

Enhancement of *Trigonopsis variabilis* D-Amino Acid Oxidase Overproduction in Fed-batch Cultivation of *Escherichia coli*

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Original scientific paper

Received: February 3, 2011

Accepted: November 7, 2011

D-Amino acid oxidase is a key enzyme in the production of semisynthetic cephalosporins. The *Trigonopsis variabilis* D-amino acid oxidase with 12- amino-acid peptide at N-terminus was expressed in *Escherichia coli* BL21(DE3), in a series of short fed-batch cultivations. The strategy of gradual induction and gradual feeding with D-methionine during expression resulted in active enzyme production twice as high as that in which the inducer and methionine were added all at once at the expression start. In optimal conditions during 14 h of aerobic cultivation, 350 mg L⁻¹ of active enzyme was produced.

Key words:

D-Amino acid oxidase, *Trigonopsis variabilis*, *Escherichia coli*, D-methionine

Introduction

D-Amino acid oxidases (DAOs) are flavoproteins that catalyze the oxidative deamination of D-amino acids to corresponding imino acids, H₂O₂ and NH₃.¹ The main applications of DAOs are industrial conversion of cephalosporin C into glutaryl-7-aminocephalosporanic acid (7-ACA),^{2–4} production of α -keto acids,⁵ analytical determination of D-amino acids,⁶ and gene therapy for tumor treatment.^{7,8} Sources of DAOs that are most thoroughly investigated and most frequently applied are microorganisms *Trigonopsis variabilis*, *Rhodotorula gracilis*, and *Fusarium solani*. Its favourable technological properties make DAO from yeast *T. variabilis* (TvDAO) the prime candidate for a high-scale industrial production of 7-ACA.⁹ Up to now, TvDAO has been expressed in *E. coli*^{10–13} and other microorganisms, e.g. *Schizosaccharomyces pombe*,⁴ *Pichia pastoris*,¹⁴ *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.¹⁵

E. coli BL21(DE3)(pTvDAOstrepN) showed high potential for industrial application.¹⁶ The recombinant enzyme, with the synthetic 12 amino-acid peptide sequence at N-terminus that exhibits affinity towards streptavidin, was identical in activity to the native one when bound to the affinity carrier. We here report on several culture conditions and feeding strategy issues during short fed-batch expressions of this enzyme.

Materials and methods

Microorganism

The construction of recombinant TvDAO bearing Strep-Tag II at N-terminus and its production in

kanamycin resistant *E. coli* BL21(DE3) have been described elsewhere,¹⁶ with *T. variabilis* ATCC10679 used originally as a DAO gene source. Cells were maintained on LB medium in a refrigerator at 4 °C.

Media and culture conditions

The 200 mL of seed culture (LB medium) was prepared in a 500 mL Erlenmeyer flask and grown in a rotary shaker overnight at 37 °C and 130 rpm. Fed-batch culture was carried out in a laboratory 5 L bioreactor (Biostat A, Braun Biotech International, Meslungen, Germany), at 37 °C. Dissolved oxygen was maintained at 30 % of air saturation by increasing stirrer speed. A pH of 6.8 was maintained with aqueous NH₃ (w = 25 %). Control and regulation of pH and dissolved oxygen were carried out by the digital control unit of the bioreactor. The initial batch medium was (per liter): Glucose, 3.5 g; (NH₄)₂HPO₄, 2.0 g; Citric acid, 0.8 g; KH₂PO₄, 4.5 g; MgSO₄·7H₂O, 0.6 g; kanamycin-sulphate, 0.1 g; trace element solution,¹⁷ 2.2 mL. The initial culture volume was 2 L. The feeding medium consisted of (per litre): Glucose, 650.0 g; (NH₄)₂HPO₄, 15 g; Citric acid, 7.5 g; KH₂PO₄, 20.0 g; MgSO₄·7H₂O, 6.0 g; kanamycin-sulphate, 0.2 g; antifoam, 0.4 mL.

The cultivations in bioreactor started with 2 L of culture volume which was inoculated by 200 mL of inoculum. After the inoculation there was a short batch-culture phase (4 hours). Exponential feeding strategy was applied for the next 10 hours of cultivation (feeding phase) in order to maintain a constant specific growth rate set to 0.12 h⁻¹. Induction started simultaneously with the feeding after 4 hours of cultivation, except for one cultivation which started after 7 hours of cultivation. Two strategies of induction were applied: a) instant addition

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of IPTG to a final concentration of 0.2 mmol L⁻¹; b) gradual addition of the same amount of IPTG during complete feeding/expression phase. The same strategies were tested for 1 mmol L⁻¹ D-methionine addition. Dissolved oxygen was maintained at 30 % of air saturation in all cultivations except one, which was performed under unsaturated conditions.

Parameters were varied in the 10- hour feeding phase according to the set-up shown in Table 1.

Table 1 – Varying parameters of expression during feeding phase of cultivations

Cultivation and expression	Parameters			
	Induction type (IPTG addition)	Induction time	Oxygen (% of air saturation)	D-methionine addition
Basic	instant	4th hour	30	–
A	instant	4th hour	30	instant
B	instant	4th hour	unsaturated	–
C	instant	7th hour	30	–
D	gradual	4th hour	30	–
E	gradual	4th hour	30	gradual

Assays

Cell density was measured at 600 nm in a “Varian” Spectra AA 300 Atomic Absorption Spectrophotometer (Mulgrave Victoria, Australia) and it was correlated to dry cell mass (DCM) using a standard curve. The dry cell mass was predetermined gravimetrically after the culture broth had been centrifuged 10 min at 6,000×g, washed with distilled water, and dried overnight at 105 °C.

Protein extraction from bacterial cells was done using B-PER reagent (Pierce, USA).

TvDAOstrepN activity was determined using DCIP-method, in which 2,6-dichlorophenol replaced dioxygen as the electron acceptor.¹⁸ Measurements were done at 30 °C in 100 mmol L⁻¹ potassium phosphate buffer, pH 8, with D-methionine as the substrate. One DAO unit corresponds to the amount of enzyme that converts 1 μmol of substrate per minute.

Protein concentrations were determined using the Bradford assay with bovine serum albumin as reference.

Plasmid stability was monitored by transferring 100 colonies (sample grown on non-selective LB-agar plate) onto selective LB agar plates (containing 50 μg mL⁻¹ kanamycin sulphate) and non-selective control plates by using sterile tooth-

picks. The ratio of the numbers of CFU (colony forming units) on both plates showed the percentage of plasmid-carrying cells.

Results and discussion

Inducer toxicity

One of possible ways of reducing the toxicity of inducer IPTG¹⁶ was its gradual addition during expression, in order to follow the cell growth curve. Induction at the 7th hour of cultivation (cultivation C), with the same amount of IPTG at higher cell concentration than in Basic fermentation (Fig. 3), clearly confirmed the expected inducer toxicity problem – induction at 7th hour allowed higher maximal change of volumetric activity (141 U L⁻¹ h⁻¹). But the best results were achieved with gradual induction (139 U L⁻¹ h⁻¹, Fig. 4) and the combination of gradual addition of IPTG and D-methionine (327 U L⁻¹ h⁻¹, Fig. 5). Besides maximal rise of volumetric activity, it was noticeable that gradual induction enabled maximal volumetric and specific activities higher than those with inductions performed at once after 4 and 7 hours of cultivation (Table 2).

Table 2 – Summary of DAO activity during cultivations

Cultivation	Volumetric activity (U L ⁻¹)	Maximal increase of volumetric activity (U L ⁻¹ h ⁻¹)	Specific activity (U g ⁻¹ of protein)
Basic	335	112	68
A	353	87	70
B	148	55	65
C	410	141	71
D	685	139	94
E	678	327	107

DAO toxicity

The characteristic time trend of both specific activities (per cell and protein mass) was observed during expressions: a fast growth at the very beginning, stagnation or a more or less dramatic drop during the next phase, and then an increase in activities again. Apparently, in cultivations A and B, as well as in the Basic cultivation (Figs. 1 and 2), the high relative quantity of inducer at the expression start caused strong DAO production and a rapid increase in activity. A few hours after the beginning of expression, a dramatic decrease in specific activities occurred. The volumetric activity also

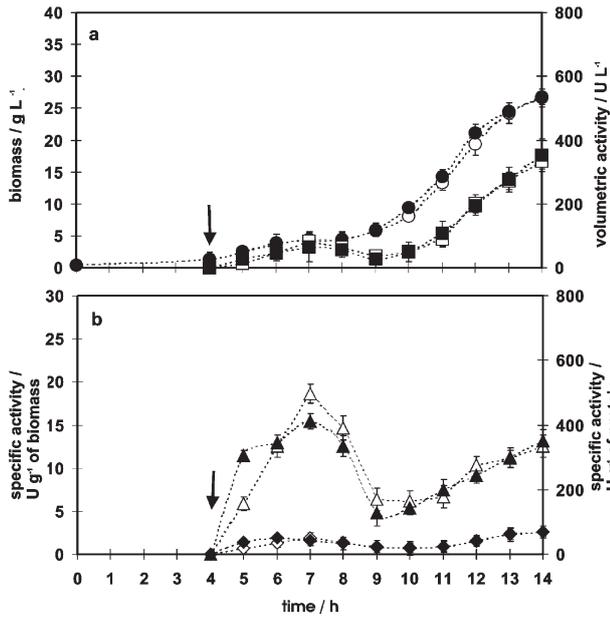


Fig. 1 – (a) Biomass growth (circles) of *E. coli* BL21(DE3) (pTvDAOstrepN), volumetric DAO activity (squares), and (b) specific DAO activity per g of biomass (triangles) and specific DAO activity per g of protein (diamonds) during 14 hours of the Basic cultivation (open symbols) and Cultivation A (closed symbols). Legend explanation: Table 1. Black arrow – expression start. The error bars represent standard deviations calculated from data obtained in three experiments.

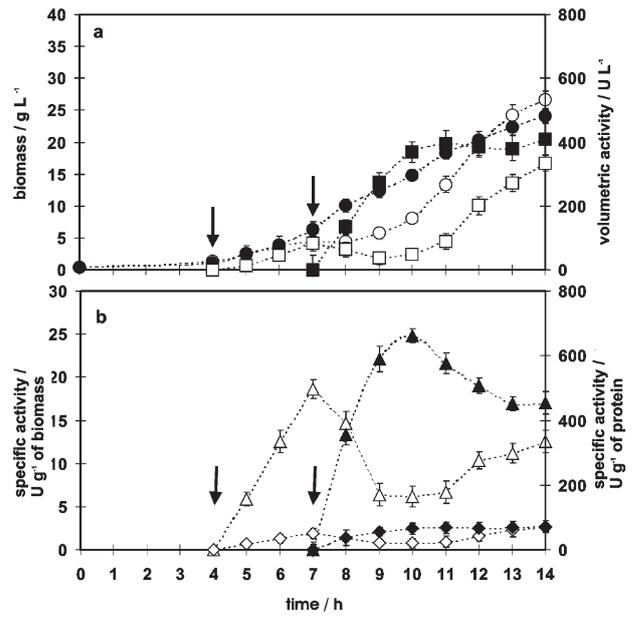


Fig. 3 – (a) Biomass growth (circles) of *E. coli* BL21(DE3) (pTvDAOstrepN), volumetric DAO activity (squares), and (b) specific DAO activity per g of biomass (triangles) and specific DAO activity per g of protein (diamonds) during 14 hours of the Basic cultivation (open symbols) and Cultivation C (closed symbols). Legend explanation: Table 1. Black arrows – expression start. The error bars represent standard deviations calculated from data obtained in three experiments.

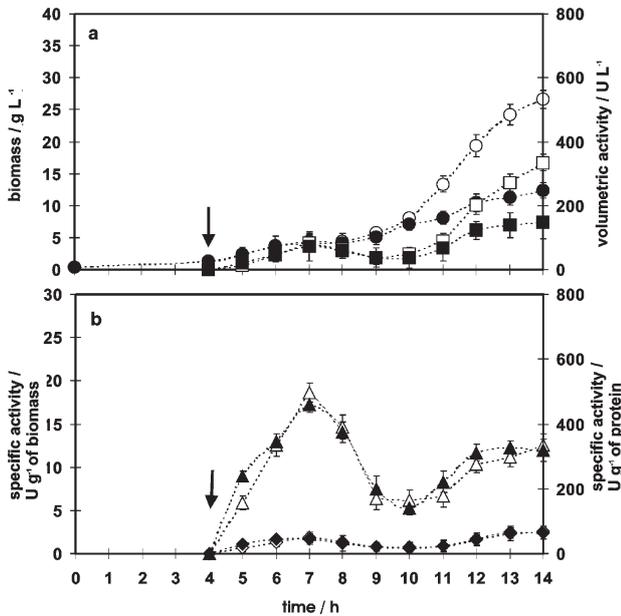


Fig. 2 – (a) Biomass growth (circles) of *E. coli* BL21(DE3) (pTvDAOstrepN), volumetric DAO activity (squares), and (b) specific DAO activity per g of biomass (triangles) and specific DAO activity per g of protein (diamonds) during 14 hours of the Basic cultivation (open symbols) and Cultivation B (closed symbols). Legend explanation: Table 1. Black arrow – expression start. The error bars represent standard deviations calculated from data obtained in three experiments.

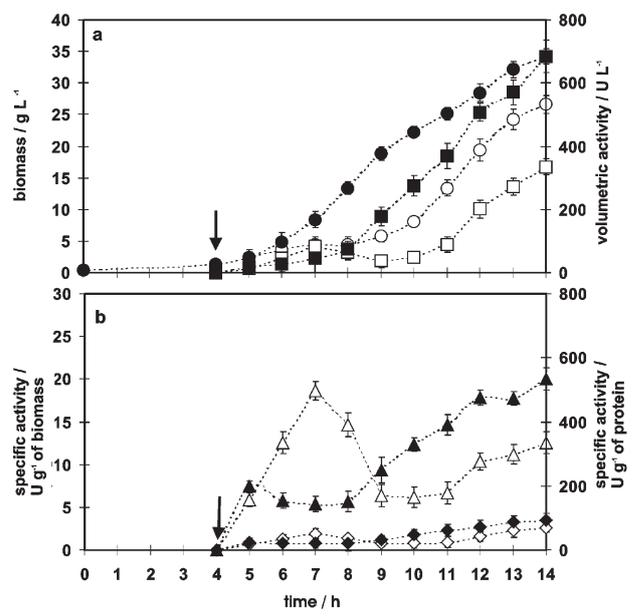


Fig. 4 – (a) Biomass growth (circles) of *E. coli* BL21(DE3) (pTvDAOstrepN), volumetric DAO activity (squares), and (b) specific DAO activity per g of biomass (triangles) and specific DAO activity per g of protein (diamonds) during 14 hours of the Basic cultivation (open symbols) and Cultivation D (closed symbols). Legend explanation: Table 1. Black arrow – expression start. The error bars represent standard deviations calculated from data obtained in three experiments.

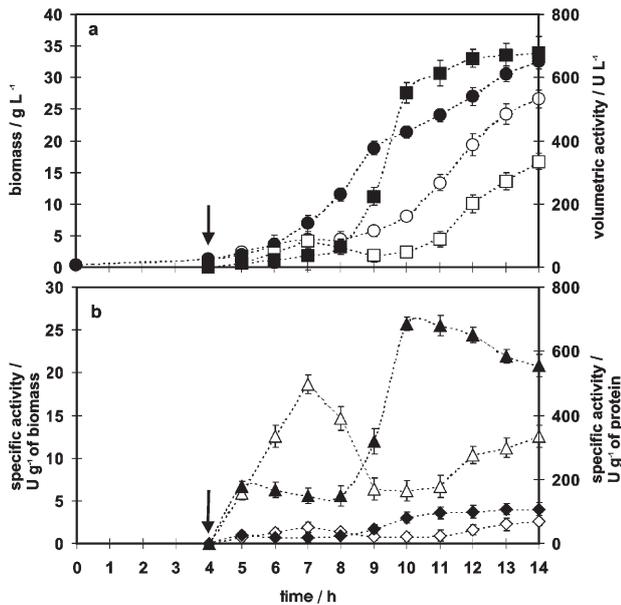


Fig. 5 – (a) Biomass growth (circles) of *E. coli* BL21(DE3) (*pTvDAOstrepN*), volumetric DAO activity (squares), and (b) specific DAO activity per g of biomass (triangles) and specific DAO activity per g of protein (diamonds) during 14 hours of Basic cultivation (open symbols) and Cultivation E (closed symbols). Legend explanation: Table 1. Black arrow – expression start. The error bars represent standard deviations calculated from data obtained in three experiments.

dropped, and the cell growth completely stopped for 1 hour. This part of expression showed that the inducer toxicity is not the single cause of lower productivity. The activity of produced DAO has an inhibitory effect also.

Oxygen supply

The toxic effect of DAO on a cell and possible inactivation of the enzyme induced by oxygen could cause a considerable decrease in the production of active DAO. Reducing oxygen concentration during expression seemed a promising strategy for tackling both causes of decreased production. Cultivation B (Fig. 2) was performed with oxygen-unsaturated media during expression. Cell growth and volumetric activity achieved in this expression were the lowest among all cultivations. The volumetric activity curve followed the cell growth curve, so both specific activities during expressions were not different from the cultivation with oxygen-saturated media (Basic cultivation).

Amino-acid supplementation

Preliminary batch experiments¹⁶ showed very promising results with D-amino acid supplementation of expression media. This strategy was further researched here by performing expressions without amino-acid supplementation, and with the addition of D-methionine, the best substrate for

TvDAO^{19,20} in two different ways. The cell growth showed a clear difference between expression with gradual addition of D-methionine (Fig. 5) and expression with addition of the same amount of D-methionine at once, at the start of expression (Fig. 1). Surprisingly, there was no difference between the expression without D-methionine addition (Basic cultivation) and the expression with D-methionine addition at the expression start (Cultivation A), neither in cell growth, nor in any activities. On the other hand, subsequent D-methionine addition, in combination with gradual induction (cultivation E), resulted in the highest volumetric activity production rate and the highest values of specific activities (Fig. 5).

The cost-effective production of recombinant protein is a primary goal of the fed-batch process. Construction and preliminary researches of *TvDAOstrepN* expressed in *E. coli* BL21(DE3), as an enzyme of high stability and activity had been done.¹⁶ The batch expressions showed a good production potential of this recombinant enzyme. It was established that this enzyme possesses good temperature tolerance and that the best volumetric activity could be achieved at 37 °C. For this reason, fed-batch expressions were performed at this temperature only. The enzyme carries the strep-tag on N-terminus that, for some reason, stabilises the enzyme and increases volumetric activity (compared to the wild type *TvDAO*). Since the basic purpose of applying the strep-tag technology was to enable simple, one-step product purification, the focus of this research was on the production of a fully active DAO and avoiding inclusion bodies formation.

From the results presented here it is apparent that the effects of toxic inducer and toxic product on active DAO production can be minimized by the strategy of gradually feeding the inducer and D-methionine. There were no differences in cell growth of cultures during cultivations with gradual addition of IPTG (cultivation D) and IPTG+methionine (cultivation E). On the other hand, the second approach (IPTG+methionine) yielded much higher productivity of active enzyme production. This indicates that the role of D-methionine is not only to replace this essential amino acid converted by active DAO during cultivation, but also to induce active enzyme production.

During these simple and short cultivations with gradual feeding of inducer and D-methionine we managed to produce approximately 700 U per litre of broth and 22 U per g of biomass. A direct comparison of this yield with other published data is very difficult, due to variations of DAO activity assay methods and conditions. However, some estimated relations have been published.²¹ According to these relations, this yield corresponds to

10500 U L⁻¹ and 323 U g⁻¹ (measured by the method referred to in Table 1 hereof), which means that approximately 350 mg of active enzyme per liter was produced during 14 hours (1200 U L⁻¹ d⁻¹, 600 mg L⁻¹ d⁻¹). The time course of DAO activity during expression is the result of active DAO production and simultaneous inactivation of one part of it. Oxidation is one of the proven causes for TvDAO inactivation.^{22,23} The attempt here to improve active DAO production by lowering the oxygen supply during the expression phase (avoiding the toxic DAO effect) failed, although our preliminary batch experiments¹⁶ had indicated a strong dependence of DAO activity/toxicity on oxygen presence (the reason probably being insufficient aeration). The question whether the process could be improved by controlled oxygen supply still remains open. There have been attempts with DO control strategy²⁴ with similar activity yield as achieved here, but in much longer fed-batch cultivation (48 hours vs. 14 hours here).

Conclusions

In this research short fed-batch cultivations *E. coli* BL21(DE3) and expressions of TvDAOstrepN under various conditions were performed. Expectations regarding obstacles were confirmed and strategies for solving these problems were pointed out. High activity was achieved in very short time periods. Combining with easy on-step DAO purification and immobilization onto the streptavidin carrier, this production system shows great potential for industrial application.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Croatian Ministry of Science, Education and Sports. We thank Prof. Dr. Bernd Nidetzky and Angewandte Biokatalyse-Kompetenzzentrum GmbH. (Graz, Austria) for E. coli BL21(DE3)(pTvDAOstrepN).

List of symbols and abbreviations

DAO – D-amino acid oxidase
 DCM – dry cell mass
 IPTG – Isopropyl-β-D-thiogalactopyranoside
 LB medium – Luria Bertani medium
 DCIP – 2,6-dichloroindophenol
 w – mass fraction, %

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