0273-2289/95/5152—0057$07.00

Lactic Acid Production by Pellet-Form *Rhizopus oryzae* in a Submerged System

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ABSTRACT

Rhizopus oryzae NRRL 395 produces optically pure L(+)-lactic acid that is highly preferred for the production of environmentally benign polymers. With xylose as the carbon source for cultivation, it can be self-immobilized as pellets with a size of about 1 mm. Repeated usage of the same pellets by transferring them into fresh media every time when the glucose was exhausted over a period of 22 d yielded 1742 or 2001 g/L lactic acid total (based on 100-mL working volume) depending on the media used. Lactic acid is known to be a strong inhibitor for both growth and production, and it can be removed continuously by the adsorption on the PVP resin. With the fermenter-adsorber system, the fermentation can be performed as effectively as the ones with added neutralizing agents, such as calcium carbonate and sodium hydroxide. One problem of the fermenter-adsorber system is that lower production was obtained than in shake flasks; hence, proper reactor design is necessary to improve the process.

Index Entries: *Rhizopus oryzae;* L(+)-lactic acid; PVP; growth medium; nongrowth medium.

INTRODUCTION

Lactic acid and its salts are being widely used in food, chemical, and pharmaceutical industries (*1*). Recently, there has been an increased interest in lactic acid because it is one of the raw materials for the production of environmentally benign polymers that have already been used in medical devices (*1,2*). The strength, biodegradability, and other properties can be controlled by changing the compositions of those polymers

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(*2,3*). For the polymerization purpose, optically pure lactic acid is highly preferred. Therefore, if the production of a large quantity of optically pure lactic acid can be achieved, it will be possible to utilize lactic acid-based polymers and copolymers in packaging and consumer goods (*2*).

Rhizopus oryzae NRRL 395 is known to be outstanding in producing optically pure L(+)-lactic acid (*4*). It has a less complicated nutritional requirement than most of lactic acid bacteria that are being used commercially for lactic acid production. Hence, it is possible to reduce the feed wastes and simplify recovery processes by *R. oryzae* (*5*).

Fungi can grow as mycelia, large clumps, or pellets depending on the growth conditions and the nature of the cells. On large scale production, it may have difficulties if the cells grow as mycelia or clumps. The Rotating Biological Contactor (RBC) (*6*) and immobilized cells systems (*7–9*) have been studied to try to overcome this problem. However, *R. oryzae* NRRL 395 can be self-immobilized as pellets by using xylose as the carbon source. Therefore, no immobilization material is needed in this case. The pellets are about the size of 1 mm; thus mixing and mass transfer can be enhanced. Hence, the pellet form of *R. oryzae* in a submerged system has been studied for lactic acid production.

R. oryzae can produce lactic acid in media without nitrogen source (*10*), but the production rate declines sharply because of cell aging after a certain period of time. Among the components in the media, urea affects the volumetric productivity owing to the increase of both cell density and specific productivity.

One of the advantages of the pellets is that they are easy to be separated from the fermentation broth and can be recycled. According to the results obtained by repeatedly using the same pellets for about 3 wk, 2001 g lactic acid were produced/L of fermentation volume. This was achieved by repeated replacement of exhausted fermentation broth with fresh media and the addition of calcium carbonate to neutralize the lactic acid produced.

Lactic acid is known to be a strong inhibitor for lactic acid production. In order to produce more lactic acid, calcium carbonate (*6,11*), sodium hydroxide (*12*), or ammonium hydroxide (*13*) is usually added to neutralize the acid and maintain the fermentation broth at a controlled pH. Lactate instead of lactic acid is obtained, and the cations have to be removed in the purification in order to produce lactic acid. This downstream processing is disadvantageous as it increases recovery costs. PVP (poly 4-vinylpyridine) is known to have the ability to adsorb undissociated lactic acid with a capacity of 0.25 g/g sorbent at room temperature (*14,15*). With a PVP column coupled to a fermenter to remove lactic acid continuously, the fermentation can proceed as effectively as those with the addition of calcium carbonate or with the pH controlled at 5 or higher. Thus, using PVP, undissociated lactic acid can be obtained as the fermentation product.

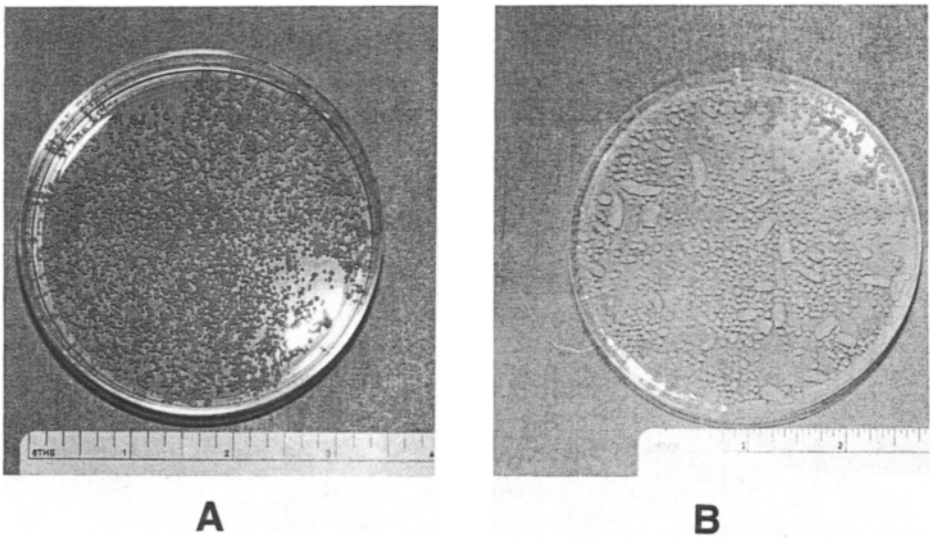

Fig. 1. The size and distribution of the pellets (**A**) after the cultivation and (**B**) after 22 d of fermentation. (The unit of the ruler shown in the figure is inches.)

METHODS

Microorganism

R. oryzae NRRL 395, obtained from the Northern Regional Research Center, Peoria, IL, was used. It is an L(+)-lactic acid producing fungal culture. The fungus was maintained on YM agar plates with growth medium. The spores were collected by washing the plates with sterile water to obtain a spore suspension before inoculation.

Culture

Culture medium consisted of 50 g xylose, 2 g urea, 0.6 g KH_2PO_4,0.25 g $MgSO_4$, and 0.088 g $ZnSO_4$/L distilled water. Spores were inoculated into 250-mL Erlenmeyer flasks containing 100 mL of culture medium sterilized by autoclaving. Cultivation was carried out at 30°C at 215 rpm in rotary shakers for 2.5–3 d. Pellets with a size of about 1 mm (~ 0.04 in) were obtained for fermentation. Figure 1A shows the size and the distribution of the pellets. For each flask after the cultivation, the pellets were separated from the broth, washed by autoclaved distilled water (dd-H_2O) and put into another 250-mL flask on the condition being studied in a shake-flask system. For the fermenter, pellets in several flasks were combined together and put into the fermenter.

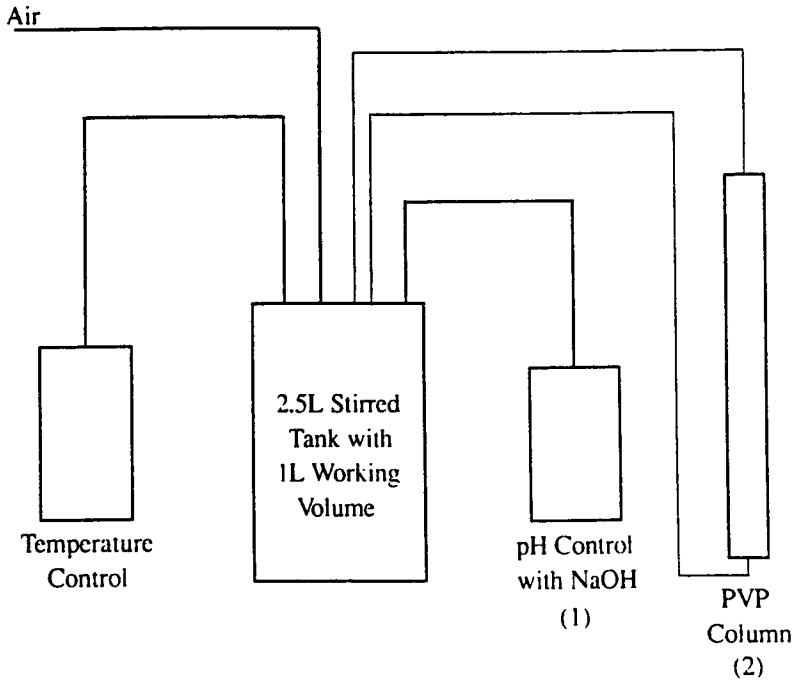

Fig. 2. Schematic diagram of the fermentation system.

Fermentation

The media for lactic acid production consisted of 80 g glucose, 0.6 g KH_2PO_4, 0.25 g $MgSO_4$, and 0.088 g $ZnSO_4$ with (growth medium) or without (nongrowth medium) urea/L distilled water. Two kinds of systems were used for lactic acid production. Shake flasks were used for the determination of the temperature and medium composition effects, as well as repeated usage of the same pellets for lactic acid production. In this system, excess calcium carbonate was added to neutralize the acid produced. Fermenters were used for the determination of the pH effect and were coupled with PVP columns to adsorb lactic acid in order to reduce the inhibitory effect caused by lactic acid. Figure 2 shows the schematic setup of the system.

Adsorbent

Reillex 425, a 25% crosslinked poly 4-vinylpyridine resin (Reilly Industries, Indianapolis, IN), was used to remove the lactic acid from the fermentation broth continuously. After the fermentation, the resins were regenerated by 0.01*N* NaOH aqueous solution until no lactic acid was in the eluent. By analysis of the eluent, the amount of lactic acid adsorbed and yield of the production were obtained. Lactic acid production during the

fermentation was estimated by the amount of glucose consumed. Then, the resin was washed by water for repeated uses. For practical purposes, hot water, ethanol, or methanol (*14,15*) can be used to regenerate the resin, and lactic acid will be obtained.

Analytical Methods

Glucose Analyzer

YSI 2700 Select Biochemistry Analyzer (Yellow Spring Instrument Co. Inc., Yellow Spring, OH) with glucose and L-lactate membranes was used for analysis of glucose and lactic acid concentrations.

HPLC

Hitachi HPLC with RI detector was used to analyze the major byproduct concentrations, as well as glucose, and lactic acid concentrations for comparison. The mobile phase used was 0.005*M* H_2SO_4 at a flow rate of 0.8 mL/min through a Bio-Rad (Richmond, CA) HPX-87H ion-exclusion column at 60°C.

Dry Weight

After the fermentation, pellets were washed by water, put into a 90°C oven for drying, and then weighed for the final dry weight.

RESULTS AND DISCUSSION

Medium

R. oryzae can utilize xylose as the carbon source for lactic acid production, but the production rate is slower than glucose. Figure 3 shows the lactic acid production with glucose or xylose as the carbon source in nongrowth medium. For glucose fermentation, the weight yield of lactic acid production was about 78% (the theoretical yield is 100%), and the major byproducts were glycerol, ethanol, or fumaric acid. The distribution of the byproducts changed depending on fermentation conditions. More ethanol was produced in growth media, and fumaric acid was found only in nongrowth medium. For xylose fermentation, the yield was about 70%, and the major byproducts were glycerol and ethanol. No fumaric acid was found under the conditions being studied.

From a preliminary study on the effect of medium concentrations on lactic acid production using the nongrowth medium (medium without nitrogen source), glucose and salt concentrations did not affect the production significantly. The reason for using nongrowth medium is that it is difficult to measure the cell density during the fermentation. The cell density remained almost constant in nongrowth medium; hence, volumetric

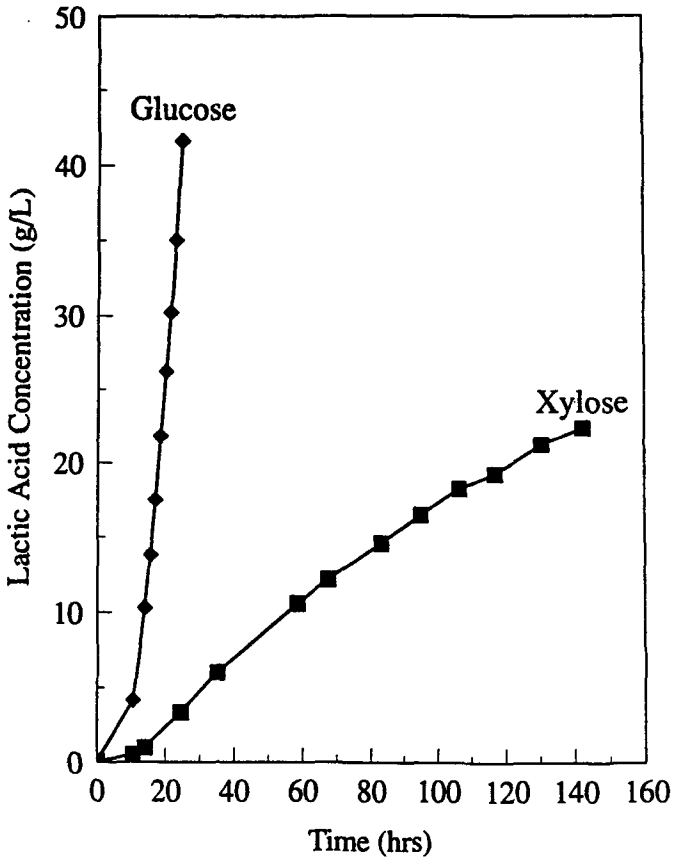

Fig. 3. Time-courses of lactic acid production with glucose or xylose as carbon source.

productivity was maintained after the lag phase. On the other hand, initial urea concentrations did affect the volumetric productivity (Fig. 4). The volumetric productivity for the ones with urea (~3.5 g/h·L) are much higher than the one without it (~1.2 g/h·L). The cell density increased from 2 to 2.9 g/L for 1 g/L urea and 3.3 g/L for 2 g/L urea owing to cell growth. Therefore, the specific productivity was slightly higher for the one with 1 g/L urea (=1.2 g/h·g biomass) than the one with 2 g/L urea (=1.07 g/h·g biomass) and was much higher than the one without urea (=0.62 g/h·g biomass). This happened probably because of the higher cell activity of the new grown cells and the necessity of the nitrogen source for maintenance. The yield of lactic acid production in nongrowth medium was 75%, and it was higher than the yields in growth media that were 65–70%. In conclusion, urea enhances volumetric productivity of lactic acid production in terms of increasing both the amount of biomass and the specific productivity.

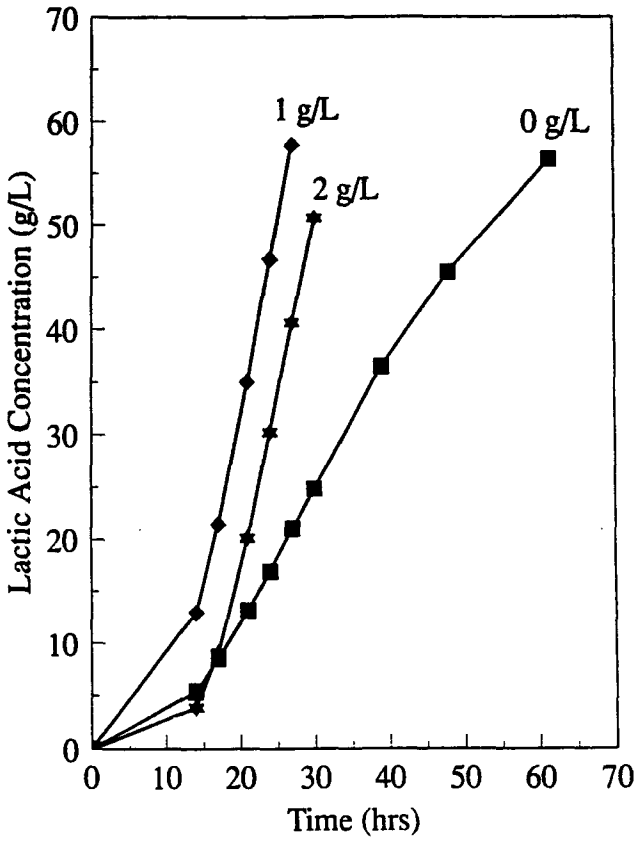

Fig. 4. Influence of the initial urea concentration on lactic acid production.

Cell Recycle

One of the major advantages of the pellet-form cells is that the pellets can be easily separated from the fermentation broth and used repeatedly for long-term fermentation. In this study, pellets were harvested from shake flasks, separated from the broth, washed by autoclaved distilled water, and transferred into fresh medium, including calcium carbonate with 100 mL medium in a 250-mL Erlenmeyer flask. Then, the same procedures were followed each time when glucose was exhausted.

R. oryzae can continuously produce lactic acid in nongrowth medium, but the volumetric productivity declined after several cycles owing to the effect of cell aging (Fig. 5). Although the lag phase was eliminated after two cycles in the same medium, nitrogen source is still necessary for an extended run with pellet recycle.

Table 1 shows the results of switching between growth and nongrowth media for lactic acid production. Nongrowth medium was used

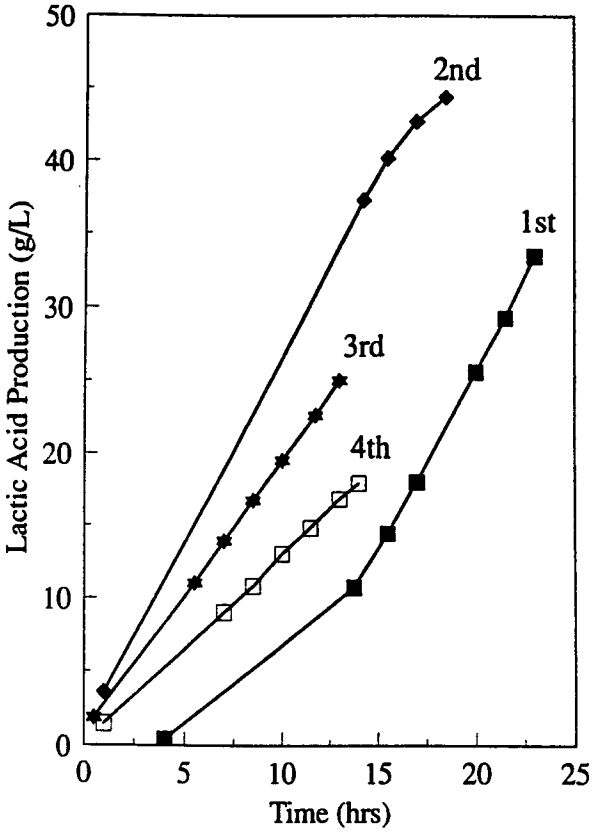

Fig. 5. The decline of lactic acid production owing to cell-aging effect.

for the first five cycles and the volumetric productivity kept on declining, as shown in Fig. 5. Then, medium with 1 g/L urea was used for the sixth to eighth cycles. The volumetric productivity did increase, but the yield was lower as expected. Again, medium was changed back to nongrowth-phase medium in order to obtain higher yield of the production. The volumetric productivity was maintained and then it declined again. By switching between the two media, the volumetric productivity was maintained at 2.7 to 6.0 g/h·L. After 22 d of fermentation, the cell density increased from 1.7 to 10.8 g/L dry wt, the total lactic acid produced was about 1742 g/L in 42 batches (total lactic acid produced was 174.2 g based on 100-mL working volume), and the production was still at a rate of about 2 g/h·L at the end of the fermentation.

As mentioned earlier, urea can increase volumetric productivity by increasing both biomass and specific productivity. Therefore, continuously using the medium with an initial 1 g/L urea to produce lactic acid was also studied (Table 2). After several cycles, the lag phase was almost eliminated, and the volumetric productivity began to increase. At the 6th d, the volumetric productivity reached the maximum, 62 g/h·L, and had remained around this value for more than 3 d. Then, it decreased steadily.

Table 1
Repeated Usage of the Same Pellets for Lactic Acid Production with Medium Switched Between Growth* and Nongrowth Media

Cycle no.	t_0, h	Initial urea conc., g/L	Volumetric productivity, g/h·L	Yield, %	Lag phase, h
1	0.0	0.0	2.5676	76.77	10.5
2	24.0	0.0	2.5790	75.77	0.0
3	43.0	0.0	1.8527	80.65	0.0
4	56.5	0.0	1.2854	74.10	0.0
5	71.0	0.0	0.9733	73.48	0.0
6	85.0	1.0	3.1240	61.61	9.1
7	100.5	1.0	4.6872	62.98	5.0
8	113.5	1.0	5.9974	57.62	5.3
10	136.5	0.0	4.7874	N/A	2.0
14	184.0	0.0	3.3879	79.77	0.0
16	207.5	1.0	4.5110	63.84	2.0
18	230.0	0.0	4.6775	67.36	1.0
20	254.5	0.0	4.3129	76.32	0.0
22	278.0	1.0	4.7579	64.55	0.6
24	300.5	0.0	4.5829	67.94	0.0
26	325.5	0.0	3.3055	65.71	1.8
28	350.0	1.0	5.0453	66.73	0.8
30	374.0	0.0	4.7263	68.10	5.0
34	422.0	0.0	2.6926	77.39	0.0
36	446.5	1.0	3.6070	65.45	0.0
42	517.5	0.0	1.9862	76.34	1.0

*Cycles 6, 7, 8, 15, 16, 21, 22, 27, 28, 35, 36, 37 were in growth phase; the others were in nongrowth phase.

Table 2
Repeated Usage of the Pellets for Lactic Acid Production with Growth Medium Only

Cycle no.	t_0, h	Initial urea conc., g/L	Volumetric productivity, g/h·L	Yield, %	Lag phase, h
1	0.0	1.0	3.7518	71.61	12.5
3	43.0	1.0	3.7905	61.53	2.6
4	56.5	1.0	3.9646	64.67	1.5
7	100.5	1.0	5.0042	69.05	0.9
10	136.5	1.0	6.1675	72.94	1.1
16	207.5	1.0	6.1092	74.35	1.0
20	254.5	1.0	5.6319	73.91	0.0
24	300.5	1.0	4.6000	67.55	0.0
28	350.5	1.0	3.9920	65.76	0.0
34	422.0	1.0	3.3919	65.80	0.6
42	517.5	1.0	2.8610	65.41	0.8

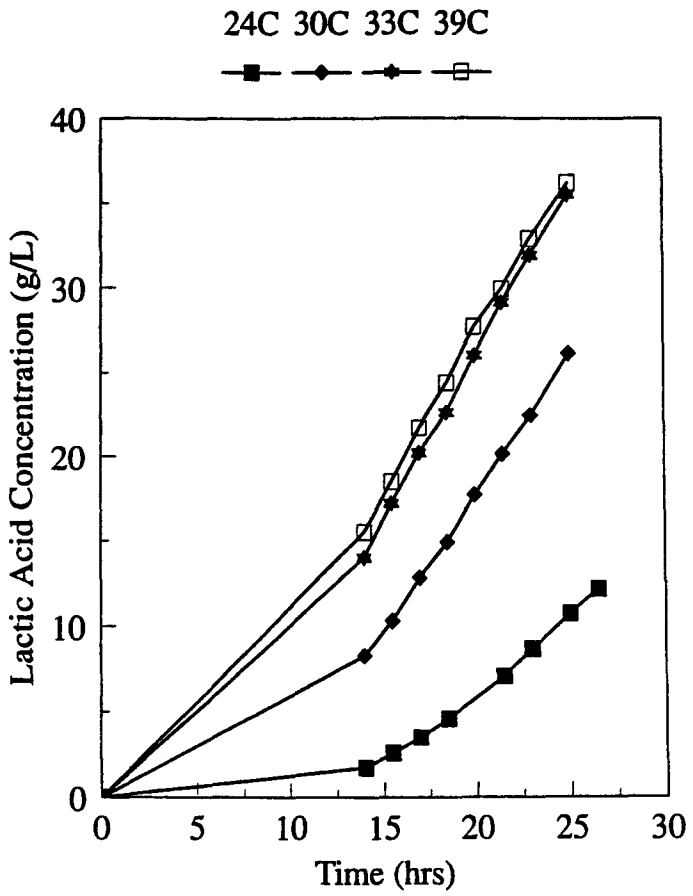

Fig. 6. Influence of temperature on lactic acid production.

After 22 d of fermentation, the yield remained at about 65–70%, the cell density increased from 1.7 g/L to the final dry wt of 10.2 g/L, the total lactic acid produced was 2001 g/L, and the production rate had dropped to about half of the highest value.

By comparison between these two conditions, the one with only growth medium had more lactic acid produced with a lower yield. Figure 1 shows that there were no obvious changes between the pellets before and after the fermentation.

Temperature

Temperature is one of the factors that may affect the production. Temperatures ranging from 24 (room temperature) to 39°C with nongrowth medium were used to study the temperature effect on lactic acid production by *R. oryzae*. The results are shown in Fig. 6. At room temperature, some of the cells apparently died after 1 d of fermentation (cell density dropped from 1.74 to 1.53 g/L), and the volumetric productivity 1 g/h·L

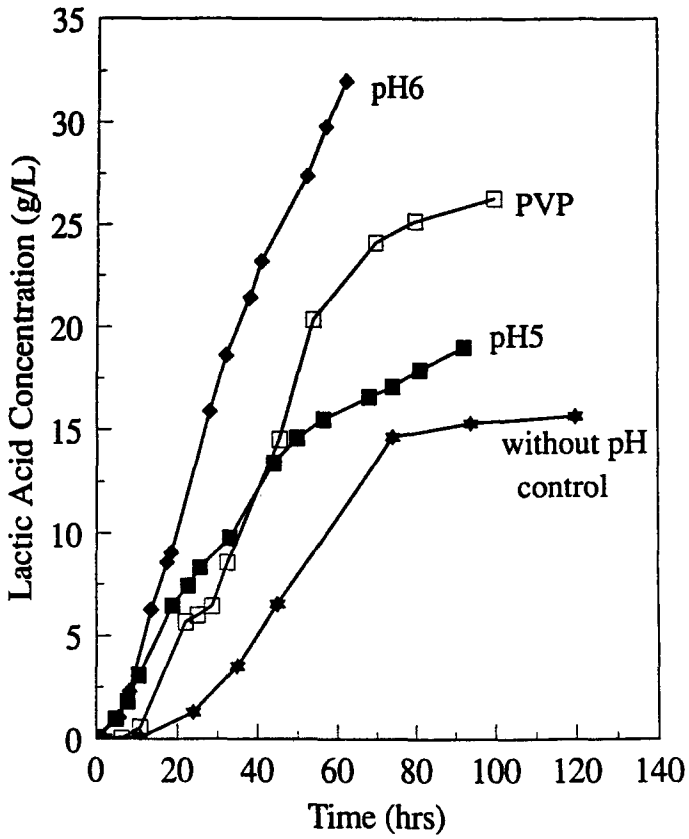

Fig. 7. Influence of pH and a fermenter with a PVP column coupled on lactic acid production.

was the lowest. At 30°C, the volumetric productivity was about 1.5 g/h·L, and the cell density remained the same throughout the fermentation. At even higher temperatures (33 and 39°C), lactic acid can be produced more effectively. The volumetric productivity was about 1.8 g/h·L at 33 and 39°C.

pH

pH is one of the most important factors that may affect the lactic acid production. Undissociated lactic acid was known to be a strong inhibitor for both growth and production in lactic acid fermentation, but lactate is not as strong. In fermentation without pH control, the pH of the fermentation broth dropped to below 3, and the volumetric productivity became slower because of the inhibitory effect of lactic acid and lowering of pH. The final lactic acid concentration obtained was about 15 g/L after 5 d of fermentation (Fig. 7).

In the cases where pH was controlled at pH 5 and 6 by the addition of sodium hydroxide, the volumetric productivities were higher than the

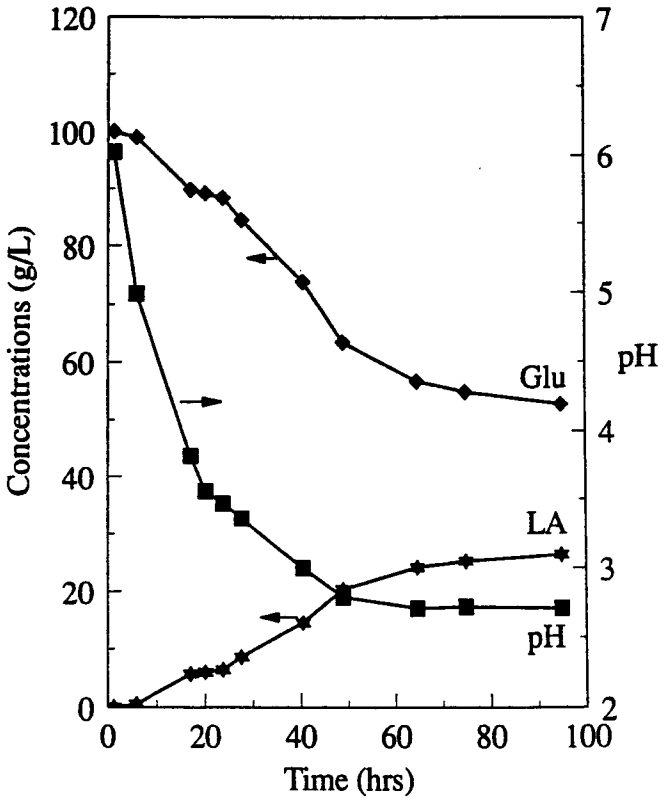

Fig. 8. Time-courses of lactic acid production, glucose consumption, and pH in the fermentation with a PVP column coupled.

one without pH control as shown in Fig. 7. The volumetric productivity was higher at pH 6 than at 5. However, there are problems with pH control by the addition of sodium hydroxide. First, lactic acid has a p*Ka* value of 3.86 at 30°C. Thus, lactate instead of lactic acid is obtained at high pH values. Second, the cations that come with the neutralizing base have to be removed after the fermentation, resulting in the increase in recovery cost.

Fermenters Coupled with PVP columns

PVP resin was known to adsorb undissociated lactic acid well with a capacity of 0.25 g LA/g sorbent at room temperature (*14,15*). Therefore, a fermenter with nongrowth medium was coupled with a PVP column (Fig. 2) to remove lactic acid continuously during the fermentation. The broth was continuously circulated through the column, lactic acid produced was adsorbed onto the resin, and the inhibitory effect on fermentation was largely reduced even though the pH was below 4 (Fig. 8). Eventually, the free lactic acid in the broth began to accumulate and the pH dropped to below 3. This coupled with the possibility of the cell-aging effect, the lactic acid production was slowed down significantly.

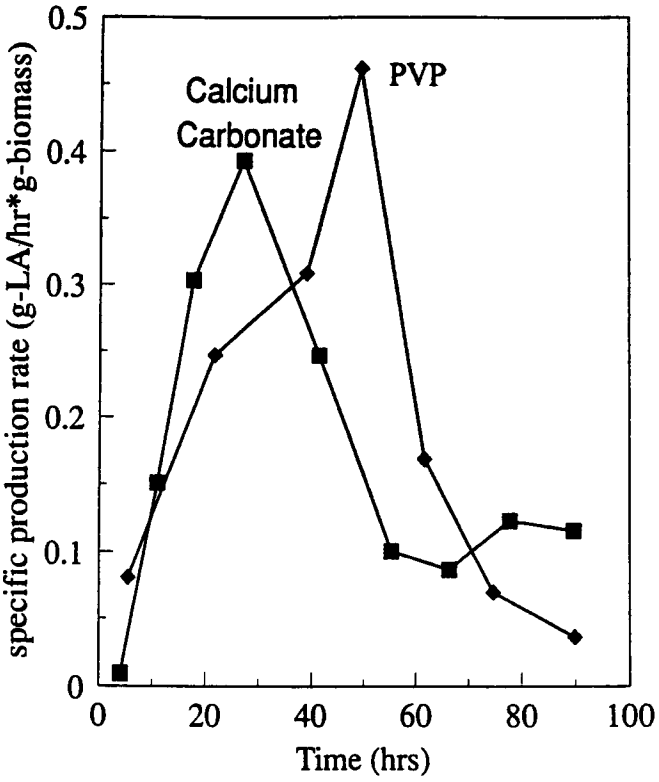

Fig. 9. The comparison of the specific production rate between two different systems.

As shown in Fig. 7, the lactic acid production in the fermenter when coupled with a PVP column can perform better than the one with pH controlled at 5. Before the volumetric productivity slowed down, it was slightly lower than the one controlled at pH 6. The advantages of the PVP column coupling are: the fermentation can be performed at low pH, thus, the chance of contamination is significantly lowered, and the product can be removed from the PVP column using hot water, ethanol, or methanol to yield undissociated lactic acid without necessity of using a neutralizing agent and the subsequent removal of cations after the recovery.

Calcium carbonate is one of the commonly used reagents to neutralize lactic acid during fermentation. Its low solubility in water makes it possible to neutralize lactic acid and maintain the pH at about 5 automatically. Comparing the specific productivity in the fermentation with the addition of calcium carbonate with that of the adsorption by PVP resin (Fig. 9), PVP can perform as well as the addition of calcium carbonate in lactic acid production in the fermenter.

The major problems with the addition of calcium carbonate are the same as when pH is controlled by sodium hydroxide, i.e., lactate was obtained and the high calcium ion content has to be removed in the subsequent purification processes.

In conclusion, undissociated L(+)-lactic acid can be obtained easily with continuous removal of the product by the PVP resin at a rate as high as the ones of neutralization by calcium carbonate and sodium hydroxide in the fermenters. One major problem of the stirred tank is that the production was lower than in shake flasks, and it is probably because of the cell damage resulting from agitation. Proper reactor design to provide gentler agitation may improve the production.

CONCLUSIONS

R. oryzae can be self-immobilized as small pellets in medium with xylose as the carbon source. With the advantage that pellets can be easily separated from the broth, it has been shown to be feasible in producing optically pure L(+)-lactic acid in extended runs. PVP resins have been shown to have the ability to remove lactic acid continuously from the fermentation broth and perform the production as well as neutralizing agents, such as calcium carbonate or sodium hydroxide in fermenters, but slower than in the shake-flask system. Proper fermenter design is necessary to improve the production.

ACKNOWLEDGMENTS

We would like to thank Dr. R. C. Ruaan for numerous useful discussions. This research is supported by a grant from the USDA Processing for Value Added Products Program.

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