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Lactic Acid Production From Cellulosic Material by Synergetic Hydrolysis and Fermentation

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Abstract

The hydrolysis process on corncob residue was catalyzed synergetically by the cellulase from *Trichoderma reesei* and the immobilized cellobiase. The feedback inhibition to cellulase reaction caused by the accumulation of cellobiose was eliminated efficiently. The hydrolysis yield of corncob residue was 82.5%, and the percentage of glucose in the reducing sugar reached 88.2%. The glucose in the cellulosic hydrolysate could be converted into lactic acid effectively by the immobilized cells of *Lactobacillus delbrueckii.* When the enzymatic hydrolysis of cellulose and the fermentation of lactic acid were coupled together, no glucose was accumulated in the reaction system, and the feedback inhibition caused by glucose was also eliminated. Under the batch process of synergetic hydrolysis and lactic acid fermentation with 100 g/L of cellulosic substrate, the conversion efficiency of lactic acid from cellulose and the productivity of lactic acid reached 92.4% and 0.938 g/(L·h), respectively. By using a fed-batch technique, the total concentration of cellulosic substrate and lactic acid in the synergetic process increased to 200 and 107.5 g/L, respectively, whereas the dosage of cellulase reduced from 20 to 15 IU/g of substrate in the batch process. The results of the bioconversion of renewable cellulosic resources were significant.

Index Entries: Cellulase; immobilized cellobiase; cellulosic material; corncob; synergetic hydrolysis; lactic acid fermentation.

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Introduction

Lactic acid is an important and multifunctional organic acid. It is widely used in food, pharmaceutical, leather, textile, and chemical industries. Recently, the polymer of lactic acid has received worldwide attention owing to its good properties, such as biodegradability, biocompatibility, transparency, and mechanical strength *(1–3)*. The demand for lactic acid has been increasing considerably, because the polylactic acid has promising applications as an environment-friendly alternative to plastics derived from petrochemical materials, and medical applications such as sutures and clips for wound closure or prosthetic devices or pharmaceutical carriers *(4–6)*.

Traditional production of lactic acid usually uses starch as the fermentation substrate, but this process is not compatible with sustainable development. As the main component of lignocellulosic materials, cellulose is an abundant and cheap renewable carbon source. Cellulose can be hydrolyzed to glucose by cellulase, and glucose may be used to produce ethanol, organic acids, and other chemicals *(7–9)*. Using cellulose as substrate instead of starch for lactic acid production is a significant project. The utilization of renewable biomass can not only save foodstuff but also reduce environmental pollution.

In China, corncob is popularly used to produce xylose by dilute-acid hydrolysis. The amount of corncob residue resulting from the manufacture of xylose is large and often causes environmental pollution *(10,11)*. Because most hemicellulose in corncob has been hydrolyzed to xylose, corncob residue is porous and easy to be degraded by cellulase. In the present work, corncob residue was used as the substrate to produce glucose for lactic acid fermentation.

In the process of bioconversion from cellulose to lactic acid, there are two main steps: hydrolysis of cellulosic material to glucose by cellulase, and lactic acid fermentation by *Lactobacillus delbrueckii* cells using glucose in the hydrolysate. Enzymatic hydrolysis is the key step, and it depends on the synergism of three components of cellulase: endo-β-glucanase, exo-β-glucanase, and cellobiase *(12–14)*. The commonly used cellulase from *Trichoderma reesei* has high activities of endo-β-glucanase and exo-β-glucanase, whereas the activity of cellobiase is quite low *(15–17)*. Thus, most of the intermediate product cellobiose cannot be converted into glucose, and the accumulation of cellobiose will cause strong feedback inhibition to the action of endo-β-glucanase and exo-β-glucanase *(18,19)*. Consequently, improving the activity of cellobiase in the cellulase system is the key to raising the hydrolysis yield *(16,20)*. In the present work, immobilized cellobiase was used to degrade cellobiose to glucose, and the hydrolysis process was performed under the synergetic action of cellulase from *T. reesei* and the immobilized cellobiase.

Another severe inhibition to the catalysis of cellulase during the hydrolysis process is caused by the accumulation of glucose, the final

hydrolysis product *(21,22)*. A suitable method to resolve this problem is the timely removal of glucose from the hydrolysate, e.g., the simultaneous saccharification and fermentation process *(22)*. In the present work, the process of cellulosic material hydrolysis was coupled with the process of lactic acid fermentation because the two processes were consistent in temperature (50°C), pH (4.8), and oxygen demand (anaerobic). By the fermentation of immobilized *Lactobacillus delbrueckii* cells, the glucose in the hydrolysate might be converted into lactic acid quickly; thus, the cellulase reaction could be accelerated, and the bioconversion process from cellulosic resources to lactic acid could be improved.

Materials and Methods

Microorganism

L. delbrueckii ZU-S2 (provided by the biochemical engineering laboratory of Zhejiang University) was used for lactic acid fermentation. The strain was stored in 20% skim milk at 4°C.

Cellulosic Material

Corncob residue was obtained from the xylose manufacturer in China. The compositions of corncob residue were as follows (w/w): 60.9% cellulose, 5.1% hemicellulose, 18.7% lignin, and 15.3% ash.

Preparation of Cellulase

Cellulase was produced by *Trichoderma reesei* under submerged fermentation according to Xia and Shen *(17)*. The filter paper activity (FPA) and the cellobiase activity (CBA) in 1 mL of cellulase preparation were 12 and 0.34 IU, respectively.

Production and Immobilization of Cellobiase

Cellobiase was produced on solid-state fermentation using *Aspergillus niger* and immobilized by being entrapped into calcium alginate gels *(20)*. The formed immobilized gel beads were 2 to 3 mm in diameter, and the CBA per milliliter of the beads was 3.6 IU.

Immobilization of L. delbrueckii *Cells*

L. delbrueckii cells were inoculated into the seed medium (2% [w/v] glucose, 1% [w/v] peptone, 1% [w/v] yeast extract, 0.05% [w/v] $MgSO_4$, 0.01% [w/v] NaCl, and 0.05% [w/v] KH_2PO_4.) at 50°C for proliferation. After 16–20 h of cultivation, the broth was centrifuged at 5000 rpm at 4°C for 10 min. The collected cells were cleansed and mixed with 2% sodium alginate solution. Then the mixture was dripped into 1% calcium chloride solution by an injector. After solidification at 4°C for 10 h, the immobilized gel beads were formed. The average diameter of the beads was 3 mm, and about 1.6×10^9 cells were immobilized in 1-mL beads *(23)*.

Hydrolysis Process of Cellulosic Material

The hydrolysis of cellulosic material was carried out synergetically by cellulase from *T. reesei* and immobilized cellobiase. The cellulase catalytic reaction was performed in a 2-L enzymatic hydrolysis jar with a working volume of 1.4 L. Corncob residue (100 g/L) was used as substrate, and the dosage of cellulase from *T. reesei* was 20 IU (FPA)/g of substrate. The enzymatic reaction was set at pH 4.8, 50°C, and 60 rpm. After 8 h of hydrolysis, the hydrolysate was filtrated and transferred to a column reactor by a peristaltic pump. The column was 250 mL (id of 2.8 cm and inside height of 40 cm) packed with 125 mL of immobilized cellobiase. The hydrolysate extruded from the immobilized cellobiase column returned to the hydrolysis jar by another peristaltic pump. The process was cycled continuously and the samples were periodically collected from the outlet of the column for analysis of sugars. The hydrolysis yield of cellulosic material was calculated as follows:

$$\text{Hydrolysis yield (\%)} = \text{concentration of reducing sugar produced (g/L)} \times 0.9 \times 100\%/\text{initial concentration of cellulose and hemicellulose in substrate (g/L)}$$

Lactic Acid Fermentation by Immobilized Cells

Batch fermentation was carried out in a 250-mL column reactor (id of 2.8 cm and inside height of 40 cm), and 125 mL of immobilized *L. delbrueckii* cells were packed in it. The hydrolysate of cellulosic material instead of pure glucose was used as the carbon source for lactic acid production *(23)*. Fermentation was kept at pH 4.8 and 50°C until the concentration of lactic acid was steady. The broth was periodically sampled for analysis.

Synergetic Process of Hydrolysis and Lactic Acid Fermentation

The enzymatic hydrolysis jar, the immobilized cellobiase column, and the immobilized *L. delbrueckii* cell column were coupled together (Fig. 1). The hydrolysate from the immobilized cellobiase column was mixed with the medium for lactic acid fermentation (except the carbon source) and pumped into the immobilized cell column. The fermentation broth extruded from the cell column was pumped back to the hydrolysis jar. The reaction temperature of the whole system was kept at 50°C, and the pH was controlled between 4.5 and 5.0 by adding calcium carbonate into the immobilized cell column to neutralize the produced lactic acid.

During the continuously cycling process, intermittent sampling from the outlet of the immobilized cellobiase column was used for sugars analysis and from the outlet of the cell column for lactic acid analysis. The conversion efficiency of lactic acid from cellulose and the productivity of lactic acid were calculated as follows:

$$\text{Conversion efficiency of lactic acid (\%)} = \frac{\text{concentration of lactic acid produced (g/L)} \times 100\%}{\text{initial concentration of cellulose in substrate (g/L)}}$$

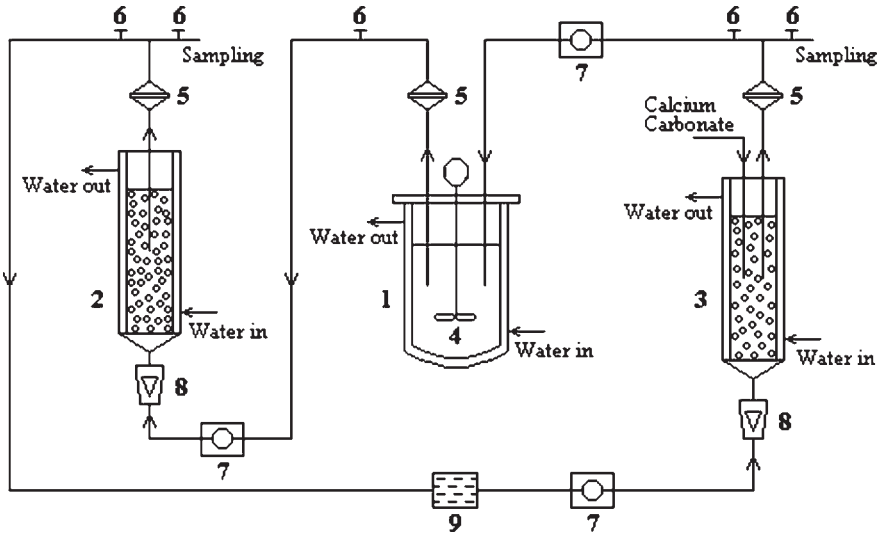

Fig. 1. Synergetic process of enzymatic hydrolysis and lactic acid fermentation from cellulosic material: 1, enzymatic hydrolysis jar; 2, immobilized cellobiase column; 3, immobilized cell column; 4, stirrer; 5, filter; 6, valve; 7, peristaltic pump; 8, flowmeter; 9, medium tank.

Productivity of lactic acid (g/[L·h]) =
concentration of lactic acid produced (g/L)/time of fermentation (h)

Fed-Batch Process

In the process of synergetic hydrolysis and fermentation, a fed-batch technique was used to increase the total concentration of cellulosic substrate. At the beginning of the reaction, 140 g of corncob residue was added to the enzymatic hydrolysis jar (working volume of 1.4 L), and the dosage of cellulase from *T. reesei* was 20 IU (FPA)/g of substrate. The feeding of substrate and enzyme was performed at 12, 24, 36, and 48 h, respectively. For each time, 35 g of corncob residue with a certain amount of cellulase was added to the hydrolysis jar. The total concentration of cellulosic substrate was 200 g/L, and the total dosages of cellulase per gram of substrate were 10, 12.5, 15, 17.5, and 20 IU (FPA), respectively, in different experiments. The time of the synergetic reaction was prolonged to 80 h.

Analysis

FPA and CBA were measured according to the method recommended by Ghose *(24)* and expressed as international units. One international unit of FPA is the amount of enzyme that forms 1 μmol of glucose (reducing sugars as glucose)/min during the hydrolysis reaction. One international unit of CBA is the amount of enzyme that forms 2 μmol of glucose/min from cellobiose.

Reducing sugar was determined using a 3,5-dinitrosalicylic acid colorimetric assay method *(25)*. Glucose, cellobiose, and xylose were analyzed by high-performance liquid chromatography (HPLC) (Waters HPX-87H ion exclusion column). The mobile phase was 0.005 mol/L of aqueous sulfuric acid with a flow rate of 0.6 mL/min at 60EC. Lactic acid was assayed using the HPLC method or the EDTA titration method *(26)*.

All experiments were performed in triplicate. The maximum difference among the three values was <5% of the mean.

Results and Discussion

Enzymatic Hydrolysis of Corncob Residue

In previous research *(16,20)*, we found that in the hydrolysis process on cellulosic material simply using the cellulase from *T. reesei*, the hydrolysis yield was relatively low because of the feedback inhibition to cellulase catalysis caused by the accumulation of cellobiose. When corncob residue (100 g/L) was used as substrate and hydrolyzed by the cellulase from *T. reesei* (20 IU/g of substrate) at pH 4.8 and 50°C for 48 h, the concentration of reducing sugar in the hydrolysate was 48.3 g/L (Table 1). The percentage of glucose in the reducing sugar was relatively low (53.2%), whereas the percentages of cellobiose and other oligosaccharides were quite high (19.5 and 19.9%, respectively). This was owing to the lack of cellobiase in the reaction system.

The previous work proved that the immobilized cellobiase could hydrolyze cellobiose to glucose continuously and efficiently *(20,27)*. In the present work the cellulase from *T. reesei* and the immobilized cellobiase were used synergetically to hydrolyze the corncob residue. After 48 h of hydrolysis, the cellulosic substrate in the hydrolysis jar decreased obviously and the concentration of reducing sugar in the hydrolysate reached 60.5 g/L. The percentage of glucose in the reducing sugar was 88.3%, whereas that of other oligosaccharides was only 4.6%, and no cellobiose was detected (Table 1).

The results indicated that under the synergism of *T. reesei* cellulase and immobilized cellobiase, cellobiose in the hydrolysate was quickly converted into glucose, and most of the other oligosaccharides were also degraded to glucose. Therefore, the concentration of reducing sugar in the hydrolysate greatly increased, and the hydrolysis yield from cellulosic material reached 82.5%, which was 25.2% higher than that in the hydrolysis process by *T. reesei* cellulase (65.9%). Moreover, the ratio of glucose in the total reducing sugar was also improved obviously. Because the glucose converted from corncob could be used further in the production of ethanol, organic acid, single cell protein (SCP), and so on, this work laid a good foundation for the fermentation process using cellulosic hydrolysate as the carbon source.

Table 1
Results of Enzymatic Hydrolysis on Corncob Residue

Enzyme	Concentration of reducing sugar (g/L)	Components of reducing sugar (g/L)				Hydrolysis yield (%)
		Glucose	Cellobiose	Xylose	Others	
T. reesei cellulase	48.3	25.7	9.4	3.6	9.6	65.9
T. reesei cellulase and immobilized cellobiase	60.5	53.4	0	4.3	2.8	82.5

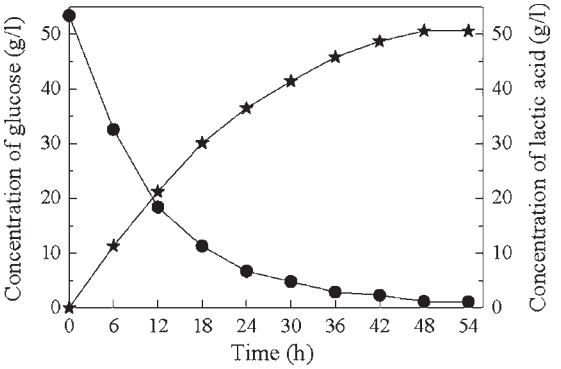

Fig. 2. Time course of lactic acid fermentation by immobilized cells of *L. delbrueckii* using cellulosic hydrolysate: (●) glucose; (★) lactic acid.

Lactic Acid Production by Immobilized Cells Using Cellulosic Hydrolysate

The cellulosic hydrolysate from synergetic hydrolysis on corncob residue was used instead of pure glucose for lactic acid production by the immobilized cells of *L. delbrueckii* ZU-S2. Figure 2 depicts the time course of fermentation. The final concentration of lactic acid reached 50.6 g/L after 48 h, and the remnant glucose was only 1.08 g/L. The results indicated that the immobilized cells of *L. delbruecki* could use cellulosic hydrolysate effectively to produce lactic acid, and about 94.8% of glucose in the cellulosic hydrolysate was converted into lactic acid.

Synergetic Hydrolysis and Lactic Acid Fermentation

The hydrolysis process by *T. reesei* cellulase and the immobilized cellobiase was coupled with lactic acid fermentation by the immobilized cells of *L. delbruecki*. In the course of the synergetic hydrolysis and fermentation, no cellobiose was accumulated, and the hydrolysis product glucose was converted into lactic acid rapidly and continuously. The feedback inhibitions to the cellulase reaction caused by the accumulation of cellobiose and glucose were both eliminated, so the hydrolysis of cellulosic material was performed more effectively. The final concentration of lactic acid reached 56.3 g/L at 60 h, the conversion efficiency of lactic acid from cellulose was 92.4%, and the productivity of lactic acid was 0.938 g/(L·h) (Fig. 3).

Effects of Cellulosic Substrate Concentration on Synergetic Hydrolysis and Fermentation

Different initial concentrations of corncob residue were used under the batch process of synergetic hydrolysis and lactic acid fermentation. The results provided in Table 2 indicate that when the initial concentration

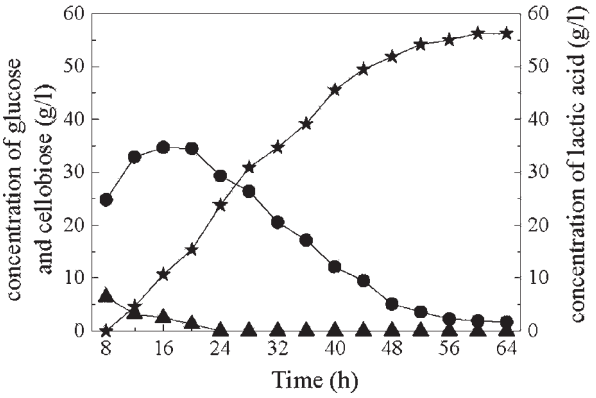

Fig. 3. Time course of synergetic hydrolysis and lactic acid fermentation using corncob residue: (●) glucose; (▲) cellobiose; (★) lactic acid.

of cellulosic substrate increased, the final concentration of lactic acid increased accordingly. However, the conversion efficiency of lactic acid from cellulose decreased and the fermentation period was prolonged. The productivity of lactic acid increased up to a cellulosic substrate concentration of 100 g/L and thereafter decreased with an increase in substrate concentration. It was more difficult to carry out the process of enzymatic hydrolysis because, at a higher concentration of corncob residue, the substrate became thicker and heavier, resulting in stronger resistance to mass transfer. The optimum initial substrate concentration was 100 g/L, at which the productivity presented the highest value of 0.938 g/(L·h), and the conversion efficiency of lactic acid from cellulose was 92.4 after 60 h of fermentation.

Effects of Cellulase Dosage in Fed-Batch Process

A fed-batch technique was used in the synergetic hydrolysis and lactic acid fermentation process to obtain higher product concentration. Different cellulase dosages in the fed-batch process were studied; Table 3 presents the results. The optimum dosage of cellulase was 15 IU (FPA)/g of substrate, which was lower than that in the batch process. Under this condition, the total concentration of cellulosic substrate reached 200 g/L, and the final concentration of lactic acid increased to 107.5 g/L. The larger cellulase dosage had no more obvious contributions to the increase in lactic acid concentration and the conversion efficiency of lactic acid from cellulose.

The process of synergetic hydrolysis and lactic acid fermentation using the fed-batch technique enhanced the utilization of cellulosic substrate and reduced the cost of cellulase. This fundamental research was helpful in accelerating the utilization of cellulosic material to produce lactic acid.

Table 2
Results of Synergetic Hydrolysis and Lactic Acid Fermentation With Different Initial Concentrations of Corncob Residue

Initial concentration of cellulosic substrate (g/L)	Fermentation time (h)	Final concentration of lactic acid (g/L)	Conversion efficiency of lactic acid from cellulose (%)	Productivity (g/[L·h])
60	52	34.8	95.2	0.669
80	56	45.7	93.8	0.816
100	60	56.3	92.4	0.938
120	68	61.2	83.7	0.900
140	80	64.6	75.8	0.808

Table 3
Results of Synergetic Hydrolysis and Lactic Acid Fermentation Under Fed-Batch Process With Different Cellulase Dosages

Total concentration of cellulosic substrate (g/L)	Total dosage of cellulase (IU/g substrate)	Final concentration of lactic acid (g/L)	Conversion efficiency of lactic acid from cellulose (%)	Productivity (g/[L·h])
200	10	84.6	69.5	1.058
200	12.5	92.8	76.2	1.160
200	15	107.5	88.3	1.344
200	17.5	107.7	88.4	1.346
200	20	107.9	88.6	1.349

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References

1. Ajioka, M., Enomoto, K., Suzuki, K., and Yamaguchi, A. (1995), *J. Environ. Polym. Degrad.* **3,** 225–234.
2. Södergård, A. and Stolt, M. (2002), *Prog. Polym. Sci.* **27,** 1123–1163.
3. Tuominen, J., Kylma, J., Kapanen, A., Venelampi, O., Itavaara, M., and Seppala, J. (2002), *Biomacromolecules* **3,** 445–455.
4. Lunt, J. (1998), *Polym. Degrad. Stab.* **59,** 145–152.
5. Vink, E. T. H., Rábago, K. R., Glassner, D. A., and Gruber, P. R. (2003), *Polym. Degrad. Stab.* **80,** 403–419.
6. Newman, K. D. and McBurney, M. W. (2004), *Biomaterials* **25,** 5763–5771.
7. Olsson, L. and Hahn-Hagerdahl, B. (1996), *Enzyme Microb. Technol.* **18,** 312–331.
8. Chen, Y., Xia, L., and Cen, P. (2000), *J. Chem. Eng. Chin. Univ.* **14,** 437–441.
9. Cao, N. J., Xia, Y. K., Gong, C. S., and Tsao, G. T. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 129–139.
10. You, X. (1984), *Production and Application of Xylitol,* China Light Industry Press, Beijing.
11. Xia, L. and Cen, P. (1999), *Process Biochem.* **34,** 909–912.
12. Tolan, J. S. and Foody, B. (1999), *Adv. Biochem. Eng. Biotechnol.* **65,** 41–67.
13. Persson, I., Tjerneld, F., and Hahn-Hagerdahl, B. (1991), *Process Biochem.* **26,** 65–74.
14. Nidetzky, B., Steiner, S., Hayn, M., and Claeyssens, M. (1994), *Biochem. J.* **29,** 705–710.
15. Stockton, B. C., Mitchell, D. J., and Grohmann, K. (1991), *Biotechnol. Lett.* **13,** 57–62.
16. Xia, L. (1998), *Chem. Ind. Forest Products (Chin. J.)* **18,** 23–26.
17. Xia, L. and Shen, X. (2004), *Bioresour. Technol.* **91,** 259–262.
18. Fujii, M., Mori, J., Homma, T., and Taniguchi, M. (1995), *Chem. Eng. J. Biochem. Eng. J.* **59,** 315–319.
19. Gusakov, A. V. and Sinitsyn, A. P. (1992), *Biotechnol. Bioeng.* **40,** 663–672.
20. Shen, X. and Xia, L. (2004), *Process Biochem.* **39,** 1363–1367.
21. Wilson, D. B. and Irwin, D. C. (1999), *Adv. Biochem. Eng.. Biotechnol.* **65,** 1–21.
22. Luo, J., Xia, L., Lin, J., and Cen, P. (1997), *Biotechnol. Prog.* **13,** 762–767.
23. Shen, X. and Xia, L. (2004), *Chem. Ind. Forest Products (Chin. J.)* **24,** 35–38.
24. Ghose, T. K. (1987), *Pure Appl. Chem.* **59,** 257–268.
25. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
26. Tianjin Light Industry College, Dalian Light Industry College, Wuxi Light Industry College. (1980), *Industry Fermentation Analysis,* China Light Industry Press, Beijing.
27. Shen, X. and Xia, L. (2005), *J. Zhejiang Univ. Eng. Sci. (Chin. J.)* **39,** 287–290.