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High expression of *Trigonopsis variabilis* D-amino acid oxidase in *Pichia pastoris*

Jian Yu[1], Dong-Yang Li, Ying-Jiu Zhang, Sheng Yang, Ren-bao Li, Zhong-Yi Yuan*

Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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Abstract

To explore a new approach of high expression of D-amino acid oxidase (DAAO) in *Pichia pastoris*, a gene encoding DAAO from *Trigonopsis variabilis* (*TvDAAO* gene) deleted intron was prepared by PCR amplification and cloned into the intracellular expression vector pPIC3.5K. The expression plasmid pPIC3.5K-DAAO linearized by *Sal*I was transformed into *Pichia pastoris* strain GS115 ($his^{-}mut^{+}$). By means of MM and MD plates and PCR, the recombinant *P. pastoris* strains ($his^{+}mut^{+}$) were obtained. Activity assay and SDS-PAGE demonstrated that DAAO was intracellularly expressed in *P. pastoris* with the induction of methanol. The recombinant strain PD27 with the highest expression of DAAO was screened through activity assay and its high-density fermentation was carried out in a 1-l fermentor. Activity assay and SDS-PAGE demonstrated that DAAO was intracellularly expressed in *P. pastoris* with the induction of methanol. The recombinant cells with high expression of DAAO were screened and the high-density fermentation was carried out in a 1-l fermentor. Interestingly, the DAAO expression level reached up to 473 U/g dry cell weight in fermentation yield. Finally, 1-hexanol was used to break recombinant cells and the specific activity of DAAO was 1.46 U/mg protein in crude extraction.
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Keywords: D-Amino acid oxidase; *Pichia pastoris*; Cloning; Expression; Fermentation

1. Introduction

D-Amino acid oxidase (oxidoreductase, DAAO, EC 1.4.3.3), a flavoprotein with molecular weight of 86 kDa is specific to the oxidation of D-amino acid. The enzyme is composed of two identical subunits [1].

DAAO combined with GL-7-ACA acylase, plays an important role in the two-step enzymatic conversion of cephalosporin C (Ceph C) to 7-aminocephalosporanic acid (7-ACA), which is a key intermediate for the industrial production of many semi-synthetic cephalospins. The enzymatic-synthetic route has some advantages of high efficiency, low cost and no pollution in comparison with traditional chemical methods of 7-ACA preparation. In addition, DAAO can also be used for production of á-keto acids and pure L-amino acids [2,3]. Therefore, many efforts have been made to obtain DAAO in large amounts. Formerly, the enzyme from hog kidney was commonly used. In recent years, great interest was focused on the enzyme from microbial sources. Among them, *Trigonopsis variabilis* appeared to be the most potential producer of DAAO [4]. Recombinant expression is a promising approach to acquire DAAO to meet its large demand in industry.

* Corresponding author. Tel.: +86-21-64374430x5289; fax: +86-21-64338357.
E-mail address: zhongyi@sunm.shcnc.ac.cn (Z.-Y. Yuan).
[1] Present address: College of Life Sciences, Jilin University, Changchun 130023, China.

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The methylotrophic yeast *Pichia pastoris* has been developed into a highly successful expression system for the efficient production of a variety of heterologous proteins. The increasing popularity of this expression system can be attributed to its important advantages: (1) strong and tightly regulated promoters, such as alcohol oxidase 1 promoter (*AOX1*) that is uniquely suited for the controlled expression of foreign genes; (2) the ability to grow to very high cell densities in simple minimal salt media [5,6]. At present, *P. pastoris* as an efficient protein expression system can be fermented routinely in large scale to meet industrial demands of interest proteins [7].

Accordingly, we cloned a gene encoding DAAO from *T. variabilis* and performed the heterologous expression of DAAO in *P. pastoris* to obtain high yield of DAAO.

2. Materials and methods

2.1. General

Restrictive enzymes and T4 DNA ligase were purchased from Gibco BRL. Pfu DNA polymerase was purchased from Takara. 2,4-Dinitrophenylhydrazin was obtained from Merck (Germany). All other reagents were made in China and of the highest quality available.

2.2. Strains, media

The *E. coli* strains used for cloning the gene were TG1 (*supE hsd*Δ 5 *thi*Δ (*lac-proAB*) *F′* [*traD*36 *PROAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]) and DH5á (*supE*44*lacU*169Δ 80*lacZM*15) *hsaR*17 *recA1 endA1 gyrA*96 *thi-relA1* [4]. *E. coli* strains were grown in LB medium.

P. pastoris GS115 (*his*$^-$*mut*$^+$), used as the expression host, was purchased from Invitrogen. The media and procedures used for *P. pastoris* growth and transformation were described in Multi-Copy *Pichia* Expression Kit from Invitrogen.

Basal salts medium (per liter): 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4{\cdot}2H_2O$, 18.2 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, 40.0 g glycerol, 100 ml YNB (10×) [8].

Trace element solutions—PTM1 (per liter): 6.0 g $CuSO_4{\cdot}5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g H_3BO_3, 0.5 g $CaSO_4{\cdot}2H_2O$, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin, 5 ml concentrated H_2SO_4.

2.3. Plasmids

The plasmid pAO6 (7.7 kb) containing *TvDAAO* gene deleted intron from *T. variabilis* CBS 4095 was kindly provided by Prof. Angel Dominguez [4]. Plasmid pPIC3.5K was purchased from Invitrogen. Plasmid pTrcHisA was from our lab. T-vector was prepared by ourselves.

2.4. PCR amplification of DAAO

The 5′ primer and the 3′ primer were as follows: 5′ primer: 5′-GGATCCATGGCTAAAATCGTTGT-3′ *Bam*HI; 3′ primer: 5′-GAATTCGTTGTTGATGGG-AGGTAA-3′ *Eco*RI.

PCR amplification was performed using Pfu DNA polymerase. The template was plasmid pAO6 (7.7 kb) containing *TvDAAO* gene without intron. The conditions for PCR were as follows: template was initially denatured at 94 °C for 5 min, followed by 5 cycles (94 °C, 30 s; 40 °C, 30 s; 72 °C, 90 s) and 30 cycles (94 °C, 30 s; 48 °C, 30 s; 72 °C, 90 s). Finally, a cycle was performed at 72 °C for 10 min.

2.5. Construction of recombinant expression plasmid

The PCR product was ligated to T-vector, then was cut out by *Bam*HI and *Eco*RI and cloned into the same enzymes digested pPIC3.5K. The resultant plasmid pPIC3.5K-DAAO contains *TvDAAO* gene under the control of *AOX1* promoter. The cloned gene was sequenced by 5′ *AOX1* primer, which contained in the kit, and confirmed to be inserted into the correct reading frame. Ligation of DNA fragments, preparation and cloning of plasmid were carried out as described in *Molecular Cloning* [9].

2.6. Transformation of P. pastoris GS115 (his$^-$mut$^+$)

Competent *P. pastoris* GS115 (*his*$^-$*mut*$^+$) cells (80 μl) were mixed with 5–20 μg of *Sal*I-linearized pPIC3.5K-DAAO or parent pPIC3.5K then transferred into an ice-cold 0.2 cm electroporation cuvette

and incubated in an ice bath for 5 min. After electroporation at 1.5 kV, 25 μF, 1 ml of ice-cold 1 M sorbitol was added to the cuvette immediately and 200 μl aliquots were spreaded on minimal dextrose medium (MD) plates. The plates were incubated at 30 °C until colonies appeared.

2.7. Screening for Mut^+ transformants

Transformants were picked out from the transformant plates and patched on both minimal methanol medium (MM) plates and MD plates and incubated at 30 °C for 2 days. The Mut^+ (methanol utilization plus) transformants were differentiated from Mut^s (methanol utilization slow) via comparison of patches growth rate on MM and MD plates. Detail procedure can be found in the kit protocol.

2.8. PCR analysis of *P. pastoris* transformants

The primers were as follows—5′ *AOX1* primer: 5′-GACTGGTTCCAATTGACAAGC-3′; 3′ *AOX1* primer: 5′-GCAAATGGCATTCTGACATCC-3′.

The genomic DNA of transformants were used as the template and isolated from *P. pastoris* as described in Multi-Copy *Pichia* Expression Kit. PCR amplification was performed as follows: initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles (1 min denaturation at 94 °C, 1 min annealing at 55 °C and 2 min elongation at 72 °C) and a final elongation at 72 °C for 10 min.

2.9. Induced expression of DAAO by recombinant *P. pastoris* in shake-flask

The recombinant *P. pastoris* strains judged by PCR analysis were inoculated to 5 ml of buffered glycerol-complex medium (BMGY), respectively, and cultured at 28–30 °C with vigorous shaking (250–300 rpm) until the OD_{600} reaches 2–6 (approximately 16–18 h). The cells were collected by centrifugation at 4000 rpm for 5 min at room temperature, resuspended in 10 ml of buffered methanol-complex medium (BMMY) and grew at 28–30 °C with shaking. Every 6 h, the samples were withdrawn to carry out activity assay. Methanol was added to a final concentration of 0.5% each day.

2.10. Measurement of DAAO activity and SDS-PAGE analysis

The assay of enzyme activity was carried out with the permeabilized cells [10]. The cells collected by centrifugation (4000 rpm, 4 °C, 10 min) were washed twice with normal saline and suspended in chilled pyrophosphate buffer (0.05 M, pH 8.5) containing 30% acetone. Then the suspended cells were shaken for 30 min at 25 °C, centrifuged (4000 rpm, 4 °C, 10 min) and washed twice with normal saline. The activity of DAAO was measured by stirring the permeabilized cells in 5 ml of 50 mM D-Ala-containing pyrophosphate buffer (pH 8.5) for 30 min at 37 °C. The reaction was terminated by adding 3 ml of trichloroacetic acid (10%). A total of 0.1 ml of the reaction mixture was diluted 10 times and reacted with 0.4 ml of 2,4-dinitrophenylhydrazine saturated (0.2%) in 2 M HCl for 10 min 1.5 ml of 3 M NaOH was added and the absorbance at 550 nm was measured after 15 min. The recombinant *P. pastoris* GS115 integrated with pPIC3.5K was used as control. One unit of DAAO activity is defined as the amount of enzyme which produces 1.0 μmol of α-keto acid per minute under the conditions described above [11].

2.11. Fermentation of the best *P. pastoris* with high expression of DAAO

The fermentation was performed in a 1-l fermentor (BIOSTAT®M, B. BRAUN). The basal salts medium had a pH 1.0–1.5 after autoclaving. After the medium reached 30 °C in the fermentor, the pH was adjusted to 6.0 with 30% NH_4OH. PTM1 was sterilized by filtering and added to basal salts medium (2 ml/l). PTM1 was also added to the methanol feed (2 ml/l).

A total of 500 μl of recombinant *P. pastoris* were inoculated to 50 ml of BMGY medium in a 250 ml flask and incubated with shaking at 28–30 °C to OD_{600} = 2–6 (approximately 16–18 h). The 50 ml of the cells were used as inoculums for 1-l fermentor media.

The standard fermentation protocol was to grow the cells in excess glycerol to repress the expression followed inducement of protein production by adding methanol after glycerol was exhausted. The temperature and pH were set at 30 °C and 6.0, respectively [8]. Every 4 h, 1 ml of culture was withdrawn to determine the activity and the dry cell weight.

2.12. Extraction of crude DAAO

Yeast cells (20 g) were carefully mixed with the indicated amount of 1-hexanol. After 3 h, 50 ml water was added, slow stirring was initiated and the pH was adjusted and kept at 8.3–8.7 over 4 h by titration with NaOH. The determination of DAAO activity was performed after another 20 h with stirring but without pH control [12].

3. Results and discussion

3.1. PCR amplification and construction of expression plasmid pPIC3.5K-DAAO

The PCR product of *TvDAAO* gene was ligated into T-vector, then into plasmid pPIC3.5K (Fig. 1). The insertion of *TvDAAO* gene into pPIC3.5K between 5′ *AOX1* and 3′ *AOX1* (TT) was proved to be correct by DNA sequencing.

3.2. Screening of Mut^+ transformants and PCR analysis of *P. pastoris* transformants

Plasmid pPIC3.5K-DAAO or pPIC3.5K was linearized by *Sal*I, respectively and electroporated into the host *P. pastoris* GS115 (his^-mut^+). Forty-nine His^+Mut^+ recombinants were identified by PCR from 52 His^+ transformants screened on MD and MM plates.

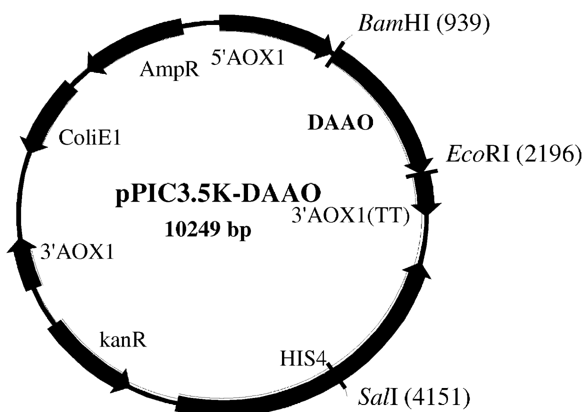

Fig. 1. Construction of expression plasmid pPIC3.5K-DAAO—5′ *AOX1*: promoter fragment; TT: transcription termination; *HIS4* ORF: a selection marker; DAAO gene was inserted between 5′ *AOX1* and 3′ *AOX1* (TT).

DAAO gene was integrated into the *P. pastoris* genome in positive recombinants (his^+mut^+) via single crossover between the *Sal*I-linearized pPIC3.5K-DAAO and genome. Most transformants should be Mut^+. However, with the presence of the *AOX1* sequences in the plasmid, there was a chance that recombination could occur at the *AOX1* locus to disrupt the wild-type *AOX1* gene and create His^+Mut^s transformants. It is worth mentioning that the recombinant cells have excellent genetic stability because of the integration.

PCR amplification results (Fig. 2) showed that parent plasmid pPIC3.5K produced a 220 bp product; plasmid pPIC3.5K-DAAO produced a 1.48 kb product containing the DAAO gene (1.26 kb) and 220 bp sequence from plasmid pPIC3.5K; positive recombinants produced the *AOX1* gene (2.2 kb) and a 1.48 kb product; negative transformants only produced the *AOX1* gene (2.2 kb) from GS115 genomic DNA; control strains transformed by plasmid pPIC3.5K produced the *AOX1* gene (2.2 kb) and a 220 bp product.

3.3. Induced expression of DAAO by recombinant *P. pastoris* in shake-flask, DAAO activity assay and SDS-PAGE

The reaction catalyzed by DAAO requires oxygen and releases NH_3 and á-keto acid from a D-amino acid substrate. The á-keto acid was used to measure DAAO activity. The strain transformed by parent plasmid pPIC3.5K was taken as control. The activity

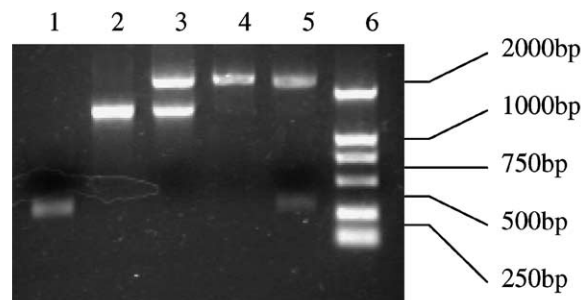

Fig. 2. PCR analysis of *P. pastoris* clones: 10 µl of a 50 µl PCR sample were run on 1% agarose gel and stained with ethidium bromide. From left to right, lane 1: PCR product of pPIC3.5K; lane 2: PCR product of pPIC3.5K-DAAO; lane 3: PCR product of positive recombinants; lane 4: PCR product of negative transformants; lane 5: PCR product of control strains; lane 6: DNA ladder (DL-2000).

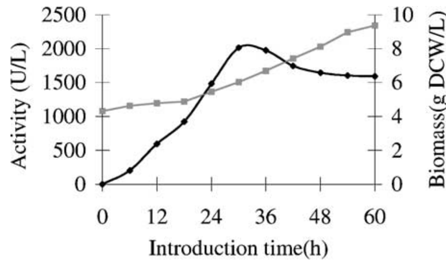

Fig. 3. The relationship between biomass and production of DAAO under induction. The "0 h" was the starting point for methanol inducing. Symbols—(■): the activity of DAAO; (◆): dry cell weight.

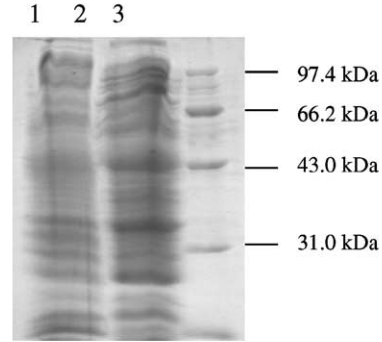

Fig. 4. SDS-PAGE analysis: lanes are numbered 1–3 from left to right. Lane 1: protein from control strain; lane 2: proteins from the best strain; lane 3: protein molecular-mass markers.

essay demonstrated that DAAO was intracellularly expressed in recombinant *P. pastoris*. In addition, activity assay was the efficient method to screen the best recombinant DAAO production strain. Among 49 positive clones screened by PCR, the recombinant cell PD27 possessed the highest activity and the activity was two to three folds higher than those from other clones. The relationship between induction time, cell density and DAAO activity is indicated in Fig. 3. SDS-PAGE of the proteins obtained from PD27 when the induction time was 30 h show a 43 kDa band (Fig. 4) and coincided with size of the two identical subunits of DAAO.

3.4. High-density fermentation of recombinant *P. pastoris*

In 1-l fermentor, at the beginning of the glycerol batch phase dissolved oxygen (DO) concentration was high (approximate 80%) then it decreased soon with the fast growth of cells. At 23 h since inoculation, the fermentation was transferred to a fed-batch phase during which glycerol was added at a growth-limiting rate to derepress the methanol metabolic machinery and allow the cells to transit smoothly from glycerol to methanol feeding, and DO concentration fluctuated in a zigzag way between 15 and 50%. Because glycerol would repress the induced expression of DAAO, methanol feeding initiated at 28th hour to ensure glycerol was exhausted, and methanol level in the fermentor was carefully judged by dissolved oxygen concentration to ensure that methanol did not accumulate in the medium during the induction phase since Mut^+ recombinants were sensitive to high concentration of residual methanol which was oxidized to formaldehyde that would kill the cells. During this phase, a peak of DAAO enzymatic activity appeared after 12 h induction and the activity reached up to a maximum

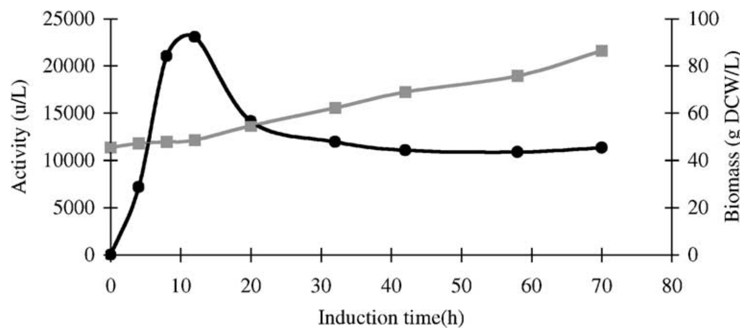

Fig. 5. High-density fermentation of recombinant *P. pastoris* PD27 in 1-l fermentor. The "0 h" was the starting point for methanol feeding. Symbols—(■): the activity of DAAO; (●): biomass (dry cell weight/l).

Table 1
Level of expression of native and recombinant D-amino acid oxidase activity from different sources

	Fermentation broth (U/l)	Cell (U/g)	Specific activity (U/mg protein)	DAAO (%)	Reference
Rhodotorula gracilis					
Using D-alanine as inducer	200	16	0.6	0.5	[13]
Recombinant (pKK-DAAO system)	800	50	0.5	0.4	[14]
Recombinant (pT7-DAAO system)	2,300	930	8.8	7.8	[14]
Pig kidney					
Recombinant (pUK-DAAO system)	85	25.8	0.15	1.6	[15]
Recombinant (pET-DAAO system)	200	n.d.	n.d.	40	[16]
Fusarium solanii					
Recombinant (pCFS315 system)	n.d.	n.d.	n.d.	8.0	[17]
Trigonopsis variabilis					
Using *N*-carbamoyl-D-alanine as inducer	4,200	450	n.d.	4.8	[15]
Recombinant (in *Kluyveromyces lactis*)	n.d.	150	n.d.	n.d.	[4]
Recombinant (in *Pichia pastoris* pPIC3.5K-DAAO system)	23,000	473	1.46	n.d.	This paper

23,000 U/l (Fig. 5). However, the cell density kept increasing slowly. The reason that intracellular DAAO did not increase continuously with the induction and the biomass increase maybe due to proteases digestion or the shortage of atom of iron and FAD. It was clear that DAAO yield from high-density fermentation were higher than that from the expression in shake-flask just because the conditions for recombinant *P. pastoris* growth was better and methanol metabolism was faster during fermentation. Furthermore, compared with the expression in shake-flask, the maximum of DAAO activity was reached faster in fermentor.

3.5. High expression of DAAO in the crude extract

The activity, protein content and specific activity of crude DAAO were determined. Compared with the DAAO from different sources both from the original source and from recombinant microorganism employed by other researchers, the level of expression of DAAO is as summarized in Table 1.

4. Conclusions

DAAO gene was heterologously expressed in *P. pastoris*. After screening on enzymatic activity, a positive recombinant strain PD27 with high expression of DAAO was obtained. This strain was cultured in 1-l fermentor and the expression level reached 23 kU/l (473 U/g dry cell) with the specific activity of 1.46 U/mg protein. More optimization of fermentation in fermentor, purification and immobilization are carried on in this lab.

Acknowledgements

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