

## D-Lactic Acid Production by *Sporolactobacillus inulinus* Y2-8 Immobilized in Fibrous Bed Bioreactor Using Corn Flour Hydrolyzate

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In this study, a fibrous bed bioreactor (FBB) was used for D-lactic acid (D-LA) production by *Sporolactobacillus inulinus* Y2-8. Corn flour hydrolyzed with  $\alpha$ -amylase and saccharifying enzyme was used as a cost-efficient and nutrient-rich substrate for D-LA production. A maximal starch conversion rate of 93.78% was obtained. The optimum pH for D-LA production was determined to be 6.5. Ammonia water was determined to be an ideal neutralizing agent, which improved the D-LA production and purification processes. Batch fermentation and fed-batch fermentation, with both free cells and immobilized cells, were compared to highlight the advantages of FBB fermentation. In batch mode, the D-LA production rate of FBB fermentation was 1.62 g/l/h, which was 37.29% higher than that of free-cell fermentation, and the D-LA optical purities of the two fermentation methods were above 99.00%. In fed-batch mode, the maximum D-LA concentration attained by FBB fermentation was 218.8 g/l, which was 37.67% higher than that of free-cell fermentation. Repeated-batch fermentation was performed to determine the long-term performance of the FBB system, and the data indicated that the average D-LA production rate was 1.62 g/l/h and the average yield was 0.98 g/g. Thus, hydrolyzed corn flour fermented by *S. inulinus* Y2-8 in a FBB may be used for improving D-LA fermentation by using ammonia water as the neutralizing agent.

**Keywords:** D-Lactic acid, corn flour hydrolyzate, ammonia water, *Sporolactobacillus inulinus*, fibrous bed bioreactor

### Introduction

D-Lactic acid (D-LA), an important precursor of many chiral materials, has various applications in pharmaceuticals, high-efficiency low-toxicity pesticides and herbicides, and cosmetics. Poly D-lactic acid (PDLA) is an important polymer, because it improves the thermostability of poly L-lactic acid (PLLA) by the stereo complex formation [6, 10]. Improvements of the D-LA production rate, product yield, and final product concentration are some of the major considerations for D-LA fermentation. Fibrous bed bioreactors (FBB) using cells immobilized in fibrous matrices have been previously used for improving the efficiency of organic acid fermentation [8, 12, 17, 21]. The FBB is a high-cell-density bioreactor with fibrous matrix as the immobilization carrier and has a better operational stability

than that of a conventional free-cell fermentation system. There have been some FBB studies focused on L-lactic acid (L-LA) production [18, 19, 21]. Previous studies reported a few wide-type D-LA-producing bacteria (DLAB) such as *Lactobacillus delbrueckii* [22], *Lactobacillus coryniformis* [24], *Sporolactobacillus* [23, 25], metabolically engineered *Saccharomyces cerevisiae* [11], and *Escherichia coli* [26], which produced D-LA with high optical purity (Table 1). However, most D-LA production processes utilize free cells for fermentation, which has several limitations such as low productivity, product inhibition, and batch-to-batch reduction of cell vitality, leading to high cost of fermentation. Thus, the FBB was developed for efficient D-LA production by using a homofermentative DLAB, *Sporolactobacillus inulinus* Y2-8, in this study.

A previous study indicated that LA fermentation is

**Table 1.** Comparison of D-LA fermentation by various strains.

Strain	Operation mode	Substrate	D-LA concentration (g/l)	D-LA yield <sup>b</sup> (g/g)	Production rate <sup>a</sup> (g/l/h)	Optical purity
<i>Lactobacillus delbrueckii</i> [22]	Batch culture	Glucose	20.1	1.005	1.67	>99.9%
	Continuous culture (dilution rate 0.87 h <sup>-1</sup> )		20.7	1.0	18.0	
<i>Lactobacillus coryniformis</i> [24]	SSF	Cellulose	24	0.89	0.5	-
<i>Sporolactobacillus</i> [23]	Fed-batch	Glucose	207	-	3.8	99.3%
<i>Sporolactobacillus</i> [25]	Repeated-batch	Glucose	80~90	0.86~0.97	1.77	-
<i>Saccharomyces cerevisiae</i> [11]	Batch fermentation	Glucose	61.5	0.612	0.854	≥99.9%
<i>Sporolactobacillus inulinus</i> (this study)	FBB-batch	Corn flour	145.8	0.965	1.62	>99.0%
	FBB-fed-batch		218.8	-	1.65	
	FBB-repeated batch		149.8	0.98	1.59~1.66	

<sup>a</sup>Production rate (g/l/h) was calculated as the ratio of D-LA concentration (g/l) to the fermentation time (h).

<sup>b</sup>D-LA yield (g/g) was calculated as the ratio of D-LA produced (g) to glucose consumed (g).

largely limited by end-product inhibition [16]. Calcium hydroxide/carbonate is used as the neutralizing agent for conventional D-LA fermentation [3]. The fermentation broth containing calcium lactate is filtered, treated with activated carbon, evaporated, and acidified with sulfuric acid to convert the salt to lactic acid and calcium sulfate; however, this process is expensive (about 50% of the total production cost) and not environmentally friendly [13]. LA production by using ammonia water as the neutralizing agent along with some *Lactobacillus* species, *Bacillus* species, and genetically engineered *Escherichia coli* has shown remarkable success [2, 4, 14, 27]; the byproduct (ammonium sulfate) of its purification process is a good nitrogen fertilizer, unlike the purification process of conventional fermentation, which produces a large quantity of calcium sulfate and gypsum as waste. Therefore, the effectiveness of ammonia water as a neutralizing agent during D-LA production was assessed in this study.

One of the primary expenses of D-LA production is the cost of raw materials. Furthermore, the industrial D-LA fermentation process is usually carried out with homofermentative bacteria, which have complex nutrient requirements that further increase the raw material expenses. In this study, corn flour was used as an economical and nutrient-rich substrate. Corn flour hydrolyzate has been used for the production of ethanol [20], organic acids such as lactic acid [21], and enzymes such as xylanase [7]. The corn flour hydrolyzate can be produced by using either enzyme or acid; however, the acid hydrolysis process produces large quantities of waste acid. From an environmental viewpoint, the enzyme hydrolysis is more sustainable for large-scale industrial production.

In this study, several factors were taken into consideration to determine the optimum fermentation conditions. Corn flour that was hydrolyzed with  $\alpha$ -amylase and saccharifying enzyme was used as a cheap, renewable fermentation substrate. The influence of pH and neutralizing agents on D-LA production were also assessed. Batch and fed-batch operation were used to evaluate the D-LA production efficiency of free cells and immobilized cells. Furthermore, repeated-batch fermentation was conducted to demonstrate the operational stability of the FBB system.

## Materials and Methods

### Microorganism and Media Composition

*Sporolactobacillus inulinus* Y2-8 (China Center for Type Culture Collection (CCTCC) No.208052), an efficient producer of D-LA with high optical purity, was used in this study. The strain was cultivated under anaerobic conditions at 37°C. The agar medium was composed of (per liter) 100 g glucose, 2 g yeast extract, 2 g peptone, 2 g KH<sub>2</sub>PO<sub>4</sub>, 2 ml corn steep liquor, 2 g sodium acetate, 0.4 g MgSO<sub>4</sub>, 0.01 g FeSO<sub>4</sub>·7H<sub>2</sub>O, and 0.01 g MnSO<sub>4</sub>·H<sub>2</sub>O. The seed culture medium was composed of (per liter) 20 g glucose, 2 g yeast extract, 2 g peptone, 5 ml corn steep liquor, 0.4 g MgSO<sub>4</sub>, 0.01 g FeSO<sub>4</sub>·7H<sub>2</sub>O, and 0.01 g MnSO<sub>4</sub>·H<sub>2</sub>O. The fermentation medium was composed of a certain concentration of corn flour hydrolyzate, 5 g/l yeast extract, 1 g/l MgSO<sub>4</sub>, 0.01 g/l FeSO<sub>4</sub>·7H<sub>2</sub>O, and 0.01 g/l MnSO<sub>4</sub>·H<sub>2</sub>O. Unless otherwise noted, the concentration of corn flour was calculated as the concentration of glucose from enzyme hydrolysis.

### Pretreatment of Corn Flour

Commercially available corn flour (Dongxu Oils and Seasoning Co, Ltd, Shandong, PRC), containing 75% of starch, was used as

raw material for D-LA fermentation. The thermostable  $\alpha$ -amylase (70,000 U/ml) and saccharifying enzyme (100,000 U/ml) used in this study were gifts from Henan Tianguan Group Co., Ltd. The pretreatment of corn flour involved two steps: liquefaction and saccharification. The liquefaction step was performed by the addition of  $\alpha$ -amylase into a mixture of 100 g of corn flour and 500 ml of water. The temperature was maintained at 90°C, and the liquefaction process was carried out for 1.5 h. The mixture was then cooled to 60°C for the saccharification process. The saccharifying enzyme was added into the mixture and the saccharification process was performed for 5 h to convert most of the starch to glucose.

### Effects of pH and Neutralizing Agents on D-LA Fermentation

The effects of pH and neutralizing agents on D-LA production were assessed using an incubator shaker equipped with a pH control system. The fermentation was performed with 300 ml of broth in a 750 ml flask that was incubated at 37°C with a shaker rotation speed of 150 rpm. D-LA production was assessed at broth pH values of 5.0, 5.5, 6.0, 6.5, and 7.0. The impact of pH maintenance by using various neutralizing agents (calcium carbonate, sodium hydroxide, or ammonia water) on D-LA fermentation was also assessed.

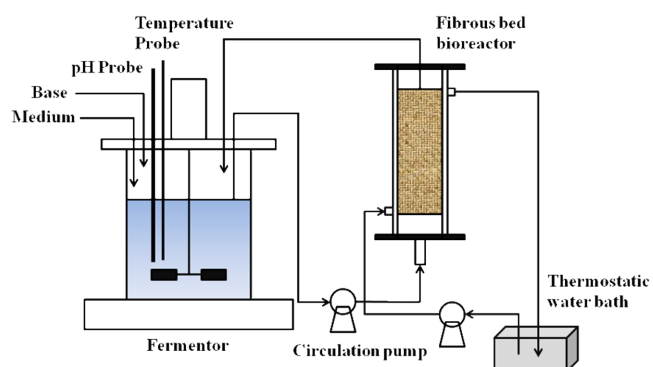
### Fermentative Production of D-LA

Free-cell fermentation was carried out in a 7.5 L fermentor (BioFlo 3000; New Brunswick Scientific Co., NJ, USA) containing 3 L of fermentation medium inoculated with 300 ml of inoculum from a serum bottle. The fermentor was equipped with a pH and temperature control system. The fermentation was performed at 37°C at 100 rpm. The pH of the fermentation broth was maintained at 6.5 by using ammonia water. Anaerobic conditions were maintained by initially treating the medium with nitrogen for 30 min.

Immobilized-cell fermentation was carried out with cells immobilized in an FBB connected to a 7.5 L fermentor through a recirculation loop (Fig. 1). The FBB, with a working volume of 1.5 L, consisted of a stainless steel column packed with spiral wound cotton towel. To maintain the temperature during fermentation, the FBB was equipped with a cooling jacket that contained warm water obtained from a thermostatic water bath. The fermentation temperature was set at 37°C, and the pH was maintained at 6.5 by adding ammonia water. Previous studies provide detailed description of the FBB construction [17, 18].

After inoculating 300 ml of seeds into the fermentor, the cells were grown for 24 h to reach an approximate optical density ( $OD_{600}$ ) of 4.0. The fermentation broth was then circulated at a flow rate of 30 ml/min through the FBB to allow cells to attach and immobilize onto the fibrous matrix. The process was carried out for 48 h to enable immobilization of the cells in the FBB.

Next, both free-cell and immobilized-cell batch fermentations were performed to evaluate the D-LA production efficiency of the two methods. Thereafter, fed-batch fermentations were carried



**Fig. 1.** Schematic diagram of the fibrous bed bioreactor system.

out by using the multi-pulse feeding strategy to determine the maximum attainable D-LA concentration. Multi-pulse feeding involved adding glucose to the fermentor when its residual glucose concentration was lower than 10 g/l, and the process was continued until the concentration of D-LA in the broth was constant. Finally, immobilized-cell repeated-batch fermentation was performed by removing the broth from the FBB and fermentor after depletion of sugars in the broth, and fresh fermentation medium was pumped into the immobilized reaction system. Samples were collected at regular intervals for the analysis of biomass and for measuring the concentrations of reducing sugars and D-LA in the broth.

### Analytical Methods

The cell density of the fermentation broth was measured by a spectrophotometer (UV-2600; Unico, Shanghai, China) at a wavelength of 660 nm, and the dry cell weight per liter (DW/l) was determined by using a linear correlation factor ( $1 OD_{660} = 0.393 \text{ g DW/l}$ ). The concentrations of D-LA and L-LA were determined by using a high-performance liquid chromatography system with a tunable UV detector at 254 nm (Agilent 1100 series; Hewlett-Packard, USA). A chiral Sumichiral OA-5000 column ( $150 \times 4.6 \text{ mm I.D.}$ ; Sumika Chemical Analysis Service, Osaka, Japan) was used with 2 mM  $\text{CuSO}_4$  for the mobile phase at a flow rate of 1 ml/min, and the column temperature was maintained at 50°C. The optical purity of D-LA was calculated as follows:  $\text{D-LA optical purity} = \text{D-LA} / (\text{D-LA} + \text{L-LA}) \times 100\%$ . The residual glucose of the corn flour hydrolyzate or the fermentation broth was measured using a SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, P. R. China).

## Results and Discussion

### Two-Enzyme Hydrolysis of Corn Flour

Various dosages of  $\alpha$ -amylase (385, 752.5, 1,120, 1,488, and 1,855 U/100 g of corn flour) and various dosages of saccharifying enzyme (5,000, 12,500, 20,000, 27,500, and

**Table 2.** Effects of different  $\alpha$ -amylase and saccharifying enzyme dosages.

$\alpha$ -Amylase dosage (U/100 g of corn flour)	Starch conversion rate <sup>a</sup> (%)	Saccharifying enzyme dosage (U/100 g of corn flour)	Starch conversion rate (%)
385	83.71	5,000	32.12
752.5	88.38	12,500	68.50
1,120	93.67	20,000	93.78
1,488	90.59	27,500	94.42
1,855	84.13	35,000	83.96

<sup>a</sup>Starch conversion rate (%) was calculated as the ratio of the quantity of glucose obtained by enzyme hydrolysis (g) to the quantity of starch of the corn flour (g).

35,000 U/100 g of corn flour) were used, as shown in Table 2. The starch conversion rate was calculated as the ratio of the quantity of glucose obtained by enzyme hydrolysis (g) to the quantity of starch of the corn flour (g). For investigating the effects of various amounts of  $\alpha$ -amylase on the corn flour hydrolysis, the dosage of saccharifying enzyme was set at 27,500 U/100 g of corn flour. For investigating the effects of various amounts of saccharifying enzyme on the corn flour hydrolysis, the dosage of  $\alpha$ -amylase was set at 1,120 U/100 g of corn flour. The optimum  $\alpha$ -amylase dosage (1,120 U/100 g of corn flour) and the optimum saccharifying enzyme dosage (27,500 U/100 g of corn flour) were determined to yield the highest starch conversion rate (94.42%). A slightly lower starch conversion rate (93.78%) was obtained using a saccharifying enzyme dosage of 20,000 U/100 g of corn flour. After considering both cost and efficacy, the optimal enzyme dosages for hydrolysis were determined to be 1,120 U/100 g of corn flour and 20,000 U/100 g of corn flour for  $\alpha$  amylase and saccharifying enzyme, respectively.

### Effects of pH on D-LA Fermentation Process

The effects of pH on D-LA production by *S. inulinus* Y2-8 are shown in Table 3. The initial concentration of the corn flour hydrolyzate was 150 g/l, and the D-LA yields at pH 5.0, 5.5, and 6.0 were 0.882 g/g, 0.898 g/g, and 0.901 g/g, respectively. The D-LA yield was calculated as the ratio of

the quantity of D-LA produced (g) to the quantity of corn flour hydrolyzate consumed (g). In addition, the D-LA production rates at pH 5.0, 5.5, and 6.0 were determined to be 0.88 g/l/h, 0.97 g/l/h, and 1.11 g/l/h, respectively. The maximum D-LA yield (0.912 g/g) was obtained at pH 6.5 with a production rate of 1.18 g/l/h. A slightly lower D-LA yield (0.908 g/g) and production rate (1.12 g/l/h) were obtained at the pH value of 7.0. Thus, the optimal pH value for D-LA production by *S. inulinus* Y2-8 was determined to be 6.5.

Although the D-LA yields and D-LA production rates at pH values above 6.0 were better than those obtained at pH 5.0 and 5.5, the cells grew better at pH 5.0 and 5.5. The maximal OD<sub>660</sub> values of the fermentation broths at pH 5 and 5.5 were above 7.5. However, the maximum OD<sub>660</sub> value of the fermentation broths at pH values above 6.0 was approximately 6.5. Thus, although relatively high pH values can promote D-LA production, they have an adverse effect on the strain growth.

The data from this study indicated that the optimum pH range for *S. inulinus* Y2-8 was 4.0–8.5. A previous study showed that for a given LA concentration, lowering the fermentation broth pH from 7.5 to 5.5 increases the concentration of undissociated LA present in the medium by 100-fold [5]. Furthermore, previous studies that compared the growth characteristics of various LA producers showed that the optimum pH for LA production varies between 5.0

**Table 3.** Effects of pH on D-LA fermentation.

pH	Production rate <sup>a</sup> (g/l/h)	D-LA yield <sup>b</sup> (g/g)	D-LA concentration (g/l)	Maximum OD <sub>660</sub>
5.0	0.90	0.882	98.54	7.76
5.5	0.97	0.898	112	7.53
6.0	1.11	0.901	121	6.68
6.5	1.18	0.911	128.9	6.66
7.0	1.12	0.908	122.5	6.53

<sup>a</sup>Production rate (g/l/h) was calculated as the ratio of D-LA concentration (g/l) to the fermentation time (h).

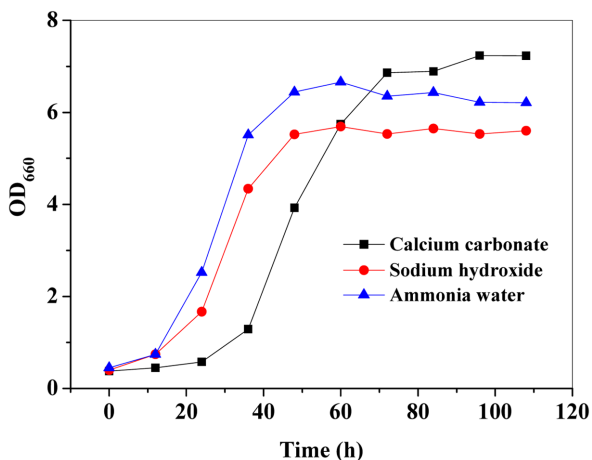
<sup>b</sup>D-LA yield (g/g) was calculated as the ratio of D-LA produced (g) to glucose consumed (g).

and 7.0, and a pH below 5.7 was only optimum for *Lactobacillus* strains, which can tolerate lower pH values than other strains [9]. Thus, effective neutralization agents should be incorporated into the fermentation broth to obtain high D-LA yields without causing detrimental effects on the fermenting bacteria.

### Effects of Various Neutralizing Agents on D-LA Fermentation Process

The pH of the fermentation broth incubated in shaker flasks was maintained at 6.5 by adding either 6 mol/l of sodium hydroxide or 6 mol/l of ammonia water. The pH of the fermentation broths was maintained at 5.0 by adding excess calcium carbonate. The data indicated that *S. inulinus* cells inoculated in fermentation broths neutralized by sodium hydroxide and ammonia water reached the logarithmic phase earlier than those inoculated in fermentation broths neutralized by calcium carbonate (Fig. 2). The maximal OD<sub>660</sub> of fermentation broth neutralized with calcium carbonate was 7.23, which was higher than that of fermentation broths neutralized by sodium hydroxide (5.59) and ammonia water (6.67). The D-LA production rate of the fermentation broths neutralized by ammonia water (1.20 g/l/h) was considerably higher than that of the fermentation broths neutralized by sodium hydroxide (1.16 g/l/h) and calcium carbonate (0.67 g/l/h; Fig. 3). Furthermore, the fermentation broths neutralized with ammonia water had the highest final D-LA concentration (131.6 g/l).

The D-LA fermentation performance obtained by using ammonia water as the neutralizing agent was better than that obtained by using sodium hydroxide or calcium

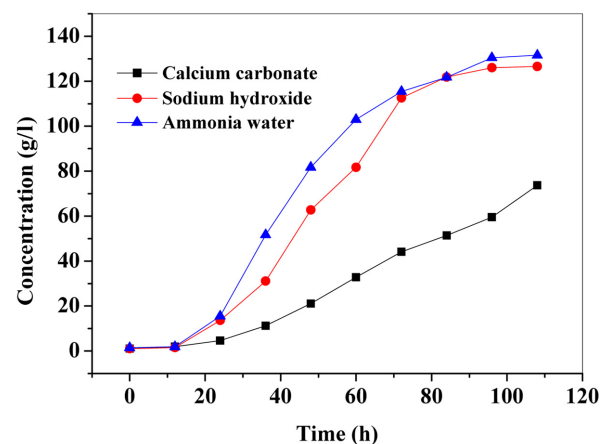


**Fig. 2.** Effects of various neutralizing agents on the growth of *Sporolactobacillus inulinus* Y2-8.

carbonate. Tashiro *et al.* [22] used *Lactobacillus delbrueckii* subsp. *lactis* QU 41 for D-LA production and found that the D-LA production rate was higher with ammonia water (1.67 g/l/h) than that obtained using sodium hydroxide (1.3 g/l/h). These results are consistent with the findings of the present study, but their optimal D-LA concentration was low (20.1 g/l). Calcium carbonate maintained the broth pH at approximately 5.0, which is not an optimum pH for D-LA production by *S. inulinus* Y2-8. Conventional D-LA fermentation, which involves adding excess calcium carbonate, is expensive and not environmentally friendly, because its purification process consumes a huge amount of sulfuric acid and produces a large quantity of calcium sulfate and gypsum as waste [13]. In this study, the fermentation broth neutralized by ammonia water (pH = 6.5) contained a mixture of LA and ammonium lactate. However, the pH of the fermentation broth can be adjusted below the pKa (3.86) of LA by adding sulfuric acid; then LA can be extracted from the broth by using macroporous adsorption resins, and its byproduct (ammonium sulfate) is a good nitrogen fertilizer. It is also worth mentioning that ammonium lactate is an animal feed that can be assimilated by rumen bacteria [15], and ammonium lactate is a feedstock for ethyl lactate, a nontoxic and biodegradable solvent [13]. Therefore, fermentation with ammonia water as a neutralizing agent is also a more economically feasible option.

### Batch Fermentation with Free Cells and Immobilized Cells

The kinetics of batch fermentation with free cells and immobilized cells were characterized by inoculating the fermentation broth with new seed broth, followed by a 12 h lag for free-cell fermentation. Extra seeds were not



**Fig. 3.** Effects of various neutralizing agents on D-LA production.

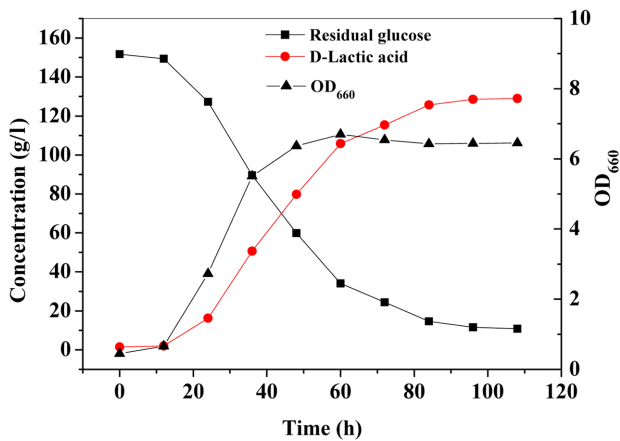


Fig. 4. Kinetics of D-LA batch fermentation with free cells.

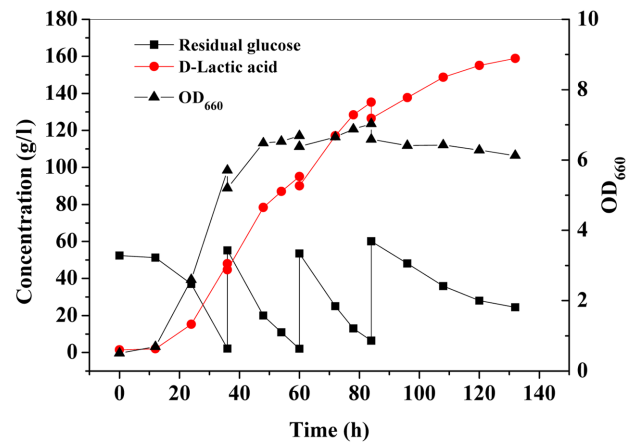


Fig. 6. Kinetics of D-LA fed-batch fermentation with free cells.

inoculated for FBB fermentation by using immobilized cells on fibrous matrix. In the FBB system, most cells attached to the fibrous matrix. Thus, the maximum  $OD_{600}$  of the free-cell fermentation broth (6.70; Fig. 4) was higher than that of the FBB fermentation broth (5.52; Fig. 5). The D-LA production rate of FBB fermentation was 1.62 g/l/h, which was 37.29% higher than the production rate of free-cell fermentation (1.18 g/l/h). The final D-LA concentration and yield of FBB fermentation were 145.8 g/l and 0.965 g/g, respectively. The final D-LA concentration and yield of free-cell fermentation were 128.9 g/l and 0.905 g/g, respectively. Thus, D-LA production was considerably improved by using FBB owing to the high cell density of the FBB system.

The optical purity of D-LA is vital to the physical properties of PDLA; the D-LA optical purities of both free-cell and FBB fermentation in this study were above 99.00%, and the results were similar to the optical purity of D-LA

produced by *Sporolactobacillus* sp. CASD [23]. The optical purity of metabolically engineered *Saccharomyces cerevisiae* could reach 99.9% or higher, but its D-LA yield was only 61.2% [11]. Yáñez *et al.* [24] obtained D-LA from cellulose by *Lactobacillus coryniformis* subsp. *torquens* using simultaneous saccharification and fermentation (SSF) with the yield of 0.89 g/g at a production rate of 0.5 g/l/h; the production rate of SSF was much lower than that of the direct fermentation from corn flour hydrolyzate in this study.

#### Fed-Batch Fermentation with Free Cells and Immobilized Cells

Fed-batch fermentations with both free cells and immobilized cells were carried out to investigate the maximum D-LA concentration attainable. Substrate inhibition was avoided in fed-batch fermentation by using the multi-pulse feeding strategy. Both fermentation reactions were

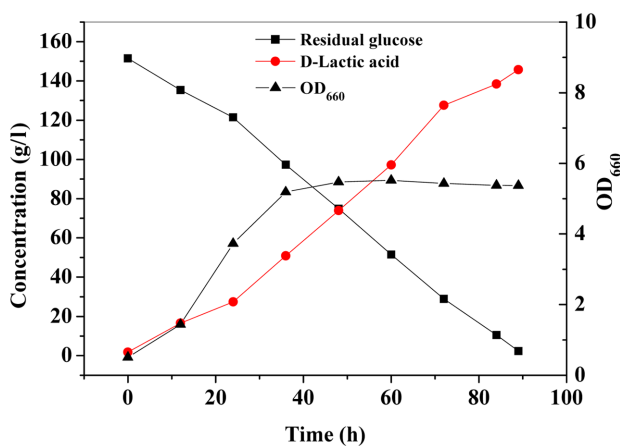


Fig. 5. Kinetics of D-LA batch fermentation with immobilized cells.

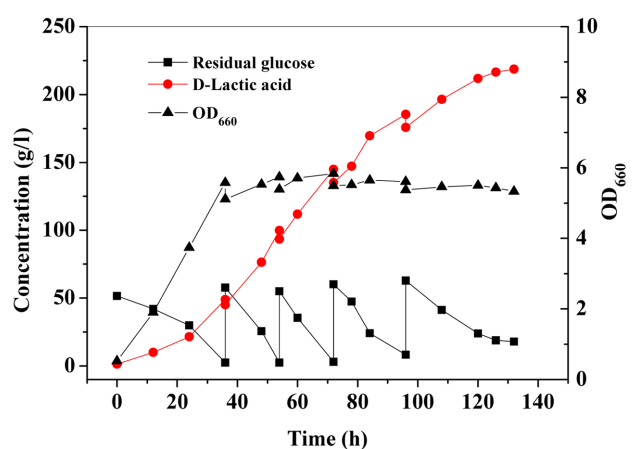
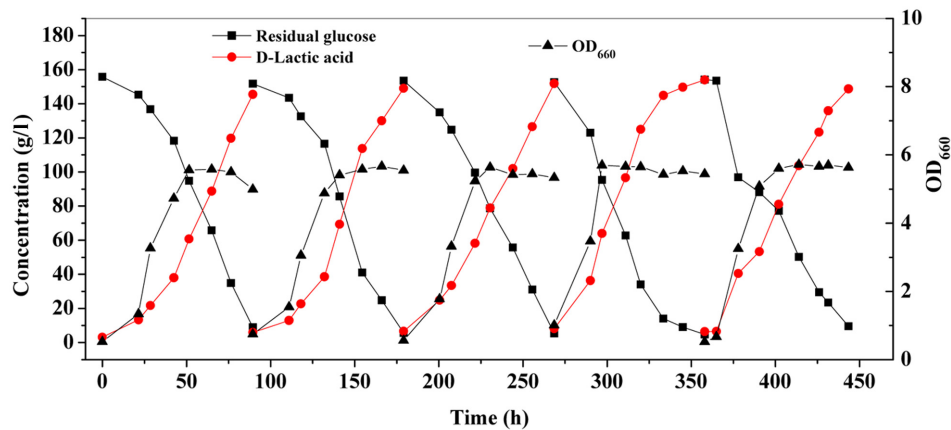


Fig. 7. Kinetics of D-LA fed-batch fermentation with immobilized cells.



**Fig. 8.** Kinetics of D-LA repeated-batch fermentation with immobilized cells.

performed for 132 h, and the maximum  $OD_{660}$  values of free-cell and FBB fermentations were 7.03 and 5.74, respectively (Figs. 6 and 7). The maximum D-LA concentration of 218.8 g/l was obtained by FBB fed-batch fermentation after five pulse feedings of 250 g/l corn flour hydrolyzate. The D-LA concentration obtained by FBB fed-batch fermentation was 37.67% higher than that (158.91 g/l) of free-cell fed-batch fermentation after four pulse feedings of 200 g/l corn flour hydrolyzate. The D-LA production rate of FBB fed-batch fermentation was 1.65 g/l/h, which was 38.66% higher than that (1.19 g/l/h) of free-cell fed-batch fermentation.

The fed-batch fermentation system maintains a low substrate concentration, because its nutrient depletion rate is higher than that of batch and continuous fermentations; therefore, the fed-batch fermentation system exhibits lower substrate inhibition [1]. In this study, after feeding of approximately 150 g/l of corn flour hydrolyzate, the D-LA production rates of free-cell and FBB fed-batch fermentations were 1.59 g/l and 1.99 g/l, respectively; both production rates were much higher than those obtained with batch fermentations with 150 g/l of initial corn flour hydrolyzate

concentration. In fed-batch fermentation, the substrate is fed continuously or sequentially to the fermentor without fermentation broth removal, which results in severe product inhibition caused by high lactic acid accumulation [4]. Wang *et al.* [23] obtained high D-LA production of 207 g/l at the production rate of 3.8 g/l/h by free *Sporolactobacillus* sp. CASD with fed-batch fermentation. Shi *et al.* [19] obtained 142 g/l of L-LA from Jerusalem artichoke hydrolyzate by using immobilized *Lactococcus lactis* ATCC19435 in a FBB with the fed-batch fermentation method; this L-LA concentration was 27.92% higher than that obtained with fed-batch fermentation using free cells (103 g/l). In this study, the maximum D-LA concentration of FBB fed-batch fermentation was 33.86% higher than that obtained by free-cell fermentation. Thus, fermentation with immobilized cells was better than that with free cells, because the immobilized cells of the FBB system had an increased tolerance to high LA concentrations.

#### Repeated-Batch Fermentation in FBB

Repeated-batch fermentation was also studied to evaluate the long-term performance of the FBB system for

**Table 4.** Kinetics of D-LA repeated-batch fermentation with immobilized cells in FBB.

Batch	Production rate <sup>a</sup> (g/l/h)	D-LA yield <sup>b</sup> (g/g)	D-LA concentration (g/l)	Maximum $OD_{660}$
1	1.59	0.969	145.53	5.58
2	1.60	0.981	149.08	5.67
3	1.62	0.98	151.78	5.64
4	1.63	0.985	153.92	5.66
5	1.66	0.984	148.68	5.72

<sup>a</sup>Production rate (g/l/h) was calculated as the ratio of D-LA concentration (g/l) to the fermentation time (h).

<sup>b</sup>D-LA yield (g/g) was calculated as the ratio of D-LA produced (g) to glucose consumed (g).

D-LA production. Cells showed robust growth and did not enter the lag phase throughout the fermentation process (Fig. 8). The maximum optical density of each batch fermentation broth was approximately 5.5 (Table 4). The average initial corn flour hydrolyzate concentration was 150 g/l, and the average D-LA concentration at the end of the fermentation reaction was 149.8 g/l. Thus, the high glucose and D-LA concentrations caused substantial substrate inhibition and product inhibition. However, the average D-LA yield of each batch was 0.98 g/g. Thus, immobilized cells were more tolerant to the high concentrations of both substrates and products after adaptation to the first batch fermentation. The D-LA production rate increased with each repeat cycle of the fermentation batches and ranged from 1.59 to 1.66 g/h/l. Our data indicated that the FBB system was stable for long-term operation (at least 450 h of fermentation process). Furthermore, the resistance of immobilized cells to the high concentration of substrates and products may be useful for developing an economical large-scale D-LA production method.

Zhao *et al.* [25] studied the kinetics of repeated-batch fermentation for D-LA production by *Sporolactobacillus* sp. strain CASD, and showed that D-LA was influenced by cell growth as the inoculum batches were used in repeat cycles. Furthermore, the study showed that the improvement in fermentation was due to the rapid overall growth of *Sporolactobacillus* sp. strain at an early stage of fermentation rather than due to the cell maintenance at the stationary phase [25]. The optical density of the broth was maintained at a certain value in the mid-to-late fermentation period of each batch owing to the cell attachment to the fibrous matrix (Fig. 5). However, D-LA was produced and corn flour hydrolyzate was consumed at a relatively high rate in this period, indicating strong vitality of the immobilized cells. Shi *et al.* [19] studied L-LA repeated-batch fermentation and showed that if the initial concentration of reducing sugars was below 100 g/l, the fermentation cycle was relatively short, and the cell density as well as the titer of L-LA could only increase moderately. Their study showed that the initial increase of fructose content prolonged the fermentation process, and that the cell density and L-LA production increased concomitantly. However, for economical purposes, the optimum concentration of reducing sugars should be in the range of 100–150 g/l. In this study, the initial glucose concentration was 150 g/l and the final residual glucose concentration was maintained below 10 g/l for each batch. Thus, a high D-LA yield and high D-LA production rates were obtained using repeated-batch fermentations with immobilized *S. inulinus* Y2-8.

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