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Enzyme and Microbial Technology 39 (2006) 1166–1172
ENZYME AND MICROBIAL TECHNOLOGY
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Single-pot conversion of cephalosporin C to 7-aminocephalosporanic acid using cell-bound and support-bound enzymes

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Received 14 December 2005; received in revised form 28 February 2006; accepted 28 February 2006

Abstract

The two enzymes in two disparate forms, D-amino acid oxidase (DAAO) in the permeabilized *Pichia pastoris* cells and immobilized glutaryl-7-aminocephalosporanic acid acylase (GA) on support, were employed to convert cephalosporin C (CPC) to 7-aminocephalosporanic acid (7-ACA) in a single reactor. As a catalyst used in the reaction, DAAO in the permeabilized cells was relatively stable and its half-life was up to 14.5 days at 30 °C. In this study, CPC could be converted to 90.9% 7-ACA, 5% α-ketoadipyl-7-ACA (AKA-7-ACA) and 4.1% unidentified by-product within 2.5 h. During the reaction process, the loss of DAAO activity in the reactor was at an average rate of 0.07 U min^{-1}, but it could be compensated by continuous addition of the new permeabilized cell suspension. At the end of reaction, the stable immobilized GA was intercepted in a specially designed reactor, and reused for the next conversions. The permeabilized cells were separated outside the reactor by centrifugation and reused. Thus, the consecutive production of 7-ACA from CPC in a single reactor is achieved by a fed-batch strategy. In the reaction system, the used permeabilized cells and immobilized GA were renewed every four cycles and every seven cycles, respectively. The yield of 7-ACA reached around 90% at each reaction cycle.

Keywords: D-Amino acid oxidase; Glutaryl-7-aminocephalosporanic acid acylase; Cephalosporin C; 7-Aminocephalosporanic acid; α-Ketoadipyl-7-ACA

1. Introduction

The 7-aminocephalosporanic acid (7-ACA) is a significant intermediate for making semisynthetic cephalosporin antibiotics in pharmaceutical industries. The traditional method for preparation of this product involves the chemical deacylation of the corresponding 7-acylaminocephalosporanic acids in organic solvents, using toxic reagents and laborious procedures [1]. In recent years, this method has been replaced by a new two-step enzymatic transformation performed in two or three reactors [2,3]. The first step involves the conversion of cephalosporin C (CPC) by D-amino acid oxidase (DAAO) to hydrogen peroxide (H_2O_2) and α-ketoadipyl-7-ACA (AKA-7-ACA), which spontaneously reacts with H_2O_2 to render 7-β-(4-carboxybutanamido)-cephalosporanic acid (GL-7-ACA) [4]. In the second step, GL-7-ACA acylase (GA) hydrolyzes GL-7-ACA to produce 7-ACA.

With the aim of lowering the production cost, some investigators have focused great efforts on a one-step protocol for 7-ACA production. The reported one-step protocols involve two aspects, i.e. the direct conversion of CPC to 7-ACA by a single enzyme, cephalosporin acylase or mutant of glutarylamidase [5–8], as well as the conversion of CPC to 7-ACA by simultaneous action of DAAO and GA [3,4,9–11]. Despite extensive attempts have been carried out to seek for a single enzyme for directly converting CPC to 7-ACA, the successful application of such an enzyme in the industrial production of 7-ACA has not been reported. Currently, simultaneous action of DAAO and GA for producing 7-ACA in a single reactor (single-pot) is studied extensively and intensively. However, the main problem of this method is the presence of H_2O_2 during the reaction process, which results in the inactivation of the employed enzymes, especially DAAO [11].

Although the single-pot production of 7-ACA in the absence of H_2O_2 by a trienzymatic system (DAAO, GA and catalase) has been recently reported, a major drawback of this method is that

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doi:10.1016/j.enzmictec.2006.02.028

the hydrolysis rate of GA with AKA-7-ACA is comparatively low [11]. In the case of the presence of H_2O_2, DAAO displays a quite low stability even in immobilized form [11]. From this point of view, if DAAO and GA being in identical forms (e.g. two immobilized enzymes on different supports or two free cells) or the two enzymes coexisting in a cell or support are simultaneously employed to produce 7-ACA in a single reactor, the active GA catalyst will be discarded along with the inactive DAAO catalyst because they cannot be separated. To solve this problem, we propose to employ DAAO and GA in two disparate forms to produce 7-ACA in a single reactor, in which the two catalysts can be separated. Thus, the inactive DAAO catalyst can be removed selectively while the active GA catalyst can be reused for next bioconversions.

On the other hand, a detailed comparison of the catalytic properties of DAAO from different sources has been made previously [12,13]. The enzymes from microorganisms appear to be much more suitable for bioconversion [12]. Therefore, cloning the DAAO gene into microorganisms for high expression of enzyme is also an approach for reducing the production cost of 7-ACA [14–16]. Additionally, the cellular DAAO can be directly employed for enzymatic reactions after the cells are permeabilized with detergents or organic solvents. As far as unstable cellular DAAO is concerned, it is highly desirable from an economic point of view to directly utilize permeabilized cells (cell-bound DAAO) for enzymatic reactions [17].

In the present work, DAAO in the permeabilized recombinant *Pichia pastoris* cells and immobilized GA on support (support-bound GA) were simultaneously employed to convert CPC to 7-ACA in a single reactor. Characterization of the two catalysts and a fed-batch strategy for the consecutive production of 7-ACA were also investigated.

2. Materials and methods

2.1. Chemicals, enzyme and strain

D-Alanine and cetyltrimethylammonium bromide (CTAB) were purchased from Fluka Co. CPC and 7-ACA were kindly provided by Shijiazhang Pharmaceutical Group Co. Ltd. (Shijiazhang, China). GL-7-ACA was synthesized in our laboratory according to a published method [18]. Its structure was analyzed by mass spectrum. All other chemicals were of analytical grade. The immobilized GA on support was commercially provided by Hunan Flag Biotech Co. Ltd. (Changsha, China), having an activity of 40 U/g wet wt. measured as the following method. The biocatalyst beads were insoluble and spherical particles with an average diameter of 200 μm. The recombinant *P. pastoris* strain was kindly provided by professor Yuan. The construction procedures of the recombinant *P. pastoris* strain and the culture conditions were described in detail in a previous paper [16].

2.2. Preparation of permeabilized cells for bioconversion

2.2.1. Cell permeabilization

One gram *P. pastoris* cells (wet wt.) was suspended uniformly in 10 ml of 0.4% CTAB in 50 mM pyrophosphate buffer, pH 8.5. The cells were incubated in the solution for 30 min at 25 °C with shaking intermittently, and then separated by centrifugation (6000 rpm, 10 min). The cell pellet was washed twice with pyrophosphate buffer (50 mM, pH 7.5) and suspended in the same buffer at a cell concentration of 0.1 g/ml. The suspension was used for the enzyme assay directly.

2.2.2. Permeabilized cells treatment

Further treatment of permeabilized cells was performed as follows. Firstly, the pH value of the suspension prepared above was adjusted to 11.5 with 1 M NaOH. The suspension was kept at 25 °C for 30 min. After that, the pH value was adjusted to 7.5 using 20% H_3PO_4 (w/v). Then the suspension was heated to 65 °C for 2 min and immediately cooled to 4 °C. Finally, cells were collected by centrifugation and suspended in pyrophosphate buffer (50 mM, pH 7.5) at a cell concentration of 0.1 g/ml. The suspension was used for subsequent enzymatic conversions directly or after dilution.

2.3. Enzyme assay

The assay of DAAO activity was carried out with the permeabilized cells by measuring the formation of keto acid according to the previous methods with slight modifications [16,19]. An appropriate amount of the permeabilized cells and about 400 U of bovine liver catalase were mixed with 5 ml of 50 mM D-alanine in pyrophosphate buffer, pH 8.5, at 37 °C, with constant shaking at 200 rpm. After 10 min, this reaction was terminated by adding 3 ml of 10% trichloroacetic acid. The reaction mixture was centrifuged and then the keto acid in the supernatant was diluted appropriately and estimated by a colorimetric method using 2,4-dinitrophenylhydrazine. One unit of DAAO activity corresponded to the formation of 1 μmol min^{-1} of pyruvate at 37 °C.

The activity of immobilized GA was measured by a previously described method [20]. One unit of acylase activity was defined as the amount of enzyme that produced 1 μmol of 7-ACA per min at 37 °C, pH 8.0. Catalase activity was assayed spectroscopically at 240 nm [21]. Esterase activity was determined by the method reported by Beck et al. [22].

2.4. Conversion of CPC to 7-ACA in a single reactor

Three milliliters of the cell solution (20 U of DAAO) and 0.5 g of immobilized GA (20 U of GA) were added to 50 ml of 1% CPC (w/v) in 0.1 M potassium phosphate buffer, pH 7.5, containing 80 μl antifoam (Dow Corning 2210). The reaction was carried out at 28 °C in a mechanically stirred reactor (150 ml) equipped with an oxygen distributor and a wire filter (25 μm of mesh size) on the bottom. The switch of wire filter was controlled by a plug. The reaction pH value was maintained at 7.5 using a pH-stat. The pure oxygen gas was sparged into the reaction mixture from the bottom of the reactor, at a flow rate of 0.2 l min^{-1}. The H_2O_2 solution (30 mM) was added continuously to the reaction mixture at a constant rate of 6 ml h^{-1}. After 2.5 h reaction time, the reaction mixture was released from the bottom of the reactor and the support-bound GA was intercepted in the reactor by the wire filter. The cells were collected by centrifugation outside the reactor and reused. The products present in the supernatant were assayed by HPLC.

2.5. High performance liquid chromatography (HPLC) assay

Reverse phase XDB C-18 column (Zorbax, 4.6 mm × 150 mm) was employed for the assay of CPC, AKA-7-ACA, GL-7-ACA and 7-ACA. The flow rate of the eluant (25 mM sodium phosphate, pH 3.5, 8% (v/v) of acetonitrile) was 1 ml min^{-1} and the peaks were monitored at 254 nm. The elution times of CPC, AKA-7-ACA, GL-7-ACA and 7-ACA were 2.1, 3.2, 8.7, and 1.7 min, respectively. The sum of peak areas was used for the calculation of the conversion rate.

All experiments were performed at least in triplicate under the same conditions and the results are presented as the mean value. The experimental errors never exceeded 5%.

3. Results and discussion

3.1. Characterization of the two catalysts

3.1.1. The permeabilized P. pastoris cells

In the present work, the high-density fermentation of the recombinant *P. pastoris* strain was carried out in a 1-l fermentor

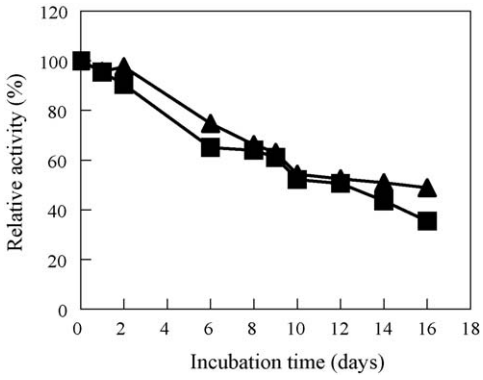

Fig. 1. Stability of DAAO in the permeabilized *P. pastoris* cells: (▲) in the untreated-permeabilized cells; (■) in the treated-permeabilized cells. The cell suspension was incubated at 30 °C, in 50 mM pyrophosphate buffer, pH 7.5.

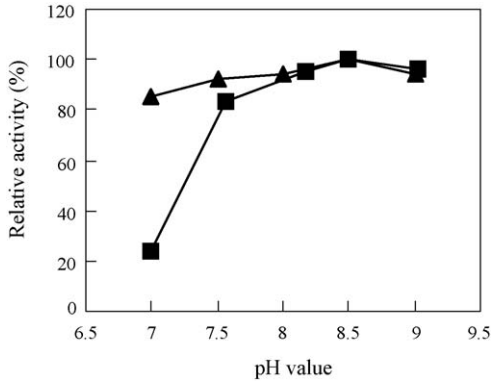

Fig. 2. Effect of pH on the activities of the catalysts: (▲) DAAO in the permeabilized *P. pastoris* cells; (■) immobilized GA on support. In these experiments, 1% CPC and 1% GL-7-ACA were used as the catalytic substrates of DAAO and GA, respectively, and the reactions were carried out at 37 °C, in 0.1 M potassium phosphate buffer.

and a maximum activity of DAAO up to 23000 U/l was obtained. Such an expression level was in agreement with that reported in a previous paper [16]. After permeabilization of *P. pastoris* cells by CTAB, these cells exhibited 91.5 U/g wet wt. of DAAO activity. Excitingly, the half-life of DAAO in the permeabilized cells reached up to 14.5 days at 30 °C (Fig. 1). The high stability of DAAO in permeabilized cells was discovered for the first time, and it was found to be much more than those investigated by other researchers [17,23,24].

Unfortunately, whole *P. pastoris* cells contained large quantity of catalase and a little esterase. In the process of the conversion of CPC by DAAO, the activities of catalase and esterase produce negative effects, which are related to the increase of by-product [17]. Therefore, it is essential that the two enzymes in the permeabilized cells are inactivated before these cells are used as a catalyst for the conversion of CPC. After treating the permeabilized cells with base (pH 11.5, 30 min) and heating (65 °C, 2 min) (see Section 2.2.2), it was observed that in these cells 99.5% of catalase activity and 100% of esterase activity were eliminated, whereas 82.6% of DAAO activity was still retained. In addition, the half-life of DAAO in the treated-permeabilized cells was very similar to that in the untreated-permeabilized cells at 30 °C (Fig. 1).

The support-bound DAAO exhibiting a better thermostability even at higher temperature has been recently reported [13,25]. Nevertheless, as an alternative, a relatively stable cell-bound DAAO as a catalyst applied for enzymatic reaction may be economically viable, because it can avoid many complicated processes of the enzyme production and consumption of a costly support, which are involved in the preparation of the support-bound DAAO.

3.1.2. Catalytic activities and stability of the catalysts

The maximum activities of the cell-bound DAAO and the immobilized GA were observed at pH 8.5 simultaneously (Fig. 2). However, the effect of pH on the former activity was found to be gentler than that on the latter activity. At pH 7.0, the cell-bound DAAO exhibited about 85% of the maximum activity, while only 23.5% of that of the immobilized GA was observed.

The effect of pH on the stability of two catalysts was also studied by assaying their activities after a period of incubation in the pH range from 7.0 to 8.5. It was found that DAAO in the permeabilized cells showed the highest stability at pH 7.0 (Fig. 3). This may be due to the fact that the rate of DAAO release from the permeabilized cells stored at lower pH values was less than those stored at higher pH values. The result was conformed to that reported by Breddam and Beenfeldt, who studied the effect of pH on the autolytic release of intracellular enzymes [26]. Thus, at low pH values, DAAO could be stabilized within the permeabilized cells and did not affect the downstream processes of 7-ACA. On the contrary, pH ranging from 7.0 to 8.5 had no deleterious effect on the stability of the immobilized GA.

Furthermore, both the cell-bound DAAO and immobilized GA displayed the maximum activities at 35–40 °C. However, when the two catalysts stored at higher temperature, the stability would be impaired.

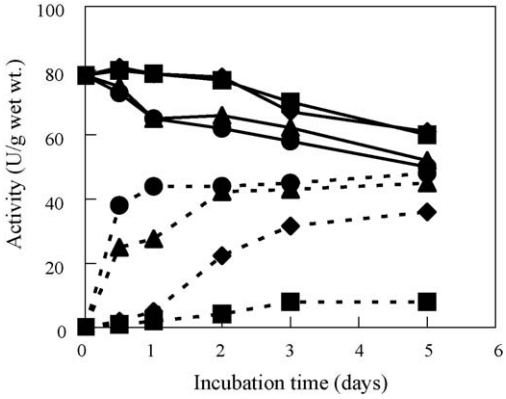

Fig. 3. Effect of pH on the stability of DAAO in the permeabilized *P. pastoris* cells: (■) pH 7.0; (♦) pH 7.5; (▲) pH 8.0; (●) pH 8.5. The solid lines, DAAO activity in the permeabilized cells; the broken lines, release of DAAO activity from the permeabilized cells. The cell suspensions containing 25 mM β-mercaptoethanol were incubated at 25 °C in these experiments.

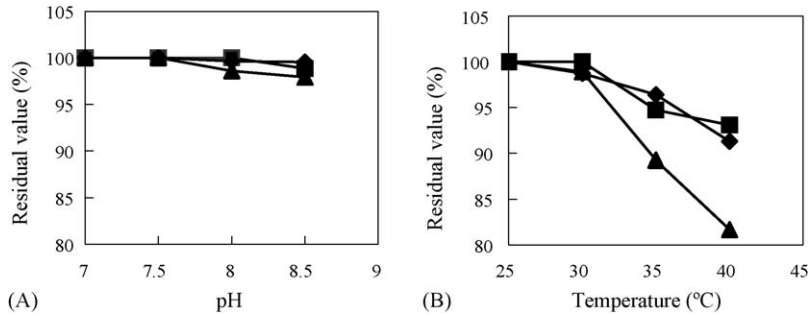

Fig. 4. Effects of pH (A) and temperature (B) on the stability of substrate and products: (♦) CPC; (▲) 7-ACA; (■) GL-7-ACA. In both experiments the substrate and products were incubated at different pH, 25 °C for 3 h (A) and at different temperature, pH 7.5 for 3 h (B), respectively. The decomposition of the determined samples was assayed by HPLC as described in Section 2.5.

3.2. Stability of the substrate and products

CPC, GL-7ACA and 7-ACA were unstable at higher pH values (>8.0), especially 7-ACA (Fig. 4A). It was indicated that 7-ACA started to be decomposed gradually when pH values exceeded 7.5. Additionally, it was previously reported that the AKA-7-ACA intermediate was decomposed more rapidly with increase in pH values, and up to 44% of the initial concentration was lost at pH 8.0 and 23 °C within 3 h [17].

The higher temperature could also exert a deleterious effect on the stability of CPC, GL-7ACA and 7-ACA (Fig. 4B). It was observed that all the determined samples were decomposed gradually when the temperature was more than 30 °C. Similarly, 7-ACA was more unstable than CPC and GL-7ACA, and about 11% of its initial concentration was lost at pH 7.5 and 35 °C within 3 h.

3.3. Determination of the reaction conditions

According to the results described above, the reaction conditions used in the subsequent experiments were determined at pH 7.5 and 28 °C. Although it has been reported that DAAO conversion rate with CPC increased linearly with the oxygen concentration [17], we refused to operate the reaction under oxygen pressure, due to added complexity and safety issues. Additionally, extra H_2O_2 was required to be added to the reaction system due to a little residual catalase present in the treated-permeabilized cells. However, the addition rate of H_2O_2 must be precisely controlled because excessive H_2O_2 can hasten the decomposition of cephalosporin nucleus and inactivate the enzymatic activities [3,10,17,27,28]. In our experiments, the H_2O_2 solution (30 mM) was added at a rate of 6 ml h^{-1}.

3.4. Single-pot production of 7-ACA

In this study, the conversion of CPC to 7-ACA with simultaneous action of DAAO in permeabilized cells and immobilized GA on support was carried out under the conditions described above. As shown in Fig. 5, CPC was converted into 90.9% 7-ACA along with 5% AKA-7-ACA intermediate and 4.1% unidentified by-product within 2.5 h. However, in another experiment, it was within 3.5 h that 87% 7-ACA yield obtained from equivalent amount of CPC by DAAO (20 U) and GA (20 U) immobilized on different supports under optimal reaction conditions. Thus, the rate of the conversion of CPC to 7-ACA performed with the two enzymes in the two different forms was higher than that performed with the two enzymes in the immobilized form. This may be due to the merits of permeabilized cells, which could be understood from the following two aspects. Firstly, permeabilized cells have larger specific surface area, making it more rapidly for substrates and products to move across the cell envelope. Secondly, permeabilized cells are much smaller than supports generally used in immobilization, as a result, they can distribute in the reaction system more uniformly. Meanwhile, permeabilized cell itself can also enter into the porous supports, which can efficiently shorten the distance between the two enzymes. The proximity of the two enzymes can decrease the product inhibition to the first enzymatic reaction and increase the rate of the second enzymatic reaction. An adequate GA activity in the reaction system plus the factors mentioned above could be the reasons to explain why no measurable GL-7-ACA was found during the reaction process by the HPLC assay.

3.5. Fed-batch production of 7-ACA in a single reactor

Since the two enzymes in the two forms were separable, their respective activities in the reaction system could be assayed anytime. As shown in Fig. 6, it was observed that DAAO in

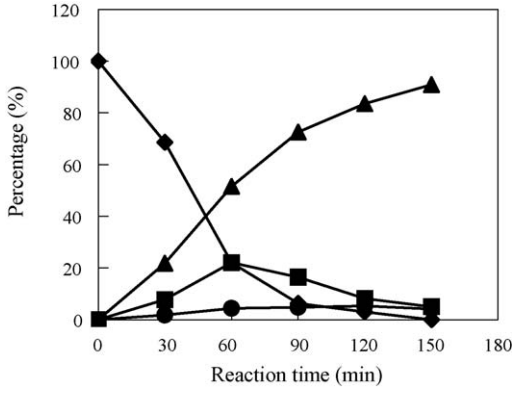

Fig. 5. Conversion of CPC to 7-ACA in a single reactor: (♦) CPC; (▲) 7-ACA; (●) by-product; (■) AKA-7-ACA. The reaction was carried out as described in Section 2.4.

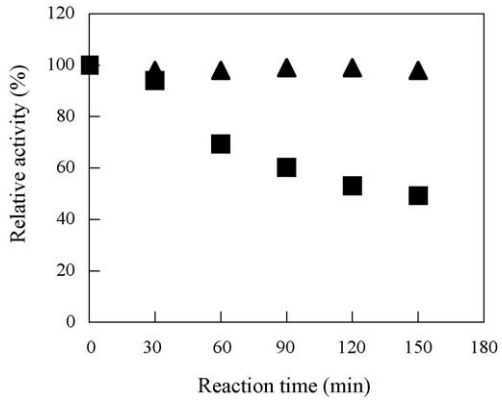

Fig. 6. Inactivation of DAAO and GA during the reaction process: (■) DAAO in the permeabilized cells; (▲) immobilized GA on support.

the permeabilized cells retained 49.3% of the initial activity at the end of reaction. The activity loss approximately ran a linear trend during the reaction process and its average rate was 0.07 U min^{-1}. However, the inactivation of immobilized GA was not observed.

The loss of DAAO activity in the reaction system could be compensated by continuous addition of the new permeabilized cell suspension. At the end of reaction, the immobilized GA granules and the permeabilized cells were methodically separated and reused for the next conversions (see Section 2.4). 7-ACA in the supernatant was further purified and crystallized. Thus, the consecutive fed-batch production of 7-ACA from CPC in a single reactor was achieved. Fig. 7 shows the schematic diagram of the reactor system. In this case, the rate of DAAO activity loss was difficult to be exactly determined, owing to the continuous addition of the cell suspension to the reaction system. Therefore, for maintaining the DAAO activity not less than its initial activity and for the convenience of operation, we decided the addition rate of the cell suspension was 6 ml h^{-1} (about 4 U h^{-1} of DAAO). As a result, the conversion rate was much faster. The equivalent amount of CPC was converted to

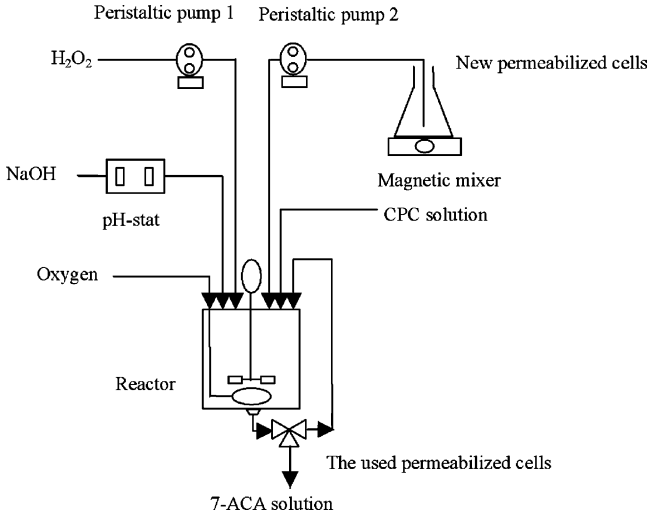

Fig. 7. Schematic diagram of reactor system for fed-batch production of 7-ACA.

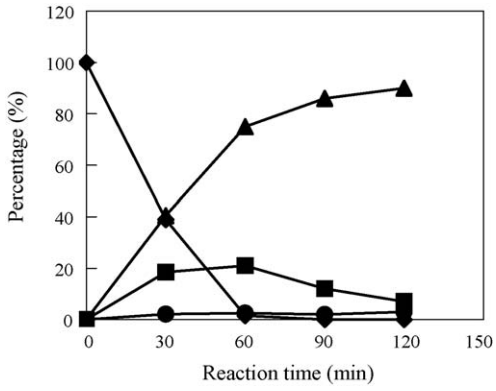

Fig. 8. Fed-batch production of 7-ACA in a single reactor: (♦) CPC; (▲) 7-ACA; (●) by-product; (■) AKA-7-ACA. The reaction conditions were described in Section 2.4. Additionally, 6 ml h^{-1} of new permeabilized cell suspension was continuously added to the reaction system.

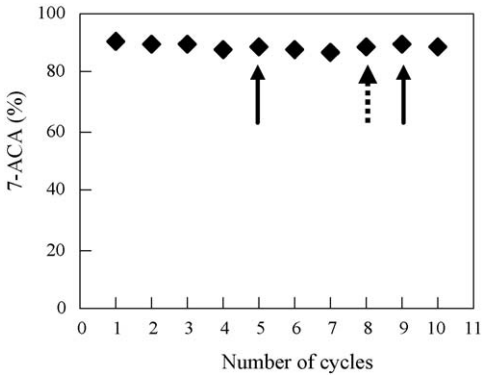

Fig. 9. Recycle of biocatalysts for consecutive batch production of 7-ACA. The solid arrows: the used permeabilized cells were replaced by the new ones at this cycle. The dotted arrow: the used immobilized GA was replaced by the new one at this cycle.

90.5% 7-ACA, 7% AKA-7-ACA and 2.5% by-product within a relatively short period of time, 2 h (Fig. 8).

When the cell suspension was added continuously at the rate of 6 ml h^{-1} to the reaction system, these cells could be reused up to four consecutive reaction cycles without any significant decrease in 7-ACA yield. However, the reaction time of the fifth reaction cycle was prolonged to 2.5 h to achieve a comparable conversion of CPC. It was inferred that the initial permeabilized cells without DAAO activity were overcrowded in the reaction system, which increased the diffusion limitation and thus resulted in the decreased conversion rate. Therefore, the used permeabilized cells were completely renewed every four reaction cycles to ensure that the reaction could be finished within about 2 h. The immobilized GA could be reused up to seven consecutive reaction cycles without a significant decrease in conversion efficiency. However, for the sake of short reaction time, the used immobilized GA was completely renewed every seven reaction cycles. The results are shown in Fig. 9.

4. Conclusions

This paper reports a new method for producing 7-ACA from CPC in a single reactor, which is performed with two enzymes in

two disparate forms, DAAO in the permeabilized *P. pastoris* cells and immobilized GA on support. In the permeabilized *P. pastoris* cells, DAAO was relatively thermostable and its half-life was up to 14.5 days at 30 °C. After treating the permeabilized cells with base and heating to inactivate the catalase and esterase, the stability of the cell-bound DAAO was not impaired significantly. Thus, it can be an approach of lowering the production cost of 7-ACA while such a relatively stable cell-bound DAAO is directly used as a catalyst in this study. Furthermore, CPC could be converted by simultaneous action of the two enzymes in the two different forms, to 90.9% 7-ACA, 5% AKA-7-ACA and 4.1% unidentified by-product within 2.5 h.

During the reaction process, an on-line assay of each individual enzyme activity can be easily conducted due to the separability of the two enzymes. The loss of DAAO activity in the reactor can be compensated by continuous addition of new cell suspension. At the end of reaction, the stable immobilized GA and the used permeabilized cells can be methodically separated and selectively reused for the next reactions. In the experiment, the conversion rate was much faster, and 90.5% 7-ACA yield was obtained within 2 h from the equivalent amount of CPC. The superiorities of such a reaction system are summarized as follows:

i. It can decrease the cost of enzyme production because the permeabilized *P. pastoris* cells are directly used for enzymatic reactions.
ii. The conversion rate is relatively high. It may be due to the better properties of permeabilized cells in the reaction system.
iii. The ratio between the activities of DAAO and GA can be easily adjusted to an optimal value for attaining a satisfactory conversion rate in the current system, although the ratio is usually difficult to control when the two enzymes are co-expressed in a recombinant strain or co-immobilized on a support to directly convert CPC to 7-ACA.
iv. Since the two enzymes are in different forms, it is possible to selectively remove the one, whose activity has been impaired. This can strategically avoid the waste of another enzyme that is still workable.

Acknowledgments

We thank professor Zhongyi Yuan for kindly providing the recombinant *P. pastoris* strains and Dr. Ming Fang, Tianwen Wang, Qiong Gao, Minjie Zhang for reading of the manuscript. This study was supported by the Key Disciplinary Foundation of Shanghai.

References

[1] Huber F, Chauvetee R, Jackson B. Preparative methods for 7-aminocephalosporanic acid and 6-aminopenicillanic acid. In: Flynn E, editor. Cephalosporins and penicillins, chemistry and biology. New York: Academic Press; 1972. p. 27–48.
[2] Parmar A, Kumar H, Marwaha SS, Kennedy JF. Recent trends in enzymatic conversion of cephalosporin C to 7-aminocephalosporanic acid (7-ACA). Crit Rev Biotechnol 1998;18:1–12.
[3] Bianchi D, Bortolo R, Golini P, Cesti P. Enzymatic transformation of cephalosporin C to 7-ACA by simultaneous action of immobilized D-amino acid oxidase and glutaryl-7-ACA acylase. Appl Biochem Biotechnol 1998;73:257–68.
[4] Luo H, Yu HM, Li Q, Shen ZY. Cloning and co-expression of D-amino acid oxidase and glutaryl-7-ACA acylase genes in *Escherichia coli*. Enzyme Microb Technol 2004;35:514–8.
[5] Nigam VK, Kundu S, Ghosh P. Single-step conversion of cephalosporin C to 7-aminocephalosporanic acid by free and immobilized cells of *Pseudomonas diminuta*. Appl Biochem Biotechnol 2005;126:13–21.
[6] Matsuda A, Atsuyama K, Yamamoto K, Ichikawa S, Komatsu KI. Cloning and characterization of the genes for two distinct cephalosporin acylases from a *Pseudomonas* strain. J Bacteriol 1987;58:5815–20.
[7] Wolf KF, Koller KP, Lange G, Liesum A, Sauber K, Schreuder H, et al. Structure-based prediction of modification in glutarylamidase to allow single-step enzymatic production of 7-aminocephalosporanic acid from cephalosporin C. Protein Sci 2002;11:92–103.
[8] Pollegioni L, Lorenzi S, Rosini E, Marcone GL, Molla G, Verga R, et al. Evolution of an acylase active on cephalosporin C. Protein Sci 2005;14:3064–76.
[9] Luo H, Li Q, Yu HM, Shen ZY. Constructure and application of fusion proteins of D-amino acid oxidase and glutaryl-7-aminocephalosporanic acid acylase for direct bioconversion of cephalosporin C to 7-aminocephalosporanic acid. Biotechnol Lett 2004;26:939–45.
[10] Nikolov A, Danielsson B. Enzymatic transformation of cephalosporin C to 7-aminocephalosporanic acid. Part II. Single-step procedure using a coimmobilized enzyme system. Enzyme Microb Technol 1994;16:1037–41.
[11] Lopez-Gallego F, Batencor L, Hidalgo A, Mateo C, Fernandez-Lafuente R, Guisan JM. One-pot conversion of cephalosporin C to 7-aminocephalosporanic acid in the absence of hydrogen peroxide. Adv Synth Catal 2005;347:1804–10.
[12] Pollegioni L, Caldinelli L, Molla G, Sacchi S, Pilone MS. Catalytic properties of D-amino acid oxidase in cephalosporin C bioconversion: a comparison between proteins from different sources. Biotechnol Prog 2004;20:467–73.
[13] Betancor L, Hidalgo A, Fernandez-Lorente R, Mateo C, Rodriguez V, Fuentes M, et al. Use of physicochemical tools to determine the choice of optimal enzyme: stabilization of D-amino oxidase. Biotechnol Prog 2003;19:784–8.
[14] Li Y, Jiang WH, Yang YL, Zhao GP, Wang ED. Overproduction and purification of glutarly 7-amino cephalosporanic acid acylase. Protein Expres Purif 1998;12:233–8.
[15] Kim DW, Yoon KH. Cloning and high expression of glutarly 7-amino cephalosporanic acid acylase gene from *Pseudomonas diminuta*. Biotechnol Lett 2001;23:1067–71.
[16] Yu J, Li DY, Zhang YJ, Yang S, Li RB, Yuan ZY. High expression of *Trigonopsis variabilis* D-amino acid oxidase in *Pichia pastoris*. J Mol Catal B: Enzym 2002;18:291–7.
[17] Vicenzi JT, Hansen GJ. Enzymatic oxidation of cephalosporin C using whole cells of the yeast *Trigonopsis variabilis* within a "cross-flow filter-reactor"'. Enzyme Microb Technol 1993;15:281–5.
[18] Shibuya Y, Matsumoto K, Fujii T. Isolation and properties of 7-β-(4-carboxybutanamide)-cephalosporinic acid acylase-producing bacteria. Agric Boil Chem 1981;45:1561–7.
[19] Brodelius P, Nisson K, Mosbach K. Production of α-ketoacids: immobilized cells of *Trigonopsis variabilis* containing D-amino acid oxidase. Appl Biochem Biotechnol 1981;6:293–308.
[20] Monti D, Carrea G, Riva S, Baldaro E, Frara G. Characterization of an industrial biocatalyst: immobilized glutaryl-7-ACA acyalse. Biotechnol Bioeng 2002;70:239–44.
[21] Aebi H. Catalase. In: Bergmayer H, editor. Methods of enzymatic analysis. 2nd ed. Deerfield Beach: Verlag Chemie International; 1981. p. 637–84.
[22] Beck S, Skrob F, Plhackova K, Kujan P, Holler P, Kyslik P. Cross-linked cell aggregates of *Trigonopsis variabilis*: D-amino acid oxidase catalyst for oxidation of cephalosporin C. Biotechnol Lett 2003;25:227–33.

[23] Upadhya R, Nagajyothi H, Bhat SD. D-Amino acid oxides and catalase of detergent permeabilized *Rhodotorula gracilis* cells and its potential use for the synthesis of α-keto acids. Process Biochem 1999;35:7–13.
[24] Moreno JA, Ruiz CA, Catalan J, Galan MA. Thermal deactivation and inhibition of D-amino acid oxidase in permeabilized cells of the yeast *Trigonopsis variabilis*. J Chem Technol Biotechnol 2004;79:321–6.
[25] Lopez-Gallego F, Betanctor L, Hidalgo A, Alonso N, Fernandez-lorete G, Guisan JM, et al. Preparation of a robust biocatalyst of D-amino acid oxidase on sepabeads supports using glutaraldehyde crosslinking method. Enzyme Microb Technol 2005;37:750–6.
[26] Breddam K, Beenfeldt T. Acceleration of yeast autolysis by chemical methods for production of intracellular enzymes. Appl Microbiol Biotechnol 1991;35:323–9.
[27] Szwajcer Dey E, Flygare S, Mosbach K. Stabilization of D-amino acid oxidase from yeast *Trigonopsis variabilis* used for production of glutarly-7-aminocephalosporanic acid from cephalosporin C. Appl Biochem Biotechnol 1991;24:239–50.
[28] Pilone MS, Pollegioni L, Buto S. Stability and kinetic properties of the immobilized *Rhodotorula gracilis* D-amino acid oxidase. Biotechnol Appl biochem 1992;16:252–62.