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# Characterization and Application of D-Amino Acid Oxidase and Catalase Within Permeabilized *Pichia pastoris* Cells in Bioconversions

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## Abstract

The high-density fermentation of recombinant *Pichia pastoris* was carried out in a 1-L fermentor. After 60 h of fermentation, the activities of D-amino acid oxidase (DAAO) and catalase assayed with the permeabilized cells attained 12,532 and 684,800 U/L, respectively. Additionally, the stability of DAAO and catalase within the permeabilized cells was relatively high. The half-life of the two enzymes reached 14.5 and 4.0 d at 30°C, respectively. Furthermore, these permeabilized cells could convert D-phenylalanine into 99% phenylpyruvate within 100 min and could be efficiently reused up to 13 cycles. After being treated with base and heating, these treated permeabilized cells could be reused up to three cycles in a batchwise conversion of cephalosporin C, and about 90% 7-β-(4-carboxybutanamido)-cephalosporanic acid was ultimately obtained at each cycle.

**Index Entries:** D-Amino acid oxidase; catalase; stability; permeabilized cells; bioconversion.

## Introduction

D-Amino acid oxidase (DAAO) (EC 1.4.3.3) is a flavoprotein that catalyzes the oxidative deamination of D-amino acids and cephalosporin C (CPC) to the corresponding α-keto acids, ammonia and $H_2O_2$. Both in the presence and in the absence of catalase, this enzymatic reaction has several

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biotechnological applications, including the production of α-keto acids *(1,2)* and 7-aminocephalosporanic acid *(3)*, the resolution of a racemic mixture of amino acids *(4)*, as well as the analytic determination of D-amino acid *(5)*. DAAO can be obtained from mammals and microorganisms. The yeasts, such as *Rhodotorula gracilis* and *Trigonopsis variabilis*, are the rich sources of this enzyme. They are also robust producers of catalase. Nevertheless, whole cells of these yeasts generally exhibited comparatively low activities of the two intracellular enzymes, owing to the permeability barriers of the cell membrane to substrates and products. Fortunately, this problem can be alleviated by treating these cells with detergents or organic solvents to remove the permeability barriers.

Many researchers have reported the application of DAAO and catalase in the immobilized form for bioconversions. However, the two enzymes in permeabilized cells directly employed for enzymatic reactions may be economically viable, because many complicated processes in the production of free enzyme and consumption of the costly supports involved in the preparation of the immobilized enzymes can be avoided. In the case of permeabilized cells, the main drawback is the instability of DAAO and catalase in these cells. To solve this problem, some investigators have carried out extensive attempts to seek efficient methods to stabilize the two enzymes within permeabilized cells for bioconversions *(6–10)*. Therefore, from an applied point of view, it is highly desirable that the expression host be able to stabilize the two cellular enzymes within the permeabilized cells. In the present work, both DAAO and catalase were highly expressed in recombinant *Pichia pastoris* in a 1-L fermentor. The stability of the two enzymes in the permeabilized cells was investigated. In addition, application of the permeabilized cells in the production of α-keto acids and 7-β-(4-carboxybutanamido)-cephalosporanic acid (GL-7-ACA) was evaluated.

## Materials and Methods

### Chemicals and Media

D-Alanine, D-phenylalanine, phenylpyruvate, and cetyltrimethylammonium bromide (CTAB) were purchased from Fluka. CPC was kindly provided by Shijiazhang Pharmaceutical Group (Shijiazhang, China). GL-7-ACA was synthesized in our laboratory according to a published method *(11)*. Its structure was analyzed by mass spectrum. All other chemicals were of analytical grade. The recombinant strains were kindly provided by Professor Zhongyi Yuan. The culture media (buffered glycerol-complex medium and basal salts medium) have been described in detail previously *(12)*.

### *Fermentation of* P. pastoris *in 1-L Fermentor*

The recombinant *P. pastoris* was cultivated in 80 mL of buffered glycerol-complex medium in a 250-mL flask and incubated with shaking at 30°C. The culture was inoculated to basal salts medium in a 1-L fermentor

after 18 h. Fermentation was performed under the conditions of 30°C, pH 5.5, 550 rpm, and 0.3 L/min aeration. After 23 h, the glycerol was fed to the medium at a rate of 4 mL/h for 6 h. During the fed-batch phase, the culture conditions were maintained as just described. Afterward, methanol was added continuously at a constant rate of 1 mL/h to the medium. The induction conditions were controlled at 30°C, pH 5.5, 650 rpm, and 0.1 L/min aeration. During the fermentation process, the culture was withdrawn at different time intervals to determine the dry cell weight (DCW) and the activities of DAAO and catalase within the permeabilized cells.

### Cell Permeabilization

*P. pastoris* cells (0.31 g DCW) were suspended uniformly in 10 mL of 0.4% CTAB in 50 m*M* pyrophosphate buffer, pH 8.5. The cells were incubated in this solution for 30 min at 25°C with intermittent shaking and then separated by centrifuging at 4000*g* for 10 min. The yeast pellet was washed twice with pyrophosphate buffer (50 m*M*, pH 8.5) and suspended in 10 mL of the same buffer. The suspension was used for enzyme assay and preparation of α-keto acids directly or after dilution.

### Treatment of Permeabilized Cells

Further treatment of permeabilized cells was performed as follows: The pH of the suspension prepared in the previous section was adjusted to 11.5 with 1 *M* NaOH. The suspension was kept at 25°C for 30 min. The pH was then adjusted to 7.5 using 20% $H_3PO_4$ (w/v). Next, the suspension was heated to 65°C for 2 min and immediately cooled to 4°C. These cells were collected by centrifugation and suspended in 10 mL of pyrophosphate buffer (50 m*M*, pH 7.5). The suspension was used for conversion of CPC directly or after dilution.

### Enzyme Assay

DAAO activity was measured by a previously described method *(1)*. One unit of DAAO activity corresponded to the formation of 1 μmol/min of pyruvate at 37°C. Catalase activity was assayed spectroscopically at 240 nm *(13)*. One unit of catalase activity was defined as the amount of enzyme required to degrade 1 μmol of $H_2O_2$/min under the determined conditions. Esterase activity was determined by the method reported by Becka et al. *(10)*.

### Preparation of α-Keto Acids

The untreated permeabilized cells (0.11 g DCW) were added to 25 mL of 25 m*M* D-phenylalanine in 50 m*M* sodium pyrophosphate buffer, pH 8.5. The reaction mixture was incubated in a 250-mL flask at 37°C in a metabolic shaker at 250 rpm. Aliquots of the reaction mixture were removed at different time intervals and the formation of keto acid was measured by 2,4-dinitrophenylhydrazine reaction and high-performance liquid chromatography (HPLC) assay. At the end of the reaction, the cells were separated by centrifugation and reused for the next reaction cycles.

### *Preparation of GL-7-ACA*

The treated permeabilized cells (0.1 g DCW) were added to 50 mL of 1% CPC in 50 m*M* potassium phosphate buffer, pH 7.5, containing 0.05 mL of Dow antifoam. This conversion was carried out at 28°C in a stirred reactor equipped with an $O_2$ distributor and a Teflon two-blade paddle stirrer. Pure oxygen gas was sparged into the reaction mixture from the bottom of the reactor at a flow rate of 0.2 L/min. After 1.5 to 2 h, the cells were collected by centrifugation and reused for the next oxidation reactions. The formed α-ketoadipyl-7-ACA (AKA-7-ACA) in this reaction was converted into GL-7-ACA by adding 5 mL of 30 m*M* $H_2O_2$ solution to the supernatant. The final yield of GL-7-ACA was calculated by HPLC assay of the supernatant.

### *HPLC Assay*

The progress of the preparation of α-keto acid was monitored by a reverse-phase XDB C-18 column (Zorbax, 4.6 × 250 mm). The mobile phase was 25% (v/v) methanol in sodium acetate solution (20 mM, pH 4.2) at a flow rate of 1 mL/min. Samples were detected at 214 nm, and the elution times of D-phenylalanine, phenylpyruvate, and phenylacetate were 4.21, 6.13, and 18.9 min, respectively. A reverse-phase XDB C-18 column (Zorbax, 4.6 × 150 mm) was also employed to follow the progress of the conversion of CPC. The flow rate of the eluent (25 mM sodium phosphate, pH 3.5; 8% [v/v] acetonitrile) was 1 mL/min, and the peaks were monitored at 254 nm. The elution times of CPC, AKA-7-ACA, and GL-7-ACA were 2.68, 3.46, and 8.17 min, respectively. The sum of peak areas was used for calculation of the conversion rate.

## Results and Discussion

### *Expression of DAAO and Catalase in Recombinant* P. pastoris *Cells*

High-density fermentation of recombinant *P. pastoris* was carried out in a 1-L fermentor. Figure 1 shows the relationship among fermentation time, biomass, and the activities of DAAO and catalase assayed with the CTAB-permeabilized cells. The cells grew quickly in the medium and the maximum biomass reached 86.8 g DCW/L after 48 h of inoculation. Afterward, the cell concentration decreased rapidly. This might be owing to the cell autolysis because of the lack of nutrients in the medium during the late phase of fermentation. Catalase was always highly expressed in the cells, even at the beginning of fermentation (e.g., 5760 U/g DCW at 12 h). During the fermentation process, the trend of catalase activity was similar to that of biomass. The maximum activity of catalase was 764,192 U/L at 48 h. However, DAAO was expressed in trace levels in the cells when glycerol was present in the medium. After glycerol was exhausted completely, the cells were induced to begin methanol metabolism by feeding methanol to the medium at an appropriate rate (1 mL/h). The maximum DAAO activity up to 12,532 U/L was obtained 60 h. Thus, the peak of DAAO activity

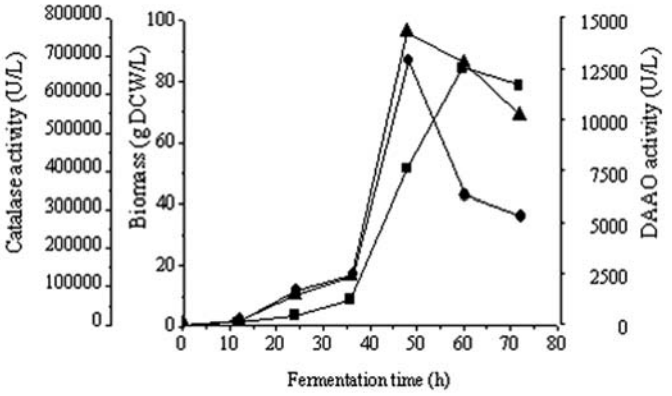


Fig. 1. Biomass and activities of DAAO and catalase as function of fermentation time. The time "0 h" was the starting time of inoculating cells into a 1-L fermentor. (●) Biomass; (▲) catalase activity; (■) DAAO activity.

lagged behind that of biomass. The probable reason is that there was a shortage of iron and FAD in the cells at the time when the maximum biomass occurred, and further accumulation of iron and FAD was required to supply the maximum activity of DAAO. In this experiment, we harvested these *P. pastoris* cells after 60 h of fermentation. The expression levels of DAAO and catalase reached up to 12,532 U/L (293 U/g DCW) and 684,800 U/L (15,984 U/g DCW), respectively.

### *Stability of DAAO and Catalase in Permeabilized* P. pastoris *Cells*

The untreated permeabilized cells were incubated at 30°C in 50 m*M* pyrophosphate buffer, pH 7.5. Aliquots of the cell suspension were withdrawn at different time intervals, and the cells were collected by centrifugation for assays of DAAO and catalase activities; Fig. 2 presents the results. The half-life of DAAO and catalase in the permeabilized cells was up to 14.5 and 4.0 d, respectively. Unexpectedly, the stability of catalase in the permeabilized cells was found to be lower than that of DAAO, which was the opposite of the result reported by Upadhya et al. *(7)*.

Whole cells of *P. pastoris* also contained a little esterase. In the conversion of CPC by DAAO, both the catalase and esterase activities generate negative effects, which are related to the increase in byproduct *(6)*. Therefore, it is essential that the two enzymes in the permeabilized cells be inactivated before these cells are used as the catalyst for the conversion of CPC. After treating the permeabilized cells with base (pH 11.5, 30 min) and heating (65°C, 2 min) (see Materials and Methods), in these cells 99.5% of catalase activity and 100% of esterase activity were eliminated, whereas 82.6% of DAAO activity was still retained. In addition, the half-life of DAAO in the treated permeabilized cells decreased to 12.2 d under the conditions described above (Fig. 2), indicating that the treatment had a slight effect on the stability of DAAO in the permeabilized cells.

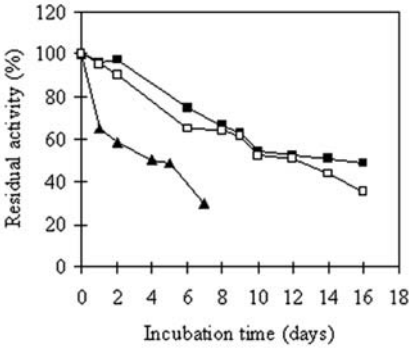


Fig. 2. Stability of DAAO and catalase in permeabilized *P. pastoris* cells. The activities of DAAO and catalase in the permeabilized cells before incubation were taken as 100%. (■) DAAO in untreated permeabilized cells; (□) DAAO in treated permeabilized cells; (▲) catalase activity in untreated permeabilized cells.

According to a previous report, permeabilized cells displayed a typical autolytic process, which led to the solubilization of cell components and engendered a decrease in the stability of the intracellular enzymes *(14)*. In the present work, the autolytic release of DAAO and catalase from the permeabilized *P. pastoris* cells was also studied. To avoid the inactivation of the released DAAO and catalase, the permeabilized cells were incubated at 25°C in 50 m*M* pyrophosphate buffer (pH 7.5) containing 25 m*M* β-mercaptoethanol. Every 12 h, these cells were collected by centrifugation, then incubated under the same conditions. The activities of DAAO and catalase present in the supernatant were assayed. As shown in Fig. 3, the rate of catalase release from the permeabilized cells was found to be much higher than that of DAAO. After 144 h, the total amount of released catalase activity was 16.7-fold more than that of DAAO. Thus, it was inferred that the intensity of catalase proteins integrated with other cell components was comparatively weak. This might be one of the reasons that catalase in the permeabilized cells showed a relatively lower stability.

## *Application of Permeabilized* P. pastoris *Cells in Bioconversions*

To assess the efficacy of both the untreated and treated permeabilized cells as the catalysts used for the bioconversions, these cells were applied for the conversion of D-phenylalanine and CPC, respectively. These reactions were carried out under the reaction conditions described in Materials and Methods. It was observed that the untreated permeabilized cells could convert D-phenylalanine to 99.0% phenylpyruvate within 100 min (Fig. 4A), and that these cells could be used up to 13 cycles without any significant decrease in phenylpyruvate yield (Fig. 4B). Additionally, no phenylacetate (a byproduct) was found during the conversion process by HPLC assay (Fig. 5). The absence of phenylacetate in this reaction might be owing to the high content of catalase along with the DAAO present in the peroxisomes,

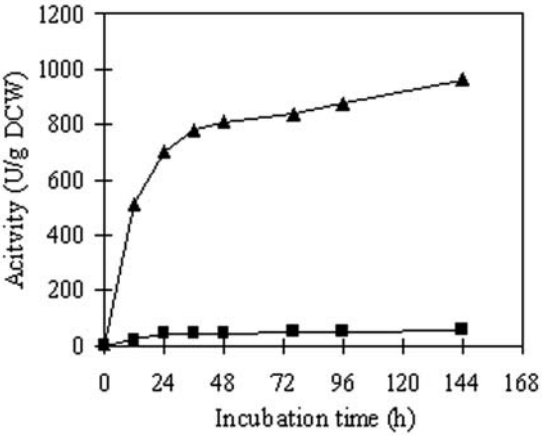


Fig. 3. Release of DAAO and catalase from permeabilized *P. pastoris* cells: (■) DAAO activity; (▲) catalase activity.

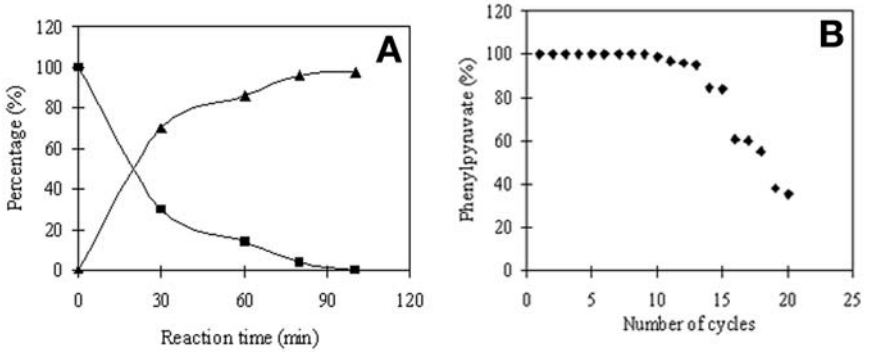


Fig. 4. Conversion of D-phenylalanine into phenylpyruvate by permeabilized *P. pastoris* cells. The initial moles of D-phenylalanine were taken as 100%. **(A)** Effect of reaction time on conversion percentage; **(B)** effect of reaction cycles on phenylpyruvate yield. (■) D-Phenylalanine; (▲) phenylpyruvate.

which resulted in the two enzymatic reactions being restricted in the microenvironment of the peroxisomes. As a result, the generated $H_2O_2$ from the DAAO reaction was removed efficiently by catalase.

Figure 6 shows that the treated permeabilized cells were applied for the conversion of CPC. It can be seen that CPC was converted into 76.5% AKA-7-ACA and 19% GL-7-ACA after a 100-min reaction time (Fig. 6A). A large amount of AKA-7-ACA formed in this reaction owing to a small amount of residual catalase activity present in the treated permeabilized cells. After treatment of the AKA-7-ACA with $H_2O_2$ solution (see Materials and Methods), 90% of the final GL-7-ACA yield was obtained. Meanwhile, these permeabilized cells could be used efficiently for three cycles without a significant decrease in conversion efficiency (Fig. 6B). Thus, the number

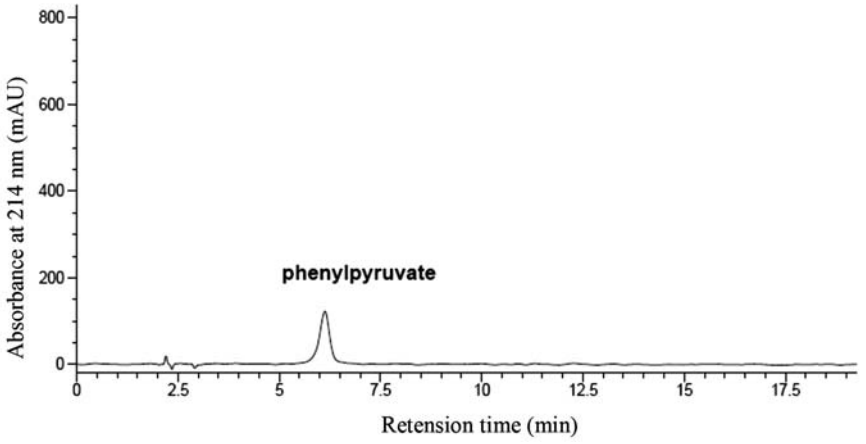


Fig. 5. HPLC assay of products from D-phenylalanine conversion.

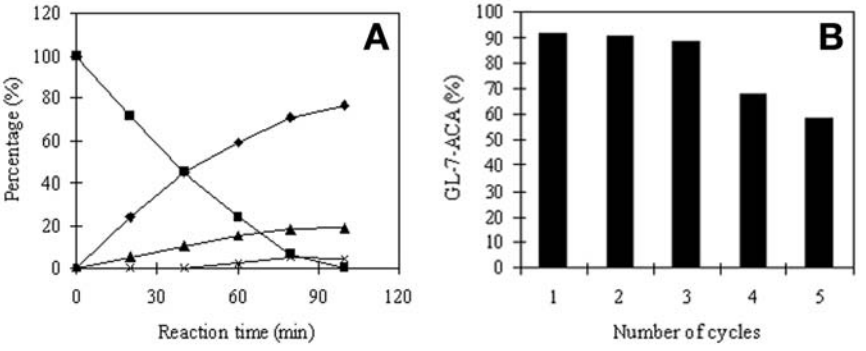


Fig. 6. Conversion of CPC into GL-7-ACA and AKA-7-ACA by permeabilized *P. pastoris* cells. The initial moles of CPC were taken as 100%. **(A)** Effect of reaction time on conversion percentage; **(B)** effect of reaction cycles on final GL-7-ACA yield. (■) CPC; (●) GL-7-ACA; (▲) AKA-7-ACA; (+) byproduct.

of permeabilized cells reused in this conversion was much less than that in the conversion of D-phenylalanine. This could be owing to the presence of oxygen tension and the rendered $H_2O_2$ in the reaction, which produce considerable inactivation of DAAO in permeabilized cells.

## *Comparison of Stability and Catalytic Efficiency of DAAO and Catalase in Permeabilized Cells of Different Yeasts*

Table 1 summarizes the stability and catalytic efficiency of DAAO and catalase in permeabilized cells of different yeasts. By comparison, the stability of the two cellular enzymes observed in our study was much higher than that reported previously *(6–8)*. The half-life of DAAO and catalase in the permeabilized *P. pastoris* cells was about 10 and 2 times more than that

Table 1
The Stability and Catalytic Efficiency of DAAO and Catalase in Permeabilized Cells of Different Yeasts

| Yeasts (DAAO gene sources) | Stability | | | Preparation of phenylpyruvate | | Reference |
|---|---|---|---|---|---|---|
| | | Half-life (d) | | | | |
| | Temperature (°C) | DAAO | Catalase | Yield (%) | No. of cycles | |
| *R. gracilis* (RgDAAO gene) | 25 | 1.5 | 2.2 | 97 | 3 | *7* |
| *R. gracilis* (RgDAAO gene) | NA | NA | NA | 48 | 1 | *17* |
| *T. variabilis* (TvDAAO gene) | 25 | 2.0 | NA | NA | NA | *6)* |
| *T. variabilis* (TvDAAO gene) | 30 | 7.6 | NA | NA | NA | *8* |
| *P. pastoris* (TvDAAO gene) | 30 | 14.5 | 4.0 | 99 | 13 | Ours |

NA, not available.

of the two enzymes in permeabilized *R. gracilis* cells *(7)*, respectively, even at a higher temperature (increased by 5°C). In addition, DAAO in the permeabilized *P. pastoris* cells was about twofold more stable than that in permeabilized *T. variabilis* cells at 30°C *(8)*. Furthermore, in the case of conversion of D-phenylalanine under the same reaction conditions, the number of cycles that the permeabilized *P. pastoris* cells could be efficiently reused was at least fourfold more than that of permeabilized *R. gracilis* cells *(7)*.

The effect of DAAO gene sources on the stability of this enzyme has been documented by other researchers *(15,16)*. It was reported that soluble DAAO from *T. variabilis* (TvDAAO) was more stable than that from *R. gracilis* (RgDAAO). According to the present comparison, it seemed that the stability of DAAO in permeabilized cells was also closely related to the nature of expression hosts.

## Conclusion

High expression of DAAO and catalase was obtained through high-density fermentation of recombinant *P. pastoris* in a 1-L fermentor. After treatment of the cells with CTAB, these permeabilized cells exhibited a good stability of DAAO and catalase. On the basis of the above-mentioned characteristics, application of the permeabilized cells in the related bioconversion was attempted. It was observed that these cells could be efficiently reused up to 13 and 3 cycles in the preparation of phenylpyruvate and GL-7-ACA, respectively, without a significant decrease in bioconversion efficiency. According to these results, it may be feasible to use permeabilized *P. pastoris* cells as catalysts for the production of α-keto acids and GL-7-ACA in order to lower production cost.

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## References

1. Upadhya, R., Nagajyothi, H., and Bhat, S. D. (1999), *Process Biochem.* **35,** 7–13.
2. Brodelius, P., Nisson, K., and Mosbach, K. (1981), *Appl. Biochem. Biotechnol.* **6,** 293–308.
3. Parmar, A., Kumar, H., Marwaha, S. S., and Kennedy, J. F. (1998), *Crit. Rev. Biotechnol.* **18,** 1–12.
4. Tu, S. and McCormick, D. B. (1972), *Sep. Sci.* **7,** 403–408.
5. Sacchi, S., Pollegioni, L., Pilone, M. S., and Rossetti, C. (1998), *Biotechnol. Tech.* **12,** 149–153.
6. Vicenzi, J. T. and Hansen, G. J. (1993), *Enzyme Microb. Technol.* **15,** 281–285.
7. Upadhya, R., Nagajyothi, H., and Bhat, S. D. (2000), *Biotechnol. Bioeng.* **68,** 430–436.
8. Moreno, J. A., Ruiz, C. A., Catalan, J., and Galan, M. A. (2004), *J. Chem. Technol. Biotechnol.* **79,** 321–326.
9. Dey, E. S., Flygare, S., and Mosbach, K. (1991), *Appl. Biochem. Biotechnol.* **27,** 239–250.

10. Becka, S., Skrob, F., Plhackova, K., Kujan, P., Holler, P., and Kyslik, P. (2003), *Biotechnol. Lett.* **25,** 227–233.
11. Shibuya, Y., Matsumoto, K., and Fujii, T. (1981), *Agric. Biol. Chem.* **45,** 1561–1567.
12. Li, D. Y., Zhang, Y. J., Yang, S., Li, R. B., and Yuan, Z. Y. (2002), *J. Mol. Catal. Ser. B: Enzym.* **18,** 291–297.
13. Aebi, H. (1984), *Methods Enzymol.* **105,** 122–127.
14. Flores, M. V., Ertola, R. J. J., and Vogel, C. E. (1996), *J. Ferment. Bioeng.* **81,** 524–529.
15. Betancor, L., Hidalgo, A., Fernandez-Lorente, G., et al. (2003), *Biotechnol. Prog.* **19,** 784–788.
16. Pollegioni, L., Caldinelli, L., Molla, G., Sacchi, S., and Pilone, M. S. (2004), *Biotechnol. Prog.* **20,** 467–473.
17. Buto, S., Pollegioni, L., Angiuro, L. D., and Pilone, M. S. (1994), *Biotechnol. Bioeng.* **44,** 1288–1294.