The Journal of Microbiology, September 2001, p.229-231 Vol. 39, No. 3

NOTE

Role of Amino Acids in Production of *D*-amino Acid Oxidase

Puneet Singh[1,3]*, Satwinder Singh Marwaha[2], and Neelam Verma[1]

[1]*Department of Biotechnology, Punjabi University, Patiala 147 002, India*
[2]*Biotechnology Division, PSCST, PB: 727, Sector 26, Chandigarh 160 019, India*
[3]*Department of Biotechnology, Guru Nanak Dev University, Amritsar 143 005, India*

(Received May 14, 2001 / Accepted July 18, 2001)

Different *DL*-amino acids were studied as inducers of *D*-amino acid oxidase (DAAO) and for their influence on the growth of *Trigonopsis variabilis*. *DL*-amino acids with non-polar side groups were found to be the best inducers of DAAO. Maximum increase in the growth of *Trigonopsis variabilis* (gram dry weight per liter culfure) was observed with *DL*-methionine (2.39 g/l) followed by *DL*-serine (2.22 g/l) and *DL*-alanine (2.21 g/l).

Key words: *D*-amino acid oxidase, *DL*-amino acids, cephalosporin C, *Trigonopsis variabilis*

The polymorphic yeast, *Trigonopsis variabilis*, contains high titers of intracellular, inducible flavoenzyme *D*-amino acid oxidase (DAAO) (E.C. 1.4.3.3). The enzyme catalyzes the oxidative deamination of *D*-amino acids to the corresponding α-keto acids. This reaction is of major biotechnological interest and can be exploited in the production of α-keto acids (2), qualitative and quantitative analysis of *D*-amino acids (5), production of L-amino acids (4), and most importantly, as the first step in the bi-enzymic conversion of cephalosporin C to 7-aminocephalosporanic (7-ACA) acid (1). The latter is a key component for the production of many (66%) semisynthetic β-lactam-cepham drugs.

The major problems in the mass production of DAAO from *T. variabilis* are low biomass (X_0) and product (P_0) yields. Although reports (6, 7, 9) are available on the use of amino acids for the induction of DAAO, these studies involve only a few components. Moreover, no report deals with the effect of amino acids on the cell biomass yield of *T. variabilis*. Keeping all the above in view, a study was planned to observe the effect of different amino acids on the growth of *Trigonopsis variabilis* and/or on the induction of DAAO.

Trigonopsis variabilis (ATCC 10679) was used in this study and cultivation was done in a chemically defined medium. The primary medium used in this study had the following components: glucose (30 g/l at 0 h and 20 g/l after 28 h), urea (3 g/l), KH_2PO_4 (5 g/l), $MgSO_4$ (1 g/l), $CaCl_2$ (0.5 g/l), $ZnSO_4$ (60 mg/l), $FeSO_4$ (40 mg/l), $MnSO_4$ (40 mg/l), $(NH_4)_6 Mo_7O_{24}$ (40 mg/l), $CuSO_4$ (40 mg/l), H_3BO_3 (100 mg/l) and biotin (0.2 mg/l).

Stock solutions for carbohydrates, basal salts and inorganic ions were prepared separately, autoclaved and mixed in defined proportions to get the primary medium. Stock solutions of different amino acids were also prepared separately and sterilized by passing through membrane filters. Different concentrations of each of nineteen different amino acids were added to the primary medium and their effect on the cell biomass (X_0) and enzyme (P_0) yields were determined. All the cultures were grown in Erlenmeyer flasks (250 ml) using 50 ml of defined medium in an incubator-shaker at 30°C, 200 rpm and using 10%(v/v) inoculum. All experiments were repeated at least six times.

At the late logarithmic phase, a sample of the culture (20 ml) was centrifuged to deposit the cells, which were washed twice with 0.85% (w/v) saline. The cell deposit was dried to constant weight in an oven at 110°C. Before each weighing, the tubes were placed in a dessicator over $CaCl_2$ until it cooled to room temperature. In the flask experiments the dry weight (DW) was estimated from a calibration curve in which OD at 550 nm was plotted against dry weight.

DAAO activity in the culture was determined *in situ*, by following a modified method of Case (3). For *in situ* assay of DAAO, *T. variabilis* cells were permeabilized with n-butanol [15% (v/v)] for 30 min at 0°C. These permeabilized cells were washed and suspended in sodium

* To whom correspondence should be addressed.
(Tel) +91-183-258802; (Fax) +91-183-258272
(E-mail) puneet461@usa.net

Table 1. Influence of different *DL*-amino acids on the biomass yield of *Trigonopsis variabilis*[a]

Amino acids	Optimized concentration (mg/l)	Maximum specific growth rate[b] (per h)	Specific biomass yield[c] (gDW/mg)	Difference in biomass yield[d,e] ± SE (gDW/l)
DL-alanine	390	0.34	0.0055	2.21 ± 0.09
DL-isoleucine	90	0.28	0.0132	1.09 ± 0.07
DL-leucine	210	0.28	0.0061	1.29 ± 0.07
DL-methionine	410	0.33	0.0059	2.39 ± 0.08
DL-phenylalanine	260	0.33	0.0078	2.12 ± 0.1
DL-valine	140	0.29	0.0102	1.44 ± 0.05
DL-asparagine	110	0.28	0.0165	1.77 ± 0.11
DL-cysteine	130	0.28	0.0110	1.42 ± 0.05
DL-glutamine	120	0.29	0.0114	1.36 ± 0.09
DL-serine	190	0.30	0.0120	2.22 ± 0.09
DL-tyrosine	130	0.28	0.0118	1.51 ± 0.07
DL-aspartic acid	70	0.28	0.0144	0.98 ± 0.06
DL-histidine	100	0.27	0.0151	1.56 ± 0.09
Control	---	0.24	---	0.00

[a]Fermentation was carried out at 30°C and 200 rpm.
[b]Growth measured hourly and μ_{max} calculated from it.
[c]Grams of dry weight per milligram of amino acid used.
[d]Grams of dry weight per liter of fermentation medium.
[e]Difference from control. Biomass yields taken at 56 h fermentation time.

pyrophosphate buffer (50 mM, pH7.2). The reaction mixture consisted of 20 µl of permeabilized cells, 880 µl of pyrophosphate buffer (50 mM, pH7.2), 100 µl of cephalosporin C and 100 µl (60 units) of catalase. The reaction mixture was incubated at 37°C for 30 min. Diphenylhydrazine (200 µl) was added and the reaction allowed to proceed for another 10 min. Finally, the reaction was terminated by addition of 1 ml of NaOH (2N) and absorbance was taken at 550 nm. One unit (U) of DAAO activity corresponds to the formation of 1 µM of α-keto acid per min at 37°C.

T. variabilis is one of the most widely studied microorganisms among those producing DAAO. To achieve higher yields of DAAO, it is necessary that the nutritional requirement of *T. variabilis* be known. In this respect, the effects of different amino acids on the biomass (X_0) and DAAO (P_0) yields of *T. variabilis* were studied. Initial experimentation (11) had shown that among different nitrogen sources namely urea, ammonium sulfate, potassium nitrate and different *DL*-amino acids, urea could support maximum cell biomass and DAAO yields. The optimum concentration of urea in the primary medium was found to be 3 g/l. From these studies it became apparent that *DL*-amino acids as the sole source of nitrogen cannot support the saturated growth of *T. variabilis*, so *DL*-amino acids were tested as supplements to urea in the primary medium.

Experimentation revealed that different amino acids had different effects on the biomass yield of *T. variabilis* under the same physical and chemical conditions. Highest specific growth rate (μ_{max}) was achieved with *DL*-alanine and *DL*-methionine followed by *DL*-phenylalanine and *DL*-glutamine. The increase in the cell biomass yield (w.r.t. control) was best observed with *DL*-methionine (2.39 gDW/l), *DL*-serine (2.22 gDW/l), *DL*-alanine (2.21 gDW/l) and *DL*-phenylalanine (2.12 gDW/l) and to a lesser extent by other amino acids (Table 1). Other amino acids like *DL*-proline, *DL*-threonine, glycine and *DL*-tryptophan could not support the growth of *T. variabilis* and hence cannot be recommended as a component of the chemically defined media. Further, among the different amino acids influencing the growth of *T. variabilis*, the highest specific biomass yield (*Ysx*) was observed with *DL*-asparagine (0.0165 gDW/mg) followed by *DL*-histidine (0.0151 gDW/mg) and *DL*-aspartic acid (0.0144 gDW/mg).

To study the effects of different amino acids on the biomass yield and specific enzyme yield, *DL*-isomers of all naturally occurring amino acids, except glutamic acid were considered. The latter was left out because it serves as a central point in amino acid metabolism, where all the metabolic reactions meet, so the use of glutamic acid was not further explored.

The influence of different *DL*-amino acids as the inducers of DAAO of *T. variabilis* is presented in Table 2. For the sake of simplicity, the results have been divided into four groups. The first group included amino acids with non-polar side groups. This group gave promising results. In the order of decreasing enzyme induction potential, the different amino acids were *DL*-methionine (351 U/gDW), *DL*-alanine (335 U/gDW), *DL*-isoleucine (280 U/gDW), *DL*-leucine (280 U/gDW) and *DL*-valine (273 U/gDW). The second group included amino acids with polar side groups while the third and fourth groups included amino acids with negatively and positively charged side groups. No

Table 2. Dependence of DAAO induction on various *DL*-amino acids[a]

Amino acids	Optimum concentration (g/l)	Specific DAAO yield[b,c] (μg/gDW)	DAAO yield[d] ± SE (U/gDW)
Amino acids with non-polar side groups			
DL-alanine	2.0	2867	335 ± 6
DL-isoleucine	1.5	2396	280 ± 4
DL-leucine	2.0	2396	280 ± 9
DL-methionine	2.0	3004	351 ± 7
DL-phenylalanine	1.0	2097	243 ± 4
DL-proline	1.0	2028	237 ± 5
DL-tryptophan	1.5	2328	272 ± 8
DL-valine	1.5	2336	273 ± 3
Amino acids with polar uncharged side groups			
DL-asparagine	0.5	1823	213 ± 7
DL-cysteine	0.5	1926	225 ± 6
DL-glutamine	0.5	1789	209 ± 2
Glycine	0.5	1785	210 ± 8
DL-serine	0.5	1908	223 ± 5
DL-threonine	0.5	1806	211 ± 6
DL-tyrosine	0.5	1789	209 ± 6
Amino acids with negatively charged side group			
DL-aspartic acid	0.5	1789	209 ± 7
Amino acids with positively charged side group			
DL-arginine	0.5	1789	209 ± 6
DL-histidine	1.5	1943	227 ± 5
DL-lysine	1.5	1943	226 ± 7
Control	0.0	1772	207 ± 4

[a]All the measurements were carried out at 40 h. Fermentation was carried out at 30°C and 200 rpm.
[b]Micrograms of DAAO protein obtained per gram of dry weight.
[c]Absolute amount of DAAO protein was calculated from the activity data assuming that all proteins in the final preparation of purification was pure DAAO. Purification experiments gave 8.56 μg protein per unit of enzyme.
[d]Units of DAAO produced per gram of dry weight.

member of the second, third or fourth group of amino acids was able to induce DAAO. On the basis of the above findings, a new fermentation medium was conceived from the primary medium by adding 2 g/l of *DL*-methionine and optimized concentration of each of the 13 different amino acids mentioned in Table 1. Cultivation of *T. variabilis* was done in this medium and different fermentation parameters were studied. Fermentation profile showed that the microorganism has a specific growth rate (μ_{max}=0.34 /h), DAAO yield (P_0=351 U/gDW), biomass yield (X_0=11.67 gDW/l) and total product yield (P_{total} =4096 U/l). Different workers have used different methodologies for the estimation of DAAO, so no true comparison can be made with different workers. However, in terms of fermentation yield, DAAO production by this medium (4096 U/l) was higher than that of Kubicek-Pranz and Röhr (7) (~2000 U/l), Mujawer and Shewale (8) (1000 U/l) and Pollegioni *et al.* (10) (800 U/l). Working on the same lines, one cannot get a true picture of specific enzyme activities obtained from different media because of the different cell disruption methods used by different workers. Even then, a specific DAAO activity of 0.6 U/mg by Pilone *et al* (9) and ~0.5 U/mg by Hörner *et al* (6) have been reported with *DL*-alanine as against 0.88 U/mg obtained using *DL*-methionine as inducer (12).

The results of this study indicate that the protein responsible for the induction of DAAO gene has a non-polar environment at its binding site that can easily accommodate the non-polar side group of *D*-amino acids. Since information concerning regulation of DAAO gene is not available, the binding site aspects of DAAO inducer can only be speculated on on the basis of this study. However to further understand the binding site environment of DAAO inducers, work is going on on the use of different derivatives of *D*-amino acids for the induction of DAAO.

References

1. Alfani, F., M. Cantarella, N. Cutarella, A. Gallifuoco, P. Golini, and D. Bianchi. 1997. Enzymatic conversion of cephalosporin C to glutaryl-7-aminocephalosporanic acid. A study in different reactor configurations. *Biotechnol. Lett.* 19, 175-178.
2. Brodelius, P., K. Nillson, and K. Mosbach. 1981. Production of α-keto acids: Part I. Immobilized cells of *Trigonopsis variabilis* containing *D*-amino acid oxidase. *Appl. Biochem. Biotechnol.* 6, 293-308.
3. Case, E.M. 1932. The determination of pyruvic acid. *Biochem. J.* 26, 753-758.
4. Fischer, L., R. Hörner, and F. Wagner. 1995. Production of L-amino acids by applying *D*-amino acid oxidase. *Ann. N.Y. Acad. Sci.* 750, 415-420.
5. Hinkkanen, A., and K. Decker. 1985. *D*-amino acids, Vol. 8., p. 329-340. *In*: H.U. Bergmeyer, J. Bergmeyer and M. Grassl (eds.), Methods of Enzymatic Analysis. VCH mbH Weinheim, Germany.
6. Hörner, R., F. Wagner, and L. Fischer. 1996. Induction of *D*-amino acid oxidase of *Trigonopsis variabilis*. *Appl. Environ. Microbiol.* 62, 2106-2110.
7. Kubicek-Pranz, E.M., and M. Röhr. 1985. D-amino acid oxidase from the yeast *Trigonopsis variabilis*. *J. Appl. Biochem.* 7, 104-113.
8. Mujawar, S.K., and J.G. Shewale. 1997. Production of *D*-amino acid oxidase by *Aspergillus* sp. Strain 020 active on cephalosporin C. *Can. J. Microbiol.* 43, 292-295.
9. Pilone, M.S., R. Verga, A. Fretto, and G.M. Hanozet. 1989. Induction of *D*-amino acid oxidase by *D*-alanine in *Rhodotorula gracilis* grown in a defined medium. *J.Gen. Microbiol.* 135, 593-600.
10. Pollegioni, L., G. Molla, S. Campaner, E. Martegani, and M.S. Pilone. 1997. Cloning, sequence and expression in *E. coli* of a *D*-amino acid oxidase cDNA from *Rhodotorula gracilis* active on cephalosporin C. *J. Biotechnol.* 58, 115-123.
11. Puneet, S., N. Verma, and S.S. Marwaha. 1999. Fermentative production of *D*-amino acid oxidase by *Trigonopsis variabilis* for the oxidative deamination of cephalosporin C. Abstr. Book. Pittsburgh Conf. Anal. Chem. Abstr. 1689P.
12. Puneet, S., N. Verma, and S.S. Marwaha. 2001. Sequential liquid-liquid extraction of *D*-amino acid oxidase from *Trigonopsis variabilis*. *Biotechnol. Lett.* (In Press).