0273-2289/99/77–79/0401/$11.75

# Optimization of L-Lactic Acid Production from Glucose by *Rhizopus oryzae* ATCC 52311

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## Abstract

The effect of nutrients on L(+)-lactic acid production from glucose was investigated using *Rhizopus oryzae* ATCC 52311. From the shake-flask experiments, the optimal medium composition was defined for improved lactic-acid production. In order to enhance lactic-acid production rate and product yield, controlled aeration in a bubble column was conducted under optimal conditions. Results showed a maximum lactic-acid production rate of 2.58 g/L/h was obtained with an initial glucose concentration of 94 g/L. Final lactic-acid concentration of 83 g/L was achieved after 32 h of fermentation with a weight of 0.88 g lactic acid/g glucose consumed.

**Index Entries:** Bubble column; lactic acid; *Rhizopus oryzae*.

## Introduction

Lactic acid is a naturally occurring multifunctional organic acid. It has been found in many food products, particularly in those involved nature or processed fermented food items. In industry, lactic acid is an intermediate-volume specialty chemical that has application in a wide range of food processing and industrial applications. Lactic acid is also the component for the production of many nonfood products such as polylactic acid (PLA). Owing to the unique property of PLA, lactic acid has the potential to become a very large-volume commodity chemical intermediate.

Recently, lactic acid has received attention because of the development of PLA plastics, which are biodegradable and have been approved for general use by US Food and Drug Administration (FDA). The potential market demand for PLA is very high. Owing to the potential of PLA, several large US corporations have been involved in the product and process development with lactic acid and PLA.

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Lactic acid is the smallest molecule that exists in two isomeric forms in nature. The synthetic method can only produce the racemic mixture of the stereoisomers. In contrast, biologically produced lactic acid can be obtained in either one or another isomer or a mixture of the two in different proportion *(1)*. Lactic acid is produced in an industrial scale through either chemical or biological means. The biological production of lactic acid, which accounts for about 50% of the current total capacity, is primarily generated through bacterial fermentation of simple sugars.

Besides lactic acid-producing bacteria, a few strains of *Rhizopus arrhizus* and *R. oryzae* are good lactic-acid producers. Unlike most bacteria, lactic acid-producing *Rhizopus* strains generated L-lactic acid as the only fermentation product *(2)*. In addition, *Rhizopus* cultures are more tolerant to low pH environment. Consequently, the pH maintenance during fungal fermentation is not as stringent as using bacterial cultures *(3)*. Furthermore, *Rhizopus* cultures are amylolytic; they can produce lactic acid from starchy substrates such as grains without prior saccharification *(4)*.

The ability of *Rhizopus* to produce only L(+)-lactic acid aerobically under nutrient-limited environment has been studied *(3,5–7)*. In this report, we examined the fermentation conditions to provide the best environment for high yield of lactic acid from glucose by *R. oryzae* ATCC 52311 in a bubble column fermentor.

## Materials and Methods

### Microorganism and Inoculum

*R. oryzae* ATCC 52311 purchased from American Type Culture Collection (Rockville, MD) was used in this work. It is a good L(+)-lactic-acid producing strain *(7)*. The fungus grows and forms spores on YMP agar plates that consist of yeast extract (0.3%), malt extract (0.3%), peptone (0.5%), glycerol (2%), and agar (2%). For fermentation study, agar plates containing sporulated fungus were washed by sterile water to obtain spore suspension.

### Preculture

Based on inoculum size, desired amount of spore suspension ($1 \times 10^6$ spores/mL) was inoculated into 500-mL Erlenmeyer flasks containing 200 mL of the cultivation medium. The cultivation medium consisted of glucose (3%), ammonium sulfate (0.1%), $KH_2PO_4$ (0.06%), $MgSO_4 \cdot 7H_2O$ (0.025%) and $ZnSO_4 \cdot 7H_2O$ (0.009%). Incubation was carried out in an incubator-shaker at 35°C and 200 rpm. After 20 h, the entire culture was filtered through Whatman filter paper to collect germinated spores.

### Culture

Germinated spores were transferred into 500-mL Erlenmeyer flasks containing 200 mL of the fermentation medium that consisted of glucose

(15%), ammonium sulfate (0.1%), $KH_2PO_4$ (0.06%), $MgSO_4 \cdot 7H_2O$ (0.025%) and $ZnSO_4 \cdot 7H_2O$ (0.009%). Neutralizing agent, $CaCO_3$, was added intermittently to maintain the pH value of the medium at around 5.5. The fermentation took place at the same incubator-shaker at 35°C and 200 rpm.

### *Effect of Metal Ions*

Five metal cations tested in this experiment were magnesium ($Mg^{2+}$), zinc ($Zn^{2+}$), iron ($Fe^{2+}$), manganese ($Mn^{2+}$), and copper ($Cu^{2+}$) that were prepared from $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, and $CuSO_4 \cdot 5H_2O$, respectively. For each experiment, fermentation medium was varied by omitting one or the other metal ions. In all the experiments, glucose solution, nitrogen sources, and salt solutions were sterilized separately and added together prior to the inoculation of spores.

### *Bubble Column Fermentor*

The batch fermentation was performed in a 750-mL bubble column with jacket. It consisted of a glass column with a diameter of 5 cm and a height of 40 cm, and a jacket for water circulation to maintain the fermentation temperature.

Preculture medium (500 mL) with glucose (30 g/L) contained 20% spore solution was added into 1000-mL Erlenmeyer flask. The flask was precultured in an incubator-shaker at 35°C for 24 h. The germinated spore medium was then transferred into the bubble column with an air flow rate of 0.5 vvm. The germinated spores were then allowed to develop for 15 h into tiny mycelial pellets of 1–2 mm in diameter. At this stage, pH of the medium was at ca. 3.0. Concentrated glucose solution was then added into the column to a final glucose concentration of 95 g/L to commence fermentation. During fermentation, $CaCO_3$ slurry was added continuously to maintain the pH at 5.5. Zero time was set at the time of glucose addition.

### *Analysis*

High-performance liquid chromatography (Hitachi Instrument, L-6200A) with a refractive index detector (Hitachi Instrument, L-3350 RI) and a Bio-Rad Aminex HPX-87H ion exclusion column (300 × 7.8 mm) was used to analyze the glucose, lactic acid, and ethanol. The mobile phase of column was 0.005 *M* $H_2SO_4$ at a flow rate of 0.8 mL/min and the column temperature was at 80°C.

## Results and Discussion

### *Effect of Nitrogen Source*

Decreasing the initial ammonium sulfate concentrations from 2 g/L, which was used in the previous experiment *(7)*, to 1 g/L influenced both total acid accumulation and acid weight yield (Table 1). The highest lactic-acid concentration obtained was at an initial ammonium sulfate concentra-

Table 1
Effect of Ammonium Sulfate Concentration
on Lactic Acid Production by *R. oryzae*[a]

| $(NH_4)_2SO_4$ (%) | Lactic acid (g/L) | Yield (g/g) | Biomass (g/L) |
|---|---|---|---|
| 0.05 | 44.5 | 0.66 | 3.56 |
| 0.1 | 56.8 | 0.62 | 4.76 |
| 0.2 | 52.5 | 0.55 | 6.39 |
| 0.3 | 52.1 | 0.57 | 6.24 |
| 0.4 | 54.1 | 0.58 | 6.16 |
| 0.5 | 55.1 | 0.59 | 6.96 |

[a]Incubation time: 60 h.

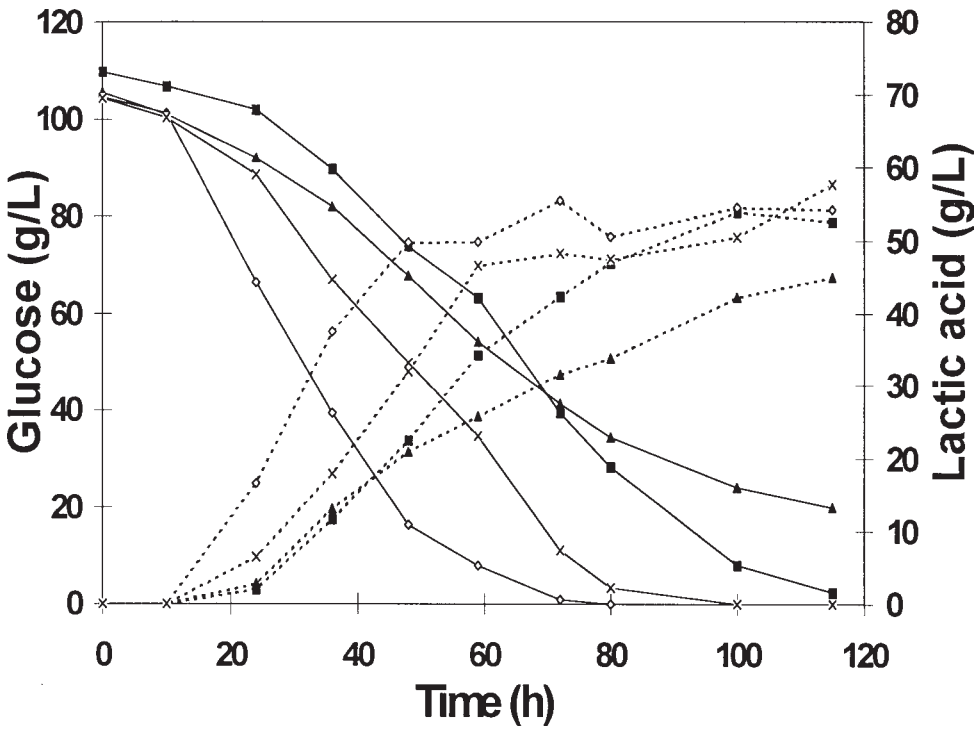


Fig. 1. Effect of nitrogen sources on lactic-acid production by *R. oryzae*. (——), Glucose; (– – – –), lactic acid; (◇), ammonium sulfate; (◆), yeast extract; (▲), ammonium nitrate; (×), urea.

tion of 0.1 g/L. At this concentration, a maximal amount of lactic acid (56.8 g/L) was obtained with a weight yield of 0.62 g/g glucose consumed. At the lowest concentration tested (0.5 g/L), lactic-acid weight yield increased with decreasing biomass yield. Increasing nitrogen concentrations, acid yield, and biomass accumulation remains more or less unchanged. Results indicated the optimal nitrogen concentration was 0.1 g/L.

Table 2
Effect of Phosphate on Lactic-Acid Fermentation[a,b]

| $KH_2PO_4$ (m*M*) | Lactic acid (g/L) | Yield (g/g) | Biomass (g/L) |
|---|---|---|---|
| 0 | 0 | 0 | 1.12 |
| 1.5 | 60.5 | 0.60 | 4.99 |
| 3.0 | 61.6 | 0.61 | 4.82 |
| 4.5 | 62.5 | 0.62 | 4.76 |
| 7.5 | 62.2 | 0.61 | 4.88 |
| 12.0 | 60.3 | 0.61 | 4.93 |

[a]Medium composition: glucose (10%), ammonium sulfate (0.1%), $MgSO_4 \cdot 7H_2O$ (0.025%), $ZnSO_4 \cdot 7H_2O$ (0.009%).
[b]Incubation time: 60 h.

For comparison, urea, ammonium nitrate, and yeast extract were used in place of ammonium sulfate. Figure 1 shows the time course of glucose consumption and lactic-acid production with various nitrogen sources. Based on the data presented, it is apparent that ammonium sulfate is the better nitrogen source for lactic-acid production by *R. oryzae*.

## *Effect of Phosphate*

Experiments described earlier in this article were obtained with a phosphate concentration of 4.5 m*M* in the form of $KH_2PO_4$. Phosphate concentration is similar to those reported previously *(7)*. Increasing the initial phosphate concentration from 1.5–12 m*M* has no noticeable effect on either biomass or lactic-acid accumulation (Table 2). When phosphate was omitted, *Rhizopus* spores failed to grow beyond the germinating stage.

## *Effect of Metal Ions*

Five metal ions, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, and $Zn^{2+}$, were tested for their effect on biomass growth and lactic-acid accumulation (Table 3). Results indicated the optimal concentration for $Mg^{2+}$, $Zn^{2+}$, and $Mn^{2+}$ were 25, 12, and 1 ppm, respectively. $Cu^{2+}$ was inhibitory at 0.5 ppm, whereas $Fe^{2+}$ is not required.

## *Kinetics of Lactic-Acid Fermentation in a Bubble Column*

The kinetics of lactic-acid production under optimal nutrient conditions was studied in a bubble-column fermentor. Typically, bubble column can provide better gas and mass transfer than the stirred tank fermentor *(7)*, which should result in an increase in productivity.

Figure 2 shows the results of the bubble-column fermentation. After 32 h, glucose (94 g/L) was completely consumed with accumulated lactic acid reached 83 g/L. The volumetric productivity was 2.59 g/L/h and the weight yield was 0.88 g/g glucose consumed. Both lactic acid produced and acid weight yield were much higher than those in shake-flask fermentation.

Table 3
Effect of Metal Ions on Lactic-Acid Fermentation[a]

| Metal ions | Concentration (ppm) | Lactic acid (g/L) | Yield (g/g) | Biomass (g/L) |
|---|---|---|---|---|
| $Mg^{2+}$ | 0 | 50.1 | 0.49 | 4.64 |
| | 25 | 55.4 | 0.54 | 4.88 |
| | 50 | 50.2 | 0.49 | 4.46 |
| | 75 | 50.3 | 0.50 | 4.34 |
| | 100 | 50.6 | 0.50 | 4.28 |
| $Zn^{2+}$ | 0 | 55.4 | 0.54 | 4.88 |
| | 4 | 66.2 | 0.66 | 4.81 |
| | 8 | 67.3 | 0.67 | 4.80 |
| | 12 | 68.1 | 0.65 | 4.79 |
| | 16 | 62.5 | 0.62 | 4.76 |
| | 20 | 42.1 | 0.59 | 3.41 |
| $Fe^{2+}$ | 0 | 66.2 | 0.66 | 4.81 |
| | 10 | 54.5 | 0.58 | 4.54 |
| | 20 | 39.0 | 0.45 | 3.85 |
| | 30 | 33.3 | 0.40 | 2.64 |
| | 40 | 21.6 | 0.37 | 2.11 |
| $Mn^{2+}$ | 0 | 66.2 | 0.66 | 4.81 |
| | 1 | 67.0 | 0.67 | 4.82 |
| | 3 | 66.5 | 0.66 | 4.84 |
| | 5 | 66.8 | 0.65 | 4.83 |
| | 7 | 64.7 | 0.63 | 4.88 |
| $Cu^{2+}$ | 0 | 67.0 | 0.67 | 4.82 |
| | 5 | 0 | 0 | 0 |

[a]Incubation time: 60 h.

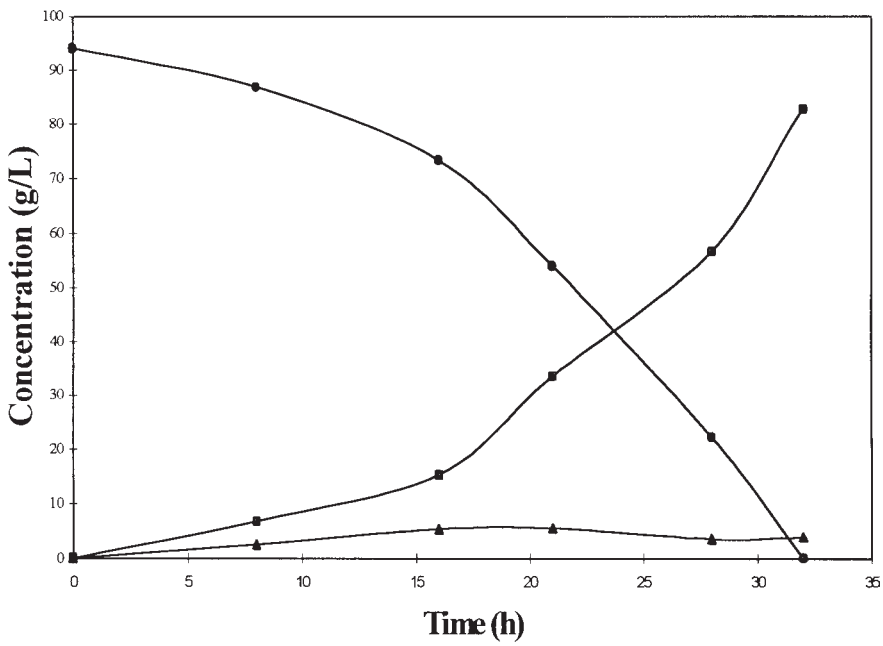


Fig. 2. Kinetics of lactic-acid fermentation in a bubble column. (●——●), Glucose; (◆——◆), lactic acid; (▲——▲), ethanol.

## Conclusion

Results of bubble-column fermentation showed *R. oryzae* ATCC 52311 is a good biocatalyst for the production of high yield of lactic acid from glucose under optimal nutrient conditions. Higher acid concentration, higher weight yield, and shorter incubation time achieved in bubble-column fermentor was probably owing to better mass and oxygen-transfer rates than in flask cultures. Further experiments using larger-scale fermentors are currently underway.

## Acknowledgments

We thank Ms. Linda Liu for analysis. This research was supported by US Department of Agriculture (Grant 96-35500-3192).

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