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Aqueous two-phase systems based on cholinium salts and tetrahydrofuran and their use for lipase purification

Ranyere L. Souza [a,c], Rafaella A. Lima [a], João A.P. Coutinho [b], Cleide M.F. Soares [a,c], Álvaro S. Lima [a,c,*]

[a] Universidade Tiradentes, Av. Murilo Dantas 300, Farolândia, 49032-490 Aracaju, SE, Brazil
[b] Departamento de Química, CICECO, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[c] ITP, Instituto de Tecnologia e Pesquisa, Av. Murilo Dantas, 300-Prédio do ITP, Farolândia, 49032-490 Aracaju, SE, Brazil

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ABSTRACT

Aqueous two-phase systems (ATPS) formed with cholinium-based ionic liquid – ILs (or salts) are a novel, low cost, and high efficient technique for the recovery of biomolecules. This study examines the formation of ATPS based on cholinium-based salts (cholinium chloride, cholinium bitartrate and cholinium dihydrogencitrate) and tetrahydrofuran (THF) for the purification of lipase from *Bacillus* sp. ITP-001, produced by submerged fermentation. The optimum conditions for this purification were determined to be 40 wt% of THF and 30 wt% of cholinium bitartrate at 25 °C. A purification factor of 130.1 ± 11.7 fold, a lipase yield of 90.0 ± 0.7% and a partition coefficient of enzyme for IL-rich phase (K_E = 0.11 ± 0.01) and protein contaminants for THF-rich phase (K_P = 1.16 ± 0.1) were achieved.

1. Introduction

Lipases are glycerol ester hydrolases (EC 3.1.1.3), and those from microbial origin, occupy a place of prominence among biocatalysts in several sectors like in oleochemistry, organic synthesis, detergent formulation, nutrition, biosensors, bioremediation, among others [1–4]. Lipase preparations with a high degree of purity are used by the fine chemical industries, for example in the biocatalytic production of the pharmaceuticals and cosmetics [3,5]. The main problem with the production of high purity enzymes is the purification process. In general it has a poor efficiency, causes loss of enzyme activity, and requires high consumption of energy and chemicals [6,7]. To overcome these limitations, a significant effort has been made to develop novel techniques in order to reduce the costs related to the purification [6,8].

Aqueous two-phase systems (ATPS) have been used for the separation and purification of a great number, of biological products as amino-acids [9,10], proteins [11] and enzymes [8,12–16] as their two phases having a rich water environment are favorable to the preservation of activity of biomolecules. These systems form two aqueous phases that coexist in equilibrium due to the dissolution, at appropriate concentrations, of pairs of solutes in water [17]. ATPS formed of polymers (namely polymer–polymer, or polymer–salt) are well-known for advantages, such as low interfacial tension, good biocompatibility, fast and high phase separation rates and low cost [8,12]. However, their performance is significantly affected due to the small difference in polarity between the coexisting phases [18]. Currently, the number of systems capable of forming two aqueous phases is increasing and the alternatives include the use of alcohol/salt [19–21], acetonitrile/carbohydrates [22,23], polymers(polyvinyl alcohol – PVA)/dextran [24] and other combinations including ionic liquids (ILs) [25,26]. The numerous combinations of cations and anions that form ILs lead to a variety of physical properties, allowing the tailoring of their polarities, and for this reason have been regarded as important constituents of ATPS [27–29]. Although good results have been achieved using ATPS formed by ILs for the extraction of amino acids, proteins, enzymes, pharmaceuticals and phenolic compounds [25,30,31], the use of these compounds may raise some issues concerning their water stability, price, and biodegradability [32–36]. Their toxicity has been shown to be (in some cases) at least equivalent to those of common organic solvents. The ILs (such as, [mim][PF6], [1-Bu-1-EtPH$_2$][(EtO)$_2$PO$_2$]) revealed comparable ecotoxicity towards freshwater algae and the freshwater invertebrate *Daphnia magna* to hydrocarbons such as toluene and xylene [37].

To overcome these issues, the search for safer and cheaper ILs for the formation of ATPS is still an imperative issue and in this context, the cholinium-based ionic liquids are a good option. This family is derived from quaternary ammonium salts described as

* Corresponding author at: Universidade Tiradentes, Av. Murilo Dantas 300, Farolândia, 49032-490 Aracaju, SE, Brazil.
E-mail address: alvaro_lima@unit.br (Á.S. Lima).

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important structures in living processes, used as precursors for the synthesis of vitamins (*e.g.* vitamin B complexes and thiamine) and enzymes that participate in the carbohydrate metabolism [38,39]. Recent works have reported the synthesis of novel cholinium-based ILs with the cholinium cation combined with a variety of different anions [40–43]. Besides the exceptional properties shared with the ionic liquids, such as, non-flammability and negligible vapour pressure at ambient conditions, and high solvation ability, the cholinium ILs also have low toxicity, excellent biodegradability and can be produced at low cost since they can be obtained from cheap raw materials [42–46]. The interest in these compounds has increased in the past few years, with applications ranging from crosslinking agents for collagen based materials, solvents in the pre-treatment and dissolution of biomass, and use as co-substrates for microorganisms in the degradation of dyes [45–48]. Moreover, a number of works have described novel cholinium-based ILs in which protein structure and the enzyme function can be maintained or even increased [47,49]. This fact, coupled with other advantages cited herein, motivated the application of these ILs to the formation of alternative ATPS, which served as a platform for the purification/separation of antibiotics [50,51] and proteins [47].

Taking into account the ability of cholinium-based ILs to promote phase separation in ATPS, their low cost and the capacity to maintain the activity of the target compounds, they will be here explored combined with tetrahydrofuran. Tetrahydrofuran (THF) is an organic solvent with excellent solvent power for numerous organic substances, and employed for the extraction of compounds from vegetables, including commercially important compounds such as carotenoids [52,53]. The formation of aqueous two-phase systems using THF and a biological buffer, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), was first reported by Taha et al. [54]. Moreover, THF + potassium phosphate buffer based ATPS, was show to be effective for the purification of extracellular lipase from *Bacillus* sp. ITP-001 [55].

This work focuses in the design of ATPS based on tetrahydrofuran (THF) and cholinium-based ionic liquids (cholinium chloride, cholinium bitartrate and cholinium dihydrogencitrate). Aiming at exploring the applicability of those novel ATPS, the lipase from *Burkholderia cepacia* (commercially obtained) is here used as a model to evaluate the profile of the enzymatic partition and efficiency of extraction, namely considering cholinium-based ILs with different anions, overall system composition and temperature of equilibrium. Subsequently, representative conditions are employed with the objective of evaluating the possibility of applying these systems for the separation and purification of lipase from *Bacillus* sp. ITP-001 produced by submerged fermentation.

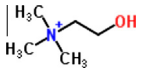
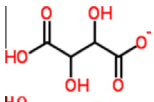
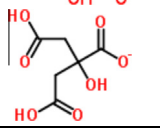
2. Materials and methods

2.1. Materials

The organic solvent tetrahydrofuran (purity ⩾99.9%), cholinium chloride (purity >98%); cholinium dihydrogencitrate (purity >98%); and cholinium bitartrate (purity >98%) were purchased from Sigma–Aldrich. Their chemical structures are shown in Table 1.

The lipase from *Burkholderia cepacia* – *BCL* (⩾30,000 U/g, pH 7.0, 50 °C – optimum pH and temperature) was also obtained from Sigma–Aldrich, and the lipase from *Bacillus* sp. ITP-001 was obtained by submerged fermentation, using $MgSO_4{\cdot}7H_2O$ (purity ⩾98%) obtained from Panreac, Triton X-100 purchased from Fisher Scientific, and $NaNO_3$ (purity ⩾99.5%), yeast extract, peptone, and starch purchased from Himedia. The ammonium sulphate (P.A.) was obtained from Synth (Brazil) and coconut oil was purchased at a local market. The protein bovine serum albumin (BSA, purity ⩾97%) was obtained from Merck.

Table 1
Chemical structure and abbreviation name of the cholinium-based ILs and THF studied in this work.

Organic solvent		Name (abbreviation)
		Tetrahydrofuran (THF)
Cholinium cation	**Anion**	**Name (abbreviation)**
		Cholinium chloride ([Ch]Cl)
		Cholinium dihydrogencitrate ([Ch][DHCit])
		Cholinium bitartrate ([Ch][Bit])

2.2. Production of the lipase by Bacillus sp. ITP-001

2.2.1. Fermentation conditions

The lipase was obtained by the fermentation of a *Bacillus* sp. ITP-001, isolated from an oil contaminated soil, stored at the Instituto de Tecnologia e Pesquisa – ITP (Aracaju–Sergipe, Brazil). The strain was cultivated in 500 mL erlenmeyer flasks containing 200 mL 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: initial pH 7; incubation temperature 37 °C, and stirring speed 170 rpm. After 72 h of cultivation, coconut oil (4%, w/v) and Triton X-100 (1%, w/v) were added as inductors as described by Feitosa et al. [56].

2.2.2. Pre-purification steps

The pre-purification steps were performed according to the methodology proposed by Barbosa et al. [8]. The fermented broth was centrifuged at 3000 rpm for 30 min, so that bottom phase was discharged (biomass) and the supernatant was used to determine the enzymatic activity and the total protein content. Protein contaminants in the cell-free fermented broth were precipitated using ammonium sulphate at 80% (w/v) saturation, the solution was prepared at room temperature and the broth was subsequently centrifuged at 3000 rpm for 30 min, separating the aqueous solution and precipitate. The aqueous phase was dialyzed using MD 25 (cut-off: 10,000–12,000 Da) against ultra-pure water for 24 h at 4 °C. The dialyzed solution containing the enzyme was then used to prepare the ATPS.

2.3. Binodal curves and tie-lines

The binodal curves were determined by the cloud-point titration method at 25 ± 1 °C and at atmospheric pressure. In a test tube, a THF aqueous solution of known concentration was added, and then a cholinium-based ILs solution of known mass fraction was added dropwise until the mixture became turbid or cloudy; then, a known mass of water was added to make the mixture clear again. This procedure was repeated to obtain sufficient data for the construction of a liquid–liquid equilibrium binodal curve. The