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Dedicated to Professor Florin Dan Irimie on the Occasion of His 65th Anniversary

7-AMINOCEPHALOSPORANIC ACID – PRODUCTION AND SEPARATION

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ABSTRACT. Cephalosporins, a large group of β-lactam antibiotics, contain a 7-aminocephalosporanic acid (7-ACA) nucleus which is derived from cephalosporin C, and substitutions of chemical groups or modifications of 7-ACA side-chains resulting in varying pharmacologic properties and antimicrobial activities, development of useful antibiotic agents, also. Cephalosporin C obtained by fungus fermentation can be transformed to 7-ACA by two-step or one step enzymatically conversion process. The most important step in 7-ACA downstream process is represented by its separation from enzymatically produced reaction mixture. Among the used methods new separation techniques have been developed and applied to bioseparations, like reactive extraction and pertraction which have considerable potential.

Keywords: *7-aminocephalosporanic acid, cephalosporin C, enzymatic reaction, pertraction, reactive extraction*

INTRODUCTION

Over the past 50 years, the industrial production of β-lactam antibiotics by fermentation is one of the outstanding examples of biotechnology. Cephalosporins are a large group of β-lactam antibiotics that are closely related to the penicillins. They are used for the treatment of infection diseases caused by gram-positive and

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gram-negative bacteria. They now comprise a group of antibiotics having a wide range of activity and low toxicity. Since their discovery in the 1950s, cephalosporins have become one of the largest classes of antibiotics. The class is divided into generations or subclasses, which are grouped by chemical properties and subsequent generalized microbiologic spectra. There are now more than 50 marketed cephalosporins [1].

Cephalosporins are widely used for treating and preventing bacterial diseases through disrupting the synthesis of the peptidoglycan layer of cell walls in both gram-positive and gram-negative bacteria [2].

Fungus *Cephalosporium acremonium* produces cephalosporin C (CPC) that is not potent for clinical use. Its molecule can be transformed to 7-aminocephalosporanic acid (7-ACA) as the intermediate compound for making semisynthetic cephalosporin derivatives that are of current interest with important applications in biopharmaceutical industries with a share of about 29% of the estimated worldwide antibiotic market.

PRODUCTION OF 7-AMINOCEPHALOSPORANIC ACID

The improved fermentation technology, by using producer microorganisms, like *Acremonium chrysogenum (syn. Cephalosporium acremonium)*, has enhanced the productivity and substantial cost reduction of the cephalosporins antibiotics production [3].

Enzymatic process technology for 7-aminocephalosporanic acid is also highly efficient (~80 - 90%), with new enzyme technology leading to major cost reductions over the past decade [4].

Cephalosporins contain a 7-aminocephalosporanic acid nucleus which is derived from cephalosporin C, and substitutions of chemical groups or modifications of 7-ACA side-chains resulting in varying pharmacologic properties and antimicrobial activities, development of useful antibiotic agents, also.

The diversity of structural types, coupled with potent antibacterial or β-lactamase inhibitory activity, provided a new incentive for expansion in the area of β-lactam chemistry directed towards semi- or total-synthesis of these new agents and analogues. The toxicity level of cephalosporin antibiotics is low and, as penicillin, hypersensitivity is the most common adverse effect.

7-aminocephalosporanic acid is the building-block chemical structure (Figure 1) of cephalosporin antibiotics and intermediates. Modifications of side-chains of 7-ACA affect the antibacterial activity and can lead to the alteration of pharmacokinetic properties and receptor binding affinity, thus creating new class of cephalosporin antibiotics with important clinical uses [5].

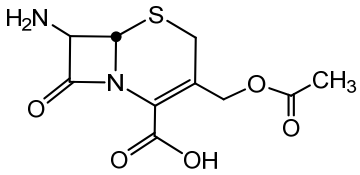

Figure 1. Chemical structure of cephalosporin C [6]

The most commonly used method to produce 7-ACA at industrially scale is chemical deacylation of chephalosporin C, a β-lactam antibiotic obtained by fermentation processes (Figure 2).

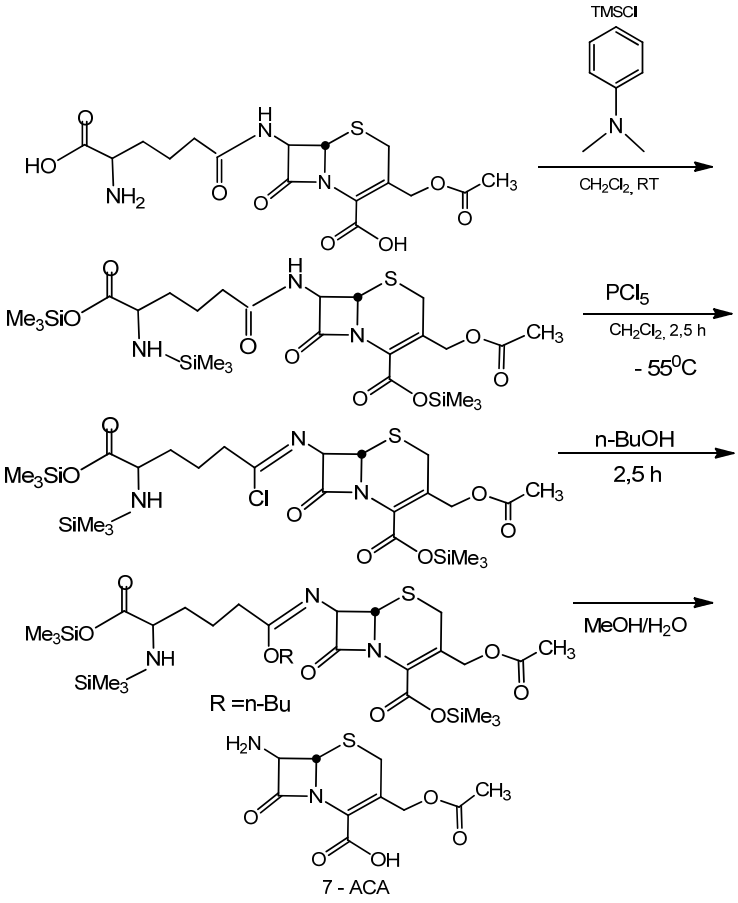

Figure 2. Chemical 7-ACA route starting from cephalosporin C [6]

However, this production method has many environmental and safety disadvantages, due to the large quantities of organic solvents and the production of toxic chemical waste. This process also utilizes many costly techniques in order to overcome environmental and ecological problems [6].

In the past decade, enzymatic methods for deacylation have attracted more attention in the manufacturing of cephalosporin antibiotics, and several enzyme-based methods have been developed. Therefore an enzymatic conversion of CPC to 7-ACA was developed, which reduce the process costs and has a positive environmental impact. Two principle enzymatic routes are proposed:

– Two-step cleavage with D-amino acid oxidase (DAAO) and glutarylacylase (GA) (Figure 3, left);

– One-step hydrolysis of CPC with a CPC acylase (CA) (Figure 3, right).

Industrial standard for 7-ACA production is represented by chemo-enzymatic hydrolysis of cephalosporin C by a two-step enzymatic process (Figure 3).

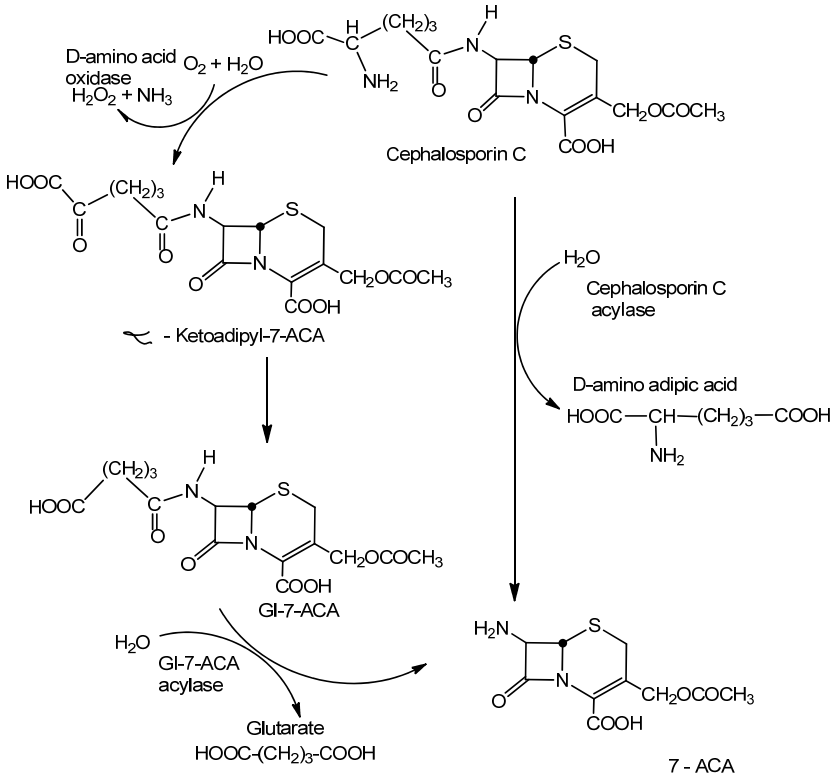

Figure 3. Chemo-enzymatic hydrolysis of cephalosporin C with 7-ACA production [7]

The transformation of cephalosporin C to 7-ACA and glutarate involves two step enzymatic conversions with the participation of three main intermediates: hydrogen peroxide (H_2O_2), α-ketoadipyl- (AKA-7-ACA), 7-β-(4-carboxybutanamido)-cephalosporanic acid (GL-7-ACA) [8].

The main problem of the method that involves one step 7-ACA bioconversion is the presence of H_2O_2 during the reaction process, which results in the inactivation of the employed enzymes, especially DAAO. In order to eliminate this main drawback of the enzymatic process, Lopez-Gallego et al. developed a system in which DAAO was co-immobilized with catalase (CAT), which is able to fully eliminate in situ the hydrogen peroxide formed by the neighboring DAAO molecules. For that, the oxidative deamination of CPC must produce only α-ketoadipyl-7-ACA, the kinetic parameters of GAC (glutaryl acylase) derivate being presented in Table 1. The conversion of cephalosporin C to 7-ACA was achieved with more than 80% yield in 180 minutes and only 2.5% of α-ketoadipyl-7-ACA was not hydrolyzed [9].

Table 1. Kinetics parameters of GAC with GL-7-ACA and α-ketoadipyl-7-ACA [9]

Kinetic parameters	Glutaryl amidase activity	α-ketoadipyl amidase activity
K_m [mM]	1.06 ± 0.05	4.9 ± 0.3
K_{cat} [min^{-1}]	97.8 ± 0.7	2.8 ± 0.5
K_{cat}/K_m [min^{-1} x mM^{-1}]	82.8 ± 0.4	0.56 ± 0.02

Another study reported the conversion of CPC to 7-ACA in a single reactor, by a fed-batch strategy, by simultaneous action of DAAO in the permeabilized *Pichia pastoris* cells and immobilized glutaryl-7-aminocephalosporanic acid acylase on support. The results indicated that CPC could be converted by simultaneous action of the two enzymes in the two different forms, to 90.9% 7-ACA, 5% AKA-7-ACA and 4.1% unidentified by-product within 2.5 h [10].

Direct conversion of cephalosporin C into 7-ACA was optimized by using cephalosporin C acylase from *Pseudomonas* N176 (named VAC). Conti et al. obtained 7-ACA as the main reaction product by converting over 98% cephalosporin C, under optimal conditions, thus obtaining approximatively 81 mg of 7-ACA in 41 hours from 15 mM cephalosporin C. All variants of VAC used were active on both substrates, cephalosporin C and Gl-7-ACA. The kinetic properties are presented in Table 2 [11].

Development of DAAO and GA into an industrial biocatalyst includes the fermentative production, the isolation, purification, and immobilization of the enzymes. Enzyme purification is needed to eliminate any unwanted catalytic activities found in cell extracts. DAAO as well as GA are both inactivated by separation of their subunits; to avoid this immobilization of the

enzymes on a solid support greatly increases their stability. Both enzymes have been immobilized as whole cells or as cell extracts in gels or on prefabricated carriers [12,13].

Table 2. Comparison of kinetic properties of VAC variants on CPC and GI-7-ACA substrates [11]

VAC variants	CPC substrate		GI-7-ACA substrate	
	K_m [mM]	V_{max}/K_m	K_m [mM]	V_{max}/K_m
VAC wild-type	9.5 ± 0.3	0.08	1.5 ± 0.2	24.3
VAC H57βS-H70βS	12.2 ± 0.9	0.24	6.9 ± 0.7	0.70
VAC HS-HS-F72βR	16.4 ± 2.3	0.10	2.0 ± 0.2	26.4
VAC HS-HS-L154βY	4.5 ± 0.7	0.73	2.0 ± 0.6	3.95

Pollegione et al. obtained ~90% conversion of cephalosporin C to 7-amino-cephalosporanic acid in a single deacylation step, starting from a known glutaryl-7-amino cephalosporanic acid acylase as the protein scaffold, an acylase gene optimized for expression in *Escherichia coli* and for molecular biology manipulations. The activity of the synthetic used gene (VAC) variants on CPC and GI-7-ACA substrates was investigated in order to determine the changes in substrate specificity. The most significant increase in activity on CPC was recorded by M4 mutant, while in the case of activity on GI-7-ACA, a significant increase in K_m was recorded for M5 and M6 mutants (Table 3) [14].

Table 3. Kinetic properties of mutant VAC on CPC and GI-7-ACA as substrates [14]

VAC mutants	CPC substrate		GI-7-ACA substrate	
	K_m [mM]	V_{max} (U/mg)	K_m [mM]	V_{max} (U/mg)
VAC wild-type	11	0.7	1.6	24.2
M1 (F270M)	6.5	0.7	2.7	24.6
M2 (A215Y)	6.9	1.8	1.7	16
M3 (D416Y)	7.2	0.7	1.7	4.9
M4 (D416Y-H417Y)	13.0	4.2	9.0	1.2
M5 (I44V-E49stop-D416Y-H417Y)	32.0	0.13	25.0	0.06
M6 (S22P-T394P-D416Y-H417Y)	24.5	0.02	37.2	0.11
M7 (E89A-A215Y)	18.9	0.10	1.1	0.66
M8 (A215Y-F270S)	10.1	1.56	2.0	23.8

Because two-step protocol shows some limitation, one-step protocol for 7-ACA production was developed, which can involve two aspects, i.e. the direct conversion of CPC to 7-ACA by a single enzyme, cephalosporin acylase or mutant of glutarylamidase [13].

A new challenge is represented by the utilization of Cephalosporin C acylase for the one-step enzymatic process to convert CPC into 7-ACA directly (Figure 4).

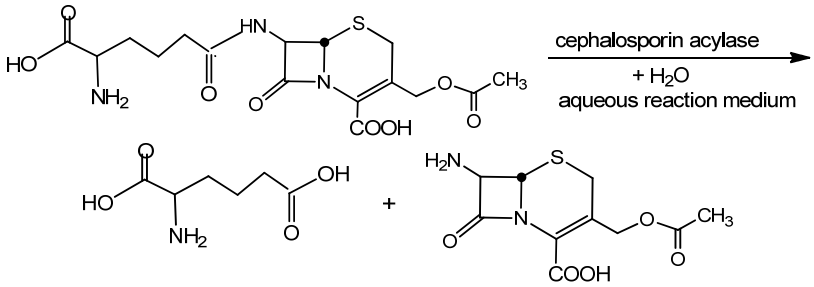

Figure 4. Mechanism conversion of CPC into D-α-aminoadipic acid and 7-ACA catalyst by CPC acylase [6]

But Cephalosporin C acylase produced by microorganisms (*Achromobacterxylosoxidans*, *Aeromonas* sp., *Arthrobacterviscosus*, *Bacillus laterosporus*, *Flavobacterium* sp., *Paecilomyces* sp., and *Pseudomonas* sp.) has low activity, therefore to increase its activity needs genetic engineering [15].

SEPARATION OF 7-AMINOCEPHALOSPORANIC ACID BY EXTRACTION

Following the enzymatic conversion process of cephalosporin C to 7-aminocephalosporanic acid, the product must be isolated from the reaction mixture. This separation process raises difficulties because the concentration of acid in the mixture is reduced and due to the presence of α-aminoadipic acid as by-product impurity. A general process flow diagram of production and separation of 7-ACA is presented below (Figure 5):

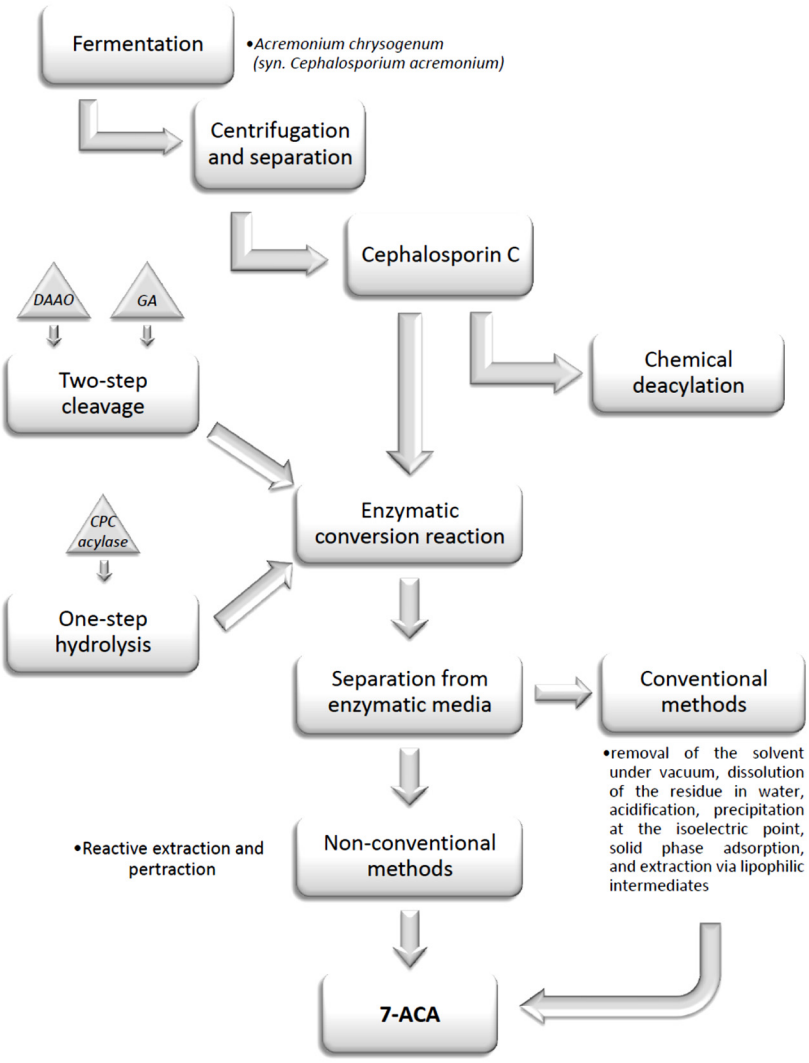

Figure 5. Process flow diagram of production and separation of 7-ACA

Since 7-ACA is an amphoteric molecule, physical solvent extraction is difficult. Various methods of extraction and purification of cephalosporin antibiotics have been studied (removal of the solvent under vacuum, dissolution of the residue in water, acidification, and precipitation at the isoelectric point of 7-ACA (pH = 3.5). These methods, along with solid phase adsorption, and extraction via lipophilic intermediates, have important materials and energy costs [16-18].

A study on the separation of 7-ACA aimed to remove the antibiotic from wastewater by using lipid-accumulating microalgae [19]. The results demonstrated that the microalgae strains that were tested, *Chlorella* sp. Cha-01, *Chlamydomonas* sp. Tai-03, and *Mychonastes* sp. YL-02, exhibited high resistance to 7-ACA in terms of cell growth and lipid production, for high 7-ACA contamination level at 100 mg 7-ACA l^{-1}. 7-ACA was mainly removed from antibiotic wastewater by hydrolysis, photolysis, and adsorption onto microalgae. The studied influential factors in microalgae cultivation were visible-light illumination, stirring and CO_2 aeration. 7-ACA removal efficiency was above 75% at 24 h in Cha-01 group.

In aqueous solutions, 7-ACA exists in ionic forms of different charges depending on the pH of the media. At pH below 2.02 (pK_{a1} = 2.02), the predominant form of 7-ACA is cationic, at pH above 4.42 (pK_{a2} = 4.42) is anionic, and in this range the zwitterion is predominant (Figure 6) [18,20].

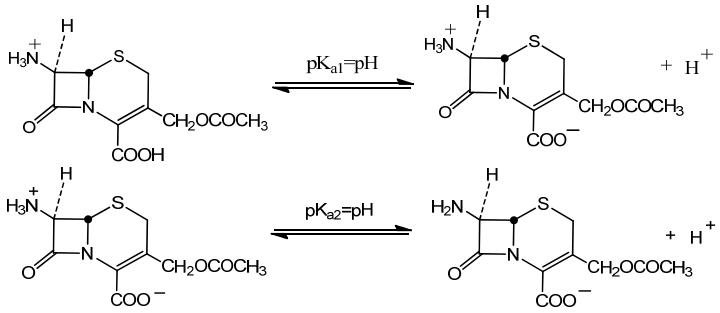

Figure 6. The chemical form of 7-ACA depending on the pH of the aqueous solution [20]

Therefore, a viable method for the separation of 7-ACA is extraction accompanied by chemical reaction with an extractant, namely reactive extraction [21]. There are two mechanisms of reactive extraction of β-lactams [17]: ion-pair extraction (the extractant dissolves in an organic phase and reacts with the β-lactam anions and a proton in the aqueous phase) and liquid-liquid ion exchange (the removal of a β-lactam anion from the aqueous phase by an ion-exchange with the anion of the extractant, typically a quaternary salt, dissolved in the organic phase).

The reactive extraction of 7-ACA using secondary and tertiary amines and a quaternary ammonium salt was studied by Bora et al. [18]. 7-ACA dissociates in the aqueous phase to give an anion and a proton. The dissolved amine in the organic phase reacts with 7-ACA anion and the proton, in the aqueous phase. The experiments were carried out using various carriers in butyl acetate: dioctylamine, Amberlite LA-1, Amberlite LA-2, trioctylamine, and Aliquat-336. According to this study, the most effective amine carrier was

Amberlite LA-2, which, however, exhibits opposite pH dependence on the distribution coefficient (K_d) to that achieved with Aliquat-336 (liquid-liquid ion-exchange extraction). As both extraction and back-extraction were easily performed at pH values of 8 and, respectively 5, Aliquat-336 was the preferred choice for the reactive extraction of 7-ACA.

Following the results of their previous study, the scientists analyzed the equilibrium and kinetics of 7-ACA extraction with Aliquat-336 dissolved in n-butyl-acetate as the solvent [22]. Varying the pH value of the aqueous solutions, the obtained equilibrium constant (K_P) for extraction of 7-ACA with Aliquat-336 was $20x10^2$ at a pH value of 8 and $5x10^2$ at a pH value of 5. The equilibrium constant increases with the pH of the aqueous solution, attributed to the increase in ionization of 7-ACA. The proposed mathematical model for the extraction of 7-ACA confirmed that the interfacial chemical reaction controls the extraction rate, the interfacial reaction between the 7-ACA anion and adsorbed solute-carrier complex playing a significant role in the extraction kinetics over the pH and concentration ranges that were studied.

In another study, 7-ACA and certain β-lactam antibiotics (7-aminodeacetoxy cephalosporanic acid (7ADCA), cephalexin, 6-aminopenicillanic acid (6-APA), and cephalosporin-C) were extracted from aqueous solution with Aliquat-336 dissolved in n-butyl acetate with a complimentary stripping kinetic study [23]. The feed phase pH value was maintained between 8 and 10 by using phosphate and carbonate buffer. The stripping phase pH varied between 3 and 5 in order to assess the pH effect on stripping rate.

Table 4. Maximum percentage stripping of various β-lactams and the corresponding pH values [22]

β-lactam	pH	% of stripping	J_S (mol/(cm^{2}s))
6-APA	4	67.4	3.74×10^{-10}
Cephalosporin-C	4	72.9	4.0×10^{-10}
Cephalexin	4	74.1	3.4×10^{-10}
7-ACA	4	75.5	3.22×10^{-10}
7-ADCA	3	83.3	5.15×10^{-10}

Table 4 offers a comparison of the extent of stripping and pH dependence of the stripping rate (*Js*), and indicates the role of the molecular structure of the β-lactams whose pK_a values are different from each other.

One of the most recent separation techniques, pertraction or permeation through liquid membrane, consists in the transfer of a solute between two aqueous phases through a liquid solvent membrane interposed between them. The extraction dynamic is based on the difference in pH value

and concentration between the feed and the stripping phase, thereby offering the possibility to transport the solute against its concentration gradient. Compared to physical extraction, this method reduces the solvent loss and requires small quantities of solvent and carrier agent, because of their constant regeneration [24].

Ghosh et al. reported some preliminary results on reactive extraction of cephalosporin C in a supported liquid membrane consisting in a mixture of Aliquat-336 as carrier and butyl acetate. The permeation rate of CPC depended on the carrier concentration in the membrane and the chloride ion concentration in the aqueous feed phase. By maintaining a fixed concentration of Cl^- ion, the increase of Aliquat-336 concentration led to an increase of the permeation coefficient (P) values, to a maximum at about 300-400 mol/m^3 of Aliquat-336 [25]. Based on this previous study, Sahoo at al. investigated the facilitated transport of 7-ACA in a bulk liquid membrane also of butyl acetate and Aliquat-336, positioned above the two aqueous phases [20].

The distribution coefficient increased up to 1.3 at a value of pH of 9.2, beyond which it decreased. The decrease of the distribution coefficient was attributed to the hydrolytic decomposition of 7-ACA at high pH values. However, since 7-ACA is stable in the pH range of 5 - 8, the experiments were carried out using aqueous solutions of pH = 8 - 9 and stripping phase pH = 5 - 6.

CONCLUSIONS

7-Aminocephalosporanic acid is an important intermediate in the production of semisynthetic cephalosporin antibiotics. The primary method for industrial production of 7-ACA is chemical deacylation of cephalosporin C, but this method has many environmental and safety disadvantages.

In recent years, enzymatic conversion of CPC to 7-ACA was developed, which reduces the process costs and has a positive environmental impact, by two-step cleavage with D-amino acid oxidase and glutarylacylase or one-step hydrolysis of CPC with a CPC acylase. Under optimized conditions, the conversion of CPC to 7-ACA was higher than 90%, with byproduct formation lower than 4%.

The separation of 7-ACA from an enzymatically produced reaction mixture was performed by numerous methods that have important materials and energy costs. Therefore, new techniques of separation were studied, namely reactive extraction and transport through bulk liquid membranes. The results published so far indicate that using Aliquat-336 as an extractant or carrier agent dissolved in different solvents may present a promising method for efficiently separate 7-ACA from enzymatic media, with over unity permeation and distribution coefficients for pH-values of 7- ACA aqueous solutions of 8.

These biotechnological achievements are industrial benchmarks in modern biocatalysis and separation processes and can lead to the industrial implementation of highly economical and sustainable production of 7-aminocephalosporanic acid.

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