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Repeated-Batch and Continuous Production of L-Lactic Acid by *Rhizopus oryzae* Immobilized in Calcium Alginat Beads: Reactor Performance and Kinetic Model*

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Abstract Repeated-batch and continuous production of L-lactic acid by immobilized *Rhizopus oryzae* with calcium alginate entrapment method in a three-phase fluidized-bed bioreactor was studied. The operation conditions were optimized. The productivity based on total reactor volume was about 3 times higher than that with free cells in a traditional stirred tank bioreactor. A mathematical model was proposed and the model predictions were in good agreement with the experimental data.

Keywords L-lactic acid, immobilized fermentation, bioreactor, kinetic model, *R.oryzae*

1 INTRODUCTION

Lactic acid is one of the most important organic acids extensively used in food, pharmaceutical and chemical industries. According to the optical activity it has two isomers, and only optical active L-lactic acid can be metabolized by the human body. An excessive uptake of D- or DL-lactic acid will result in hyperacidity phenomenon in one's urine and other diseases. Therefore, it is desirable that L-lactic acid should be used in food and pharmaceutical industries instead of those in D- or DL- form. In addition, studies on the potential applications of polymer derived from L-lactic acid monomer in the manufacture of biodegradable plastics indicate that it will probably become an important commodity chemical in the future[1,2].

Production of L-lactic acid using *Rhizopus* species in fermentation accounts for most of processing at the present time because *Rhizopus* species can produce almost solely the L-form of the acid and grow on minimal media. Conventionally free-cell fermentation in a stirred tank bioreactor is employed. *Rhizopus oryzae*, a filamentous fungus, grows aerobically with long mycelia which tends to form large-size pellets and causes dramatic increase in oxygen mass transfer resistance. The mycelia are also liable to anchor on the internal elements of the reactor, such as heat exchanger, bafflers and stirrers. Lin *et al.*[3] studied L-lactic acid fermentation with immobilized cell in a rotating-disc-contactor bioreactor. Hang *et al.*[4] studied the feasibility of producing L-lactic acid by immobilized *R. oryzae* with calcium alginate entrapment. Hamamci *et al.*[5] simulated this process in a tapered-column fluidized-bed batch reactor using the kinetic data taken independently from shake-flask cultures, in which both extra- and intra-particle mass transfers were neglected.

The objectives of the present work were to develop a new process to perform L-lactic acid

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fermentation using immobilized mycelia in a three-phase fluidized-bed bioreactor and to establish the fermentation kinetic model of the process.

2 MATERIALS AND METHODS

2.1 Microorganism and fermentation media

Rhizopus oryzae NRRL 395 was maintained on potato dextrose agar slants and transferred to fresh ones every 2 months.

Fermentation medium consisted of (per liter) 1.0 g urea, 0.2 g KH_2PO_4, 0.2 g $MgSO_4$, 0.05 g $ZnSO_4 \cdot 7H_2O$ and glucose was added as required in the experiments.

2.2 Immobilization Procedure

Spores, which were produced with steamed rice as medium[6], were washed off from the molded rice by vigorous shaking. The spore suspension was mixed with Na-alginate solution to obtain a mixture containing 30 $g \cdot L^{-1}$ of Na-alginate. The mixture was then added dropwisely into a sterile solution with 20 $g \cdot L^{-1}$ $CaCl_2$. The average diameter of the beads was 3.5 mm.

2.3 Experimental Apparatus

The experimental apparatus is shown in Fig.1. A porous glass plate is located at the lower part of the bioreactor to serving as a support for the beads. Sterile air, providing power for fluidizing the beads as well as supplying oxygen for cell growth, is fed at the bottom of the bioreactor and the exhausted air exits at the top. To alleviate production inhibitory effect, sterilized $CaCO_3$ powder is added to the broth from the reservoir at the top of the bioreactor to neutralize the L-lactic acid produced.

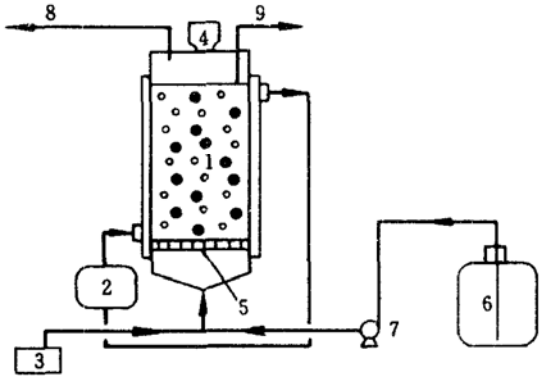


Figure 1 Bioreactor assembly used for L-lactic acid fermentation
1—three-phase fluidized-bed bioreactor, 2.5 L, with 80 mm i.d. and 500 mm high; 2—temperature control system; 3—air supply system; 4—$CaCO_3$ reservoir; 5—porous glass plate; 6—feed reservoir; 7—feed pump; 8—outlet for air; 9—outlet for product

2.4 Analytical Methods

Glucose concentration is determined by Fehling's method[7] and the calcium lactate concentration measured by the EDTA method[7].

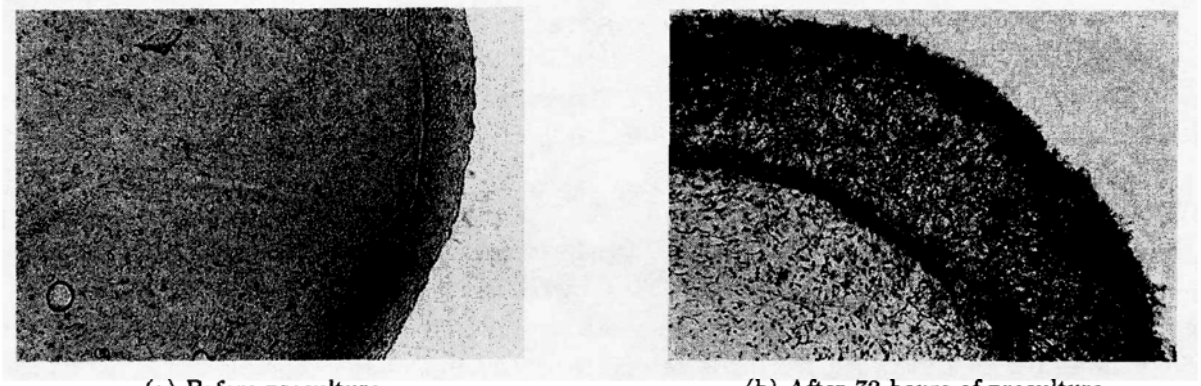
3 RESULTS AND DISCUSSION

3.1 Repeated-batch Production of L-lactic Acid

Immobilized *R. oryzae* beads were precultured at 30 °C in 500 ml Erlenmeyer flasks shaken at 160—180 $r \cdot min^{-1}$. Photographs of the beads before and after preculture were shown in Fig.2. It can be seen that before preculture the spores are uniformly distributed in the beads, but after preculture a dense thin shell of mycelia is formed on the surface of the beads, which is favorable for oxygen transfer and L-lactic acid production.

Immobilized mycelia, precultured in shaking flasks for up to 72 h, are transferred into the three-phase fluidized-bed bioreactor. As shown in Fig.3, in the first cycle, there is a lag phase for

the immobilized mycelia to be adapted in the new environment, then the glucose consumption and L-lactic acid formation begin to behave almost linearly with time. After the first cycle, fermentation broth is withdrawn from the bioreactor and fresh medium is added for starting a new cycle. Immobilized mycelia are reused in the new cycle. Five subsequent repeated cycles are being processed, keeping almost similar fermentation rate. The stability of the fermentation rate can be maintained for more than two weeks without trouble. From Fig.3 we can see that the fermentation rate is almost in zero-order reaction kinetics. This is due, possibly, to the fixed surface area of the beads, leading to unchanged amount of biocatalyst, and may also due to the fact that fermentation rate is relatively insensible to the glucose and calcium L-lactate concentration.



(a) Before preculture (b) After 72 hours of preculture

Figure 2 Photographs of Ca-alginate gel beads before and after preculture

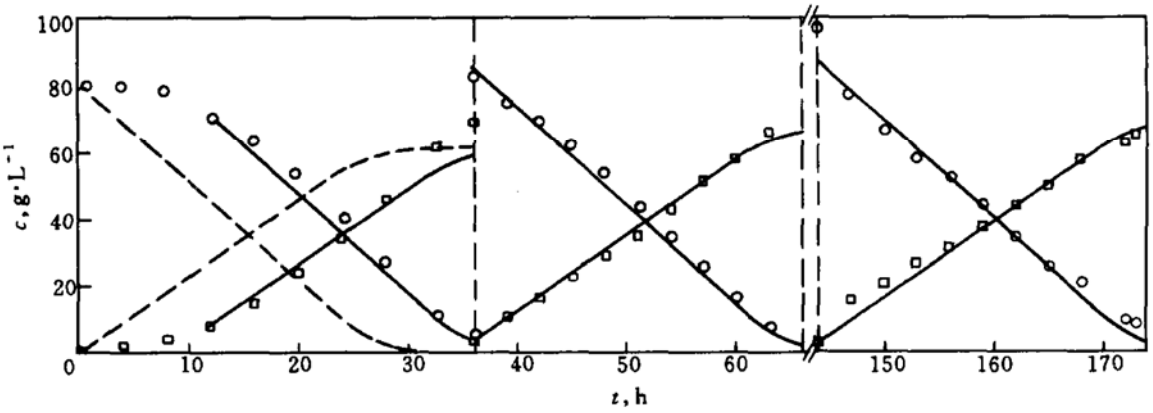


Figure 3 Time courses of repeated batch L-lactic acid production by immobilized *R. oryzae* in the three-phase fluidized-bed bioreactor

(solid lines predicted by the mathematical model) (temperature 33 °C, aeration rate v_a 1.0 vvm, beads volume 140 ml, culture media volume 1000 ml)

---- predicted from $t = 0$ h; — from $t = 12$ h, *i.e.* lag phase has deducted

○ glucose; □ L-lactic acid.

A series of repeated-batch fermentations in the three-phase fluidized-bed bioreactor were performed to determine the optimal operation conditions including the liquid/solid ratio, aeration rate and fermentation temperature.

3.1.1 *Effect of liquid/solid ratio*

As shown in Fig.4, the rate of L-lactic acid formation is directly proportional to the volume of immobilized beads added into the bioreactor. This also means that the fermentation rate is proportional to the total surface area of the beads or the amount of biocatalyst present. More

beads should be added to obtain higher productivity, as related to total bioreactor volume. But a much too small liquid/solid ratio will bring about problems in fluidization and oxygen supply. Experimental results indicate that the appropriate liquid/solid ratio should be around 5/1—6/1.

3.1.2 *Effect of aeration rates*

As mentioned air supply is necessary for the fluidization of beads as well as for the growth of the *R. oryzae*. The critical fluidization rate is relatively low(about 0.7 vvm) because of small density difference between liquid and solid phases. Fig.5 shows the effect of aeration rate on the specific rate of L-lactic acid formation. It can be seen that with the increase of the aeration rate from 0.8 vvm to 1.0 vvm, the rate of L-lactic acid formation per unit volume of beads increases from about 13 to 17 $g \cdot L^{-1} \cdot h^{-1}$. Any further increase in aeration rate beyond this, apparently, will not lead to any increase in L-lactic acid fermentation rate, which explains that oxygen supply is no longer the limiting factor. It has been observed through the retention time distribution measurements, however, that bioreactor may be considered as a perfect mixing vessel when the aeration rate is 1.0 vvm, therefore, the aeration rate of 1.0 vvm is adopted in the subsequent experiments.

3.1.3 *Effect of fermentation temperature*

Fig.6 shows that the maximum specific rate of L-lactic acid formation will be obtained when immobilized *R. oryzae* was cultured at 38 ℃, which is higher than the optimal temperature for free cell culture (30 ℃)[7]. But higher temperature tends to accelerate the ageing of immobilized mycelia and lead to the production of other organic acids. Therefore fermentation temperature should be set at about 33 ℃.

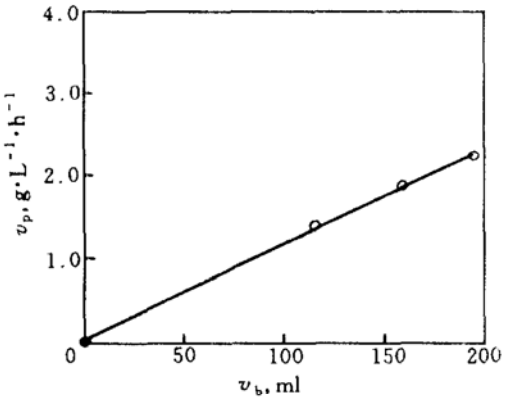


Figure 4 Effect of volume of immobilized beads added into the bioreactor on the rate of L-lactic acid formation
(temperature 28 ℃, aeration rate 1.0 vvm, culture media volume 1000 ml)

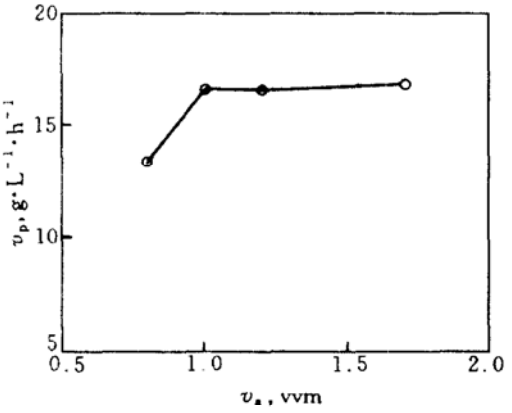


Figure 5 Effect of aeration rate on the specific rate of L-lactic acid formation
(temperature 33 ℃)

3.2 Continuous production of L-lactic acid

Fresh medium is continuously fed into the bioreactor by a peristaltic pump and then withdrawn from the top of the bioreactor. The working volume in the bioreactor is maintained unchanged by the location of outlet tube. The L-lactic acid production is carried out in continuous operation mode for over ten days. Then the fermentation rate tends to slow down, the process is then terminated.

Different dilution rate and inlet glucose concentration are evaluated during the continuous fermentation to determine the optimal operation conditions. Time courses are shown in Fig.7

and Fig.8. After an initial lag phase, with the decrease of inlet glucose concentration, the effluent glucose concentration decreased gradually, but the L-lactic acid concentration was almost unchanged because glucose supply was in excess and the fermentation rate was constant.

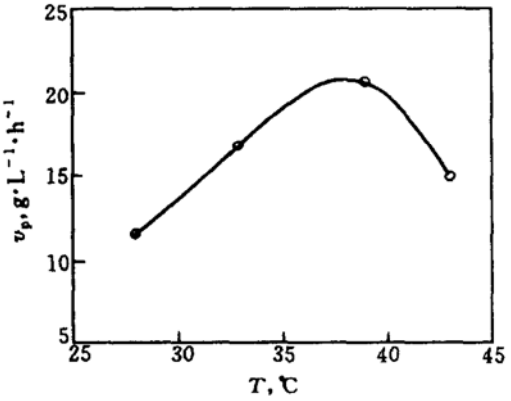


Figure 6 Effect of fermentation temperature on the specific rate of L-lactic acid formation
(aeration rate 1.0 vvm)

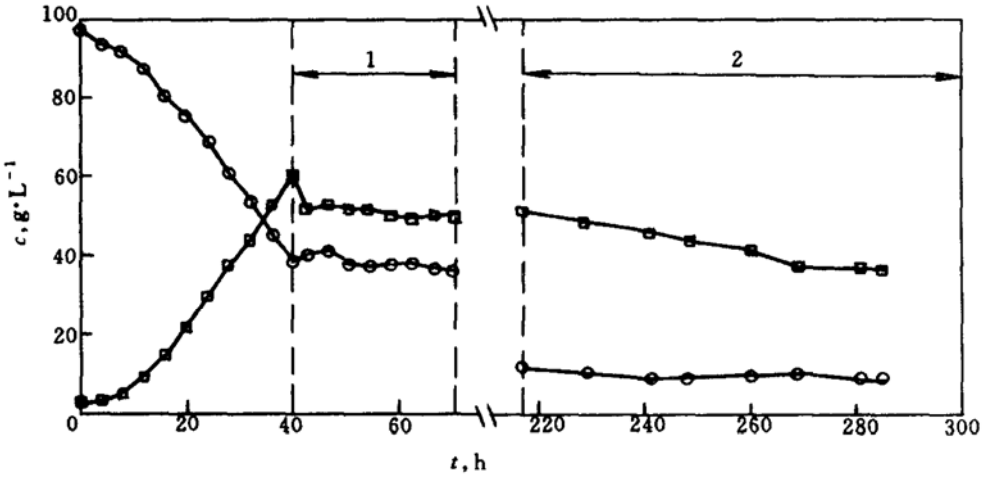
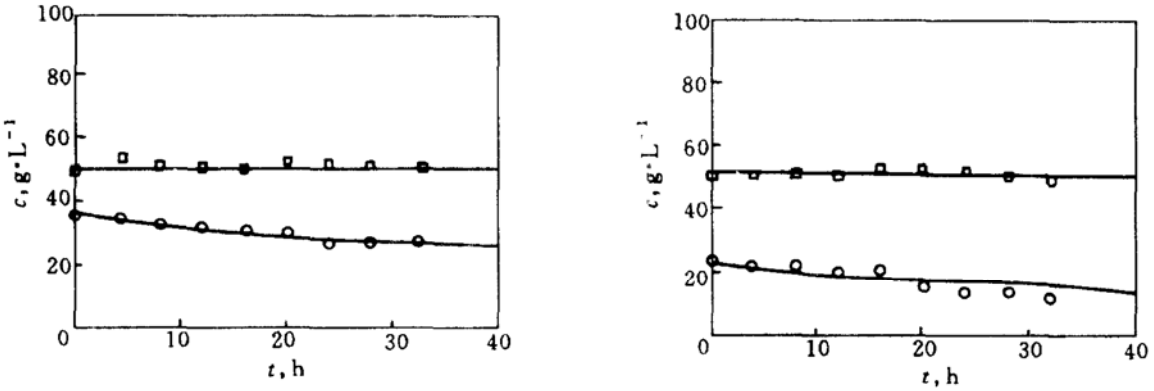


Figure 7 Time courses of continuous L-lactic acid production with immobilized *R.oryzae* in the three-phase fluidized-bed bioreactor
(temperature 33 °C, aeration rate 1.0 vvm, beads volume 170 ml,
culture media volume 1540 ml, dilution rate 0.035 h^{-1}, inlet glucose concentration: 1—101.00 $g \cdot L^{-1}$;
2—67.05 $g \cdot L^{-1}$) ○ glucose; □ lactic acid

The effects of dilution rate and inlet glucose concentration on the continuous fermentation are listed in Table 1. It can be seen that with increases in dilution rate, the specific rate of L-lactic acid formation and yield remain almost constant while the glucose utilization ratio and effluent L-lactic acid concentration tend to decrease. Lower inlet glucose concentration will promote glucose utilization ratio and effluent L-lactic acid concentration. And the low solubility limitation of calcium L-lactate should always be kept in mind, crystalization of calcium lactate in the fermenter should be prevented. Therefore an optimal rate of glucose supply should be found. For example if the glucose-to-L-lactic acid yield is 75%, the inlet glucose concentration should be 90 $g \cdot L^{-1}$ and the dilution rate should be 0.057 h^{-1}.



(a) From 101.00 g·L^{-1} to 91.96 g·L^{-1} (b) From 91.96 g·L^{-1} to 80.89 g·L^{-1}

Figure 8 Time courses of continuous *l*-lactic acid production after a step change in inlet glucose concentration

(Operation conditions were similar to that for Fig.7)

——predicted with the model; ○ glucose; □ lactic acid

Table 1 Effect of dilution rate and inlet glucose concentration on the continuous fermentation with immobilized *R.oryzae*

Dilution rate h^{-1}	Inlet glucose concentration $g \cdot L^{-1}$	Effluent glucose concentration $g \cdot L^{-1}$	Effluent lactic acid concentration $g \cdot L^{-1}$	Liquid/solid ratio	Specific rate of lactic acid formation $g \cdot L^{-1} \cdot h^{-1}$	Glucose utilization ratio %	Yield %
0.035	101.00	36.16	50.21	9.0/1	15.82	64.2	77.4
0.039	91.96	27.52	51.15	9.0/1	17.95	70.1	79.4
0.034	80.89	9.64	55.42	9.0/1	16.96	88.1	77.8
0.073	91.96	36.56	41.97	5.4/1	16.54	60.2	75.8
0.140	78.10	41.78	27.01	4.7/1	17.77	46.5	74.4

4 MATHEMATICAL MODEL

4.1 Model Assumptions

(1) The immobilized biocatalysts are spherical particles with an average diameter of 3.5 mm. The mycelia of *R. oryzae* is uniformly distributed on the surface of the particle to form a shell with a thickness of 0.5 mm and the fermentation will take place on the shell layer. The activity of the mycelia is kept stable throughout the fermentation process.

(2) A Monod equation considering both substrate and product inhibitions is used to describe the L-lactic acid production.

$$v_p(s,p) = \frac{v_m}{1 + K_s/s + s/K_{si}}(1 - \frac{p}{K_{pi}})^n \tag{1}$$

(3)The three-phase fluidized-bed bioreactor is regarded as a perfect CSTR and the volume of fermentation broth stays constant.

4.2 Model Development

The mass balance equations of glucose and lactic acid in three-phase fluidized-bed bioreactor can be described as follows

$$V_R \frac{ds}{dt} = F(S_i - S) - V_b \eta v_p / Y_{p/s} \tag{2a}$$

$$V_R \frac{dp}{dt} = F(p_i - p) + V_b \eta v_p \tag{2b}$$

where the specific rate of product formation, v_p, is described by a Monod kinetics as shown in Eq.(1).

The mass balance eqution of glucose and lactic acid on shell layer of the mycelia are expressed by

$$D_{e,s}\left(\frac{d^2 s_g}{dr^2} + \frac{2}{r}\frac{ds_g}{dr}\right) = v_s(s_g, p_g) \tag{3a}$$

$$D_{e,p}\left(\frac{d^2 p_g}{dr^2} + \frac{2}{r}\frac{dp_g}{dr}\right) = -v_p(s_g, p_g) \tag{3b}$$

The boundary conditions are

$$\left.\frac{ds_g}{dr}\right|_{r=R-\delta} = 0 \tag{3c}$$

$$\left.\frac{dp_g}{dr}\right|_{r=R-\delta} = 0 \tag{3d}$$

$$D_{e,s}\left.\frac{ds_g}{dr}\right|_{r=R} = k_{F,s}(s_b - s_g|_{r=R}) \tag{3e}$$

$$D_{e,p}\left.\frac{dp_g}{dr}\right|_{r=R} = k_{F,p}(p_b - p_g|_{r=R}) \tag{3f}$$

According to the definition of the effectiveness factor, an equation for the effectiveness factor is

$$\eta = \frac{4\pi R^2 D_{e,s}\left.\frac{ds_g}{dr}\right|_{r=R}}{\frac{4}{3}\pi\left[R^3 - (R-\delta)^3\right] v_s(s_b, p_b)} = \frac{-4\pi R^2 D_{e,p}\left.\frac{dp_g}{dr}\right|_{r=R}}{\frac{4}{3}\pi\left[R^3 - (R-\delta)^3\right] v_p(s_b, p_b)} \tag{4}$$

4.3 Kinetic parameters estimation

Two sets of independent experiments in shake flasks with free cell were carried out to determine the kinetic parameters of Eq.(1). The effects of glucose and L-lactic acid concentration on the fermentation rate are shown in Figs.9 and 10 respectively. The parameters in Eq.(1) correlated with experimental data are listed in Table 2. The sufficient $CaCO_3$ has been added to the broth to meutralize lactic acid produced, the product should be calcium lactate rather than free acid. The inhibition caused by calcium lactate is negligible, therefore, the product inhibition term in Eq.(1) can be omitted and the Monod equation could be written in the following simplified form

$$v_p(s, p) = \frac{v_m}{1 + K_s/s + s/K_{si}} \tag{5}$$

The mass transfer coefficients of glucose and lactic acid are to be calculated using the Sangar's equation[9]. Intraparticle diffusivity of glucose, D_e, is taken from literature[10], whereas the intraparticle diffusivity of lactic acid is not reported in literature, and has to be assumed $D_{es} = D_{ep}$. The obtained K_f and D_e values are listed in Table 2.

Table 2 The summary of the kinetic parameters

D_e, $cm^2 \cdot s^{-1}$*	3.0×10^{-7}
$k_{F,s}$, $m \cdot s^{-1}$**	11.1×10^{-5}
$k_{F,p}$, $m \cdot s^{-1}$	14.3×10^{-5}
v_m, $g \cdot L^{-1} \cdot h^{-1}$	25.36
K_s, $g \cdot L^{-1}$	0.7077
K_{si}, $g \cdot L^{-1}$	752.3237
K_{pi}, $g \cdot L^{-1}$	11.785
n	0.5

* obtained from Ref. [10].

* * calculated by the method introduced by Ref. [9].

4.4 Model prediction

The model developed above had been applied to predict the experimental data for different fermentation conditions. The predicted curves for repeated-batch fermentation are shown in Fig.3. The continuous fermentation with step change in inlet glucose concentration are also predicted with the model as shown in Fig.8. The results indicates that the proposed mathematical model with the parameters obtained from independent experiments with free cells as well as from literature is capable of predicting the experimental data with good agreement except for the initial lag phase in the first cycle of the repeated-batch fermentation.

From Table 2, we can find that the parameter K_s is small whereas K_{si} is large. According to Eq.5, the product formation rate v_p is indeed a zero-order reaction kinetics if the substrate concentration is in the range of 10—100 $g \cdot L^{-1}$. In the repeated fed batch process, the substrate concentration falls within this range of the time in a cycle, therefore the time courses are almost straight lines.

The relationship among the effectiveness factor η and substrate concentration and product concentration is shown in Fig.11. The results indicate that with increases in substrate concentration, the effectiveness factor η will tend to increase, because of the relative higher value of $ds/dr|_{r=R}$ at higher S value. But if $S > 23.07\,g \cdot L^{-1}$, the increase of η value becomes less notable. On the other hand, increasing lactic acid concentration will bring about the decrease in the effectiveness factor η due to product inhibition. The value of the effectiveness factor η is in the range of 0.5—0.7, which explains that interparticle mass transfer resistance is an important issue for the overall fermentation rate.

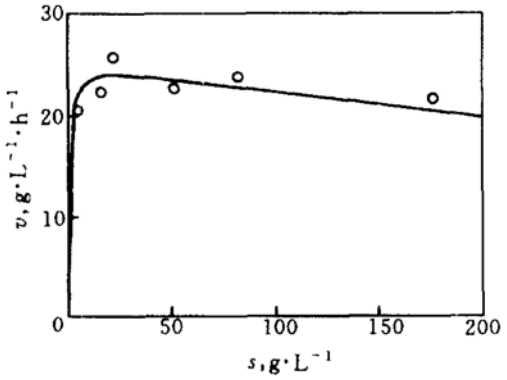


Figure 9 Effect of glucose concentration on specific rate of product formation

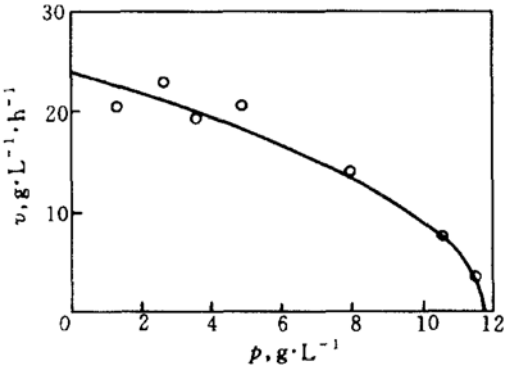


Figure 10 Effect of lactic acid concentration on specific rate of product formation

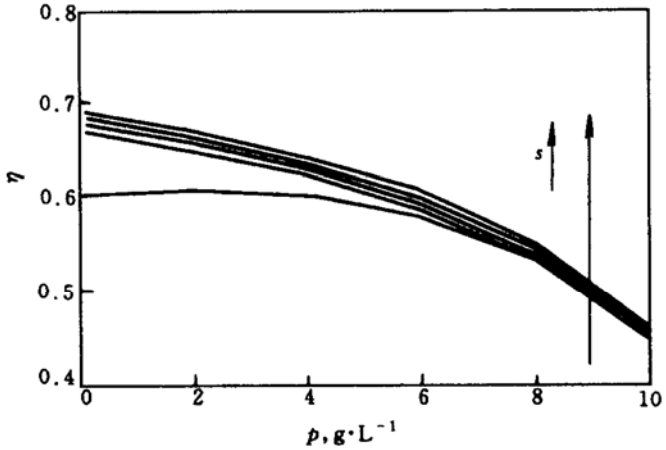


Figure 11 Relationship among the effectiveness factor η, substrate concentration and lactic acid concentration

5 CONCLUSIONS

In summary, L-lactic acid production by employing immobilized *Rhizopus oryzae* with alginate calcium entrapment method in a three-phase fluidized-bed bioreactor can be carried out either in repeated batch or in continuous operation modes, with a lifetime of more than two weeks. The optimal operation conditions are as follows: for the beads, culture media volume=1:(5—6); aeration rate 1.0 vvm; temperature 33°C. The specific rate of L-lactic acid formation per volume of beads will be 16—18 $g \cdot L^{-1} \cdot h^{-1}$, and glucose-to-L-lactic acid yield is 70%—80%. The productivity based on total reactor volume is 3—4 $g \cdot L^{-1} \cdot h^{-1}$, which is about 3 times higher comparing with free-cell fermentation in traditional stirred tank bioreactor(0.65—1.53 $g \cdot L^{-1} \cdot h^{-1}$ [8]). The results indicate that the immobilized cell fermentation in a three-phase fluidized-bed bioreactor can be an alternative method for L-lactic acid fermentation.

A mathematical model to describe the process is suggested. In the model, the kinetics of immobilized *R.oryzae* is expressed by a Monod kinetics taking both substrate and product inhibitions into consideration, and the extra- and intra-particle mass transfer are being simplified by employing an effectiveness factor method. The model predictions with parameters determined from independent experiments are in good agreement with the experimental data.

NOMENCLATURE

c	concentration in fermentation solution, $g \cdot L^{-1}$
D_e	effective diffusivity, $cm^2 \cdot s^{-1}$
F	flow rate of feed media, $L \cdot h^{-1}$
K_{pi}	product inhibition constant, $g \cdot L^{-1}$
K_s	saturation constant, $g \cdot L^{-1}$
K_{si}	substrate inhibition constant, $g \cdot L^{-1}$
k_F	Michaelis-Menten constant, $m \cdot s^{-1}$
p	concentration of lactic acid, $g \cdot L^{-1}$
R	radius of a particle, mm
r	radial distance in a spherical particle, mm
s	concentration of glucose, $g \cdot L^{-1}$
t	time, h
V_b	volume of immobilized beads packed in the bioreactor, L
V_R	volume of culture medium in the bioreactor, L

v rate of product formation per volume of beads, $g \cdot L^{-1} \cdot h^{-1}$
v_a aeration rate, vvm
v_m maximum rate of product formation per volume of beads, $g \cdot L^{-1} \cdot h^{-1}$
$Y_{p/s}$ yield of lactic acid from glucose, $g \cdot g^{-1}$
δ thickness of the shell layer of mycelia, mm
η effectiveness factor

Subscripts

b in bulk solution
g in immobilized beads
i inlet
p lactic acid
s glucose

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