Production of 6-Aminopenicillanic acid in aqueous two-phase systems by recombinant *Escherichia coli* with intracellular penicillin acylase

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Running title: Production of 6-penicillanic acid in aqueous two-phase systems

Abstract

Bioconversion of Penicillin G in PEG 20000-Dextran T 70 aqueous two-phase systems was achieved using the recombinant *Escherichia coli* A56 (ppA22) with intracellular penicillin acylase as catalyst. The best conversion conditions were attained for: 7%(w/v) substrate (penicillin G), enzyme activity in bottom phase 52 U/ml, pH 7.8, temperature 37°C, reaction time 40 min. Five repeated batches could be performed in these conditions. Conversions ratios between 0.902-0.985mol of 6-aminopenicillanic acid (6-APA) per mol of penicillin G, were obtained and specific productivity was 3.6-4.6 μmol/min•ml. In addition the product 6-APA could directly be crystallized from the top phase with a purity of 96.2%.

Key words: 6-aminopenicillanic acid production; Aqueous two-phase systems; Bioconversion; Recombinant *E. coli* A56; penicillin acylase.

INTRODUCTION

Aqueous two-phase systems (ATPS) have been used for the purification of proteins for several decades (Albertson, 1986).. Their mild environment and low interfacial tension combined with the ease of scale-up and continuous operation makes them well suited for the large-scale purification of biological macromolecules with special relevance to proteins. Bioconversion is another possible practical application of ATPS in which the goal is to partition, the biocatalyst and substrate(s) to one of the phases, and the product(s) to the other phase. This will reduce inhibition of biocatalyst by the products, increase biocatalyst stability, improve mass transfer, enhance specific productivity and facilitate product(s) recovery (Andersson *et al.* 1990).

Penicillin acylase (EC 3.5.1.11) is an enzyme produced by several bacteria and fungi. Although its metabolic role is not completely understood it is widely used for the production of 6-aminopenicillanic acid (6-APA) due to its ability to catalyse the hydrolysis of penicillin G in this compound and phenylacetic acid (Ma *et al.*, 1992). 6-APA is an intermediate product with special importance to pharmaceutical industry as it is the starting material for the semisynthesis of β-lactam antibiotics such as ampicillin and amoxicillin. This enzyme could also be used to catalyse the reverse reaction, the synthesis of the amide bond (Pessina *et al.*, 1988 and Stambolieva *et al.*, 1992).

Andersson *et al.*, (1984) applied penicillin acylase to the production of 6-APA in PEG 20000-potassium phosphate ATPS, but the enzyme could not be completely partitioned to one phase and was unstable in concentrated phosphate solutions. In the present study we used a recombinant strain of *E. coli* with intracellular penicillin acylase for the production of 6-APA in PEG-Dextran ATPS to overcome the aforementioned disadvantages.

MATERIALS AND METHODS

Materials

All reagents used were of analytical grade. Dextran T 70, T 500, T 2000 were purchased from Pharmacia (Uppsala, Sweden) and polyethylene glycol (PEG) 6000, 10000, 20000, from Carbowax (Union carbide, Japan). 6-APA standard was obtained from National institute for Control of Pharmaceutical and Biological Products, Beijing. Penicillin G (potassium salt) and the recombinant *E. coli* A56 (ppA22) with intracellular penicillin acylase were generous gifts from Dong Feng pharmaceutical corporation (Jiang Xi , China).

Analytical methods

The concentration of bacterial cells in ATPS was determined by solution optical density (O.D.) at 600nm. A calibration curve between cell dry weight and O.D. at 600nm was constructed to determine cell concentration. The method of Balasinghan *et al*., 1972 was both used for the determination of 6-APA concentration and intracellular penicillin acylase activity of recombinant *Escherichia coli* A56 (ppA22). Enzymatic assays were done under continuous stirring to assure mass transfer. A unit of enzyme specific activity was defined as the wet bacterial mass weight (g) required to produce 1 μmol 6-APA per minute under assay conditions. Concentration of phenylacetic acid in ATPS was determined by titration with NaOH. Penicillin G was determined by the iodimetric method (Chen *et al*., 1991). PEG in aqueous two-phase systems was determined by a spectrophotometric method (Xuejun *et al*., 1993). A sample (10 μl) from the top or bottom phases of a PEG-Dextran ATPS was withdrawn and diluted 1000 to 10000-fold with water. 5ml of the diluted solution was mixed with 1 ml of 5% $BaCl_2$ and 1 ml of 2% KI/I_2, and after 15 min, the absorbance was measured at 535 nm. Dextran in ATPS was determined by a polarimetric method after removal of

penicillin G by adsorption with an ion exchanger (Xuejun *et al.*, 1994).

Preparation of the catalyst

The recombinant *Escherchia coli* cells were crosslinked with glutaraldehyde to prevent cell breakage. The method used was as follows: 50g of previously centrifuged cells were suspended under stirring for two hours in 200ml of a solution containing 0.1 M sodium acetate and 1% glutaraldeyde. The cross-linked cells were harvested by centrifugation, washed with 0.1 M phosphate pH 7.5 and stored at 4ºC until needed.

Screening of ATPS

Several PEG-Dextran systems were tested to determine the best conditions for bioconversion. A volume of 50ml of each of the following nine systems were prepared: PEG 6000 8.3%(w/w)-Dx T 70 4.8%(w/w); PEG 6000 7.4%-Dx T 500 3.7%; PEG 6000 6.4%-Dx T 2000 3.6%; PEG 10000 4.7%-Dx T 70 4.2%; PEG 10000 8.2%-Dx T 500 3.5%; PEG 10000 6.6%-Dx T 2000 3.8%; PEG 20000 4.0%-Dx T 70 3.4%; PEG 20000 8.0%-Dx T 500 3.5%; PEG 20000 5.6%-Dx T 2000 3.1%. The concentration of PEG and Dextran in the previous ATPS were choosen to obtain a 4:1 volume ratio.

Determination of partition coefficients

For each of the previous systems under stirring four 10 ml aliquots were taken. Penicillin G (0.7g), 6-APA (0.5g), phenylacetic acid (0.2g), wet bacterial mass of the recombinant *E. coli* A56 with intracellular penicillin acylase (3.0g) were separately added to the four aliquots. The systems were mixed and centrifuged for 15 min. Samples were taken from the top and bottom phase and concentration of the component under analysis were determined. Partition coefficients (K) were calculated as:

$$K = \frac{\text{Top phase concentration}}{\text{Bottom phase concentration}} \qquad (1)$$

Optimum amount of bacterial cells

Penicillin G (10g) was added to three stirred flasks, each containing 100 ml of 4.0% PEG 20000-3.4%Dx T 70 ATPS in 0.05 M sodium phosphate at pH 7.8, 37°C. The bacterial cells were added to the flasks in proportions of 10:1, 10:2, 10:3 (total system volume to bacterial mass weight) respectively. The pH was mantained at 7.8 with 7.5 M NaOH during reaction. 6-APA concentration in the total system was determined at every 5 min. When reaction was finished the conversion ratio (C) of penicillin G was calculated as:

$$C = \frac{[6\text{-}APA]_{top\ phase} \times \text{top volume}}{\text{Amount of penicillin added}} \times 100 \qquad (2)$$

Repeated batch conversions

Repeated batch conversions were performed in the aforementioned systems with a ratio of total system volume to bacterial mass weight equaling 10:3. Bioconversion was followed by the determination of 6-APA concentration in 10 min intervals. After each run a sample of the system was taken, and the top phase concentrations of PEG 20000 and Dextran T 70 were determined. The top phase was removed and a new one with the same volume and composition and 7% penicillin G was mixed with the original bottom phase. The conversion process was repeated four times.

Crystallization of 6-APA

One-third volume of methyl butyl ketone was added to the removed top phase. After

stirring the pH was adjusted to 3.9-4.1 with 6 M HCl . The solution was kept at 4°C overnight and filtered. The filter cake was washed with water and acetone, and dried under vacuum.

RESULTS AND DISCUSSION

Screening of ATPS

In all the experimental conditions used the bacterial cell accumulates totally in the bottom phase. When the bottom phase could not contain all the bacterial mass, some bacteria cells acumulated at the interface between the two phases. This one side partition behavior had been previously described by Albertsson (1986) and is advantageous to bioconversion in ATPS as it allows the easy separation of cells from the products.

The partition of penicillin G , phenyalcetic acid and 6-APA in nine PEG/Dextran ATPS are shown in Table 1. Partition coefficients of these three substances changed little with the system and are close to one with the exception of phenylacetic acid which shows a slightly trend to acumulate in the top phase. Although the ideal situation would be penicillin G concentrated in the bottom phase, while 6-APA and phenylacetic acid concentrated in top phase, this was not possible to achieve. The best compromise was obtained for the system containing PEG 20000 4.0%-DxT 70 3.4% where K values of penicillin G, phenylacetic acid and 6-APA, are 0.96, 1.31, 0.94, respectively. Given the low phase viscosity and phase separation time (data not shown) this system was chosen for the bioconversion studies.

Bioconversion of penicillin G to 6-APA

In ATPS bioconversion, when the product is recovered in the top phase, the yield could be calculated by the following formula (Andersson *et al.* 1990):

$$Y_t = V_t/V_b \times K/(V_t/V_b \times K+1) \times 100 \qquad (3)$$

where Y_t is product yield , V_t and V_b are the volumes of top phase and bottom phase, respectively and K is the partition coefficient.

According to this formula a high volume ratio should be employed to achieve a high yield. In the present case a volume ratio of 4:1 was chosen as the highest that allow the bottom phase to contain the total amount of the bacterial cells. However the volume of the bacterial cells increase the bottom phase volume changing the volume ratio and consequently the original phase composition.

The ratio ATPS volume/bacterial mass is also an important parameter as this determine the time needed to achieve equilibrium. In this case 10:3 was chosen as for lower ratios the bottom phase could not contain all the bacterial cells. Fig 1 shows a typical reaction progress curve in these conditions. The conversion of Pen G to 6-APA was 0.962 and the reaction was complete after 40 minutes. This high conversion ratio in a short time was possibly due to the high enzyme activity in bacterial cells and high cell concentration in bottom phase. In fact, the enzyme activity in the bottom phase was 52 U/ml, which led to a specific productivity of 4.6 μmol/min•ml. This value is much higher than the ones previous reported by Andersson *et al.*, 1984; 0.31-1.47 μmol/mg protein•min leading to 0.37-1.76 μmol/min•ml.

Repeated batch conversion in this system was attempted. The results of five runs are shown in Fig 2, and the bottom phase-based specific productivity and top phase-based conversion ratio are shown in Table 2.

In the first run the amount of substrate, 7%(w/v), penicillin G, added was calculated according to the total volume of the two phases. However in later runs according just to the volume of the top phase. The top phase conversion ratio in the several runs was similar to that obtained in the stand alone bioconversion study ranging from 0.902 to 0.985 mol 6-APA per

mol penicillin G. The specific activity is also maintained very high ranging from 3.6 to 4.6 μmol/min•ml. However decrease of specific activity is observed during the all process. The initial specific activity of bacterial cells with the penicillin acylase was 118U/g wet bacterial mass, with 75 U/g remaining after 96 hour run (including work and interval time). Enzyme activity decreased when 7.5 M NaOH was added for adjusting pH. This problem was overcome, by adjusting the pH of the fresh top phase before mixing with the bottom phase in the reactor. Alternatively the loss of enzyme activity can be compensated by adding new bacterial cells.

As mentioned above, the phase composition of ATPS changed to some extent after penicillin G, and the bacterial cells were added. The bottom phase volume was almost equal to top phase volume, while the initial volume ratio of blank ATPS was 4:1. Therefore, the composition of the top phase had to be determined before replacing this phase. The composition and volume of fresh top phase should be the same as those of the replaced top phase, or the phase systems would be change. In addition, a high concentration of NaOH should be used for adjusting pH, to avoid too much water being added to the phase system

The drawback of the repeated batch conversion is the large volume of the bottom phase owing to the bacterial cells partitioned to the bottom phase. To overcome this, an attempt will be done to immobilize penicillin acylase on the PEG, the polymer that constitutes the ATPS. In this way, penicillin acylase can be retained in one phase, and its stability could be improved due to immobilization Alternatively bacterial cells with higher penicillin acylase activity can be used.

6-APA crystallization

The final 6-APA concentration in the repeated batches was 162-179mM (Table 2). Using the same method as for the crystallization from water, it was possible to crystallized

this product from the top phase without prior concentration. The final product is a white powder with clean appearance with a purity of 96.2% (PDAB) and an yield of 83%-88%.

CONCLUSION

6-APA was produced by a recombinant *Escherichia coli* with intracellular penicillin acyalse in an ATPS. The substrate with high concentration, 7% (w/v) of penicillin G was used in this study, and the conversion ratio was excellent and reaction time was short due to the high enzyme activity in bottom phase. The product, 6-APA could directly be crystallized from top phase in a high purity, without prior concentration.

Acknowledgements This work was supported by the Science & Technological Commission of Shanghai Municipal People's Government. The helpful suggestions of senior engineer Liu Jiang are gratefully acknowledged. Special thanks also to Dong Feng pharmaceutical Factory, Jiangxi Province for the provision of recombinant *E. coli* strain.

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Table 1 Partition coefficients (K) of PG, 6-APA and phenylacetic acid in PEG-Dextran ATPS.

Aqueous two phase systems	Penicillin G	Phenylacetic acid	6-APA
PEG 6000 8.3%-Dx T 70 4.8%	0.98	1.54	0.79
PEG 6000 7.4%-Dx T500 3.7%	1.0	1.66	0.77
PEG 6000 6.4%-Dx T2000 3.6%	0.96	1.36	0.76
PEG10000 4.7%-Dx T70 4.2%	0.95	1.36	0.85
PEG10000 8.2%-Dx T500 3.5%	1.02	1.58	0.88
PEG10000 6.6%-Dx T2000 3.8	1.06	1.30	0.68
PEG 20000 4.0%-Dx T70 3.4%	0.96	1.31	0.94
PEG 20000 8.0%Dx T 500 3.5%	1.06	1.54	0.97
PEG 20000 5.6%-Dx T2000 3.1%	0.95	1.41	0.79

Table 2 Results of repeated batch conversion.

Batch No.	1	2	3	4	5
volume of top phase (ml)	70	65	59	55	50
volume of bottom phase (ml)	68	66	66	60	49
amount of PG added (g)	9.70	4.55	4.13	3.85	3.50
6-APA concentration in top phase (mM)	179	177	162	178	175
conversion ratio of PG in top phase (mol 6-APA per mole PG)	0.923	0.985	0.902	0.947	0.950
Specific productivity of bottom phase (μmol/min•ml)	4.6	4.4	3.6	4.1	4.5

Figure legends

Figure 1- Reaction progress curve of Pen G hydrolysis in ATPS. Flask containing 100 ml of 4.0% PEG-3.4% Dx T70 system in 0.05 M sodium phosphate at pH 7.8, and 37ºC. Initial concentration of Penicillin G was 7% and total volume to bacterial mass weight ratio was 10:3. 6-APA concentration was determined at 5 min interval.

Figure 2 - Repeated batch bioconversion of penicillin G. Reaction conditions as in figure 1. After each run the top phase was removed and substituted by solution with the same volume and composition with the addition of 7% PenG. Five repeated runs were carried out. 6-APA concentration was determined at 10 min interval

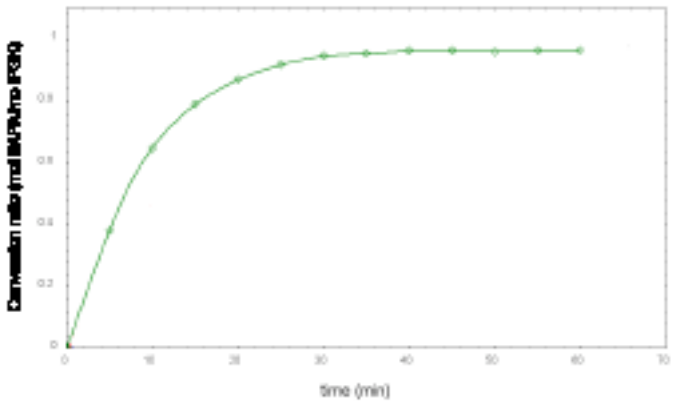

Figure 1

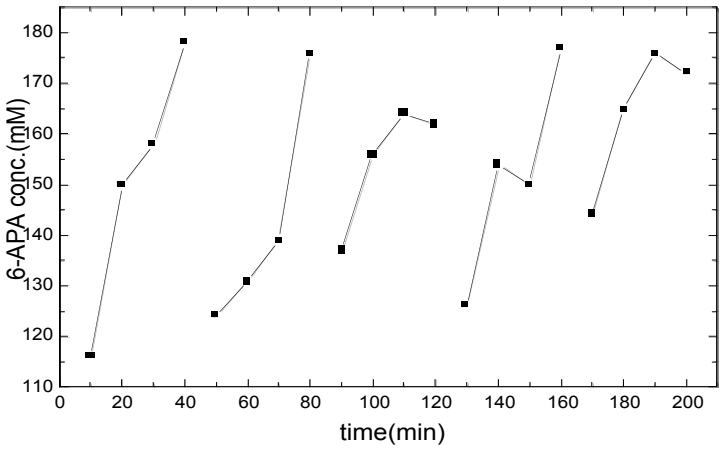

Figure 2