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Coimmobilization of D-amino acid oxidase and catalase by entrapment of *Trigonopsis variabilis* in radiation polymerised Polyacrylamide beads

ASHWINI DESHPANDE, S. F. D'SOUZA and G. B. NADKARNI
Food Technology and Enzyme Engineering Division, Bhabha Atomic Research Centre, Bombay 400 085, India

Abstract. *Trigonopsis variabilis* induced for D-amino acid oxidase and catalase was immobilized by entrapment in Polyacrylamide beads obtained by radiation polymerisation. Permeabilization of the cells was found to be essential for optimal activity of the enzymes in free cells. However, the process of entrapment itself was found to eliminate the permeability barrier of cells immobilized in Polyacrylamide. The two enzymes exhibited a differential response on Polyacrylamide entrapment. Thus, D-amino acid oxidase activity was stabilized to heat inactivation whereas catalase in the same cells showed a destabilization on entrapment in Polyacrylamide. The coimmobilized enzyme preparation showed an operational half life of 7-9 days after which the D-amino acid oxidase activity remained stable at a value 35–40% of that of the initial activity for a study period of 3 weeks. Coimmobilization of MnO_2 was not effective in enhancing the operational life of the enzyme preparation.

Keywords. Coimmobilization; D-amino acid oxidase; *Trigonopsis variabilis;* Polyacrylamide beads.

Introduction

Recently keto acid therapy has been found to be useful in the management of chronic uremia (Walser, 1978; Aobi *et al.*, 1984). The practical use of this therapy, however, has been limited due to the lack of suitable methods for the production of the keto analogues of all amino acids. Enzymatic methods now investigated for the preparation of keto acids include, the use of either L or D-amino acid oxidases (Wikström *et al.*, 1982; Szwajcer *et al.*, 1982). The use of D-amino acid oxidase [D-amino acid:oxygen oxidoreductase (deaminating); EC 1·4·3·3] has the advantage of simultaneous separation of the natural L-isomer from chemical DL-racemates along with the keto acids.

Of the several micro-organisms screened, the yeast *Trigonopsis variabilis* has been found to be one of the most potent sources of D-amino acid oxidase which could act on most of the amino acids (Brodelius *et al.*, 1981; Kubicek-Pranz and Röhr, 1985). Earlier studies have shown the feasibility of immobilizing this enzyme in Ca-alginate using intact cells (Brodelius *et al.*, 1981). However, H_2O_2, the by-product of D-amino acid oxidase has been found to inhibit the enzyme as well as decarboxylate the keto acid thus resulting in low operational stability. Several methods directed at the improvement of the operational stability include coimmobilization of H_2O_2 degrading agents like MnO_2 and activated charcoal (Brodelius *et al.*, 1981; Szwajcer *et al.*, 1982). In the present studies, the yeast *T. variabilis* grown under conditions optimum for the induction of D-amino acid oxidase were also found to contain large excess of catalase which could not only be useful in detoxifying H_2O_2 but can simultaneously release utilizable oxygen for the D-amino acid oxidase reaction.

Materials and methods

Materials

Acrylamide, N,N'-methylene-bis-acrylamide (Bis) and DL-methionine were obtained from Sisco Research Laboratories, Bombay. The various other DL-amino acids were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Other chemicals were obtained from standard sources.

Culture conditions and indution of enzymes

The strain *T. variabilis* NCIM 3344 was obtained from the National Chemical Laboratory, Pune. It was maintained by a biweekly transfer on MGYP agar slants containing malt extract—0·2%, glucose—1%, yeast extract—0·3%, peptone—0·5% (pH 6·4 to 6·8) in 2% agar. For the induction of D-amino acid oxidase, the yeast was grown aerobically for 3 days in a liquid medium containing 0·3% DL-methionine (Berg and Rodden, 1976). The cells were harvested by spinning in cold (0–4°C) at 8,000 *g* for 5 min and washed twice with cold isotonic saline. The cells grown under these conditions were also found to contain high levels of catalase.

Permeabilization of yeast cells

To 10 ml of 10% cell suspension, 5 mL chloroform and 2 mg Na-deoxycholate were added and the mixture was incubated at 37°C for 10 min with intermittent stirring. The cells were then washed free of the permeabilizing agents with cold saline and resuspended in saline to a 50% cell concentration. Unless otherwise stated, these cells are referred to as the treated cells. The amount of cells used in these studies has been indicated as wet weight of packed cells.

Enzyme assays

D-Amino acid oxidase was assayed by stirring 100 mg of yeast cells (immobilized or otherwise) in 5 ml of 50 mM DL-methionine contained in 50 mM pyrophosphate buffer (pH 8·5) for 30 min at room temperature. The reaction was terminated by heating in a boiling water bath for 10 min, the cells sedimented by centrifugation and the α-keto acids in the supernatant estimated using dinitrophenylhydrazine reagent (Brodelius *et al.,* 1981). One unit of D-amino acid oxidase activity has been defined as μ mol of α-keto acid formed per hour.

Catalase activity was assayed by the method described earlier (D'Souza and Nadkarni, 1980). One unit of catalase activity has been defied as μ mol of H_2O_2 decomposed per min.

Immobilization of yeast cells in Polyacrylamide beads

An aqueous solution of acrylamide (20%), Bis (0·8%) and yeast cells (10% w/v) was mixed well with or without MnO_2 (l00mg/ml) and dropped through a syringe in

toluene at – 75°C maintained by means of dry ice-acetone bath. The frozen beads obtained were exposed to 400 kR of ^{60}Co γ-rays in a Gamma cell–220 (AECL) at a dose rate of 2·5 kR/min with air as the gas phase (Kawashima and Umeda, 1976). After irradiation, the toluene was discarded and the beads were allowed to thaw slowly in ice-cold water. The beads obtained were washed with cold distilled water, resuspended in 50 mM pyrophosphate buffer (pH 8·5) and stored at 0–4°C.

Results

Effect of pretreatment on enzyme activities of yeast cells

D-Amino acid oxidase in *T. variabilis* is an intracellular enzyme and exhibits a permeability barrier for the substrate. Different methods were investigated for the permeabilization of the yeast cells. It can be seen from table 1 that D-amino acid oxidase activity of yeast cells increases upto 10-fold on treatment with permeabilizing agents. Unlike D-amino acid oxidase, considerable amount of catalase activity in the same cells could be detected in whole cells eventhough for expression of total catalase activity, permeabilization of the cells was found to be essential. Catalase activity of yeast cells also increased from 2,800–15,400 units/g on treatment with chloroform-Na-deoxycholate which were routinely used for permeabilizing the cells.

Table 1. Permeability of yeast cells.

Permeabilizing agent	Activity (u/g)
None	19
Toluene (5% v/v)	107
Toluene (50% v/v)	152
Toluene + Na-deoxycholate (25 mg%)	194
Chloroform (50% v/v)	188
Chloroform + Na-deoxycholate	200
Sonication	85
Freeze-thawing	240

The cells were permeabilized with organic solvents as described in the text. Cell suspension (50%) was sonicated in an MSE ultrasonic disintegrator model 60 W at 17–20 KC for 15 min at 4°C. Freezing and thawing was carried out by rapidly freezing the yeast cells suspensions (50%) in saline in acetone solid CO_2 mixture and thawing in tap water. This process was repeated 12 times.

The yield of D-amino acid oxidase and catalase activities in various cell preparations after entrapment are presented in table 2. Intact cells on entrapment showed a greater yield of D-amino acid oxidase activity as compared to the permeabilized entrapped cells. Although permeabilized cells showed a 5–6-fold increase in catalase activity, the yield of catalase activity on entrapment of these cells was comparable to that of the untreated entrapped cells. These results suggest that permeabilization is not essential for expression of optimal activities of both the enzymes on immobilization, as the process of immobilization alters the permeability barrier. Thus in further studies, non-permeabilized (intact) cells have been used for immobilization.

Table 2. Yield of enzyme activity on entrapment.

	D-Amino acid oxidase activity of yeast cells (μmol/h/g of cells)	Catalase activity of yeast cells (μmol/min/g of cells
None (intact cells)	5	2,800
Permeabilized	200	15,400
Intact cells + entrapment	240	2,150
Permeabilized cells + entrapment	180	2,380

The cells were permeabilized using chloroform and Na-deoxycholate as stated in the text. The units of activity are expressed in the text.

Optimal conditions for Polyacrylamide entrapment

The immobilized preparation with optimum D-amino acid oxidase activity was obtained when a mixture containing 20% acrylamide and 0·8% Bis was exposed to a radiation dose of 400 kR. The scanning electron micrograph of the lyophilized cross-sectioned bead showed a porous, spongy infrastructure. The D-amino acid oxidase activity varied inversely with the bead diameter (figure 1). Beads with an average diameter of 2 mm were used in the studies. The cells immobilized in Polyacrylamide beads obtained under optimal conditions also retained considerable amount of catalase activity. Catalase activity of these preparations was about 500 times more than that of D-amino acid oxidase.

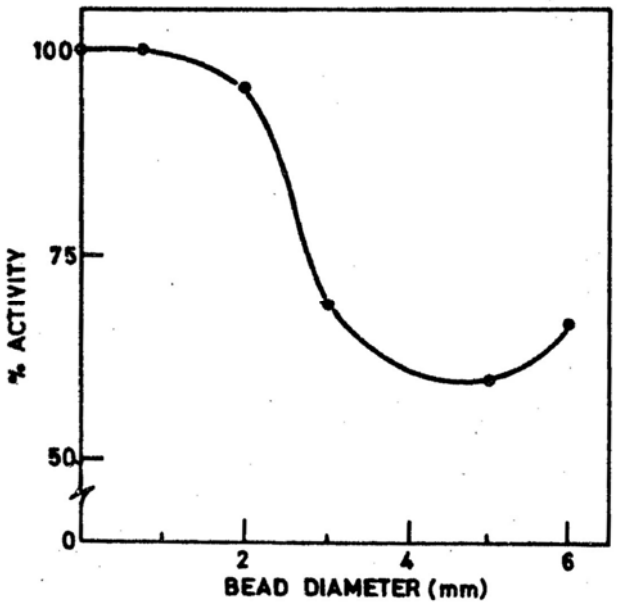

Figure 1. Effect of bead diameter on D-amino acid oxidase activity of immobilized *T. variabilis*.

Properties of immobilized enzymes

The kinetic and stability characteristics of the enzymes in immobilized cells were compared to those of the free cells. D-amino acid oxidase in both the preparations was optimally active between pH 8·0–8·5. At this pH, the coimmobilized catalase also showed retention of 90% of its optimal activity (figure 2). K_m for the activity with D-methionine as substrate was found to be altered from $3{\cdot}6 \times 10^{-3}$ M to $5{\cdot}2 \times 10^{-3}$ M on entrapment.

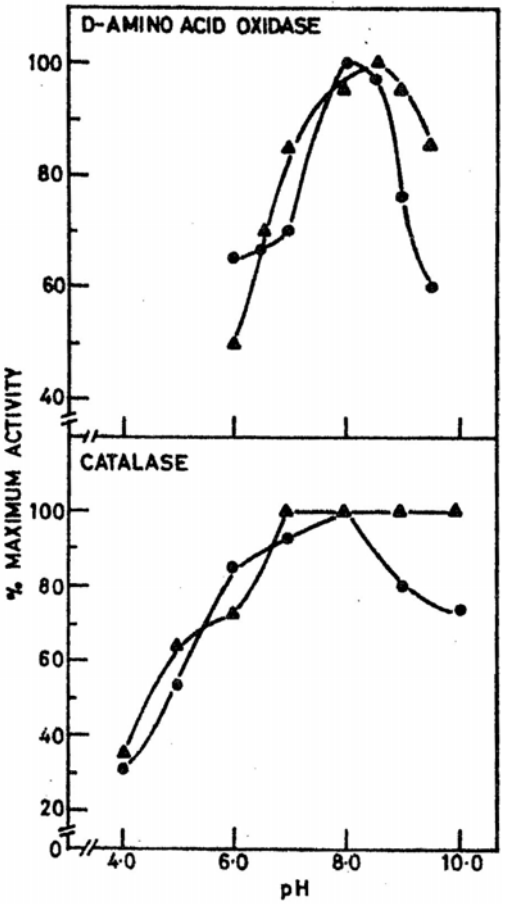

Figure 2. pH activity profiles of D-amino acid oxidase and catalase. The buffers (50 mM) used were: acetate (pH 40–5·0) phosphate (pH 6·0–8·0)and pyrophosphate (pH 8·0–10·0). (●), Free cells; (▲), immobilized cells.

The coimmobilized enzyme preparation showed a differential response to temperature on polyacrylamide entrapment. Thus, temperature optimum of D-amino acid oxidase increased from 37°–55°C on immobilization; however, catalase in the same cells showed a decrease in the temperature optimum on entrapment (figure 3). Immobilized D-amino acid oxidase was found to be more thermostable as compared to the native cells. On the other hand, a reduction in the thermostability was observed in the catalase activity (figure 4).

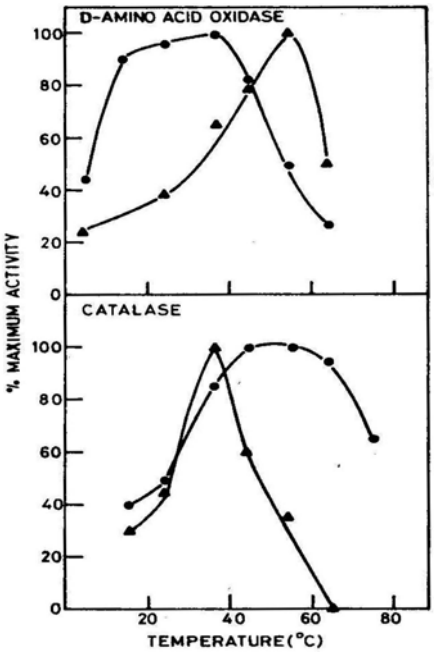

Figure 3. Temperature activity profiles of D-amino acid oxidase and catalase. (●), Free cells; (▲), immobilized cells.

Operational stability

Operational stability was studied by continuously passing 20 mM DL-methionine solution through a column containing the immobilized cell beads (5 g cells) at room temperature. For comparison, the same studies were simultaneously carried out using immobilized yeast cells containing coimmobilized MnO_2 (figure 5). Both the columns showed an operational half-life of about 7–9 days. Inclusion of an additionnal H_2O_2 degrading agent like MnO_2 failed to prolong the operational half-life even though the initial rates of conversion in the MnO_2 columns were about 1·5 times higher than those without MnO_2. The eluants from columns containing MnO_2 beads were coloured. Both the columns retained a constant conversion rate of about 35–40% of the original activity for a study period of 3 weeks.

Discussion

T. variabilis cells grown under aerobic conditions for the induction of D-amino acid oxidase were also found to induce high amounts of catalase. D-amino acid oxidase of *T. variabilis* is intracellular. Catalase from the same cells showed a part of its activity with the whole cells (patent catalase), whereas a major part was found to be intracellular (cryptic catalase). Such a distribution of catalase is known to occur in *Saccha-*

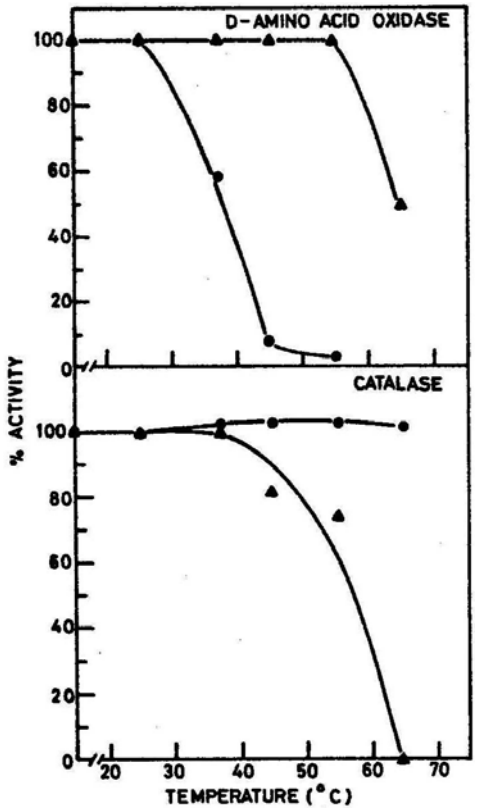

Figure 4. Thermostability of D-amino acid oxidase and catalase. The cell suspension was subjected to the desired temperature for 20 min and 10 min for D-amino acid oxidase and catalase respectively followed by rapid cooling by immersion in ice. Residual enzyme activity was determined. (●), Free cells; (▲), immobilized cells.

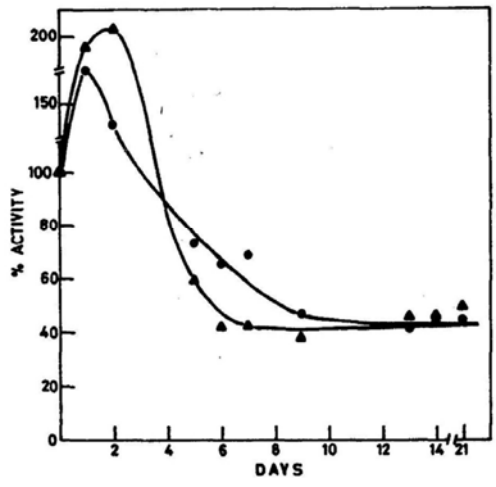

Figure 5. Operational stability of immobilized *T. variabilis*. (●), Without MnO_2; (▲) with MnO_2.

romyces cervisiae (Kaplan, 1963). Unlike the studies reported earlier (Brodelius *et al.,* 1981), present observations indicate that permeabilization of cells was very essential for expression of optimal D-amino acid oxidase and catalase activities in native cells eventhough permeabilization was not found to be essential for entrapment in polyacrylamide. Entrapment in Polyacrylamide is known to bring about changes in the permeability barrier (D'Souza and Nadkarni, 1980; Godbole *et al.,* 1980). However, unlike Polyacrylamide, entrapment in various other supports like Ca-alginate, agar, agarose, hen egg white etc. required permeabilized cells for expression of D-amino acid oxidase (unpublished work).

Eventhough the pH compatibility of both enzymes was satisfactory, one of the notable characteristics of the coimmobilized system was the differential response of the two enzymes in the same cells to temperature. The D-amino acid oxidase of *T. variabilis* was stabilized to heat inactivation whereas catalase in the same cells showed a destabilization on entrapment in Polyacrylamide. Similar destabilization of catalase in yeast *S. cerevisiae* entrapped in Polyacrylamide has also been reported (D'Souza and Nadkarni, 1980).

One of the limitations reported so far for the use of immobilized D-amino acid oxidase for large scale operation has been the low operational stability which ranges from a few hours to about 5 days. The reasons for the comparatively short half lives of D-amino acid oxidase observed in Ca-alginate has been mainly attributed to the inactivating effect of H_2O_2. In addition, however the low intrinsic stability of D-amino acid oxidase itself may be responsible for the elicitation of a short half life since efficiency combination H_2O_2 degrading agents like MnO_2 and catalase failed to improve the operational stability in the present studies. At present, we are identifying the factors responsible for the instability of the system in order to enhance the operational stability.

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