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High Soluble Expression of D-Amino Acid Oxidase in *Escherichia coli* Regulated by a Native Promoter

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Abstract To express high-active soluble D-amino acid oxidase (DAAO), a constitutive plasmid that is regulated by a native hydantoinase promoter (P_{Hase}), was constructed. A D-amino acid oxidase gene (*dao*) was ligated with the P_{Hase} and cloned into pGEMKT to constitutively express protein of DAAO without the use of any inducer such as isopropyl β-D-1-thiogalactopyranoside which is poisonous to the cells and environment. The ribosome binding site region, host strain, and fermentation conditions were optimized to increase the expression level. When cultivated in a 5-m^3 fermenter, the enzyme activity of JM105/pGEMKT-R-DAAO grown at 37 °C was found to be 32 U/mL and increase 16-fold over cells of BL21(DE3)/pET-DAAO grown at 28 °C. These results indicate the success of our approaches to overproducing DAAO in soluble form in *Escherichia coli*.

Keywords D-Amino acid oxidase · Hydantoinase promoter · Constitutive expression · Ribosome binding site · Fermentation

Introduction

D-amino acid oxidase (DAAO) catalyzes the oxidative deamination of D-amino acid to produce corresponding keto acid and H_2O_2. As an industrial enzyme, DAAO has several other current and potential biotechnological applications, such as the production of α-ketoacids, chiral intermediates, and antibiotics [1, 2]. Its most important use is in the production of 7-aminocephalosporanic acid (7-ACA) from cephalosporin C (CPC), in conjunction with 7-aminocephalosporanic acid acylase (GLA) [3–5].

The DAAO activity has been found in pig kidney [6] and a number of microorganisms belonging to genus *Fusarium solami* [7], the yeasts *Rhodotorula gracilis* [8, 9], and *Trigonopsis variabilis* [10, 11]. Unfortunately, the low-specific activity renders the wild strains unsuitable for industrial uses, so, typical production of DAAO is from genetic

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recombinant microbial strains. However, this method has a tendency to form aggregates under overexpression conditions that is considered one of the limiting factors in the industrial process development [6]. Thus, it is necessary to construct a recombinant strain that can produce a large amount of soluble active DAAO.

In our previous work, a gene of D-amino acid oxidase was obtained by reverse transcription-polymerase chain reaction (PCR) from *T. variabilis*, and the *dao* gene was expressed in recombinant *Escherichia coli* BL21(DE3)/pET-DAAO with a DAAO activity level of 1.98 U/mL by a fed-batch culture [12]. In the present paper, a constitutive plasmid, regulated by a native hydantoinase promoter that expressed high-active soluble DAAO, was constructed. In order to obtain the highest expression level in the shortest possible time, the ribosome binding site (RBS) region, host strain, and fermentation conditions were optimized and this resulted in the increase of the activity of DAAO to 32 U/mL when cultivated in 5-M^3 fermenter without the use of any inducer.

Materials and Methods

Materials

Cephalosporin C (CPC) and 7-(4-carboxybutanamido)-cephalosporanic acid (GL-7-ACA) were supplied by JiuPai Co., Ltd. Restriction enzymes, T4 DNA ligase, the Taq DNA polymerase, and DNA maker were obtained from Takara Biotechnology (Dalian) Co., Ltd. Protein molecular weight makers were purchased from Tiangen (Beijing) Co., Ltd. All other chemicals were reagent grade and obtained from commercial sources.

Plasmids and Strains

Plasmid pGEMKT used as the cloning and expression vector was constructed by previous work from pGEMT with kanamycin resistance instead of ampicillin. Plasmid pET-DAAO and the recombinant *E. coli* BL21(DE3)/pET-DAAO were constructed previously (*12*). *E. coli* strain TOP10F' (Invitrogen, The Netherlands) was used as host strain for genetic cloning, while the strains JM109, BL21, and JM105 (Invitrogen, The Netherlands) for expression.

DNA Manipulation and Construction of pGEMKT-DAAO

The fragment containing the native hydantoinase promoter (P_{Hase}) and the ribosome binding site from *A. radiobacter* TH572 (GenBank accession number X91070) was amplified by PCR using primers Hp-1 (5′-AAGG*ACTAGT*TTACATGCAGCACGACAATAACAGG-3′) and Hp-2 (5′-GAGCGACATCATGGCTAAAATCGTTGTT-3′), and a *Spe*I site (underlined) was added upstream of the P_{Hase}. The resulting PCR product was named Hp-r. The *dao* gene from the recombinant plasmid pET-DAAO was amplified by PCR using primers DAAO-1 (5′-GAGCGACATCATGGCTAAAATCGTTGTT-3′) and DAAO-2 (5′-AAAA*CTGCAG*CCCT AAAGGTTTGGACGAGTAAG-3′). A region overlap with the RBS region of Hp-r was added upstream of the start condon of *dao*, and a *Pst*I site (underlined) was added downstream of the termination condon. The PCR product was named r-D.

The above two fragments Hp-r and r-D were linked by overlapping PCR with primers Hp-1 and DAAO-2. The resulting fragment Hp-r-D was cloned into *Spe*I/*Pst*I site of pGEMKT. The sequence engineered by PCR was verified by DNA sequencing. The resulting plasmid pGEMKT-DAAO was transformed into *E. coli* TOP10F'.

Expression of DAAO

Cells of *E. coli* strains carrying the recombinant plasmids were grown in Luria–Bertani broth (LB) medium supplemented with kanamycin (50 μg/mL) at 37 °C. A single colony was transferred into 5 mL of LB medium with kanamycin at the above-mentioned concentration in a 50-mL flask. This culture was incubated overnight at 37 °C with shaking. In a 1-L flask, 100 mL of LB with kanamycin was inoculated with 1 mL of the overnight culture. This culture was incubated at 37 °C with vigorous shaking (230 rpm) for 12 h before harvested.

The batch culture for production of recombinant proteins was performed in a 5-L fermenter (B. Braun Company, Germany) containing 2.9 L semidefined medium: corn steep liquor (48 g/L), yeast extract (3.0 g/L), NH_4Cl (8.5 g/L), glycerol (1.5 g/L), $MgSO_4$ (3.5 g/L), $FeSO_4.7H_2O$ (0.3 g/L), $MnCl_2.4H_2O$ (0.2 g/L), $CaCl_2$ (0.4 g/L), NaCl (3.0 g/L). The cultivation temperature was 37 °C.

The feed solution of nitrogen sources used for the fed-batch culture contained, per liter: 240 g corn steep liquor (end concentration of 6.5%), 20 g yeast extract, and carbon sources, per liter: 250 g glycerol (end concentration of 1.6%). The temperature was maintained at 37 °C for 20 h and the pH value was controlled at 7.3 with carbon sources and nitrogen sources. Agitation and aeration were set to 750 rpm and 3.0 L/min, respectively.

Assay of the Activity of DAAO

The major method of assay of DAAO activity was measuring the production of keto acid according to the Freidemann method [13]. This method is simple and directly observable, but it is not very accurate and the values obtained are much higher than the actual ones because the substrate is not CPC but D-alanine. In this study, we used CPC as substrate and the activity of the enzyme was determined by assay the concentration of GL-7-ACA.

The standard assay for determination of DAAO activity was carried out as following: 2 mL cells were harvested, then washed, and resuspended in 1 mL 50 mM Tris-HCl buffer (pH 8.0). Cell walls were disrupted by sonication and cell debris was removed by centrifugation (13,000 rpm, 10 min). Five hundred microliters of crude extract was mixed with 1.5 mL prewarmed (37 °C) 1.5% cephalosporin C. The reaction was incubated at 37 °C for 30 min and 20 μl H_2O_2 was added to the reaction solution once every 10 min. The reaction was ended by the addition of 6 M HCl.

The concentration of Gl-7-ACA was determined using high performance capillary electrophoresis (HPCE), which was equipped with P/ACE™ MDQ Capillary Electrophoresis System (Beckman Coulter). Phosphate buffer of 10 mM (pH 6.5) was used as the running buffer, D-*p*-hydroxyphenylglycine (D-HPG) as an internal standard, detection wavelength 254 nm, separating voltage 30 kV. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of Gl-7-ACA from CPC per minute at 37 °C.

Results and Discussion

Construction of Recombinant Plasmid of pGEMKT-DAAO and pGEMKT-R-DAAO

The fragments of Hp-r (P_{Hase} including RBS region) and r-D (*dao* gene with RBS region upstream of it) were successfully cloned by use of PCR and linked together by overlapping PCR. The resulting ~1.3 kb *Spe*I/*Pst*I fragment of Hp-r-D was ligated into plasmid pGEMKT which was transformed into *E. coli* strain TOP10F'.

It is now known that the ribosome binding site is one of the important signals for the identification of genes in a DNA sequence and, thus, it is very important for efficiency of translation. To improve the expression level of DAAO, the native RBS (RBS_1) region of P_{Hase} from *A. radiobacter* was substituted with a new one (RBS_2) from *E. coli*. The sequence of RBS_2 was CTGAGGAGGATCTGGAA, whereas that of RBS_1 was CTGAGAGCGACATC.

The procedure for the construction of pGEMKT-R-DAAO started with the plasmid pGEMKT-DAAO, through a series of DNA manipulation, including PCR, overlapping PCR, ligation, and transformation (Fig. 1). In the amplification of the P_{Hase} and DAAO gene, the RBS_2 sequence was introduced into the downstream and upstream of the two genes, respectively. The resulting two fragments were ligated by use of overlapping PCR and then inserted into the plasmid pGEMKT which was transformed into *E. coli* Top10F'.

The expression plasmids of pGEMKT-DAAO and pGEMKT-R-DAAO were each transformed into *E. coli* cells Top10F' to express the *dao* gene under control of P_{Hase}. The clones were then screened for DAAO activity. The enzyme activity of Top10F'/pGEMKT-R-DAAO grown in LB broth at 37 °C was found to be 0.3 U/mL and increased threefold

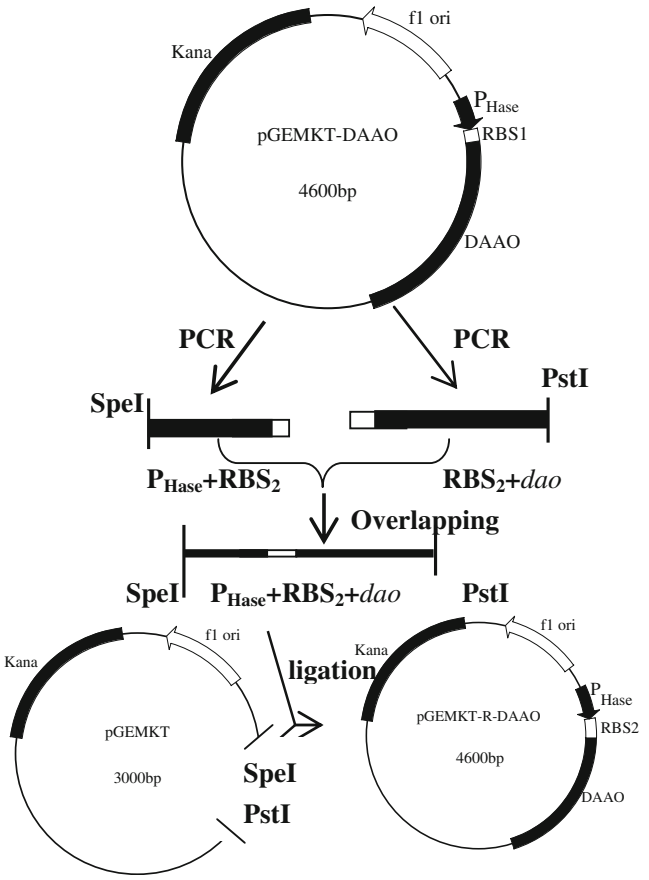

Fig. 1 Construction scheme of recombinant plasmids pGEMKT-R-DAAO. *Symbols* denote: f1 ori, phage f1 region; *Kana* kanamycin resistance gene

over cells of Top10F'/pGEMKT-DAAO. This suggested that the RBS_2 could really improve the active protein production in *E. coli*, compared to RBS_1. The solubilities of the constitutive and inducible expression DAAO were compared and the result was shown in Fig. 2. Only a very small part of the DAAO produced by BL21(DE3)/pET-DAAO even when cultivated at 25 °C was present in soluble fraction (Fig. 2), whereas almost all of the enzymes produced constitutively by Top10F'/pGEMKT-DAAO and Top10F'/pGEMKT-R-DAAO were expressed as the soluble form and the insoluble fraction could hardly been seen in Fig. 3. This could explain why the enzyme activity of Top10F'/pGEMKT-R-DAAO grown in flask culture was even higher than that of BL21(DE3)/pET-DAAO by a fed-batch culture.

Optimal Host Cell Strain

To find the most optimal host cell strain for DAAO expression, several *E. coli* strains were analyzed as hosts to the recombinant plasmids. The plasmid of pGEMKT-R-DAAO was transformed into four *E. coli* strains (Top10F', JM109, JM105, and BL21). The expression levels at 37 °C varied in different host cells, and *E. coli* strain JM105 proved to be the best as judged by measuring their respective enzyme activities (data shown in Fig. 4). The enzyme activity of JM105/pGEMKT-R-DAAO was 0.66 U/mL.

Optimal Fermentation Conditions

In order to obtain the highest expression level in the shortest possible time, the fermentation conditions for the recombinant cells were optimized. The fermentation studies comprised experiments with medium composition, cultivation temperatures between 25 °C to 42 °C

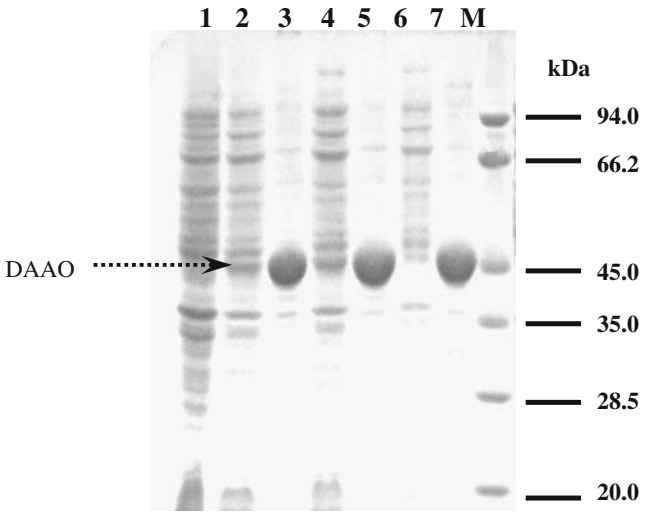

Fig. 2 The solubility of recombinant DAAO expressed by BL21 (DE3)/pET-DAAO at different culture temperature: *Lane 1* uninduced and grown at 37 °C; *lane 2* soluble fraction of cells grown at 25°C; *lane 3* insoluble fraction of the same; *lane 4* soluble fraction of cells grown at 28 °C; *lane 5* insoluble fraction of the same; *lane 6* soluble fraction of cells grown at 37 °C; *lane 7* insoluble fraction of the same; *lane 8* protein size marker

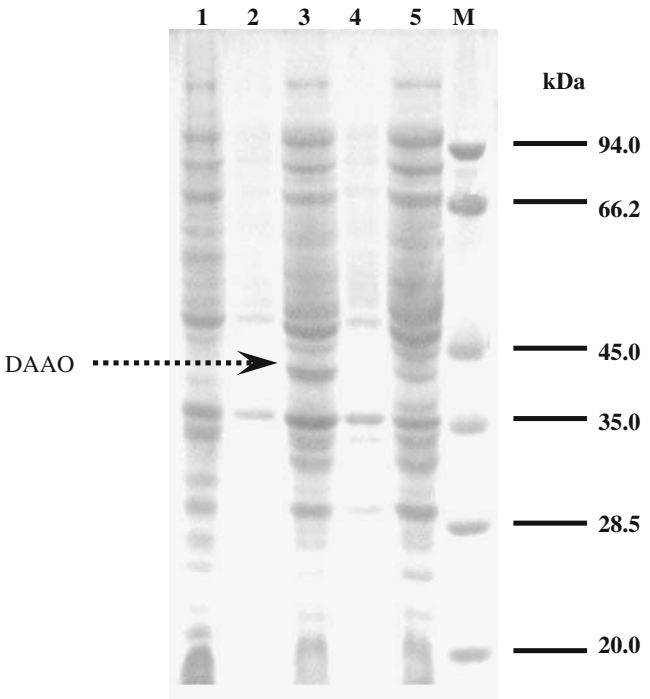

Fig. 3 The solublility of constitutive recombinant DAAO expressed at 37 °C: *lane 1*, the whole cell of *E. coli* strain Top10F' without any plasmid; *lane 2* insoluble fraction of *E. coli* strain Top10F' carrying pGEMKT-R-DAAO; *lane 3* soluble fraction of the same; *lane 4* insoluble fraction of *E. coli* strain Top10F' carrying pGEMKT-DAAO; *lane 5* soluble fraction of the same; *lane 6* protein size marker

and effect of inoculation volume. These studies were made for the system in *E. coli* strain JM105/pGEMKT-R-DAAO.

The optimization of the fermentation conditions used for preparing the D-amino acid oxidase was first investigated in flask culture at 37 °C. Comprehensive consideration of the

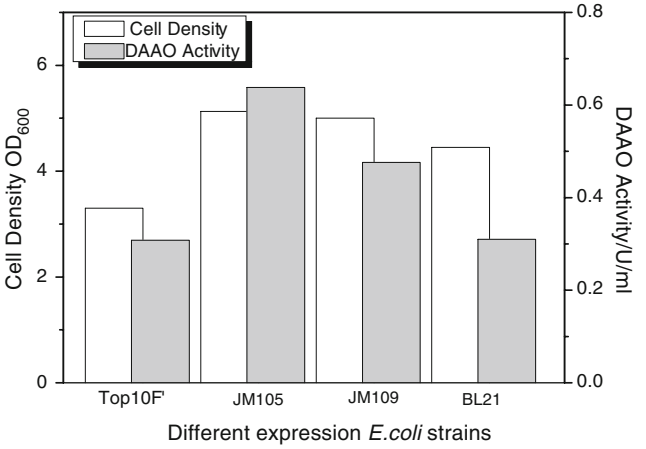

Fig. 4 D-amino acid oxidase activities of different expression *E. coli* host strains

growth of the organism, nutrition for the expression of DAAO and feasibility of industrial application, a scheme of orthogonal test for optimizing culture medium, which includes seven factors and three levels, was designed, with corn steep liquor and yeast extract used as nitrogen sources and glycerol used as carbon sources. By analyzing the results of orthogonal experiments (data not shown), the suitable concentrations of carbon and nitrogen sources and other necessary compositions such as NH_4Cl, $MgSO_4$, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $CaCl_2$, NaCl were determined: corn steep liquor (48 g/L), yeast extract (3.0 g/L), NH_4Cl (8.5 g/L), glycerol (1.5 g/L), $MgSO_4$ (3.5 g/L), $FeSO_4.7H_2O$ (0.3 g/L), $MnCl_2.4H_2O$ (0.2 g/L), $CaCl_2$ (0.4 g/L), and NaCl (3.0 g/L).

Based on the optimal culture medium, effects of the initial pH of medium on the activity of enzyme and growth of cells were investigated. It was found that the best initial pH was 7.5 and that the enzyme activity and cell density would decrease when the initial pH was lower than 7.0 (data not shown). In order to find the most optimal culture temperature, the cells were cultivated at different temperatures ranging from 25 °C to 42°C. As shown in Fig. 5, 37 °C was suitable not only for cells growth but for the expression of enzyme as well. The effect of inoculation volume on the activity of DAAO was also studied and the optimal inoculation volume was 2.0%, as shown in Fig. 6.

Batch and Fed-Batch Fermentations

The batch fermentation for DAAO production was performed in a 5-L fermenter containing 2.9 l semidefined medium and 100 mL inoculum. The initial pH value of the culture medium was adjusted to 7.5 by 3 M NaOH and the cultivation temperature was 37 °C. When the fermentation time was above 6.5 h, the pH value was maintained at 7.5 by addition of HCl. Aliquots were taken during the fermentation for assay. OD_{600} (cell density) achieved the maximum value 25.8 after 10 h and the activity of DAAO reached 3.78 U/mL after 13–14 h, as shown in Fig. 7a. These results are little higher than those obtained in the flask culture (Figs. 5 and 8), but the fermentation time was shortened from 30 to 14 h.

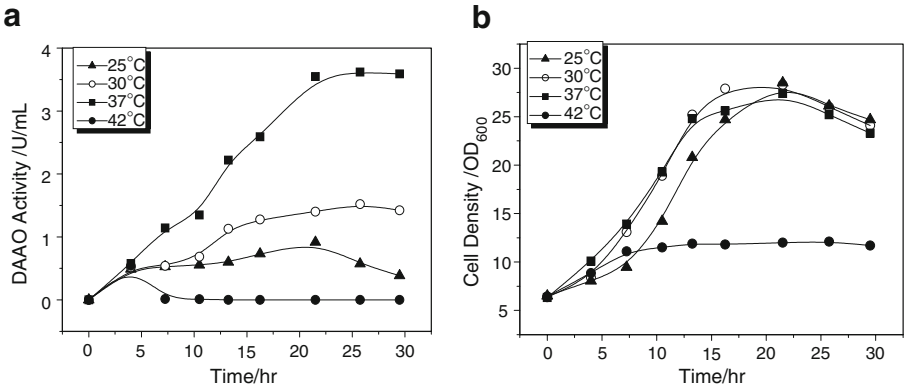

Fig. 5 Growth curves (**a**) and D-amino acid oxidase activity accumulation (**b**) of JM105/pGEMKT-R-DAAO cultivated at different temperature. Cell concentrations and DAAO activity were measured and plotted at 25 °C (*filled triangle*), 30 °C (*unfilled circle*), 37 °C (*filled square*), and 42 °C (*filled circle*)

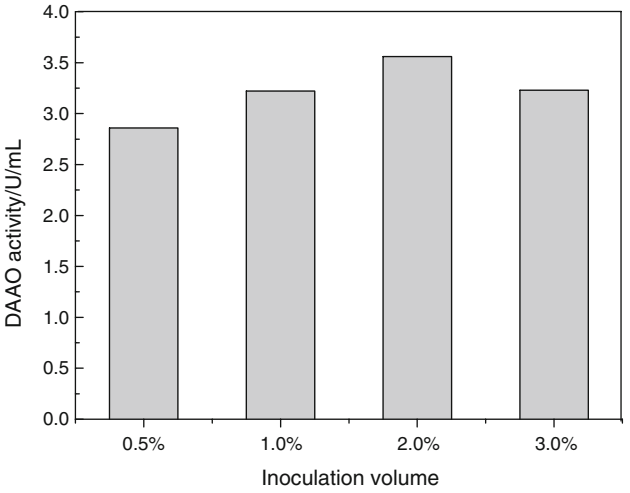

Fig. 6 Effect of different inoculation volume on DAAO activity

In order to further improve the DAAO activity and cell concentration, the fed-batch fermentation was done based on the results of the batch fermentation. The feed solution of nitrogen sources used for the fed-batch culture contained corn steep liquor, yeast extract, and carbon sources containing glycerol. The cultivation temperature was 37 °C and initial pH value was adjusted to 7.3 by consideration of the growth feature of the recombinant cells. Agitation was set to 750 rpm. The carbon sources feeding period was 6–11.5 h and the feeding rate was depended on the pH value that was maintained between 7.3 and 7.4. The feeding period of the nitrogen sources was 10–15 h. The total fermentation time was 20 h. Aliquots were taken during the fermentation for assay. The OD_{600} reached 54.96 and the activity was found to be 14.4 U/mL in the end (Fig. 7b). Not only the activity of DAAO per unit volume but also the cell concentration was improved sharply, compared to the batch fermentation and flask cultivation (Fig. 8).

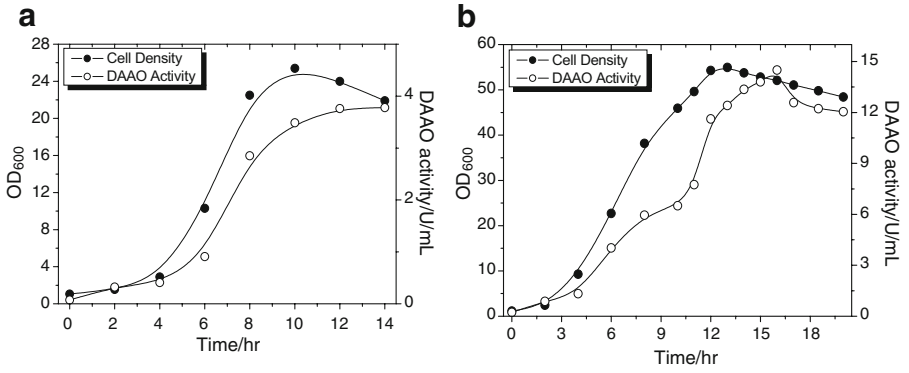

Fig. 7 Time profiles of cell concentration and DAAO activity by batch culture (**a**) and fed-batch culture (**b**). Cell concentrations (*filled circle*) and DAAO activity (*unfilled circle*) were measured

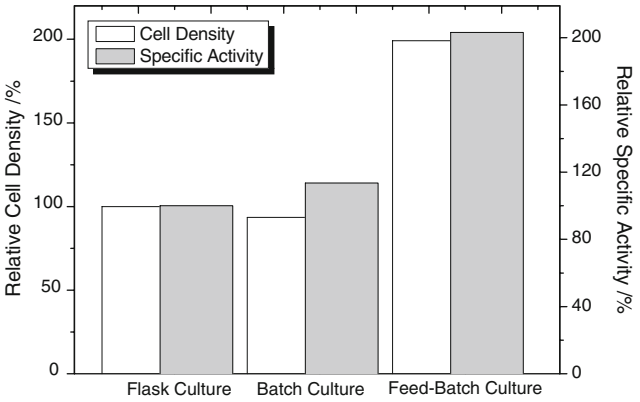

Fig. 8 Relative cell density and specific activity of D-amino acid oxidase by different culture methods including flask culture, batch, and feed-batch culture

Scaling Up of Production to 5 M^3

Based on the optimal results found in the small-scale process, scaling up experiment was done in a 5-M^3 fermenter. The only difference with the 5-L process was that a 300-L seed fermenter was used before the 5-M^3 main fermenter. One hundred milliliter inoculum from flask culture was initially grown in the seed fermenter with 150 L LB broth and subsequently all transferred into the main fermenter with 3-M^3 fermentation medium. The cultivation was done with an aeration rate of 0.5 vvm and agitation rate of 110 rpm. As shown in Table 1, the expression level and cell concentration were much higher than those obtained in the small-scale process, without adding kanamycin into the seed and the main fermenter. Furthermore, the fermentation period was shortened to 14–16 h.

This study shows that the recombinant strain JM105/pGEMKT-R-DAAO constructed can be used to produce D-amino acid oxidase with high activity and stability. The notable advantages of this protocol are no need of induction, using only cheap nitrogen and carbon sources, and simple controlling process. All these have made its possible to obtain DAAO for further research on extraction and immobilization in a cheap way, which would then reduce the cost of 7-ACA production possibly on a large scale. Thus, the strain and the process demonstrated by this work show great potential for the pharmaceutical industry.

Table 1 Results of cultivating the recombinant strains JM105/pGEMKT-R-DAAO in 5-M^3 fermenter.

Batch number	1	2	3	4	5	Average
Fermentation period (h)	14	15	14	13	16	14.4
Cell concentration (g/L)	42	40	39	42	43	41.2
DAAO activity(U/mL broth, assayed by HPCE)	28	26	32	34	40	32
DAAO activity(U/mL broth, assayed by measuring the production of keto acid)	182	176	213	225	263	212
Enzyme activity ratio(U/g dry cells)	101	98	123	122	140	116

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