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Fermentative intensity of L-lactic acid production using self-immobilized pelletized *Rhizopus oryzae*

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L-Lactic acid is a promising three-carbon building-block chemical, widely used in the food, pharmaceutical, leather and textile industries and *Rhizopus oryzae* is an important filamentous fungus for the production of L-lactic acid with high optical purity. This study investigated the medium compositions for the maximum biomass cultivation of *R. oryzae* L-lactic acid fermentation, and optimized the operation parameters for semi-continuous repeated fermentation in a stirred tank fermentor using response surface method (RSM) analysis. The results indicated that a higher biomass cultivation of 3.750±0.05 g/L was achieved when the medium was composed of 12% (w/v) glucose, 0.4% (w/v) ammonium sulfate and 0.045% (w/v) monopotassium phosphate. The optimal fermentation conditions for the initial batch were as follows: the aeration was 0.75 L/(L·min), inoculation of germs was 11% and agitation speed was 560 rpm. The fermentative intensity of the initial batch and the sequentially repeated batches with self-immobilized pelletized *R. oryzae* were 2.162 g/(L·h) and 3.704 g/(L·h), respectively.

Key words: Self-immobilized, *Rhizopus oryzae*, pellet, lactic acid, response surface method (RSM).

INTRODUCTION

L-Lactic acid is a natural chemical, widely used as acidulant, flavor and preservative in the food, pharmaceutical and leather industries (Efremenko et al., 2006; Rojan et al., 2007; Gullon et al., 2008). It can further be polymerized into polylactic acid, which is applied in biodegrable plastics and textile fiber production. Currently, this environmental friendly polymer attracts the interest of the researchers all over the world (Zhang et al., 2007; Sauer et al., 2008; Maneeboon et al., 2010).

Rhizopus oryzae belongs to the family of filamentous fungus and possess excellent fermentation capability in producing optically pure L-(+)-lactic acid from biomass. The morphology of *R. oryzae* vary from dispersed filamentous clumps to pellet mycelia during the fermentative process to produce L-lactic acid when produced by submerged fermentation technology. Physical forms of fungal growth are influenced by the strain of fungi, nutrient compositions, spore concentrations, pH of medium, agitation and aeration (Yang et

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al., 1995; Zhou et al., 2000; Bai et al., 2003; Buyukkileci et al., 2006; Liu et al., 2008; Zhao et al., 2010; Mohamed et al., 2013). The relationship between morphology of *R. oryzae* and lactate productivity was investigated for the purpose of enhancing the conversion rate of glucose.

R. oryzae mycelia having floc and pellet forms are biologically active at the end of the submerged batch fermentation and these two forms have been demonstrated to be the feasible morphologies to achieve a more efficient conversion of lactate (Kosakai et al., 1997; Liu et al., 2006; Yu et al., 2007). Therefore, *R. oryzae* mycelia with floc and pellet forms have been utilized to produce L-lactic acid in the semi-continuous fermentation. In order to obtain *R. oryzae* mycelia with floc and pellet forms, an initial fermentation was performed, in which at the end, the liquid broth was drained and the mycelia were separated and kept in the fermentor for reuse. The fresh medium was then supplied into the fermentor, and the next cycle of fermentation started. This semi-continuous fermentation had some merits such as immobilization carrier-independence, lower viscosity of the suspension in favor of mass transfer, shorter fermentation cycles and good production stability (Yang et al., 1995; Yin et al., 1998; Yu et al., 2007). Pellet was an appropriate form for recycling use of *R. oryzae* mycelia (Liu et al., 2006, 2008; Liao et al., 2007).

In the semi-continuous fermentation, the biomass cultivation is another important factor besides the optimal medium compositions and operation parameters. Since more mycelia are formed in the initial batch fermentation, the fermentation period for the following repeated batches would be shortened and the productivity of lactate would be enhanced (Du et al., 1998; Yin et al., 1998; Martak et al., 2003; Yu et al., 2007; Liu et al., 2008; Wang et al., 2013).

In the present study, the medium constituents were optimized to form more biomass cultivation and the initial batch fermentation parameters in a 4 L fermentor were established by using response surface methodology. Moreover, the fermentative intensity of the initial and the repeated production of L-lactic acid were also comparatively investigated.

MATERIALS AND METHODS

Microorganism and media

R. oryzae As 3.819, a highly optical purity L-(+)-lactic acid producing strain was provided by China General Microbiological Culture Collection Center. The strain was maintained on potato dextrose agar (PDA) slants.

Seed culture

Spores of *R. oryzae* were grown on a PDA slant at 32°C for 3 days. They were collected with a platinum loop and suspended in sterilized distilled water. 1 mL (2% (v/v) of the sterile seed medium broth) of the fungi spore suspension (the concentrations were adjusted to be $5\times l0^6$ spores/mL) were inoculated into 50 mL sterile seed medium in a 250 mL flask with the composition of 12% (w/v) glucose, 0.4% (w/v) ammonium sulfate, 0.045% (w/v) monopotassium phosphate, 0.044% (w/v) heptahydrate and 0.025% (w/v) magnesium sulfate. The culture was incubated in a rotary shaker for 12 or 24 h (12 h for stirred tank fermentor, 24 h for flask) at 32°C, with the agitation speed of 200 rpm. 0.1% (w/v) sterilized calcium carbonate was added to the seed medium at the beginning of the culture in order to prevent a decrease in pH.

Biomass culture in the flask

5 mL (10% (v/v) of the sterile production medium broth) seed culture of *R. oryzae* As 3.819 was inoculated in 50 ml production medium in a 250 mL flask with the composition of 12% (w/v) glucose, 0.3% (w/v) ammonium nitrite, 0.0214% (w/v) dihydrogen phosphate ions with 1:1 of the concentration ratio of K^+/Na^+, 0.022% (w/v) heptahydrate, 0.025% (w/v) magnesium sulfate. The rotary shaker ran at the speed of 200 rpm under 32°C for 60 h, and the broth was taken out for the analysis of biomass content. 0.6% (w/v) sterilized calcium carbonate was added to the production medium at the beginning of the culture in order to prevent a decrease in pH.

Biomass culture in the fermentor

A 4 L stirred tank (Zhenjiang East Biotech Equipment and Technology Co., Ltd, P.R.C.) was used in this study. Culture was performed in a working volume of 2.5 L production medium at 32°C, 250 mL seed culture were inoculated. The fermentation was incubated at 32°C for 60 h, with the aeration rate of 1.0 L/(L·min) and agitation speed of 300 rpm. 0.6 % (w/v) sterilized calcium carbonate was added to the culture medium at the beginning of the fermentation in order to prevent a decrease in pH.

The repeated fermentation in the fermentor

In the end of each batch, the agitation and aeration were stopped, five sixth volume of broth was flowed out through a given outlet due to inner pressure from sterilized air and the vegetative mycelia were kept in the fermentor. Then, the sterile repeated fermentation medium was added into the tank to the volume of the initial batch with the composition of 8% (w/v) glucose, 0.3% (w/v) ammonium nitrite, 0.0075% (w/v) monopotassium phosphate, 0.022% (w/v) heptahydrate, 0.025% (w/v) magnesium sulfate and 0.4% (w/v) calcium carbonate. The operation parameters of the fermentor for the repeated batches were the same as the biomass culture in the fermentor.

Effect of glucose, ammonium sulfate and monopotassium phosphate on the fungal biomass cultivation

To optimize the medium compositions of glucose, ammonium sulfate and monopotassium phosphate (Wu et al., 2011) and obtain higher biomass cultivation, an orthogonal test of L_9 (3^4) with 3 replicates was designed. The factors and their levels of the orthogonal test were designed according to the single-factor experiments results in advance and listed in Table 1. The results were treated with the variance analysis of Pan and Chen (2008).

Effect of operation parameters on the fermentative intensity of initial batch in stirred tank

In order to study the productivity in the initial batch of fermentation

Table 1. Factors and levels for orthogonal test.

Factors	Levels of each factor		
	1	2	3
A: Glucose (%(w/v))	9	12	15
B: Ammonium sulfate (%(w/v))	0.25	0.4	0.55
C: Monopotassium phosphate (%(w/v))	0.015	0.030	0.045

Table 2. Factors and levels of the fermentation for the principle of central composite design of Box-Behnken.

Factor	Coding	Value of factor	Level
(A) aeration (L/(L·min))	X_1[a]	+1	1.0
		0	0.6
		-1	0.2
(B) inoculation (%(w/v))	X_2[b]	+1	15
		0	10
		-1	5
(C) agitation (rpm)	X_3[c]	+1	700
		0	500
		-1	300

[a] X_1 = (A-0.6)/(0.6-0.2), [b] X_2 = (B-10)/(10-5), [c] X_3 = (C-500)/(500-300).

in fermentor, an experiment based on the principle of central composite design Box-Behnken with three factors of aeration, inoculation and agitation speed, each in three levels was designed (Table 2) (Thana et al., 2008; Wei et al., 2009). The response surface and contour plots were generated to understand the interaction of various variables and then used to find the optimum operation parameters mainly affecting the response.

Analytical methods

L-lactic acid was extracted from the fermented medium with 0.5 M H_2SO_4, diluted with distilled-water and filtered through a 0.22 μm membrane. L-lactic acid concentration was analyzed by HPLC equipped with Purospher STAR C18 (Merck, USA) 250×4.6(5 μm) column and UV detector at 210 nm. The eluent was 5 mM H_2SO_4 with a flow rate of 0.8 mL/min. The residual glucose concentration was determined by the 3,5-dinitrosalicylate method. Dry biomass was monitored by harvesting culture samples, filtering and washing the mycelia with 0.01 M HCl to remove potential calcium carbonate, then washed with distilled water to pH 6. The washed mycelia were dried at 80°C until constant weight was achieved, then weighed (Zheng et al., 2009; Wu et al., 2011). The formula of calculating the productivity was:

$$I_F = \frac{D_L}{T}$$

Where I_F is the productivity (g/(L·h), D_L is the concentration of lactic acid (g/L) and T is the time of fermentation (h).

Statistical analysis

All the determinations reported in this work were carried out in triplicate and experiments were executed at least in duplicate; the results are given as the mean values. In the experiment design of Box-Behnken, the software package SAS9.0 (SAS Institute, USA) was used for experimental design, data analysis and the quadratic model building.

RESULTS AND DISCUSSION

Optimization of the culture medium for fungal biomass cultivation

Previous study showed that the concentrations of glucose, ammonium sulfate and monopotassium phosphate in medium showed significant impact on the biomass cultivation and formation of pellet. High biomass cultivation in initial batch fermentation and the pelletized mycelia are beneficial to the enhancement of lactic acid productivity (Liao et al., 2007). The effects of glucose, ammonium sulfate and monopotassium phosphate in the production medium on biomass were investigated by using orthogonal experiment $L_9(3^4)$ in Table 3.

With the computational analysis in Table 4, it was not difficult to show that the optimal combination was $A_2B_2C_3$ from the values of K_1, K_2 and K_3 (the sum of the results with each level) of each factor in Table 3, which was the same for the result of the directly analysis, and it means that when the glucose was 12% (w/v), ammonium sulfate was 0.4% (w/v) and monopotassium phosphate was 0.045% (w/v), the achieved L-lactic acid concentration reached the highest level. The results of verification tests with the optimum conditions from direct analysis and computational analysis (test results were not shown) suggested that the biomass was 3.750±0.05 g/L in triplicate experiments, and the optimized results were tested to be consistent with a further independent experiment.

Optimization of operation parameters of initial batch in stirred tank

The initial batch fermentation played an important role in the total process with self-immobilized *R. oryzae*. Under the feasible fermentation conditions, mycelia can flocculate into proper size of compact pellets together

Table 3. The optimization of the culture medium by orthogonal experimental design.

Number	Factor				Biomass
	A (%)	B (%)	C (%)	D	(g/L)
1	1 (9)	1 (0.25)	1 (0.015)	1	1.530
2	1	2 (0.4)	2 (0.030)	2	1.964
3	1	3 (0.55)	3 (0.045)	3	2.096
4	2 (12)	1	2	3	2.610
5	2	2	3	1	3.790
6	2	3	1	2	2.306
7	3 (15)	1	3	2	1.924
8	3	2	1	3	1.824
9	3	3	2	1	1.564
K_1	5.590	6.064	5.660	6.884	
K_2	8.706	7.578	6.138	6.194	
K_3	5.312	5.966	7.810	6.530	
$K_1/3$	1.863	2.021	1.887	2.295	T=19.604
$K_2/3$	2.902	2.526	2.046	2.065	
$K_3/3$	1.771	1.989	2.603	2.177	
SS	2.367	0.544	0.850	0.079	

Table 4. Analysis of variance for the orthogonal design.

Source of variance	SS	f	MS	F	Significant
A	2.367	2	1.184	29.827	**
B	0.544	2	0.272	6.860	*
C	0.850	2	0.425	10.705	*
E	0.079	2	0.040		
sum	3.840	8			

$F_{0.01}(2,8) = 8.65$, **, $F_{0.05}(2,8) = 4.46$,*, $F_{0.1}(2,8) = 3.11$, $F_{0.25}(2,8) = 1.66$. SS, sum of square for each factor; f, freedom; MS, mean of sum of square for each factor; F, F-ratios, e, total of error; sum, total of sum of square.

with the production terminal arrival and is promising for further fed-batch fermentation. The impacts of operation parameters of initial batch in stirred tank on lactic acid productivity are shown in Table 5. The following mathematical regression model in terms of coded factors was built to analyze the data by the SAS9.0 software.

$$Y=2.09+0.25X_1+0.13X_2+0.069X_3-0.31X_1^2-0.042X_1X_2-0.02X_1X_3-0.24X_2^2+0.028X_2X_3-0.11X_3^2$$

And the best optimal regression equation was as follows.

$$Y=2.09+0.25X_1+0.13X_2+0.069X_3-0.31X_1^2-0.042X_1X_2-0.24X_2^2-0.11X_3^2$$

Where *Y* is the response, productivity of the lactic acid, X_1, X_2 and X_3 are the factors of aeration, inoculation and agitation speed, respectively.

The analysis results showed that there was a highly significant relationship of multiple regression between the dependent variable and the independent variable (R^2=0.9877). This indicated that the experiment was designed reliably. The pictures of response surface was drawn based on the analysis data of RSM, which visually showed the effects of aeration and inoculation of germs (Figure 1). An order partial derivative of the nonlinear regression model was based on the optimum of each independent variable, and then let the result to be zero, to obtain the maximum of the surface,

$$0.25-0.62X_1-0.042X_2=0$$

$$0.13-0.042X_1-0.48X_2=0$$

$$0.069-0.22X_3=0$$

The results calculated from the equations are: X_1=0.387, X_2=0.237 and X_3=0.314. These numerical values

Table 5. Arrangements and results of the fermentative strength by the principle of central composite design of Box-Behnken.

Number	X_1	X_2	X_3	Y (productivity) (g/(L·h))
1	-1	-1	0	1.15
2	-1	1	0	1.51
3	1	-1	0	1.66
4	1	1	0	1.85
5	0	-1	-1	1.58
6	0	-1	1	1.64
7	0	1	-1	1.79
8	0	1	1	1.96
9	-1	0	-1	1.29
10	1	0	-1	1.89
11	-1	0	1	1.49
12	1	0	1	2.01
13	0	0	0	2.10
14	0	0	0	2.08
15	0	0	0	2.08

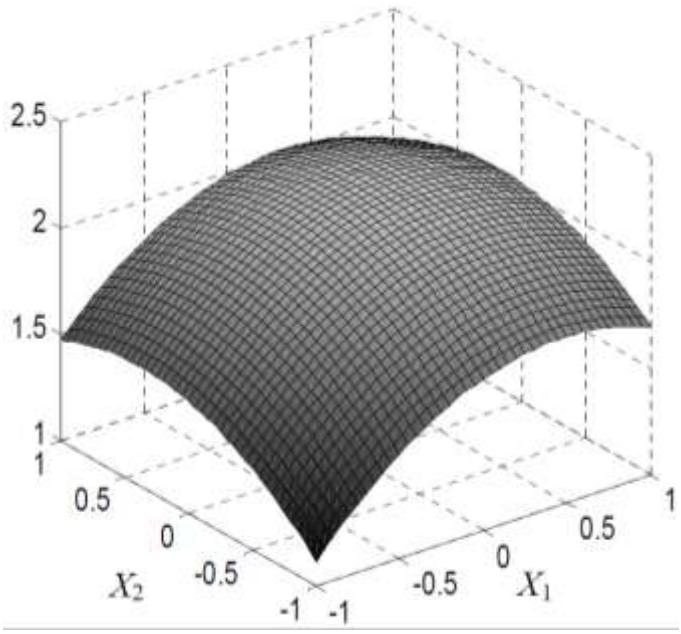

Figure 1. 3D graph of response surface for fermentative intensity (Y) of L-lactic acid between the aeration (X_1) and inoculation of germs (X_2) at agitation speed (X_3=0).

corresponded to the fermentation conditions of aeration of 0.755 L/(L·min), inoculation of 11.18%, and agitation speed of 562.8 rpm. The estimated productivity (Y) upon these solutions was (2.16±0.12) g/(L·h). Based on the feasible of operation, the aeration was 0.75 L/(L·min), the inoculation was 11%, and agitation speed of 560 rpm. The productivity of testing experiment was 2.10±0.05) g/(L·h), which was about 5% of relative error when g/(L·h), which was about 5% of relative error when compared with model predicted values. This showed that using RSM to realize better fermentation parameters was feasible.

Productivity of repeated batches fermentation

During the semi-continuous fermentation by self-immobilized *R. oryzae*, five sixth volume of the fermented broth was drained, the pellets remained and fresh medium was supplied to reach the former volume. The semi-continuous fermentation conditions were kept as those of the initial batch: aeration 0.75 L/(L·min), inoculation 11% and agitation speed off 560 rpm. Under these conditions, the mycelia morphology was maintained as pellet form with diameter of about 1.5 mm, except for a small portion adsorbed to the baffle and impellers. The L-lactic acid productivity was stable at 3.704 g/(L·h).

The fermentative intensity is an important index for the evaluation of fermentation technology and product cost. As for L-lactic acid production by *R. oryzae*, the fermentative intensity of batch submerged fermentation varied from 0.70 g/(L·h) (Liu et al., 2006), 1.80 g/(L·h) (Park et al., 1998) to 2.73 g/(L·h) (Yu et al., 2007). The combination of immobilization technology and semi-continuous fermentation is a good way to enhance the fermentative intensity. Production of lactic acid from glucose with loofa sponge immobilized *R. oryzae* RBU2-10 showed that repeated batch fermentation could be carried out for 10 cycles and the maximum productivity (1.84 g/(L·h)) was obtained at the third cycle of fermentation (Ganguly et al., 2007). The average calculated productivity of L-Lactic acid production from glucose with a semi-batch process using poly(vinyl alcohol)-cryogel-entrapped *R. oryzae* was 2.8±0.4 g/(L·h) (Efremenko et al., 2006). The fungal mycelia of *R. oryzae* NRRL 395 was immobilized on cotton cloth in a rotating fibrous-bed bioreactor to produce L-lactic acid from glucose and a high productivity of 2.50 g/(L·h) was obtained in fed-batch fermentation (Tay and Yang, 2002). In this study, L-lactic acid production using self-immobilized pelletized *R. oryzae* and fed-batch fermentation can reach a higher productivity of 3.704 g/(L·h).

Conclusion

The biomass was achieved (3.75±0.05 g/L) in flask with the optimum medium by self-immobilized *R. oryzae*. Combination theoretical analysis with the actual test, the best fermentation parameters of stirred tank fermentations by self-immobilized *R. oryzae* was: aeration 0.75 L/(L·min), inoculation 11%, agitation speed 560 rpm. Under these conditions, the L-lactic acid productivity of the initial patch was 2.10±0.05 g/(L·h). In the repeated semi-continuous fermentation with the same fermentation conditions as the initial batch, the mycelia morphology was kept in proper pellet size, and the L- lactic acid

productivity was sustained at 3.704 g/(L·h), which is higher than that of other reports.

Conflict of interests

The authors have not declared any conflict of interests.

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