African Journal of Food Science Vol. 3(9). pp.257-261, September, 2009
Available online http://www.academicjournals.org/AJFS
ISSN 1996-0794 © 2009 Academic Journals

Full Length Research Paper

Response surface optimization of D(-)-lactic acid production from *Lactobacillus* SMI8 using corn steep liquor and yeast autolysate as nitrogen sources

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Accepted 07 August, 2009

The production of D(-) lactic acid from *Lactobacillus* LMI8 sp. was optimizated by central composite design, using two low-cost nitrogen sources: corn steep liquor (CSL) and yeast autolysate (YA). The fermentation were performed in 250 mL Erlenmeyer flasks containing 100 mL of production medium, maintained at 200 rpm and 37±1°C for 48 h. After data analysis by surface response method, it was revealed that Lactic acid production was significantly affected by the isolated CSL as well as the interaction between CSL and YA and the maximal production of D(-) lactic acid was 41.42 g/L – a value located at the central point, which corresponded to 15 g/L of CSL and 5 g/L of YA.

Key words: D(-)-Lactic acid, *Lactobacillus*, Corn steep liquor, Yeast autolysate, Response surface methodology.

INTRODUCTION

Lactic acid is the common name given to 2-hydroxypropanoic acid (Datta et al., 1995), which is a versatile product applied in diverse fields, including as an acidulant in the food and pharmaceutical industries (Schepers et al., 2002). With the development and commercialization of biopolymers, poly(lactic acid) use has an increasing use in these new applications (Datta and Henry, 2006).

The high chemical resistance of poly(lactic acid) is advantageous for the manufacture of fibers, non-woven fabrics, and films while the high heat resistance is suitable for producing the cutlery and deli trays/cups. Thus, microbial production of optically pure lactic acid has extensively been studied because chemically synthesized lactic acid is racemic. Recently, the development of stereocomplex of poly(L-lactic acid) and poly(D-lactic acid) is one of the topics in the application of the poly(lactic acid) because its melting point (225ºC) is higher than that of poly(L-lactic acid) (177ºC) (Ikada et al., 1987; Tsuji and Fukui, 2003).

Lactic acid bacteria make up a group of diverse microorganisms that are widely distributed in nature and associated with plants (kale, corn, barley), meat, milk products, porridge and silage. These Lactobacilli are micro-organisms in great need of fermentable carbohydrates, amino acids, B complex vitamins, nucleic acids and minerals for growth as well as other nutrients that are specific to each strain (Gomes and Malcata, 1999).

A number of byproducts and raw materials from the food and/or agriculture industries have been employed to feed Lactobacilli due to their considerable availability and low cost, including cheese whey, corn steep liquor, corn syrup, distillery yeast and molasses (Moraes et al., 1991). In Brazil, lactic acid production from sugarcane and corn residuals has considerable economic appeal due to the abundance and low cost of these byproducts.

Molasses, a by-product of the sugar manufacturing process, is used as an animal feed as well as for ethanol and yeast production (Focus, 1975). It can be also used for lactic acid production (El-Sherbiny et al., 1986; Tiwari et al., 1979). The most abundant sugar is sucrose, which raises the viscosity of the liquid at high concentration (Focus, 1975). Moreover, molasses and corn steep liquor are prominent culture media in fermentative processes due to the high content of sugars and nitrogen, respectively (Beaulieu et al., 1995).

The aim of the present study was to optimize the production medium of lactic acid by *Lactobacillus* sp. LMI8 by employing a central composite design (CCD) and response surface method, using sugarcane molasses of low cost enriched with corn steep liquor and yeast autolysate as the culture medium.

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Table 1. Experimental range and levels of the independent variables (Xi, i = 1 and 2) used in central composite design.

Independent Variables		Range and levels				
		-α	-1	0	+1	+α
Production medium optimization (g/L)						
Corn steep liquor (CSL)	X_1	0	4.3	15	25.7	30.1
Yeast autolysate	X_2	1.47	2.5	5	7.5	8.52

MATERIALS AND METHODS

Microorganisms

The *Lactobacillus* sp. LMI8 was isolated from a decantation tank of residuals from the flour industry (Plaza Starch Mill - Santa Maria-SP, Brazil). The culture was stored in Man, Rogosa and Sharpe (MRS) medium with 20% (v/v) glycerol at -20ºC.

Isolation of LMI8 culture

The successive dilution method was employed and plating was performed using the "pour plate" method. Bacterial strain selection was based on the greatest production of the D(-) lactic acid isomer from the culture medium used in the fermentation process.

Sugarcane molasses

Sugarcane molasses was obtained from the Santa Lucia Sugar Processing Plant located in the region of Araras-SP, Brazil. The molasses was diluted and hydrolyzed at a ratio of 1 mL of H_2SO_4 (20%) in 100 mL of molasses solution and heated for 20 min. After hydrolysis, the pH of the molasses was adjusted to 6.5 with 4.0 M of KOH.

Medium and growth conditions

The inoculum was prepared through the transference of 2 mL of stock culture to Erlenmeyer flasks containing 100 mL of growth medium (MRS). The MRS growth medium was made up of peptone (10 g/L), yeast extract (5 g/L), meat extract (10 g/L), glucose (20 g/L), sodium acetate (5 g/L), ammonium citrate (2 g/L), K_2HPO_4 (5 g/L), $Na_2HPO_4.2H_2O$ (2 g/L), $MgSO_4.7H_2O$ (0.1g/L) and $MnSO_4.4H_2O$ (0.05g/L). The inoculated medium was incubated at 37 ± 1ºC for 18 h in a refrigerated incubator (New Brunswick, USA) at 200 rpm. Initial pH of the medium was adjusted to 6.7. A total of 10% (v/v) of the inoculum was transferred to 250-mL Erlenmeyer flasks containing 100 mL of production medium (modified MRS medium) agitated at 200 rpm in a refrigerated incubator at 37 ± 1ºC for 48 h. Calcium carbonate (100 g/L) was added to the medium in order to maintain the pH constant. The modified MRS medium consisted of the same salts as in the growth medium, with the addition of 70 g/L of molasses, corn steep liquor ranging from 0 to 30.1 g/L and yeast autolysate ranging from 1.475 to 8.525 g/L.

Central composite design (CCD)

In order to study the influence of the corn steep liquor (CSL) and yeast autolysate (YA) in the synthesis of lactic acid by the *Lactobacillus* sp. LMI8 isolate, a central composite design (CCD) was used. For the two factors, this design was made up of a full 2^2 factorial design with its four cube points, augmented with three replications of the center points and the four star points, that is, points having an axial distance to the center of (± 1.41) for one factor, whereas the other factor is at level 0, resulting in a total of 11 experiments. Thus, the influence of all experimental variables, factors and interaction effects on the response was investigated. The experimental design was determined using the Statistic 7.0 software program. The independent variables, experimental range and levels investigated for the CCD is given in Table 1. In performing the regression equation, the test variables were coded according to the following equation 1:

$$x_i = \frac{\left(X_i - X_{cp}\right)}{\Delta X_i} \qquad (1)$$

where *xi* is the coded value of an independent variable, *Xi* is the real value of an independent variable, Xcp is the real value of an independent variable at the center point, and *ΔXi* is the step change value.

Analytical methods

Substrate consumption and lactic acid concentrations (sample of 20 µL) were determined using a high performance liquid chromatography system (Waters Co., Milford, MA) equipped with a tunable UV detector set at 210 nm. An Aminex HPX-87H ion-exchange column (300×7.8 mm, Bio-Rad, Hercules, CA) was eluted with 0.005 N of H2SO4 as a mobile phase at a flow rate of 0.6 ml/min. Column temperature was maintained at 60ºC and a refraction index (RI) detector was used.
D(-)Lactic acid concentrations were determined by an enzyme test kit (R-biopharm AG - Roche, Darmstadt, Germany), as reported elsewhere (Yun et al, 2003). Cell growth was measured by a UV-160A spectrophotometer (Shimadzu Co., Tokyo, Japan) set at 650 nm.

RESULTS AND DISCUSSION

Optimization of fermentative production of lactic acid

The influence of corn steep liquor and yeast autolysate on the fermentation of sugarcane molasses was studied using CCD with three replicates at the center, totaling 11 experiments. The results are displayed in Table 2.
Analyzing Table 2, the best results of all the responses evaluated were obtained in the experiments located at the central point (Experiments 9, 10 and 11). Comparing Experiment 5 with Experiments 6, 9, 10 and 11, performed with the lowest and highest concentrations of CSL (0, 15 and 30 g/L) and equal concentrations of YA (5

Table 2. Results of lactic acid production obtained in experiments employing the LMI8 isolate.

Experiments	CSL (g/L)	YA (g/L)	Lactic acid (g/L)
1	4.3	2.5	15.32
2	4.3	7.5	20.43
3	25.7	2.5	35.23
4	25.7	7.5	22.7
5	0	5	12.11
6	30.1	5	25.39
7	15	1.47	24.16
8	15	8.52	31.27
9	15	5	41.42
10	15	5	41.23
11	15	5	39.98

Concentration of corn steep liquor (CSL); Concentration of yeast autolysate (YA).

Table 3. Stationary point for lactic acid production and coded x_1 and x_2 values at the optimization point.

P0	Lactic acid	Coordinates	Lactic acid
λ_1	-12.233	x_1	0.139
λ_2	-5.833	x_2	-0.029

g/L), it is evident that lactic acid production was directly proportional to the increase in CSL, reaching a maximal value of 25.39 (Experiments 6) and 41.42 g/L (Experiments 9), both in 32 h of fermentation. Moreover, the increase in the nitrogen concentration in the fermentative medium led to a reduction in fermentation time, reaching maximal lactic acid production. Wee et al. (2006) studied the effect of CSL on lactic acid production using different concentrations (15 to 60 g/L) and obtained the same final concentration of lactic acid in all experiments, but the time needed to achieve this concentration decreased significantly when using greater concentrations of CSL. Yu et al. (2007) used sugarcane molasses ranging from 12 to 60 g/L and CSL ranging from 24 to 56 g/L and report that the increase in molasses and CSL provided greater lactic acid production in a shorter fermentation time. Selmer-Olsen and Sorhaug (1998) and Altaf et al. (2006) report that peptone and yeast extract are the main nitrogen sources used for the production of lactic acid because yeast extract is an excellent source of B complex vitamins and is often used to provide these factors to the bacteriological culture media, which are often considered indispensable to obtaining faster growth and production rates of lactic acid by lactic bacteria. The high cost of yeast extract, however, has a negative impact when used in industrial processes (Hurok et al., 2005).

The precision of the results of the experiments can be seen in the assessment of the results obtained at the central point of the design, where the difference between responses was close to 1%.

The identification of significant parameters was performed through testing the hypotheses using the Student's t-test. The maximal likelihood of error on the test was established as 5%. Thus, parameters with a level of significance greater than 5% were discounted. The adjusted empirical equation that represents the synthesis of lactic acid is expressed in equation 2:

$$\text{Lactic acid} = 40.876 + 5.12X_1 - 4.41X_1X_2 - 11.01X_{12} - 6.53X_{22} \quad (2)$$

In Experiment 5 (Table 2), there was lactic acid production in the absence of CSL. Despite being less significant within the fermentation process (Eq. 1), YA provided a good interaction with CSL, generating greater lactic acid production.

The fitness of the model was checked by the determination coefficient (R^2) and multiple correlation coefficients (R). In this case, the value of the R^2 (0.96) for equation 2 indicates that the sample variation of 96.0% for lactic acid was attributed to the independent variables and only 4% of the total variation cannot be explained by the model. The value of the adjusted determination coefficient (adjusted R^2 = 0.935) is also high, which stresses the significance of the model. The high value of R (0.98) demonstrates a high degree of agreement between the experimental observations and predicted values. This correlation is also proven by the plot of predicted versus experimental values of lactic acid in Figure 1, as all the points cluster around the diagonal line, which means that no significant violations of the model were found.

The effects of the independent variables and their interactions on the formation of the product are illustrated in the analysis of the response surfaces (Figure 2) constructed from equation 2.

Analyzing Figure 2, maximal lactic acid production occurred when nitrogen concentrations were near 15 g/L (CSL) and 5 g/L (YA). For greater and lower concentrations of CSL and YA, there was a reduction in response, likely due to either a lack or excess of nitrogen in the fermented medium. According to Wood and Holzapfel (1995) the genus *Lactobacillus* has complex nutritional requirements. As lactic acid synthesis through fermentation is associated cell growth, there is no formation of product if the medium does not have an adequate concentration of nitrogen in order to promote this growth (Pritchard and Coolbear, 1993). On the other hand, high concentrations of nitrogen may lead to cell death and inhibition of the product.

An algorithm carried out on the Maple 9.5 program (Waterloo Maple, Inc., Canada) was used to calculate the stationary point (P_0) for synthesis of lactic acid. These values are displayed in Table 3. The λ values indicate that these responses have a maximal point, as they have equal and negative signs. Lactic acid production was 41.52 g/L at the optimization point from the coded x_1 and

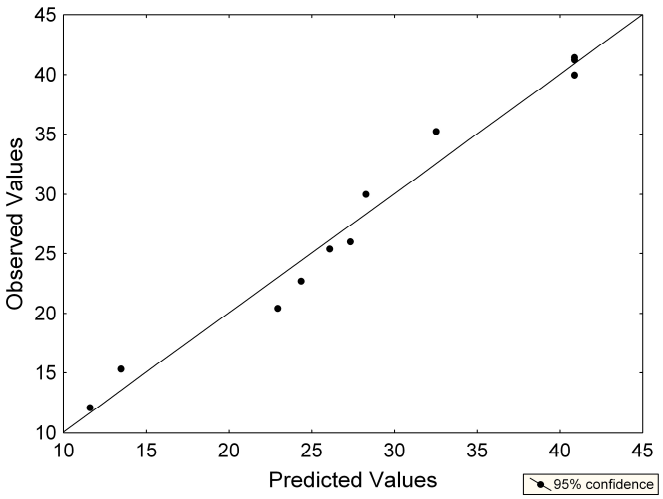

Figure 1. Predicted vs. experimental values plot for lactic acid.

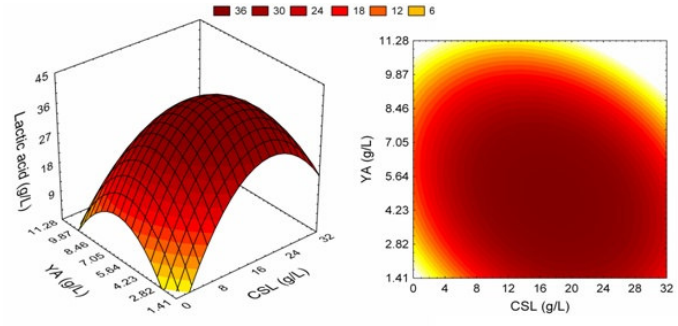

Figure 2. Response surface and contour curve representing the effects of corn steep liquor and yeast autolysate concentrations on lactic acid production.

x_2 values (Table 3).

Comparing this value with lactic acid production values in experiments 9, 10 and 11 (Table 2), the results were practically the same, as the conditions of the maximal point were near to those of the central point. Thus, the corresponding values for the optimal concentrations of CSL and YA were 16.48 and 4.93 g/L, respectively. The model predicted a maximal response of 41.38 g/L of lactic acid at this point.

Wee et al. (2006) produced 48.6 g/L of lactic acid using wood hydrolysate (equivalent to 50 g/L of glucose) supplemented with 60 g/L of CSL in 36 h of fermentation. Bustos et al. (2004) obtained maximal lactic acid production (58.9 g/L) in 96 h of culturing using 5 g/L of CSL together with 3.6 g/L of yeast extract and 10 g/L of peptone. Using a mathematical model furnished the response surface method, Hauly et al. (2003) determined maximal concentrations values tested for sugarcane molasses (100 g/L), yeast extract (20 g/L) and peptone (4 g/L) as the best medium composition for the production of lactic acid. In practice, these conditions provided a production of 30.5 g/L of lactic acid.

Conclusion

The central composite design was proved to be a useful and applicable tool for determining the behavior of the variables studied in the production of lactic acid. The *Lactobacillus* sp. LMI8 strain using organic nitrogen source (corn steep liquor and yeast autolysate) achieved significant results regarding lactic acid production. Under optimal conditions (16.48 g/L of CSL and 4.93 g/L of YA) a lactic acid concentration of 41.52 g/L was obtained.

ACKNOWLEDGEMENTS

We thank the Fundação de Amparo à Pesquisa de Estado de São Paulo (FAPESP) for fellowships and financial support.

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