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Cultivation of Recombinant *Escherichia coli* to Achieve High Cell Density with a High Level of Penicillin G Acylase Activity

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ABSTRACT

A mutant strain of *E. coli* EP1 harbouring pGL-5 was employed to develop a process for producing penicillin G acylase (PGA). In comparison with different carbon sources in the medium, it was found that the specific levels of PGA activity obtained in the glucose medium were the lowest, which was likely due to catabolic repression. Phenylacetic acid (PAA) was previously reported to be an regulatory inducer for PGA production, whereas in this study, the addition of PAA repressed both cell growth and enzyme expression. In a fed-batch culture, the increase of specific PGA activity followed the pattern of the cell concentration during the early to middle cell growth phase. With application of pure oxygen aeration and an appropriate medium design, the cell concentration reached 162 (g wet weight/l), which was 2.4 times higher compared to that of the original operation, and a specific PGA activity of 37 (IU/g wet weight) was achieved after 12 h of cultivation.

Key Words: penicillin G acylase, high cell density fermentation, *Escherichia coli*, sorbitol

I. Introduction

Penicillin G acylase (PGA) (EC 3.5.1.11) hydrolyzes penicillin G to 6-aminopenicillanic acid (6APA), which is used in the semi-synthetic antibiotic industry. PGA can be produced by a variety of bacteria, molds and yeasts. However, recombinant *E. coli* is preferentially used (Sobotkova *et al*., 1995).

The expression of the intracellular PGA activity of *E. coli* occurs under several regulatory controls, such as induction, catabolic repression, temperature, pH, oxygen level, etc. (Shiloach and Bauer, 1975; Mori *et al*., 1983; Reiling *et al*., 1985; Pavlasova *et al*., 1986; Panbangred *et al*., 1990; Babu and Panda, 1991). All these characteristics complicate PGA production at high cell densities since the schemes for nutrient supplementation have to be carefully arranged so as to trigger the induction effect while avoiding catabolic repression.

The fermentation process for PGA production is economically attractive when high cell density culture (HCDC) is possible. Consequently, the major task in recombinant *E. coli* fermentation is to obtain a high level of genetic protein expression in HCDC (Konstantinov *et al*., 1991; Chou *et al*., 1994). Fed-batch cultivation is a useful technique not only for cultivation of microorganisms showing substrate inhibition or catabolic repression, but also for achieving high biomass densities within short cultivation times. For feeding nutrients into the fermenter, various techniques have been developed (Gleiser and Bauer, 1981; Tompson *et al*., 1985; Tsai *et al*., 1987; Zabriskie *et al*., 1987; Rinas *et al*., 1989; Paalme *et al*., 1990), such as constant feeding, exponential feeding, and feeding with the respiratory quotient or pH as the control indicator. In HCDC, the dissolved oxygen concentration (DOC) is usually another important factor. In a stirring tank, DOC can be increased by adjusting the agitation, aeration and tank pressure. Pure oxygen gas and oxygen gas mixed with air are also proper alternatives for increasing the DOC. The DOC can be applied as a control indicator for the feeding of nutrients since it is always a growth-limiting factor in HCDC (Pan *et al*., 1987; Fass *et al*., 1989; Riesenberg *et al*., 1991).

It is important to develop HCDC along with a high level of enzyme expression. In this study, a mutant strain, *E. coli* EP1, derived from the parent strain ATCC 9637 and harbouring the pGL-5 plasmid (Liu *et al*., 1991;

Chou, 1992[1]), was used to produce PGA. The purpose of this study was to obtain a high level of enzyme expression in HCDC of recombinant *E. coli* EP1.

II. Materials and Methods

1. Bacterial Strain

A mutant strain, *E. coli* EP1, derived from *E. coli* ATCC 9637 and harbouring pGL-5 plasmid (Liu *et al*., 1991; Chou, 1992[1]) with chloramphenicol resistance, was employed in this work.

2. Media

The Luria-Bertani medium (LB) was composed of yeast extract 1.0% (weight/volume, w/v), NaCl 0.5% (w/v) and tryptone 1.0% (w/v). The seed medium (SM) consisted of LB medium along with a trace element 0.3% (v/v) and chloramphenicol 30 μg/ml. The trace element contained $FeCl_2 \cdot 6H_2O$ 2.7% (w/v), $ZnCl_2 \cdot 4H_2O$ 0.2% (w/v), $CoCl_2 \cdot 6H_2O$ 0.2% (w/v), $Na_2MoO_4 \cdot 2H_2O$ 0.2% (w/v), $CaCl_2 \cdot 2H_2O$ 0.1% (w/v), $CuCl_2$ 0.1% (w/v), H_3BO_3 0.05% (w/v), and concentrated HCl 10% (v/v) (Bauer and Shiloach, 1974). Two types of complex medium (FB1, FB2) were prepared, the composition of which is shown in Table 1. The production medium (PM) used in flask study was identical to the FB1 medium except for the feeding medium, and the carbon source of PM was as mentioned in the test. Carbon and nitrogen sources in all the media were sterilized separately. Chloramphenicol was added after sterilization as an antibiotic selection for the production of plasmid-encoded PGA. All the media were supplemented with 1% silicone antifoam (LG109, Asahidenka ltd. Co., Japan) to prevent foaming. The base medium used in the flask studies was the same as that of FB1 with the carbon source concentration mentioned in the test.

3. Cultivation Conditions

For incubation, 50 ml Erlenmeyer flasks containing 10 ml LB seed medium were inoculated with 2 – 3 loops of cells from the preservation slant following by cultivation on an orbital shaker at 37˚C and 200 rpm for 16 – 18 h. Flask studies were performed with 100 ml base medium in 500 ml Hinton flasks. The medium was inoculated with 5 ml LB culture, and cultivated on the orbital shaker at 28˚C and 200 rpm. Fed-batch cultivation was carried out in a 28l fermenter (NBS Co., New Brunswich, NJ, U.S.A.) at 28˚C. 500 ml of seed medium, inoculated with 50 ml LB culture, was operated at 28˚C and 200 rpm for 18 h; then, this seed culture was used as an inoculum in a 1:10 ratio to the production medium. The pH of the broth was controlled at 7.0 using 33% ammonia in water. The feeding strategy was applied to maintain the sorbitol concentration at around 3%. In the operation of FB1, the DOC was maintained at a 20% saturation level by adjusting the aeration, agitation, tank pressure. In contrast, pure oxygen gas was supplied to keep the DOC at the 20% saturation level throughout the FB2 process.

4. Assay

The optical density (OD) of the cells was determined by diluting the sample 100 times and measuring at 600 nm. The cell gram wet weight (gww) was measured by centrifuging 1ml of broth at 12000 rpm for 5 min and then the precipitate was washed twice with distilled water. The obtained OD value of the cells was correlated with the cell wet weight concentration via the calibration line. The penicillin G acylase activity was determined using the colorimetric method proposed by Kornfeld (1978), and one unit (IU) of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of 6APA per min.

5. Chemicals

The chemicals used in this study were at least reagent grade for medium preparations and analytical grade for analysis purposes, and all of them were purchased from conventional commercial sources.

Table 1. Compositions of Media

Type of Medium	Fed-Batch1 (FB1)	Fed-Batch2 (FB2)
Base Medium:		
Yeast extract (g/l)	5	5
Casamino acid (g/l)	5	5
KH_2PO_4 (g/l)	0	4
K_2HPO_4 (g/l)	5	4
$Na_2HPO_4 \cdot 12H_2O$ (g/l)	0	7
$MgSO_4 \cdot 7H_2O$ (g/l)	2.4	2.4
NH_4Cl (g/l)	0.2	0.2
$(NH_4)_2SO_4$ (g/l)	1.2	1.2
$CaCl_2$ (g/l)	0.02	0.02
Trace element (ml/l)	3	3
Sorbitol (g/l)	40	40
Chloramphenicol (μg/ml)	30	30
Feeding Medium:		
yeast extract (g/l)	40	40
KH_2PO_4 (g/l)	0	35
K_2HPO_4 (g/l)	0	35
$Na_2HPO_4 \cdot 12H_2O$ (g/l)	0	85
Trace element (ml/l)	3	3
Sorbitol (g/l)	500	350
Chloramphenicol (μg/ml)	30	30

[1] Chou, C.C. (1992) *The Function of Promoter and Spacer Sequence of pac Gene from E. coli ATCC 9637*. M.S. Thesis. Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, R.O.C.

Table 2. Effect of Concentration of Carbon Sources on the Cell Densities and Specific PGA Activity with Addition of Chloramphenicol

carbon source	carbon source concentration (% w/v)	cultivation time (h)	cell concentration (gww/l)	specific PGA activity (IU/gww)
Glucose	0.5	7	24.7	13.7
Glucose	1.0	7	33.9	15.6
Glucose	3.0	12	53.5	17.5
Sorbitol	0.5	7	26.1	28.4
Sorbitol	1.0	7	38.3	26.0
Sorbitol	3.0	12	51.3	24.3
Sorbitol	5.0	12	70.0	17.8
Glycerol	0.5	7	30.5	26.0
Glycerol	1.0	8	42.5	30.0
Glycerol	3.0	12	55.6	22.8
Glycerol	5.0	12	66.0	20.7

Table 3. Effect of Concentration of PAA on the Cell Densities and Specific PGA Activity

PAA concentration (% w/v)	Cell concentration (gww/l)	Specific PGA activity (IU/gww)
0	40.0	23.4
0.5	39.8	12.1
1	32.8	9.4
1.5	29.0	10.8
2	25.5	9.8

III. Results and Discussion

1. Effects of Carbon Sources

Three different carbon sources, (glucose, sorbitol and glycerol) were employed to investigate their effects on PGA accumulation in *E. coli*. The experimental results for the final cell densities and specific PGA activities are shown in Table 2. In all cases, higher cell concentrations were obtained with higher concentrations of carbon sources. However, the media which employed sorbitol and glycerol at concentrations less than 3% gave specific PGA activity levels higher than 22.0 IU/gww. On the other hand, the media with glucose as the carbon source had an average specific PGA activity level of only about 15.6 IU/gww. The lower specific PGA activity level obtained in the glucose medium was possibly due to catabolic repression, as glucose was the most favorable carbon source. The experimental results also indicated that at the highest concentrations of sorbitol and glycerol (5%), the enzyme activities were repressed. In the following fed-batch culture, sorbitol was employed as the carbon source with its concentration controlled at around 3%.

2. Effect of the Addition of Phenylacetic Acid (PAA)

PAA usually acts as an inducer in the cultivation of *E. coli* producing PGA (Babu and Panda, 1991). In this work, PAA was added to the production medium after three hours of cultivation to prevent inhibition of initial cell growth. The concentration of PAA was varied from 0 – 2% g/ml. As shown in Table 3, PAA repressed both the cell concentration and specific PGA activity of *E. coli* EP1. This result is apparently contrary to that reported by and Panda *et al*. (1991). It is probable that the mutant strain was fully released from dependence on PAA as a regulatory inducer in PGA formation.

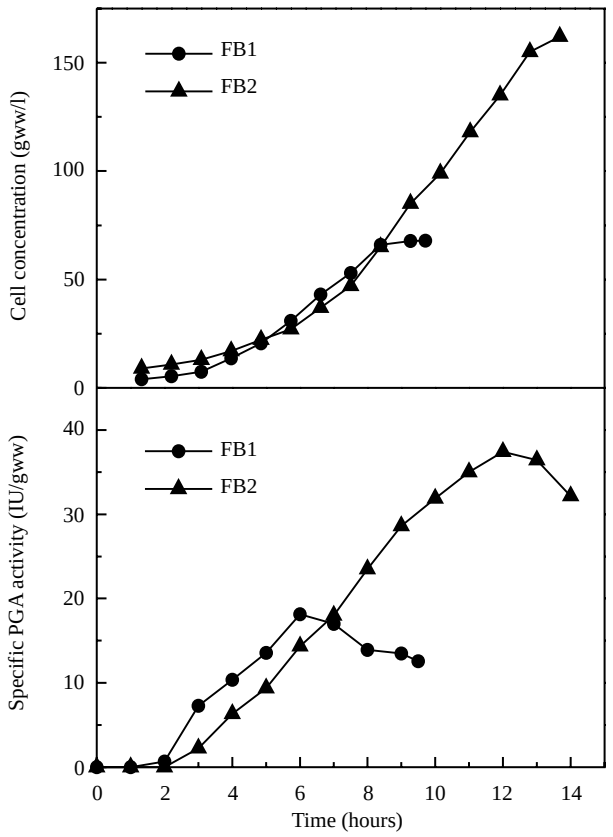

Fig 1. Time courses of cell concentrations and specific PGA activities for two different cultivative conditions in *E. coli* EP1 fed-batch fermentation.

3. Fed-batch Cultures of *E. coli* EP1

Figure 1 shows the time courses of the cell concentration and specific PGA activity obtained during the cultivation of *E. coli* EP1. The PGA activity accumulation was found to follow a growth-associated pattern during the early to middle growth phase of *E. coli*. In the FB1 fed-batch culture, in which only air was supplied during aeration, the highest biomass density obtained was 68.0 gww/l after 9.5 h. Meanwhile, the highest level of specific PGA activity (18.1 IU/gww) occurred after 6 h and then decreased gradually due to reduction of the cell growth rate. It was observed that DOC could not be maintained at a 20% saturation level with aeration using air alone after 6 h. The starvation of dissolved oxygen was apparently a critical factor affecting both the cell growth rate and PGA production rate. To avoid a shortage of dissolved oxygen, pure oxygen aeration was applied to maintain the DOC at 20% saturation during the whole fermentative process in FB2. In addition, the phosphate concentration in the FB1 medium was found to be another factor which limited cell growth. To obtain a higher level of specific PGA activity, the FB2 medium was designed to have a higher phosphate concentration in both the base medium and feeding medium. With the aforementioned modifications, the experimental results showed that the final cell concentration (162.4 gww/l) of FB2 was 2.4 times higher than that of FB1, and the maximum level of specific PGA activity (37.4 IU/gww at 12 h) was also higher than that of FB1.

IV. Conclusion

In the course of cultivating of *E. coli* EP1 for recombinant enzyme production, it was found that PGA production was catabolically repressed by glucose and was not induced by PAA. The media containing sorbitol or glycerol as the carbon source gave better results in terms of specific enzyme activity. The specific PGA activity of the cells increased at a constant rate during the early to middle cell growth phase. This growth-associated recombinant enzyme production pattern of the mutant strain has seldom been mentioned in the literature. When a high phosphate concentration was employed together with pure oxygen supplementation, a higher level of specific PGA activity was obtained in HCDC. The merit of using pure oxygen gas for the production of a recombinant enzyme has been demonstrated.

References

Babu, P.S.R. and Panda, T. (1991) The role of phenylacetic acid in biosynthesis of penicillin amidase in *E. coli*. *Bioproc. Eng*., **26**:71-74

Bauer, S. and Shiloach, J. (1974) Maximal exponential growth rate and yield of *E. coli* obtainable in a bench-scale fermentor. *Biotechnol. Bioeng*., **14**:933-941.

Chou, C.H., Bennett, G.N. and San, K.Y. (1994) Effect of modified glucose uptake using genetic engineering techniques on high-level recombinant protein production in *Escherichia coli* dense cultures. *Biotechnol. Bioeng*., **44**:952-960.

Fass, R., Clem, T.R. and Shiloach, J. (1989) Use of a novel air separation system in a fed-batch fermentative culture of *Escherichia coli. Appl. Enviorn. Microbiol*., **55**:1305-1307.

Gleiser, I.E. and Bauer, S. (1981) Growth of *E. coli* W to high cell concentration by oxygen level linked control of carbon source concentration. *Biotechnol. Bioeng*., **23**:1015-1021.

Konstantinov, K.B., Nishio, N., Seki, T. and Yoshida, T. (1991) Physiologically motivated strategies for control of the fed-batch cultivation of recombinant *Escherichia coli* for phenylalanine production. *J. Ferment. Bioeng*., **71**:350-355.

Kornfeld, J.M. (1978) A new colorimetric method for the determination of 6-aminopenicillanic acid. *Anal. Biochem*., **86**:118-126.

Liu, Y.T., Liao, G.L., Chao, H.Y., Jee, D.D. and Han, S.H. (1991) Application of the recombinant DNA plasmid for penicillin G acylase production. R.O.C. Patent 44304.

Mori, H., Kobayashi, T. and Shimizu, S. (1983) Effect of carbon dioxide on growth of microorganisms in fed-batch culture. *J. Ferment. Technol*., **61**:211-213.

Paalme, T., Tiisma, K., Kahru, A., Vanatalu, K. and Vilu, R. (1990) Glucose-limited fed-batch culture of *Escherichia coli* with computer-controlled fixed growth rate. *Biotechnol. Bioeng*., **35**:312-319.

Pan, J.G., Rhee, J.S. and Lebeault, J.M. (1987) Physiological constraints in increasing biomass concentration of *Escherichia coli* B in fed-batch culture. *Biotechnol. Lett*., **9**:89-94.

Panbangred, W., Udombunditkul, M. and Meevootisom, V. (1990) High expression of the penicillin Gacylase gene in genetically engineered *Escherichia coli. Enzyme Eng*., **10**:455-459.

Pavlasova, E., Stejskalova, E. and Sikyta, B. (1986) Stability and storage of *E. coli* mutants hyperproducing galactosidase. *Biotechnol. Lett*., **8**:475-478.

Reiling, H.E., Laurila, H. and Fiechter, S. (1985) Mass culture of *E. coli*: Medium development for low and high density cultivation of *E. coli* B/r in minimal and complex media. *J. Biotechnol*., **2**:191-206.

Riesenberg, D., Schulz, V., Knorre,W.A., Pohl, H.D., Korz, D., Sanders, E.A., Rob, A. and Deckwer, W.D. (1991) High cell density cultivation of *Escherichia coli* at controlled specific growth rate. *J. Biotechnol*., **20**:17-28.

Rinas, U., Kracke-Helm, H.A. and Schugerl, K. (1989) Glucose as a substrate in recombinant strain fermentation technology. By-product formation, degradation and intracellular accumulation of recombinant protein. *Appl. Microbiol. Biotechnol*., **31**:163-167.

Shiloach, J. and Bauer, S. (1975) High-yield growth of *E. coli* at different temperatures in a bench-scale fermentor. *Biotechnol. Bioeng*., **17**: 227-239.

Sobotkova, L., Stepanek, V., Plhackova, K. and Kyslik, P. (1995) Cloning of penicillin G acylase-encoding gene from *Escherichia coli* high-producing strain RE3. *Biotechnol. Lett*., **17**: 723-728.

Tompson, B.G., Kole, M. and Gerson, D.F. (1985) Control of ammonium concentration in *E. coli* fermentation. *Biotechnol. Bioeng*., **27**:818-824.

Tsai, L.B., Morris, M.F., Rotgers, C. and Fenton, D. (1987) The effect of organic nitrogen and glucose on the production of recombinant human insulin-like growth factor in high cell density *Escherichia coli* fermentation. *J. Ind. Microbiol*., **2**:181-187.

Zabriskie, D.W., Wareheim, D.A. and Ploansky, M.J. (1987) Effects of fermentation feeding strategies prior to induction of expression of a recombinant malaria antigen in *Escherichia coli. J. Ind. Microbiol*., **2**:87-95.

應用基因改質大腸桿菌之高密度培養生產盤尼西林醯胺酵素

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摘　　要

本研究中以一經基因改變帶有pGL-5 質體之大腸桿菌，進行醱酵生產盤尼西林醯胺酵素（PGA）之研究。在培養基中進行不同碳源之比較，結果顯示葡萄糖有代謝抑制效應，故其PGA 之比活性最低。據文獻報導苯乙酸爲PGA 酵素生產之誘導劑，但在此菌株之培養基中加入苯乙酸反而會抑制菌體生長及酵素之活性。在饋料批式醱酵中，菌體生長之前期至中期，其PGA 比活性會隨著菌體生長濃度增加而增加。若以純氧氣進行饋料批式醱酵並適度的調整培養基配方，在12 小時內可獲得高菌體濃度達162（克菌濕重/升），此較原操作高2.4 倍，另外PGA 之比活性亦可增加至37（IU/克菌濕重）。