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# Induction of the D-Amino Acid Oxidase from *Trigonopsis variabilis*

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**Induction of the D-amino acid oxidase (EC. 1.4.3.3) from the yeast *Trigonopsis variabilis* was investigated by using a minimal medium containing glucose as the carbon and energy source, $(NH_4)_2SO_4$ as the nitrogen source, and various D- and DL-amino acid derivatives as inducers. The best new inducers found were *N*-carbamoyl-D-alanine, *N*-acetyl-D-tryptophan, and *N*-chloroacetyl-D-α-aminobutyric acid; when the induction effects of these compounds were compared with the effects of D-alanine as the nitrogen source and inducer, the resulting activities of D-amino acid oxidase per gram of dried yeast were 4.2, 2.1, and 1.5 times higher, respectively. The optimum concentration of the best inducer, *N*-carbamoyl-D-alanine, was 5 mM. This inducer could also be used in its racemic form. The induction was pH dependent. After cultivation of the yeast in a 50-liter bioreactor, D-amino acid oxidase activity of about 3,850 μkat (231,000 U) was obtained. In addition, production of the D-amino acid oxidase was found to be significantly dependent on the metal salt composition of the medium. Addition of zinc ions was required to obtain high D-amino acid oxidase levels in the cells. The optimum concentration of $ZnSO_4$ was about 140 μM.**

The flavoprotein D-amino acid oxidase (D-AO) (EC. 1.4.3.3) catalyzes the oxidation of D-amino acid to the corresponding α-imino acid. The resulting α-keto acid and ammonia are produced after uncatalyzed hydrolysis. Reoxidation of reduced flavin adenine dinucleotide, which is bound tightly to the biocatalyst, occurs by reduction of molecular oxygen, and hydrogen peroxide is released. D-AOs are widely distributed in nature; for example, they occur in vertebrates, especially mammals (8, 17, 18), and in microorganisms, such as the fungi *Cephalosporium acremonium* (1), *Neurospora crassa* (32), *Fusarium solani* (15), and *Fusarium oxysporum* (9), the alga *Chlorella vulgaris* (28), the yeasts *Candida tropicalis* (37), *Trigonopsis variabilis* (30), and *Rhodotorula gracilis* (33), and the bacterium *Alcaligenes denitrificans* (9).

The D-AOs are highly enantioselective enzymes and have a very broad substrate spectrum. This makes them biocatalysts that are in demand in several areas of biochemistry and biotechnology; for instance, they are used in qualitative and quantitative analyses of D-amino acids (13), in biosensors (12, 21), in the production of L-amino acids (10, 22), in the production of α-keto acids (5), and, most importantly, in the conversion of cephalosporin C to 7-glutarylcephalosporanic acid (26, 35). The latter process takes place on an industrial scale, and the D-AO from *T. variabilis* is used as one of the two biocatalysts that take part in the enzymatic two-step conversion of cephalosporin C to 7-aminocephalosporanic acid (7), which is a key compound for the production of many semisynthetic β-lactam drugs (world market, $\$20.5 \times 10^9$).

The production of D-AO by *T. variabilis* has to be induced; this can be done by several D-amino acids, which have to be present in the culture medium (19). The best results for induction of the enzyme have been obtained with D-methionine or D-alanine in a minimal medium (19). By using recombinant DNA techniques, a *T. variabilis* strain containing multiple copies of the D-AO gene was generated, and this strain exhibited increased cell activity (11).

However, the aim of our study was to screen for new inducers which result in higher enzyme levels in wild-type *T. variabilis* and can be used at low concentrations. A good inducer should also be utilized as a racemic mixture to reduce production expenses. Since the first investigations of D-AO production in *T. variabilis*, which were performed by Sentheshanmuganathan and Nickerson (30), all other workers have used almost the same metal salt composition in their media. During our induction experiments, the influence of the metal salt composition on D-AO production was evident. For this reason, the metal salt composition of the medium was varied and investigated in more detail in this study.

## MATERIALS AND METHODS

**Microorganism.** *T. variabilis* DSM 70714 was used in all experiments.

**Chemicals.** The chemicals used were commercially available and reagent grade. The substances tested as inducers were purchased from Sigma, Fluka, ICN, or Degussa or were synthesized as described elsewhere (34).

**Media and culture conditions.** All suspension cultures were grown in 500-ml shaking flasks with baffles containing 100 ml of medium on a rotary shaker at 30°C and 100 rpm.

**(i) Preculture 1 (rich medium).** *T. variabilis* was cultivated in rich medium (20 g of malt extract per liter, 20 g of glucose · $H_2O$ per liter, 10 g of Bacto Peptone per liter, 5 g of yeast extract, per liter; pH 6.0) until the optical density at 546 nm ($OD_{546}$) of the suspension was 65 to 70 (after approximately 55 h).

**(ii) Preculture 2 (minimal medium).** *T. variabilis* was cultivated by using the following ingredients in standard medium: 20 g of glucose · $H_2O$ per liter, 4 g of $KH_2PO_4$ per liter, 20 μg of biotin per liter, 100 μg of thiamine HCl per liter, and 4 g of $(NH_4)_2SO_4$ per liter. The following three media with different metal salt compositions were tested: a generally recommended basic medium (medium A) (23), a previously used basic medium (medium B) (19), and, after optimization, the most suitable medium (medium C), which contained 4.1 mM $MgSO_4 \cdot 7H_2O$, 99 μM $CaSO_4 \cdot 2H_2O$, 139 μM $ZnSO_4 \cdot 7H_2O$, and 90 μM $FeSO_4 \cdot 7H_2O$. To prepare a medium, $(NH_4)_2SO_4$ and the mineral salts were sterilized by heating them for 15 min at 121°C. The glucose and phosphate were sterilized separately, and the vitamins were passed through filters (pore size, 0.2 μm; Sarstedt, Nümbrecht, Germany). After the medium had been inoculated with 5 ml of preculture 1, the culture was grown until the $OD_{546}$ was 35 to 40 (after approximately 20 to 25 h).

**(iii) Main culture (minimal medium and inducer).** *T. variabilis* was cultivated by using a medium described above (basic medium C) supplemented with various inducers (each at a concentration of 5 mM) which had been passed through

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filters (pore size, 0.2 μm). This medium was inoculated with 5 ml of preculture 2. In all cases, the cells were harvested at the beginning of the stationary growth phase (detected by determining the $OD_{546}$).

**Bioreactor cultivation.** *T. variabilis* was cultivated in a 50-liter bioreactor (Braun-Diessel Biotech, Melsungen, Germany) without pH correction. The stirrer used was a three-stage rushton turbine. The standard medium and basic medium C were used (see above). In order to get more biomass, the concentrations of the components were increased 1.65-fold. *N*-Carbamoyl-DL-alanine (5 mM; 0.66 g liter$^{-1}$) was used as the inducer. The bioreactor was inoculated with a 5-liter suspension of preculture 2. Cultivation was carried out at 30°C, the air stream was 0.6 v/vm (30 liters per min), and the stirrer speed was 400 rpm.

**Determination of growth and biomass.** The yeast dry weight (YDW) was determined gravimetrically after a 20-ml sample had been dried for 48 h at 110°C. In the flask experiments, the YDW of the culture broth was estimated from a calibration curve in which $OD_{546}$ was plotted versus YDW for concentrations ranging from 0 to 24 g liter$^{-1}$.

**Preparation of the cell extract.** The cells were harvested by centrifugation at 6,000 rpm (Varifuge F; Heraeus Sepatech, Hannover, Germany) for 20 min, resuspended in 0.9% NaCl, and then centrifuged at 3,000 rpm for 10 min at 4°C. Then the cells (wet biomass, 3 g) were suspended in 7 ml of 50 mM potassium phosphate buffer (pH 8.0) containing 0.1 M sucrose and 3 mM EDTA. To disrupt cells, 0.6 ml of this suspension and 1.2 g of glass beads (diameter, 0.75 mm) were shaken in reaction tubes (volume, 2 ml) with a bead mill (model MM2; Retsch, Haan, Germany) at 4°C for 15 min. The resulting crude extract was centrifuged, and the clear supernatant was used for further investigations.

**Enzyme assay.** The D-AO activity was estimated photometrically by performing a coupled enzyme test at 30°C in 50 mM potassium phosphate buffer (pH 8.0), in which D-alanine was used as the substrate (3); 1 nkat of activity was defined as the amount of enzyme that resulted in consumption of 1 nmol of D-alanine per s under the conditions used (16.67 nkat = 1 U). The activities of crude extracts of D-AO are expressed in microkatals per gram of YDW.

**Analytical procedures.** The protein concentrations of the crude extracts were determined by the Bradford method (4). *N*-Carbamoyl-alanine contents were determined by thin-layer chromatography. Samples were spotted onto silica gel plates, and *n*-butanol–acetic acid–water (65:13:22, vol/vol/vol) was used as the liquid phase. Quantification was performed after the plates were sprayed with *p*-dimethylaminobenzaldehyde (0.4 g in 75 ml of methanol and 25 ml of 26% hydrochloric acid) by scanning the spots at 420 nm with a DESAGA model CD 60 densitometer.

## RESULTS

**Screening for new inducers of D-AO.** Screening for new D-AO inducers was done by using glucose as the energy and carbon source, $(NH_4)_2SO_4$ as the nitrogen source, and basic medium C. Each of the substances tested for induction was added at a concentration of 5 mM (Table 1). The yeast cells that were used as the inoculum were previously cultivated in the same medium without any inducing substances (preculture 2) to ensure that only the compound tested was responsible for the differences in growth and D-AO activities. The substances used for induction of D-AO were D-configured amino acid derivatives. As pure chiral inducers are unacceptably expensive for large-scale production, the racemates of several chiral amino acid derivatives were also used in the comparison, and, in some cases, the inducers tested were only available as racemic mixtures.

All cultivations were completed at the beginning of the stationary phase, which was ascertained by measuring the $OD_{546}$ of the cell suspension periodically. The cells were harvested by centrifugation, washed, and subsequently disrupted with a bead mill. The cell-free, clear enzyme solution was tested for D-AO activity by using D-alanine as the substrate. The results are summarized in Table 1. Three reference preparations, one containing no inducer (reference 1), one containing D-alanine as the nitrogen source and inducer (reference 2), and one containing D-alanine as the inducer (reference 3), were included in this study.

The highest D-AO activities were obtained when *N*-carbamoyl-D-alanine, *N*-acetyl-D-tryptophan, or *N*-chloroacetyl-DL-α-aminobutyric acid was used as the inducing compound; Table 2 shows the structures and relative activities (compared with reference 2) of these compounds. *N*-Carbamoyl-D-alanine resulted in 4.2 times higher D-AO activity per gram of dry

TABLE 1. Screening for inducers of D-AO in *T. variabilis* by using the standard medium with basic medium C in 500-ml shaking flasks with baffles at 30°C[a]

| Inducer[b] | YDW (g liter$^{-1}$) | Length of cultivation (h) | D-AO activity (μkat g of YDW$^{-1}$) |
|---|---|---|---|
| Reference 1 (without inducer) | 6.3 | 20.5 | 0.5 |
| Reference 2[c] | 7.0 | 24.0 | 3.3 |
| D-Alanine (reference 3) | 5.5 | 27.0 | 1.0 |
| *N*-Carbamoyl-D-alanine | 5.1 | 33.0 | 14.0 |
| *N*-Carbamoyl-DL-alanine | 5.4 | 33.0 | 11.3 |
| *N*-Carbamoyl-DL-methionine | 6.1 | 23.0 | 3.0 |
| *N*-Carbamoyl-D-methionine | 5.9 | 22.5 | 2.1 |
| *N*-Acetyl-D-tryptophan | 5.5 | 27.0 | 6.8 |
| *N*-Acetyl-DL-tryptophan | 5.4 | 23.0 | 5.0 |
| *N*-Acetyl-DL-valine | 6.3 | 22.5 | 2.2 |
| *N*-Chloroacetyl-DL-α-aminobutyric acid | 3.0 | 25.0 | 4.8 |
| D-α-Amino-*n*-butyric acid | 6.0 | 23.0 | 1.0 |

[a] Substances that did not have a significant effect ($<$1.0 μkat g of YDW$^{-1}$) when they were used at a concentration of 5 mM were *N*-acetyl-D-alanine, *N*-acetyl-DL-methionine, *N*-acetyl-DL-methionineamide, *N*-formyl-DL-methionineamide, *N*-carbamoyl-DL-valine, *N*-carbamoyl-DL-phenylalanine, *N*-carbamoyl-DL-methionineamide, *N*-carbamoyl-DL-serine, D-methylthioethylhydantoin, DL-5-hydroxymethylhydantoin, DL-isopropylhydantoin, D-5-methylhydantoin, D-alaninole, α-methyl-DL-serine, cephalosporin C, and D-alanine-glycine.

[b] Each inducer was tested at a concentration of 5 mM.

[c] D-Alanine (30 mM) was used as the nitrogen source as well as the inducer.

biomass than reference 2, but the total amount of biomass produced was somewhat lower and the growth velocity was also a little lower. Lower growth velocities were also observed with *N*-acetyl-D-tryptophan (which resulted in D-AO activity that was 2.1 times higher) and with *N*-chloroacetyl-DL-α-aminobutyric acid (which resulted in D-AO activity that was 1.5 times higher). The D-AO activity decreased about 20% when the racemates of *N*-carbamoyl-alanine and *N*-acetyl-tryptophan were used instead of the pure enantiomers. In addition, significant inducing effects were observed with *N*-carbamoyl-DL-methionine, *N*-acetyl-DL-valine, and D-α-amino-*n*-butyric acid compared with reference 3 when the same concentration of D-alanine (5 mM) was used.

**Optimum concentration of *N*-carbamoyl-DL-alanine.** In view of the production of D-AO by *T. variabilis*, further investigations were carried out with *N*-carbamoyl-DL-alanine. This com-

TABLE 2. Structures of the three best inducers of D-AO activity

| Inducer | Structure | % Induced D-AO activity compared with reference 2[a] |
|---|---|---|
| *N*-Carbamoyl-D-alanine | | 420 |
| *N*-Acetyl-D-tryptophan | | 210 |
| *N*-Chloroacetyl-D-α-aminobutyric acid | | 150 |

[a] See Table 1.

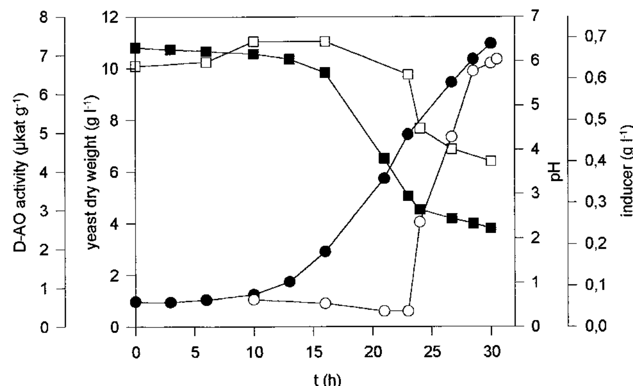


FIG. 1. Cultivation of *T. variabilis* in a 50-liter bioreactor by using *N*-carbamoyl-DL-alanine as the inducer. Symbols: ○, D-AO activity; ■, pH; ●, YDW; □, inducer concentration.

pound can be synthesized easily by reacting DL-alanine with cyanate (34). The optimum concentration of *N*-carbamoyl-DL-alanine (range tested, 0 to 50 mM) was 5 mM, which resulted in an enzyme activity of about 14 μkat g of $YDW^{-1}$. At a concentration of only 0.5 mM this inducer had a strong effect on D-AO production by the yeast cells (enzyme activity, about 5 μkat g $YDW^{-1}$), and concentrations greater than 5 mM resulted in minor decreases in D-AO activity (data not shown).

**Use of *N*-carbamoyl-DL-alanine as the sole nitrogen source.** To ensure that *N*-carbamoyl-DL-alanine was not used as the nitrogen source, the standard medium and basic medium C without $(NH_4)_2SO_4$ were used to grow *T. variabilis*, and various parameters, including the inducer (the pure D enantiomer or the racemate of *N*-carbamoyl-alanine), the concentration (5 or 20 mM), and the starting pH (pH 6.0 or 3.0; the latter should have made the uptake of the compound by the yeast cells possible [Fig. 1]), were varied. In all of these investigations, the microorganism was not able to utilize *N*-carbamoyl-DL-alanine as a nitrogen source and only slight growth took place (in 10 days the YDW increased from 0.27 g $liter^{-1}$ to 0.5 to 1.2 g $liter^{-1}$), but, surprisingly, resting cells exhibited high levels of D-AO activity after 10 days of cultivation (about 5 μkat g of $YDW^{-1}$; the initial cell activity was about 0.5 μkat g of $YDW^{-1}$). In addition, *N*-carbamoyl-alanine (5 or 20 mM; pH 3.0 or 6.0) was tested as a carbon source in the presence of $(NH_4)_2SO_4$; in this experiment no growth at all was observed.

**Influence of pH and phosphate concentration.** During the cultivation experiments performed with the new inducers, it was observed that the pH decreased from 6.0 to about 2.2. To get more information about the influence of pH on growth and induction, we tried to keep the pH constant (Table 3). To do this, either the pH in the culture flasks was permanently controlled and corrected by titration (Table 3, experiment 2) or higher phosphate concentrations were used with the hope that they would maintain the pH of the medium (Table 3, experiments 3 and 4).

Under the conditions used, the highest level of D-AO activity was observed with the standard medium (basic medium C) with no pH correction (experiment 1 [reference]). The more phosphate the medium contained, the higher the biomass yield obtained (experiments 3 and 4), but at the same time the D-AO activity decreased significantly. When the standard medium with pH correction was used (experiment 2), the activity was only 25% of the reference activity. Subsequently, a low pH had to be reached for maximal production of D-AO when *N*-carbamoyl-DL-alanine was the inducer. The most likely reason for this was pH-dependent uptake of the inducer into the cells (Fig. 1).

**Bioreactor cultivation.** *T. variabilis* was cultivated in a 50-liter bioreactor containing *N*-carbamoyl-DL-alanine (5 mM; 0.66 g $liter^{-1}$) as the inducer (Fig. 1). The yield of biomass was about 11 g of YDW $liter^{-1}$ after 30.5 h of cultivation, and the maximum growth rate was 0.11 $h^{-1}$ (between 16 and 26.75 h). The logarithmic growth phase and the decrease in pH of the medium started at the same time, after approximately 15 h. The inducer was taken up by the cells when the pH was less than approximately 3.5, and, production of D-AO occurred simultaneously. These results indicate that membrane transport of the inducer is most likely pH dependent. Later, when the inducer is present inside the yeast cells, strong production of D-AO is induced. The resulting D-AO activity of the yeast cells was about 7 μkat g of $YDW^{-1}$. The total yield of enzyme activity obtained by fermentation was 77 μkat $liter^{-1}$.

**Variation of the metal salt composition.** The generally recommended basic medium for microorganisms (medium A) (23) and the previously used basic medium (medium B) (2, 19, 36) were tested by performing induction experiments. We found that significant induction of D-AO occurred only when the latter medium was used (data not shown). These two media differ in metal salt composition. Medium A contains only $Mg^{2+}$, $Ca^{2+}$, and $Fe^{2+}$. In order to find the metal salt(s) which was needed for D-AO production, we added the metal salts that were present in medium B but missing in medium A one by one (Table 4). The results demonstrated that $ZnSO_4$ was the metal salt that was necessary for high biomass yields (5.4 g of YDW $liter^{-1}$), as well as D-AO activity (approximately 10 μkat g of $YDW^{-1}$). The effect of $ZnSO_4$ on D-AO induction was about 20-fold compared with cultivation without zinc (Table 4).

**Influence of the zinc ion concentration.** To estimate the optimal concentration of zinc ions for D-AO production, various concentrations of $ZnSO_4$ (0 to 7 mM) were tested by using the standard medium (basic medium C) and *N*-carbamoyl-DL-alanine (5 mM) as the inducer. It turned out that the optimal concentration for D-AO production was approximately 140 μM $ZnSO_4$ (which resulted in an enzyme activity of 11.8 μkat g of $YDW^{-1}$). $ZnSO_4$ concentrations that were less than 90 μM and more than 500 μM resulted in significant decreases in D-AO activity (for concentrations of approximately 90 to 500 μM, the differences in D-AO activity were about 15%).

A strong dependence of oxidase production on zinc ions was also observed when a rich medium was used. The complex components contained some zinc ions; thus, without zinc supplementation an activity of approximately 3.5 μkat g of $YDW^{-1}$ was observed when *N*-carbamoyl-DL-alanine was the inducer. However, addition of 139 μM $ZnSO_4$ resulted in an 1.8-fold increase in activity, to 6.2 μkat g of $YDW^{-1}$. Without any inducer, only a little D-AO activity was observed (0.8 μkat g of $YDW^{-1}$), as expected.

## DISCUSSION

Previously, induction of D-AO activity in microorganisms was achieved by adding D- or DL-amino acids, which were simultaneously used as nitrogen sources (19, 27, 33). Our goal was to cultivate *T. variabilis* in a minimal medium containing $(NH_4)_2SO_4$ as the nitrogen source and to introduce a more or less unmetabolizable effector molecule to induce D-AO. In our investigations, the most effective inducer was *N*-carbamoyl-D-alanine (Table 1). When this amino acid derivative was used, D-AO production was about 15 times higher than the production obtained with D-alanine under the same conditions and

TABLE 3. Influence of pH and $KH_2PO_4$ concentration on the production of D-AO when the standard medium with basic medium C was used[a]

| Expt | Medium and conditions | YDW (g liter$^{-1}$) | Final pH | Length of cultivation (h) | D-AO activity (μkat g of YDW$^{-1}$) |
|---|---|---|---|---|---|
| 1 | Standard medium (reference) without pH control | 4.6 | 2.2 | 37.0 | 14.2 |
| 2 | Standard medium with pH control and correction | 5.1 | 6.0 | 30.0 | 3.6 |
| 3 | Standard medium containing 12 g of $KH_2PO_4$ per liter without pH control | 5.2 | 3.8 | 28.5 | 4.6 |
| 4 | Standard medium containing 24 g of $KH_2PO_4$ per liter without pH control | 6.6 | 5.3 | 27.0 | 1.6 |

[a] Preparations were cultivated by using an initial pH of 6.0 and 5 mM *N*-carbamoyl-DL-alanine as the inducer.

about 4 times higher than the production obtained when D-alanine was the nitrogen source. The other D-alanine derivatives tested, including the *N*-acetyl, hydantoin, and alcohol forms, caused no significant induction of the enzyme (Table 1). This could be due to one of two reasons; either these compounds are easily metabolized, or they do not fit into the binding site of the protein which is responsible for the expression of the D-AO gene. Interestingly, *N*-acetylated D-valine had a significant induction effect, but *N*-carbamoyl-valine did not. Thus, the carbamoyl residue did not ensure successful induction. When both functionalities, the amino group and the carboxyl group, were blocked (in the case of various hydantoins, *N*-carbamoyl-DL-methionineamide, and *N*-acetyl-DL-methionineamide), no induction of D-AO was observed. Since information concerning regulation of the D-AO gene is not available, the structure of a tailor-made inducer could not be predicted accurately on the basis of our results. However, the general features of the new inducers found were as follows: the carboxyl group was unprotected, the amino protection group was small ($-COCH_3$; $-CONH_2$), and the residue was hydrophobic [$-CH_3$; $-CH_2-CH_3$; $-CH-(CH_3)_2$; $-CH_2-CH_2-S-CH_3$; $-CH_2$-indole]. Furthermore, it was found that *T. variabilis* was almost unable to grow when the new inducer *N*-carbamoyl-D-alanine was the nitrogen source, that the concentration of inducer used was not toxic, and that this inducer was not a substrate for D-AO (data not shown).

The uptake of *N*-carbamoyl-D-alanine is assumed to be pH dependent (Fig. 1). This could be explained by protonation of the free carboxyl group, which might be necessary to pass through the biomembrane.

The absolute activity data which we obtained (14 μkat g of YDW$^{-1}$; 840 U g of YDW$^{-1}$) are difficult to compare with the results published by other groups of workers. The reason for this is that different assay methods, conditions, and/or substrates were used. The importance of the assay method used for comparison was demonstrated by workers in our laboratory (9). Various assay methods were used to determine the D-AO activity of *T. variabilis* under the same conditions with D-alanine as the substrate (the $H_2O_2$-peroxidase–*o*-dianisidine method; the α-keto acid–hydrazine method; the pyruvate-lactate dehydrogenase method, and the oxygen electrode method). The results showed that the data obtained with the different methods varied up to 300% (9). When *F. oxysporum* (induced with D-alanine as the nitrogen source), a recently identified D-AO producer, was compared with *T. variabilis* (induced with 5 mM *N*-carbamoyl-D-alanine) by using the same assay and same conditions, the *F. oxysporum* activity was 72% of the *T. variabilis* absolute D-AO activity (9).

However, other workers obtained their best results (about 5 μkat g of YDW$^{-1}$) by using D-methionine or D-alanine as the inducer and N source, D-methionine as the substrate, and a temperature of 37°C (19). With 30 mM D-alanine as the inducer and nitrogen source we obtained an enzyme activity of about 3.5 μkat g of YDW$^{-1}$, and with 5 mM *N*-carbamoyl-DL-alanine as the inducer and nitrogen source we obtained an enzyme activity of about 14 μkat g of YDW$^{-1}$; in both cases D-alanine was used as the substrate and the temperature used was 30°C (Table 1). This system can be compared with the D-AO activities of *R. gracilis* by considering the specific activities of the crude enzyme extracts (25, 27). The *Rhodotorula* extract had a D-AO activity of about 10 to 15 nkat mg of protein$^{-1}$ (with D-alanine as the substrate at 37°C), and in our investigations, crude extracts of *T. variabilis* had D-AO activities of about 70 to 80 nkat mg of protein$^{-1}$ (with D-alanine as the substrate at 30°C).

The dependence of D-AO induction in *T. variabilis* on the zinc ion concentration is reported for the first time in this paper. The D-AO of *T. variabilis* is not a metalloenzyme (20, 29, 36). Previously, Sentheshanmuganathan and Nickerson used a minimal medium containing $ZnSO_4$ when they investigated D-AO production by this yeast (30), as did many other authors (2, 20, 31, 36). However, zinc ions are usually available in minimal media from impurities which are present in the reagent grade chemicals and from the abundant release of zinc from borosilicate glass. The resulting zinc ion concentration is high enough to achieve high biomass yields with most microorganisms (14). In our experiments, growth of *T. variabilis* without additional zinc ions was possible, but the amount of growth was much less than the amount of growth in the media which were supplemented with $ZnSO_4$. As the reason for the zinc requirement remains unknown, only general considerations can be discussed. For instance, zinc is required for nucleic acid metabolism and cell division and can be a component of transcription factors (14). In the case of the yeast *R.*

TABLE 4. Influence of various metal ions on induction of D-AO in *T. variabilis* determined by using the standard medium with basic medium A (4.1 mM $MgSO_4 \cdot 7H_2O$, 99 μM $CaSO_4 \cdot 2H_2O$, 90 μM $FeSO_4 \cdot 7H_2O$)[a]

| Metal salt added | YDW (g liter$^{-1}$) | Length of cultivation (h) | D-AO activity (μkat g of YDW$^{-1}$) |
|---|---|---|---|
| | 3.7 | 42.0 | 0.6 |
| $CaCl_2 \cdot 2H_2O$ (3.4 mM) | 3.5 | 56.5 | 0.5 |
| $Na_2MoO_4 \cdot 2H_2O$ (165 μM) | 3.0 | 42.0 | 0.5 |
| $H_3BO_3$ (1.6 mM) | 3.0 | 42.0 | 0.7 |
| $ZnSO_4 \cdot 7H_2O$ (139 μM) | 5.4 | 46.0 | 10.0 |
| $CuSO_4 \cdot 5H_2O$ (180 μM) | 2.0 | 42.0 | 0.3 |

[a] *N*-Carbamoyl-DL-alanine (5 mM) was used as the inducer, and the metal salts of basic medium B were added one by one.

*gracilis*, which also produces D-AO (see above), zinc deficiency led to reduced amounts of ribosomes and mitochondria and to minor protein production (6). In *Ustilago sphaerogena* zinc seems to be required for the production of the δ-aminolevulinate dehydratase (16). Recently, Percheron et al. established that $ZnCl_2$ plays a fundamental role in significantly increasing exoprotease production by *Burkholderia pseudomallei* (24). All of these facts and our own results indicate that zinc-dependent processes have a role in the regulation of certain proteins. Whether this regulation takes place at the level of transcription or the level of translation should be investigated in the near future.

Our results demonstrate that even when a protein is produced in relatively large amounts, further improvement of induction may still be possible and, in troublesome cases, may be an alternative to gene technology.

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