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Full Length Research Paper

Optimization of lactic acid production with immobilized *Rhizopus oryzae*

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Lactic acid production by *Rhizopus oryzae* NRRL 395 immobilized in polyurethane foam was investigated by using response surface methodology. A 2^3 full-factorial central composite design was chosen to explain three independent variables; glucose concentration, pH and agitation rate. The model *F-value* (17.01) shows that predicted model is suitable for good fitting. Linear and quadratic effects of glucose concentration and quadratic effect of agitation rate were shown to be significant for lactic acid production. Maximum lactic acid production 93.2 g/l was obtained using a glucose concentration of 150 g/l, pH 6.39 and agitation rate 147 rpm. Glucose concentration and agitation rate were found as limiting parameters. So, little variation of these parameters alters production of lactic acid. Initial pH has no effect on lactic acid production due to neutralizing agent. Production of lactic acid from immobilized whole cells which are under optimum conditions was determined about 55% that is higher than production of lactic acid from suspension culture systems.

Key words: Lactic acid, *Rhizopus oryzae*, immobilization, response surface methodology.

INTRODUCTION

Lactic acid is the most widely utilized organic acid in the food, pharmaceutical, cosmetics and chemical industries. One of its most promising applications is for used biodegradable and biocompatible polylactate polymers, such as poly-lactic acid (PLA), an environmentally friendly alternative to biodegradable plastics (Datta et al., 1995; Hofvendahl et al., 1999). Physical properties of PLA are strongly influenced by the isomeric composition of lactic acid. Lactic acid occurs in two optical isomers which are D-(-)- and L-(+)-lactic acids naturally. Since elevated levels of the D-isomer are harmful to humans, L-(+)-lactic acid is the preferred isomer of food and pharmaceutical industries (Hofvendahl and Hahn-Hagerdal,2000). Microbial production of lactic acid produces either separately or a mixture in different proportions of two isomers depending on the microorganism, substrate and growth conditions used whereas the chemical production only results in a mixture of the two isomers (Tsao et al., 1999). Another significant advantage over the chemical synthesis is that biological production can use cheap raw materials (Huang et al., 2005).

Lactic acid production by fungi, such as *Rhizopus oryzae* has draw attention recently (Zhang et al., 2007). The immobilization of microorganisms has generally been attractive for industrial fermentation to improve the yield of the desired product. In contrast to ordinary suspension culture systems, immobilized whole cells have the merits of: Avoiding wash-out of cells at a high dilution rate, higher cell concentration in the reactor and easy separation of cells from the system or the product containing solution (Frusaki and Seki, 1992). Hence, the cells have been immobilized by means of adsorption on polymer supports, by embedding with natural polymers like alginate gels and synthetic polymers (Tamada et al., 1992). Several researchers have attempted to use immobilization techniques for L(+)-lactic acid production with *R. oryzae*. The entrapment methods using soft gels such as Ca-alginate have mostly been employed in these studies (Hang et al., 1989). In gel-entrapping methods, the limitation of oxygen supply because of diffusional resistance might decrease the fermentation rate and/or L(+)-lactic acid transformation efficiency (Dong et al.,

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1996). The problems, associated with filamentous fungal fermentations can be overcome with cell immobilization on support polymer matrix. In this work, the cells were immobilized by physical entrapment in the open pore network of reticulated polyurethane foam which provides less diffusional resistance to substrate transfers. Spores would enter the loose matrices and grow inside the cubes. Then the mycelia were embraced by the matrices after growing up (Dong et al., 1996).

The one factor at a time is the most frequently used operation in optimization process. This technique involves changing one independent variable while keeping the other factors constant. The conventional methods for multifactorial experimental design are time-consuming and incapable of detecting the true optimum, especially due to the interactions among the factors (Liu and Tzeng, 1998). In contrast, experimental design offers a number of important advantages as the researchers could easily determine effects of factors with considerably less experimental effort, find real optimum value and facilitate system modeling (Bandaru et al., 2006).

In the present study, we described optimized fermentation medium and conditions to obtain maximum lactic acid production with immobilized *Rhizopus oryzae* using response surface methodology (RSM).

MATERIALS AND METHODS

Microorganism, media and culture conditions

A lactic acid producing strain of *R. oryzae* NRRL 395 was maintained on nutrient agar plates. It was incubated at 30°C for 96 h and then stored at 4°C. After growth and sporulation, 10 ml of distilled water was aseptically added to each agar plates which were then scraped to release the spores. This spore suspension was centrifuged at 4000 rpm for 10 min; the spores were washed and resuspended in 1 ml distilled water. Then, 500 µl spore suspension was used to provide spore inoculum for each of 250 ml shake-flask containing 50 ml of the medium. The flasks were then incubated on a rotary shaker at 30°C and 150 rpm. The fermentation medium contained per liter of distilled water: glucose variable (75 to 175 g), $MgSO_4.7H_2O$ 0.25 g, KH_2PO_4 0.65 g, $(NH_4)_2SO_4$ 2 g. To avoid pH decrease due to lactic acid production, 50 g/l of sterile $CaCO_3$ in powder form was added to each flask approximately 24 h after inoculation (Hamamci and Ryu, 1994).

Analytical procedure

At the end of fermentation, the fermented materials were centrifuged and supernatants were analyzed for L(+)-lactic acid and residual carbohydrate. Lactic acid was analyzed using high performance liquid chromatography (HPLC) (Cecil Instruments 1100 series, Cambridge, UK) with A Bio-Rad (Torrance, CA) Aminex HPX 87C column and an IR detector at 210 nm. An Hewlett-Packard model 3395 integrator was used to record and analyze the data. The eluant, 4 mM H_2SO_4 was used at a flow rate of 0.6 ml/min. Glucose was determined by using Beckman type glucose analyzer. The result of each point was determined as average value from different three flasks.

Polyurethane foam preparation

Foam matrices (15 ppi; pore per inch) were used throughout the work. Prior to use, the support materials submerged in distilled water were autoclaved three times for 15 min at 121°C, the distilled water being replaced each time to remove any chemical that might have otherwise leached out into the culture medium. One foam slab (55 × 20 × 8 mm) was placed in each flask and held stationary by fixing onto a stiff L-shaped stainless steel wire. Each flask was placed in the incubator shaker after sterilization.

Experimental design and statistical analysis

RSM is a collection of experimental strategies, mathematical methods, and statistical inference which enable an experimenter to make efficient empirical exploration of the system of interest. According to this design, 20 experiments were conducted containing six replications at the center point. The independent variables selected for the study of production of lactic acid were: glucose concentration, initial pH and agitation rate. Actual variables and their corresponding coded levels are presented in Table 1. The response variable was fitted by a second order model in order to correlate the response variable (Lactic acid concentration) to the independent variables. The model equation is represented as:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Where, Y is the predicted response; β_o is the intercept; β_i is the linear coefficient; β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's *F-test*, correlation coefficient R, determination coefficient R^2 which measures the goodness of fit regression model. It also includes the student's *t*-value for the estimated coefficients and the associated probabilities *p(t)* (Dey et al., 2001). Analysis of variance (ANOVA) was performed and three-dimensional response surface curves were plotted by Design Expert (version 6.0, Stat-Ease, Inc., Minneapolis, USA) statistical package to study the interaction among components.

RESULTS AND DISCUSSION

In the present study, the relationship between four criteria of lactic acid production and three independent variables (glucose concentration, initial pH and agitation rate) were investigated. The optimum values of parameters for maximum lactic acid production were determined using statistical central composite design according to design matrix which is given in Table 1 and 2. For achieving a more realistic model in this method, prior knowledge obtained from previous studies are required. In our previous works, we showed that glucose concentration, initial pH and agitation rate are important factors for lactic acid production by immobilization on polyurethane foam matrices in fermentation medium (Bulut et al., 2004).

The results obtained after CCD were analyzed by ANOVA which gave the following regression equation for the level of lactic acid production:

Table 1. Experimental range and levels of the independent variables.

Variable	Symbol coded	Range and level				
		-α	-1	0	+1	+α
Glucose (g/l)	X_1	83	100	125	150	167
Initial pH	X_2	4.3	5	6	7	7.7
Agitation rate (rpm)	X_3	65	100	150	200	235

Table 2. Experimental design used in RSM studies by using three independent variables with six center points showing observed lactic acid production.

Run order	Glucose X_1	Initial pH X_2	Agita. rate X_3		Lactic acid production (g/l)
1	-1.00	-1.00	-1.00		52.5
2	1.00	-1.00	-1.00		90
3	-1.00	1.00	-1.00	Fractional 2^3 factorial design	58.75
4	1.00	1.00	-1.00		92
5	-1.00	-1.00	1.00		46.25
6	1.00	-1.00	1.00		80
7	-1.00	1.00	1.00		52.5
8	1.00	1.00	1.00		90
9	-1.68	0.00	0.00		50
10	1.68	0.00	0.00		86.25
11	0.00	-1.68	0.00	Star points	76.25
12	0.00	1.68	0.00		77.5
13	0.00	0.00	-1.68		52.5
14	0.00	0.00	1.68		52.5
15	0.00	0.00	0.00		87.5
16	0.00	0.00	0.00		86.25
17	0.00	0.00	0.00		88.75
18	0.00	0.00	0.00	Central points	85
19	0.00	0.00	0.00		86.75
20	0.00	0.00	0.00		87.15

$$Y = 86.71 + 14.86\,X_1 + 1.95\,X_2 - 1.79\,X_3 - 5.43\,X_1^2 - 2.34\,X_2^2 - 10.96\,X_3^2 - 0.062\,X_1X_2 + 0.063\,X_1X_3 + X_2 \quad (2)$$

Where, Y is the response that is lactic acid (g/l) and X_1, X_2, X_3 are coded values of the test variables, glucose (g/l), initial pH, agitation rate (rpm), respectively.

The ANOVA of quadratic regression model demonstrates that the model is highly significant, as is evident from the *Fisher's F-test* with a very low probability value [($P_{model}>F$) =0.01)] (Table 3). The model *F-value,* determined as 17.01, shows that predicted model is suitable for good fitting. For goodness of fit of the regression equation, the multiple correlation coefficient R and the determination coefficient R^2 (93.9 %) are sufficient. Adjusted R^2 is a modification of R^2 that adjusts for the number of explanatory terms in a model. Unlike R^2, the adjusted R^2 increases only if the new term improves the model more than what would be expected by chance. The adjusted R^2 was 0.88. The coefficient of variation (CV) which indicates the degree of precision with which the treatments were compared, was 7.84%. Relatively lower value of CV indicates a better precision and reliability of the experiments carried out. The adequate precision which measures the signal to noise ratio was 12.2 and this ratio was greater than 4 as it indicates an adequate signal. The "Lack of fit *F-Value*" of 41.55 implies that it is significant, which means that the order of the regression was not secondary (the model may have not included all appropriate functions of independent variables or the experimental region may be too large for the quadratic model used) (Martinez and Pilosof, 2012). However, when a large amount of data was included in the analysis, a model with significant lack of fit could still be used (Box and Drapper, 1987). The high coefficient R^2 shows the applicability of the

Table 3. ANOVA for quadratic model.

Source	SS	DF	MS	*F-value*	*Prob(p)>F*
Model	5136.94	9	570.77	17.01	0.0001
Residual (error)	335.63	10	33.56		
Lack of Fit	327.74	5	65.55	41.55	0.0004
Pure Error	7.89	5	1.58		
Total	5472.57	19			

R^2= 0.9387; CV= 7.84 %; SS, sum of squares; DF, degrees of freedom; MS, mean square; *Adj* R^2= 0.8835.

Table 4. The least-squares fit and parameter estimates (significance of regression coefficient).

Model term	Parameter estimate	Standard error	P-value
Intercept	86.71	2.36	-
X_1	14.86	1.57	<0.0001
X_2	1.95	1.57	0.2424
X_3	-1.79	1.57	0.2791
X_1^2	-5.43	1.53	0.0052
X_2^2	-2.34	1.53	0.1560
X_3^2	-10.96	1.53	<0.0001
X_1X_2	-0.063	2.05	0.9763
X_1X_3	0.063	2.05	0.9763
X_2X_3	1.00	2.05	0.6359

regression model between the ranges of variables included.

The parameters which have small value of "*Prob > F*" less than 0.05 indicate that model terms are significant for obtaining higher lactic acid production. According to Table 4, the variables with largest effect were linear term of glucose consentration (X_1), squared term of glucose concentration (X_1^2) and agitation rate (X_3^2) on response (Y). These showed that the concentration of glucose has direct relationship with the production of lactic acid. It is well known that glucose is a readily metabolizable carbon source by many organism. Carbon source is a necessary microbial growth and lactic acid production but lactic acid production is known to be limited by product inhibition (Elibol, 2004). The squared term of glucose concentration can act as limiting factor. So, little variations in glucose concentration will alter the production of lactic acid. Although, initial pH is one of the most important factors affecting the fermentation process, the *p-value* of initial pH given in Table 4 shows that it has no significant effect on the production of lactic acid. In this study, calcium carbonate was used as a neutralizing agent to control the growth pH during fermentation. Therefore, the effect of initial pH on lactic acid production was not found significant. Different agitations seemed to provide different distribution and transportation of air and nutrients to the cells. Metabolic products are susceptible to mechanical force, which may disturb the elaborate shape of complex molecule to such a degree that denaturation occurs (Tanyıldızı et al., 2007). Agitation rate has a squared effect on lactic acid production as shown in Table 4.

The relationships between independent variables can be better understood by examining the series of the response surface plot and contour plots. Figures 1 to 3 represent the isoresponse contour and surface plots for the optimization of fermentation conditions of lactic acid. The main goal of response surface is to efficiently determine the optimum values of the variables to either maximize or minimize the response. Each contour curve represents an infinite number of combination of two test variables with the other two maintained at their respective zero level. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram (Tanyıldızı et al., 2005).

The effects of the concentration of glucose and pH on the lactic acid production were shown in Figure 1. High production of lactic acid was observed at higher level of concentration glucose and intermediate level of pH. The interaction effect of agitation rate and glucose concentration on the lactic acid production in Figure 2 indicate that there is no significant effect on the response,

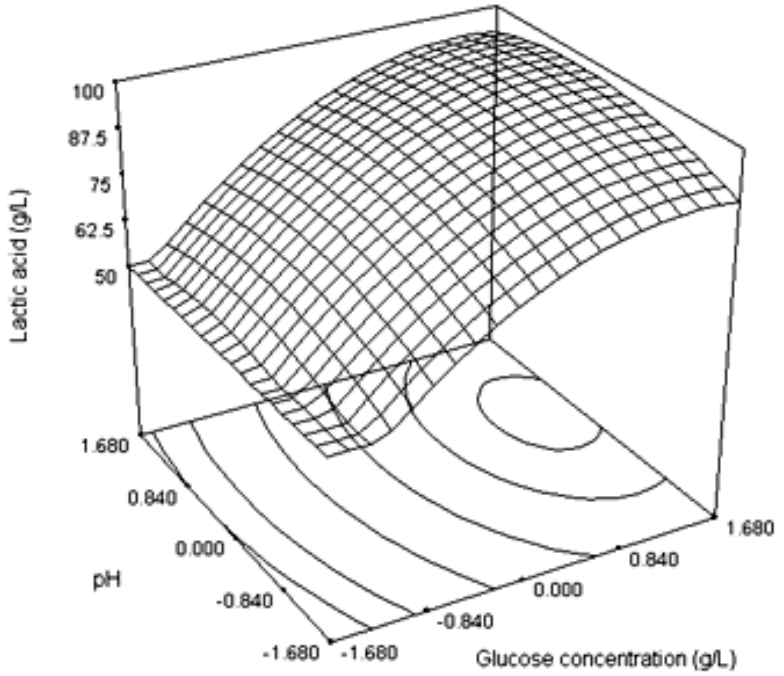

Figure 1. Response surface plot showing the effect of glucose concentration, pH and their mutual effect on the production of lactic acid (g/l).

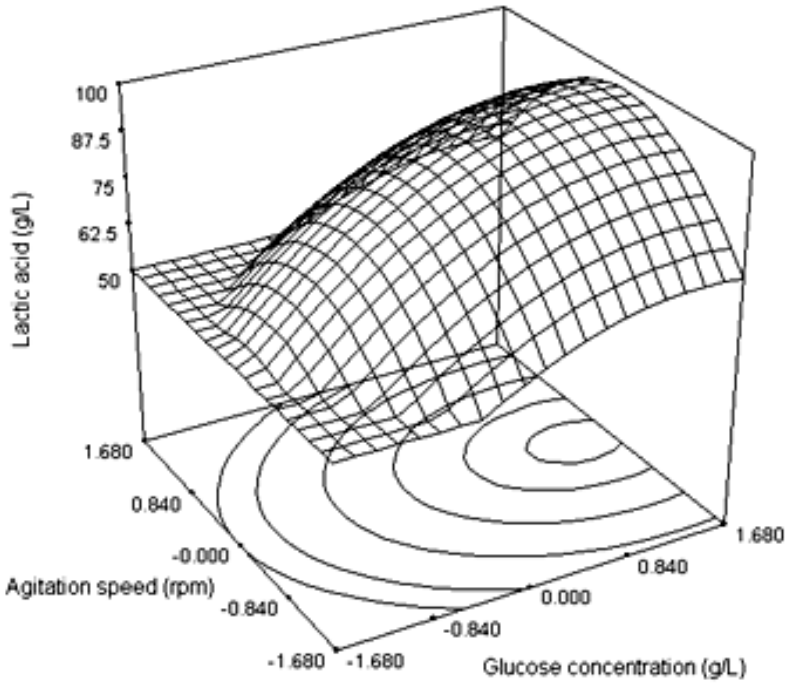

Figure 2. Response surface plot showing the effect of glucose concentration, agitation rate and their mutual effect on the production of lactic acid (g/l).

whereas, the lactic acid production increased with increase in glucose concentration as shown in Figure 1. It was found that maximum lactic acid production was obtained at the higher levels of glucose concentration as shown in Figures 1 and 2. The 3D plot in Figure 3 and relatively smaller *P-value* (0.64) shows that the interact-

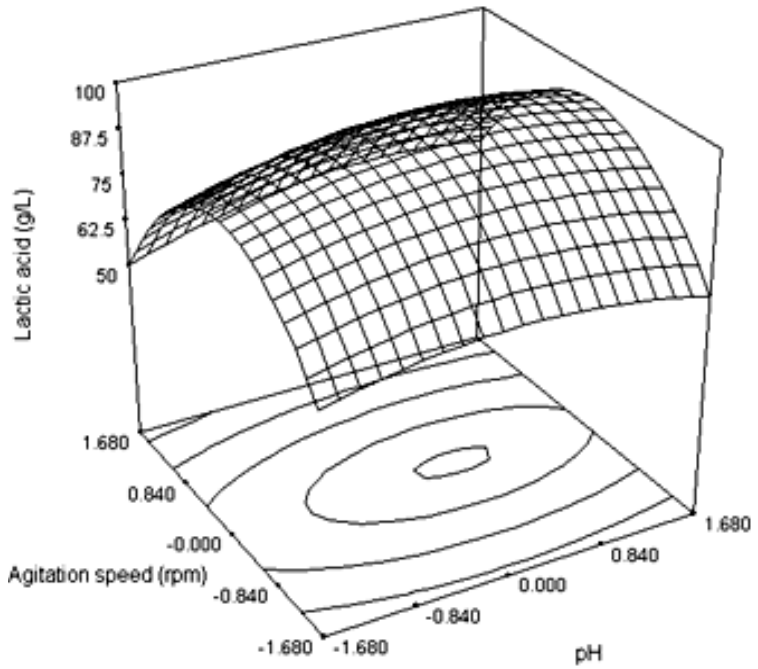

Figure 3. Response surface plot showing the effect of pH, agitation rate and their mutual effect on the production of lactic acid (g/l).

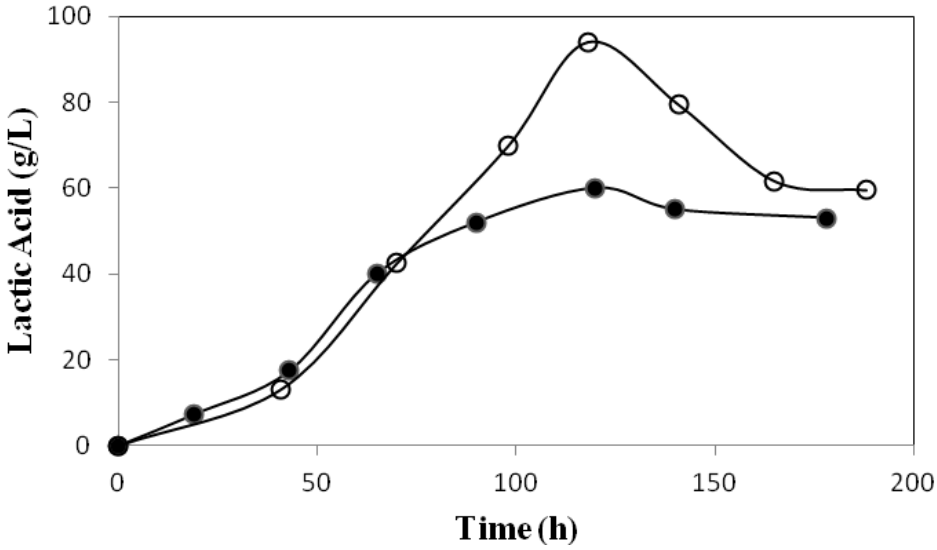

Figure 4. Lactic acid production in optimized (○) and non-optimized medium (●).

tion between pH and agitation rate is more significant than the others. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar, 2001). The effect of pH on lactic acid production can be hindered by calcium carbonate which is used as a neutralizing agent. The optimum values of pH and agitation rate for maximum lactic acid production were observed near the central point.

A numerical method given by Meyers and Montgomery was used to solve the regression equation (Equation 2). The optimal natural values of the test variables are: glucose =150 g/l, pH =6.39, agitation rate=147 rpm. The maximum lactic acid production obtained by using the above optimized concentrations of the variables is 96.56 (g/l). The maximum enzyme activity obtained experimentally was found to be 93.2 (g/l) in Figure 4. This

is obviously in close agreement with the model prediction.

Conclusion

The use of an experimental design where the main point was to reveal the influence of variables on lactic acid production allowed rapid screening of large experimental domain in search of the optimum glucose concentration and fermentation conditions for maximum lactic acid production. In this study, lactic acid production with *R. oryzae* by using RSM with CCD was successfully immobilized on polyurethane matrix. In our previous study, maximum lactic acid production was found to be 60 (g/l) by using suspension culture systems. Lactic acid production from *R. oryzae* which was immobilized on polyurethane foam matrix by using RSM with central composite design was found about 55% higher than lactic acid production obtained in the medium where free cells are used (Figure 4) (Bulut et al., 2004).

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