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Production of L(+)-Lactic Acid Using Immobilized *Rhizopus oryzae*

Reactor Performance Based on Kinetic Model and Simulation

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ABSTRACT

The production of L(+)-lactic acid using alginate immobilized *Rhizopus oryzae* in tapered-column fluidized-bed batch reactor was tested and simulated using the kinetic data taken independently in shake-flask cultures. The data show saturation kinetics with substrate and product inhibitions in linear form. Analysis of the kinetic data gave kinetic constants: V_m, 11.04 g lactic acid/(L-bead. h); K_m, 20.9 g glucose/L; and K_i, 365 g glucose/L for lactic acid production. The product inhibition constant, K_p, was found to be 316 g lactic acid/L. The simulation results showed a good agreement with the experimental results when the initial lag phase was taken into account in the simulation model. Without the adjustment for the initial lag period, the kinetic model showed higher conversion. Starting with a glucose concentration of 150 g/L, it was possible to produce 73 g/L of L(+)-lactic acid in 44.5 h. The lactic acid yield was 64.8% by weight based on the amount of glucose consumed.

Index Entries: L(+)-lactic acid; *Rhizopus oryzae;* tapered-column, fluidized-bed reactor; immobilized mycelia; bioreactor performance; fermentation kinetics; bioprocess simulation.

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Nomenclature: C_g = glucose concentration, g/L; C_l = lactic acid concentration, g/L; K_i = substrate inhibition constant, g glucose/L; K_m = substrate saturation constant, g glucose/L; K_p = product inhibition constant, g lactic acid/L; V_{bead} = volume of beads in the tapered column; V_{total} = total active volume in the tapered column; ν_l = lactic acid production rate, g lactic acid/L-bead·h; ν_{lm} = maximum lactic acid production rate, g lactic acid/L-bead·h.

INTRODUCTION

Worldwide demand and capacity of lactic acid production is constantly increasing. Petrochemical process and bacterial fermentation account for most of world production of lactic acid at the moment, but its production using *Rhizopus* species seems to be a viable alternative. The fermentation processes using *Rhizopus* species offer several advantages. Among the most important advantages are: solely the L(+)-form of the acid is produced, they can grow on minimal media, and recovery and purification will be more economical. The recovery is one of the major problems in lactic acid production. There is also a continuing interest in using immobilized organisms for organic acid production.

In the recent years Horitsu et al. (*1*) tested the effects of the concentration of various ions on lactic acid production using immobilized *R. oryzae* in continuous cultures and Hang et al. (*2*) tested the stability of immobilized *R. oryzae* for lactic acid production in repeated batch fermentations. Kristofikoya et al. (*3*) and Rosenberg et al. (*4*) showed that there was the possibility of producing valuable chemicals along with the lactic acid fermentation using *Rhizopus* species in submerged cultures. Vassilev and Vassileva (*5*) reviewed the literature on organic acid production using immobilized fungi and Goncalves et al. (*6*) reviewed the possible immobilization matrices for lactic acid production.

In this work a fluidized-bed tapered-column bioreactor was used for batch production of L(+)-lactic acid using *R. oryzae* immobilized in Ca-alginate gels, using glucose as the carbon source. The concentrations of the substrate and the product were monitored and simulated by numerical integration using the kinetic model developed. The kinetic data taken from the shake-flask cultures were used as part of the simulation parameters.

MATERIALS AND METHODS

Microorganism

Rhizopus oryzae NRRL 395 (2) was used. The organism was maintained in a medium containing 3% Difco potato-dextrose agar, and transferred to fresh slants every 2 mo.

Spore Production Medium

Spores were produced on steamed rice, and washed off from the molded rice by vigorous shaking in distilled water.

Experimental Methods

The volume of tapered-column bioreactor was about 1 L. The bioreactor diameters at the bottom and the top were 37 mm and 100 mm, respectively. The total (liquid and solid) volume in the column was 350 mL, of which 100 mL was made up of beads of immobilized mycelia. Fluidization was accomplished by air fed at the bottom through a sparger at a rate of 1 vvm. Air was first saturated by passing it through sterilized distilled water. The whole system was set up in an incubator that was kept at 30°C. The results of three different experiments using the tapered-column bioreactor showed reproducible results, and one set of representative data was used for the simulation study.

The kinetic data were taken in 250-mL flasks containing 25 mL of medium and 25 mL of the beads. The beads used in each experiment were preequilibrated at the same concentration of glucose and/or lactic acid to be used in the respective experiment for 2–3 h. At the starting time of the experiment the conditioning media were replaced by the fresh media.

As is discussed below, a quick adaptation of the microorganisms to high lactic acid concentrations used for the product inhibition test proved to be difficult. In order to overcome this problem, a high lactic acid concentration was obtained by the fermentation and sterilized glucose in powder form was added to adjust the glucose concentration to the desired level. All flask experiments were carried out at least in duplicate. Although one set of representative data obtained from well-controlled experimental conditions was used for the analysis of kinetic data, many experiments were carried out under varying conditions of the experiments for different purposes. The temperature of the incubator shaker was controlled at 30°C and the shaking speed was set at 175 rpm.

Immobilization Procedure

The spore suspension obtained by washing the molded rice with distilled water was mixed with 30 g/L Na-Alginate solution to obtain a mixture of 20 g/L. This mixture was pumped into a sterile 30 g/L solution of $CaCl_2$ through a glass capillary tubing, while the salt solution was stirred continuously. The beads obtained were on the average 2.8 mm in diameter.

Growth and Lactic Acid Production Media

The activation medium for the beads contained: 50 g/L glucose, 2 g/L $(NH_4)_2SO_4$, 0.65 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.025 g/L $ZnSO_4 \cdot 7H_2O$ (2). Twenty grams per liter of $CaCO_3$ was sterilized in dry form and added to the medium after sterilization.

Lactic acid production medium used in the tapered-column bioreactor contained (all g/L); 150 g/L of glucose, 2.0 $(NH_4)_2SO_4$, 0.65 KH_2PO_4, and 0.25 $MgSO_4 \cdot 7H_2O$. Fifty grams per liter of $CaCO_3$ was sterilized in powder form and added to the medium later.

The medium used in the shake flask cultures for the determination of the kinetic parameters contained, in addition to the salts present in the production media, varying concentrations of glucose and lactic acid.

Analytical

Lactic acid was analyzed using an HPLC (Hewlett-Packard model 1090, Palo Alto, CA) with a Bio-rad (Richmond, CA) Aminex-HPX-HX ion-exclusion column and a RI detector. The eluant, 0.005 *N* H_2SO_4, was used at a flowrate of 0.6 mL/min. A Shimadzu (Kyoto, Japan) CR3-A integrator was used to record and analyze the data. Glucose was determined by a Yellow Springs (Yellow Springs, OH) enzymatic analyzer. The cell content in the beads was estimated to be 52 g dry biomass/L bead by weighing a measured volume of the beads, after drying them under vacuum at 80°C before and after growth.

RESULTS AND DISCUSSION

Kinetic Data and Model

The dependence of the rate of lactic acid production on the glucose concentration was determined by taking data from shake-flask fermentation experiments at varying initial glucose concentrations of 5, 10, 20, 50, 100, 150, and 200 g/L. The rate of production of lactic acid was followed for about 2–5 h depending on the concentration range indicated above, and the data for 5–20 g/L glucose concentration range are shown in Fig. 1. The rates of lactic acid production were estimated based on the results of regression analysis of the data shown in Fig. 1. Figure 2 shows the effect of glucose concentration on the lactic acid production rate. The kinetic data presented in Fig. 2 suggested the kinetic model of the form:

$$\nu_1 = (\nu_{lm} C_g) / (C_g + K_m + C_g^2 / K_i) \quad (1)$$

The saturation constant, K_m, and maximum rate of production, ν_{lm} values were determined from the double reciprocal plot shown in Fig. 3. The substrate inhibition constant, K_i, was determined from the slope of the regression line in Fig. 4 which is equal to V_m/K_i when evaluated using high substrate concentration range (7). The glucose concentration range from 50 to 200 g/L was used in this regression analysis. The kinetic parameters determined are presented in Table 1. These kinetic constants determined were used for simulation according to the Eq. (1) and the results are shown in Fig. 2.

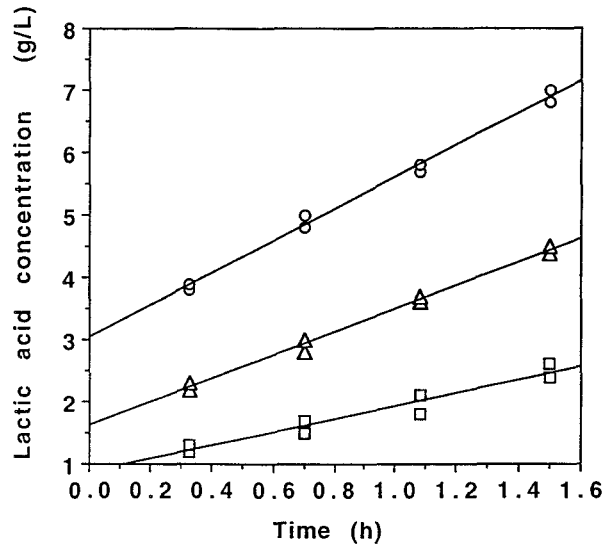

Fig. 1. The change of lactic acid concentration with time in shake flask cultures. Starting glucose concentrations: (□) 5 g/L, (△) 10 g/L, and (○) 20 g/L.

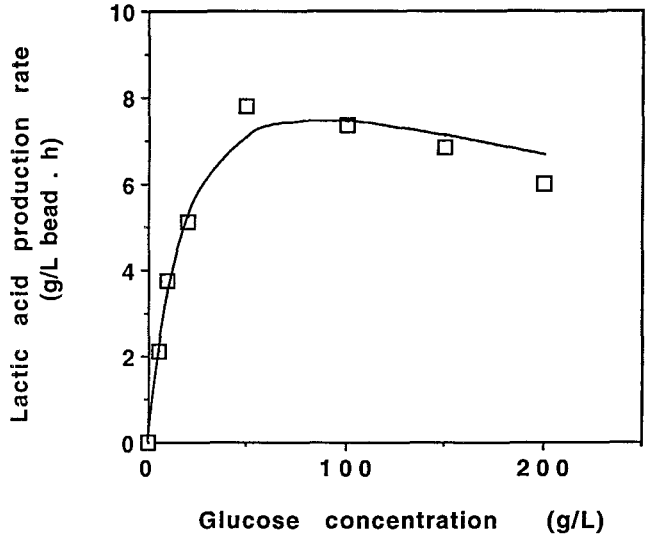

Fig. 2. Lactic acid production rate as a function of initial glucose concentration. Each data point in this figure corresponds to a line in Fig. 1 or to a similar set of data. The solid line is drawn by using Eq. (1).

The product inhibition kinetics were also determined by carrying out similar experiments at a fixed glucose concentration (50 g/L) and varying lactic acid concentrations. For these experiments, the beads were first allowed to produce lactic acid to desired levels (10–55 g of lactic acid/L) and glucose in powder form was added to make 50 g of glucose/L final concentration; after which point the kinetic data were taken. The results

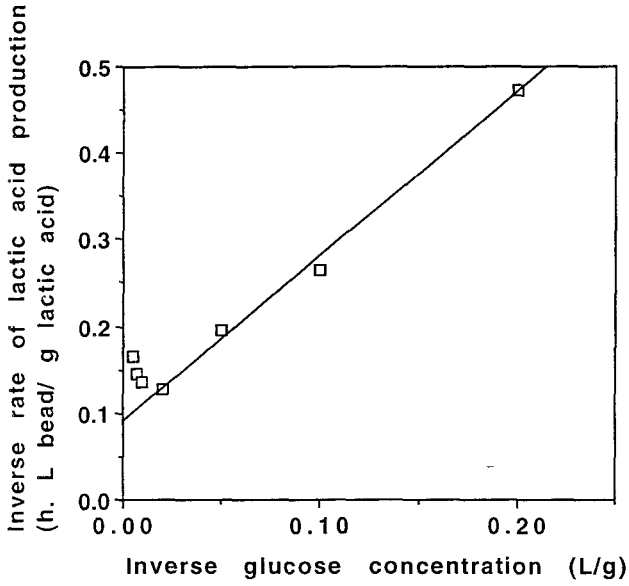

Fig. 3. Inverse rate of lactic acid utilization vs inverse of glucose concentration.

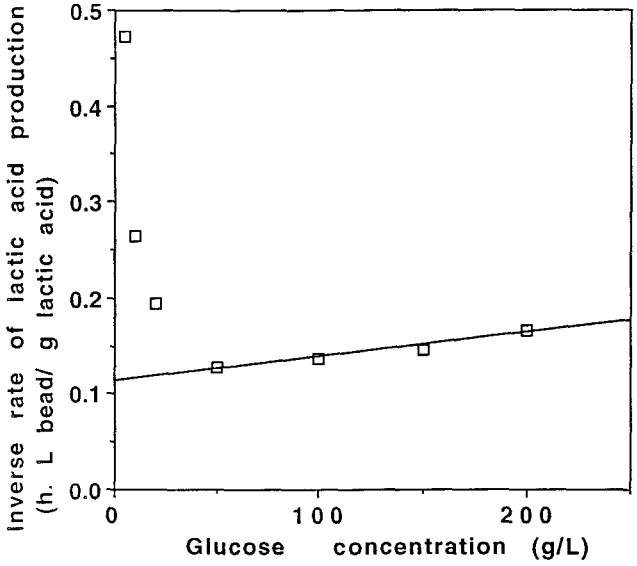

Fig. 4. Inverse rate of lactic acid production vs glucose concentration. The four data points were used to evaluate substrate inhibition constant.

of these analyses are shown in Fig. 5. The results indicated that the product inhibition, in this concentration range of lactic acid, can be expressed by a kinetic model of the form:

$$[1 - (C_1 / K_p)] \quad (2)$$

which led us to choose the general rate equation as:

Table 1
The Summary of the Kinetic Parameters

ν_{lm}	11.04 g lactic acid/L-bead h
K_i	365 g glucose/L
K_m	20.9 g glucoce/L
K_p	316 g lactic acid/L

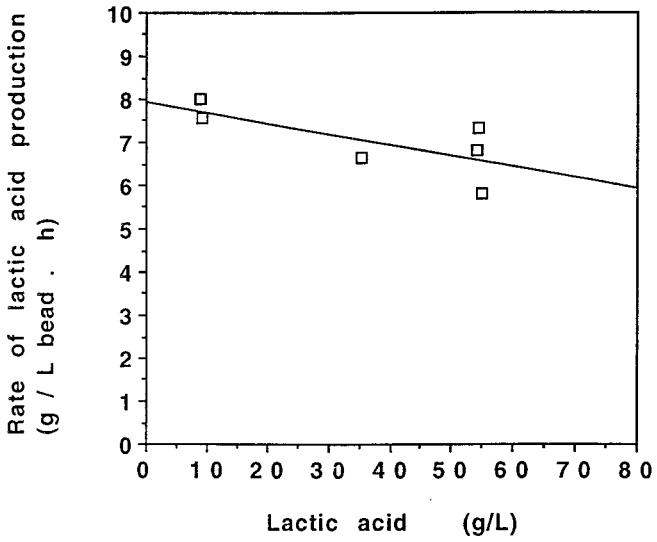

Fig. 5. Lactic acid production rate as a function of lactic acid concentration.

$$\nu_1 = \{[\nu_{1m} C_g] / [C_g + K_m + (C_g^2 / K_i)]\} [1 - (C_1 / K_f)] \quad (3)$$

This equation was used to numerically integrate the fluidized-bed tapered-column data which is presented in Fig. 6. In this analysis, a uniform concentration in the fluidized bed was assumed. The rate equation was based on the bead volume and a function of apparent kinetic parameters and the following equation was used:

$$(V_{total} / V_{bead}) (dC_1 / dt) = \nu_1 \quad (4)$$

Integration was performed using a Runge-Kutta algorithm (*8*), of sixth order and step size was chosen as 0.1 h. A smaller step size of 0.05 h gave results that differed by only 1.0% of the final value. Throughout the analysis of 65% yield of lactic acid on glucose consumed was assumed. The results of this analysis are shown in Fig. 6, where the broken lines represent the simulation of lactic acid and glucose profiles.

The simulation result for the later fermentation phase showed a good agreement with the experimental results as compared to that for the initial lag phase. Since the kinetic data were taken using beads containing cells that were preconditioned before the actual experiments, the model

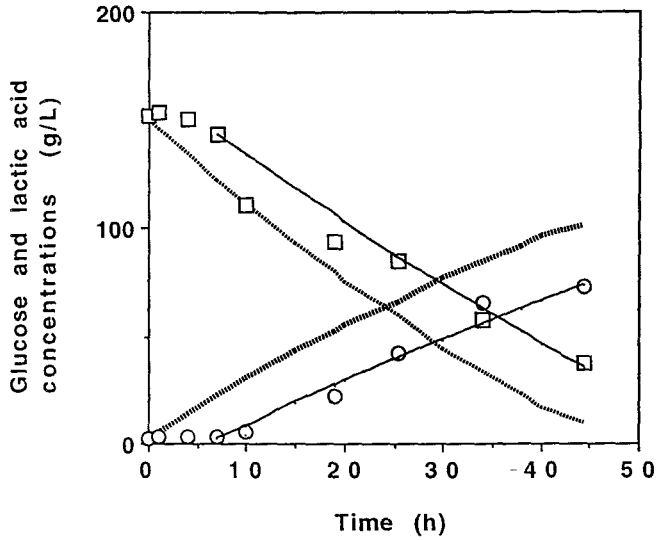

Fig. 6. Fluidized-bed tapered column data for batch production of lactic acid. (□) Glucose, (○) lactic acid, broken lines correspond to simulation when it was started at zero time, solid lines are simulations when it was started with the conditions prevailing at the 8th hour.

may be less accurate in predicting the bioreactor performance during the initial lag phase. This lag phase is believed to be owing to both a physiological lag and to the time it takes to achieve diffusional equilibrium between the beads and the solution. In order to improve the simulation model, the lag time was adjusted by using initial conditions corresponding to the C_g (144 g/L) and C_l (2.4 g/L) at the end of the lag phase. The results of this analysis are given in Fig. 6 as the solid lines. As can be seen the model can simulate the later stages of the fermentation much better.

In conclusion, it is possible to produce lactic acid from glucose solutions efficiently in a fluidized tapered-column bioreactor containing immobilized cells of *R. oryzae*. The fermentation process was simulated using a kinetic model that showed substrate and product inhibition. The simulation result showed a good agreement with the experimental results when the initial lag time is taken into account in the simulation model.

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