0273-2289/05/126/0177/$30.00

Microencapsulated Mycelium-Bound Tannase From *Aspergillus niger*

An Efficient Catalyst for Esterification of Propyl Gallate in Organic Solvents

Xiao-Wei Yu and Yong-Quan Li*

Zhejiang University, College of Life Science, Yuquan Campus, Hangzhou 310027, P.R. China, E-mail: lyq1962@yahoo.com

Received January 20, 2005; Revised May 22, 2005; Accepted May 24, 2005

Abstract

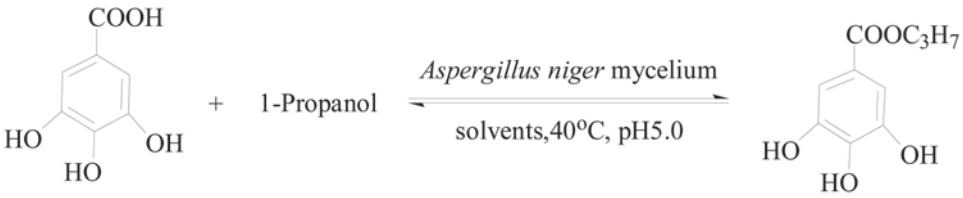
Microencapsulated *Aspergillus niger* with mycelium-bound tannase activity was employed to investigate the esterification of propyl gallate from gallic acid and propanol in organic solvents. The effects of various organic solvents (log *P*: –1.0 to 6.6) on the enzymatic reactions showed that benzene (log *P*: 2.0) was the suitable solvent, for which the conversion reached 26.8%. The optimum catalyst concentration and water concentration was found at 25 capsules in 10 mL of benzene and 0.04 g of water/capsule. The external mass transfer effect could be eliminated at stirring speeds of 180 rpm or higher. Both substrates 1-propanol and gallic acid had significant inhibition effects on the tannase activity. Maximum molar conversion (36.2%) was achieved with 9.1% (v/v) 1-propanol and 8 m*M* gallic acid and decreased with increasing amounts of substrates.

Index Entries: Biosynthesis; immobilized cells; *Aspergillus niger*; tannase; organic solvents; optimization; propyl gallate; production kinetics.

Introduction

The advantages of employing enzymes in organic solvents as opposed to aqueous media have been well known *(1)*. Some of these include dramatically higher substrate solubility; the ability to use enzymes synthetically, rather than degradatively; and the capability to modify native selectivity by simply tailoring the reaction medium, rather than the enzyme itself.

*Author to whom all correspondence and reprint requests should be addressed.



Scheme 1. Esterification of propyl gallate by free or microencapsulated *A. niger* with mycelium-bound tannase activity in organic solvents.

Tannin acyl hydrolases (EC 3.1.1.20) are esterases able to hydrolyze the ester bonds of tannin in aqueous media and synthesize gallic acid esters in organic media. Toth and Hensler *(2)* disclosed the ability of soluble tannase to produce gallic acid esters. Gaathon et al. *(3)* synthesized propyl gallate in a reverse micelle system. Researchers also used immobilization methods to improve the esterification activity of tannase *(4–6)*. However, there is no report on using mycelium-bound tannase as catalyst. Whole-cell biocatalysis in organic solvents offers economical and technical benefits, such as improved stability to the inhibitory effects of substrate/product/solvent and the avoidance of costly and time-consuming purifications *(7)*. In the course of our earlier investigations on reverse hydrolytic processes, we found that *A. niger* with mycelium-bound tannase has significant esterification activity in organic solvents but that the lyophilized free mycelia showed very low activity, probably owing to denaturation of membrane-bound enzymes by contact with organic solvents directly. Here the microencapsulated mycelia were used for the synthesis of propyl gallate, which is widely used as a food additive antioxidant. Immobilization of microbial cells in a high density not only improves the productivity of a bioreactor, but also provides many benefits over free cells, such as protection from harsh environmental conditions (pH, temperature, organic solvent, and poison), and recovery from the solution without difficulty *(8–10)*. Among various immobilization methods, microencapsulation with a liquid core gives a larger space for microbial cell immobilization, reduces mass transfer resistance inside the capsule, and allows the permeable polymer membrane to resist the toxicity of the organic media efficiently *(11)*.

In the present work, *A. niger* with mycelium-bound tannase microencapsulated within chitosan-alginate complex coacervate membrane was employed to investigate the synthesis of propyl gallate in organic solvents. Scheme 1 depicts the esterification scheme.

Materials and Methods

Microorganism, Media, and Culture Conditions

A. niger (China General Microbiological Culture Collection Center, no. 3.315) was grown in 500-mL Erlenmeyer flasks containing 100 mL of medium (20 g/L of tannic acid, 4 g/L of starch, 6 g/L of saccharose, 2 g/L of

glucose, 9 g/L of soybean flour, 1 g/L of K_2HPO_4, 1 g/L of NH_4NO_3, 0.5 g/L of KCl, 0.5 g/L of $MgSO_4$, 0.2 g/L of $CaCl_2$, 0.01 g/L of $FeSO_4$; pH 6.0) at 30°C, 180 rpm. Suspensions of spores (1×10^7) were used as inoculums. After cultivation for 72 h, the mycelia were collected by centrifugation, washed thoroughly with 8.5 g/L of NaCl solution, and touch-dried with filter paper.

Immobilization Method

Twenty milliliters of 15 mg/mL chitosan solution containing 20 mg/mL of $CaCl_2$ and 10 mL of 0.1 mol/L acetic buffer (pH 5.0) containing 2 g of mycelia (0.4 g dry wt) were mixed and then extruded dropwise via a syringe into a stirred 14 mg/mL alginate solution. After a reaction period of up to 20 min, the beads were collected and washed three times with distilled water, then hardened for 30 min in 20 mg/mL of $CaCl_2$. Finally, the beads were suspended in 0.1 mol/L of pH 5.0 acetic acid buffer and 2 mg/mL of $CaCl_2$ solution for 1 h, and the pH of the beads was adjusted to pH 5.0. The chitosan-alginate capsules without mycelia were used as control beads.

Determination of Water

The immobilized capsules were touch-dried with filter paper, and then the capsules were freeze-dried for 24 h. At regular intervals, 10 capsules were taken out and weighed; the difference between the touch-dried weight and the freeze-dried weight of the capsules was considered as the water content of the chitosan-alginate beads. Significant differences ($p < 0.05$) were measured.

Enzymatic Reactions

Typical reactions were carried out by adding 20 mycelia capsules to the reaction mixture, which consisted of 10 mL of organic solvent, 10 m*M* gallic acid, and 9.1% (v/v) 1-propanol in 25-mL glass flasks shaken at 40°C, 200 rpm. All solvents had been dried over 3- Å molecular sieves for 72 h. The molar conversion was defined as (molar of propyl gallate/molar of the initial fed gallic acid) × 100%. The initial reaction rate was defined as the amount of propyl gallate formed by 1 g of dry mycelia within capsules/h under the assay conditions.

Partition Experiments

The partitioning of propyl gallate between control chitosan-alginate beads and organic reaction mixture was measured by mixing them in the presence of propyl gallate. After 3 h the concentration of propyl gallate in the organic phase was measured. The concentration of propyl gallate in the beads was analyzed according to the difference between its initial fed concentration and equilibrated concentration after mixing.

Analytical Procedures

High-performance liquid chromatography analysis was performed on a LiChrospherC18 column (LiChroCART 125 × 4, Merck KGaA, Darmstadt, Germany). Samples (200 µL) removed from the systems were vacuum evaporated, diluted with 0.15 mmol/dm^3 of ethyl *p*-hydroxybenzoate in methanol, and injected with a volume of 20 µL. The solvent system/mobile phase comprised methanol:water in a ratio of 55:45 adjusted to pH 3.0 with phosphoric acid at a flow rate of 1 mL/min for 8 min. Ethyl *p*-hydroxybenzoate was the internal standard substance. Absorbance analysis was carried out at 275 nm. The reaction product was calculated from a calibration curve plotted ($Y = 0.03144X - 0.00194$; $R = 0.9999$) as propyl gallate concentration (X, µg/mL) vs the ratio (Y) between propyl gallate peak area and internal standard substance peak area.

Qualitative analysis was performed by thin-layer chromatography. The coating was silica gel-G plant. The solvent system was chloroform:methanol in a ratio of 8:2 with two drops of glacial acetic acid added per 100 mL of solvent. Visualization was by iodine vapor. All the assays were done in duplicate, and significant differences ($p < 0.05$) were measured.

Results and Discussion

Time Course Profiles of Synthesis of Propyl Gallate in Hexane

The time course of the synthesis of propyl gallate in benzene by microencapsulated mycelia is shown as an equilibrium-controlled reaction (Fig. 1). The catalytic rate rose quickly in the first hours of incubation, and the reaction equilibrium was reached at about 72 h. Thus, the reaction terminal in further experiments was determined at 72 h. In the control test, no product could be detected with the control beads.

Effect of Organic Solvents on Synthesis of Propyl Gallate

In biocatalytic reactions, proper selection of organic solvent is essential. One of the most important criteria for solvent selection is biocompatibility. Many organic solvents are toxic to living organisms because of their devastating effects on biologic membranes *(12)*. This toxicity correlates with the hydrophobic character of the solvent, expressed by the logarithm of its partition coefficient between octanol and water (log *p* value). Researchers found that the polar solvents with log $p < 1.0$ are not suitable for tannase esterification and that with log $p > 2.0$ are generally preferred for biocatalysis *(6)*. The experimental results (Table 1) were consistent with this rule. The microencapsulated mycelia gained very low conversion in high polar solvents (log $p < 1.0$), such as *N*,*N*-dimethylformamide, acetone, 1-propanol, and pyridine. The poor enzyme activity may be the result of the polar solvents incorporating within membrane lipids, which may cause disruption of essential membrane functions, inactivation or denaturation of membrane-bound enzymes, and at high concentrations lysis of the cells

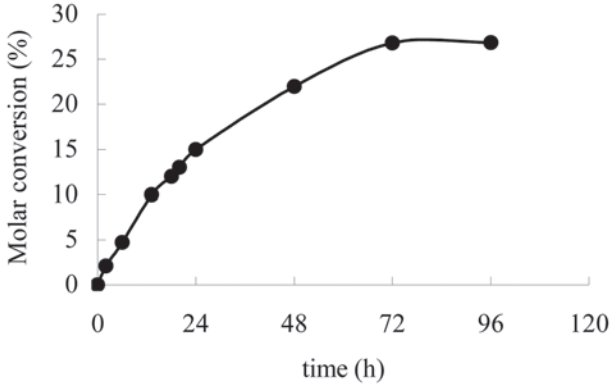


Fig. 1. Time course of synthesis of propyl gallate by microencapsulated mycelia of *A. niger*. Reaction conditions: 10 m*M* gallic acid, 9.1% (v/v) 1-propanol and 10 mL of benzene, 20 capsules, pH 5.0, 40°C at 200 rpm.

Table 1
Effect of Organic Solvents on Synthesis
of Propyl Gallate by Microencapsulated Mycelia of *A. niger*[a]

Solvent	Log *P*	Molar conversion (%)
N,*N*-Dimethylformamide	–1.0	1.8
Acetone	–0.23	2.6
1-Propanol	0.28	0.6
Pyridine	0.71	3.2
Benzene	2.0	26.8
Hexane	3.4	4.9
Octane	4.5	0.2
Dodecane	6.6	0.9

[a]Reaction conditions: 10 m*M* gallic acid, 9.1% (v/v) 1-propanol, and 10 mL of organic solvent; 20 capsules; pH 5.0; 40°C; incubated for 72 h at 200 rpm.

(13–15). Additionally, alcohol may react as a strong competitive inhibitor, whose effect has usually been found to be significant in lipase-catalyzed esterification. The highest yield reached was 26.8% conversion in benzene, even though the log *p* was only 2. Although with an increase in the log *p* value the organic solvent will be less harmful to biocatalysts, [13]*n*-hexane (log *p* = 3.4), octane (log *p* = 4.5), and dodecane (log *p* = 6.6) used as reaction medium did not give encouraging results, and the more hydrophobic the medium, the lower the tannase activity. Esterifications of propyl gallate by tannase also showed the same relationship between yields and log *p (6)*. Although the log *p* value could not be considered the only parameter responsible for the extent of conversion, some investigators proposed a solvent toxicity model based on the notion of a solvent critical membrane concentration that was independent of the solvent type. Beyond this con-

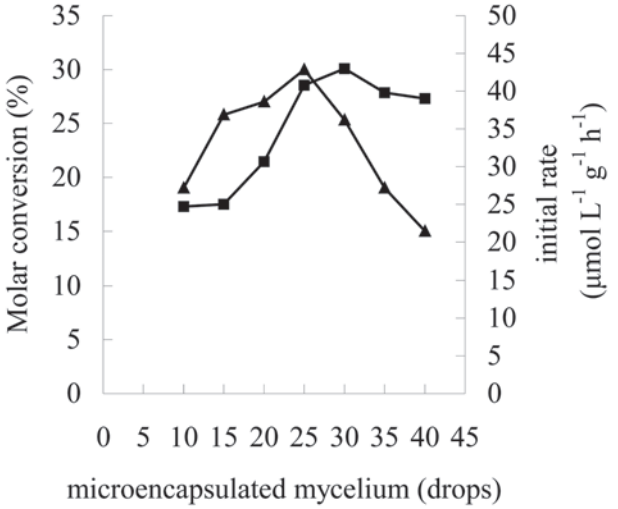


Fig. 2. Effect of concentrations of microencapsulated mycelia of *A. niger* on synthesis of propyl gallate: (■) initial rates; (▲) molar conversion. Reaction conditions: 10 m*M* gallic acid, 9.1% (v/v) 1-propanol and 10 mL of benzene, pH 5.0, 40°C, incubated for 72 h at 200 rpm.

centration, whole-cell catalytic activity is lost *(16,17)*. The molar conversion of propyl gallate was still low in benzene; thus, further experiments were carried out to improve the performance of esterification by optimizing water content and the amount of catalysts detecting substrates and diffusion effects.

Effect of Biocatalyst Concentration on Synthesis of Propyl Gallate

The microencapsulated mycelia and control beads without mycelia were both dried at 80°C until reaching constant weight. The mycelium in calcium alginate beads was about 1.25 mg dry wt/capsule according to the difference between the weight values. The water content in touch-dried capsules was measured at 0.1 g of water/capsule. The results in Fig. 2 suggest that the optimum catalyst concentration was 25 capsules in 10 mL of organic solvent, with a maximum yield of 30%. Although the use of capsules in excess decreased the molar conversion sharply, increasing the capsules from 25 to 40 drops introduced more catalyst in the reaction system, and the water content also increased greatly, from 25 to 40% (w/v), which led to a shift in the thermodynamic equilibrium toward hydrolysis. Furthermore, the partition experiments showed that about 15% of propyl gallate remained inside the capsules. Therefore, the real concentration of the product was higher than the apparent concentration in the organic solvent. The more capsules, the higher the apparent concentration compared with the real concentration. The initial rate also increased with an increase in the catalyst concentration from 10 to 25 capsules and thereafter almost remained constant, which was not the same as the trend of molar

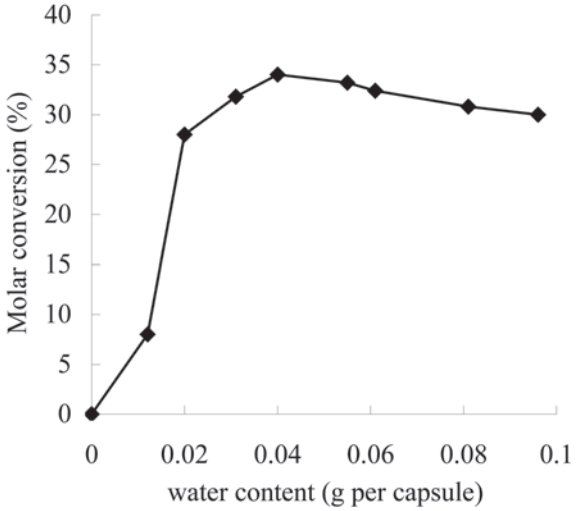


Fig. 3. Effect of water content of microencapsulated mycelia on synthesis of propyl gallate. Reaction conditions: 10 m*M* gallic acid, 9.1% (v/v) 1-propanol and 10 mL of benzene, 25 capsules, pH 5.0, 40°C, incubated for 72 h at 200 rpm.

conversion, because the water content did not have a significant effect on the synthesis of propyl gallate at the beginning of the reaction.

Effect of Water Content on Synthesis of Propyl Gallate

The role of water in the esterification reaction in organic solvents is one of the major areas of question in nonaqueous enzymology. It has been proven that water molecules provide an essential ingredient for the enzyme to retain the activity, but, on the other hand, the enzyme also catalyzes the hydrolysis reaction, which mitigates successful synthesis of esters. The previous section suggested that the water content had a significant effect on the synthesis of propyl gallate by varying the amount of capsules. In this section, the effect of the water content in the capsule is discussed. The water in the capsules was controlled from 0.1 to 0 g/capsule by freeze-drying, and, thus, the total water content varied from 25% (w/v) to 0, fixing 25 capsules in the reaction mixture. Figure 3 shows that microencapsulated mycelium-bound tannase was not very sensitive to the water content between 0.02 and 0.1 g/capsule. The maximum yield (34%) was gained at a water content of 0.04 g/capsule, whereas for values <0.02 g of water/capsule, a steep decrease in catalytic activity was observed. Moreover, when the mycelia were completely dehydrated, no synthesis reaction occurred. The results suggested that a proper amount of water was essential for esterification by microencapsulated mycelia. The water requirement of catalyst might be influenced by considering the distribution of water between solvent and enzyme, and high concentrations of water initiated the reverse hydrolysis reaction and decreased the net production of esters. On the other hand, lack of water would cause excessive cell dehydration or the loss of the water barrier

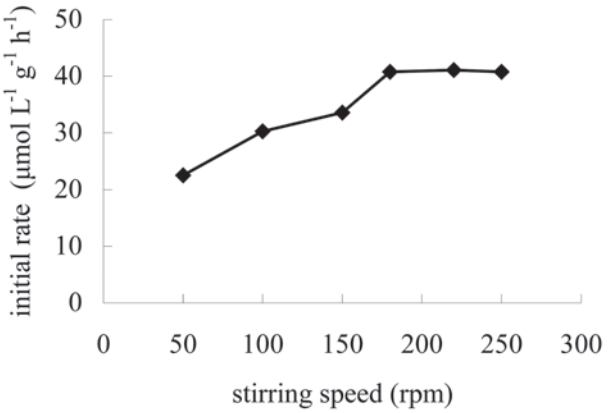


Fig. 4. Effect of stirring speed on synthesis of propyl gallate. Reaction conditions: 10 m*M* gallic acid, 9.1% (v/v) 1-propanol and 10 mL of benzene, 25 capsules, pH 5.0, 40°C.

between the biocatalyst and the solvent; however, high enzyme activity in the presence of just a little amount of water has been reported by several researchers *(18–20)*. It may therefore be concluded that not all enzymes are retained in their active conformation in freeze-dried form; the active site of tannase may be hindered by neighboring proteins. An appropriate amount of water will help the enzyme conformation between the enzyme and proteins, thereby creating a more "effective" enzyme concentration.

Mass Transfer Effect in Immobilized Cell System

Diffusional resistance usually is observed in immobilized biocatalyst systems. Diffusional resistance is generally described as external resistance from the fluid motion around the particles or internal resistance as the result of intraparticle diffusion *(21)*. External mass transfer limitations, however, can be prevented if adequate hydrodynamic conditions are chosen *(22)*. To assess the extent of external diffusional resistance in the present study, reaction rates were measured with different shaking speeds, from 50 to 250 rpm. The experimental results (Fig. 4) show that external mass transfer was apparently not the limiting factor at stirring speeds of 180 rpm or above. Thus, further experiments were performed with a 180-rpm stirring speed.

Effect of Substrate Concentration on Synthesis of Propyl Gallate

The substrate concentration afforded by a biologic catalyst is an important parameter affecting enzyme activity and potential application. The effects of 1-propanol and gallic acid concentration were investigated by varying one of the substrate's concentration, whereas the other was held constant. As shown in Fig. 5A, molar conversion increased with an increase in 1-propanol concentration and reached a maximum yield (34%) at 9.1% (v/v) 1-propanol. With a further increase in the concentration of alcohol, the

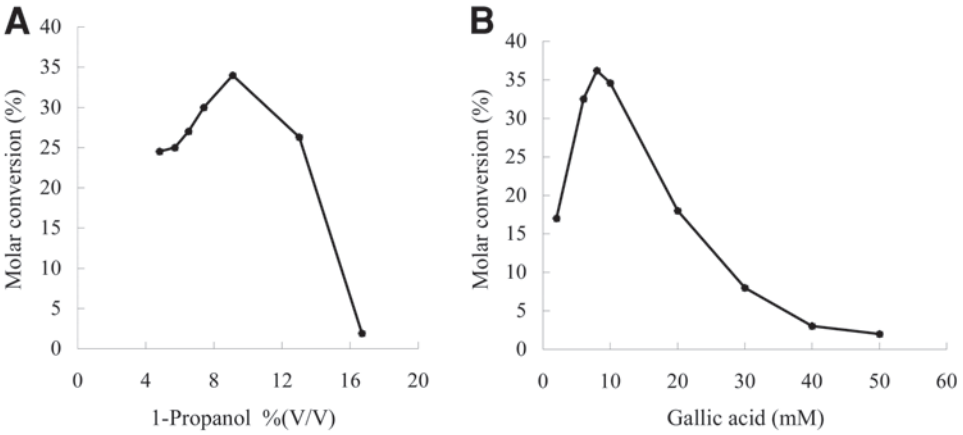


Fig. 5. Effect of substrate concentration on synthesis of propyl gallate: **(A)** 1-propanol concentration varied at fixed gallic acid concentration of 10 m*M*; **(B)** gallic acid concentration varied at fixed 1-propanol concentration of 9.1% (v/v). Reaction conditions: 10 mL of benzene, 25 capsules, pH 5.0, 40°C, 180 rpm, incubated for 72 h.

yield started to decrease quickly and the tannase nearly lost its activity completely at an alcohol concentration of 16.7% (v/v). The loss of activity at high alcohol concentrations might be influenced by the toxic effect of ethanol on the microorganism, which would cause inhibition of lipase activity. Similarly, gallic acid (Fig. 5B) also showed an inhibiting effect on the reaction. An increase in acid concentration might introduce a pH effect into the system and change the catalytic environment. The maximum molar conversion (36.2%) was achieved at a gallic acid concentration of 8 m*M*. Taking into account that whole cells were used in the study, it can be concluded that the specific activity of the enzymes involved must be very high.

Conclusion

Propyl gallate is a safe antioxidant and is widely used in foods, cosmetics, hair products, adhesives, and lubricants. Now it is mainly produced in large scale by a chemical method. Whereas the chemical method has several disadvantages, including the production of byproducts and the requirement of critical reaction conditions such as strong acid or strong base and high temperature and pressure, the biologic method is more efficient and could avoid these disadvantages. Tannase specialized in the catalysis of the ester bonds in tannin, and the reaction is carried out under mild conditions.

Our study allowed a better understanding of the enzymatic synthesis of propyl gallate in organic solvents by encapsulated *A. niger* mycelia, and it could help to define ways to further improve the performance of the synthetic reaction. The mycelium-bound tannase activity could be improved by medium engineering and biocatalyst engineering. On the one hand, regarding the importance of medium engineering, it would be helpful to

attempt to use more kinds of organic solvents or other nonaqueous media such as ionic liquids and supercritical fluid. *Candida rugosa* lipase showed enhanced enantioselectivity in ionic liquids compared to organic solvents *(23)*. *Pseudomonas cepacea* lipase was used to catalyze the transesterification reaction between 1-phenylethanol and vinyl acetate in supercritical sulfur hexafluoride and showed increased reaction rates and high stability of the enzyme *(24)*. In addition, enzyme activity could be improved by optimizing the reaction conditions of pH and temperature, and adding crown ethers *(25)* and lyoprotectants *(26)*. On the other hand, immobilization and protein engineering are long known for improving biocatalyst efficiency or stability of enzymes. Although the microencapsulation method has been proved to have many benefits (microencapsulation gives a larger space for microbial cell immobilization, reduces mass transfer resistance inside the capsule, and allows the permeable polymer membrane to resist the toxicity of the organic media efficiently *[11]*), it is difficult to control the water content of the catalyst using this method. Other immobilization methods such as absorption and ligand coupling may improve the enzyme activity. Llanes et al. *(27)* reported that *Mycobacterium* sp. cells immobilized on celite used for the selective side-chain cleavage of sitosterol to androstenedione reached 45.8% conversion in phthalate derivative solution. Wax ester synthesis by lipase-catalyzed esterification with fungal cells immobilized on cellulose particles gained >90% molar conversion in a solvent-free system *(28)*. Regarding *A. niger* itself, the growth conditions were optimized to obtain the maximal activity of mycelium-bound tannase. To improve tannase activity further, protein engineering is a promising way to tailor the organism. There have been many successful reports on improving the enzyme's activity, stability, and selectivity in nonaqueous media by rational design or directed evolution *(29,30)*.

Acknowledgment

This research work was supported by grants from the Natural Science Fund of China (no. 20172046).

References

1. Carrea, G. and Riva, S. (2000), *Angew. Chem. Int. Ed.* **39,** 2226–2254.
2. Toth, G. and Hensler, D. (1952), *Acta Chimica II* **10,** 209–212.
3. Gaathon, A., Gross, Z., and Rozhanski, M. (1989), *Enzyme Microb. Technol.* **11,** 604–609.
4. Weetal, H. H. (1985), European patent application 0137601.
5. Sharma, S. and Gupta, M. N. (2003), *Bioorg. Med. Chem. Lett.* **13,** 395–397.
6. Yu, X. W., Li, Y. Q., and Wu, D. (2004), *J. Mol. Catal. B: Enzym.* **30,** 69–73.
7. León, R., Fernandes, P., Pinheiro, H. M., and Cabral, J. M. S. (1998), *Enzyme Microb. Technol*. **23,** 483–500.
8. Queiroz, N. and Nascimento, M. G. (2002), *Tetrahedron Lett.* **43,** 5225–5227.
9. Park, J. K. and Jung, J. Y. (2002), *Enzyme Microb. Technol*. **30,** 726–733.
10. Green, K. D., Gill, I. S., Khan, J. A., and Vulfson, E. N. (1996), *Biotechnol. Bioeng*. **49,** 535–543.
11. Park, J. K. and Chang, N. H. (2000), *Biotechnol. Adv.* **18,** 303–319.

12. Sikkema, J., de Bont, J. A. M., and Poolman, B. (1995), *Microbiol. Rev.* **59,** 201–222.
13. Vermü, M., Sikkema, J., Verheul, A., Bakker, R., and Tramper, J. (1993), *J. Biotechnol. Bioeng.* **42,** 747–758.
14. Inoue, A. and Horikoshi, K. (1991), *J. Ferment. Bioeng.* **71,** 194–196.
15. Sikkema, J., Weber, J. J., Heipieper, H. J., and de Bont, J. A. M. (1994), *Biocatalysis* **10,** 113–122.
16. Bassetti, L., Hagendoorn, M., and Tramper, J. (1995), *J. Biotechnol.* **39,** 149-155.
17. Osborne, S. J., Leaver, J., Turner, M. K., and Dunnill, P. (1990), *Enzyme Microb. Technol.* **12,** 281–291.
18. Converti, A., Del Borghi, A., Gandolfi, R., Molinari, F., Palazzi, E., Perego, P., and Zilli, M. (2002), *Enzyme Microb. Technol.* **30,** 216–223.
19. Molinari, F., Gandolfi, R., Converti, A., and Zilli, M. (2000), *Enzyme Microb. Technol.* **27,** 626–630.
20. Molinari, F., Gandolfi, R., Villa, R., and Occhiato, E. G. (1999), *Tetrahedron: Asymmetry* **10,** 3515–3520.
21. Shuler, M. L. and Kargi, F. (1992), *Bioprocess Engineering*, Prentice-Hall, London.
22. Ballesteros, A., Boross, L., Bucholz, K., Cabral, J. M. S., and Kasche, V. (1994), *Applied Biocatalysis*, Harwood Academic, New York.
23. Ulbert, O., Fráter, T., Bélafi-Bakó, K., and Gubicza, L. (2004), *J. Mol. Catal. B: Enzym.* **31,** 39–45.
24. Celia, E., Cernia, E., Palocci, C., Soro, S., and Turchet, T. (2005), *J. Supercritical Fluids* **33,** 193–199.
25. van Unen, D. J., Engbersen, J. F. J., and Reinhoudt, D. N. (2002), *Biotechnol. Bioeng.* **77,** 245–255.
26. Dabulis, K. and Klibanov, A. M. (1992), *Biotechnol. Bioeng.* **39,** 176–185.
27. Llanes, N., Fernandes, P., Léon, R., Cabral, J. M. S., and Pinheiro, H. M. (2001), *J. Mol. Catal. B: Enzym.* **11,** 523–530.
28. Chen, J. P. and Wang J. B. (1997), *Enzyme Microb. Technol.* **20,** 615–622.
29. Lutz, S. and Patrick, W. M. (2004), *Curr. Opin. Biotechnol.* **15,** 291–297.
30. Bornscheuer, U. T. and Pohl, M. (2001), *Curr. Opin. Chem. Biol.* **5,** 137–143.