

Enzymatic Synthesis of Amoxicillin with Immobilized Penicillin G Acylase

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Abstract. *The synthesis of amoxicillin with immobilized penicillin G acylase (PGA) in aqueous medium was investigated. The parameters studied were: time course of amoxicillin production, concentration of substrates: hydroxyphenylglycine methyl ester (HPGM) and 6-aminopenicillanic acid (6-APA) and the effect of enzyme (PGA) content and pH, under variable and constant conditions and temperature variations. In the study of two substrate concentration on amoxicillin production, impressive results were obtained for a 1/3 ratio of 6-amino penicillanic acid (6-APA) and hydroxyl-phenylglycine methyl ester (HPGM). The synthesis of amoxicillin was preferable at constant pH rather than a variable one. Other optimal conditions obtained were: enzyme concentration: 5 g/L with 100U, process time: 480 min and temperature: 35° C. The yield for amoxicillin synthesis under prescribed conditions showed up to 50%.*

Keywords: *Amoxicillin; Penicillin G acylase; Aqueous medium; Enzyme content .*

INTRODUCTION

Semi-synthetic B-lactam antibiotics are produced mostly by chemical synthesis. In this process, an amino B-lactam such as 6-aminopenicillanic acid (6-APA), usually having its carboxyl group protected, reacts with an activated side-chain derivative followed by the removal of the protecting group by hydrolysis. In spite of the high yields that this process has achieved, it has been criticized for several disadvantages. These reactions typically involve costly steps, such as low temperatures, and toxic organic solvents, such as methylene chloride and silylation reagents. Also, high volumes of waste and byproducts have made this process undesirable [1].

Amoxicillin is one of the major B-lactam antibiotics, with sales of US \$2200 million as a bulk formulated drug in 1994 [2]. Furthermore, as a broad-spectrum antibiotic, this semi-synthetic penicillin is applicable against a wide variety of bacterial infections [3]. Nowadays, amoxicillin is produced in industry through a chemical route. Due to the

significant disadvantages of this process, the enzymatic synthesis of amoxicillin has become more interesting due to the increasingly tight environmental regulations. Mild reaction conditions and aqueous solutions may be used in the enzymatic reactor. The industrial enzymatic synthesis of semi-synthetic penicillin and cephalosporins is only taking its first steps [4].

This work is an optimization study of the enzymatic synthesis of amoxicillin, to identify process conditions under which this “green process” might become economically competitive. The kinetically controlled synthesis of amoxicillin from hydroxyl-phenylglycine methyl ester (HPGM) and 6-amino penicillanic acid (6-APA) catalyzed by penicillin G acylase (PGA) was studied. PGA from *Escherichia coli* requires that the phenylglycine carboxyl group be protonated while, at the same time, the amino group of the B-lactam nucleus be neutral, available for nucleophilic interactions. However, for the range of pHs where the enzyme is active (pH 6-8), the number of substrates molecules having reactive groups with the proper charge is negligible.

The use of derivatives of p-hydroxyphenylglycine (either esters or amides) is necessary because the direct, thermodynamically controlled synthesis of amoxicillin is not favored. The kinetically controlled synthesis of amoxicillin is a strategy presented by several studies [5,6]. Many investigators have studied the catalytic mechanism of PGA and its hydrolytic pathways, which

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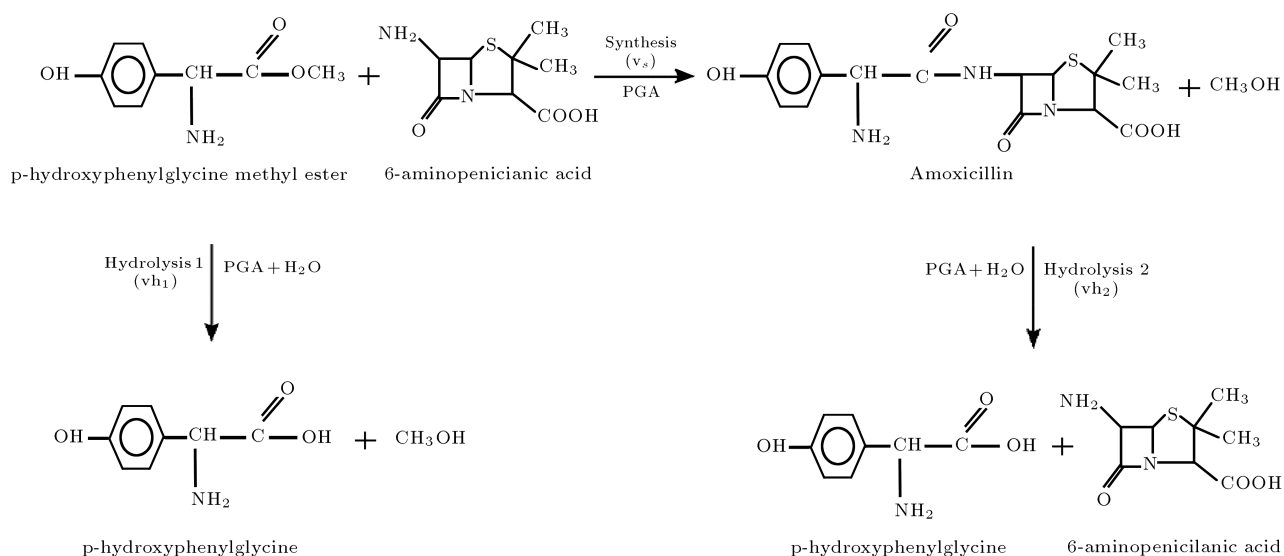


Figure 1. Kinetically controlled synthesis of amoxicillin using Penicillin G acylase as catalyst.

already been elucidated. Also, a fully mechanistic kinetic model is available for both the hydrolytic or synthetic reaction. Figure 1 shows the kinetically controlled synthesis of amoxicillin from HPGM and 6-APA. Two side reactions, also catalyzed by PGA complete with the synthesis of amoxicillin.

The enzyme penicillin acylase immobilization is studied in some research [7-10], which catalyze the synthesis (reaction I): This reaction is irreversible, but if amoxicillin hydrolysis increases, the amount of product will be reduced. By coupling HPGM and APA, the undesired reactions are: substrate hydrolysis, HPGM (reaction II) and product hydrolysis, amoxicillin, (reaction III). Both side reactions lead to hydroxyphenylglycine (HPG) [11-14]. Other antibiotics, such as cephalixin synthesized by enzymatic processes are studied by other researchers [15-20].

- I) Synthesis:

$$\text{APA} + \text{HPGM} \rightarrow \text{Amox} + \text{MeOH},$$
- II) Substrate hydrolysis:

$$\text{HPGM} + \text{H}_2\text{O} \rightarrow \text{HPG} + \text{MeOH},$$
- III) Product hydrolysis:

$$\text{Amox} + \text{H}_2\text{O} \rightarrow \text{HPG} + \text{APA}.$$

In a batch reactor, both substrates may initially be mostly undissolved. In addition, enzyme activity is limited to a narrow range of temperature and pH.

As a first step, the concentrations of the two substrates, HPGM and 6-APA, have to be known in order to reach an acceptable production of products. For choosing these concentrations, several points should be noticed, such as the solubility of the two substrates. According to the low solubility of these substrates, a

narrow range of concentrations (10-90 mM) can be examined. But, the key point is the ratio of their concentration. In this study, several concentrations of HPGM and 6-APA have been examined and the suitable ratio is introduced.

Secondly, the concentration of enzyme is one of the determining factors in the enzymatic synthesis of amoxicillin, since, with the low amount of enzyme, the reaction does not reach a good yield for amoxicillin production. Also, for high amounts of enzyme, the hydrolysis velocity is high and the kinetic control of amoxicillin production will be difficult. In other words, before achieving the highest yield, the hydrolysis of amoxicillin accelerates the synthesis and the productivity decreases. Temperature is the other parameter which can be studied in amoxicillin production.

In this investigation, optimization of the enzymatic synthesis of amoxicillin including the parameters affecting amoxicillin production such as time, substrate and enzyme concentration, pH and temperature were studied.

MATERIALS AND METHODS

Materials

The immobilized Penicillin G acylase (PGA, EC 3.5.1.11) from *Escherichia coli* imported material (Germany): Enzyme immobilization: Glyoxyl-agarose gel was prepared as reported by Guisan [21]. Penicillin acylase was immobilized in glyoxyl-agarosegel beads, based on the procedure described by Alvaro et al. [22], but using phenylacetic acid (PAA) instead of penicillinG sulfoxide as the protecting agent during immobilisation; time of immobilization was extended to 20 h, determined as the optimum for biocatalyst

stability [23]. The glyoxyl-agarose immobilized penicillin acylase was stored as a wet gel at 5°C. No enzyme inactivation or leakage has been detected during prolonged storage. Hydroxyphenylglycine methyl ester (HPGM), 6-aminopenicillanic acid (6-APA) and Amoxicillin trihydrate were obtained from the Zakari-aye Razi Pharmaceutical Company (Iran). All other chemicals were of laboratory grade prepared from different commercial suppliers. All materials were of pure analytical grade.

Methods

Enzyme Activity

The titration of 6-APA, released during the hydrolysis of penicillin G with 0.1 M NaOH, provided the basis for the evaluation of enzyme activity. To determine enzyme activity, a known amount of enzyme was added to a stirred 20 ml of 10% Penicillin GK in phosphate buffer, pH 8. The amount of 6-APA produced within 10 minutes was titrated with 0.1 M NaOH. One International Unit (U) of enzymatic activity was defined as the amount of enzyme that hydrolyzes 2 g of penicillin GK in 10 minutes; pH 8.0 and at 28°C [24].

Synthesis Reaction in Water

Synthesis reactions were performed by adding 6-APA and HPGM to water in a continuously stirred, thermo jacketed vessel at different temperatures. The desirable amount of enzyme was added. The stirring rate was 100-300 rpm. The pH was monitored and samples of 20-50 μ l were taken from the reaction mixture in the course of the synthesis and added to the corresponding amount of eluent, in order to dilute the sample. The samples were removed by a 0.45 μ m filter, in order to separate solids, stop the enzymatic reaction and analyze the composition of the solution. This enzyme is intracellular and immobilization is economic. Also, it is easy to separate an immobilized enzyme by filtration from substrates and products. The samples include: hydroxyphenylglycine methyl ester, amoxicillin, hydroxyphenylglycine and 6-amino penicillanic acid subjected to HPLC analysis. The reaction yield is defined as follows:

$$\text{Yield}(\%) = \left[\frac{\text{Concentration of Amoxicillin}(\text{mM})}{\text{Concentration of 6-APA}(\text{mM})} \right] 100.$$

Analysis

Concentration of amoxicillin(amox) was determined using HPLC : C18 column (Waters Nova-pak C18 60 A°4 μ m, 3.6 * 150 mm) with 1.5 mL/min of mobile phase containing 5% methanol, 0.01 M phosphate buffer of pH 5 at 25°C, and $\lambda = 230$ nm.

RESULTS AND DISCUSSIONS

Amoxicillin Production

Firstly, it is necessary to know the time course of amoxicillin production during the reaction to estimate the time needed to reach the maximum yield. According to Figure 2, the maximum yield is achieved after 400 minutes. We have continued all the tests up to 500-600 minutes.

Optimization of Initial Substrates

A number of experimental variables may influence the enzymatic synthesis of B-lactam antibiotics. However, not all these variables exert a strong influence. One of the most important factors influencing the production of amoxicillin is the substrate concentrations. To find the best ratio of substrates, nine reactions at different concentrations (different initial amounts of APA and HPGM) have been tested. Results from the experiments are given in Figure 3. The results show the amount of amoxicillin production as a function of time. In these reactions, the initial pH was set at 6.5, while no more pH control was performed during the reaction. 0.25 g of enzyme (in 50 cc water) was used for all tests. It is shown in Figure 3 that the optimal is reached when the ratio of 6-APA/HPGM concentration is 1/3. There are several reasons for why increasing the ester concentration (HPGM), increases amoxicillin production. Firstly, with more acyl-enzymes forming, the possibilities of increasing antibiotic formation increase. In addition, the ester was taken as a powerful competitive inhibitor of amoxicillin hydrolysis, mainly at pH 6.5 (L.R.B. Goncalves, 2003). Furthermore, HPGM attends in both the synthesis reaction and hydrolysis reaction and, so, an excess amount of HPGM prevents the

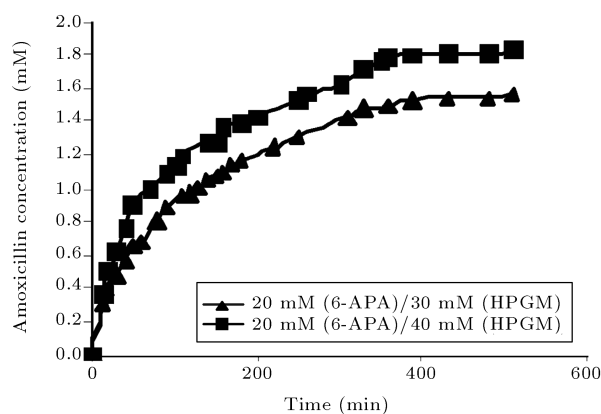


Figure 2. Amoxicillin synthesis catalyzed by immobilized PGA at 25°C, initial pH 6.5, 0.5% of enzyme C_{initial} of 6-APA = 20 mM and HPGM= 30 mM (▲) and C_{initial} of 6-APA = 20 mM and HPGM = 40 mM (■).

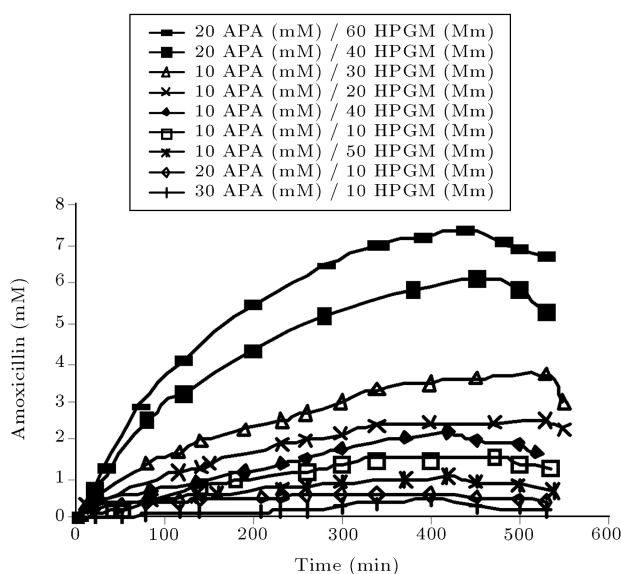


Figure 3. Time curves of amoxicillin synthesis with different initial concentrations of substrates. Reaction conditions: initial pH 6.3, 25°C and 0.5% of enzyme.

decrease in the synthesis yield because of the hydrolysis taking place parallel to synthesis.

In order to maximize the beneficial effect of increasing effective concentrations of starting substrates, we have investigated the different ratios of two substrates and the amount of amoxicillin formed after 300 minutes. From the results, summarized in Table 1, one can see that with excess amounts of 6-APA, the reaction yield decreases significantly. The highest values were obtained for a 1/3 ratio of 6-APA and HPGM, which gets to 37% yield at room temperature.

Table 1. Yields for the enzymatic synthesis of amoxicillin with different ratio of substrates, pH 6.3, 25°C, 0.5% enzyme. Reaction conditions: initial pH 6.3, 25°C, 400 min.

Initial Concentrations (mM: 6-APA/HPGM)	Maximum Yield* (%)
30/10	2
20/10	3
10/50	11
10/10	16
10/40	22
10/20	25
10/30	37
20/40	31
20/60	37

* Yield represents the molar ratio of the product (amoxicillin) formed to initial 6-APA.

Optimization of Initial Amount of Enzyme

One of the most important parameters in improving the enzymatic synthesis of amoxicillin is the initial amount of enzyme. The amount of enzyme is explained by its activity. To test the effect of enzyme concentration on the production of amoxicillin, seven reactions under different conditions (different initial amounts of enzyme) have been tested. Results from the experiments are given in Figure 4. In all tests, the initial pH is set on 6.3. The temperature is 25°C and the initial amount of substrates is 6-APA = 20 mM and HPGM = 40 mM. Although, in the next sections, we came to the conclusion that the best ratio of substrates is 1/3 (6-APA/HPGM), the tests have been done with a 1/2 ratio of substrates, since the HPLC results will be erratic with an excess amount of HPGM.

According to Figure 4, by increasing the initial amount of enzyme, the amoxicillin production increases. At low amounts of enzyme, the progress curve of amoxicillin is ever-increasing, up to 400 minutes. However, after a significant concentration (which is 4 g/L with our enzyme), the amoxicillin production comes to a constant amount after 300 minutes, which means that the hydrolysis velocity becomes equivalent with the synthesis of amoxicillin. The key point in these results is the amoxicillin production with enzyme concentration more than 5 g/L. As shown in Figure 4, with 6 g/L enzyme, the turning point of the amoxicillin production curve takes place in about 200 minutes indicating that the yield never reaches its maximum amount. In other words, the velocity of reaction is out of control and the hydrolysis velocity becomes too high. As a result, according to the summarized results shown in Table 2, the optimum concentration for the enzyme will be 5 g/L.

It is important to indicate that different kinds of enzyme (produced from different sorts of fungi or bacteria) immobilized on different polymers have shown

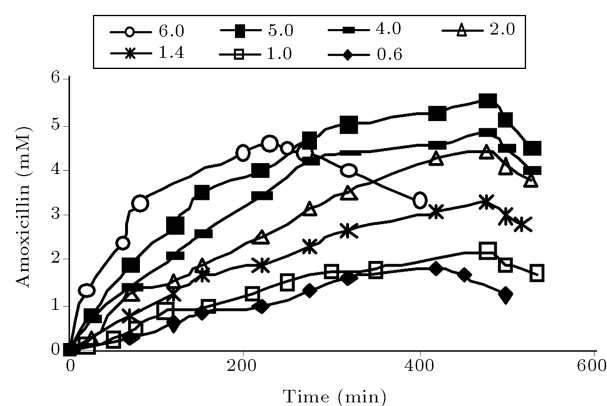


Figure 4. Progress curves of amoxicillin synthesis with different initial concentrations of enzyme. Reaction conditions: initial pH 6.3, 25°C.

Table 2. Maximum yields for the enzymatic synthesis of amoxicillin with different concentrations of enzyme. Reaction conditions: initial pH 6.3, 25°C.

Initial Concentration of Enzyme (g/L)	Maximum Yield* (%)
0.6	8
1	12
1.4	16
2	22
4	25
5	28
6	24

* Yield represents the molar ratio of the product (amoxicillin) formed to initial 6-APA.

very different behavior for the enzymatic production of amoxicillin [7,10,11].

Effect of pH on Amoxicillin Production

Enzyme activity, the stability of HPGM and amoxicillin, and the solubility of substrates are the determining factors for choosing the best pH to start the reaction. According to results shown in Figures 5 and 6, HPGM and Amoxicillin are both hydrolyzed in a wide range of pH. As a result, the reaction cannot take place in this range of pH.

Furthermore, penicillin G acylase has its highest activity in the range of pH 6 to 7.5 [7]. According to these three factors, we have a narrow range of pH to perform the experiments which is 6 to 7. In addition, to have the high solubility of substrates, the pH should be near to its iso-electric pH which is 6.5. As a result, we have started all the tests with pH = 6.3. The course of pH during the reaction time has been investigated before [6]. However, to reach a constant pH, we have tested this course and the result is shown in Figure 7.

The pH takes a very different course at different initial substrate amounts. This is described as being quite reasonable by models in other work [6]. However, we needed to predict the pH course at the condition of our reactions to prevent hydrolysis during the tests. The next step is to study the effect of pH on enzymatic synthesis of amoxicillin to find out whether there is a need to stabilize the pH during the reaction or if there is not a very high variation in the yield. Figure 8 shows the effect of stabilizing pH during the reaction on the production of amoxicillin.

As shown in Figure 8, by stabilizing the pH at constant pH = 6.3, the amoxicillin production increases and, consequently, the yield of reaction increases about

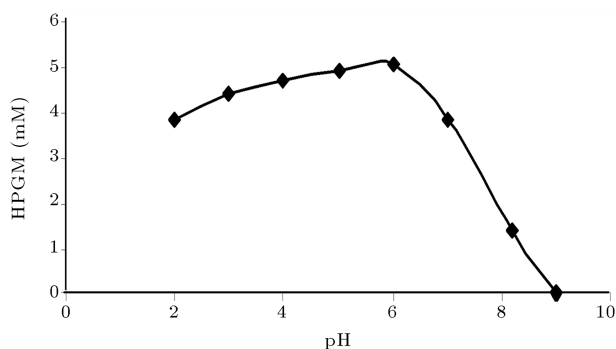


Figure 5. Hydrolysis of HPGM at different pH, 25°C.

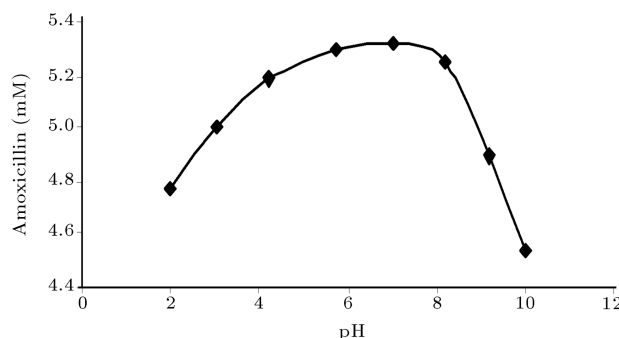


Figure 6. Hydrolysis of amoxicillin at different pH, 25°C.

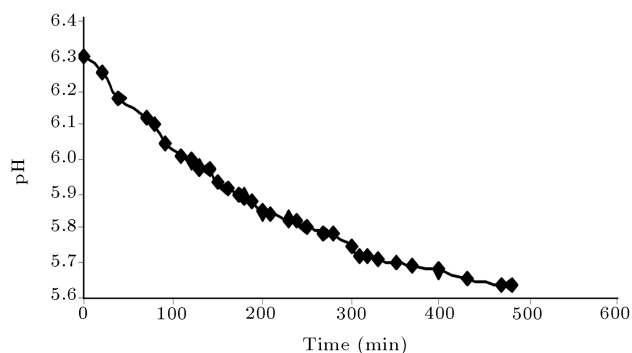


Figure 7. pH profile as a function of time during the synthesis of amoxicillin. Reaction condition: 25°C, initial pH 6.3, C_{initial} of 6-APA = 20 mM and HPGM = 40.

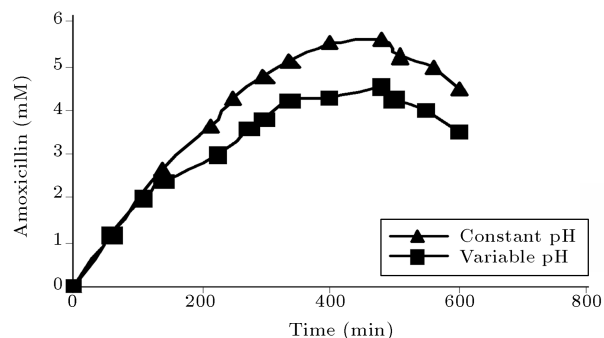


Figure 8. Amoxicillin production with constant (\blacktriangle) pH=6.3 and variable (\blacksquare) pH. Reaction condition: 25°C, initial pH 6.3, C_{initial} of 6-APA = 20 mM and HPGM = 40, 5g/L of enzyme.

10%. So, it is preferable to perform the tests at a constant pH rather than at a variable one.

Effect of Temperature on Amoxicillin Production

The effect of low temperature on the synthesis of β -lactam antibiotics has been previously determined [8]. However, amoxicillin synthesis at high temperatures has not been performed yet. To investigate the effect of different temperatures on production, three reactions under different temperatures (different temperatures) have been tested. The time for optimal amoxicillin production was reached after 480 min. Results from the experiments are given in Figure 9 (5°C, 25°C and 35°C). Also, the yield for each reaction is given in Table 3. Productivity under three different temperatures is also presented in Table 3; increasing temperature from 5 to 35 results in productivity increase. Productivity is the amount of product (amoxicillin) formed per unit time. Other investigators for enzymatic synthesis of amoxicillin under prescribed conditions: 0.1M APA, 0.1 M HPGM, T 25°C and pH 6 showed the yield about 10% [6].

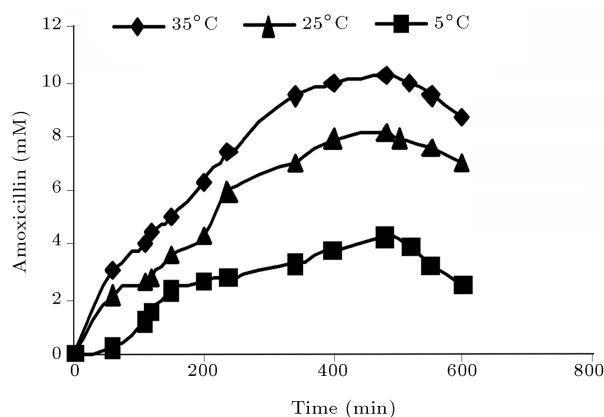


Figure 9. Progress curves of amoxicillin synthesis at different temperatures. Reaction condition: initial pH 6.3, C_{initial} of 6-APA = 20 mM and HPGM = 40, 5g/L of enzyme.

Table 3. Maximum yields for the enzymatic synthesis of amoxicillin at different temperatures. Reaction conditions: initial pH 6.3.

Different Temperature (°C)	Maximum Yield (%)*	Productivity (mM/min)*
5	21	0.009
25	40	0.017
35	50	0.021

* Yield represents the molar ratio of the product (amoxicillin) formed to initial 6-APA.

For enzymatic synthesis of ampicillin, other investigators studied the effect of pH and substrate concentration on synthesis. The yield for reaction reached 75% [25-27]. The effect of co-solvent on the Kinetically Controlled Synthesis of Amoxicillin with Immobilized Penicillin G Acylase was studied using ethylene glycol as co-solvent (50%) v/v with a yield of 69.13 [28].

Yield represents the ratio of the product, amoxicillin, formed to initial substrate, 6-APA consumed. Productivity is the amount of amoxicillin produced per time interval.

The results shown in Table 3 indicates that increasing the temperature from 25° to 35°C, increases the yield to 50%, which has not been predicted in any previous work. As the experiment shows, high temperature is beneficial to the synthesis of amoxicillin.

Finally, a test including 20 mM 6-APA, 60 mM HPGM 5 g/L enzyme with constant pH 6.3 and temperature 35°C was performed and a yield of 50% was achieved.

CONCLUSION

The effect of different parameters on the enzymatic synthesis of amoxicillin in an aqueous medium was investigated. Obtained data demonstrated that the ratio of substrates is very important and effective to the yield of the reaction. In addition, the concentration of initial enzyme plays an integral role in the trend of the reaction and the time needed for maximum yield. Furthermore, constant pH shows better results than variable pH during the reaction. Also, results are significantly better at 35°C than those at low temperatures, such as 5°C or even room temperature. Finally, yield was substantially increased by performing all the optimized parameters in one test. The test including 20 mM 6-APA, 60 mM HPGM and 5 g/L enzyme with constant pH 6.3 and the temperature 35°C was performed and a yield of 50% was achieved.

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