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Optimization of Culture Condition for the Production of D-Amino Acid Oxidase in a Recombinant *Escherichia coli*

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Abstract The gene encoding D-amino acid oxidase (DAAO) from *Trigonopsis variabilis* CBS 4095 has been cloned and expressed in *Escherichia coli* BL21 (DE3). Unfortunately, it was observed that the host cell was negatively affected by the expressed DAAO, resulting in a remarkable decrease in cell growth. To overcome this problem, we investigated several factors that affect cell growth rate and DAAO production such as addition time of inducer and dissolved oxygen (DO) concentration. The addition time of lactose, which was used as an inducer, and DO concentration appeared to be critical for the cell growth of *E. coli* BL21 (DE3)/pET-DAAO. A two-stage DO control strategy was developed, in which the DO concentration was controlled above 50% until specific stage of bacterial growth (OD_{600} 30-40) and then downshifted to 30% by changing the agitation speed and aeration rate, and they remained at these rates until the end of fermentation. With this strategy, the maximum DAAO activity and cell growth reached 18.5 U/mL and OD_{600} 81, respectively. By reproducing these optimized conditions in a 12-m^3 fermentor, we were able to produce DAAO at a productivity of 19 U/mL with a cell growth of OD_{600} 80. © KSBB

Keywords: D-amino acid oxidase, Escherichia coli *BL21 (DE3), fermentation, dissolved oxygen*

INTRODUCTION

DAAO (EC 1.4.3.3) is a flavoprotein that catalyzes the oxidation of D-amino acids to the corresponding α-keto acids with the concomitant production of ammonia and hydrogen peroxide. DAAOs are used in the separation process of racemic mixtures of amino acids, in the production of α-keto acid, and in the quantitative analyses of D-amino acids [1-4]. Most importantly, DAAO has been used for the production of 7-aminocephalosporanic acid (7-ACA) from cephalosporin C (CPC) in cooperation with glutaryl-7-aminocephalosporanic acid (GL-7-ACA) acylase [5-8].

Commercially, 7-ACA, a starting compound for the production of semisynthetic β-lactam antibiotics, is produced through chemical synthesis or by enzymatic conversion. Because of stringent environmental regulations against the use of organic solvents and increasing production cost, it is becoming more attractive for the pharmaceutical industry to produce 7-ACA by enzymatic conversion method [9-12]. DAAO involves the conversion of CPC to GL-7-ACA and many efforts have been directed in recent years to obtain this enzyme in large amounts from various microorganisms [10,13-18]. Among them, *Trigonopsis variabilis* has been reported to be a good producer of DAAO [8,15]. However, the production of DAAO using the *T. variabilis* for the commercial use has many problems such as high production cost, complex purification process, and low purification yield [19,20]. To overcome these problems, we investigated the possibility of overexpression of this enzyme in *E. coli* which seems to be a logical candidate. Thus, we have cloned and expressed the gene encoding DAAO of *T. variabilis* in *E. coli*, but toxicity of the enzyme to this bacterium has hampered efficient expression in the scale-up process.

Although there are many evidences from the literature that DAAO gene from *T. variabilis* can be expressed in *E. coli*, little information is available about the optimum fermentation condition which is important for an industrial scale [21-23].

The object of this study was to investigate the optimum fermentation condition that would yield the efficient DAAO production for industrial application by recombinant *E. coli*.

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MATERIALS AND METHODS

Strains

T. variabilis CBS 4095 obtained from CBS (Centraalbureau voor Schimmelcultures, The Netherlands) was used as the source of DNA to clone the DAAO gene. *E. coli* JM109 (TaKaRa Bio Inc., Japan) was used for the amplification of pET-DAAO expression plasmid and *E. coli* BL21 (DE3) (Novagen Inc., USA) was employed to overproduce the enzyme.

DNA Manipulation and Expression

Total DNA of *T. variabilis* cells was isolated with DNeasy Tissue Kit (Qiagen GmbH, Germany) after treating the culture broth with 5 mg/mL of Novozyme 234. We modified the polymerase chain reaction (PCR) method used by Jorge Alonso *et al.* [21] to amplify the DAAO gene from isolated total DNA of *T. variabilis* and identified with gel electrophoresis. The DAAO gene of *T. variabilis* has an intron which disrupts the reading frame. The upper exon is composed of only 24 nucleotides and followed by the intron. To eliminate the intron region, we designed the primers as follow: TvNF; 5′-ATACATATGGCTAAAATCGTTGTTATTGGG**GCCGGTGCCGGTTTAAC** and TvXR; 5′-TATACACTCGAGCTAA AGGTTTGGACGAG. TvNF has the whole upper exon sequence (underlined) and 17 nucleotides of downstream exon (bald).

The PCR reaction mixture, in 100 μL, contained 5 ng template DNA, 0.2 mM each dNTP, 250 pmole each primer, 10 μL PCR buffer and 5 U Taq polymerase (TaKaRa Bio Inc., Japan). The PCR reaction was performed in a GeneAmp PCR System 2700 (Applied Biosystems, Singapore) for 30 cycles under the following conditions: 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1.5 min, followed by a final extension at 72°C for 5 min.

The PCR product of DAAO gene and pET-24a(+) harboring the T7 promoter (Novagen Inc., USA) were digested by *Nde*I and *Xho*I and isolated by agarose gel electrophoresis, respectively. These fragments were purified with GENECLEAN SPIN Kit (Qbiogene Inc., USA) and ligated by T4 DNA ligase (TaKaRa Bio Inc., Japan). The pET-DAAO expression plasmid was amplified in the *E. coli* JM109 and then transferred in the host *E. coli* BL21 (DE3). Cloning and transformation techniques were essentially performed by the method of Sambrook *et al.* [24].

Culture Media

The medium used for the cultivation of *T. variabilis* was composed of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L glucose. The seed medium for the cultivation of recombinant *E. coli* contained 5 g/L yeast extract, 10 g/L sodium chloride, 10 g/L tryptone, 0.02 g/L kanamycin, and 0.1 g/L antifoaming agent. The production medium for fermentation study was as follows: 10 g/L glucose; 5 g/L yeast extract; 7.5 g/L KH_2PO_4; 7.5 g/L $Na_2HPO_4 \cdot 12H_2O$; 3 g/L $MgSO_4 \cdot 7H_2O$; 0.01 g/L $FeSO_4 \cdot 7H_2O$; 0.0675 g/L $CaCl_2 \cdot 2H_2O$; 0.00375 g/L $MnSO_4 \cdot 5H_2O$; 0.00065 g/L $CoCl_2 \cdot 6H_2O$; 0.00375 g/L $ZnSO_4 \cdot 7H_2O$; 0.00065 g/L $CuCl_2 \cdot 2H_2O$; 0.00065 g/L $Na_2MoO_4 \cdot 2H_2O$; 0.02 g/L kanamycin; 0.2 g/L antifoaming agent. The feed solution contained: 500 g/L glucose; 5 g/L $MgSO_4 \cdot 7H_2O$; 0.02 g/L $FeSO_4 \cdot 7H_2O$; 0.135 g/L $CaCl_2 \cdot 2H_2O$; 0.0075 g/L $MnSO_4 \cdot 5H_2O$; 0.0013 g/L $CoCl_2 \cdot 6H_2O$; 0.0075 g/L $ZnSO_4 \cdot 7H_2O$; 0.0013 g/L $CuCl_2 \cdot 2H_2O$; 0.0013 g/L $Na_2MoO_4 \cdot 2H_2O$; 0.1 g/L kanamycin; 0.2 g/L antifoaming agent.

Cultivation Conditions

The seed culture was carried out in a 5-L round flask containing 500 mL of the seed medium for the lab-scale experiments and in a 200-L pilot fermentor with a working volume of 120 L for the large-scale experiment. Both cultures were incubated at 36°C for 5 to 8 h and then transferred to the main fermentors when an optical density of 3 to 6 at 600 nm (OD_{600}) and the pH of 6.5 to 7.0 were achieved. The lab-scale experiment for optimizing the culture conditions was performed in a 30-L jar fermentor (Korea Fermentor Co., Ltd., Korea) with a 15 L working volume. The main fermentation was carried out with 1% of the inoculum and the pH of the culture broth was controlled at 7.0 with 25% ammonia water. The substrate feeding strategy was glucose concentration control method based on pH-stat and the glucose concentration in the culture broth was maintained below 0.05%. DO concentration was monitored by a polarographic oxygen electrode (InPro® oxygen sensor, Mettler Toledo, Switzerland) and controlled according to the study design by varying the agitation speed and aeration rate. The culture temperature was carried out initially at 35°C and then changed to 21°C according to cell growth (28°C at OD_{600} 20, 21°C at OD_{600} 30). For the induction of DAAO enzyme, 20 g/L lactose was added at specific stages of bacterial growth according to the experimental design. The fermentor used for the large-scale experiment was a regular stirred tank with 3 Rushton turbines, 4 baffles and a total volume of 12 m^3 (Jung Hyun Plant Co., Ltd., Korea). The mature seed (OD_{600} 3~6, pH 6.5~7.0) in 200-L pilot fermentor was injected pneumatically into 12-m^3 fermentor through a pipe connecting the two fermentors. The large-scale fermentation was carried out with the same control method used at lab-scale experiment.

Analytical Methods

Cell growth was monitored by the broth OD using a UV-Vis spectrophotometer (Hewlett Packard, Waldbronn, Germany) at 600 nm. The glucose concentration was monitored using a glucose analyzer (YSI model 2700, Yellow Springs Instrument Incorporated, Yellow Springs, OH, USA). The acetic acid concentration in the culture broth was quantified with a Roche enzymatic kit (Mannheim, Germany). The DAAO activity was assayed using a polarographic oxygen electrode (InPro® oxygen sensor, Switzerland), measuring the rate of oxygen consumption in the presence of CPC [25].

The reaction was carried out at 25°C in 100 mM potassium phosphate buffer, pH 8.0, containing 20 mM CPC and an appropriate amount of crude extract. One unit of enzyme was defined as the amount of enzyme which consumes 1 μmol of oxygen per min.

RESULTS AND DISCUSSION

Effect of Induction Time

For the expression of foreign protein with the T7 promoter, the use of isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose as an inducer is required [23,26,27]. Because IPTG is not only costly but it may also be toxic to humans, IPTG-induction system is unfeasible on the industrial scale. In the preliminary experiment, it showed that lactose was as effective as IPTG for inducing DAAO enzyme (data not shown). Optimal induction with lactose is dependent on the time at which the inducer is added [28,29]. To determine the effect of induction time with lactose on the DAAO production and cell growth in recombinant *E. coli*, following investigation was carried out.

A set of fermentation was carried out with DO concentration above 50% by changing agitation speed and aeration rate, with a temperature downshift control method [30,31] which had been very effective to maximize DAAO productivity and cell growth rate in the preliminary test (data not shown), and with various application of induction time with lactose: initial stage of fermentation, OD_{600} 30, OD_{600} 50, and no induction (Fig. 1). Throughout the fermentation, the glucose concentration was maintained below 0.05% to reduce acetic acid formation in the culture broth [32]. When the lactose was added at initial stage of fermentation, the cell growth and DAAO production were very poor and the phenomenon of rapid cell lysis was observed (Fig. 1A). A similar result was achieved when the lactose was added at a broth OD_{600} of 30, although the time of cell lysis was slightly delayed (Fig. 1B). When the lactose was added at a broth OD_{600} of 50, the ordinary cell growth pattern was observed until the lactose was added, but a significant cell lysis was happened with the formation of target protein after adding the lactose (Fig. 1C). However, when the fermentation was carried out without induction, the phenomenon of cell lysis did not happen until the end of fermentation, but the DAAO activity in culture broth was not observed (Fig. 1D). These results indicate that the cell growth of recombinant *E. coli* is strongly influenced by the addition time of lactose and the cause of cell lysis may have a close relation to the DAAO formation.

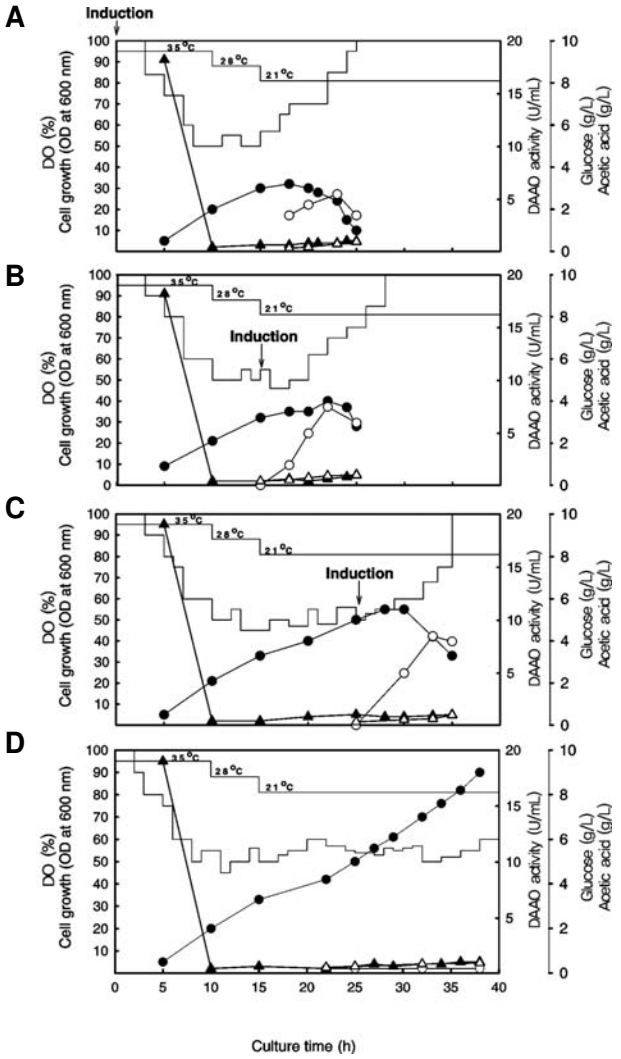

Fig. 1. Effects of the induction time of lactose on the DAAO production and cell growth in recombinant *E. coli* BL21 (DE3)/pET-DAAO. (A) Induction at initial stage of fermentation; (B) induction at a broth OD_{600} of 30; (C) induction at a broth OD_{600} of 50; (D) no induction. The arrows indicate the addition of lactose (●, cell growth; ○, DAAO activity; —, DO; ▲, glucose; △, acetic acid).

Effect of DO Concentration

Since the expression of a target protein and cell growth rate of recombinant *E. coli* were generally known to be significantly influenced by DO concentration [26,33,34], the effect of various DO concentration was investigated by maintaining it from 20 to 50% (Fig. 2). The higher DO concentration was maintained, the faster cell growth was observed before induction with lactose. In all cases, however, as the DAAO was expressed, cell growth rate was decreased or retarded. The decline of cell growth curve in high DO concentration case was greater than that of in low DO concentration case after induction. These results indicate that a relatively high DO concentration is favorable for the cell growth in the early stage of fermentation but negatively affected on the cell growth after the addition of lactose. Therefore, a new set of experiment was performed, where DO concentration was maintained above 50% until the desired cell mass has been achieved. When the OD_{600} of the culture broth reached 30 to 40, the lactose was added and then the

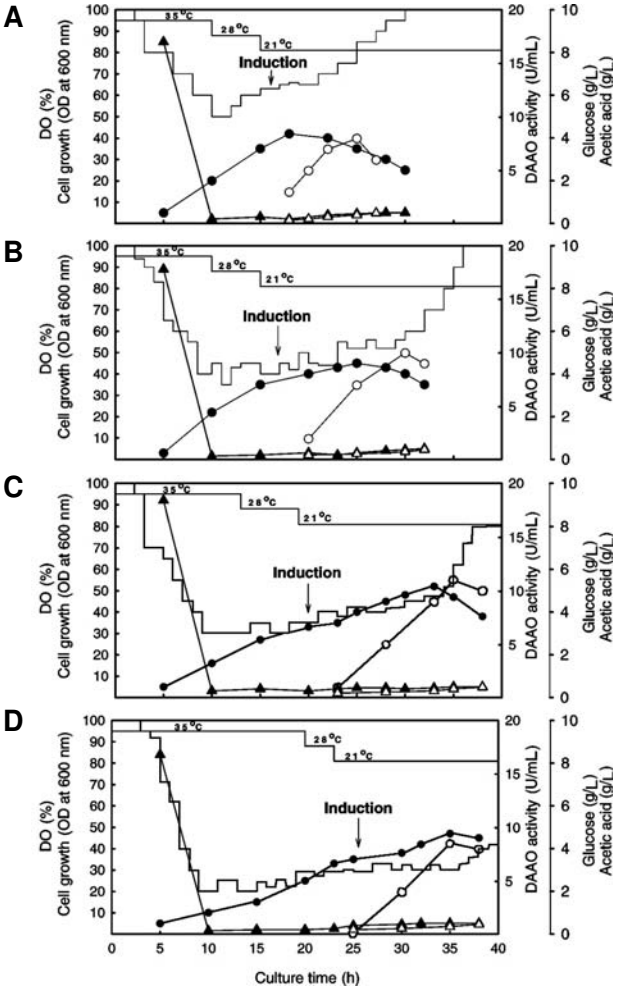

Fig. 2. Effects of DO concentration on the DAAO production and cell growth in recombinant *E. coli* BL21 (DE3)/pET-DAAO. (A) Maintain DO at 50%; (B) maintain DO at 40%; (C) maintain DO at 30%; (D) maintain DO at 20%. The arrows indicate the addition of lactose (●, cell growth; ○, DAAO activity; —, DO; ▲, glucose; △, acetic acid).

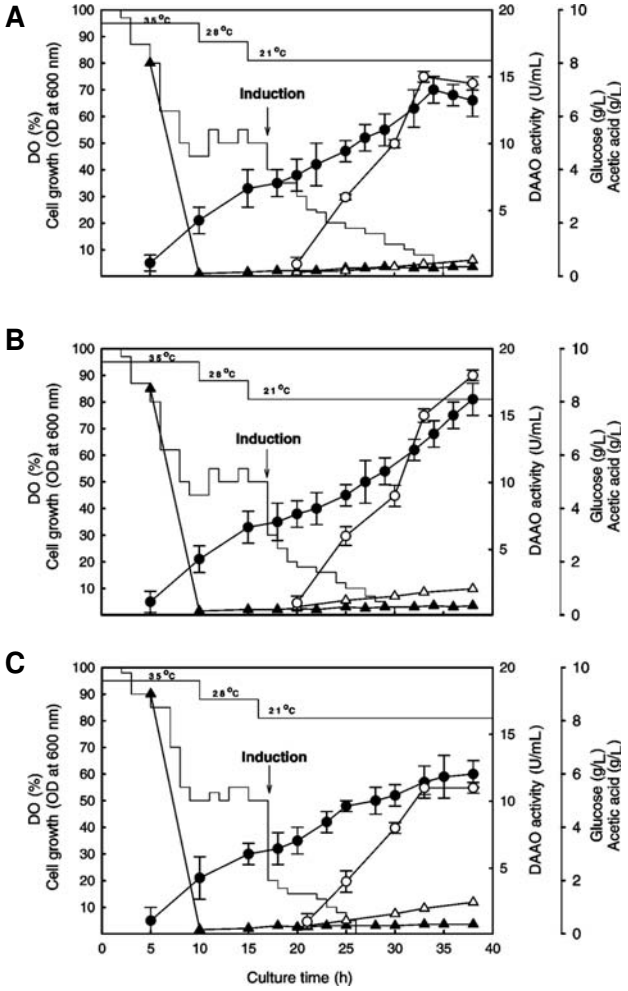

Fig. 3. Effects of DO concentration downshift on the DAAO production and cell growth in recombinant *E. coli* BL21 (DE3)/pET-DAAO. (A) Maintain DO above 50% until OD_{600} 30 to 40, and then reduce DO to 40%; (B) maintain DO above 50% until OD_{600} 30 to 40, and then reduce DO to 30%; (C) maintain DO above 50% until OD_{600} 30 to 40, and then reduce DO to 20%. The arrows indicate the addition of lactose. The error bars represent the standard deviations calculated from the data obtained in three independent experiments (●, cell growth; ○, DAAO activity; —, DO; ▲, glucose; △, acetic acid).

DO concentration was downshifted to 40, 30, and 20% by changing agitation speed and aeration rate, and they remained at these rates until the end of fermentation (Fig. 3). As shown in Fig. 3, this control method allowed us to achieve a typical cell growth pattern without a significant cell lysis, although DO concentration decreased gradually according to cell growth, even reached zero and the acetic acid concentration increased gradually due to oxygen-limited condition, which inevitably resulted in acetic acid accumulation. When DO concentration was downshifted to 40%, the fastest cell growth was achieved, but the phenomenon of slight cell lysis was observed at 35 h, resulting in a decrease in cell growth (Fig. 3A). When DO concentration was downshifted to 30%, a DAAO productivity of 18.5 U/mL and cell growth yielding a broth OD_{600} of 81 were achieved at 38 h (Fig. 3B). When DO concentration was downshifted to 20%, the cell growth rate was decreased after 28 h, resulting in a broth OD_{600} of 60 and a DAAO productivity of 11 U/mL (Fig. 3C). These results indicate that the downshift in the DO concentration at specific stage of bacterial growth (OD_{600} 30~40) is a very effective way to maximize productivity and cell mass.

Based on these results, we can conclude that the lysis of host cell is due to the potential toxicity of the expressed DAAO. Not only the consumption of D-amino acids by the activity of expressed DAAO could interfere the biosynthesis of *E. coli* cell wall, but the hydrogen peroxide produced by the oxidation of D-amino acids could also be harmful to the

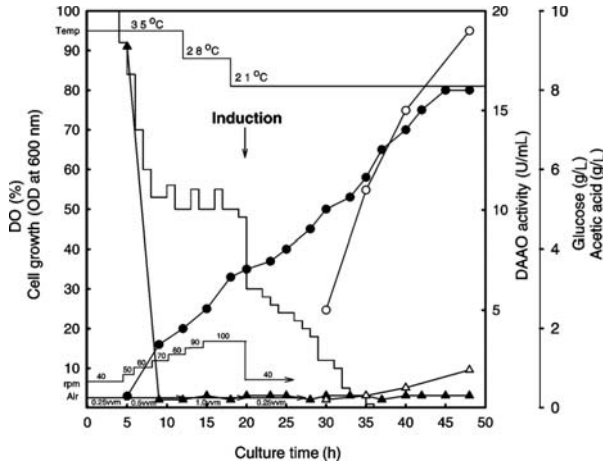

Fig. 4. Typical time course for DAAO production by recombinant *E. coli* BL21 (DE3)/pET-DAAO in a 12-m^3 fermentor. The arrow indicates the addition of lactose (●, cell growth; ○, DAAO activity; —, DO; ▲, glucose; △, acetic acid).

cells [12,19,35]. Therefore, as shown in Fig. 3, the fermentation process, adding the lactose after the desired cell mass has been achieved and then reducing the oxygen supply needed for oxidation of intracellular D-amino acids by the expressed DAAO, appears to be optimal for the production of DAAO by recombinant *E. coli*.

Large-scale Cultivation

The large-scale production of DAAO enzyme was carried out in a 12-m^3 fermentor with 8 m^3 of working volume, based on the optimal condition found thus far (Fig. 3B). The fermentation process was carried out initially at a temperature of 35°C, an agitation speed of 40 rpm, an aeration rate of 0.25 vvm, and operating pressure of 0.5 kg/cm^2. DO concentration was maintained above 50% until the cell growth resulted in a broth OD_{600} of 30 to 40. When the OD_{600} of the culture broth reached 35, 20 g/L of lactose was added and then the DO concentration was downshifted to 30% by reducing the agitation speed and aeration rate, and they remained at these rates until the end of fermentation. This control method allowed us to achieve a typical cell growth pattern with a broth OD_{600} of 80 reached at 45 h (Fig. 4). The maximum productivity of 19 U/mL was obtained at 48 h, which was found to be 7.5-fold higher than that obtained with *T. variabilis* CBS 4095, the source of DNA to clone the DAAO gene.

In the present study, we successfully developed the fermentation process for mass production of DAAO in recombinant *E. coli*, one of the industrially useful enzymes, by optimizing the induction time with lactose and a two-stage DO control strategy.

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