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Extraction of tetracycline from fermentation broth using aqueous two-phase systems composed of polyethylene glycol and cholinium-based salts

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ABSTRACT

Aiming at developing not only cheaper but also biocompatible and sustainable extraction and purification processes for antibiotics, in this work it was evaluated the ability of aqueous two-phase systems (ATPS) composed of polyethylene glycol (PEG) and cholinium-based salts to extract tetracycline from the fermented broth of *Streptomyces aureofaciens*. Conventional polymer/salt and salt/salt ATPS were also studied for comparison purposes. The novel systems here proposed are able to extract tetracycline directly from the fermentation broth with extraction efficiencies higher than 80%. A tailored extraction ability of these systems can also be achieved, with preferential extractions either for the polymer- or salt-rich phases, and which further depend on the cholinium-based salt employed. The gathered results support the applicability of biocompatible ATPS in the extraction of antibiotics from complex matrices and can be envisaged as valuable platforms to be applied at the industrial level by pharmaceutical companies.

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1. Introduction

Antibiotics are chemical compounds that can be totally or partly synthesized by living microorganisms that either inhibit the growth or even kill other microorganisms. Therefore, they are currently used worldwide for an effective control of the levels of pathogenic bacteria in humans and animals. At a global level, the antibiotic market reached US$42 billion (≈34 billion €) in 2009 [1]. Nevertheless, in the past few years, a number of patents regarding the synthesis and applications of antibiotics and other drugs have expired and they fall into the public domain [2]. The pharmaceutical industry is facing the growth of generic drugs and there is a crucial need to minimize operational costs by optimizing the antibiotics' production and their purification steps. The production cost of antibiotics derives, by a large extent, from the extraction and associated purification processes. In this context, it is imperative to find and evaluate new extractive/purification techniques which could be scaled-up by biopharmaceutical companies.

There are several classes of antibiotics such as β-lactams, aminoglycosides, macrolides and tetracyclines (TCs). Tetracyclines are bacteriostatic antimicrobials produced by *Streptomyces aureofaciens* or *Streptomyces rimosus*. They are a broad-spectrum antibiotic since they can be used against Gram positive and negative bacteria, *Coccidian*, *Trichomonas*, *Mycoplasma*, *Chlamydia* and *Rickettsia*. Tetracyclines inhibit the synthesis of bacterial proteins by binding to the small unit (30 S) of bacterial ribosome while preventing the access of aminoacyl tRNA to the acceptor site on the mRNA-ribosome complex [3–6]. Besides their antibiotic properties, tetracyclines also possess anti-inflammatory, anti-apoptotic and anti-neurodegenerative properties [6]. Due to all their benefits tetracyclines are currently added to animal feed in order to prevent diseases as well as a feed additive to accelerate growth [7].

Chromatographic techniques (particularly ion exchange chromatography), liquid–liquid or solid–liquid extractions are generally used for the extraction and purification of common antibiotics from the fermentation broth [8–10]. Among these techniques, the most used is the liquid–liquid extraction which has been carried out using organic solvents, namely ethyl acetate, acetonitrile and methanolic trichloroacetic acid (TCA) [11]. This type of liquid–liquid extraction is a useful technique which involves low costs and leads to a high purity level. However, these organics compounds present some drawbacks since they are volatile and hazardous to human health [8]. Taking into account the sustainability and biocompatibility of extraction processes, the use of aqueous two-phase systems (ATPS) can represent a viable option.

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ATPS are considered a low cost, gentler and biocompatible alternative to other extraction techniques since they are mainly composed of water [12–14]. In the last decade, novel ATPS composed of ionic liquids (ILs) + water + organic/inorganic salts, amino acids, polymers or carbohydrates have also been proposed in literature [15]. The main advantages of polymer–salt or polymer–IL ATPS, when compared to polymer–polymer systems, relay on their lower viscosities and possibility of changing the polarities of the coexisting phases. Moreover, they usually display a quick phase separation and high extraction efficiencies which can be easily manipulated by a proper selection of the ions composing a given IL or salt [15]. Indeed, IL-based ATPS have been successfully used in the separation, concentration and purification of proteins, antioxidants, metal ions, alkaloids and antibiotics [15].

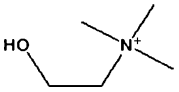
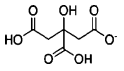
Most hydrophilic ILs exhibit unique properties that make them environmentally friendly solvents such as negligible vapor pressures, non-flammability and high thermal and chemical stabilities [16]. Their chemical diversity offers unique opportunities to develop solvents for specific purposes with tailored characteristics by the combination of proper ions. Due to their attractive physicochemical properties, ILs have additionally been applied for bio-purification and bio-extraction processes [15,17], in enzymatic catalysis [18], bioprocess operations [19] and in biofuel production [20]. However, most ILs are poorly biodegradable and of low biocompatibility. In this context, ionic liquids and salts based on the cholinium ion can be a valuable alternative; yet, poorly studied. Cholinium chloride is an essential nutrient, as considered by the Institute of Medicine in 1998, due to its role in the human body (used for neurotransmitter synthesis, cell-membrane signaling, etc.) [21]. It is of easy preparation, relatively cheap, stable in water and air, biocompatible and biodegradable. The cholinium-based salts, or ionic liquids (ILs) when their melting point is below 100 °C, are constituted by the 2-hydroxyethyl-*N*,*N*,*N*-trimethylammonium cation combined with anions as diverse as chloride, bicarbonate, acetate, levulinate, malate, glycolate, among others. Hence, the cholinium-based salts/ILs are a feasible option to be used in the formation of ATPS. We demonstrated recently the possibility of creating aqueous two-phase systems of the type polymer–salt or salt–salt either by the combination of PEG and cholinium-based salts or by the addition of an inorganic salt to mildly hydrophobic cholinium-based ILs [22,23]. Aiming at exploring the applicability of those novel ATPS, this work is focused on the extraction of tetracycline directly from the fermentation broth of *S. aureofaciens*. Systems composed of polyethylene glycol 600 and cholinium-based salts were investigated by means of the partition coefficients and extraction efficiencies obtained for tetracycline. To further ascertain on the enhanced ability of these novel systems to extract tetracycline, further experiments were carried out with conventional polymer/salt and salt/salt ATPS for comparison purposes.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG) with an average molecular weight of 600 g mol^{-1} (abbreviated as PEG 600) and tetracycline (TC), ≥98 wt% pure, were supplied by Fluka. The cholinium-based salts were acquired at Sigma–Aldrich: cholinium chloride, [Ch]Cl, cholinium bicarbonate, [Ch]Bic, and cholinium dihydrogencitrate, [Ch]DHcit, with purities of ≥98 wt%, 80 wt% (in aqueous solution), and 99 wt%, respectively. The cholinium acetate, [Ch]Ac, and cholinium dihydrogenphosphate, [Ch]DHph, were purchased from Iolitec with purity levels of 98 wt% and ≥98 wt%, respectively. These last two cholinium-based salts fall within the category of ILs since they present melting temperatures below 100 °C. Nevertheless, to avoid any ambiguity, all these materials will be thereinafter referred as cholinium-based salts. The chemical structures of the investigated cholinium-based salts are presented in Table 1. ^{1}H and ^{13}C NMR spectra were performed to evaluate the purity of each sample. All samples are of high purity and agree with the purity levels indicated by the suppliers. The inorganic salts K_3PO_4 (≥98 wt%) and Na_2SO_4 (>99.0 wt%) were acquired from Sigma–Aldrich and LabSolve, respectively. All the other reagents are of analytical grade and were used as received.

Table 1
Chemical structures of the studied cholinium-based salts.

Cholinium-based salts	Cation	Anion
[Ch]Bic		
[Ch]Cl		Cl^-
[Ch]Ac		
[Ch]DHcit		
[Ch]DHph		

2.2. Microorganism maintenance and fermentation processes

S. aureofaciens was kindly provided by the Microorganism Collection of the Department of Antibiotics from Federal University of Pernambuco, Recife, PE, Brazil. The frozen microorganism was maintained at −70 °C with glycerol in a cryotube. According to the procedure described by Darken et al. [24], a thick spore suspension contained in the cryotube was transferred to 25 mL of reactivation medium in 250 mL-Erlenmeyer flasks. After an incubation period of 24 h in an orbital shaker at 30 °C and 200 rpm, 5 mL of this suspension were added to 45 mL of the fermentation medium and re-incubated under the same conditions of reactivation during 48 h (at this condition the pH value was 4.50 ± 0.04). For the fermentation processes, 5.0 mL of the resulting cell suspension were added to 45 mL of fermentation medium in 500 mL-Erlenmeyer flasks and incubated during 120 h at the same operational conditions. At the end of the fermentation process, the fermented broth was filtrated through a Whatman N. 4 paper and then centrifuged at 3720 × *g* for 15 min at 5 °C. The supernatant obtained from this process presented a final pH of 4.27 ± 0.09 and a TC concentration of 0.175 g/L (quantified as described below). The supernatant was frozen and stored in an ultrafreezer at −70 °C and further used in all the partitioning studies.

2.3. Media composition

A modified liquid ISP-2 medium [25] with a constitution of 4.0 g L^{-1} of yeast extract and 10.0 g L^{-1} of malt extract was used for reactivation. The medium proposed by Darken et al. [24] for the preparation of the *S. aureofaciens* inoculum was used and it is composed as follows (g L^{-1} in deionized water): sucrose (30.0), soybean meal (5.0), $Na_3C_6H_5O_7{\cdot}5H_2O$ (1.0), $(NH_4)_2SO_4$ (3.3), $MgSO_4{\cdot}7H_2O$ (0.25), KH_2PO_4 (0.10), K_2HPO_4 (0.10), $CaCO_3$ (1.0), $MnSO_4{\cdot}4H_2O$ (0.01), $ZnSO_4{\cdot}7H_2O$ (0.04), $K_2Cr_2O_7$ (0.000016) and CH_3COOH (0.40). The fermentation medium was prepared according to the description given by Darken et al. [24] and it is composed of: $H_3C_6H_5O_7{\cdot}H_2O$ (12.8), sucrose (40.0), $(NH_4)_2SO_4$ (6.0), $MgSO_4{\cdot}7H_2O$ (0.25), KH_2PO_4 (0.15), $CaCO_3$ (11.0), $MnSO_4{\cdot}4H_2O$ (0.01), $ZnSO_4{\cdot}7H_2O$ (0.04) and $K_2Cr_2O_7$ ($0.016.10^{-3}$). All media were autoclaved at 121 °C for 15 min.

3. Methods

3.1. Phase diagrams and tie-lines

Phase diagrams for each ternary system composed of PEG600 + cholinium-based salt + H_2O at 25 °C were previously determined by us [23] using the cloud point titration method. Other ATPS used for comparison purposes were taken from literature [22,26]. The knowledge of these phase diagrams allows the choice of mixture points which correspond to a liquid–liquid two-phase system.

3.1.1. Optimization of the TC partitioning in ATPS composed of PEG and cholinium-based salts

In order to optimize the experimental conditions and phase compositions to be applied in the extraction of TC from the fermented broth, several model systems were initially investigated making use of commercial TC of high purity. Three different mixture compositions at the biphasic region, and for each ATPS, were selected. Small amounts of commercial TC, 0.25–0.45 mg, were added to glass tubes containing the appropriate weights of PEG, [Ch]-salt and water to form a system with a total mass of 5 g. After the equilibration time, 12 h at 25 (± 1) °C, the phases were carefully separated and the quantification of TC in both phases was carried out. The quantification of tetracycline is described below. All the assays were performed in triplicate and the respective standard deviations were determined.

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