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Original Article

Optimization of Enzymatic Synthesis of Ampicillin Using Cross-Linked Aggregates of Penicillin G Acylase

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Abstract

Penicillin G acylase from E. coli TA1 was immobilized by Cross-Linked Enzyme Aggregates (CLEA), a new method for immobilization. This biocatalyst and commercial immobilized penicillin G acylase (PGA-450) were used to study the effect of pH, temperature and substrate concentration on the synthesis of ampicillin from phenyl glycine methyl ester (PGME) and 6-aminopenicillanic acid (6-APA). Compared with PGA-450, this immobilized enzyme showed a high synthesis activity. The optimum conditions for synthetic activity was at pH 6, 25°C and 2:6 (6-APA:PGME) substrate ratio.

Keywords: Penicillin G acylase; *E. Coli;* Cross-linked Enzyme Aggregates; Ampicillin.

Introduction

Ampicillin is one of the most widely used -Lactam antibiotics with an annual production of 5600 tons per year (1).

Ampicillin is produced by a chemical synthesis process requiring protection of the amino group of phenylglycine (PG), very low temperature (-30°C), anhydrous conditions and the use of highly toxic compound (pyridine, dimethylaniline, dichloromethane) (2, 3). Currently, the industrial production of -Lactam antibiotics and their intermediates are under a remarkable transformation (4). Therefore, traditional chemical conversions based on stoichiometry are being replaced by enzyme-catalyzed processes using enzymes of the group of penicillin acylase (5).

The enzymatic synthesis of -lactam antibiotics such as ampicillin offers several benefits compared to the conventional chemical methods. The enzymatic procedure is carried out under mild conditions in water, with no need for protection/deprotection schemes and is environmentally benign (5-7).

Penicillin acylases (EC 3.5.1.11) are a group of enzymes that cleave the acyl chain of penicillins to yield 6-Amino penicillanic acid (6-APA) and can also be used in the reverse synthesis of -lactam antibiotics from the corresponding -Lactam nuclei and suitable acyl donor (figure 1)(8).

Although microbial conversion of penicillin G into 6-APA has been known for almost five decades, industrial application of the enzyme involved penicillin acylase has been introduced successfully only in the past two decades. This is primarily a result of the fact that efficient enzyme production and recovery was unavailable (9).

Penicillin acylase is widely distributed among bacteria, yeast and filamentous fungi. The enzyme is a heterodimer with a 20.5 KDa β-subunit and a 69 KDa -Subunit (10). The Crystal structure of E. coli penicillin acylase indicates the catalytically active centre to be the N-terminal serine residue of the -Subunit (11) . A more narrow substrate specificity is found in a

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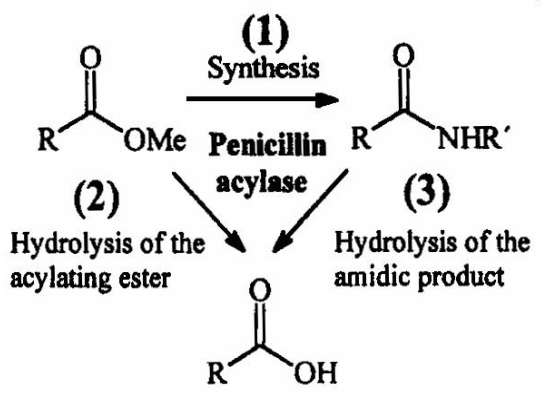

Figure 1. Reaction involved in the kine tically controlled synthesis of â -lactam antibiotic.

second class of enzymes: the ampicillin synthesizing enzyme from Pseudomonas melanogenum has especially been known to be an interesting Penicillin amidase (12, 13) which shows activity only for the synthesis of ampicillin and cephalexin containing a side chain of D-phenylglycine (14, 17).

The term immobilized biocatalyst refers to a system or preparation in which an enzyme is confined or localized in a relatively defined region of space that can be easily separated from a reaction mixture and can be used continuously in flow processes or repetitively in batch contacts as long as the enzyme preparation is active. Many techniques for the preparation of immobilized forms of enzymes have been developed such as carrier-binding, cross-linking and entrapping (15, 18).

Based on the studies carried out by Abedian et al. (19), we tried to immobilize penicillin acylase form E. coli TA1 for the production of ampicillin, using a Cross-Linked Enzyme Aggregation (CLEA) method described by Sheldon et al. (20, 21) which involves the physical aggregation of the enzyme under non-denaturing conditions followed by cross-linking, to cross-link enzyme aggregates.

Table 1. Effect of substrate concentration on the production of ampicillin by free enzyme and CLEA (5 U/ml) at pH 6,), a temperature of 25ºC.

	Max [Ampicillin](mM)		Conversion %	
6-APA:PGME(mM)	Free enzyme	CLEA	Free enzyme	CLEA
50:50	24	22	48	44
50:100	28	27	56	54
50:150	35	37	70	74
50:200	40	38	80	76
50:250	43	39	86	78
100:150	60	50	60	50
100:200	67	63	67	63
100:300	87	84	87	84
200:300	106	101	53	50
200:400	120	110	60	55
200:600	176	168	88	84

Experimental

Materials

D-Phenyl Glycine Methyl Ester (PGME) was purchased from Aldrich Chemical Company. tert-Butyl alcohol was purchased from Fluka Chemical, K2HPO4, KH2PO4, and glutaraldehyde were purchased from Merck company. PGA-450 was a gift from Roche Diagnostic GmbH, 6-Aminopenicillanic Acid (6-APA), Phenyl Acetic Acid (PAA) and Penicillin G potassium salt were kindly donated by Antibiotic-sazi Iran Company. E. coli TA1 was obtained from the stock culture from department of pharmaceutical biotechnology, Faculty of Pharmacy, Tehran University of Medical Sciences.

Microorganism and cultivation conditions

E. coli TA1 was grown in an improved medium described by Zahedi et al. (22). The medium consisted of yeast extract (0.7% *w/v*), phenyl acetic acid (0.15% *w/v*), ammonium chloride (0.3% *w/v*), K2HPO4 (0.2% *w/v*), and MgSO4 (0.02% *w/v*).

The inoculum was prepared from 10 ml of grown cells in 100 ml of medium. Cultures were shaken at 60 rpm for 44 h at 25°C and cells were harvested during the exponential growth phase by centrifugation at 7000 rpm for 5 min at 5°C; pellets obtained were washed and resuspended in phosphate buffer (0.1 M, pH 7.0).

Preparation of cell-free extract

Cells were disrupted by a labsonic (Bandelin Sonoplus UW2200) sonicator to provide continuous application of acoustic waves, with a power input of 300 w and a frequency of 20 KHz for 1-2 min, with 30 sec cooling intervals. For determination of the percentage of cell lysis, samples were stained with crystal violet. Homogenates were clarified by centrifugation (Beckman , 36000 g) for 30 min at 5°C to yield

crude extract (23,24).

Precipitation with Ammonium sulfate

Ammonium sulfate (50%) was added to the cell extract. To complete the precipitation, the solution was stored at 4°C for 12 h. After centrifugation for 5 min at 13000 rpm, protein pellets were resuspended in the primary volume of phosphate buffer (0.1 M, pH 7.0) (13).

Immobilization procedure

CLEAs were prepared by slowly adding tert-butanol to the penicillin G acylase solution under gentle stirring at 0°C. When no more activity was detected in the supernatant, the physically aggregated penicillin G acylase was subjected to chemical cross-linking, using gluteraldehyde (25% aqueous solution) at 0°C. The CLEAs were then collected by filtration and washed with phosphate buffer (0.1 M, pH 7) and dispersed in buffer and stored at 4 °C before use (20, 21).

Synthetic activity

The synthesis activity of biocatalysts was determined in a batch reactor at 25 °C. The reaction was started by adding biocatalysts (6 U/ml) to 50 ml of an aqueous solution containing 50 mM 6-APA and 150 mM PGME. The pH was kept constant by titration with 6 N H2SO4, using a Metrohm 654 pH-meter. The ampicillin concentration was determined by High Performance Liquid Chromatography (HPLC).

HPLC Analysis

Substrates and products of enzymatic synthesis were analyzed by HPLC, using a Waters chromatograph (model 515) with a UV-detector at 220 nm. The HPLC system was equipped with a 4 □m (4.6 mm × 250 mm) Bondapak C18 column. Samples were eluted with methanol 20% in a solution containing 50 mM Ammonium phosphate buffer (25).

The Amount of reactant and products were calculated from the calibration curves, using the stock solutions. One unit of synthesis activity was defined as the amount of biocatalyst synthesizing 1 □mol of ampicilin/min at 25°C and pH 7, from 45 mM 6-APA and 135 mM PGME in a 0.1 phosphate buffer (26).

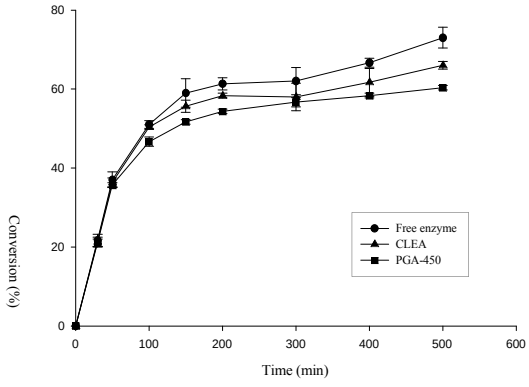

Figure 2. Comparison of biocatalysts (5 U/ml) synthesis activity at 25ºC, pH 7 and a [6 -APA]:[PGME] ratio of 50:150.

Results And Discussion

Activity of the CLEAs

In general, enzyme immobilization causes degradation reactions during the immobilization process resulting in partial loss of activity, which is due to changes in the enzyme environment, physicochemical properties of the matrix surrounding the enzyme or the cell, and matrix-substrate interactions (27, 28).

Comparing synthesis activity of different forms of the biocatalyst, the immobilized enzyme based on the CLEA method showed a better synthesis activity compared to that of PGA-450 (figure 2). Free enzyme showed the highest synthesis activity, but could not be recycled. Hence, it has no economical value.

Effect of pH

The effect of pH on the activities of enzymes is shown in figure 3. The optimal pH for

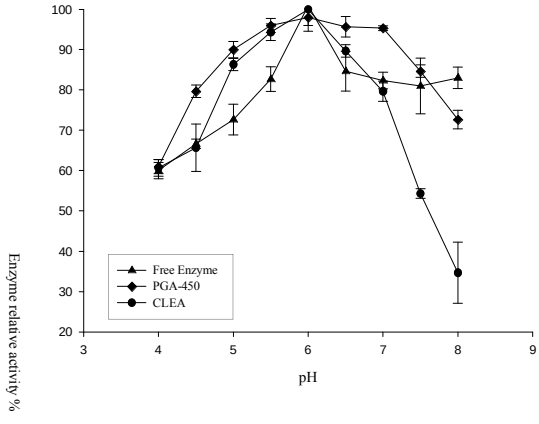

Figure 3. Effect of pH on synthesis activity of biocatalysts (5 U/ml) at 25ºC, and a [6 -APA:PGME] ratio of 50:150.

(a)

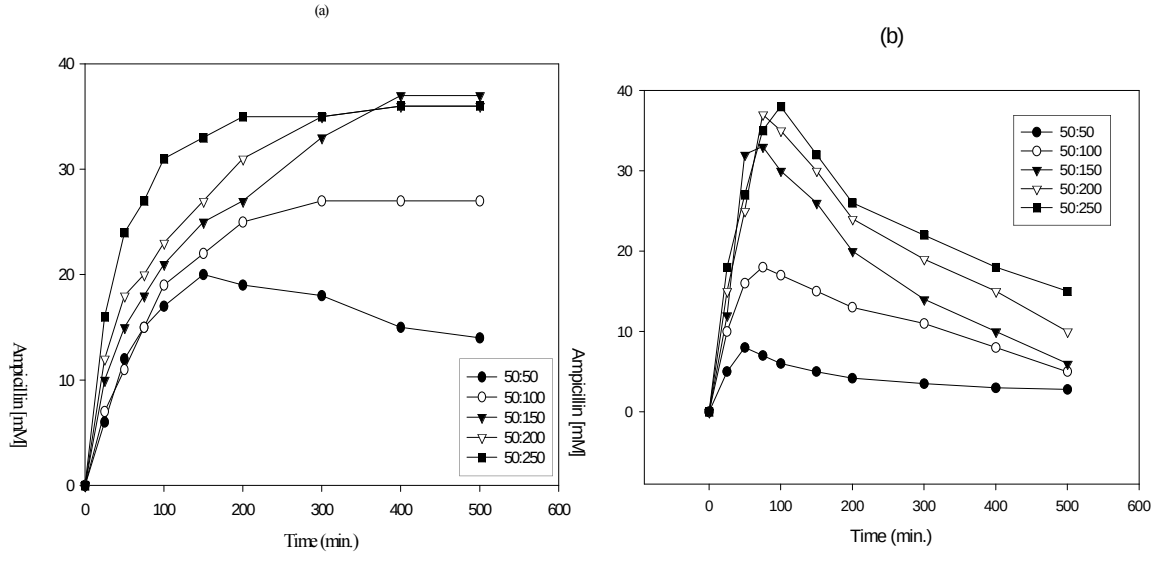

Figure 4. Comparison of the efficiency of PGME concentration on the production of ampicillin by CLEA (5 U/ml) at pH 6 (a) and 7 (b), a temperature of 25ºC and a [6 -APA] concentration of 50 mM.

synthesis reaction was slightly acidic. The optimum pH for synthesis activity was between 5.5 to 6.5, which confirms previous results (19, 20). In the immobilized enzyme obtained from the CLEA method, the extent of ampicillin producion in pH 6 was much more than of pH 7 (figures 4, 5 and 6). Therefore, it could be concluded that by increasing pH, the reaction switches in favor of hydrolysis. Ospina et al. (25) have also reported a similar finding in the immobilized E. coli. After the free enzyme, the immobilized enzyme obtained from the CLEA method showed the maximum synthesis activity. The immobilized enzyme prepared by the CLEA method and the commercially immobilized enzyme (PGA-450) showed a slightly wider range of pH (5.5- 6.5) for the maximum synthesis activity. Therefore, it seems that the synthesis activities of these two enzymes are slightly less sensitive to minor pH changes.

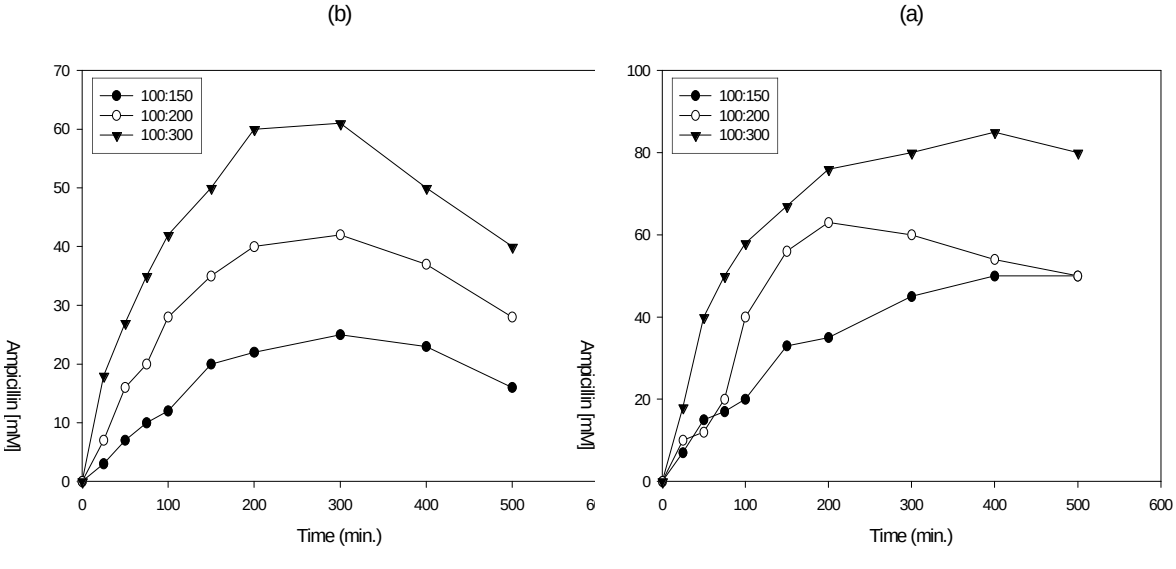

Figure 5. Comparison of the efficiency o f PGME concentration on the production of ampicillin by CLEA (5 U/ml) at pH 6 (a) and 7 (b) ,), a temperature of 25ºC and a [6 -APA] concentration of 100 mM.

Effect of temperature

The maximum synthesis activity was observed at 25-30 °C (Figure 7). At this temperature the highest synthesis activities were observed in all the tested biocatalysts. Therefore, temperature is an important environmental factor, affecting the activity toward the optimization of synthesis. The CLEA based immobilized enzyme showed a better synthesis activity compared to that of PGA-450. This enzyme kept its maximum activity up to 30°C, showing the stability of this enzyme.

Ryu and Kim (13) reported that a temperature of 35°C is suitable for the activity of immobilized P. melangenum cells. In comparison to the free enzyme, immobilized cells show a better thermal stability but on the other hand a lower thermal resistance than the immobilized enzymes (13, 29).

Effect of substrate concentration

In all the tested biocatalysts, a ratio of 1:3 (6-APA: PGME) produced the highest ampicillin yield (Table1). The conversion percentage for the free enzyme and the immobilized enzyme prepared by the CLEA method were 88% and 84% respectively. Ospina et al (25) have also reported a ratio of 1:3 as the best ratio and a conversion percentage of 75%, using immobilized E. coli. Increasing the PGME proportion is not justified economically. By comparing the ratios of 50:150, 100:300 and 200:600, the latter one was found to be better for ampicillin production and a ratio of 2:6 has been announced as the best one.

Conclusion

We managed to immobilize penicillin G acylase based on the CLEA method and showed that the product acts even better than the commercial sample. Although the results obtained in this study are far from the industrially acceptable biocatalyst, nevertheless could be used for the development of a CLEA based penicillin G acylase in industrial production. Comparison of the different forms of the enzyme and optimization of the activities, especially ampicillin production, are the other important outcomes of this research, which could be used as further researches in this field.

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