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GREEN TECHNOLOGY FOR 6-AMINOPENICILLANIC ACID PRODUCTION - STUDY OF PENICILLIN G HYDROLYSIS IN A BIOREACTOR WITH MOBILE BED OF IMMOBILIZED *PENICILLIN AMIDASE* UNDER SUBSTRATE INHIBITION

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Abstract

The efficiency of enzymatic hydrolysis of Penicillin G to 6-Aminopenicillanic acid under substrate inhibition has been analyzed for a bioreactor with mobile bed of immobilized *penicillin amidase*. The results indicated that the optimum values of temperature and pH remained the same as for homogeneous hydrolysis using free enzyme. The inhibitory effect induced at higher Penicillin G amount in liquid phase was attenuated by increasing the size of the biocatalyst particles. For this reason, at substrate concentration over 150 mol/m^3, the highest volumetric productivity of hydrolysis process was recorded for the larger particles of immobilized enzyme. The proposed mathematical correlation between the volumetric productivity, Penicillin G concentration and biocatalyst diameter offers a good concordance with the experimental data, the average deviation being of ±4.53%.

Key words: bioreactor, 6-Aminopenicillanic acid, Penicillin, *penicillin amidase*, productivity

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1. Introduction

6-Aminopenicillanic acid is the precursor for semi-synthetic beta-lactamic antibiotics production (amoxycillin, ampicillin, etc.). This acid can be obtained by chemical synthesis, but this method requires a lot of intermediary stages with high consumption of materials and energy, offering rather low overall yield (Wu et al., 2010).

The 6-Aminopenicillanic acid could be obtained also by *Penicillium sp.* fermentation without precursor, but the degree of substrate conversion to the desired product is unsatisfactory (the biosynthetic mixture contains up to 50% 6-Aminopenicillanic acid, the rest being non-bioactive Penicillins) (Cao et al., 2011; Caşcaval et al., 2012). For this reasons, the current method applied at industrial scale for 6-Aminopenicillanic acid production consists on the deacylation of natural Penicillins (Penicillins G and V) with immobilized *penicillin amidase* (Bianchi et al., 1996). By using renewable substrates, as well as low-expensive and eco-friendly technical solutions, this technology respects the concept of "white biotechnology", the interest in producing 6-Aminopenicillanic through enzymatic method being increased in the last decade.

Penicillin amidase is produced by various microorganisms (*Escherichia coli, Bacillus megatherium, Arthrobacter viscosus, Streptomyces sp.*), the main commercial producer being *E. coli*. For

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increasing enzyme stability, as well as for facilitating its recovery and re-using in successive hydrolysis cycles, several inorganic and organic supports have been developed for *penicillin amidase* immobilization by cross-linking, adsorption, physical entrapment, covalent binding (polyacrylamide, agarose, chitosan, epoxy-activated, etc.) (Bianchi et al., 1996; Fang et al., 2010; Huang et al, 1996; Knezevi et al. 2006; Nabais and Cardoso, 2000). Among them, the covalent immobilization of *penicillin amidase* in the epoxy-activated support Eupergit C is efficiently applied at large scale (Huang et al, 1996; Nabais and Cardoso, 2000).

As it was previously stated, the use of immobilized enzymes offers the advantages of the increase of the thermal, chemical and to the shear forces resistance of the biocatalysts. Other advantages are the attenuation of the substrates inhibition process, the easier recovery of the biocatalysts from the final medium, and, implicitly, the increase of number of the repeated enzymatic reaction cycles which use the same particles of biocatalysts (Lupăşteanu et al., 2007). The bioreactors using immobilized biocatalyst can be designed as stirred, column, gas-lift or membrane bioreactors, being operated in batch, continuous or semicontinuous systems, with fixed, mobile/stirred, expanded or fluidized bed. Most of the industrial processes for 6-Aminopenicillanic acid synthesis have been carried out using bioreactors with partially or completely mobile bed of immobilized *penicillin amidase*. These bioreactors are operated using the "classical" kinetic models and physical laws describing the heat and mass transfer in stirred bioreactors.

But, these models either are valid only for the homogeneous systems, with free enzymes, or are partially adapted to the heterogeneous systems, with immobilized enzymes, without taking into consideration the geometrical characteristics of the biocatalysts particles.

Moreover, as it was previously stated, for certain operating parameters of the stirred bioreactor, the biocatalyst activity and physical integrity depend strongly on its size and enzyme concentration inside it (Galaction et al., 2007, 2011).

Therefore, the rate and efficiency of enzymatic hydrolysis of Penicillin G to 6-Aminopenicillanic acid by immobilized *penicillin amidase* in a bioreactor with mobile bed of biocatalyst has to be analyzed in correlation with the specific factors influencing this process.

Because the enzymatic process is affected by substrate inhibition (Illanes, 2008; Kumar et al., 1996), this paper reports firstly the study on the cumulated influences of the medium parameters (pH-value, temperature) and biocatalyst particle size on the Penicillin G hydrolysis in a batch bioreactor with mobile bed of immobilized *penicillin amidase* under inhibitory effect.

2. Materials and methods

The experiments have been carried out in 5 l (4 l working volume) batch laboratory bioreactor (Biostat A, B. Braun Biotech International), with computer-controlled and recorded parameters (Caşcaval et al., 2011). The mixing equipment consists on two pitched bladed turbines of 64 mm diameter and three baffles. The lower impeller has been placed at 64 mm from the bioreactor bottom. The upper impeller was placed on the same shaft at a distance of 32 mm from the inferior one.

The rotation speed was maintained at 250 rpm, this value avoiding the "cave" formation at the broths surface, solid phase deposition at the bioreactor bottom and mechanical disruption of the biocatalysts particles. According to the previous results, these impellers combination and rotation speeds were found to be the optimum ones for the investigated fermentation system (Galaction et al., 2007). Any mechanical damage of the biocatalyst due to the shear forces was recorded during the experiments.

Penicillin amidase from *E. coli* (Fluka), with 180 UI, was immobilized in Eupergit C, according to the covalent binding method described by Torres-Bacete et al. (Torres-Bacete et al., 2000). The following sizes of the biocatalysts have been used: 1.0, 1.5 and 2.0 mm, respectively. In all cases, the volumetric fraction of the immobilized enzymes into the medium was 0.28.

The medium was a solution of Penicillin G potassium salt (Merck) with concentration 80 - 300 mol/m^3. This solution pH has been varied between 7 and 9, being maintained at the prescribed value with phosphate buffer. The enzymatic hydrolysis has been carried out at different temperatures varying between 20 and 40^oC. The hydrolysis rate and efficiency have been calculated by means of the variation of Penicillin G and 6-Aminopenicillanic acid concentrations in the liquid bulk volume during the enzymatic conversion.

These compounds concentrations have been measured by high performance liquid chromatography technique (HPLC) using an UltiMate 3000 Dionex system with a Acclaim 120 C18 column (4.6 mm diameter, 150 mm length), provided with the Variable Wavelength RS Detector at 220 nm (Caşcaval et al., 2012).

The mobile phase was a mixture of 28% acetonitrile and 72% solution of 0.64 g/l KH_2PO_4 with a flow rate of 0.7 mL/min. The analysis temperature was 30^oC. The hydrolysis end has been considered when the Penicillin G conversion degree reached minimum 90 - 95%. Each experiment has been repeated for two or three times for identical conditions, the average value of the considered parameters being used. The average experimental error was of ±6.08 %.

3. Results and discussion

The influence of pH-value of hydrolysis medium on enzymatic conversion rate has been analyzed for the three diameters of immobilized *penicillin amidase* particles, d_P, for process without and with inhibitory effect of Penicillin G (according to Kumar et al. (1996) and Caşcaval et al. (2012), the Penicillin G concentration over 80 mol/m^3 can be considered to exhibit an inhibitory effect on free enzymes activity; the inhibition occurs at higher substrate concentration for immobilized enzymes (Caşcaval et al., 2012; Kumar et al., 1996)). In all cases, Figs. 1 and 2 indicate that the optimum pH-value is 8, as well as the fact that the lowest rate of 6-Aminopenicillanic acid are reached for neutral or acidic pH-domain. This result is also suggested in Fig. 3 by means of the dependence between the hydrolysis yield and pH-value. Moreover, from Fig. 3 it can be observed that the negative effect of the increase of pH-value over pH = 8 on Penicillin G hydrolysis yield is less important than that of the pH decrease below the optimum pH-value.

The variation of Penicillin G hydrolysis yield with temperature, plotted in Fig. 4 for the considered biocatalysts particles sizes and two substrate initial concentrations, C_{PG}, respects the general shape of the dependence between the efficiency of enzymatic conversions, or enzymes activity, with temperature. In this case, the optimum temperature was found to be 30^{o}C. But, indifferent of the Penicillin G concentration, the influence of the lower temperatures on 6-Aminopenicillanic acid formation efficiency is stronger than that of the higher ones. The attenuation of the temperature effect for temperatures over 30^{o}C is more important by increasing the size of immobilized *penicillin amidase* particles. Thus, for the biocatalysts with 2 mm diameter, the values of the hydrolysis yield recorded for the temperature variation domain between 30 and 40^{o}C become rather similar.

This phenomenon is the result of the amplification of the resistance to the heat transfer inside the biocatalyst by increasing the particle diameter. Consequently, the internal temperature is lower than that of the liquid phase, this counteracting the negative effect of the medium heating over 30^{o}C. For this reason, the value of the hydrolysis yield reached at 40^{o}C for the largest particles of immobilized *penicillin amidase* exceeds those corresponding to the smaller biocatalyst particles. Therefore, by increasing the biocatalyst diameter it is possible to move the optimum temperature, related to the liquid phase, to higher values. From Figs. 3 and 4 it can be observed that the hydrolysis yield is reduced by increasing the Penicillin G concentration.

Thus, considering the hydrolysis process duration of 5 hours, the yield corresponding to the less concentrated solution of Penicillin G is for about 1.4 - 1.6 times greater than that obtained for the solution with 200 mol/m^3 substrate. In these circumstances, the time needed for overall consumption of substrate increases from 5 - 5.5 hours for 80 mol/m^3 Penicillin G to over 10 hours for 200 mol/m^3 Penicillin G.

Although this variation seems to be due to the increase of substrate amount per enzyme UI, in the case of Penicillin G enzymatic hydrolysis the inhibitory effect of substrate is significant and controls the rate of 6-Aminopenicillanic acid formation (Kumar et al., 1996; Illanes, 2008). This assertion is supported by the order of the biocatalyst particle sizes corresponding to the decrease of the hydrolysis yield.

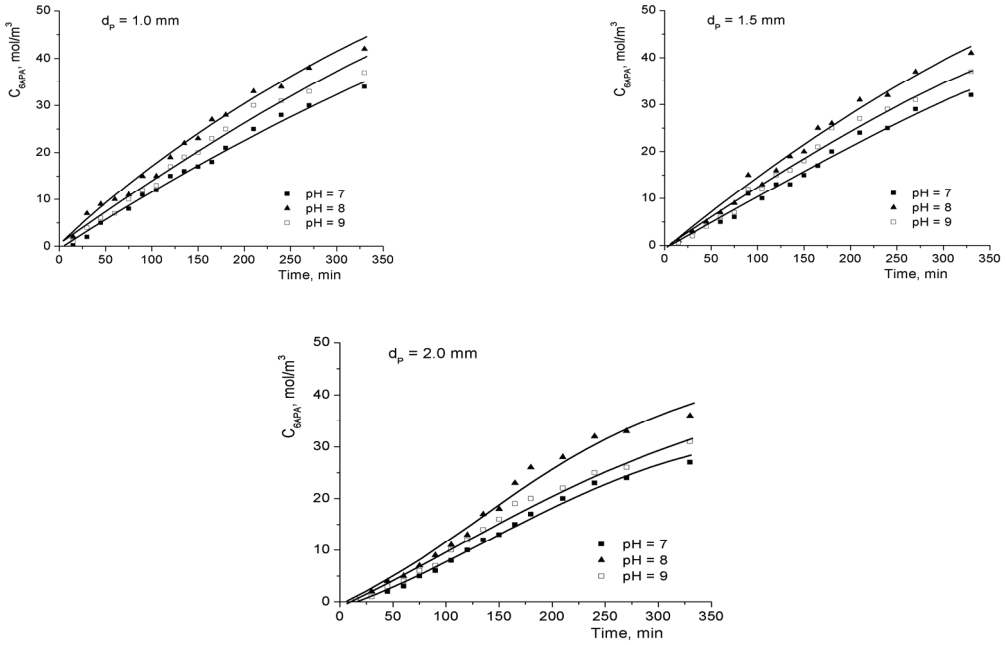

Fig. 1. Influence of pH-value and biocatalyst particle size on 6-Aminopenicillanic acid formation rate (Penicillin G concentration = 80 mol/m^3, temperature = 30^{o}C)

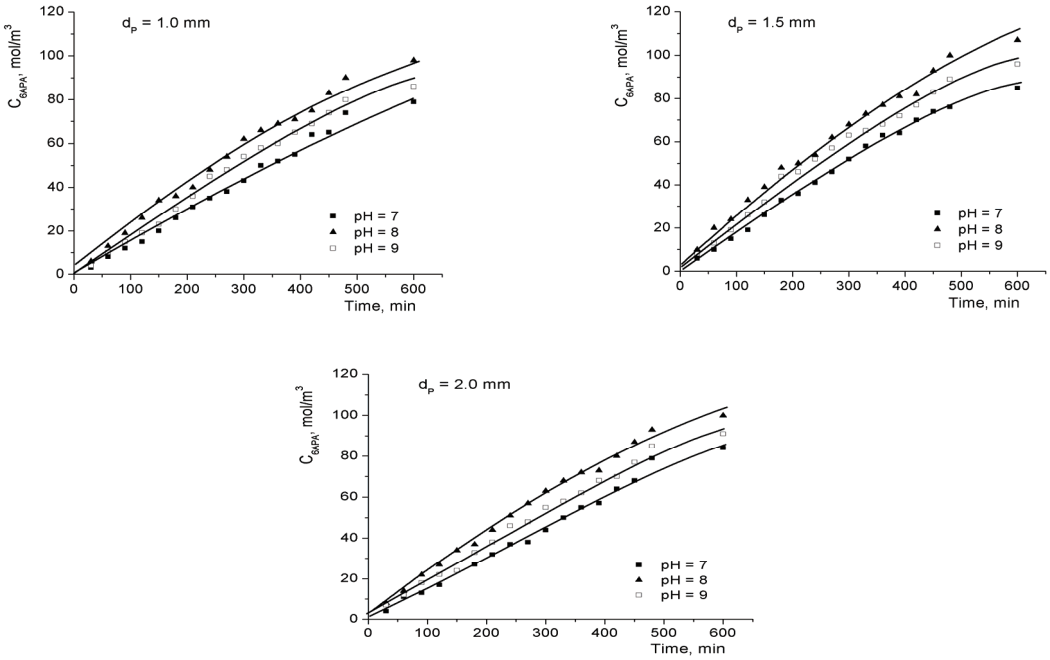

Fig. 2. Influence of pH-value and biocatalyst particle size on 6-Aminopenicillanic acid formation rate (Penicillin G concentration = 200 mol/m^3, temperature = 30^{o}C)

Therefore, either by varying pH-value or temperature, for Penicillin G concentration of 200 mol/m^3 the highest yield is reached for the intermediary immobilized enzyme particles (diameter of 1.5 mm), while the lowest one for the smallest biocatalysts (Figs. 3 and 4). This result is contrary to that obtained for the medium containing 80 mol/m^3 substrate, the highest efficiency of enzymatic conversion corresponding to the smallest biocatalyst particles.

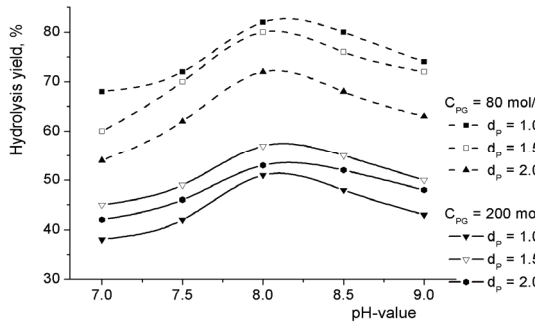

Fig. 3. Influence of pH-value and biocatalyst particle size on hydrolysis yield (temperature = 30^{o}C, process duration = 5 h)

At high amount of substrate in the liquid phase, due to the internal diffusion of substrate inside the biocatalyst, the inhibitory effect of Penicillin G is attenuated, this leading to the increase of the hydrolysis yield with the variation of particles diameter from 1 to 1.5 mm (Fig. 5). For larger biocatalyst particles, the internal diffusion through the inert matrix used for enzyme immobilization becomes an important limiting step, its related resistance hindering supplementary the Penicillin G conversion to 6-Aminopenicillanic acid. In conclusion, at higher Penicillin G concentration, the equilibrium between the phenomena of diminution of substrate inhibition and of enhancement of resistance to substrate internal diffusion leads to reaching the maximum hydrolysis yield for the biocatalyst with the intermediary size.

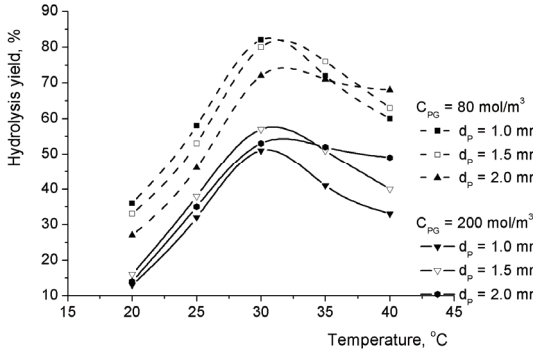

Fig. 4. Influence of temperature and biocatalyst particle size on hydrolysis yield (pH = 8, process duration = 5 h)

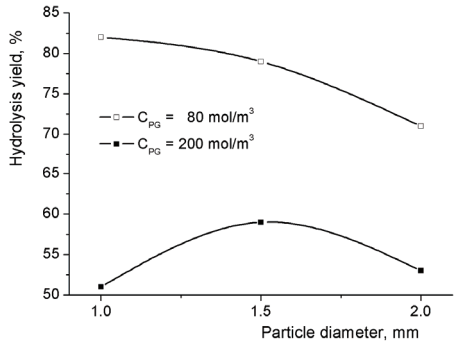

Fig. 5. Influence of biocatalyst particle size on hydrolysis yield (pH = 8, temperature = 30^{o}C, process duration = 5 h)

Because for a given moment of the conversion process the hydrolysis yield depends on the ratio between the Penicillin G amount in the medium and enzyme UI inside the biocatalyst particle, the productivity represents the most criterion relevant for analyzing the performance of the enzymatic process. In this case, the volumetric productivity, P, is defined by Eq. (1):

$$P = \frac{C_{6APA}}{t}, \text{ mol/m3h} \qquad (1)$$

being calculated at the end of the hydrolysis cycle.

The dependence between the volumetric productivity and the size of immobilized *penicillin amidase* particles at different initial concentration of Penicillin G in the liquid phase is graphically presented in Fig. 6 for the optimum process conditions. For all experimented sizes of biocatalyst particles, the plotted variations indicate the improvement of the volumetric productivity by increasing the initial concentration of substrate up to 150 mol/m^3.

For this domain of Penicillin G concentration, the highest volumetric productivity is reached for the smallest biocatalyst particles, as the result of the lowest magnitude of diffusional resistance to the substrate diffusion inside the inert matrix.

The supplementary increase of Penicillin G concentration in medium over 150 mol/m^3 leads to the significant differentiation of the variations of hydrolysis volumetric productivity related to the biocatalysts diameter. Thus, for the immobilized enzyme particles with 1 mm diameter, the hydrolysis volumetric productivity decreases for about 1.5 times by varying the substrate concentration from 150 to 300 mol/m^3.

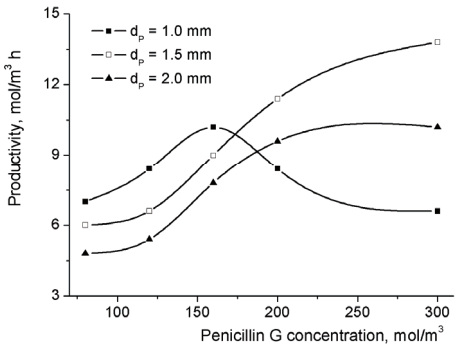

Fig. 6. Influence of Penicillin G concentration on hydrolysis volumetric productivity (pH = 8, temperature = 30^{o}C)

The negative influence of Penicillin G concentration increase is the consequence of the inhibitory phenomenon generated by the high substrate concentration both in the liquid phase and inside the particle.

As it was above mentioned, the enlargement of the biocatalyst particles counteracts the substrate inhibition. This positive effect on the volumetric productivity is underlined by the continuous increase of this parameter with the amount of Penicillin G for the biocatalyst with intermediary size, its value corresponding to 300 mol/m^3 substrate being for about 2.2 times greater than that recorded for the smallest particles.Owing to the internal diffusional resistance, which becomes important for the largest particles of immobilized *penicillin amidase*, the volumetric productivity reached for the largest biocatalyst at higher substrate concentrations is inferior to that for the particles with 1.5 mm diameter, but higher than that for the smallest biocatalysts.

The cumulated influences of penicillin G concentration and diameter of immobilized enzyme particles on hydrolysis productivity is given in Fig. 7 and has been included in following mathematical correlation between these parameters (Eq. 2):

$$P = 1.17 \cdot \frac{C_{PG}^{\ 0.37}}{d_P^{\ 3.21 \cdot 10^{-3}}}, \text{ mol/m}^3\text{h} \qquad (2)$$

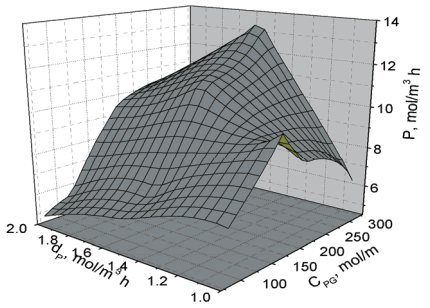

Fig. 7. Cumulated influences of Penicillin G concentration and biocatalyst particle size on hydrolysis volumetric productivity (pH = 8, temperature = 30^{o}C)

This equation is valid for Penicillin G concentration domain between 80 and 300 mol/m^3 and variation of biocatalyst particle diameter from 1 to 2 mm, the enzymatic process occurring at optimum values of temperature and pH. Using the proposed equation (2), the calculated values of hydrolysis volumetric productivity are in concordance with the experimental ones, the maximum deviation being +9.15% and the average one ± 4.53% (Fig. 8).

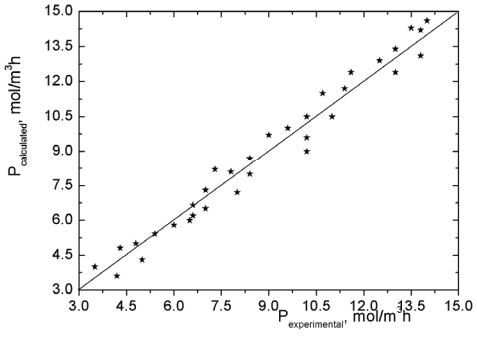

Fig. 8. Correlation between the calculated and experimental value of volumetric productivity (pH = 8, temperature = 30^{o}C)

4. Conclusions

The enzymatic hydrolysis of Penicillin G to 6-Aminopenicillanic acid with *penicillin amidase* immobilized in Eupergit C particles indicated that the optimum pH-value (pH = 8) and temperature (30^{o}C) were the same as for homogeneous hydrolysis with free enzyme. However, by enzyme immobilization the optimum temperature is moved to higher value and the inhibitory effect of Penicillin G is attenuated.

At substrate concentration below 150 mol/m^3, the maximum volumetric productivity corresponds to the biocatalyst with the diameter of 1 mm, due to the lowest resistance to the Penicillin G diffusion inside the inert matrix of support. At substrate concentration over 150 mol/m^3, as the consequence of the diminution of the Penicillin G inhibition inside the larger particles, the highest efficiency of 6-Aminopenicillanic acid production was recorded for biocatalysts particles with 1.5 mm diameter, while the lowest one for the smallest biocatalyst (1 mm diameter). Therefore, the larger size of immobilized *penicillin amidase* particles is recommended for concentrated solutions of Penicillin G, but the process duration increases significantly.

By taking into consideration the cumulated effects of substrate concentration and biocatalysts size, the hydrolysis process has been mathematically described by means of its volumetric productivity. The calculated values are in concordance with the experimental ones, the average deviation being of ±4.53%.

The aim of the future work is to complete this study with the analysis of 6-Aminopenicillanic acid production costs in a stirred bioreactor for selecting the combination Penicillin G concentration - biocatalyst size which could be optimum from technical and economical points of view simultaneously.

Acknowledgements

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