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OPTIMIZATION OF CHEESE WHEY BASED MEDIA COMPONENTS FOR LACTIC ACID PRODUCTION BY LACTOBACILLUS CASEI USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

The present study was carried out to optimize cheese whey media composition for maximum lactic acid production by Lactobacillus casei using Response Surface Methodology. Four media components i.e. CSL, K_2HPO_4, $MgSO_4$, and Sodium acetate were subjected for optimization Response surface methodology (RSM) a central composite design to determine the optimal concentration of these components for lactic acid production by Lactobacillus casei. A satisfactory fit of the model was realized. Lactic acid production was significantly affected by interaction of CSL, K_2HPO_4, $MgSO_4$, and Sodium acetate. Very close correlationship between experimental and predicted value was observed. The expected yield of lactic acid under this optimal condition (CSL 0.10%, K_2HPO_4 0.36%, $MgSO_4$ 0.42%, and Sodium acetate 0.32%) was 15.43 g/L and actual yield under this condition was 15.85 g/L. The subsequent validation experiment confirmed the validity of the statistical model. From the results obtained, it is concluded that Lactobacillus casei showed the maximum production of lactic acid 24.90 g/L after 48 hours of fermentation in static mode. In the present study found approximately 50% efficiency for lactic acid production in cheese whey based media. Cheese whey can be used as cheap carbon source for lactic acid production and it also gives further potential for industrial production of lactic acid.

INTRODUCTION

Whey is a major by-product of dairy industry and contains approx. 60 to 65 % (*m/V*) lactose as well as moieties of protein, fat and mineral salts. The increased demandfor cheese on the market has led to a rise in whey production. Due to the low nutrient concentration, whey was discarded in the environment for a number of years without causing harm, but has become an environmental problem due to the volume produced and biochemical oxygen demand (BOD, 30 000 to 50 000 mg/L). Lactose is the main component responsible for high BOD values, as there is a BOD reduction of only 1000 mg/L when removing protein from cheese whey. The use of lactose in biotechnological processes reduces the BOD of cheese whey by as much as 75 % (Mawson, 1994). In order to reduce the BOD level and acquire useful compounds, the whey can be used as a cheap carbohydrate source for the production of lactic acid by bacteria (Büyükkileci 2010; Kisaalita *et al,* 1990). However, lactic acid bacteria have complex nutrient requirements, as they have a limited capacity to synthesize vitamin B and amino acids (Van Niel *et al.,* 2002). A number of studies have shown that the lactic acid production of most lactobacilli is significantly improved by the addition of yeast extract, amino acids, protein concentrates, hydrolysates, vitamins and inorganic compounds such as $(NH4)_2SO_4$ and $(NH4)_2HPO_4$ (Amrane *et al.,*1998, Demirci *et al.,*1998, Champagne *et al.,* 1992, Cheng *et al*., 1991). Other studies have demonstrated the need to supplement cheese whey with commercially available growth supplements, such as corn steep liquor, yeast extract, casamino acids, peptone, neopeptone, molasses and trypticase (Cheng *et al*., 1991, Gupta *et al*., 1995, Roy *et al*., 1986). Corn steep liquor is a by-product of the corn milling industry and has been used as an inexpensive nutrient source for fermentation. It is an excellent source of nitrogen for most microorganisms, as it has high content of amino acids and polypeptides, with considerable amounts of B-complex vitamins (Cardinal *et al.,* 1984). Lactic acid is a natural organic acid, which has many applications in pharmaceutical, food and chemical industries. These include:

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uses as an acidulant, preservative and in recent years as a substrate for the production of biodegradable plastics. These plastics besides its application in food and cosmetic industry (Tashiro *et al.,* 2011) are applied as drug-carriers (Lipinsky, 1986) and for biodegradable packaging (Hashitani, 2002).

Lactic acid is produced by chemical synthesis and microbial fermentation. Chemical synthesis results in racemic mixture of lactic acid whereas specific stereo isomeric form can be obtained by microbial fermentation (Naveena *et al.,* 2005). The fermentative production of lactic acid is interesting due to the prospect of using cheap raw materials. The most attractive method for lactic acid production is fermentation of lactose in whey, a typical effluent from dairies. Hence, two problems could be solved at the same time wastewater treatment due to its high biochemical oxygen demand (BOD) 30000-50000 ppm (Tango, 1999) and useful product recovery. There are many reports on the lactic acid fermentation potential of Lactobacillus bacteria using lactose as substrate (Kisaalita *et al,* 1990, Rao *et al,* 2006). Lactic acid production from various substrates like corn (Montgomery, 1997), sugarcane (Tsuji, 1991), cassava (Lee, 1997), beets (Champagne *et al.,* 1992), paper sludge (Gupta *et al*.,1995), biodiesel (Yun *et al.,* 2003) and green microalga, Hydrodictyon reticulum (Cardinal *et al.,* 1984) has been studied to meet the increasing demands for lactic acid. In this article, lactic acid production from whey using Lactobacillus casei (NCDC 297) has been studied. The optimal conditions for the production of lactic acid were determined in flask culture. Response surface methodology has been successfully used to model and optimize biochemical and biotechnological processes related to food systems (Parajó *et al*., 1995, Vázquez *et al*., 1998, Ramírez *et al*., 2000). This methodology could be employed to optimize media for lactic acid fermentation.Initial screening of the process variables affecting the yield of lactic acid by Lactobacillus casei was done by employing fractional factorial design. After choosing the important variables, optimization was carried out using response surface methodology. Response surface methodology (RSM) is a powerful technique to achieve global optimization. In this work, central composite design of experiments with 6 center points were carried out.

MATERIALS AND MEDTHODS

Microorganism

The lactic acid bacterium used was Lactobacillus casei (NCDC 297). The strain was stored in De Man, Rogosa and Sharpe (MRS) broth with 50 % (v/v) glycerol at – 18 °C till further use.

Inoculum Preparation

The inoculum was prepared by transferring 100 µl of glycerol stock to 100 ml of sterile lactobacillus MRS broth (pH 7.0; Hi-Media, India), in a 250-ml Erlenmeyer flask and incubated at 37^0C for 24 h. Cells were harvested in a sterile centrifuge tube by centrifugation at 9,000 rpm for 10min. The pellet obtained was resuspended in sterile distilled water to an optical density of 1.0 at 660 nm. One ml of thus prepared inoculum was transferred to 100 ml of production media.

Media and Culture Conditions

Cheese whey containing 50 % (w/v) lactose was obtained from Amul Dairy, Anand, INDIA. The inoculum was prepared by transferring glycerol stock culture (1 ml) to an Erlenmeyer flask containing 100 ml of MRS medium and incubated at 37°C. Experiments were performed in 250 ml Erlenmeyer flasks containing 100 ml of cheese whey production medium along with the significant components (CSL, K_2HPO_4,$MgSO_4$ and sodium acetate). The medium was adjusted to pH 6.5 and sterilized by autoclaving at 121^0C for 15 min. It was maintained by 5gm% of sterile $CaCO_3$. After inoculation, the flasks were incubated at 37^0C under static condition. Samples were collected at 48 h. Cells were harvested by centrifugation at 9,000 rpm for 10 min, and clear supernatant was subjected to lactic acid estimation. The composition of modified MRS medium was (g/l) : Peptone 10, Beef extract 10, Yeast extract 5, Ammonium citrate 2,$MnSO_4$ 0.05, Dextrose 20, Polysorbate 80 1gm.CSL, K_2HPO_4,$MgSO_4$ and Sodium acetate was added in varying concentration to each flask as per the design.

Analysis of Media Constituents and Estimation of Lactic Acid

Factorial Design and Analysis of Results Design-Expert version 7.0 (State-Ease Inc., Minneapolis, U.S.A.) was used for experimental design (Central Composite Design, CCD), regression and graphical analysis of the data obtained. Four independent variables, including CSL, K_2HPO_4,$MgSO_4$ and Sodium acetate were studied at five different levels (Table 1).Lactic acid estimation was carried out by a colorimetric method (Taylor,1996).

Table 1. Range of values for the response surface method

Independent variables	Levels				
	- α	-1	0	+1	+α
CSL	-0.35	0.10	0.55	1.00	1.45
Sodium acetate	-0.38	0.25	0.88	1.50	2.13
K_2HPO_4	-0.20	0.20	0.60	1.00	1.40
$MgSO_4$	-0.20	0.20	0.60	1.00	1.40

Value are expressed as g/100ml

A set of 30 experiments was performed. The minimum and maximum ranges of variables were used, and the full experimental design with respect to their coded values is listed in (Table 2). The data on lactic acid production obtained from RSM were subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second-order polynomial [Eq. (1)].

$$Y = \beta0 + \beta1A + \beta2B + \beta3C + \beta4D + \beta1\beta2AB + \beta1\beta3AC + \beta1\beta4AD + \beta2\beta3BC + \beta2\beta4BD + \beta3\beta4CD + \beta1\beta1A2 + \beta2\beta2B2 + \beta3\beta3C2 + \beta4\beta4D2 \quad (1)$$

where Y is the response variable (dependent variable), β0 is the intercept (constant), β1, β2, β3, and β4 are linear coefficients β1β2, β1β3, β1β4, β2β3, β2β4, and β3β4 are interaction coefficients, β1β1, β2β2, β3β3, and β4β4 are squared coefficients, and A, B, C, D, AB, AC, AD, BC, BD, CD, A2, B2, C2, and D2 are levels of independent variables. Statistical significance of the above model equation was determined by Fisher's test value, and the proportion of

variance explained by the model was given by the multiple coefficient of determination, R squared (R2) value. Later, an experiment was run using the optimum values for variables given by response optimization to confirm the predicted value and the lactic acid production was confirmed.

Table 2. CCD design with actual value and predicted value of lactic acid (response) produced

Run	CSL	Sodium acetate	K_2HPO_4	$MgSO_4$	Lacticacid g/L(Actual)	Lacticacid g/L(Predicted)
1	0.1	0.25	1	0.2	17.55	16.66
2	1	0.25	1	1	20.63	20.65
3	0.55	0.88	0.6	0.6	18.35	18.13
4	0.55	0.88	0.6	0.6	18.51	18.13
5	0.1	1.5	0.2	0.2	14.57	14.68
6	0.1	1.5	1	1	16.91	17.02
7	1	1.5	1	0.2	10.23	10.29
8	1	0.25	0.2	0.2	11.64	11.67
9	0.1	0.25	0.2	1	16.88	16.96
10	1	1.5	0.2	1	14.41	15.43
11	1	1.5	1	1	14.84	14.68
12	0.1	1.5	1	0.2	14.78	15.21
13	0.1	0.25	1	1	24.9	24.7
14	0.55	0.88	0.6	0.6	21.52	20.31
15	1	0.25	0.2	1	18.35	18.24
16	1	1.5	0.2	0.2	14.57	15.09
17	0.55	0.88	0.6	0.6	20.42	20.31
18	0.1	1.5	0.2	1	20.47	21.17
19	0.1	0.25	0.2	0.2	12.5	12.98
20	1	0.25	1	0.2	19.14	18.76
21	0.55	0.88	0.6	0.6	14.38	18.03
22	0.55	0.88	0.6	0.6	19.81	18.03
23	-0.35	0.88	0.6	0.6	17.68	17.49
24	0.55	-0.38	0.6	0.6	15.39	16.09
25	0.55	0.88	-0.2	0.6	15.95	14.75
26	0.55	0.88	0.6	-0.2	11.22	11.26
27	1.45	0.88	0.6	0.6	14.14	13.85
28	0.55	0.88	1.4	0.6	16.96	17.68
29	0.55	2.13	0.6	0.6	13	11.82
30	0.55	0.88	0.6	1.4	20.15	19.63

RESULTS AND DISCUSSION

This experiment suggested four components, CSL, K_2HPO_4,$MgSO_4$ and sodium acetate, as having significance of more than 95% confidence level. In the present study, these variables was selected for further optimization by CCD of response methodology. The results of the CCD experiments for studying the effect of independent variables are presented in Table 2. CCD is based on three basic points with respect to concentration of components; full factorial points 2k where k is the number of variables, center points η0 (η0>1) and two axial points for each variable (α=2k/4, =2 for k=4). The center points are usually replicated six times to give five degrees of freedom for purely experimental error. In the present study, we selected 6 center points in order to have a better idea of the experimental error. Thus, for the present study with four variables and 6 center points, total design points (experiments) would be N=2k+2k+η0=30 The results showed that, among the four variables tested, CSL, K_2HPO_4, $MgSO_4$ and sodium acetate were highly significant for lactic acid production, whereas interaction among CSL and MgSO4 and Sodium acetate and MgSO4 are significant considering the p value. A 2D contour plot was generated for the response (lactic acid) at any two independent variables while keeping the others at their 0 level. Thus, six contour graphs were obtained by considering all the possible combinations (Fig. 1).

Both CSL and Sodium acetate at higher concentrations resulted in increased production of lactic acid (Figs. 1A, 1B). A similar effect was observed for MgSO4 (Fig. 1C). In all the above cases, the concentration of MgSO4 significant effect on lactic acid production, whereas higher lactic acid production was achieved at a lower concentration of CSL by increasing

This can be attributed to the better utilization of Cheese whey sugar by the organism for lactic acid production in the presence of elevated levels of these nutrients. Increased concentration of sodium acetate have higher significant effect, K2HPO4 was present at concentration above 15.0 g/l (Fig. 1D). Elevated levels of sodium acetate along with increased concentration of MgSO4 resulted in no higher lactic acid production (Fig. 1E). The concentration of MgSO4 had no significant effect; however, it resulted in a higher production of lactic acid with increased concentration of K2HPO4 (Fig. 1F). By applying multiple regression analysis on the experimental results, a second-order polynomial model (Eq. 2) was derived explaining the role of each variable and their interaction in lactic acid production.

$$\text{Lactic acid} = +18.831 - 0.9095\,A - 1.066B + 0.733\,C + 2.094\,D - 0.663\,AB - 0.240\,AC - 0.444\,AD - 1.881\,BC - 0.465\,BD - 0.078\,CD - 0.590\,A2 - 1.019\,B2 - 0.454\,C2 - 0.647\,D2 \quad (2)$$

where A is CSL, B is sodium acetate, C is K2HPO4, and D is MgSO4. ANOVA test (Table 3) showed that the coefficient of determination (R2) for production of lactic acid was 0.9154, implying that 91.54% variance can be explained by the model. The R2 value should be in between 0 and 1. The closer the R2 is to 1, the stronger the model and the better it predicts the

response (Haaland, 1989). Adequate precision measures the signal-to-noise ratio. An adequate precision for lactic acid production was 13.63, and the predicted R2 of 0.6967 was in reasonable agreement with the adjusted R2 of 0.8244. This indicated a good agreement between the experimental and predicted values of lactic acid production. The model F-value of 10.05 and value of p of less than 0.0001 indicated that the model was highly significant. The lack of- fit value of 0.20 and p-value of 0.9771 implied that lack of fit was not significant: Lack of fit is the variation of the data around the fitted model. If the model does not fit the data well, this will be significant. Independent variables that are not significant were neglected considering their pvalue, and the model equation was modified to the reduced fitted model.

$$\text{Lactic acid} = +18.831 - 0.9095\,A - 1.066B + 2.094\,D - 1.881\,BC - 1.019\,B2 \quad (3)$$

This reduced fitted model is considerably simpler and fits the data as well as the model (Eq. 3) with all the terms. Hence, it can be used for further validation. Lactic acid bacteria are fastidious microorganisms and have complex nutrient requirements. Moreover, a considerable amount of expensive complex organic nitrogen sources, such as yeast extract, must be added to the medium (Chapin, 1993, Fitzpatrik *et al.,* 2001). If inorganic nitrogen sources have to be used in the medium, it must be supplemented with vitamins (Nancib *et al., 2005)* Sodium acetate is reported to enhance the cell growth, thereby indirectly increasing the product yield (Krishnan *et al.,* 1998, Peters *et al.,* 1954). Inorganic phosphates have an effect on lactic acid production by Lactobacillus helveticus (Abdeltif, 2000). In the present study, we observed that CSL, sodium acetate, K2HPO4 and MgSO4 had significant effect on high production of lactic acid, when Cheese whey was used as a sugar source.

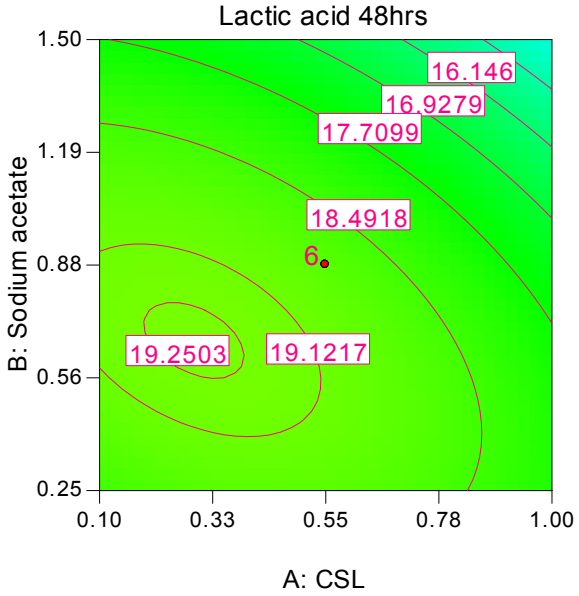

Fig. A.

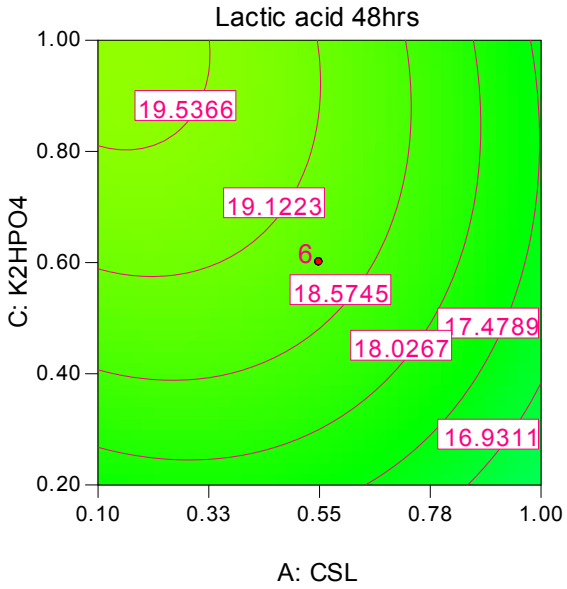

Fig. B.

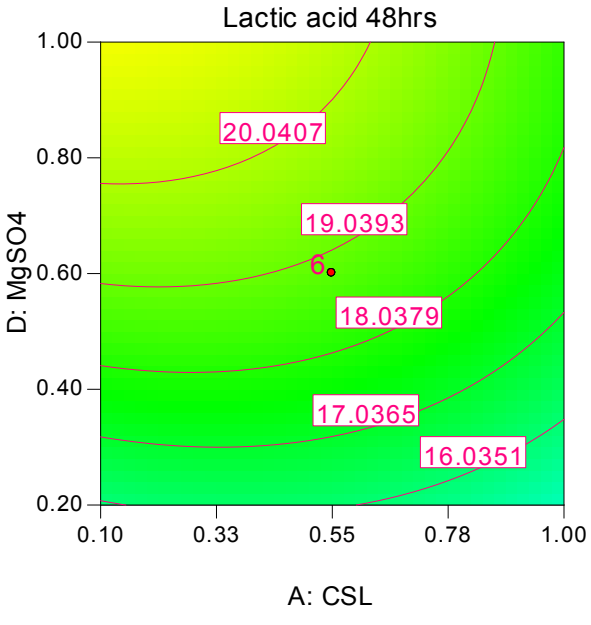

Fig. C

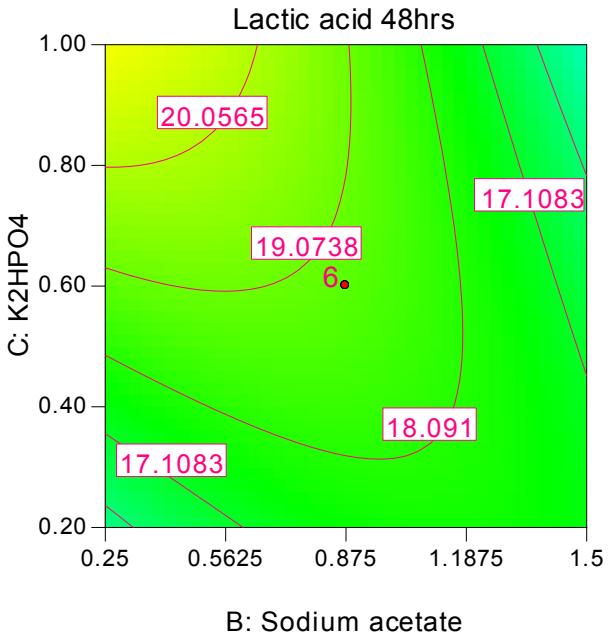

Fig. D

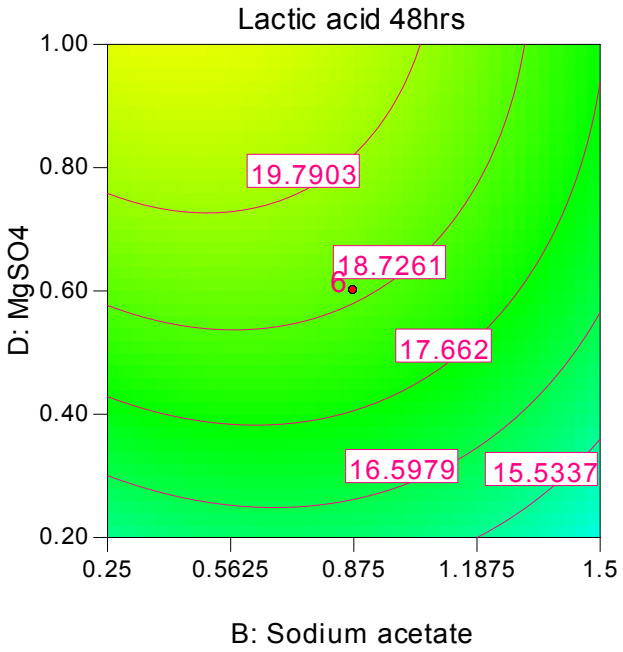

Fig. E

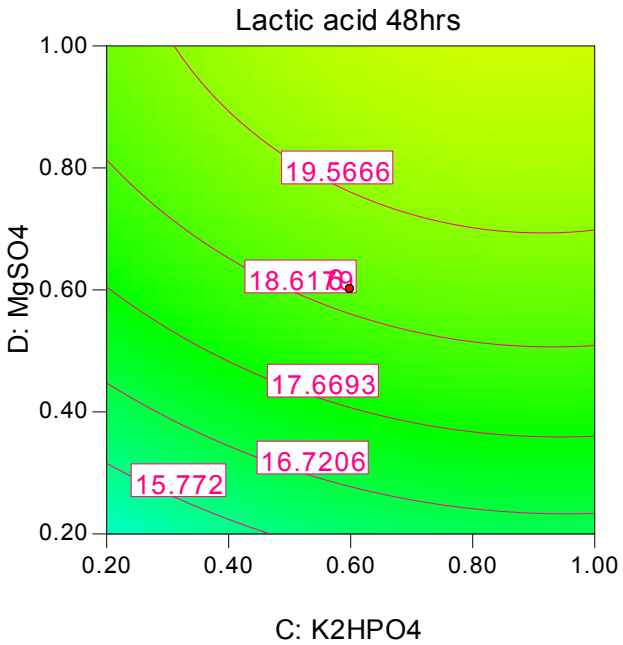

Fig. F

Fig. 1. Contour plots showing the effect of CSL and sodium acetate (A), CSL and K_2HPO_4 (B), CSL and $MgSO_4$ (C), sodium acetate and K_2HPO_4 (D), sodium acetate and $MgSO_4$ (E) and K_2HPO_4 and $MgSO_4$ (F) with other independent variables at 0 level

Table 3. ANOVA for response surface design evaluation

Source	Sum of Squares	df	Mean Square	F value	p-value
Model	277.63	14	19.83	10.05	<0.0001
A-CSL	19.85	1	19.85	10.06	0.0073
B-Sodium acetate	27.28	1	27.28	13.83	0.0026
C- K_2HPO_4	12.92	1	12.92	6.55	0.0238
D- $MgSO_4$	105.29	1	105.29	53.38	<0.0001
AB	7.03	1	7.035	3.56	0.0815
AC	0.92	1	0.92	0.46	0.5052
AD	3.15	1	3.15	1.60	0.2279
BC	56.66	1	56.66	28.72	0.0001
BD	3.46	1	3.46	1.75	0.2076
CD	0.09	1	0.09	0.04	0.8274
A2	9.57	1	9.57	4.85	0.0462
B2	28.51	1	28.51	14.45	0.0022
C2	5.67	1	5.67	2.87	0.1138
D2	11.48	1	11.48	5.82	0.0313
Residual	25.64	13	1.97		
Lack of Fit	10.28	10	1.02	0.20	0.9771
Pure Error	15.36	3	5.12		
Cor Total	336.50	29			

Adequate precision=13.63
R^2= 0.9154
Adujsted R^2=0.8244
Predicted R^2=0.6967

Validation was carried out under conditions predicted by the model. Validation of the statistical model and regression equation was performed by running the optimization program with Design Expert within the experiment range investigated, and the following optimum values were obtained: CSL 0.10%; sodium acetate: 0.32% ; K_2HPO_4: 0.36%, and $MgSO_4$: 0.42%. The predicted response for lactic acid production was 15.43 g/l. Hence, the parameters given by the response surface optimization were used for confirmation of the predicted response of 15.43 g/l lactic acid. The organism produced 15.85 g/l lactic acid, confirming the validity.

RSM has been employed for the production of lactic acid from wheat bran by using Lactobacillus amylophilus GV6 (Naveena *et al.*, 2005) It has also been applied for the production of various enzymes, such as cyclodextrin glucanotransferase (CGTase) (Gawande *et al.*, 1999; Mahat *et al.*, 2004), chitinase (Gohel *et al.,* 2005), α-amylase (Rao *et al.,* 2003) and pectinase (Nair *et al.,* 1997), and vitamin riboflavin (Pujari *et al.,* 2000) RSM can also be useful in optimizing the enzyme reaction conditions (Murthy *et al.,* 2000). Response surface methodology can be used to determine optimum concentration of significant medium components. In the

present study, CSL, sodium acetate, K_2HPO_4, and $MgSO_4$ were identified to be significant, and individual as well as interaction effects of these components were studied. Finally, a higher lactic acid production (50%) was achieved by using RSM.

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