FRACTIONATION OF THE MIXTURE OBTAINED BY THE ENZYMATIC HYDROLYSIS OF PENICILLIN G

Using the experimental data on the individual physical and reactive extraction of GAPA with 1,2-dichloroethane, D2EHPA and Amberlite LA-2 together with data on the influence of the pH value of the aqueous phase and the Amberlite LA-2 concentration on the selective separation of GAPA, PG and PAA from a mixture, a process flow sheet for the fractionation of the solution obtained by the enzymatic hydrolysis of PG has been elaborated and applied. Thus, at pH=10, GAPA was selectively separated by four extraction stages, the overall extraction degree being 98.8%. PG was selectively extracted from the raffinate at pH=6 by three extraction stages, adjusting the molar concentration of Amberlite LA-2 to the value of the PG molar concentration for each extraction stage. The antibiotic extraction degree was 99.6%.

6-Aminopenicillanic acid (GAPA) is the main component of semi-synthetic Penicillins, antibiotics that are obtained by GAPA acylation and formation of amide bonds differing from the natural ones. GAPA is the biosynthetic product of some Penicillin producing fungus grown on nutritive media without precursors. Thus, in the absence of phenylacetic acid, at the end of the P. chrysogenum fermentation cycle, the broth contains about 50% GAPA, 30% Penicillin K, 15% Penicillin DF and 5% Penicillin F [1]. GAPA could be obtained if the precursors are added into the media (phenylacetic acid for Penicillin G biosynthesis, phenoxycetic acid for Penicillin V), but at very low concentration levels (generally, 0.2 – 0.4%, rarely 2 – 3%). Because the biosynthesis of this compound is economically inefficient, some chemical or enzymatic methods for Penicillin G hydrolysis to GAPA were proposed and applied on the industrial scale [2-5].

The chemical hydrolysis of Penicillin G requires a lot of stages and uses special chemical agents and low temperature (≤60°C), which limits the application of the method [2,3]. The enzymatic hydrolysis on the other hand, is catalyzed by penicillinamidase and can be described by the following reaction:

\[
\text{Penicillin G} \xrightarrow{\text{Penicillinamidase}} \text{GAPA} + \text{Phenylacetic acid}
\]

The penicillinamidase most used on the industrial scale is produced by E. coli. This enzyme could be used after biomass separation, free or immobilized on different polymeric supports, or without biomass filtration, the Penicillin G hydrolysis occurring simultaneously with enzyme production. The best results were obtained for immobilized enzyme (the Penicillin G conversion exceeds 90%), the process being carried out at 35 – 40°C and pH=8.2 [2-4]. Recently, penicillinamidases biosynthesized by Bacillus megaterium, Streptomyces lavendulace, Achromobacter sp., Proteus retiger, Actinoplanes sp., Bovista plumee, Klyvera strophile, Pseudomonas melanogenum, Fusarium sp., Chaenia, have been isolated for Penicillin G or V hydrolysis [5,6].

The solution obtained by enzymatic hydrolysis of Penicillin G contains about 4–5% GAPA, 1.6–3% phenylacetic acid (PAA) and 0.8–1% unhydrolyzed Penicillin G (PG). The industrial separation of GAPA is achieved by acidification to pH=2.5, extraction of PG and PAA with butyl acetate, concentration and acidification with hydrochloric acid for GAPA precipitation [2,3]. This separation technology needs large amounts materials and high energy consumption, thus increasing the GAPA cost.

For this reason, the aim of this work was to establish the conditions for the selective separation of GAPA by reactive extraction from the mixture obtained by enzymatic hydrolysis of Penicillin G. In order to select the most efficient extractions system, the individual extractions of GAPA with organophosphoric acids (di-(2-ethylhexyl) phosphoric acid, D2EHPA) and high molecular weight amines type (lauryl-trialkyl-methylamine, Amberlite LA-2) extraction agents were initially studied. In the second step of the study the optimum conditions for the selective separation of GAPA from mixture containing PG and PAA by reactive extraction was analyzed.

Using the experimental data, a process flow sheet for the selective separation of GAPA, PG and PAA from
the mixture obtained by the enzymatic hydrolysis of Penicillin G has been elaborated and applied.

EXPERIMENTAL

The experiments were carried out in two steps. In the first step, the individual reactive extraction of 6APA was analyzed. For this purpose, an extraction column with vibratory mixing was used. This laboratory equipment has been described in detail in previous papers [7,8]. Phase mixing was achieved by means of a perforated disk of 45 mm diameter and 20% free section. The vibrations had a frequency of 50 s⁻¹ and an amplitude of 4 mm. The position of the mixer was maintained at the initial contact interface between the aqueous and organic phases. The extraction time was 1 minute. The resulting emulsion was removed through the base of the column and broken in a centrifugal separator at 5000 rpm.

For the first experiments step, the aqueous phase consisted on a solution of 3.3 – 3.5 g·l⁻¹ 6APA. In the second step, the aqueous solution contained mixtures of 6APA, PG and PAA. The concentrations of the components were varied as follows:

- 6APA: 4.32 – 45 g·l⁻¹
- PG: 7.49 – 10 g·l⁻¹
- PAA: 2.72 – 24 g·l⁻¹

The values of the maximum concentration correspond to the composition of the solution obtained by the enzymatic hydrolysis of Penicillin G.

The solvent was 1,2-dichloroethane, being used individually or as a solution of two types of extracting agents types: D2EHPA and Amberlite LA-2. The concentrations of the extractants in the organic phase were varied between 0 and 80 g·l⁻¹. The volume ratio between the aqueous solution and organic solvent was 1, each phase volume being 50 ml.

The pH of the initial aqueous solution was adjusted using a solution of 0.1M hydrochloric acid or 0.1M sodium hydroxide, depending on the desired pH value (the pH values were determined using a Consort C832 digital pH-meter). The pH values were recorded throughout each experiment and any pH change was noted.

The re-extraction of the extracted components from the organic phase was performed using the same laboratory equipment and a solution of 5–7% sodium carbonate. The volume of each phase was 50 ml.

The degree of extraction was calculated by means of the mentioned concentrations of the components in the initial solution and in the raffinate:

\[ Y = \left(1 - \frac{C_r}{C_0}\right) \times 100\% \]

The concentrations of 6APA, PG and PAA were measured using the high performance liquid chromatography technique (HPLC) with a Lichrospher 100 RP – 18 column (5 μm) and an UV detector at 225 nm. The mobile phase was a mixture of phosphate buffer (pH = 6) and acetanilide in a volume ratio of 4 : 1.

RESULTS AND DISCUSSION

Individual reactive extraction of 6APA

Owing to the presence of amine and carboxylic groups in the chemical structure of 6APA chemical, this compound exhibits amphoteric character, measuring the pH-values of the aqueous solution inducing the following dissociation equilibriums:

\[ \text{pK}_\text{COOH} = 2.3 \quad \text{pK}_\text{1} = 3.8 \quad \text{pK}_\text{NH}_2^+ = 8 \]

The corresponding pH-values were determined by potentiometric titration with solutions of \(10^{-3}\) M hydrochloric acid and \(10^{-3}\) M sodium hydroxide. The obtained values were closed to those given in literature [4,9]:

\[ \text{pK}_\text{COOH} = 2.3 \quad \text{pK}_\text{1} = 3.8 \quad \text{pK}_\text{NH}_2^+ = 8 \]

The formation of these ionic species considerably reduces the solubility of 6APA in nonpolar solvents. However, due to its acidic and basic properties, 6APA can react with acidic or basic extractants, thus allowing its solubilization in an organic phase. For this reason, the reactive extraction with D2EHPA and Amberlite LA-2 was studied. Thus, the extraction mechanism was comparatively analyzed by means of the influence of the pH-value of aqueous phase and of the concentration of the extracting agent in organic phase on the extraction degree.

As can be seen from Figure 1, increasing the pH initially leads to a reduction of the efficiency of the physical or reactive extraction reaching a minimum value at pH=6-7. This is followed by an increase of the extraction yield. This phenomenon is more pronounced during reactive extraction with Amberlite LA-2, the extraction degree being significantly higher compared to those obtained for physical or reactive extraction with D2EHPA.

The variation of the separation efficiency for the considered extraction systems can offer information about the extraction mechanism. Therefore, physical extraction, which is based only on the physical
solubilization of 6APA in 1,2-dichloroethane, is strongly limited by the ionization of this compound in aqueous solution. The physical extraction yield is very low, reaching maximum values only in the pH domain corresponding to the formation of the mono-ionized species (Y=7.7% at pH=2 and 14% at pH=12). For the domain of pH favorable to the appearance of zwitterions, the extraction efficiency is reduced, becoming 0 at pH=6.

The reactive extraction with D2EHPA is possible only in the acidic domain and occurs by means of the following interfacial ionic exchange reaction:

\[
\text{H}_3\text{N}^+ + \text{HP} \rightarrow \text{H}_2\text{N}^+\text{COOH} + \text{H}^+ \]

with ion-pair formation in the organic phase (X is the counter ion which exists in aqueous solution and is the anion of the acid used for pH correction). This mechanism is similar to the extraction of amino acids with Amberlite LA-2 [8].

Increasing the pH leads to a reduction of the extraction yield, due to the appearance of zwitterions in the aqueous solution. At pH-values greater than pH=6, the extraction efficiency increases strongly and reaches a maximum level at pH=10. In this pH domain, the extraction mechanism is different, the interfacial reaction between 6APA and Amberlite LA-2 becoming similar to that for Penicillins extraction [12]:

\[
\text{H}_2\text{N}^+\text{COOH} + \text{H}^+ + \text{A}^- (aq) \rightarrow \text{H}_2\text{N}^+\text{COOH} (o) + \text{A}^- (aq)
\]

The decrease of the degree of extraction at pH>10 could be the result of both the rapid chemical deactivation of 6APA in strong alkaline media, and of the formation of its sodium salt to a significant amount, a process which induces 6APA re-extraction from the solvent to the aqueous phase.

The proposed mechanisms were verified by analyzing the influence of the concentration of the extraction agent in 1,2-dichloroethane on the degree of extraction. As can be seen from Figure 2, the separation efficiency continuously increases with the concentration of extractants. However, for both extraction agents, the increase of the extraction degree is initially pronounced, but then diminishes. The modification of the evolution of the extraction yield is produced at an extractant concentration value corresponding to the equimolecular ratio between 6APA and Amberlite LA-2, respectively D2EHPA. This result confirms the formation of 6APA-extractant complex in organic phase by chemical reaction between one molecule of each compound.

Fractionation of the mixture obtained by the enzymatic hydrolysis of Penicillin G

The experimental data of the preliminary study on the reactive extraction of 6APA showed that the highest
degrees of extraction are obtained using Amberlite LA-2 as extracting agent (at pH=10, the extraction yield with Amberlite LA-2 is over 4.7 times greater compared with physical extraction or the reactive extraction with D2EHPA). Moreover, the literature indicates that the maximum extraction efficiency of PG and PAA with Amberlite LA-2 is reached at pH<5, decreasing at higher pH-values and becoming 0 at pH=9 for PG, respectively pH=8 for PAA [12].

By means of these assumptions, the reactive extraction of 6APA, PG and PAA from mixtures at different pH-values was studied. The experimental results are depicted in Figure 3 and suggest that the separation of PG and PAA is more effective in the pH domain below 7, over this pH-value only 6APA extraction being possible.

Figure 3. Influence of the pH of the aqueous phase on the degree of extraction of 6APA, PG and PAA from a mixture with Amberlite LA-2 (C_{6APA,0} = 5.04 g l^{-1}, C_{PG,0} = 8.70 g l^{-1}, C_{PAA,0} = 3.13 g l^{-1}, C_{Am,LA-2} = 80 g l^{-1}).

The separation selectivity can be described using a selectivity factor. For the studied extraction system, the selectivity factor, S1, is defined as the ratio between the degree of extraction of 6APA and the overall degree of extraction of PG and PAA:

\[ S_1 = \frac{Y_{6APA}}{Y_{(PG+PAA)}} \]

Analyzing the above results through the selectivity factor, it was observed that at pH≤7 S1 is lower than 1 but increases significantly with increasing pH and tending to ∞ for pH=9 (Figure 4). This evolution suggests the possibility of the selective separation of 6APA by reactive extraction with Amberlite LA-2 at pH=10. As the extraction degree of 6APA with 80 g l^{-1} Amberlite LA-2 at pH=10 is about 67%, four extraction stages are required for its total recovery from aqueous solution. Under these circumstances, the final raffinate will contain only PG and PAA.

The supplementary fractionation of the raffinate is based on the pH effect on the reactive extraction of PG and PAA with Amberlite LA-2. From Figure 3 it can be seen that both components are extracted in the acidic pH domain, the degree of PG extraction being superior. For this extraction system, the selectivity factor, S2, was calculated as the ratio between the yields of reactive extraction of PG and PAA:

\[ S_2 = \frac{Y_{PG}}{Y_{PAA}} \]

The selectivity factor S2 maintains at a value close to 1 for pH≤4, then increases strongly with increasing pH and tending to ∞ for pH=8 (Figure 4). Irrespective of the pH domain PG reactive extraction occurs simultaneously with PAA reactive extraction. Therefore, consideration of pH-value as the unique parameter which controls the extraction selectivity is not sufficient. Owing to the higher
acidity of PG ($k_a = 1.74 \times 10^{-6}$ for PG, whereas $k_a = 4.88 \times 10^{-4}$ for PAA [12]). Amberlite LA-2 will react mainly with PG. For this reason, limitation of PAA coextraction is possible by using a concentration of the extracting agent inferior to the stoichiometric amount needed for chemical reaction with both components in the aqueous phase.

By plotting the effect of the Amberlite LA-2 concentration on the selectivity factor $S_2$ two regions can be observed (Figure 5). Thus, for PG and PAA concentrations in the aqueous phase corresponding to those obtained by the enzymatic hydrolysis of PG (9.81 g l$^{-1}$ PG, 24 g l$^{-1}$ PAA), pH=6 and an extractant concentration below 10 g l$^{-1}$, the selectivity factor $S_2$ increases with increasing extractant concentration, reaches a maximum value of 15 for 10 g l$^{-1}$ Amberlite LA-2, and then decreases with higher values of the extractant concentration. By means of this evolution and interfacial reaction stoichiometry, it can be affirmed that in order to obtain a high selectivity of PG separation from PAA a molar concentration of Amberlite LA-2 in the organic phase equal to that of the concentration of PG in the aqueous phase is required.

The most pronounced difference between the degrees of extraction of PG and PAA was reached at pH=6 (64% for PG, 20% for PAA). Therefore, the selective separation of the two compounds can be carried out at this pH–value, using three stages of reactive extraction and adjusting the molar concentration of Amberlite LA-2 to the value of the molar concentration of PG for each extraction stage.

Using these data, a process flow sheet for the selective separation of the mixture obtained by enzymatic hydrolysis of PG (45 g l$^{-1}$ 6APA, 10 g l$^{-1}$ PG, 24 g l$^{-1}$ PAA) has been elaborated and applied (Figure 6). The separation conditions are given in Table 1.

The proposed method allows the initial selective separation of 6APA and then of PG with overall separation yields of over 98%, the final aqueous phase containing only PAA. The re-extraction of 6APA and PG from the solvent phase was performed with a 5–7% sodium carbonate solution, the process needing one stage. The re-extraction yield was about 97–98% and the regenerated solvent was used for subsequent separation cycle.

**CONCLUSIONS**

Owing to its amphoteric character, 6APA can react with both acidic and basic extracting agents. The study on the individual reactive extraction of 6APA with D2EHPA and Amberlite LA-2 indicated that the separation is more effective with Amberlite LA-2, in both cases the separation occurring by means of an
interfacial reaction between the components at equimolecular ratio.

Using the influences of the pH of the aqueous phase and the concentration of Amberlite LA-2 in 1,2-dichloroethane on selective separation of a mixture of 6APA, PG and PAA, a process flow sheet for the fractionation of solution obtained by the enzymatic hydrolysis of PG has been elaborated and applied. Thus, at pH=10, 6APA was selectively separated using four extraction stages, the overall extraction degree being 98.8%. PG was selectively extracted from the raffinate at pH=6 using three extraction stages, with the molar concentration of Amberlite LA-2 being adjusted to the value of the molar concentration of PG for each extraction stage. The antibiotic extraction degree was 99.6%. The final aqueous phase contained only PAA which can be used as a precursor for PG biosynthesis.

NOTATIONS

\( A \) — Amberlite LA-2

\( C_{6APA} \) — initial concentration of 6APA in the aqueous phase, g.l\(^{-1}\)

\( C_{PAA} \) — initial concentration of PAA in the aqueous phase, g.l\(^{-1}\)

\( C_{PG} \) — initial concentration of PG in the aqueous phase, g.l\(^{-1}\)

\( C_{AmberLA2} \) — Amberlite LA-2 concentration in the organic phase, g.l\(^{-1}\)

\( C_{D2EHPA} \) — D2EHPA concentration in the organic phase, g.l\(^{-1}\)

\( k_0 \) — acidity index

\( pK_i \) — pK-value corresponding to the isoelectric point

\( S_i \) — selectivity factor for 6APA separation from PG and PAA

\( S_2 \) — selectivity factor for PG separation from PAA

\( Y \) — extraction degree, %

REFERENCES


IZVOD

RAZDVAJANJE SMEŠE NASTALE ENZIMSKOM HIDROLIZOM PENICILINA G

(Naučni rad)

Dan Cascaval¹, Corneliu Oniscu¹, Anca-Irina Galaction²

¹ Tehnični univeritet "Gh Asachi", ladi, Rumunija
² Univerzitet medicine in farmacije, ladi, Rumunija

U radu je objašnjen in primenjen postopak fraksioniranja smeše dobijene nakon enzimske hidrolize Penicilina G koriščenjem eksperimentalnih podatka individualnih procesa ekstrakcije in reaktivne ekstrakcije 6APA sa 1,2 dihloroethanom, D2EHRA in amberlitom LA-2, kao in podatka o učinku pH vrednosti vodenog rastvora in koncentracij Amberlite LA-2 na selektivnost razdvajanja 6APA, PG in PAA iz složene smeše. Utvrdjeno je je, na pH=10, moguće selektivno izdvojiti 6APA primerno četiri stupnja ekstrakcije, pri čemeru se ostvaruje izdvajanje ob 98,8%. PG se selektivno izdvaja iz raffinate na pH=6 u tri stupnja ekstrakcije, kada se podeli molarna koncentracija Amberlite LA-2 koja odgovara koncentraciji PG u svakom stupnju ekstrakcije. Ovim putem može se izdijeliti 98,8% ovog antibiotika.