Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 41 (2007) 326–330
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

High-level production penicillin G acylase from *Alcaligenes faecalis* in recombinant *Escherichia coli* with optimization of carbon sources

Shiwei Cheng, Qingxun Song, Dongzhi Wei *, Bingxue Gao

State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, PR China

Received 9 October 2006; received in revised form 24 February 2007; accepted 25 February 2007

Abstract

Different carbon sources were investigated for overproduction penicillin G acylase from *Alcaligenes faecalis* in recombinant *Escherichia coli* strains. The results indicated that the enzyme was optimally produced with 45 g/l of dextrin, and about 43,385 and 79,880 U/l for the highest enzyme activities were obtained in batch cultivations of shaken flasks and a 3.7 l bioreactor, respectively. Active site titration and SDS-PAGE electrophoretic analysis demonstrated that the maximum yield of the active enzyme was 2.54 g/l, which was about 40% of total soluble proteins. The highest specific activity of *A. faecalis* penicillin G acylase obtained was above 10 U/mg protein, and there was almost no plasmid lost in the whole batch cultivations. Furthermore, the cultivation process was relatively simple and suitable for large-scale production.

Keywords: *Alcaligenes faecalis*; Batch cultivation; Carbon source; Overexpression; Penicillin G acylase

1. Introduction

Penicillin acylase (E.C. 3.5.1.11) has been widely used for the production of 6-aminopenicillianic acid (6-APA), 7-amino-3-desacetoxycephalosporanic acid (7-ADCA) and important semi-synthetic antibiotics [1,2]. It is also used in peptide synthesis [3] and resolution of racemic compounds [4,5].

The relatively unknown penicillin G acylase (PGA) from *Alcaligenes faecalis* shares only 49% of protein sequence homology to *Escherichia coli* PGA [6], which has several unique outstanding characteristics such as a broad pH optimum, higher enantioselectivity [7–9]. Especially, the thermal stability of *A. faecalis* PGA is significantly higher than that of other penicillin G acylases because it contains a disulfide bridge [9]. Accordingly, more and more attentions have been paid to its new application though *E. coli* PGA is the most frequent source recently in industrial application.

E. coli is the most common prokaryotic expression system for production of homologous and heterologous proteins. Penicillin acylase in *E. coli* is formed by a series of post-translational steps (i.e., periplasmic processing/folding steps, intracellular proteolysis [10] and cofactors for the maturation [11]), which is unusual for prokaryotic proteins. So the complex posttranslational level must be considered in order to attain a high level of pga gene expression [12]. It was reported that, glucose is an important parameter to affect significantly the rate of the proteolytically sensitive pre-pro-protein of penicillin acylase (ppPA) [13]. Furthermore, several environmental conditions, such as carbon source [14], can regulate the expression of the *E. coli* pga gene. In this paper, a nonrepressing and noninducing carbon source instead of glucose was studied for high-level expression and overproduction of *A. faecalis* PGA in recombinant *E. coli*.

2. Materials and methods

2.1. Bacterial strain and plasmid

E. coli strain DH5α was used as the host for the production of *A. faecalis* PGA. The recombinant plasmid, pSMLFPGA, is a medium copy-number plasmid, which carries the *A. faecalis* pga gene, rrnB transcript terminator, medium-copy replicon (p15A) and a tetracycline resistant gene. The expression of pga gene is controlled by the *trc* promoter, which needs not be induced by isopropyl-β-thio-galactopyranoside (IPTG) because of no $lacI^q$ gene.

2.2. Conditions of seed culture

Seed culture was first prepared by inoculating the −20 °C glycerol-containing stock culture (200 μl) into 25 ml of Luria-Bertani medium (yeast

* Corresponding author. Tel.: +86 21 64252981; fax: +86 21 64250068.
E-mail address: dzhwei@ecust.edu.cn (D. Wei).

0141-0229/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2007.02.011

extract 0.5%, w/v, NaCl 1.0%, w/v, tryptone 1.0%, w/v, pH 7.0) and incubated on a rotary shaker for 16 h at 37 °C and 200 rpm. 80 μg/ml of tetracycline was as a selection marker. The pre-culture at about 2 ml was inoculated into the improved medium (50 ml) and continued to incubate at 37 °C for 10 h with agitation (200 rpm), and 40 μg/ml of tetracycline was supplemented in the medium. The following compositions were used as the improved medium (per litre): 5 g yeast extract, 10 g tryptone, 2 g KH_2PO_4, 5 g $K_2HPO_4{\cdot}3H_2O$, 7 g $Na_2HPO_4{\cdot}12H_2O$, 1.2 g $(NH_4)_2SO_4$, 0.2 g NH_4Cl, 0.1 g NaCl, and the initial pH was 7.0.

2.3. Batch cultivations in shaken flasks and a bioreactor

Batch cultivation in shaken flasks was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of the producing enzyme medium inoculated with 4% (v/v) seed culture. The producing enzyme medium (per litre) contained: different carbon sources, 5 g yeast extract, 20 g tryptone, 1 g $MgSO_4{\cdot}7H_2O$, 3.5 g $Na_2HPO_4{\cdot}12H_2O$, 2.5 g $K_2HPO_4{\cdot}3H_2O$, 1 g KH_2PO_4, 0.05 g NaCl, 0.6 g $(NH_4)_2SO_4$, 0.1 g NH_4Cl, 0.2 mg $FeSO_4{\cdot}7H_2O$, 40 μg $CoCl_2{\cdot}6H_2O$, 20 μg $ZnCl_2$, 20 μg $Na_2MoO_4{\cdot}2H_2O$, 10 μg $CuSO_4{\cdot}5H_2O$, 7 μg $MnSO_4{\cdot}H_2O$, 5 μg H_3BO_4.

The bioreactor cultivation was carried out in a 3.7 l bioreactor (Bioengineering, Switzerland) containing 2.5 l working volume of the producing enzyme medium. 100 ml of seed culture was used as the inoculum. The producing enzyme medium was supplemented with 1 ml/l of antifoam to avoid excessive foaming. Temperature was controlled automatically at 30 °C. Filter-sterilized air at 2.5 l/min was purged into the culture for aeration, and the dissolved oxygen concentration was kept above 30% by increasing the stirrer speed.

2.4. Treatment of samples

Cell culture samples (10 ml) were centrifuged at 4 °C and 12,000 × *g* for 5 min. The cell pellets were resuspended in 30 ml phosphate buffer (50 mM, pH 7.8) and disrupted using the ultrasonic cell disruptor (Ningbo Scientz, JY88-II, China) in an ice bath at 400 W for 15 min. They were centrifuged again at 12,000 × *g* for 10 min, and the supernatants were assayed for PGA activity, protein content and active site titration. Protein contents of the cell lysate, the pellet (as insoluble fraction) and the supernatant (as soluble fraction) were analyzed by SDS-PAGE.

2.5. Determination of PGA activity and protein content

The enzyme activity was measured using potassium penicillin G (PGK) solution. The hydrolysis of PGK by PGA yields 6-APA which is followed by a spectrophotometric assay with para-dimethylaminobenzadehyde (PDAB) as a colorimetric substrate [15]. One enzyme activity unit (U) is defined as the amount of enzyme required to produce one μmol of 6-APA per min in 4% (w/v) solution of PGK at pH 7.8 and 37 °C. Protein content was determined by Lowry method using Folin phenol reagent [16].

2.6. Assay for cell density and dextrin concentration

Growth of the microorganism was determined by optical density measurement at 600 nm (OD_{600}). The optical density was also converted to dry cell weight (DCW) using a standard curve. One OD_{600} unit corresponded to 0.32 g/l of dry cell weight. Dextrin concentration was measured with the former method [17], and the dextrin concentration was expressed as the equivalent glucose concentration.

2.7. Active site titration and electrophoretic analysis

Molar concentration of *A. faecalis* PGA was determined by active site titration [7,11]. Equal amounts of PGA were incubated with different amounts of phenylmethanesulfonyl fluoride (PMSF) in phosphate buffer (pH 7.8, 50 mM) for 30 min, and the residual activity was measured with PDAB. SDS-PAGE analysis (15% gel) was by the reported method [18]. Protein bands were stained with Coomassie Blue and densitometry was carried out using the Furi Smart View software (China).

2.8. Determination of plasmid stability

Culture samples taken at different time were diluted to 10^{-5} to 10^{-7} in fresh LB medium, which were spread on a non-selective LB agar plate and grown at 37 °C for 16 h. At least 100 individual colonies selected randomly were replica plated on selective LB agar plates (containing 100 μg/ml tetracycline) and non-selective control plates by using sterile toothpicks, and then incubated at 37 °C for 24 h. The ratio of the number of colony forming units on the selective agar plate to that on the non-selective agar was used to determine the percentage of plasmid-carrying cells [19,20].

3. Results and discussion

3.1. Effect of carbon sources on overexpression A. faecalis PGA

The effect of carbon sources on the production of *A. faecalis* PGA was performed at 30 °C and 200 rpm in 250-ml Erlenmeyer flasks, which contained 50 ml of the producing enzyme medium supplemented with 4% (w/v) carbon sources. The results of the present investigation demonstrated that dextrin was the best carbon source for the production of heterologous *A. faecalis* PGA, which could make the host/vector system have a higher level of pga gene expression, and the enzyme activities obtained were 11,939 and 24,781 U/l respectively at 48 and 96 h (Table 1). It was notable that the biomass and enzyme activity were both lower using starch as the carbon source, which might be that

Table 1
Influence of carbon sources on the production of *A. faecalis* PGA

Carbon sources	Enzyme activity (U/l)		Biomass (OD_{600})		Specific activity (U/mg protein)		Final pH	
	48 h	96 h	48 h	96 h	48 h	96 h	48 h	96 h
Dextrin	11,939	24,781	10.1	16.4	5.2	9.3	7.26	7.08
Sucrose	1,009	829	3.6	3.5	1.3	1.3	8.16	8.28
Mannitol	3,933	3,213	8.1	8.0	2.3	2.4	4.60	4.74
Lactose	1,459	1,549	5.7	5.5	1.6	1.6	7.14	7.54
Starch	1,054	1,324	6.4	6.8	0.9	1.7	8.02	8.13
Maltose	3,078	2,538	10.1	9.2	1.7	1.6	4.77	4.66
Glycerol	1,369	1,414	8.2	9.5	0.9	1.1	4.70	4.85
Glucose	1,054	784	5.5	5.5	0.7	0.8	4.18	4.20
Sorbitol	379	1,189	3.9	7.8	0.5	0.7	7.84	5.33

Conditions: 4% (w/v) carbon source; culture temperature, 30 °C; initial pH, 7.0. Data are means of duplicates. Standard errors were less than 5% of the means.

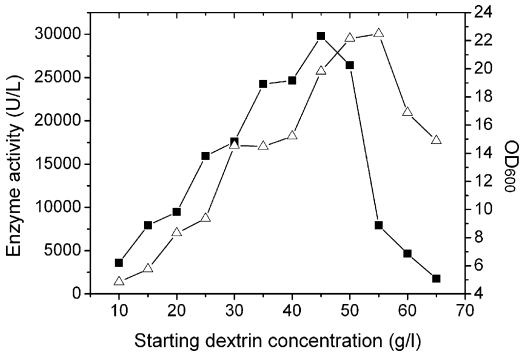

Fig. 1. Effect of dextrin concentration on cell growth and *A. faecalis* PGA activity. (△) OD_{600}; (■) enzyme activity.

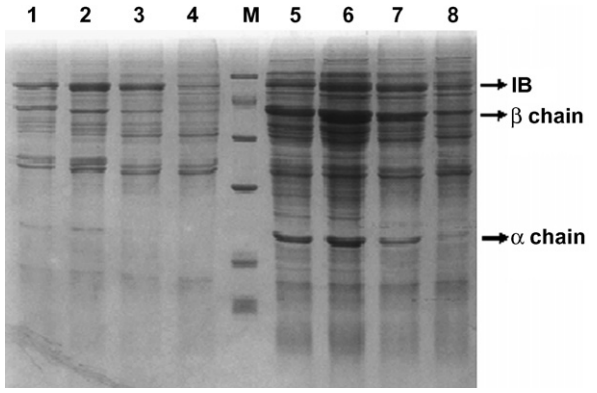

Fig. 2. SDS-PAGE analysis on the *A. faecalis* PGA expression with different dextrin concentration. Lane M—protein marker, from bottom to top: 14, 20, 31, 43, 66 and 97 kDa. From lines 1 to 4: the precipitation of disrupted cells, where the dextrin concentration was 25, 45, 55 and 65 g/l, respectively. From lines 5 to 8: the disrupted cells were analyzed, where the dextrin concentration was 25, 45, 55 and 65 g/l, respectively. IB: *A. faecalis* PGA precursors existing in cells as inclusion bodies; α chain: α subunit of *A. faecalis* PGA; β chain: β subunit of *A. faecalis* PGA.

the formed tremellose deposition and higher pH (Table 1) in the cultivation system were not propitious to *A. faecalis* pga gene expression. Though relatively higher biomasses (OD_{600}) were obtained with mannitol, maltose and glycerol as carbon sources, SDS-PAGE electrophoretic analysis demonstrated that their *A. faecalis* PGA expression levels were all lower than that of with dextrin (data not shown). At the same time, the plasmid pSMLF-PGA was rather stable and almost not lost from the recombinant (data not shown). So it might be that the culture pH declined fleetly in the system and then could not be propitious to the production of *A. faecalis* PGA.

3.2. Effect of dextrin content on producing A. faecalis PGA

The influence of dextrin content on the production of *A. faecalis* PGA was summarized in Fig. 1. The relatively better yields were obtained at the dextrin concentration from 35 to 50 g/l, and the highest enzyme activity was observed at 45 g/l of dextrin. The maximum cell density was 22.5 (OD_{600}) when the dextrin concentration was 55 g/l, while the active enzyme yield was little. SDS-PAGE electrophoretic analysis indicated that the *A. faecalis* PGA protein expression level was relatively lower at higher dextrin concentration, furthermore, the inactive proenzyme increased with the augment of the PGA protein expression level (Fig. 2). So low *A. faecalis* PGA yield in high dextrin concentration was because the protein expression level decreased while not the accumulation of the inactive proenzyme in a short period of time.

3.3. Batch cultivation in shaken flasks

Batch cultivation of the host/vector system of DH5α harboring pSMLFPGA with dextrin as the sole carbon source was conducted in shaken flasks. The results were summarized in Fig. 3. The maximum enzyme activity obtained was about 43385 U/l at 192 h, where 11.4 U/mg protein and 23.6 for the specific activity and OD_{600} were attained, respectively, and the results were higher than the former reports [21]. It was infrequent that the fermentation time was relatively longer in the cultivation of the recombinant (Fig. 3), but which might conduce a more efficient posttranslational processing of the PGA precursor and result in higher plasmid copy numbers due to reduce the growth rate [22–24], so high activity of *A. faecalis* PGA was obtained in the cultivation process of the recombinant *E. coli*.

3.4. Batch cultivation in a bioreactor

As the pH change of the batch cultivation in shaken flasks was little except at the phase of cell lysis (Fig. 3), the bioreactor cultivation for producing *A. faecalis* PGA was also investigated without controlling pH (Fig. 4). Compared to the cultivation in shaken flasks, the cell density and *A. faecalis* PGA activity both increased about two-fold while the specific activity increased little, which were 44.7 (OD_{600}), 79,880 U/l and 12.8 U/mg protein, respectively, at 120 h. After 144 h, OD_{600} decreased gradually because of autolysis of the bacterial cells. The fermentation time was shortened about three days as compared to that of the shake-flask cultivation, which might be due to dissolved oxygen concentration increased in the system. Similar change about

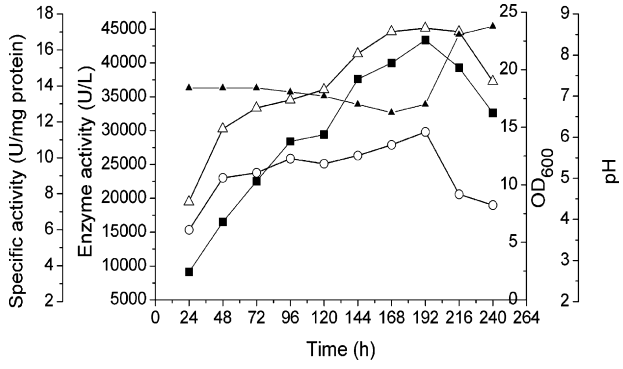

Fig. 3. Batch cultivation profiles in shaken flasks with dextrin as the sole carbon source. (■) Enzyme activity; (◯) specific activity; (△) OD_{600}; (▲) pH. Conditions: starting dextrin concentration, 45 g/l; initial pH, 7.0; shaker speed, 200 rpm; culture temperature, 30 °C.

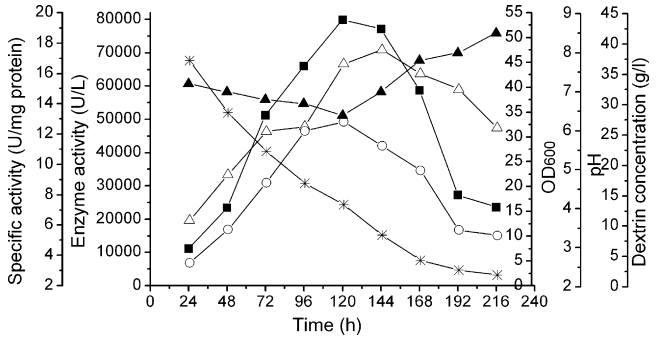

Fig. 4. Batch cultivation profiles in a 3.7 l bioreactor with dextrin as the sole carbon source. (■) Enzyme activity; (◯) specific activity; (△) OD_{600}; (▲) pH; (*) dextrin concentration. Conditions: starting dextrin concentration, 45 g/l; initial pH, 7.0; culture temperature, 30 °C.

the culture pH was observed, and the lowest pH was 6.43 at 120 h.

The biochemically active *A. faecalis* PGA is composed of two dissimilar subunits (α, approximately 23 kDa; β, approximately 63 kDa) [9,25], and the molar concentration of the active enzyme can be determined by the method of active site titration [7]. The maximum yield of the active *A. faecalis* PGA obtained was 2.54 g/l or 153 mg/g dry cell weight (DCW), which was 40.7% of total soluble proteins in the cells, and the result was higher than the former in the literature [11]. SDS-PAGE electrophoretic analysis indicated that the highest active *A. faecalis* PGA level was about 40% of total cellular soluble proteins (Fig. 5, lane 2), which was agreed with the result of active site titration.

Production of functional recombinant proteins is often limited with the formation of large protein aggregates called inclusion bodies in bacterial cells [26]. In order to avoid the accumulation of polypeptide intermediates and improve the active enzyme production, a "balanced" protein synthesis flux throughout the gene expression steps (i.e., transcription, translation, and post-translational steps) should be properly maintained [23]. In our study, SDS-PAGE electrophoretic analysis indicated that little *A. faecalis* PGA existed in the precipitation of disrupted cells (Fig. 5, lane 1) and relatively low amount of the inactive PGA precursors produced in the process of the *A. faecalis* PGA protein expression, so the host/vector system had a better "balanced" *A. faecalis* PGA synthesis flux with dextrin as the carbon source and then high activity of the aim enzyme was obtained.

3.5. Plasmid stability

The batch cultivations in this work were done under no selective pressure, and the reason was that the producing enzyme medium contained Mg^{2+} which could make tetracycline lose its function. So plasmid stability was measured in the whole cultivations of the shaken flasks and 3.7 l bioreactor. The results indicated that the recombinant was rather stable in the absence of tetracycline selection (data not shown), and the loss of plasmid pSMLFPGA from the recombinant DH5α could be negligible in course of the batch cultivations.

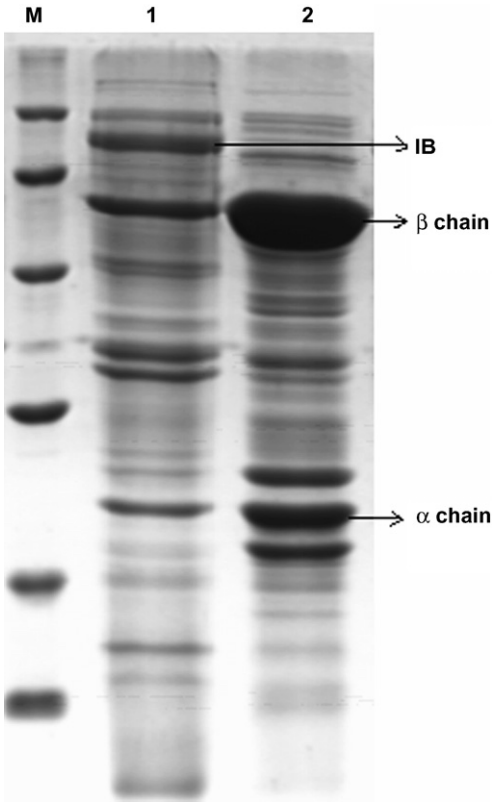

Fig. 5. SDS-PAGE analysis on protein contents for the fractions of disrupted cells with the bioreactor cultivation at 120 h. Lane M—protein marker, from bottom to top: 14, 20, 31, 43, 66 and 97 kDa; lane 1: the precipitation of disrupted cells; lane 2: the supernatant of disrupted cells. IB: *A. faecalis* PGA precursors existing in cells as inclusion bodies; α chain: α subunit of *A. faecalis* PGA; β chain: β subunit of *A. faecalis* PGA.

4. Conclusions

Dextrin was a better carbon source for cell growth and overexpression of *A. faecalis* PGA in recombinant *E. coli*. In the cultivated process, the change of culture pH was little except at the phase of cell lysis. The maximum yield of the active enzyme obtained was about 40% of total soluble proteins, and relatively little inactive pro-protein of *A. faecalis* PGA produced. To our knowledge, our results were higher than the reported yields in the literature.

Acknowledgements

This work was supported by the grant from the Ministry of Science and Technology, Basic Research project No.: 2002CCA400, and Program for New Century Excellent Talents in University, project No.: NCET-04-0411. We thanked Prof. Zhongyi Yuan (Shanghai Institute for Biological Sciences, China) for the strain and plasmid.

References

[1] Baldaro E, Fuganti C, Servi S, Tagliani A, Terreni M. The use of immobilized penicillin G acylase in organic synthesis. In: Servi S, editor. Microbial

reagents in organic synthesis. The Netherlands: Kluwer Academic Publishers; 1992. p. 175–88.
[2] Bruggin K, Roos EC, Devroom E. Penicillin acylase in the industrial production of β-lactam antibiotics. Org Process Res Dev 1998;2:128–33.
[3] Khimiuk AY, Korennykh AV, van Langen LM, van Rantwijk F. Penicillin acylase-catalyzed peptide synthesis in aqueous medium: a chemo-enzymatic route to stereoisomerically pure diketopiperazines. Tetrahedron-Asymmetr 2003;14:3123–8.
[4] Basso A, Braiuca P, Martin LD, Ebert C, Gardossi L. D-Phenylglycine and D-4-hydroxyphenylglycine methyl esters via penicillin G acylase catalysed resolution in organic solvents. Tetrahedron-Asymmetr 2000;11:1789–96.
[5] Fadnavis NW, Sharfuddin M, Vadivel SK. Resolution of racemic 2-amino-1-butanol with immobilised penicillin G acylase. Tetrahedron-Asymmetr 1999;10:4495–500.
[6] Ignatova Z, Stoeva S, Galunsky B, Hörnle C, Nurk A, Piotraschke E. Proteolytic processing of penicillin amidase from *Alcaligenes faecalis* cloned in *E. coli* yields several active forms. Biotechnol Lett 1998;20:977–82.
[7] Svedas V, Guranda D, van Langen L, van Rantwijk F, Sheldon R. Kinetic study of pencillin acylase from *Alcaligenes faecalis*. FEBS Lett 1997;417:414–8.
[8] van Langen LM, Oosthoek NHP, Guranda DT, van Rantwijk F. Penicillin acylase-catalyzed resolution of amines in aqueous organic solvents. Tetrahedron-Asymmetr 2000;11:4593–600.
[9] Verhaert RMD, Riemens AM, van der Lann JM. Molecular cloning and analysis of the gene encoding the thermostable penicillin G acylase from *Alcaligenes faecalis*. Appl Environ Microb 1997;63:3412–8.
[10] Ignatova Z, Taruttis S, Kasche V. Role of the intracellular proteolysis in the production of the periplasmic penicillin amidase in *Escherichia coli*. Biotechnol Lett 2000;22:1727–32.
[11] Kasche V, Ignatova Z, Märkl H, Plate W, Punckt N, Schmidt D, et al. Ca^{2+} is a cofactor required for membrane transport and maturation and is a yield-determining factor in high cell density penicillin amidase production. Biotechnol Prog 2005;21:432–8.
[12] Ignatova Z, Mahsunah A, Georgieva M, Kasche V. Improvement of posttranslational bottlenecks in the production of penicillin amidase in recombinant *Escherichia coli* strains. Appl Environ Microbiol 2003;69:1237–45.
[13] Ignatova Z, Enfors SO, Hobbie M, Taruttis S, Vogt C, Kasche V. The relative importance of intracellular proteolysis and transport on the yield of the periplasmic enzyme penicillin amidase in *Escherichia coli*. Enzyme Microb Technol 2000;26:165–70.
[14] Merino E, Balbás P, Recillas F, Becerril B, Valle F, Bolivar F. Carbon regulation and the role in nature of the *Escherichia coli* penicillin acylase (pac) gene. Mol Microbiol 1992;6:2175–82.
[15] Shewale JG, Kumar KK, Ambekar GR. Evaluation of 6-amino penicillanic acid by *p*-dimethylaminobenzadehyde. Biotechnol Technol 1978;1:715–8.
[16] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin-phenol reagent. J Biol Chem 1951;193:265–75.
[17] Zhang M, Shi M, Zhou Z, Yang S, Yuan Z, Ye Q. Production of *Alcaligenes faecalis* penicillin G acylase in *Bacillus subtilis* WB600 (pMA5) fed with partially hydrolyzed starch. Enzyme Microb Technol 2006;39:680–5.
[18] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[19] Zhang Y, Taiming L, Liu J. Low temperature and glucose enhanced T7 RNA polymerase-based plasmid stability for increasing expression of glucagon-like peptide-2 in *Escherichia coli*. Protein Exp Purif 2003;29:132–9.
[20] Hägg P, Pohl JW, de, Abdulkarim F, Isaksson LA. A host/plasmid system that is not dependent on antibiotics and antibiotic resistance genes for stable plasmid maintenance in *Escherichia coli*. J Biotechnol 2004;111:17–30.
[21] Deak PM, Lutz-Wahl S, Bothe H, Ficher L. Bioreactor cultivation of *Escherichia coli* for production of recombinant penicillin G amidase from *Alcaligenes faecalis*. Biotechnol Lett 2003;25:397–400.
[22] León AD, Hernóndez V, Galindo E, Ramírez OT. Effects of dissolved oxygen tension on the production of recombinant penicillin acylase in *Escherichia coli*. Enzyme Microb Technol 2003;33:689–97.
[23] Lin WJ, Huang SW, Chou CP. DegP-coexpression minimizes inclusion-body formation upon overproduction of recombinant penicillin acylase in *Escherichia coli*. Biotechnol Bioeng 2001;73:484–91.
[24] Ospina S, Merino E, Ramirez OT, López-Munguía A. Recombinant whole cell penicillin acylase biocatalyst: production, characterization and use in the synthesis and hydrolysis of antibiotics. Biotechnol Lett 1995;7:615–20.
[25] Verhaert RMD, van Duin J, Quax WJ. Processing and functional display of the 86 kDa heterodimeric penicillin G acylase on the surface of phage fd. Biochem J 1999;342:415–22.
[26] Sriubolmas N, Panbangred W, Sriurairatana S, Meevootisom V. Localization and characterization of inclusion bodies in recombinant *Escherichia coli* cells overproducing penicillin G acylase. Appl Microbiol Biotechnol 1997;47:373–8.