0273-2289/98/70–72—0323$9.75

Production of L-Lactic Acid by *Rhizopus oryzae* in a Bubble Column Fermenter

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ABSTRACT

Two distinctive forms of growth (mycelial filamentous and mycelial pellets) of *Rhizopus oryzae* were obtained by manipulating the initial pH of the medium with the controlled addition of $CaCO_3$ in a bubble fermenter. In the presence of $CaCO_3$, diffused filamentous growth was obtained when the initial pH of the substrate was 5.5. In the absence of $CaCO_3$, mycelial pellet growth was obtained when the initial pH was 2.0. The fermentation study indicated that the mycelial growth has a shorter lag period before the onset of acid formation. Both physical forms of growth of *Rhizopus* exhibited a high yield of L-lactic acid in the bubble fermenter when the initial glucose concentration exceeded 70 g/L. A final lactic acid concentration of 62 g/L was produced by the filamentous form of *Rhizopus* from 78 g/L glucose after 27 h. This showed a weight yield of 80% of glucose consumed, with an average specific productivity of 1.46 g/h/g. Similarly, the pellet form of *Rhizopus* produced a final lactic acid concentration of 66 g/L from 76 g/L glucose after 43 h, with a weight yield of 86% and an average specific productivity of 1.53 g/h/g.

Index Entries: Bubble fermentor; L-lactic acid; mycelial pellet; *Rhizopus oryzae*.

INTRODUCTION

Lactic acid is an intermediate-volume specialty chemical that is used in a wide range of food-processing and industrial applications. Because of its chemical properties, lactic acid has the potential of becoming a large-volume, commodity-chemical intermediate that can serve as feedstock for

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biodegradable polymers, oxygenated chemicals, environmentally friendly solvents, and other intermediates. Polylactate is the feedstock for the biodegradable polymer that is synthesized from lactic acid. For polylactate synthesis, L-lactic acid is preferred.

Rhizopus oryzae is known to produce L-lactic acid *(1–3)*. Similar to many other mycelial fungal species, *Rhizopus* cultures are morphologically complex. They can grow as mycelial mats, mycelial clumps, or mycelial pellets, depending on the growth conditions and the strain of fungus. Different morphological growth forms can have a significant effect on the rheology of the fermentation broth that will affect the performance of the bioreactors. Large-scale production of lactic acid in a traditional stirred tank may be difficult, if the fungal cells were grown in the filamentous form. This is because filamentous growth results in highly viscous broth. The high viscosity will have a negative impact on the mass-transfer properties of the broth *(4)*. The problem is more profound when the fermentations require high oxygen transfer, such as in lactic acid production by *R. oryzae*. As a rule, there are two types of growth in the submerged cultures of mycelial fungi; the mycelial pellet form, and the extended filamentous form. The size of mycelial pellet varied from 1 mm to as large as 10 mm in diameter; the filamentous growth forms a homogeneous suspension that can disperse throughout the liquid medium. The growth of *R. oryzae* in the shake flasks varied from extensive mycelial growth to small pellets to conglomerates. The physical forms of fungal growth are influenced by the strains of fungi, nutrients, pH of the medium, agitation, aeration, inoculum size, and substrate concentrations. In order to obtain a uniform size of mycelial pellets (1–2 mm in diameter) from *R. oryzae*, the authors have formulated a growth medium with xylose as the sole carbon source. The characteristics of *Rhizopus* pellets were examined and the results were documented *(3)*.

In this report, the relationship among growth conditions, the morphology of the mycelial growth, and lactic acid production in a bubble fermenter were studied.

MATERIALS AND METHODS

Microorganism and Inoculum

Culture of *R. oryzae* ATCC 52311, purchased from American Type Culture Collection (Rockville, MD), was chosen for this study because it is a good sporangiospore producer. The culture was maintained on YMP (Difco, Detroit, MI) agar slants and propagated by growing in Erlenmeyer flasks, in YMP agar, which consists of 0.3% yeast extract, 0.3% malt extract, 0.3% peptone, 1% glycerol, and 1.5% agar to obtain sporangiospores. For fermentation study, spores were collected by washing spore-bearing cultures with sterile water and collecting this as a spore suspension.

Preculture

For each experiment, the spore suspension (1×10^6 spores/mL) was inoculated into three 2.5-L Erlenmeyer flasks containing 1 L cultivation medium. The medium consisted of 20 g glucose, 3 g urea, 0.6 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, and 0.088 g $ZnSO_4 \cdot 7H_2O$ in 1 L dH_2O. Incubation was carried out at 35°C and 200 rpm in an incubator–shaker for 20 h.

Bubble Column

The batch fermentations were performed in a 5-L bubble column with an operating volume of 3.5 L. The fermenter was constructed with transparent polycarbonate (Meyer Plastics, Lafayette, IN) plastic pipe. It consisted of a column having a diameter of 6 cm and a height of 90 cm, with a perforated plate (10 holes of 1 mm in diameter), located at the bottom of the column, to serve as the air sparger. Sterile air was supplied at 0.2–0.3 vvm.

Fermentation

After the germ tubes were formed (up to 4 mm in length) in the preculture step, the germinated spores were transferred aseptically into the sterilized bubble column containing only glucose in water. Depending upon the experiment, sterilized $CaCO_3$ was added whenever needed to maintain the pH at 5.5. The fermentation was operated at an aeration rate of 0.2–0.3 vvm at 31°C. Samples were taken periodically for HPLC analysis.

Analytical Methods

Lactic acid, glycerol, ethanol, and glucose were determined and quantified by high-performance liquid chromatography (Hitachi, L-6200A (Hercules, CA)), using a Bio-Rad Aminex HPX-87H ion exclusion column (300 × 7.8 mm) with a refractive index detector (Hitachi, L-3350 RI). The column was eluted with dilute sulfuric acid (0.005 *M*) at a column temperature of 80°C and a flow rate of 0.8 mL/min over an 18-min period.

RESULTS

Fermentation of Lactic Acid by Diffused Filamentous Form of *Rhizopus*

When the germinated spores of *R. oryzae* obtained from preculture were transferred into the bubble column in the presence of $CaCO_3$ (pH = 5.5) and glucose, cells grew into the distinctive diffused filamentous form with extended hyphae. The length of mycelial growth extended to 10 mm

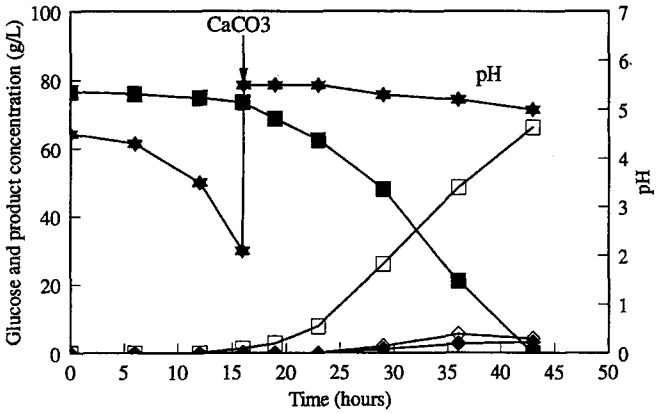

Fig. 1. Kinetics of lactic acid fermentation by filamentous form of *Rhizopus oryzae* in a bubble fermenter. (■, Glucose; □, L-Lactic acid; ✻, pH; ◇, Ethanol; ◆, Glycerol).

long. Lactic acid was detected after a 10 h lag period (Fig. 1). After 27 h of incubation, glucose was entirely consumed. An average specific lactic acid productivity of 1.46 g/h/g, and a final lactic acid concentration of 62.5 g/L, were obtained. Throughout the fermentation period, lactic acid yield was constant and reached a value of 0.8 g/g. Ethanol (3 g/L) and glycerol (2 g/L) were also detected during the fermentation.

Fermentation of Lactic Acid by Mycelial Pellet Form of *Rhizopus*

The growth of pellet form of *R. oryzae* in the bubble column was achieved after transferring the germinated spores into liquid glucose medium without $CaCO_3$. During the first 16 h of incubation with bubbling air, broth dropped from about pH 5.0 to 2.0, because of the production of a small amount of lactic acid (Fig. 2). During this period, the germinated spores grew into more or less uniform sized elliptical pellets (1–2 mm). The growth of the pellets stopped after reaching this size, presumably because of the exhaustion of the available nitrogen source. A small amount of $CaCO_3$ was then pumped into the column after the completion of pellet development, to maintain the broth in the range of 5.0 to 5.5 pH in order to enhance the lactic acid production. During the fermentation stage, aeration was increased from 0.2 to 0.3 vvm, to maintain fermentation rate.

After 43 h of incubation, glucose was consumed to produce a final lactic acid concentration of 66 g/L, with an average specific lactic acid productivity of 1.53 g/h/g. The longer incubation time was required for *Rhizopus* pellets to complete the fermentation. This is a result of the long lag period (21 h). Throughout the acid production period, the product yield was constant and reached a value of 0.8 g/g. Ethanol and glycerol were detected during later stages of fermentation.

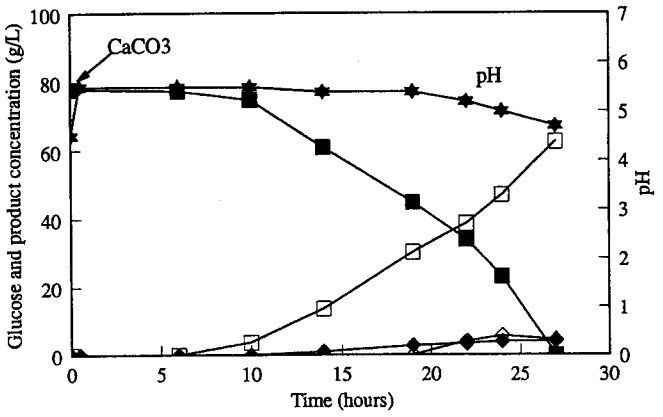

Fig. 2. Kinetics of lactic acid fermentation by pellet form of *Rhizopus oryzae* in a bubble fermenter. (■, Glucose; □, L-Lactic acid; *, pH; ◇, Ethanol; ◆, Glycerol).

Cell Recycle

One of the advantages of fermentation in the bubble fermenter is that the mycelia and the pellets can be easily separated from the fermentation broth by simply allowing the cells to settle. In the cell-recycle study, fresh glucose solution was supplied after about 75% of the fermented broth was drained. The repeat fermentation was initiated by supplying oxygen. Cells of *Rhizopus* can continue to produce lactic acid from glucose under this nongrowth condition, with much shorter lag period and higher volumetric productivity than the first cycle fermentation. However, the fermentation activity declined after the second cycle. The results of cell recycle experiments are summarized in Table 1.

DISCUSSION

L-Lactic acid fermentation by *R. oryzae* can be separated into two distinctive stages, cell growth and product formation. During the growth stage, in the presence of a nitrogen source, *R. oryzae* grows with extended hyphae and forms large-sized pellets or mycelial aggregates. Once the cells are fully grown, the fermentation stage can be augmented by changing the medium to one without a nitrogen source. When fermentation was carried out in the conventional stirred-tank fermenter, the growing mycelia were adsorbed onto the heat exchanger and impellers, and formed mycelial clumps. The problem was accentuated when $CaCO_3$ was used as the neutralizing agent. Added $CaCO_3$ intermingled with mycelial aggregates and further complicated the clumping problem. Under the conditions described, the fermentation time was long, and ethanol was accumulated in place of lactic acid. One way to avoid this problem is by conducting the fermentation in a bubble fermenter. The bubble fermenter can provide better gas and mass transfer than the stirred-tank fermenter (5).

Table 1
Summary of Results for Lactic Acid Production by *Rhizopus oryzae* in Bubble Column

Forms of *Rhizopus oryzae*	Pelleted forms			Filamentous forms		
Cycle no.	1	2	3	1	2	3
Initial glucose concentration (g/L)	76.5	70.8	63.0	77.9	73.2	57.1
Final lactic acid concentration (g/L)	66.2	65.8	55.6	62.5	63.2	47.2
Fermentation time (hr)	43	15	19	27	12	18
Lag phase (hr)	21	0.5	1.0	10	0.5	2.0
Biomass dry weight (g/L)	1.97	–	2.80	2.52	–	3.93
Yield (%)	86.5	92.9	88.2	80.2	86.4	82.6
Volumetric productivity (g-lactic acid/h/L)	1.54	4.39	2.93	1.69	5.06	2.36
Specific productivity (g-lactic acid/h/g biomass)	1.53	–	1.10	1.46	–	0.75

The authors' previous study on lactic acid fermentation by *R. oryzae* indicates that pellets with a loose structure and a diameter of 1–2 mm provide the best physical characteristics for producing high yields of lactic acid *(3)*. In this study, two different physical forms (filamentous or pellet) of cell growth can be realized by controlling the timing of addition of the neutralizing agent, $CaCO_3$, to the bubble fermenter. The controlled $CaCO_3$ addition can bypass the difficulties encountered in the stirred tank fermentation. It can also avoid the complication of using a specially formulated growth medium to achieve pellet formation before subjecting the cells to the nongrowth fermentation condition.

In the authors' experience, a higher concentration (>10%) of calcium lactate can be obtained by supplying a higher initial glucose concentration. However, this is undesirable, because the limited solubility of the calcium lactate resulted in the *in situ* crystallization of the product during operation. In this study, the substrate concentration was kept at under 80 g/L to avoid the crystallization of calcium lactate.

CONCLUSION

The results of this study indicate that two distinctive forms of mycelial growth can be obtained by controlling the timing of $CaCO_3$ addition. The bubble column fermentation results indicate that both the filamentous and the pellet forms of *Rhizopus* exhibited good fermentation rates and high product yield.

ACKNOWLEDGMENTS

The authors thank Linda Liu for analysis. This research was supported in part by US Department of Agriculture Grant 96-35500-3192.

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