Biochemical Engineering Journal 56 (2011) 75–83

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Liquid–liquid extraction by mixed micellar systems: A new approach for clavulanic acid recovery from fermented broth

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ARTICLE INFO

Article history:
Received 13 December 2010
Received in revised form 29 March 2011
Accepted 17 May 2011
Available online 23 May 2011

Keywords:
Clavulanic acid
Aqueous two-phase mixed micellar systems
Extraction purification

ABSTRACT

This work is the first attempt to apply aqueous two-phase mixed micellar systems (ATPMS) of the nonionic surfactant Triton X-114 and the anionic one AOT to extract clavulanic acid (CA) from broth fermented by *Streptomyces clavuligerus*. Cloud points were determined in McIlvane buffer pH 6.5 with or without NaCl, and diagram phases/coexistence curves were constructed. CA partition was investigated following a 2^4-full factorial design in which AOT (0.022, 0.033 and 0.044% w/w), Triton X-114 (1.0, 3.0 and 5.0% w/w) and NaCl (0, 2.85 and 5.70% w/w) concentrations and temperature (24, 26 and 28 °C) were selected as independent variables, and CA partition coefficient (K_{CA}) and yield in the top phase (η_{CA}) as responses. CA partitioned always to the top, micelle-poor phase. The regression analysis pointed out that NaCl concentration and interaction between temperature and Triton X-114 concentration had statistically significant effects on K_{CA}, while η_{CA} was mainly influenced by temperature, Triton X-114 concentration and their interaction. Different ATPMS compositions were then needed to maximize these responses, specifically 0.022% (w/w) AOT, 5% (w/w) Triton X-114 for K_{CA} (2.08), and 0.044% (w/w) AOT, 1% (w/w) Triton X-114 for η_{CA} (98.7%), both at 24 °C without NaCl. Since at 0.022% (w/w) AOT, 1% (w/w) Triton X-114 and 28 °C without NaCl the system was able to ensure satisfactory intermediate results (K_{CA} = 1.48; η_{CA} = 86.3%), these conditions were selected as the best ones. These preliminary results are of concern for possible industrial application, because CA partition to the dilute phase can simplify the subsequent purification protocol.

1. Introduction

Antibiotics are encompassing a broad group of chemical compounds with a wide range of different molecular targets in fighting infectious diseases [1]. The β-lactam family is the biggest and most important class of clinical antibiotics [2].

Clavulanic acid (CA) is a bicyclic β-lactam compound made up of a β-lactam ring and an oxazolidine one (Fig. 1A), which inactivates bacterial β-lactamases by entering their active sites [3]. The reaction of CA with these enzymes is an irreversible one, because the reaction products keep attached to them [4]. Its application in conjunction with penicillin has proven to be successful against bacterial resistance to β-lactam antibiotics and makes CA very important both clinically and economically [5,6].

Commercial products such as Augmentin™ and Timetin™, combinations of CA with amoxicillin or ticarcillin, respectively, have made CA a product valued in excess of US$ 1 billion/year and created a powerful incentive to study the production and extraction of this antibiotic [5].

CA is produced industrially by batch fermentation and isolated and purified from the fermented medium in several steps. The first step, consisting in clarification of the medium by filtration or centrifugation, is followed by either adsorption or liquid–liquid extraction with organic solvents and further purification by anion-exchange chromatography or other chromatographic techniques. Owing to the unstable nature of its free acidic form, CA is separated as lithium, potassium or sodium salt [7]. Thus, the development of more efficient and cost-effective separation and purification processes is crucial to improve process efficiency and economics, keeping the required high standards of quality for market approval [8].

In the last years, the liquid–liquid extraction by aqueous systems has been used to extract many biomolecules, either biologically active or not. For this purpose, the addition of hydrophilic polymers or nonionic surfactants in aqueous solution was proposed to obtain two immiscible phases. Among these, aqueous two-phase mixed

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doi:10.1016/j.bej.2011.05.011

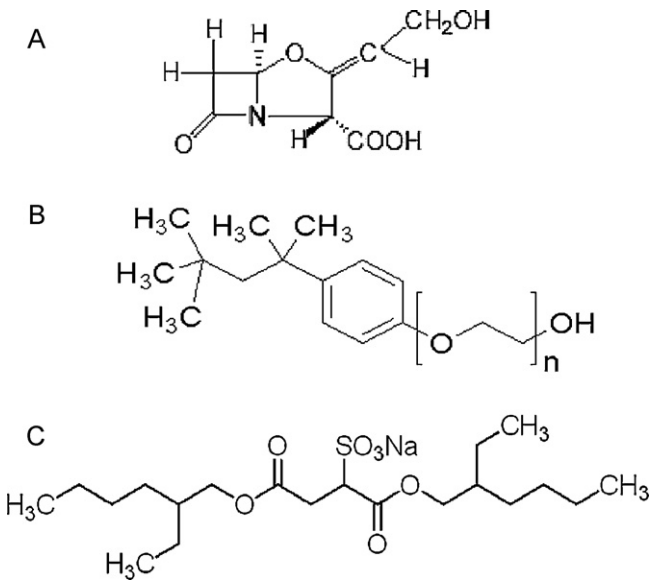

Fig. 1. Chemical structures of (A) clavulanic acid, (B) polyethylene glycol *tert*-octylphenyl ether (Triton X-114), (C) sodium bis(2-ethylhexyl) sulphosuccinate (AOT).

micellar systems (ATPMS) exploit the fact that some aqueous micellar solutions, under appropriate conditions, can spontaneously separate into two aqueous, yet immiscible, liquid phases, between which proteins and other biomolecules can distribute unevenly [9].

When compared to the extensively studied aqueous two-phase polymer systems (ATPS), ATPMS offer a number of unique and desirable features, including the self-assembling property of micelles, which enables us to control and optimize the partitioning behaviour by tuning the micellar characteristics, along with their ability to ensure an amphiphilic environment, i.e. hydrophobic and hydrophilic at the same time. These characteristics can be exploited to selectively partition a target biomolecule depending on its hydrophobicity [10]. In addition, the partition selectivity can be improved by using mixed micelles, made up of charged surfactants or surfactant-type affinity ligands in mixture with nonionic surfactants [11–14]. A mixed system almost invariably brings about enhanced interfacial properties compared to a simple one. However, it is the rich polymorphism in bulk solution that has attracted increasing interest in recent years [15].

In ATPMS, the electrostatic interactions between charged biomolecules and oppositely charged mixed micelles can be exploited to enhance the yield and selectivity of partitioning [14,16,17]. So, electrostatic interactions often play an important role in determining the phase behaviour of charged surfactant mixtures [18,19].

Although ionic surfactants can bind and improve biomolecules stability, this effect has been shown to depend on the type and concentration of the ionic surfactant [14,20]. If from the one hand, the amount of ionic surfactant should be sufficiently large to induce a significant change in the biomolecule partitioning behaviour, from the other it should be sufficiently low to prevent severe stability loss [16,17].

The addition of inorganic salts may result in a modification of both intramicellar and intermicellar interactions because of electrostatic screening [19,21]; therefore, it often leads to changes in the phase behaviour of aqueous mixtures of oppositely charged surfactants [19], thereby improving the biomolecules partitioning.

CA recovery was successfully performed in ATPS using PEG and salts; however, under these conditions, it partitions towards the PEG phase, and its subsequent separation is difficult [22]. In order to overwhelm this bottleneck, CA transfer to the aqueous buffer/salt phase is hoped for. To this purpose as well as to reduce the number of downstream processing steps, an ATPMS of the nonionic surfactant polyethylene glycol *tert*-octylphenyl ether (Triton X-114) (Fig. 1B) and the anionic surfactant sodium bis(2-ethylhexyl) sulphosuccinate (AOT) (Fig. 1C) was investigated for the first time in this work as an alternative ATPS to be used as a first step of CA recovery/purification protocol.

The experimental design methodology is very important in liquid–liquid extraction studies performed in aqueous systems, because it allows reducing the number of experiments as well as identifying both the independent variables with statistical significance for the process and their significant interactions [22–27]. So, extraction experiments were performed in this work according to a 2^4-full factorial design, where the concentrations of Triton X-114, AOT and NaCl and temperature were selected as the independent variables, and the CA partition coefficient, K_{CA}, and CA yield in the top phase, η_{CA}, as the responses.

2. Material and methods

2.1. Materials

Polyethylene glycol *tert*-octylphenyl ether (Triton X-114) and sodium bis(2-ethylhexyl) sulphosuccinate (AOT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Two different sources of CA were tested, specifically CA produced by *Streptomyces clavuligerus* ATCC 27064 fermentation and commercial CA (potassium salt) from Sigma–Aldrich (St. Louis, MO, USA) (purity 99%). Imidazole used in CA determinations was provided by Sigma–Aldrich (St. Louis, MO, USA). All the other reagents were of analytical grade and were used as received. All the solutions were prepared in McIlvaine's buffer, pH 6.5, consisting of 1.38 mM citric acid and 5.3 mM disodium phosphate in water purified by filtration through a Millipore Milli-Q ion-exchange system (Bedford, MA, USA). All glassware used in the experiments was washed in a 50:50 ethanol/1 M sodium hydroxide bath, washed in a 1 M nitric acid bath, rinsed copiously with Milli-Q water and dried in an oven at 90 °C.

2.2. Microorganism maintenance and fermentations

Streptomyces clavuligerus ATCC 27064 was kindly provided by the Microorganism Collection of the Department of Antibiotics of the Federal University of Pernambuco, Recife, PE, Brazil. The microorganism was maintained frozen at −70 °C with glycerol in a cryotube. A thick spore suspension contained in cryotube was transferred to 25 mL of reactivation medium in 250 mL-Erlenmeyer flasks. After incubation in an orbital shaker at 28 °C and 250 rpm for 24 h, 2.5 mL of this suspension were added to 22.5 mL of fermentation medium and again incubated in an orbital shaker under the same conditions.

For fermentations, 5.0 mL of the resulting cell suspension were added to 45 mL of fermentation medium in 500 mL-Erlenmeyer flasks and incubated in orbital shaker under the same conditions for 120 h. After fermentation, the broth was centrifuged at 3720 × *g* for 15 min at 5 °C in a centrifuge, model BR4i (Jouan, Saint Herblain, France). The supernatant was frozen in ultrafreezer at −70 °C to be used in the partitioning studies.

2.3. Media composition

To get vegetative cell from the spores, we used the reactivation medium proposed by Ortiz et al. [28], having the following composition (g/L in deionised water): glycerol (15), bacteriologic peptone (10), malt extract (10), K_2HPO_4 (2.5), $MgSO_4.7H_2O$ (0.75),