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Evaluation of L-Lactic Acid Production in Batch, Fed-batch, and Continuous Cultures of *Rhizopus* sp. MK-96-1196 Using an Airlift Bioreactor

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Abstract Various processes which produce L-lactic acid using ammonia-tolerant mutant strain, *Rhizopus* sp. MK-96-1196, in a 3 L airlift bioreactor were evaluated. When the fed-batch culture was carried out by keeping the glucose concentration at 30 g/L, more than 140 g/L of L-lactic acid was produced with a product yield of 83%. In the case of the batch culture with 200 g/L of initial glucose concentration, 121 g/L of L-lactic acid was obtained but the low product yield based on the amount of glucose consumed. In the case of a continuous culture, 1.5 g/L/h of the volumetric productivity with a product yield of 71% was achieved at dilution rate of 0.024 h^{-1}. Basis on these results three processes were evaluated by simple variable cost estimation including carbon source, steam, and waste treatment costs. The total variable costs of the fed-batch and continuous cultures were 88% and 140%, respectively, compared to that of batch culture. The fed-batch culture with high L-lactic acid concentration and high product yield decreased variable costs, and was the best-suited for the industrial production of L-lactic acid.

Keywords: *Rhizopus*, L-lactic acid, airlift bioreactor, ammonia-tolerant strain, fed-batch culture

INTRODUCTION

We have already reported a mutant strain, *Rhizopus* sp. MK-96-1196, which produced L-lactic acid with a high yield under a pH controlled with ammonium hydroxide, and a purification of free L-lactic acid from the culture broth with a butanol esterification method [1,2]. For industrial-scale production of L-lactic acid as a feedstock for biodegradable plastics, *i.e.*, poly lactic acid (PLA), further reduction of the production cost is urgently required in order to compete with traditional synthetic chemical commodities [3]. Many researchers have already investigated a number of different methods by which the total production cost may be reduced. Hamamuci *et al.* produced L-lactic acid using alginate-immobilized *R. oryzae* in a tapered-column fed-batch reactor [4]. They demonstrated that it was possible to produce 73 g/L of L-lactic acid in 44.5 h with a yield of 64.8% by weight based on the amount of glucose consumed. Sun *et al.* also produced L-lactic acid with continuous culture by immobilized *R. oryzae* in polyurethane foam cubes using an airlift bioreactor, giving 1~6 g/L/h of L-lactic acid volumetric productivity at 0.04~0.2 h^{-1} of dilution rates [5]. In contrast, Yuru *et al.* studied the production of L-lactic acid by immobilizing *R. oryzae* in polyurethane particles using a three-phase fluidized bed, showing the average yield of 8 repeated batches to be higher than 75% based on the amount of glucose consumed [6].

To date, however, there has been no report on the economical evaluation of the production of L-lactic acid by fermentation. In this paper, batch, single stage steady-state continuous and fed-batch cultures of the *Rhizopus* sp. MK-96-1196 ammonium-tolerant fungi were carried out in a 3 L airlift bioreactor and these culture processes were economically evaluated.

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MATERIALS AND METHODS

Microorganism and Culture Media

Rhizopus sp. MK-96-1196 was used throughout this study. The medium compositions of the pre-culture and production, and their preparation were described in the previous study [1,2].

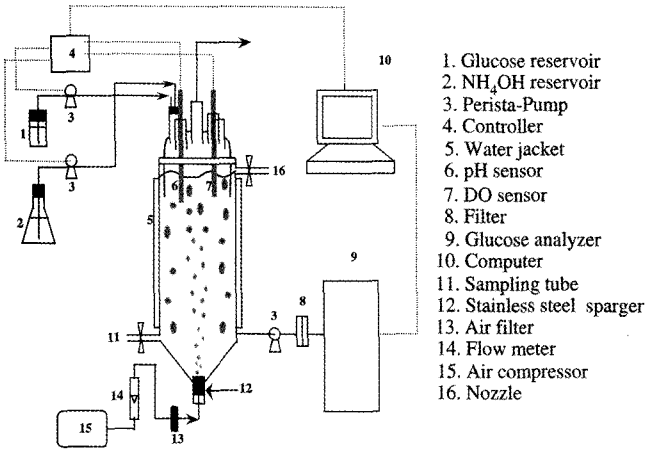

Fig. 1. A schematic diagram of the 3 L airlift bioreactor and its control system.

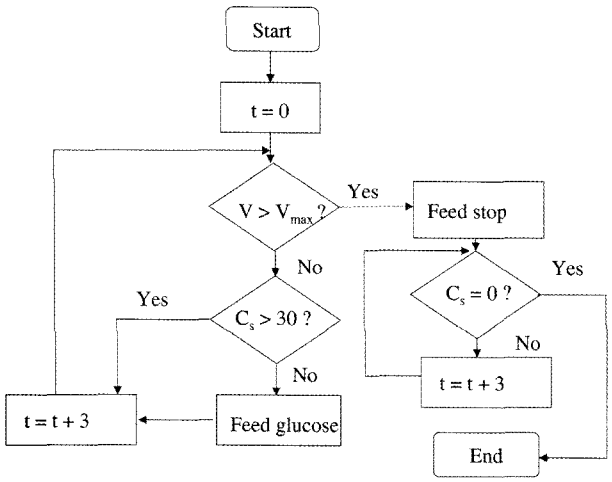

Fig. 2. The glucose feeding policy in the fed-batch L-lactic acid fermentation in a 3 L airlift bioreactor. t, V, and C denote time, culture volume, and concentration, respectively. Subscript max, s, and f denote maximum, glucose, and final respectively.

Culture Method

Batch and fed-batch cultures were carried out in a 3 L airlift bioreactor as shown in Fig. 1. Culture conditions, such as temperature and pH, were described in the previous study [1,2].

Continuous cultures were carried out employing the airlift bioreactor used in batch and fed-batch cultures. When the culture had reached a mid-exponential phase, the fresh medium was supplied from a feeding pump at a desired dilution rate and the culture broth was allowed to overflow from the nozzle (port 16 in Fig. 1) in order to keep the liquid volume at 2.0 L. Once the fermentation had reached a steady state, the dilution rate was maintained until at least three working volumes had been changed.

Fed-batch culture was carried out with 1.7 L starting medium containing 60 g/L glucose. The 75% concentrated glucose solution was fed to keep the glucose concentration in the broth above 30 g/L until the broth volume in the reactor reached a maximum value (2.3 L) in fed-batch culture using the FermExpert 2.1 system (Marubishi, Tokyo, Japan). A connecting glucose on-line sensor (BF-400, ABLE, Tokyo, Japan) was also used. The glucose feeding algorithm used in this system is shown in Fig. 2.

Batch, continuous, and fed-batch cultures were carried out in duplicate, and the average was presented.

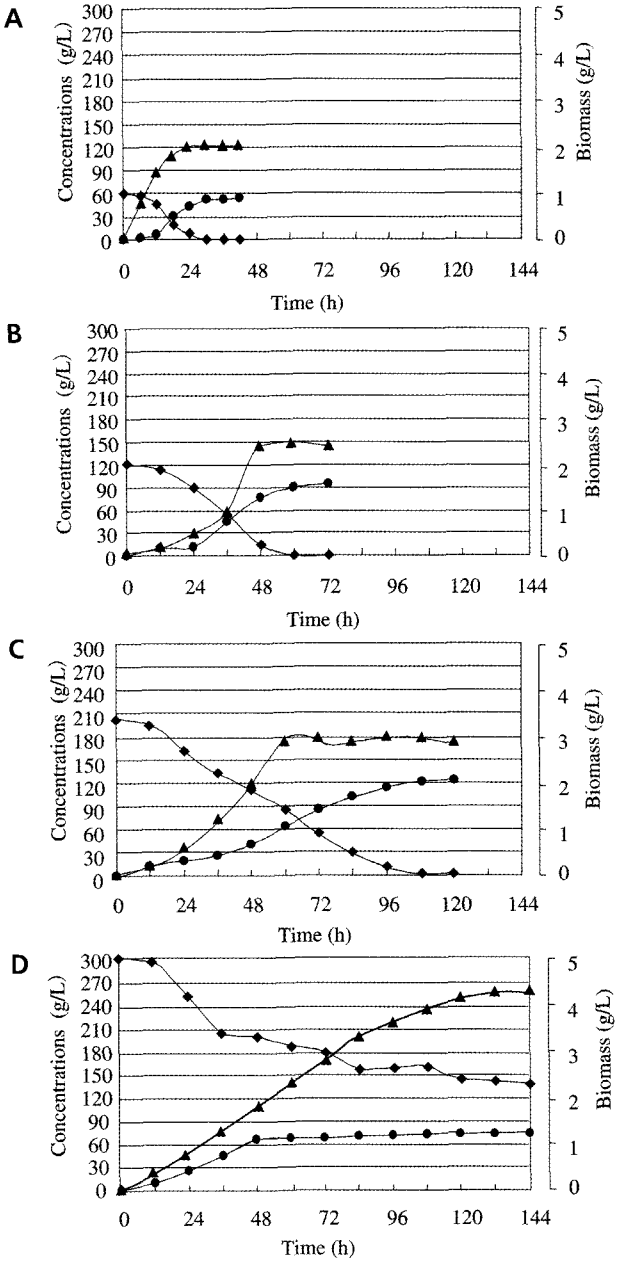

Fig. 3. The batch L-lactic acid fermentations in a 3 L airlift bioreactor at different initial glucose concentrations: 60 g/L (A), 120 g/L (B), 200 g/L (C), and 300 g/L (D). Symbols: (●), L-lactic acid; (▲), biomass; (◆), glucose.

Table 1. Yield coefficients in the batch cultures

Concentration (g/L)				Specific growth rate ($\times 10^{-2}$ h^{-1})	Product yield ($Y_{P/S}$) ($\times 10^{-1}$ g/g)	Biomass yield ($Y_{x/s}$) ($\times 10^{-2}$ g/g)
Initial glucose	Residual glucose	Biomass	L-Lactic acid			
60	0	2.1	53	13.0	8.8	3.5
120	0	2.5	91	6.6	7.6	2.1
200	0	3.0	121	5.8	6.1	1.5
300	140	4.3	73	3.7	2.4	1.4

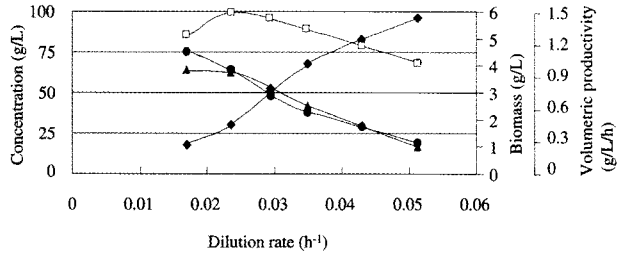

Fig. 4. L-Lactic acid fermentation with continuous culture in different dilution rates. Symbols: (●), L-lactic acid; (▲), biomass; (◆), glucose; (□), L-lactic acid volumetric productivity.

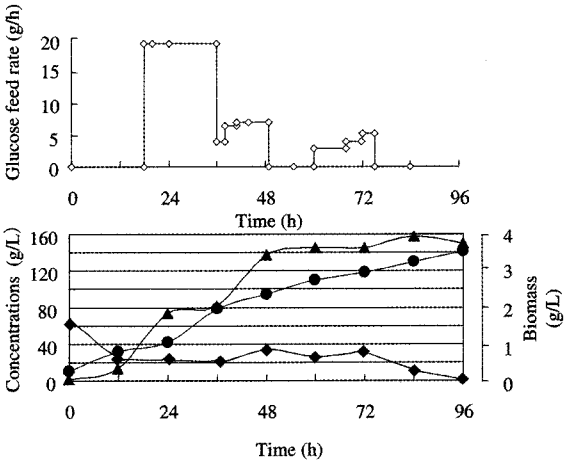

Fig. 5. Fed-batch L-lactic acid fermentation. Symbols: (●), L-lactic acid; (▲), biomass; (◆), glucose; (◇), glucose feeding rate.

Analytical Methods

The biomass, lactic acid, and residual glucose concentrations have been described in separate papers [1,2].

RESULTS

Batch Production of L-Lactic Acid

The L-lactic acid fermentations were carried out in varying initial glucose concentrations of 60, 120, 200, and 300 g/L. The corresponding fermentation time-courses are shown in Fig. 3. The yield coefficients of the biomass ($Y_{x/s}$) and product yields based on initial glucose ($Y_{P/S}$), and L-lactic acid concentrations are summarized in Table 1. When the 200 g/L of initial glucose concentration was used, the maximum L-lactic acid concentration was 121 g/L, but the $Y_{P/S}$ was lower than those with initial glucose concentrations of 60 and 120 g/L. On the other hand, the cell growth was inhibited by high glucose concentration, because the specific growth rate of *Rhizopus* sp. MK-96-1196 at the initial glucose concentration of 120 g/L was half to that at 60 g/L (Table 1).

Continuous Production of L-Lactic Acid

The single stage steady-state continuous culture was carried out with different dilution rates from 0.017 to 0.051 h^{-1}. The concentrations of biomass, L-lactic acid, glucose, and L-lactic acid volumetric productivity are shown in Fig. 4. When the dilution rate was 0.024 h^{-1}, the highest volumetric productivity, 1.5 g/L/h was obtained with concentrations of L-lactic acid and residual glucose of 65 and 31 g/L, respectively.

L-Lactic Acid Production in Fed-batch Culture

In the continuous culture described in the previous section, the volumetric productivity was the highest at a dilution rate of 0.024 h^{-1}, where the remaining glucose concentration was 31 g/L. Referring to these continuous culture data, fed-batch culture with an initial glucose concentration of 60 g/L was carried out by feeding 75% glucose solution to maintain the glucose concentration at around 30 g/L, according to the feeding algorithm as shown in Fig. 2. When the working volume reached 2.3 L, the glucose feeding was stopped, but the fermentation was continued until glucose was completely consumed. As shown in Fig. 5, 141 g/L of L-lactic acid was produced for the culture time of 96 h with $Y_{P/S}$ of 83%.

Economic Evaluation of L-Lactic Acid Production

The total production cost is generally divided into fixed and variable costs. If the production of L-lactic acid is assumed in an existing plant, the fixed costs of labor and investment are assumed to be almost equal irrespective of the culturing processes. Here, the three kinds of culture processes were economically evaluated only by the variable costs of raw materials, steam, and waste treatment.

The process of fermentation, filtration, and evapora-

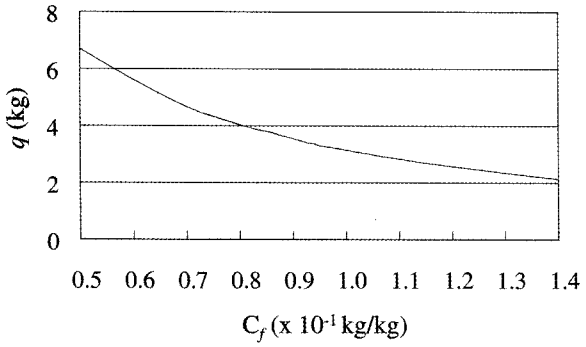

Fig. 6. Simulation of the correlation between L-lactic acid concentration and a required amount of steam for evaporation.

tion for producing a 90% concentrated L-lactic acid solution was considered. After harvesting, the fermented broth was filtered and the resulting filtrate was concentrated up to 90% (w/w) in an L-lactic acid solution. Therefore, the energy for evaporation, *i.e.*, the amount of steam required, increased inversely with the L-lactic acid concentration. Based on the mass balance before and after the evaporation of the filtrate, the amount of the evaporated water per unit weight of L-lactic acid, $w(kg/kg)$ is given by Eq. (1)

$$w = \frac{1}{C_f}\left(1 - \frac{C_f}{0.90}\right) \quad (1)$$

On the other hand, the amount of required steam for evaporation is given by Eq. (2).

$$q\lambda_s = wc_p(t_s - t_w) + w\lambda_w \quad (2)$$

Substituting Eq. (1) into Eq. (2)

$$q = \frac{\frac{1}{C_f}\left(1 - \frac{C_f}{0.90}\right)(C_p(t_s - t_w) + \lambda_w)}{\lambda_s} \quad (3)$$

Fig. 6 shows the correlation between L-lactic acid con-

Table 2. Mass balance and cost evaluation of the cultures

Process	C_f (g/L)	V (L)	Consumed glucose (g)	Produced L-lactic acid (g)	$Y_{P/S}$ (g/g)	PD[a] (g/L/h)	Cost (₩/kg of L-lactic acid)			
							Carbon source	Steam	Waste treatment	Total
Fed batch culture	141	2.35	400	331	0.88	1.47	72.5	4.5	3.6	80.6
Batch culture										
C_o[a] = 60	53	2.09	120	111	0.93	1.26	64.9	13.2	9.4	87.5
120	94	2.17	244	204	0.84	1.18	71.8	7.7	6.5	86.0
200	121	2.15	404	260	0.64	1.41	93.2	5.3	3.7	91.3
300	72	2.00	334	144	0.43	0.50	139.2	9.5	6.9	155.6
Continuous culture										
D[b] = 0.017	75	2.00	4.1	2.6	0.63	1.28	95.2	9.1	6.7	111.0
0.023	65	2.00	5.5	3.0	0.55	1.50	111.1	10.6	7.9	129.6
0.029	48	2.00	7.0	2.8	0.40	1.42	146.3	14.7	10.4	171.4
0.034	39	2.00	8.2	2.7	0.33	1.34	181.8	18.3	13.5	213.6
0.043	28	2.00	10.3	2.4	0.23	1.22	250.0	25.8	15.2	291.6
0.051	21	2.00	12.2	2.1	0.17	1.04	352.9	34.6	17.2	404.7

[a] L-Lactic acid volumetric productivity. [b] Initial glucose concentration (g/L). [c] Dilution rate (h^{-1}).

Table 3. Comparison of L-lactic acid concentration

Process	Cell	pH control	Concentration(g/L)		Product yield ($Y_{P/S}$) ($\times 10^{-1}$ g/g)	Volumetric productivity (g/L/h)	Reference
			Initial glucose	L-lactic acid			
Batch culture in flask	Free[a]	$CaCO_3$	120	89	7.4	1.26	[3]
in jar-fermentor	Free[a]	$CaCO_3$	120	93	7.2	1.29	[3]
	Mycelial flocs[a]	$CaCO_3$	120	104	8.6	1.70	[8]
in airlift bioreactor	Free[b]	NH_4OH	120	95	8.4	1.31	This work
	Mycelial flocs[a]	$CaCO_3$	120	105	8.7	1.80	[9]
	Immobilized[a]	$CaCO_3$	150	73	4.8	1.69	[2]
Fed-batch culture in jar-fermentor	Free[b]	NH_4OH	60[a]	140	8.3	1.47	This work
Continuous culture	Immobilized[a]	$CaCO_3$	102[a]	43	4.7	1.50	[4]
in airlift bioreactor	Free[b]	NH_4OH	120[a]	63	5.4	1.50	This work

[a] *R. oryzae* NRRL395. [b] Ammonia-tolerant mutant strain *Rhizopus* sp. MK-96-1196. [c] Initial concentration.

centration in the fermentation and the amount of steam required for evaporation.

Filtered cake from the filtration of culture broth and effluent water from the evaporation process were all treated in a conventional active sludge method.

Table 2 shows the comparison of the estimated total variable costs among production processes, *i.e.*, batch, continuous, and fed-batch cultures. Economic estimation was according to the method described in the previous paper [7]. In the case of batch culture with an initial glucose concentration of 120 g/L, the variable cost was the lowest because of the high product yield. Although the highest L-lactic acid concentration (121 g/L) was obtained in the batch culture with an initial glucose concentration of 200 g/L, the carbon source cost increased due to a decrease in $Y_{P/S}$. In contrast, although the batch with an initial glucose concentration of 60 g/L gave the highest $Y_{P/S}$, the evaporation and waste treatment costs increased significantly due to low L-lactic acid concentration, resulting in an increase in the variable cost.

In the case of the fed-batch culture, the total variable cost was the lowest among all of the experiments (Table 2), because of both high L-lactic acid concentration and product yield. These reduced the costs relating to carbon source, steam, and waste treatment costs, resulting in a significant decrease in the variable cost. In the case of continuous culture, however, although the volumetric productivity was the highest among all of the production processes, the total variable cost was the highest due to the low L-lactic acid concentration and the residual glucose concentration. The former increased the steam cost for concentration of L-lactic acid and the latter increased the waste treatment cost for the decomposition of residual glucose.

DISCUSSION

L-Lactic acid concentration and yield coefficients are summarized in Table 3. In the case of conventional batch culture with calcium carbonate as a pH-controlling agent using free cells in the jar fermentor or shaking flask, L-lactic acid concentrations, volumetric productivity, and the $Y_{P/S}$ were similar; 89~93 g/L, 1.26~1.29 g/L/h, and 72~74%, respectively. When mycelial flocs formed by mineral support addition were used, L-lactic acid concentrations and volumetric productivity were 104 g/L and 1.7 g/L/h, respectively. However, neither the use of calcium carbonate as pH-controlling agent nor the addition of any supplementation in the industrial production of L-lactic acid is desirable pointing terms of economics and engineering, including recovery and purification. Because when calcium carbonate is used, produced lactic acid is existed in the form of calcium lactate in the culture broth. Harvested calcium lactate is substituted by sulfuric acid to achieve a pure lactic acid. In this purification process calcium sulfate as a byproduct is generated, which is recalcitrant waste in the purification process of lactic acid production [10] and is also an obstacle of efficient purification. Therefore, we isolated an ammonia-tolerant *Rhizopus* sp. MK-96-1196 mutant strain for pH control [1,2]. In the recovery and purification of L-lactic acid, ammonia can be recovered from culture broth after harvesting and subsequently recycled for successive culture, and can in part be used as a nitrogen source. The batch culture using an airlift bioreactor controlling pH with ammonium water gave both a product yield and a production rate as high as those in the jar fermentor. Furthermore, in the case of the fed-batch culture using an airlift bioreactor controlling pH with ammonium water, a L-lactic acid concentration of 141 g/L and a product yield of 83% were achieved with a comparatively high production rate, 1.47 g/L/h. In contrast, if the immobilized cells were used in either batch or continuous culture, the product yields were only 56% to fed-batch culture using free cells in this research, irrespective of high production yield.

In conclusion, for low cost production of L-lactic acid, batch, continuous, and fed-batch cultures were investigated with an ammonia-tolerant *Rhizopus* sp. MK-96-1196 mutant strain. As determined by simple cost evaluation, the fed-batch culture is the best-suited for the industrial production of L-lactic acid. A new, highly purified L-lactic acid production process without recalcitrant waste, *e.g.*, $CaSO_4$, may be realized in the near future.

NOMENCLATURE

t	Culture time
V	Volume of the broth
V_{max}	Maximum volume of the broth
Cs	Glucose concentration detected with glucose sensor
C_f	Final L-lactic acid concentration (kg/kg)
C_p	Specific heat of water (kcal/kg/°C)
q	Amount of steam for 1 kg of water removal (kg)
t_s	Temperature of steam (°C)
t_w	Temperature of water (°C) (equal to the temperature of the culture broth)
w	Amount of removed water (kg) per 1 kg of L-lactic acid
$Y_{P/S}$	Product yield coefficient based on consumed glucose (g/g)
$Y_{x/s}$	Biomass yield coefficient based on consumed glucose (g/g)
λ_s	Enthalpy of steam (kcal/kg)
λ_w	Enthalpy of water (kcal/kg)

REFERENCES

[1] Miura, S., T. Arimura, M. Hoshino, D. Lies, and M. Okabe (2003) Optimization and scale-up of L-lactic acid fermentation by mutant strain *Rhizopus* sp. MK-96-1196 in airlift bioreactors. *J. Biosci. Bioeng.* 96: 65-69.

[2] Miura, S., L. Dwiarti, T. Arimura, M. Hoshino, T. Liu, and M. Okabe (2004) Enhanced production of L-lactic acid by ammonia tolerant mutant strain *Rhizopus* sp. MK-96-1196. *J. Biosci. Bioeng.* 97: 19-23.

[3] Ohara, H. (1994) Poly-L-lactic acid as biodegradable plas-

tic. *Biosci. Ind.* 52: 642-644.
[4] Hamamuci, H. and D. Y. Ryu (1993) Production of L(+)-lactic acid using immobilized *Rhizopus oryzae*. *Appl. Biochem. Biotechnol.* 44: 125-132.
[5] Sun, Y., L.-Y. Li, and S. Bai (1999) Modeling of continuous L(+)-lactic acid production with immobilized *Rhizopus oryzae* in an airlift bioreactor. *Biochem. Eng. J.* 3: 87-90.
[6] Yuru, C., X. Liming, and C. Peilin (2000) L-Lactic acid by immobilization *Rhizopus oryzae* in a three-phase fluidized-bed. *Acta Microbiol. Sinica* 40: 415-419.
[7] Futamura, T., H. Ishihara, T. Takayoshi, T. Yasutake, G. Huang, M. Kojima, and M. Okabe (2001) Kojic acid production in an airlift bioreactor using partially hydrolyzed raw corn starch. *J. Biosci. Bioeng.* 92: 360-365.
[8] Kosakai, Y., Y. S. Park, and M. Okabe (1997) Enhancement of L(+)-lactic acid production using mycelial flocs of *Rhizopus oryzae*. *Biotechnol. Bioeng.* 55: 461-470.
[9] Park, E. Y., Y. Kosakai, and M. Okabe (1998) Efficient production of L(+)-lactic acid using mycelial cotton-like flocs of *Rhizopus oryzae* in an airlift bioreactor. *Biotechnol. Prog.* 14: 699-704.
[10] Buchita, K. (1983) Lactic acid. pp. 409-417. In: H.-G. Rehm and G. Reed (eds.). *Biotechnology* 1st Edn. Verlag Chemie, Weinheim, Germany.

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