed by ILs and inorganic salts are reported to present higher recoveries of the proteins studied than the conventional systems employing polyethylene glycol (PEG) and inorganic salt systems.[47,49] In addition, IL-based ATPS also display better process properties, such as lower viscosity, little emulsion formation and fast phase separation.[47,49] Considering the whole picture, it seems that the exploitation of hydrophilic ILs in the context of the separation of macromolecules, can be considered as an economical and efficient extraction approach for those separation systems.[23,42,44,45,50]

This work reports the production and purification of a lipolytic enzyme produced by *Bacillus* sp. ITP-001. The separation and purification steps were performed on the fermentation broth after the end of the production phase, aiming to avoid toxicity,[35,38,51] biocompatibility[41] and biodegradability[52] problems derived from the direct contact of the ILs with the bacterium.[53] This is, to the best of our knowledge, the first report of a comprehensive study showing the integration of IL-based ATPS in the process of production and purification of an enzyme. The pre-purification and purification stages are performed using an integrated process which comprises a salt precipitation with ammonium sulphate $(NH_4)_2SO_4$. The pre-purification is followed by a purification step using ATPS based in hydrophilic ILs and the phosphate buffer solution composed of K_2HPO_4 and KH_2PO_4, with the capacity to maintain a neutral pH.[33,46,54]

2. Results and discussion

2.1. Production and pre-purification of the extracellular lipolytic enzyme

The present work addresses the process for the separation and purification of an extracellular lipolytic enzyme produced by *Bacillus* sp. ITP-001. The process, since the production until the final step of purification is depicted in Fig. 1. It details the whole route of production and pre-purification of lipase while Table 1 reports enzymatic activity (EA - U mL^{-1}), total protein concentration (C - mg mL^{-1}), specific activity (SA - U mL^{-1}), and purification factor (PF - fold) on the fermentation broth and dialysate.

During the salt precipitation by $(NH_4)_2SO_4$ a great deal of contaminant proteins are removed and the lipase is concentrated in the supernatant. Following the salt precipitation the supernatant was dialyzed to decrease the amount of low molecular weight compounds, including inorganic salts from salt precipitation and fermentation processes. In this step, it was observed a small decrease in the enzymatic activity (EA = 4,245.40 U mL^{-1}), probably due to losses of enzyme during the dialysis process.

2.2. Enzyme partition and purification on IL-based ATPS

As described in Fig. 1, after the pre-purification stage, the IL-based ATPS were used for further purification of the enzyme. The ATPS studied were based in four different ILs. Those were chosen by taking into account a previous study based in the partition and purification of *Candida antarctica* lipase B (CaLB) in IL-based ATPS.[46] Fig. 2 shows the binodal curves of the ATPS used for the preparation of the extraction systems.[33] The compositions of the extraction system (25 wt% of IL + 30 wt% of phosphate buffer solution at pH 7.0) are the same for all the ILs studied to exclude the influence of the IL and salt compositions, since it is reported in the literature[42,43,55] that these conditions can significantly affect the purification/separation performances. Table 2 shows the purification and partition parameters obtained after the extraction of the enzyme and total protein present in the dialysate. The data suggest that the purification of the enzyme was controlled mainly by the alkyl chain length, followed by the cation core and finally, by the anion moiety. The increase in the alkyl chain length leads to a stronger increase in PF and K_P than on K_E, which remains consistently very low. This happens due to an increase in the hydrophobic nature of the ILs from $[C_4mim]$ to $[C_8mim]$, that decreases the Coulombic interactions and increases the dispersive forces that occur between the enzyme

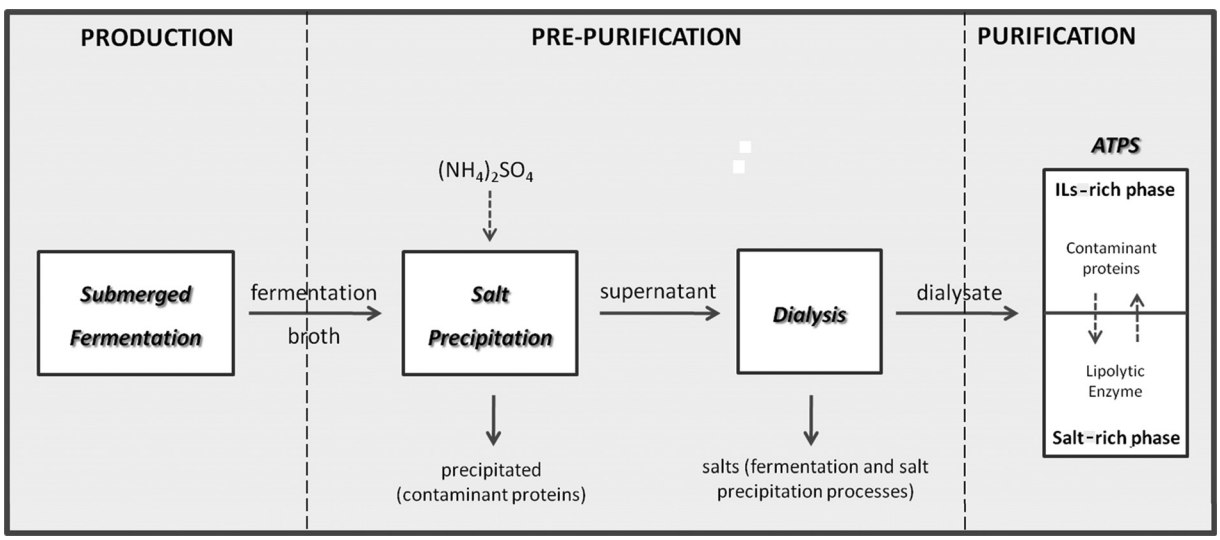

Fig. 1 Representative scheme of the different steps followed for the production and purification of the lipolytic enzyme produced by *Bacillus* sp. ITP-001 *via* submerged fermentation.

Published on 18 January 2012 on http://pubs.rsc.org | doi:10.1039/C2GC16428K
Downloaded by Universidade de Aveiro (UAveiro) on 05 March 2012

and the ILs at the IL-rich phase. These factors seem to favor the partitioning of the contaminant proteins into the IL-rich phase. Due to its very low isoelectric point (pI = 3.0),[56] the lipase is negatively charged at this pH 7.0,[57–59] which results in the increase of its hydrophilic character, creating a higher affinity of the enzyme for the salt-rich phase. The differences observed in the partitioning behavior between the contaminant proteins and the lipolytic enzyme must result from differences in their hydrophobic/hydrophilic nature that will allow their partition into different phases. Note that in this work, the increase in the purity and, consequently in the PF, is entirely defined by the enzymatic activity. The increase in the PF from 40.8 ± 0.4 to 51 ± 2 fold, must thus result mainly from the removal of the contaminants which act as inhibitors.[46]

The influence of the anion in the enzyme partitioning was investigated using the ILs [C_4mim][$N(CN)_2$] and [C_4mim]Cl as shown in Table 2. The [C_4mim][$N(CN)_2$] was responsible for the poorest partition coefficient of the proteins (K_P = 0.063 ± 0.003) and the lowest purification performance (PF = 26 ± 1). It presents low values of K_P and K_E that were significantly lower than unit (≪ 1), which indicates that with this IL both enzyme and contaminant proteins partition preferentially into the salt-rich phase (bottom phase), leading to low PF.

The results in Table 2 also provide information on the effect of the cation core on the purification by comparing the systems based on [C_4mim]Cl and [C_4mpyr]Cl. In what concerns the ability for the lipase purification, the cation core seems to play a small role, when compared with the effect of the other IL features. Indeed, the substitution of the imidazolium ring by a pyridinium is responsible for a small decrease in the purification factor from 40.8 ± 0.4 to 36.7 ± 0.9. The enzyme and protein partition coefficients for the pyridinium-based IL suggest that the migration of the contaminant proteins for the top phase decreased when comparing with [C_4mim]Cl, while the enzyme remained in the bottom phase (salt-rich phase). The high purification factors were also followed by high enzyme recovery efficiencies at the bottom phase (90.6 ± 0.1 < R_B^E < 96.14 ± 0.08)%.

To support our interpretation of the results concerning the purification capacity of the IL-based ATPS a 2D electrophoresis analysis was performed using samples of the bottom and top phases from the ATPS based in the [C_8mim]Cl (considered in this work the best purification/separation system). The three lanes shown in Fig. 3 correspond to the molecular mass standard (Lane P), the bottom phase (Lane B) and top phase (Lane T). The presence of multiple light bands in Lane T confirms the presence of some contaminant proteins, designated as ***i***, ***ii*** and ***iii***. In Lane B, it is possible to see the presence of the target enzyme with a molecular weight of around 54 kDa (here abbreviated as ***Enz***) and the absence of contaminant compounds. The results from the electrophoresis show the excellent purification ability of the [C_8mim]Cl ATPS that made possible the complete separation of the enzyme from the contaminant compounds.

Table 1 Purification factor, enzymatic activity, specific activity, and protein concentration at the end of each step of the production and pre-purification

Process	EA (U mL^{-1})	C (mg mL^{-1})	SA (U mL^{-1})	PF (fold)
Fermentation	4662.06	1.19	3922.53	1.00
Dialysate	4245.40	0.06	76 501.46	19.50

2.3. Comparison of the performance of polymer and IL-based ATPS

Since the aim of IL-based ATPS is to replace the polymer based ATPS, the purification achieved using these two systems was here compared. The case study performed in this work (production and purification of the enzyme produced by *Bacillus* sp. ITP-001) was also investigated elsewhere using ATPS based in polyethylene glycol (PEG 8000) and potassium phosphate.[56] Despite some differences in the operation conditions of both systems (inorganic salt, temperature, and pH), the results obtained in this work are in general superior, with PF ranging from 37 to 51, than those using PEG-based ATPS that have purification factors below 30.[56] This comparison was carried out considering similar conditions in both works (20% (w/v) of PEG 8000 + 30% (w/v) potassium, pH 6.0, and 4 °C of temperature). As described in the literature,[8,46,56] the pH of the extraction system is a very important parameter. The work with PEG-based ATPS was carried out with a pH of 6.0 while the pH used in this work is 7.0. As described by Barbosa *et al.*[56] the increase in the pH leads to a strong decrease in the PF, which means that the purification factor of 30 fold here described, would be lower at the pH value used in this work.

A further advantage of the IL-based ATPS in comparison with PEG-based systems is the low viscosity of the aqueous phases on these systems. To make a comparison between the two systems, the dynamic viscosity of the top and bottom phases was measured at 25 °C for the four IL-based and two polymer-based ATPS (PEG 600 and PEG 4000). The viscosity of the salt-rich phase, the bottom phase (6.11–19.70 mPa s) is low and similar for the IL and polymer-based ATPS (6.96–8.27 mPa.s). While the viscosity of the IL-rich phases (4.96–8.91 mPa s) is comparable with those of the salt-rich phases (6.11–19.70 mPa s), the viscosity of the polymer-rich phase is larger by an order of

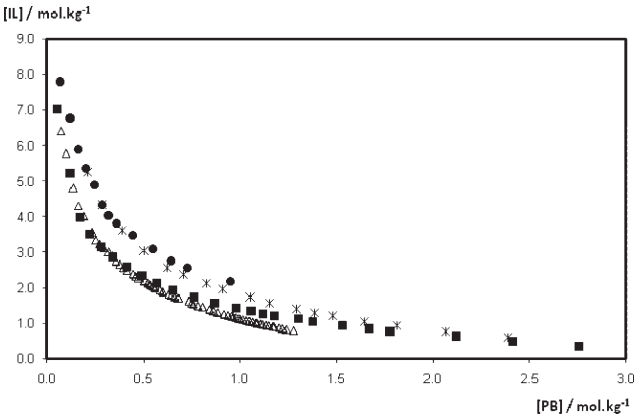

Fig. 2 Phase diagrams for the ILs used in the partitioning and purification studies: (△) - [C_4mim][$N(CN)_2$], (■) - [C_4mpyr]Cl, (✳) - [C_8mim]Cl, and (●) - [C_4mim]Cl.

This journal is © The Royal Society of Chemistry 2012

View Online

Table 2 Effect of the different IL structural features in the partition and purification of the enzyme before the pre-purification steps, using 25% (w/w) of IL and 30% (w/w) of potassium phosphate buffer solution, at pH 7.0 and 25 °C

Ionic Liquid	$R_v \pm$ std	$K_p \pm$ std	$K_E \pm$ std	SA_T (U mL^{-1})	SA_B (U mL^{-1})	$R_B^E \pm$ std (%)	PF ± std (fold)
[C_4mim][N(CN)$_2$]	0.90 ± 0.09	0.063 ± 0.003	0.049 ± 0.003	151386.43	101583.10	96.14 ± 0.08	26 ± 1
[C_4mpyr]Cl	1.04 ± 0.04	1.137 ± 0.008	0.059 ± 0.003	67799.08	143776.15	94.2 ± 0.3	36.7 ± 0.9
[C_4mim]Cl	1.35 ± 0.04	0.27 ± 0.02	0.082 ± 0.007	294746.13	161693.56	90.6 ± 0.1	40.8 ± 0.4
[C_8mim]Cl	1.11 ± 0.04	14 ± 2	0.076 ± 0.001	5614.01	187485.13	92.2 ± 0.2	51 ± 2

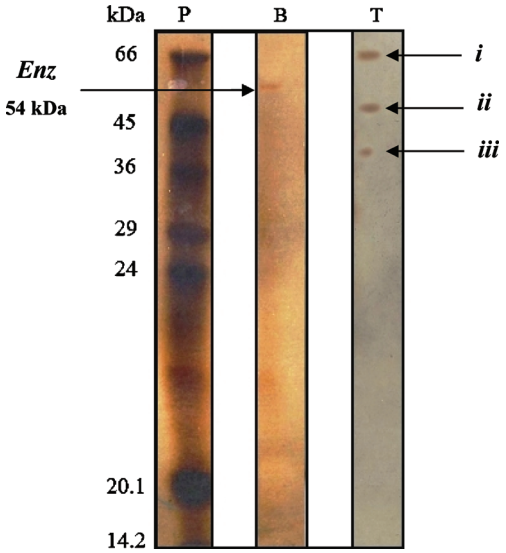

Fig. 3 Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) patterns of the enzyme produced by *Bacillus* sp. Lane P: molecular mass standard (14.2–66 kDa), Lane B: bottom phase obtained from the [C_8mim]Cl-based ATPS; Lane T: top phase obtained from the [C_8mim]Cl-based ATPS. 12.5% acrylamide gel stained with silver nitrate solution.

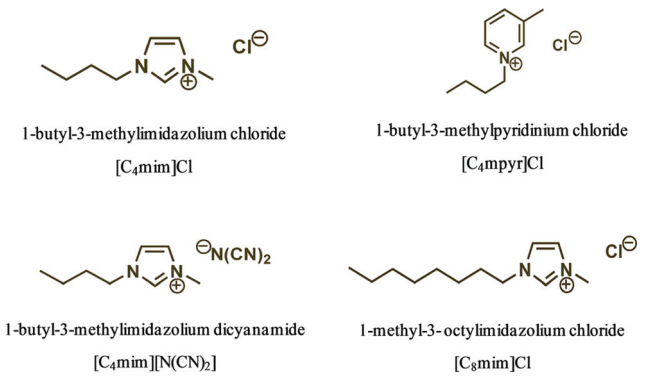

Fig. 4 Chemical structure of the ILs used in the present work.

magnitude (26.67–134.57 mPa s). This appears as an excellent advantage of the IL-based ATPS since the lower viscosities make the fluid transport and the mass transfer between the phases easier.

This work shows that IL-based ATPS are successful in the purification of proteins from real systems, constitute an improvement over the polymer-based systems, and may become an important operation in the design and future industrial implementation of new biocompatible purification techniques.

3. Experimental section

3.1. Materials

The present study was carried out using the inorganic salts K_2HPO_4 and KH_2PO_4 purchased at Sigma-Aldrich with purities higher than 98% (w/w). The ILs used are: 1-butyl-3-methylimidazolium chloride [C_4mim]Cl, 1-butyl-3-methylimidazolium dicyanamide [C_4mim][N(CN)$_2$], 1-methyl-3-octylimidazolium chloride [C_8mim]Cl, and 1-butyl-3-methylpyridinium chloride [C_4mpyr]Cl. They were all acquired at IoLiTec (Ionic Liquid Technologies, Germany) with mass fraction purities higher than 98%, confirmed by us using ^{1}H-NMR and ^{13}C-NMR. Their molecular structures, respective names and abbreviations are reported in Fig. 4. Poly(ethylene glycols) of average weights 600 g mol^{-1} and 4000 g mol^{-1} (abbreviated as PEG 600 and PEG 4000) were supplied by Fluka and Sigma-Aldrich, respectively.

3.2. Methods

3.2.1. Fermentation conditions. *Bacillus* sp. ITP-001 is a bacterium strain isolated from soils contaminated with petroleum, stored in the Institute of Research and Technology (Aracaju-Sergipe, Brazil). The bacterium was maintained on nutrient agar slants at 37 °C, stored at 4 °C and cultured once a month.

The production of the extracellular lipase was carried out during a submerged fermentation conducted in 500 mL Erlenmeyer flasks and containing 200 mL of the fermentation medium, whose composition (%, w/v) is the following: 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 was carried at 37 °C, pH 7.0 and continuous agitation of 170 rpm. After 72 h of cultivation, coconut oil (4%, w/v) was added as inducer.

3.2.2. Pre-purification steps

3.2.2.1. Centrifugation of fermentation broth. A sample of approximately 8 mL was centrifuged at 3000 rpm for 15 min. The bottom phase was discharged (biomass) and the supernatant was used to determine the enzymatic activity and the total protein.

3.2.2.2. Salt Precipitation and Dialysis. The fermented broth was centrifuged at 10 000 *g* for 30 min, to separate the biomass. Protein contaminants in the cell-free fermented broth were

Published on 18 January 2012 on http://pubs.rsc.org | doi:10.1039/C2GC16428K
Downloaded by Universidade de Aveiro (UAveiro) on 05 March 2012

View Online

Table 3 Values of the extraction systems compositions in mol kg^{-1}. The composition of the phosphate buffer was the same for all the experiments, 2.75 mol kg^{-1}

Ionic liquid	[IL] (mol kg^{-1})
$[C_4mim][N(CN)_2]$	1.62
$[C_4mpyr]Cl$	1.80
$[C_4mim]Cl$	1.91
$[C_8mim]Cl$	1.44

precipitated using a solution of ammonium sulphate $(NH_4)_2SO_4$ at 80% (w/v) and the broth was subsequently centrifuged at 10000 *g* for 30 min, aiming at separating the supernatant (with the target enzyme) from the precipitate (with the contaminant compounds). This procedure was performed several times until a much reduced amount of precipitate is achieved. All the supernatant phase obtained was dialyzed using dialysis membranes MD 25 (cut-off: 10 000–12 000 Da) against ultra-pure water. The dialysate solution containing the enzyme was then used to prepare the ILs-based ATPS.[56]

3.2.3. Purification stage

3.2.3.1. Phase diagrams and tie-line determination. The binodal data were determined for all the ILs studied using the cloud point titration method[30,31] at 25 (± 1) °C. Repetitive drop-wise addition of the aqueous inorganic salt solution to the aqueous solution of IL (≈ 60 wt%) was carried out until the detection of a cloudy solution, followed by the drop-wise addition of ultra-pure water until the detection of a monophasic region (limpid solution). The whole procedure was performed under constant stirring. The ternary system compositions were determined by the weight quantification of all components added within an uncertainty of ± 10^{-4} g. All these systems were already reported in the literature.[54] These were all prepared at 25 °C and pH 7.0.
Tie-lines (TLs) were determined by a gravimetric method previously described by us[30,31] and adapted from Merchuck *et al.*[60] A mixture at the biphasic region was prepared, vigorously stirred, and allowed to reach equilibrium by phase separation of both phases for 24 h, at 25 °C, using small ampoules (10 cm^3) especially designed for this purpose. After the separation step, both top and bottom phases were weighed. Each individual tie-line (TL) was determined by application of the lever arm rule[60] as previously described.[46]

3.2.3.2. Partition of the extracellular lipolytic enzyme. The extraction systems were prepared by adding 25 wt% of IL + 30 wt% of potassium phosphate buffer + 45 wt% of dialysate solution containing the lipolytic enzyme produced by *Bacillus* sp. ITP-001. The mixture point was the same for all the systems studied aiming to maintain the same compositions of IL and inorganic salt for all the experiments. The compositions in mol kg^{-1} are reported in Table 3. The potassium phosphate buffer was obtained from the mixture of two inorganic salts, the K_2HPO_4 and KH_2PO_4 in the ratio of 1.087 (w/w) for the pH 7.0. In this case, the phosphate buffer solution was prepared with the dialysate solution and directly in the extraction system. Thus, the extraction systems were composed of 25 wt% of IL + 30 wt% of phosphate buffer aqueous solution at pH 7.0 (the total mass of the extraction systems prepared is 5.0 g). All systems were prepared in graduated glass centrifuge vials of *circa* 10 cm^3. The mixture was gently stirred and centrifuged at 3000 rpm for 20 min. The graduated tubes were placed at 25.0 (± 0.1) °C, for at least 12 h to reach equilibrium, using a thermostatic bath (Marconi MA-127). After reaching equilibrium, both phases become clear and transparent and the interface was well defined. The two aqueous phases were carefully separated and cautiously collected for the determination of their volume and weight. For the determination of the viscosity data, two systems were prepared using the polymers PEG 600 and PEG 4000. The concentration used was the same as for the IL-based ATPS, but replacing the IL by the polymers
In this study, the partition coefficient was defined as the ratio of protein concentration or enzyme activity in the bottom and top phases, as described 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 are the enzyme activity (U mL^{-1}) of the top and bottom phases, respectively. These partition experiments were carried out in triplicate, being the results reported in this work, the average of these assays. It should be remarked that for all studied ATPS, the top phase is the IL-rich phase while the bottom phase is the phosphate-buffer-rich phase. To further evaluate this process as a purification technique, the enzyme specific activity (SA, expressed in U mg^{-1} of protein), the enzyme recovery in bottom phase (R_B^E), and the purification factor (PF) were calculated for all systems as,

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

$$R_B^E = \frac{100}{1 + R_V K_E} \quad (4)$$

$$PF = \frac{SA}{SA_i} \quad (5)$$

where R_V represents the volume ratio between V_T and V_B that are, respectively, the volumes of top and bottom phases. The enzyme specific activity (SA) (eqn (3)) can be evaluated for both phases through the ratio of the enzyme activity (EA) and the protein concentration (*C*) in each one of the phases. The purification factor (PF) was calculated by the ratio between the SA after and before (SA_i) the extraction procedure. This procedure was already used and validated before.[46]

3.2.3.3. Enzymatic activity. Lipolytic activities were assayed using the oil emulsion method according to a modification proposed by Soares *et al.*[61] The reaction was carried at 37 °C and pH 7.0. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid per minute (μmol min^{-1}) under the assay established conditions (37 °C, pH 7.0 and 100 rpm). The mentioned experimental techniques followed the protocols previously described by Carvalho *et al.*[62] The possibility of the hydrolysis of olive oil by the ILs

Published on 18 January 2012 on http://pubs.rsc.org | doi:10.1039/C2GC16428K
Downloaded by Universidade de Aveiro (UAveiro) on 05 March 2012

 This journal is © The Royal Society of Chemistry 2012

was tested in control assays at the measurement conditions in the absence of enzyme and verified to be negligible.[46]

3.2.3.4. Protein determination. Total protein concentration was determined by the Bradford method,[63] using a Varian 50 Bio UV-Vis Spectrophotometer at 595 nm, and a calibration curve previously established for the standard protein bovine serum albumin (BSA). To cancel the influence of the IL's presence on the protein concentration analysis, a control system for each IL-based ATPS without enzyme was prepared under the same conditions. Equilibrium conditions (24 h, 25 °C) and the phase separation procedure were those previously described.[46]

3.2.3.5. Polyacrylamide gel electrophoresis. The molecular mass and purity level of the lipase were analyzed by a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass standard was composed of bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.2 kDa) and were used as protein markers in this experimental method.[64] The experimental procedure was focused in the protein silver staining method.[65,66]

3.2.3.6. Viscosity determination. Measurements of viscosity for each one of the ATPS and both top and bottom phases, were performed in the temperature of 25 °C at atmospheric pressure, using an automated SVM 3000 Anton Paar rotational Stabinger viscometer. The temperature uncertainty is 0.02 °C. The relative uncertainty of the dynamic viscosity obtained is less than 0.5% for the standard fluid SHL120 (SH Calibration Service GMbH), for the studied temperature. This viscometer was previously tested for similar systems and presented a very good reproducibility.[67,68]

4. Conclusion

Ionic liquid based aqueous two-phase systems were here successfully applied to the purification of lipase, produced by the bacterium *Bacillus* sp. ITP-001, from a fermentation broth. Both high purification factors and enzyme recovery efficiencies at the salt-rich phase were obtained for all systems ($90.6 \pm 0.1 < R_B^E < 96.14 \pm 0.08$)%. It is shown that IL-based ATPS can be more efficient and significantly improve the extraction capacity of the commonly used polymer-based ATPS. To fully evaluate the potential of this purification technique its application to other enzymes or proteins must be object of further studies.

Acknowledgements

The authors are grateful for financial support from Fundação para a Ciência e a Tecnologia for the Ph.D. grant SFRH/BD/37830/2007 of S.P.M. Ventura. Moreover, the authors are grateful for the financial support from CAPES for the Ph.D. grant 551223/2010-3 of José M. P. Barbosa and grant PIBIC from FAPITEC for the grant of Rafael L. F. de Barros.

References

1 R. Sharma, Y. Chisti and U. C. Banerjee, *Biotechnol. Adv.*, 2001, **19**, 627–662.
2 R. Gulati, J. Isar, V. Kumar, A. K. Parsad, V. S. Parmar and R. K. Saxena, *Pure Appl. Chem.*, 2005, **77**, 251–262.
3 E. Zatloukalova and Z. Kucerova, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2004, **808**, 99–103.
4 A. R. Piergiovanni, *J. Agric. Food Chem.*, 2007, **55**, 3850–3856.
5 *Protein purification: Principles and practice*, ed. R. K. Scopes, Springer Verlag, New York, 1994.
6 S. Sasakawa and H. Walter, *Biochemistry*, 1972, **11**, 2760–2765.
7 J. G. Huddleston, H. D. Willauer and R. D. Rogers, *J. Chem. Eng. Data*, 2003, **48**, 1230–1236.
8 M. Perumalsamy, A. Bathmalakshmi and T. Murugesan, *J. Chem. Eng. Data*, 2007, **52**, 1186–1188.
9 R. M. Banik, A. Santhiagu, B. Kanari, C. Sabarinath and S. N. Upadhyay, *World J. Microbiol. Biotechnol.*, 2003, **19**, 337–348.
10 J. Sinha, P. K. Dey and T. Panda, *Appl. Microbiol. Biotechnol.*, 2000, **54**, 476–486.
11 S. Saravanan, J. R. Rao, B. U. Nair and T. Ramasami, *Process Biochem.*, 2008, **43**, 905–911.
12 K. Naganagouda and V. H. Mulimani, *Process Biochem.*, 2008, **43**, 1293–1299.
13 S. Klembt, S. Dreyer, M. Eckstein, U. Kragl, *Biocatalytic reactions in ionic liquids*, ed. P. Wasserscheid, Wiley VCH, Weinheim, 2007.
14 F. van Rantwijk and R. A. Sheldon, *Chem. Rev.*, 2007, **107**, 2757–2785.
15 U. Kragl, M. Eckstein and N. Kaftzik, *Curr. Opin. Biotechnol.*, 2002, **13**, 565–571.
16 A. K. Burrell, R. E. D. Sesto, S. N. Baker, T. M. McCleskey and G. A. Baker, *Green Chem.*, 2007, **9**, 449–454.
17 R. E. Del Sesto, T. M. McCleskey, C. Macomber, K. C. Ott, A. T. Koppisch, G. A. Baker and A. K. Burrell, *Thermochim. Acta*, 2009, **491**, 118–120.
18 P. Wasserscheid and W. Keim, *Angew. Chem., Int. Ed.*, 2000, **39**, 3772–3789.
19 T. Welton, *Chem. Rev.*, 1999, **99**, 2071–2083.
20 J. Dupont, R. F. de Souza and P. A. Z. Suarez, *Chem. Rev.*, 2002, **102**, 3667–3691.
21 L. I. N. Tomé, V. R. Catambas, A. R. R. Teles, M. G. Freire, I. M. Marrucho and J. A. P. Coutinho, *Sep. Purif. Technol.*, 2010, **72**, 167–173.
22 Y. Jiang, H. Xia, J. Yu, C. Guo and H. Liu, *Chem. Eng. J.*, 2009, **147**, 22–26.
23 Z. Li, Y. Pei, H. Wang, J. Fan and J. Wang, *TrAC, Trends Anal. Chem.*, 2010, **29**, 1336–1346.
24 S. P. M. Ventura, R. L. Gardas, F. Gonçalves and J. A. P. Coutinho, *J. Chem. Technol. Biotechnol.*, 2011, **86**, 957–963.
25 S. P. M. Ventura, A. M. M. Gonçalves, F. Gonçalves and J. A. P. Coutinho, *Aquat. Toxicol.*, 2010, **96**, 290–297.
26 K. E. Gutowski, G. A. Broker, H. D. Willauer, J. G. Huddleston, R. P. Swatloski, J. D. Holbrey and R. D. Rogers, *J. Am. Chem. Soc.*, 2003, **125**, 6632–6633.
27 N. J. Bridges, K. E. Gutowski and R. Rogers, *Green Chem.*, 2007, **9**, 177–183.
28 Y. Deng, J. Chen and D. Zhang, *J. Chem. Eng. Data*, 2007, **52**, 1332–1335.
29 Y. Deng, T. Long, D. Zhang, J. Chen and S. Gan, *J. Chem. Eng. Data*, 2009, **54**, 2470–2473.
30 C. M. S. S. Neves, S. P. M. Ventura, M. G. Freire, I. M. Marrucho and J. A. P. Coutinho, *J. Phys. Chem. B*, 2009, **113**, 5194–5199.
31 S. P. M. Ventura, C. M. S. S. Neves, M. G. Freire, I. M. Marrucho, J. Oliveira and J. A. P. Coutinho, *J. Phys. Chem. B*, 2009, **113**, 9304–9310.
32 C. Z. Wu, J. J. Wang, Y. C. Pei, H. Y. Wang and Z. Y. Li, *J. Chem. Eng. Data*, 2010, **55**, 5004–5008.
33 S. P. M. Ventura, S. G. Sousa, L. S. Serafim, Á. S. Lima, M. G. Freire and J. A. P. Coutinho, *J. Chem. Eng. Data*, 2011, **56**, 4253–4260.
34 H. Zhao, S. Xia and P. Ma, *J. Chem. Technol. Biotechnol.*, 2005, **80**, 1089–1096.
35 S. Oppermann, F. Stein and U. Kragl, *Appl. Microbiol. Biotechnol.*, 2011, **89**, 493–499.
36 A. P. de los Rios, F. J. Hernandez-Fernandez, L. J. Lozano, S. Sanchez, J. I. Moreno and C. Godinez, *J. Chem. Eng. Data*, 2009, **55**, 605–608.
37 A. P. Abbott, G. Capper, D. L. Davies, R. K. Rasheed and P. Shikotra, *Inorg. Chem.*, 2005, **44**, 6497–6499.
38 M. Matsumoto, K. Mochiduki, K. Fukunishi and K. Kondo, *Sep. Purif. Technol.*, 2004, **40**, 97–101.

Published on 18 January 2012 on http://pubs.rsc.org | doi:10.1039/C2GC16428K
Downloaded by Universidade de Aveiro (UAveiro) on 05 March 2012

View Online

39 M. Domínguez-Pérez, L. I. N. Tomé, M. G. Freire, I. M. Marrucho, O. Cabeza and J. A. P. Coutinho, *Sep. Purif. Technol.*, 2010, **72**, 85–91.
40 J. J. Wang, Y. C. Pei, Y. Zhao and Z. G. Hu, *Green Chem.*, 2005, **7**, 196–202.
41 X. Chen, J. Liu and J. Wang, *Anal. Methods*, 2010, **2**, 1222–1226.
42 Q. Cao, L. Quan, C. He, N. Li, K. Li and F. Liu, *Talanta*, 2008, **77**, 160–165.
43 F. J. Deive, A. Rodriguez, A. B. Pereiro, J. M. M. Araujo, M. A. Longo, M. A. Z. Coelho, J. N. C. Lopes, J. M. S. S. Esperanca, L. P. N. Rebelo and I. M. Marrucho, *Green Chem.*, 2011, **13**, 390–396.
44 S. Dreyer and U. Kragl, *Biotechnol. Bioeng.*, 2008, **99**, 1416–1424.
45 S. E. Dreyer, P. Salim and U. Kragl, *Biochem. Eng. J.*, 2009, **46**, 176–185.
46 S. P. M. Ventura, S. G. Sousa, M. G. Freire, L. S. Serafim, Á. S. Lima and J. A. P. Coutinho, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 2679–2687.
47 Y. Pei, W. Jianji, K. Wu, X. Xuan and X. Lu, *Sep. Purif. Technol.*, 2009, **64**, 288–295.
48 Y. Pei, Z. Li, L. Liu, J. Wang and H. Wang, *Chem. Mat. Sci.*, 2010, **53**, 1554–1560.
49 Z. Du, Y. L. Yu and J. H. Wang, *Chem.–Eur. J.*, 2007, **13**, 2130–2137.
50 W. Meindersma, F. Onink and A. B. de Haan, in *Handbook of Green Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, 2010.
51 J. Ranke, K. Molter, F. Stock, U. Bottin-Weber, J. Poczobutt, J. Hoffmann, B. Ondruschka, J. Filser and B. Jastorff, *Ecotoxicol. Environ. Saf.*, 2004, **58**, 396–404.
52 N. Gathergood, P. J. Scammells and M. T. Garcia, *Green Chem.*, 2006, **8**, 156–160.
53 G. Quijano, A. Couvert and A. Amrane, *Bioresour. Technol.*, 2010, **101**, 8923–8930.
54 S. P. M. Ventura, S. G. Sousa, L. S. Serafim, Á. S. Lima, M. G. Freire and J. A. P. Coutinho, *J. Chem. Eng. Data*, in press.
55 Q. Liu, J. Yu, W. Li, X. Hu, H. Xia, H. Liu and P. Yang, *Sep. Sci. Technol.*, 2006, **41**, 2849–2858.
56 J. M. P. Barbosa, R. L. Souza, A. T. Fricks, G. M. Zanin, C. M. F. Soares and Á. S. Lima, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 3853–3858.
57 D. Forciniti, C. K. Hall and M. R. Kula, *Biotechnol. Bioeng.*, 1991, **38**, 986–994.
58 Q. K. Shang, W. Li, Q. Jia and D. Q. Li, *Fluid Phase Equilib.*, 2004, **219**, 195–203.
59 G. Bassani, B. Farruggia, B. Nerli, D. Romanini and G. Picó, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2007, **859**, 222–228.
60 J. C. Merchuck, B. A. Andrews and J. A. Asenjo, *J. Chromatogr., Biomed. Appl.*, 1998, **711**, 285–293.
61 C. M. F. Soares, H. F. Castro, F. F. Moraes and G. M. Zanin, *Appl. Biochem. Biotechnol.*, 1999, **79**, 745–758.
62 N. B. Carvalho, R. L. de Souza, H. F. de Castro, G. M. Zanin, Á. S. Lima and C. M. F. Soares, *Appl. Biochem. Biotechnol.*, 2008, **150**, 25–32.
63 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.
64 U. K. Laemmli, *Nature*, 1970, **227**, 680–685.
65 M. Chevallet, S. Luche and T. Rabilloud, *Nat. Protoc.*, 2006, **1**, 1852–1858.
66 C. R. Merril, D. Goldman, S. A. Sedman and M. H. Ebert, *Science*, 1981, **211**, 1437–1438.
67 M. J. Pratas, S. Freitas, M. B. Oliveira, S. C. Monteiro, Á. S. Lima and J. A. P. Coutinho, *J. Chem. Eng. Data*, 2010, **55**, 3983–3990.
68 P. J. Carvalho, T. Regueira, L. M. N. B. F. Santos, J. Fernandez and J. A. P. Coutinho, *J. Chem. Eng. Data*, 2009, **55**, 645–652.

Published on 18 January 2012 on http://pubs.rsc.org | doi:10.1039/C2GC16428K
Downloaded by Universidade de Aveiro (UAveiro) on 05 March 2012

 This journal is © The Royal Society of Chemistry 2012