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LACTIC ACID PRODUCTION BY NEW *Lactobacillus plantarum* LMISM6 GROWN IN MOLASSES: OPTIMIZATION OF MEDIUM COMPOSITION

L. F. Coelho[1], C. J. B. de Lima[1], C. M. Rodovalho[2], M. P. Bernardo[1] and J. Contiero[1*]

[1]Department of Biochemistry and Microbiology, Institute of Biological Sciences,
Phone: + (55) (19) 3526-4180, Fax: + (55) (19) 3526-4176, Universidade Estadual Paulista,
(UNESP), Zip Code 13506-900, Rio Claro - SP, Brazil.
E-mail: jconti@rc.unesp.br
[2]Department of Biology, Institute of Biological Sciences, Universidade Estadual Paulista,
(UNESP), Zip Code 13506-900, Rio Claro - SP, Brazil.

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Abstract - A Plackett-Burman experimental design was used to evaluate seven medium components added to molasses (corn steep liquor, sodium acetate, magnesium sulfate, manganese sulfate, ammonium citrate, potassium phosphate and Tween 80). Corn steep liquor (CSL), K_2HPO_4 and Tween 80 increased lactic acid production. The concentrations of these three components as well as the molasses were further optimized using the response surface method. A maximal lactic acid production of 94.8 g L^{-1} was obtained when the concentrations of molasses, CSL, K_2HPO_4 and Tween 80 were 193.50 g L^{-1}, 37.50 mL L^{-1}, 2.65 g L^{-1} and 0.83 mL L^{-1}, respectively. However, in both shaker and bioreactor, approximately one fourth of the sugar added initially was not utilized after 48 hours of fermentation. Future studies that consider high conversion of sugar into final product as well as high volumetric productivity are necessary to improve the fermentation process and to reduce the downstream costs.
Keywords: Plackett-Burman; Corn steep liquor; Response surface methodology; Medium optimization; Potassium phosphate; Tween.

INTRODUCTION

Lactic acid is an organic acid with a wide range of applications in the food, pharmaceutical and cosmetics industries (Datta et al., 1995). It has recently been studied with great interest as a biodegradable polylactic acid (PLA) that can be used to improve physical properties in the production of food packaging, plastic utensils, garbage bags and agricultural plastic sheeting, thereby replacing products made from petroleum (Ohara, 2003).

Lactic acid can be obtained either by the action of fermentative microorganisms or chemical synthesis. The fermentation process has the advantage of being more cost effective (Silva and Mancilha, 1991). Approximately 90% of all lactic acid worldwide is produced by bacterial fermentation (Zhou et al., 2006).

Lactic acid bacteria are traditionally fastidious microorganisms and have complex nutrient requirements due to their limited ability to biosynthesize B-vitamins and amino acids (Fitzpatrick and Keeffe, 2001).

Refined sugars, such as glucose or sucrose, have been used more frequently as a carbon source to produce lactic acid than raw starchy substrates, such

*To whom correspondence should be addressed

as barley, corn, or wheat (Hofvendahl and Hahn-Hägerdal, 1997). Furthermore, a considerable amount of an expensive complex nitrogen source, such as yeast extract, must be added to the medium in order to produce lactic acid within a reasonable timeframe. However, this is economically unfavorable. According to Tejayadi and Cheryan (1995), raw materials account for 68% of the overall cost of lactic acid production from whey and yeast extract using *Lactobacillus bulgaricus*.

A number of industrial by-products or wastes have been evaluated as substrates for lactic acid production with the aim of decreasing the cost of the process, such as sugarcane (Calabia and Tokiwa, 2007), molasses (Dumbrepatil et al., 2008) and whey (Buyukkileci and Harsa, 2004) as carbon sources and CSL (Bustos et al., 2004) as a nitrogen source. CSL is an excellent source of nitrogen for most microorganisms due to its high concentration of amino acids and polypeptides, with considerable amounts of B-complex vitamins (Cardinal and Hedrick, 1948). Sugarcane molasses is an industrial by-product of sugar and alcohol processing and is rich in fermentable sugars (Lima et al., 1975), nitrogen and vitamins. This substrate is inexpensive and highly available in Brazil, with an annual production of 17.9 million tons during the sugar manufacturing process.

The aim of the present study was to optimize lactic acid production by *Lactobacillus plantarum* LMISM6 grown in molasses.

MATERIALS AND METHODS

Microorganism

Lactobacillus plantarum LMISM6 was isolated from cassava wastewater. The stock cultures were maintained in Man, Rogosa and Sharpe (MRS) growth medium with 20% (v/v) glycerol at -20 ºC. The MRS medium had the following composition (g L^{-1}): glucose (20.0), peptone (10.0), yeast extract (5.0), meat extract (10.0), sodium acetate (5.0), ammonium citrate (2.0), K_2HPO_4 (5.0), $MgSO_4.7H_2O$ (0.1) and $MnSO_4.4H_2O$ (0.05). The pH was adjusted to 6.0 prior to sterilization at 121°C for 15 min.

Inoculum Preparation

The inoculum was prepared through the transference of 2 % of stock culture to Erlenmeyer flasks containing growth medium (MRS). Incubation temperature was 35 ± 1ºC for 18 hours at 150 rpm.

Substrate

The cane molasses was obtained from the Santa Lucia plant located in Araras-SP, Brazil and this substrate was hydrolyzed by adding 1 ml of H_2SO_4 (20%) to 100 ml of molasses solution. The acidified molasses solution was heated in a boiling water bath for 20 min. The pH of the medium was adjusted to 6.0 with 4.0 M KOH prior to sterilization. The sugarcane molasses contained 100% reducing sugar.

Analysis

Lactic acid concentrations were determined by a high performance liquid chromatography system equipped with a UV detector at 210 nm. A Rezex ROA (300 x 7.8 mm, Phenomenex) column was eluted with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.4 mL/min and the column temperature was maintained at 60°C. Reducing sugars were measured using the 3.5-dinitrosalicylic acid method (Miller, 1959). Cell growth was determined using a spectrophotometer at 650 nm (OD650) following centrifugation and washing of the cells. The dry mass was determined from a standard curve of optical density versus dry mass.

Plackett-Burman Experimental Design

The purpose of this first step of the optimization was to identify the medium components with a significant effect on lactic acid production by *Lactobacillus plantarum* LMISM6 using molasses. The sugar concentration from molasses was maintained constant (150 g L^{-1}) and twelve experiments were generated from seven factors: CSL, sodium acetate, magnesium sulfate, manganese sulfate, ammonium citrate, potassium phosphate and Tween 80. The variables with a confidence level greater than 95% were considered to have a significant influence on lactic acid production. The Plackett-Burman experimental design (PB) was based on the first-order model, with no interaction among the factors (Plackett and Burman, 1946). The concentrations used for each variable are displayed in Table 1.

The experiments were done in 125 mL Erlenmeyer flasks containing 20 mL of production medium and 100 g L^{-1} of calcium carbonate at 150 rpm for 48 h.

A central composite design (CCD) was performed with the variables that significantly increased the production of lactic acid.

Table 1: Variables and levels used in Plackett-Burman design

Variables	Codes	Range and levels	
		-1	+1
Sodium acetate (g L^{-1})	X_1	0	5
$MgSO_4$ (g L^{-1})	X_2	0	0.2
$MnSO_4$ (g L^{-1})	X_3	0	0.1
Citrate (g L^{-1})	X_4	0	2
K_2HPO_4 (g L^{-1})	X_5	0	2
Tween 80 (mL L^{-1})	X_6	0	1
CSL (mL L^{-1})	X_7	0	70

Central Composite Design and Optimization Using Response Surface Methodology

A central composite design (CCD) with four independent variables – each at five levels with eight star points (α = 2.0) and four replicates at the center points – was used to develop a second-order polynomial model that determined the optimal values of variables for lactic acid production. Screened through previous work, CSL, K_2HPO_4 and Tween 80 were taken as variables for investigation, along with molasses.

The variables of the experiments were coded according to the following equation:

$$x_i = (X_i - X_{CP}) / \Delta X_i \quad (1)$$

in which x_i is the coded value of an independent variable; X_i is the real value of an independent variable; X_{CP} is the real value of an independent variable at the center point; and ΔX_i is the step change value.

The behavior of the system was described by the following quadratic equation:

$$Y = b_0 + \Sigma\, b_i x_i + \Sigma\, b_{ii} x_i^2 + \Sigma\, b_{ij} x_i x_j \quad (2)$$

in which Y is the predicted response, i.e., lactic acid concentration; b_0 is the offset term; b_i is the linear effect; b_{ii} is the squared effect; b_{ij} is the interaction effect; and x_i is the independent variable.

Using the CCD method, a total of 28 experiments with various combinations of molasses, CSL, K_2HPO_4 and Tween 80 were conducted. Table 2 displays the range and levels of the variables investigated.

The experiments were done in 125 mL Erlenmeyer flasks containing 20 mL of production medium and 100 g L^{-1} of calcium carbonate at 150 rpm for 48 h.

Table 2: Real values of variables used in central composite design

Variables	Codes	Ranges and levels				
		- 2	-1	0	+1	+ 2
Molasses (g L^{-1})	X_1	70	120	170	220	270
CSL (mL L^{-1})[a]	X_2	0	15	30	45	60
K_2HPO_4 (mL L^{-1}) [a]	X_3	0	1.0	2.0	3.0	4.0
Tween 80 (g L^{-1}) [a]	X_4	0	0.5	1.0	1.5	2.0

[a] Variables identified as significant in increasing the lactic acid production using a Plackett-Burman design

The Statistica 7.0 software package (StatSoft, Tulsa, USA) was used for the experimental design and regression analysis of the experimental data. The response surface was generated to understand the interactions among the variables. The optimal points for the variables were obtained from Maple 9.5 (Waterloo Maple Inc., Ontario, Canada).

In order to validate the optimization of the medium composition, tests were carried out using the optimized condition to confirm the results of the response surface analysis.

Scale-Up Fermentation of Lactic Acid Production

Scale-up fermentation of lactic acid production with the optimal medium was carried out in a 13 L glass vase bioreactor with an initial medium volume of 4.0 L. Agitation speed and culture temperature were controlled at 150 rpm and 35°C, respectively. The pH was controlled at 6.0 by the automatic addition of 10 M NaOH. Samples of 1 mL were withdrawn from the fermentation broth every 3 hours for 96 hours and centrifuged at 7,800 g for 10 minutes.

RESULTS AND DISCUSSION

Plackett-Burman Experimental Design

Table 3 displays the Plackett-Burman design matrix (real and coded values) of the 12 experiments with seven variables added to molasses (X_1= Acetate, X_2=$MgSO_4$, X_3=$MnSO_4$, X_4=Citrate, X_5=K_2HPO_4, X_6=Tween 80, X_7= CSL) and the respective results (lactic acid).

CSL was the most influential variable in the production of lactic acid, followed by K_2HPO_4 and Tween 80. All three variables had a significant positive effect on lactic acid production at a 95%

confidence level. Figure 1 (Pareto chart) illustrates the effects of these variables, which were therefore used to optimize the production of lactic acid.

K_2HPO_4 had a positive effect on lactic acid production (Fig. 1). According to Honorato et al. (2007), the addition of phosphate to the culture medium increases the growth of the microorganism and enhances lactic acid production, as this component maintains the pH near the optimal growth value, thereby allowing the conduction of fermentation for a longer time.

The Plackett-Burman design is a two-level multifactorial design based on the rationale known as balanced incomplete blocks (Stanbury et al., 1986). The key to this technique is forming various combinations (which are called assemblies) of the components with varying amounts. Plackett-Burman design is an efficient way to screen for the important factors among a large number of variables. As there were seven parameters to be evaluated for lactic acid production, the Plackett-Burman was the most appropriate design.

There are a number of reports in which the Plackett-Burman design has been used to screen the factors in a fermentation medium to be optimized in subsequent experiments (Krishnan et al., 1998; Reddy et al., 1999; Son et al., 1998; Srinivas et al., 1994; Yu et al., 1997).

After finding the critical factors (CSL, K_2HPO_4 and Tween 80), the next step was to optimize the concentrations of these components in the growth medium. In this work, a response surface methodology (RSM) using a central composite design was used.

Table 3: Plackett-Burman design (real and coded values) with the respective results

Run	Independent variables[a]							Response
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Lactic acid (g L^{-1})
1	5 (1)[b]	0 (-1)	0.1 (1)	0 (-1)	0 (-1)	0 (-1)	70 (1)	78.64
2	5 (1)	0.2 (1)	0 (-1)	2(1)	0 (-1)	0 (-1)	0 (-1)	5.44
3	0 (-1)	0.2 (1)	0.1 (1)	0 (-1)	2 (1)	0 (-1)	0 (-1)	17.92
4	5 (1)	0 (-1)	0.1 (1)	2(1)	0 (-1)	1 (1)	0 (-1)	13.96
5	5 (1)	0.2 (1)	0 (-1)	2(1)	2 (1)	0 (-1)	70 (1)	85.04
6	5 (1)	0.2 (1)	0.1 (1)	0 (-1)	2 (1)	1 (1)	0 (-1)	42.64
7	0 (-1)	0.2 (1)	0.1 (1)	2(1)	0 (-1)	1 (1)	70 (1)	82.40
8	0 (-1)	0 (-1)	0.1 (1)	2(1)	2 (1)	0 (-1)	70 (1)	81.32
9	0 (-1)	0 (-1)	0 (-1)	2(1)	2 (1)	1 (1)	0 (-1)	40.64
10	5 (1)	0 (-1)	0 (-1)	0 (-1)	2 (1)	1 (1)	70 (1)	96.48
11	0 (-1)	0.2 (1)	0 (-1)	0 (-1)	0 (-1)	1 (1)	70 (1)	85.20
12	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	6.00

[a] X_1= Acetate, X_2=$MgSO_4$, X_3=$MnSO_4$, X_4=Citrate, X_5=K_2HPO_4, X_6=Tween 80, X_7=CSL
[b] (-1) and (1) are coded levels.

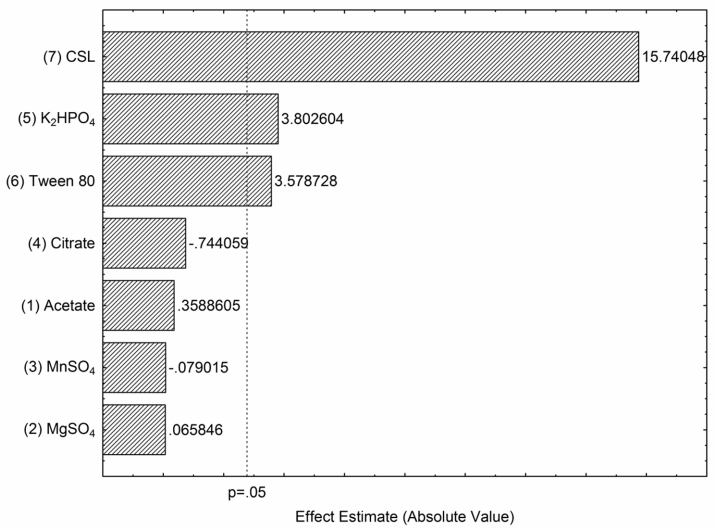

Figure 1: Pareto chart for lactic acid production

Response Surface Methodology

The three above-mentioned components and molasses were further optimized using response surface optimization. Table 4 displays the design matrix of the variables in coded units and real values with the respective results.

The highest production of lactic acid was 90.2 g L^{-1}, obtained from 220 g L^{-1} of molasses, 45 mL L^{-1} of CSL, 3 g L^{-1} of K_2HPO_4 and 1.5 mL L^{-1} of Tween 80 (Table 4). The application of multiple regression analysis methods yielded the following regression Eq. (3) for the experimental data.

$$Y = 85.5 + 3.91X_1 + 8.19X_2 + 4.38X_3 + 2.69X_4 - 3.58X_1^2 - 6.42X_2^2 - 3.38X_3^2 - 0.96X_4^2 - 1.8X_1X_2 - 0.39X_1X_3 + 0.44X_1X_4 - 0.19X_2X_3 - 0.51X_2X_4 + 0.25X_3X_4 \quad (3)$$

The quadratic model in Equation (3) contains four linear terms, four quadratic terms and six factorial interactions, in which Y is the predicted response (lactic acid concentration) and X_1, X_2, X_3 and X_4 are the coded values of molasses, CSL, K_2HPO_4 and Tween 80, respectively.

Table 5 displays the Student's t-distribution and the probability (*p*) values that serve as a tool to check the significance of each coefficient. Smaller *p*-values denote a more significant corresponding coefficient.

The results show that the variables X_1 (molasses), X_2 (CSL), X_3 (K_2HPO_4) and X_4 (Tween 80) had a significant effect, based on *p*-values lower than 0.05. Moreover, as these variables have positive coefficients (Table 5), an increase in their concentrations results in an increase in the production yield. The squared variables X_1^2, X_2^2, X_3^2, X_4^2 and the X_1X_2 interaction were also significant to different extents. The non-significant terms (X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4) were discarded. The Eq. (3) model was modified to the reduced Eq. (4) fitted model.

$$Y = 85.55 + 3.9X_1 + 8.19X_2 + 4.38X_3 + 2.68X_4 - 3.85X_1^2 - 1.8X_1X_2 - 6.42X_2^2 - 3.38X_3^2 - 0.95X_4^2 \quad (4)$$

The response surface quadratic model was performed in the form of analysis of variance (ANOVA) and the results are summarized in Table 6. The *F*-test was used to check the statistical significance of Eq. (4).

Table 4: Central composite design and results

Run	Independent variables				Response
	X_1	X_2	X_3	X_4	Lactic acid (g L^{-1})
1	120 (-1)[a]	15 (-1)	1 (-1)	0.5 (-1)	48.9
2	120 (-1)	15 (-1)	1 (-1)	1.5 (1)	54.9
3	120 (-1)	15 (-1)	3 (1)	0.5 (-1)	59.8
4	120 (-1)	15 (-1)	3 (1)	1.5 (1)	66
5	120 (-1)	45 (1)	1 (-1)	0.5 (-1)	71.4
6	120 (-1)	45 (1)	1 (-1)	1.5 (1)	75
7	120 (-1)	45 (1)	3 (1)	0.5 (-1)	80
8	120 (-1)	45 (1)	3 (1)	1.5 (1)	83
9	220 (1)	15 (-1)	1 (-1)	0.5 (-1)	61.6
10	220 (1)	15 (-1)	1 (-1)	1.5 (1)	68.9
11	220 (1)	15 (-1)	3 (1)	0.5 (-1)	69.3
12	220 (1)	15 (-1)	3 (1)	1.5 (1)	76.3
13	220 (1)	45 (1)	1 (-1)	0.5 (-1)	75.7
14	220 (1)	45 (1)	1 (-1)	1.5 (1)	79.15
15	220 (1)	45 (1)	3 (1)	0.5 (-1)	82
16	220 (1)	45 (1)	3 (1)	1.5 (1)	90.2
17	70 (-2)	30 (0)	2 (0)	1 (0)	63.4
18	270 (2)	30 (0)	2 (0)	1 (0)	78.3
19	170 (0)	0 (-2)	2 (0)	1 (0)	43
20	170 (0)	60 (2)	2 (0)	1 (0)	76
21	170 (0)	30 (0)	0 (-2)	1 (0)	63.1
22	170 (0)	30 (0)	4 (2)	1 (0)	80.2
23	170 (0)	30 (0)	2 (0)	0 (-2)	76.4
24	170 (0)	30 (0)	2 (0)	2 (2)	86.3
25	170 (0)	30 (0)	2 (0)	1 (0)	85.2
26	170 (0)	30 (0)	2 (0)	1 (0)	86
27	170 (0)	30 (0)	2 (0)	1 (0)	85.1
28	170 (0)	30 (0)	2 (0)	1 (0)	85.9

[a] (-2), (-1), (0), (1) and (2) are coded levels.

Table 5: Coefficients of t-values for lactic acid production using a composite rotatable design

Factor	Coefficient	Standard error coefficient	*t*-value	Probability
Intercept	85.55000	0.487286	175.5641	0.000000
X_1	3.91458	0.198934	19.6778	0.000000
X_2	8.19792	0.198934	41.2093	0.000000
X_3	4.38542	0.198934	22.0446	0.000000
X_4	2.68958	0.198934	13.5200	0.000000
X_1^2	-3.58385	0.198934	-18.0153	0.000000
X_1X_2	-1.80313	0.243643	-7.4007	0.000005
X_1X_3	-0.38438	0.243643	-1.5776	0.138669
X_1X_4	0.44688	0.243643	1.8341	0.089618
X_2^2	-6.42135	0.198934	-32.2789	0.000000
X_2X_3	-0.19688	0.243643	-0.8080	0.433602
X_2X_4	-0.51562	0.243643	-2.1163	0.054183
X_3^2	-3.38385	0.198934	-17.0100	0.000000
X_3X_4	0.25313	0.243643	1.0389	0.317781
X_4^2	-0.95885	0.198934	-4.8200	0.000335

$R^2 = 0.9970$; adjusted $R^2 = 0.9933$; $R = 0.9984$;

Table 6: Analysis of variance for the second-order polynomial model

Source	Sum of squares	Degrees of freedom	Mean square	F-value	$p > F$
Model	3853.152	9	428.1280	323.7185	0.0000
Residual	23.806	18	1.3225		
Lack of fit	23.156	15	1.544	7.125	0.06574
Pure error	0.650	3	0.217		
Total	3876.957	27			

$R^2 = 0.9938$; Adj $R^2 = 0.9907$; $R = 0.9969$

ANOVA of the quadratic regression model demonstrates that the model is highly significant, which is evident from the *F*-test (*F* model, the ratio of mean square regression to mean square residual is 323.7185), and has a very low probability value [($p_{model} > F$) = 0.0000]. The fit of the model was checked by the coefficient of determination (R^2) and the multiple correlation coefficient (R). The R^2 value (0.9938) for Eq. (4) indicates that the sample variation of 99.38% for lactic acid was attributed to the independent variables and only 0.62% of the variation cannot be explained by the model. The value of the adjusted coefficient of determination (adjusted R^2 = 0.9907) is also high, which demonstrates the high significance of the model. The high R value (0.9969) demonstrates strong agreement between the experimental observations and predicted values. This correlation is also confirmed by the plot of predicted versus experimental values of lactic acid production in Fig. 2, as all points cluster around the diagonal line, demonstrating that no significant violations of the model were found. Moreover, the value of lack of fit for regression Eq. (4) was not significant at the 5% level (p>0.05), indicating the good predictability of the model. Fig. 3 presents a plot of residuals versus the predicted response and displays no pattern or trend, suggesting that the variance of the original observation is constant. All residuals were smaller than 2%, which indicates that the model is adequate for describing lactic acid production over the experimental ranges studied.

The 3D response surface is the graphic representation of the regression equation and is plotted to understand the interaction of the variables and locate the optimal level of each variable for maximal response (Fig. 4 and Fig. 5). Each response surface and contour plotted for lactic acid production represents the different combinations of two test variables at one time while keeping the other two variables at their respective zero level. The convex response surfaces suggest that there are well-defined optimal variables.

Molasses and CSL were the dominant nutrients controlling lactic acid production (Fig. 4). Hence, a strong interaction between these nutrients for lactic acid fermentation is inevitable. However, both may cause inhibition of lactic acid production at higher concentrations due to significant carbon and nitrogen repression. As lactic acid bacteria are nutritionally fastidious and require several amino acids and vitamins for growth, it is very important to choose the right nitrogen and carbon sources. Nitrogen is necessary for the synthesis of amino acids, enzyme cofactors, some carbohydrates and other substances. The nitrogen source is a major factor of influence on the growth of *Lactobacillus* (Wood and Holzapfel, 1995). On the other hand, high concentrations of nitrogen can lead to cell death (De Lima et al., 2009).

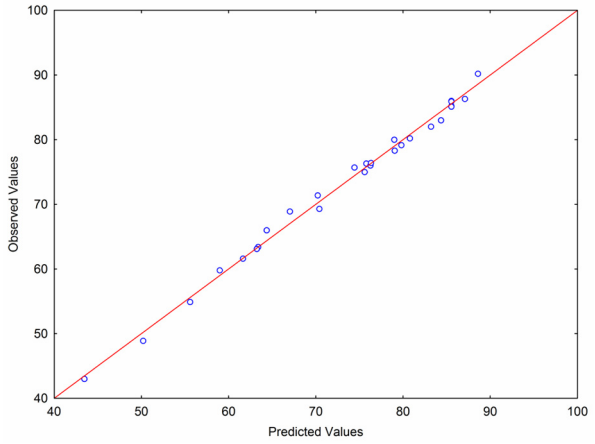

Figure 2: Plot of predicted vs. observed values of lactic acid production

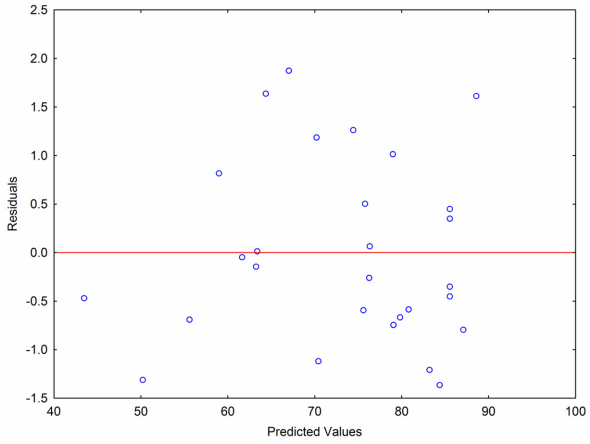

Figure 3: Plot of residuals vs. predicted values for lactic acid production.

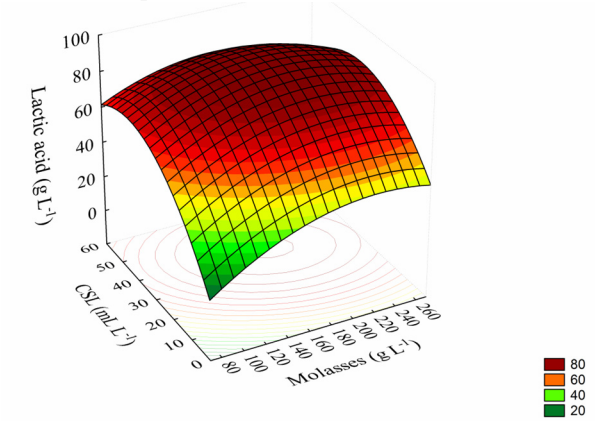

Figure 4: Response surface and contour plots of lactic acid production by *L. plantarum* LMISM6 showing the interaction between molasses and CSL at constant levels of K_2HPO_4 (2 g L^{-1}) and Tween 80 (1 mL L^{-1})

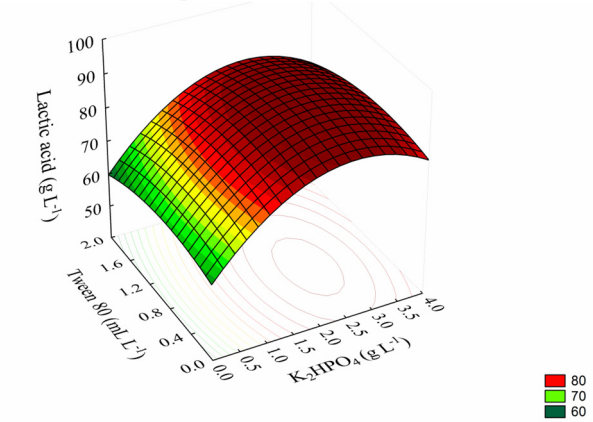

Figure 5: Response surface and contour plots of lactic acid production by *L. plantarum* LMISM6 showing the interaction between K_2HPO_4 and Tween 80 at constant levels of molasses (170 g L^{-1}) and CSL (30 mL L^{-1})

CSL has long proven to be an inexpensive alternative to materials such as yeast extract (YE) and peptone (Liggett and Koffler, 1948). The use of a cheap nitrogen source for the complete replacement of YE has been widely discussed. Supplementation with whey containing lactose at 55 g/L and 5% malt reaches a lactic acid yield similar to YE supplementation at around 55 h (Pauli and Fitzpatrick, 2002). Yu et al. (2008) found that CSL not only replaces YE as the sole nitrogen source in an optimized medium, but also helps to enhance lactic acid production when associated with other beneficial medium components.

The effect of molasses and CSL on lactic acid production was positive, as molasses is rich in trace elements and vitamins (Beaulieu et al., 1995). Yu et al. (2008) reported the same for L(+) lactic acid production by *L. rhamnosus*. The addition of phosphate to the culture medium increased lactic acid production (Fig. 5). The use of K_2HPO_4 is reported to provide K^+ and phosphate for microorganism growth and also acts as a buffering agent in the medium (Honorato et al., 2007). Lactic acid production increased with the increase in concentration to slightly more than 2.5 g L^{-1} of K_2HPO_4 and 0.8 mL L^{-1} of Tween 80.

The positive effect of Tween 80 on lactic acid production is also described by Belhocine (1987), who reported that the addition of 1 g L^{-1} of Tween 80 significantly increased *L. helveticus* growth and lactic acid production. Yu et al. (2008) found that the addition of 1.5 mL L^{-1} of Tween 80 to a fermentation medium containing molasses and CSL significantly increased the production of lactic acid by

Lactobacillus rhamnosus CGMCC 1466. This is likely due to the fact that Tween 80 can dissolve lipid structures in the cell membrane, thereby improving membrane permeability and enhancing the release of intracellular enzymes. Tween 80 has also been found to promote the migration of nutritive compounds into cells and has therefore been added to bacterial media to promote growth (Reese and Maguire, 1969). Thus, Tween 80 serves as an essential growth factor and has proven beneficial in the cultivation and fermentation of Lactobacillus (Duggan et al., 1959). However, a higher concentration of Tween 80 [1.4% (w/v)] decreased lactic acid production (Fig. 5). This was likely due to the fact that the surfactant Tween 80 becomes toxic at higher concentrations, leading to the destruction of the cell membrane and/or loss of the cell membrane function caused by the solubility of lipid bilayer (Ben-Kun Q. et al., 2009).

The point of maximal lactic acid production was determined through canonical analysis of the adjusted model. A study was carried out to identify the nature of the stationary point (maximal point, low response or saddle point). An algorithm carried out with the Maple 9.5 program (Waterloo Maple, Inc., Canada) was used to calculate the stationary point (P0) for the synthesis of lactic acid. These values are displayed in Table 7. The λ values referring to molasses, CSL, K_2HPO_4 and Tween 80 indicate that these responses have a maximal point, since they have equal, negative signs (Table 7). The analysis determined that the maximal predicted lactic acid concentration was 91.61 g L^{-1}, with the corresponding optimal values of the test variables at 193.50 g L^{-1} of molasses, 37.50 mL L^{-1} of CSL, 2.65 g L^{-1} of K_2HPO_4 and 0.83 mL L^{-1} of Tween 80.

Table 7: Stationary point for lactic acid production and coded values of the variables X_1, X_2, X_3 and X_4.

P_0	Lactic acid	Coordinates	Lactic acid
λ_1	- 6.69	X_1	0.47
λ_2	- 3.54	X_2	0.50
λ_3	- 3.19	X_3	0.65
λ_4	- 0.90	X_4	1.46

All optimal points were located within the experimental region. To confirm the adequacy of the model for predicting maximal lactic acid production, three additional experiments were performed in a shaker with this optimal medium composition. The mean value of lactic acid concentration was 94.8 g L^{-1}, which is in agreement with the predicted value of 91.61 g L^{-1}. Thus, the model proved adequate. Yu et al. (2008) studied the addition of CSL in molasses as the sole nitrogen source and obtained 112.5 g L^{-1} of lactic acid in 36 hours of fermentation by *Lactobacillus rhamnosus*.

Plackett-Burman design was appropriate to make a preliminary assessment of the significant components (CSL, K_2HPO_4 and Tween 80) in the production of lactic acid. These same components were also significant by using central composite design.

Comparison of the Time Course of Lactic Acid Production in the Optimized Medium Run with the Shake-Flask Method and in a Bioreactor

The scale-up fermentation of lactic acid in the optimized medium was carried out in the bioreactor (4 L of medium). The time courses are displayed in Fig. 6. After 48 hours of fermentation, lactic acid production in the bioreactor and in the shake-flask was 89.88 g L^{-1} and 94.8 g L^{-1}, respectively. However, productivity in the bioreactor (3.43 g $L^{-1}h^{-1}$) was higher than that achieved with the shake-flask method (2.89 g $L^{-1}h^{-1}$) after 12 h of fermentation. Yu et al. (2008) state that this is due to the addition of different neutralizing agents.

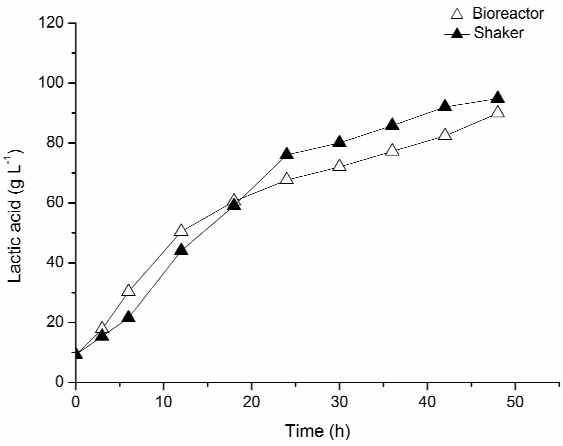

Figure 6: Time course of lactic acid production in the shaker compared to that in the bioreactor: in the shaker:(▲); in the bioreactor (△).

Molasses is an economically feasible raw material for industrial production of lactic acid and it has enough necessary nutrients for growth of *Lactobacillus plantarum* LMISM6. However, it is important to note that the quality of molasses depends on the maturity of the sugar cane or sugar beet, the amount of sugar extracted, and the method of extraction. The fermentable sugar content of molasses varies inversely with the purity of the raw sugar produced at the factory (Gopal and Kammen, 2009). Consequently, molasses is subject to a large batch-to-batch variation that could influence the lactic acid production.

CONCLUSIONS

Under optimized conditions, the best result for lactic acid production (94.8 g L^{-1}) was obtained after 48 hours with 193.50 g L^{-1} of molasses, 37.50 mL L^{-1} of CSL, 2.65 g L^{-1} K_2HPO_4 and 0.83 mL L^{-1} of Tween 80. Thus, the use of molasses for fermentation by *L. plantarum* LMISM6 is feasible and yields considerable lactic acid production, requiring only supplementation with a cheap nitrogen source (CSL), K_2HPO_4 and Tween 80. This work focused on high titers; however in both, shaker and bioreactor, approximately one fourth (50 g L^{-1}) of the sugar added initially was not utilized. Future studies that consider high conversion of sugar into final product as well as high volumetric productivity are necessary to improve the fermentation process and to reduce the downstream costs.

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