

Optimized lactic acid production from whey using hybrid design and ridge analysis

A Swathi, V Sridevi, GH Rao*

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Abstract

Cheese whey, a byproduct of dairy industry, is usually considered as a high strength wastewater from environmental point of view because of its high chemical oxygen demand. Production of valuable chemicals from cheese whey has been considered as an attractive option because of its rich nutrient content. This investigation aimed at the optimized production of lactic acid from cheese whey using isolated *Lactobacillus plantarum* JX183220 under submerged fermentation by applying response surface methodology (RSM) that involved 4-variable hybrid design and ridge analysis. The effect of different process variables such as whey concentration, yeast extract concentration, inoculum size, and pH of the medium were monitored so as to enhance the lactose conversion in whey to lactic acid. By applying 416C version of hybrid design along with ridge analysis, the maximum production of lactic acid was found to be 36.09 g/L at the optimum combinations of process variables: whey concentration (% v/v) 76.96, yeast extract concentration (% w/v) 1.163, inoculum percentage (% v/v) 4.47, and pH 6.8.

Keywords: Lactic acid, *Lactobacillus plantarum* JX183220, Hybrid design 416C, ridge analysis, experimental optimization

Introduction

Lactic acid (2-hydroxypropionic acid), a naturally occurring multifunctional organic acid, is a valuable industrial chemical used as an acidulant and preservative in food industry, pharmaceutical, leather, and textile industries, as well as a chemical feedstock. Recently, lactic acid consumption has been increased considerably because of its role as a monomer in the production of biodegradable

poly lactic acid (PLA) which is well-known as a sustainable bioplastic material (Datta et al. 1995; Litchfield 1996). Racemic DL-lactic acid is always produced by chemical synthesis from petrochemical resources, while an optically pure L (+) or D (-) lactic acid can be obtained by microbial fermentation of renewable resources when the appropriate microorganism that can produce only single isomer is selected (Hofvendahl and Hahn-Hagerdal 2000). Therefore, the biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative to environmental pollution caused by the petrochemical industry.

Lactic acid bacteria (LAB) are a group of gram-positive, non-sporing, cocci or rods which are anaerobic but aerotolerant that produce lactic acid as the major end-product during fermentation of carbohydrates. The representative genera of LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. Most of the strains used in industry belong to the genus *Lactobacillus*. *Rhizopus* species such as *R. oryzae* and *R. arrhizus* have amylolytic enzyme activity which enables them to convert starch directly to L (+) lactic acid (Yin et al. 1997; Oda et al. 2002). Sucrose from cane and beet sugar, whey containing lactose, maltose and dextrose from hydrolyzed starch are presently used as substrates for the production of lactic acid commercially.

Cheese whey, the greenish translucent liquid effluent generated during the cheese making process, is a clean, abundant food-grade material and a potential environmental pollutant due to its high lactose content (Ghaly and Ramkumar 1999). Production of cheese whey in the world is estimated to be over 108 tons per year. Cheese whey contains 5 - 6% lactose, 0.8 - 1% protein and 0.06% fat (Marwaha and Kennedy 1988; Mawson 1994; Siso 1996). The availability of carbohydrate reservoir of lactose in whey and presence of other essential nutrients for the growth of microorganisms makes the whey one of the potential substrate for the production of different bio-products through biotechnological means. The production of lactic acid through LAB could be an alternative processing route for whey lactose utilization (Kisaalita et al. 1990; Buyukkileci et al. 2004). Studies have demonstrated the need

A Swathi, V Sridevi, GH Rao*

Department of Chemical Engineering & Biotechnology, Anil Neerukonda Institute of Technology & Sciences (ANITS), Sangivalasa -531162, Visakhapatnam, AP, India.

*E-mail: ghrao77@gmail.com

to supplement cheese whey with commercially available growth supplements, such as corn steep liquor, yeast extract, casamino acids, peptone, neopeptone, molasses and trypticase (Roy et al. 1986; Cheng et al. 1991; Gupta and Gandhi 1995).

Response surface methodology (Box and Draper 2007) has been successfully used to model and optimize biochemical and biotechnological processes related to food systems (Vazquez and Martin 1998). This methodology could be employed to optimize media for lactic acid fermentation. Thus, the aim of the present study is to apply for the first time, the 4-variable hybrid design 416C of RSM along with ridge analysis to optimize lactic acid production, from isolated *Lactobacillus plantarum* JX183220 at controlled concentrations of whey, yeast extract, inoculum percentage, and pH under submerged fermentation.

Hybrid Design of RSM

Hybrid design (HD) is a superior and more economical (in terms of experimental runs) modeling technique when compared to the other traditional designs like central composite design (CCD), Box-Behnken design (BBD), or Dohrlert design (DD) that are being widely used by the investigators for experimental optimization. When the experiments are expensive to carry out, this is the most economical experimental design requiring minimum runs. For a 4-variable process, this design needs only 16 runs while the other methods require more than 25 runs. Therefore, this design can be conveniently adopted to predict optimum process conditions for the submerged state fermentation of *Lactobacillus plantarum* JX183220 for lactic acid production from cheese whey. An attempt was made to increase the product yield by optimizing the four chosen process parameters such as whey concentration, yeast extract concentration, inoculum percentage and pH.

Hybrid response surface designs were developed (Roquemore 1976) to achieve the same degree of orthogonality as the CCD, to be near-minimum-point in size, and to be near rotatable. They are created using a CCD for k-1 factors, and the levels of the kth factor are chosen to create certain symmetries within the design. The result is a class of designs that are economical and either rotatable or near-rotatable. For k = 3, there are three hybrid designs which are denoted as 310, 311A and 311B. For k = 4, there are three hybrid designs which are denoted as 416A, 416B and 416C. The first digit indicates the number of factors (i.e. independent variables), the next two digits indicate the number of runs while the alphabet denotes the variant of the particular design.

Four independent variables, (x_1, x_2, x_3, x_4), would be chosen and scaled according to a predetermined matrix that would consist of 16 runs.

Table 1. Scaling matrix for hybrid design

x_1	x_2	x_3	x_4
0	0	0	1.7658
0	0	0	0
+/- 1	+/- 1	+/- 1	0.5675
+/- 1.4697	0	0	-1.0509
0	+/- 1.4697	0	-1.0509
0	0	+/- 1.4697	-1.0509

The notation +/- 1 in Table 1 indicates a 2³ factorial in x_1, x_2 and x_3 with x_4 fixed. The design points, following the 2³ factorial, are axial points in x_1, x_2 and x_3 with x_4 fixed. A centre run is added in 416C design to avoid near similarity. The notation +/- 1.4697 implies 2 axial points. When possible, one or two more centre runs should be added to the hybrid design. The first three variables are situated with

five levels while the last one would be studied with four levels for 416C version of hybrid design.

Hybrid designs are not used as much in industrial application as they should be, even though the use of saturated or near saturated response surface designs should be avoided. Hybrid designs provide a good choice when the cost of experimentation is prohibitive.

A regression equation for the case of 4 independent variables is given by:

$$y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{14}x_1x_4 + \beta_{23}x_2x_3 + \beta_{24}x_2x_4 + \beta_{34}x_3x_4 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{33}x_3^2 + \beta_{44}x_4^2 \quad (1)$$

where y is the measured response and x_i represent scaled independent variables.

Location of the Stationary point

The stationary point, if exists, will be the set of x_1, x_2, x_3 , and x_4 for which partial derivatives will be equated to zero:

$$\partial y / \partial x_1 = \partial y / \partial x_2 = \partial y / \partial x_3 = \partial y / \partial x_4 = 0 \quad (2)$$

and the corresponding differentiated equations will be solved for stationary point.

The set of points, say, $x_{1,s}, x_{2,s}, x_{3,s}, x_{4,s}$, is called stationary point that might represent :

1. a point of maximum response,
2. a point of minimum response,
3. a saddle point

Writing the second-order model in matrix notation, we have,

$$y = \beta_0 + \mathbf{x}'\mathbf{b} + \mathbf{x}'\mathbf{B}\mathbf{x} \quad (3)$$

where,

$$\mathbf{x} = \begin{bmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \end{bmatrix} \quad \mathbf{b} = \begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \\ \beta_4 \end{bmatrix}$$

$$\mathbf{B} = \begin{bmatrix} \beta_{11} & 0.5\beta_{12} & 0.5\beta_{13} & 0.5\beta_{14} \\ 0.5\beta_{21} & \beta_{22} & 0.5\beta_{23} & 0.5\beta_{24} \\ 0.5\beta_{31} & 0.5\beta_{32} & \beta_{33} & 0.5\beta_{34} \\ 0.5\beta_{41} & 0.5\beta_{42} & 0.5\beta_{43} & \beta_{44} \end{bmatrix}$$

That is, \mathbf{b} is a (4 by 1) vector of the first-order regression coefficients and \mathbf{B} is (4 by 4) symmetric matrix whose main diagonal elements are the pure quadratic coefficients (β_{ii}) and whose off-diagonal elements are one-half the mixed quadratic coefficients (β_{ij}). The derivative of y w.r.t elements of vector \mathbf{x} equated to 0 is:

$$\partial y / \partial \mathbf{x} = \mathbf{b} + 2\mathbf{B}\mathbf{x} = 0 \quad (4)$$

The stationary point is the solution to the above equation

$$\mathbf{x}_s = (-1/2) \mathbf{B}^{-1} \mathbf{b} \quad (5)$$

Furthermore, by substituting the above Eq. 5 in Eq. 3 we can find the predicted response at the stationary point as:

$$y_s = \beta_0 + 0.5\mathbf{x}_s' \mathbf{b} \quad (6)$$

The eigen values of matrix **B** would give an indication of the nature of response surface as shown in Table 2.

Table 2. Relation between eigen values and the nature of response surface

Eigen values	Nature of response surface
All positive	Minimum
All negative	Maximum
Mixed signs (some positive and some negative)	Saddle surface, where optimum is not identified
Zeros	Flat surface

Comparison of hybrid design with other popular designs

A central composite design combines a fractional factorial design with additional points (star points) and some points at the center of the experimental region. The total no. of design points (N) is determined by the formula

$$N = 2^k + 2K + C_0 \quad (7)$$

where K is the number of variables and C_0 is the number of center points. The CCD has five levels for a four variable design.

Box-Behnken designs are three level rotatable second order designs. The number of experimental points (N) is given by

$$N = 2K(K - 1) + C_0 \quad (8)$$

Doehlert designs have different number of levels (7, 7, 5, 3 levels for a four-variable designs) for the process variables and is based on spherical experimental design with uniform space filling. The no. of experiments required,

$$N = K^2 + K + C_0 \quad (9)$$

Hybrid designs are compared with the other three popular designs in Table 3, where the efficiency of experimental design is defined as the ratio of number of coefficients in the quadratic response surface model to the no. of experiments. The no. of experiments, N, is equal to no. of coefficients in the quadratic model plus one. The four-variable hybrid design has five levels for the first three variables and four levels for the last variable.

Table 3: Comparison of efficiency of different designs of RSM

Variable	No. of Coefficients	CCD	BBD	DD	HD
K = 4	No of Runs. N	25	25	21	16
	Efficiency (P/N)	0.60	0.60	0.71	0.94

(Only one centre point is included in all the above designs)

The efficiency of hybrid design is far better than any one of the three popular designs. The reported applications of hybrid design

are meager in the literature. Only one article has been published about the application of 416C design which dealt with the monoxygenase production (Yan and Le-he 2007).

Ridge analysis

When unique optimum is not indicated by response surface diagrams/contours or eigen value analysis, ridge analysis will be employed to locate the optimum conditions. Ridge means a long narrow raised line along the surface of something. Ridge analysis is an optimization technique that finds factor settings \mathbf{x} such that Eq. 3 is optimized subject to $\mathbf{x}'\mathbf{x} = \rho^2$.

The solution \mathbf{x} to this problem provides operating conditions that yield an estimated absolute maximum or minimum response on a sphere of radius ρ . Different solutions can be obtained by trying different values of ρ .

The original formulation of ridge analysis was based on the eigen values of a stationary system. With the wide availability of non-linear programming codes, ridge analysis problems can be solved without recourse to eigen value analysis. For ridge analysis, the above constraint can be written as,

$$0 \leq \sqrt{\mathbf{x}'\mathbf{x}} \leq \rho \quad (10)$$

where radius ρ is taken as 1.

A good introduction to theory and applications of RSM and Ridge analysis is provided by Box and Draper (2007). Ridge analysis was applied in extraction of polysaccharides from *Cordyceps sinensis* (Dong et al. 2009).

Materials and methods

Microorganism

Lactobacillus plantarum JX183220, previously isolated from goat milk in the Department of Biotechnology, ANITS, Visakhapatnam, India was used throughout the study (Sridevi 2012; Sridevi et al. 2015).

Growth conditions

The culture was maintained on MRS agar slants having the composition (w/v): protease peptone - 10g, beef extract - 10g, yeast extract - 5g, D-glucose - 20g, tween 80 (1ml), sodium acetate - 5g, tri-ammonium citrate - 2g, magnesium sulphate - 0.1g, manganese sulphate - 0.05g, dipotassium phosphate - 2g and agar - 15g per liter of distilled water. The pH of the medium was adjusted to 6.5, inoculated with culture and incubated at 37 °C for 48h. The culture slants were stored at 4 °C and sub culturing was carried out for every 15 days.

Inoculum preparation

A loopful of *Lactobacillus plantarum* JX183220 from agar slants was inoculated into 50 ml of autoclaved MRS medium and incubated at 37 °C with shaking at 120 rpm for 48 h to obtain active culture.

Preparation and clarification of whey

Whey clarification was carried through protein precipitation induced by heating the whey at 90 °C for 20 min. Precipitated

proteins were removed by centrifugation at 4,000 rpm for 15 min (Panesar et al. 2010).

Lactose estimation

Lactose present in whey was estimated following dinitro salicylic acid (DNS) method.

Fermentation medium

The production medium consisted of clarified cheese whey added with yeast extract and inorganic salt, $MnSO_4$ (20 mg/L). The medium was sterilized, inoculated with 48 h old culture of *Lactobacillus plantarum* JX183220, and incubated in an orbital shaker at 37 °C and 120 rpm for 96h.

Estimation of lactic acid

The fermented broth was collected, filtered and centrifuged at 5000 rpm for 5 min. The cell free supernatant was used for determination of lactic acid. Lactic acid estimation was done following Kimberley and Taylor method (Kimberley et al. 1996). To the 0.5ml of sample, 3ml of 96% H_2SO_4 was added and incubated at 95-100 °C for 10min 50µl of 4% $CuSO_4$ followed by 100µl of p- phenyl phenol reagent were added. The tubes were left at room temperature for 30min and the absorbance was read at 570 nm.

Optimization of process parameters

As a first optimization step, the critical variables for lactic acid production along with their ranges were estimated based on classical 'one-variable-at-a-time' approach. The effect of fermentation time (24-168 h), whey concentration (70-100% v/v), yeast extract concentration (0.2-1% w/v), inoculum percentage (1-5% v/v) and pH (5-9) were investigated. All experiments were conducted in duplicate, and the mean values were calculated.

Secondly, hybrid design was used to describe the nature of the response surface in the experimental region, to search optimal medium composition for maximizing lactic acid yield and to apply ridge analysis in case a saddle stationary point is identified.

Results and discussion

The optimum conditions for lactic acid production obtained in the preliminary studies were found to be incubation time - 96 h, whey concentration - 90% v/v, yeast extract concentration - 0.8% w/v, inoculum percentage - 3% v/v and pH - 7.0. All the further experiments were carried out by fixing incubation time to 96 h.

A 16-run hybrid design of version 416C (with 2 additional center points: 16A, and 16B) for 4 variables (having 5 levels for first three variables and 4 levels for last variable) was employed for fitting the response surface. Based on the results obtained in preliminary experiments and from available literature, four potential independent variables such as whey concentration, yeast extract, inoculum level and pH were chosen for optimization by hybrid design. The selected range of the four process variables for hybrid design was shown in the Table 4. The relation between natural variable, X, and the scaled variable, x, is given by

$$X = X_{mid} + x * (X_{max} - X_{mid})/x_{max} \quad (11)$$

where $X_{mid} = 80, 0.8, 3, 7$;

$X_{max} = 94.967, 1.3879, 4.4697, 8.7654$; and

$x_{max} = 1.4697, 1.4697, 1.4696, 1.7654$ for the four variables respectively.

Table 4: Scaling and range of process variables for Hybrid design

Scaled Parameter code (5 levels) ($x_1, x_2, \& x_3$)	Levels				
	-1.4697	-1	0	1	1.4697
X_1 : Whey concentration (% v/v) (Step = 10)	65.303	70	80	90	94.697
X_2 : Yeast extract concentration (%w/v) (Step = 0.4)	0.2121	0.4	0.8	1.2	1.3879
X_3 : Inoculum percentage (%v/v) (Step = 1)	1.5303	2	3	4	4.4697
x_4: Scaled Parameter code (4 levels)	-1.0509	0	0.5695	1.7654	
X_4 : pH (Step =1)	5.9491	7	7.5675	8.7654	

Experimental design for optimization

Experiments were performed according to the hybrid design 416C as given in Table 5 in order to determine the optimum combination of selected components in the medium. Fitting the experimental data of lactic acid yield values with the four scaled variables resulted in the following second order response surface model:

$$y = 28.833 + 4.3474x_1 - 1.0380x_2 + 3.4330x_3 - 6.0233x_4 - 6.1875x_1x_2 - 0.5625x_1x_3 + 4.7843x_1x_4 - 6.0625x_2x_3 + 1.2784x_2x_4 + 3.7524x_3x_4 - 3.5003x_1^2 - 3.7318x_2^2 - 1.0698x_3^2 - 0.5454x_4^2 \quad (12)$$

whose results were listed in Table 5. 'regstats' program of MATLAB7 software was used for estimating the coefficients of Eq. 12.

The model adequacy was checked by F-test and the coefficient of determination, R^2 . The high value of R^2 , 0.9882, indicates that 98.82% of the variability in the response could be predicted by the model. The p-values of estimated coefficients indicate the significance of each coefficient. The smaller the value of p, the more significance is the corresponding coefficient. Usually, the coefficients with p-values <0.05 are included in the fitted equation. A model is said to be hierarchical if the presence of higher-order terms (such as interaction and second order terms) requires the inclusion of all lower-order terms contained within those of higher order. Many regression model builders believe that hierarchy is a reasonable model building practice when fitting polynomials. So in this model all the terms were included using the hierarchical principle even though values of p for certain coefficients were high.

The results of the analysis of variance (ANOVA) for the quadratic model were presented in Table 6. The F value indicates the significance of the fitted model. From the table, it was evident that Eq. 12 is highly significant, as evident from the F-value (p model, $F < 0.02$). The eigen values of the matrix (formed from the coefficients of interaction terms and squared terms of the coefficients) were found to be: -7.69, -4.4118, 0.99772, 2.2523. As the eigen values have different signs, the response surface was a saddle one and hence unique optimum values cannot be specified with Eq. 12. The contour plots showing the mutual effect of any two process variables on lactic acid production were shown in Fig. 1.

Table 5. Hybrid design 416C with experimental and predicted values of lactic acid yield by *Lactobacillus plantarum* JX183220

Run	Scaled Variables				Lactic acid yield (g/L)	
	x ₁	x ₂	x ₃	x ₄	Experimental	Predicted
1	0	0	0	1.7654	16.5000	16.5000
2	-1	-1	-1	0.5675	3.0000	3.9375
3	1	-1	-1	0.5675	32.5000	31.5625
4	-1	1	-1	0.5675	4.5000	3.5625
5	1	1	-1	0.5675	5.5000	6.4375
6	-1	-1	1	0.5675	5.0000	4.0625
7	1	-1	1	0.5675	28.5000	29.4375
8	-1	1	1	0.5675	27.0000	27.9375
9	1	1	1	0.5675	29.5000	28.5625
10	1.4697	0	0	-1.0509	26.0000	26.0000
11	-1.4697	0	0	-1.0509	28.0000	28.0000
12	0	1.4697	0	-1.0509	23.0000	23.0000
13	0	-1.4697	0	-1.0509	30.0000	30.0000
14	0	0	1.4697	-1.0509	31.5000	31.5000
15	0	0	-1.4697	-1.0509	33.0000	33.0000
16	0	0	0	0	32.0000	28.8333
16A	0	0	0	0	28.0000	28.8333
16B	0	0	0	0	26.5000	28.8333

Table 6. Analysis of variance in the regression model for optimization of lactic acid

Source of variation	Degrees of freedom	Sum of squares	Mean square	F value	P value
Regressions	14.0000	1942.9132	138.7795	17.9472	0.0180
Residual	3.0000	23.1979	7.7326		
Total	17.0000	1966.1111			

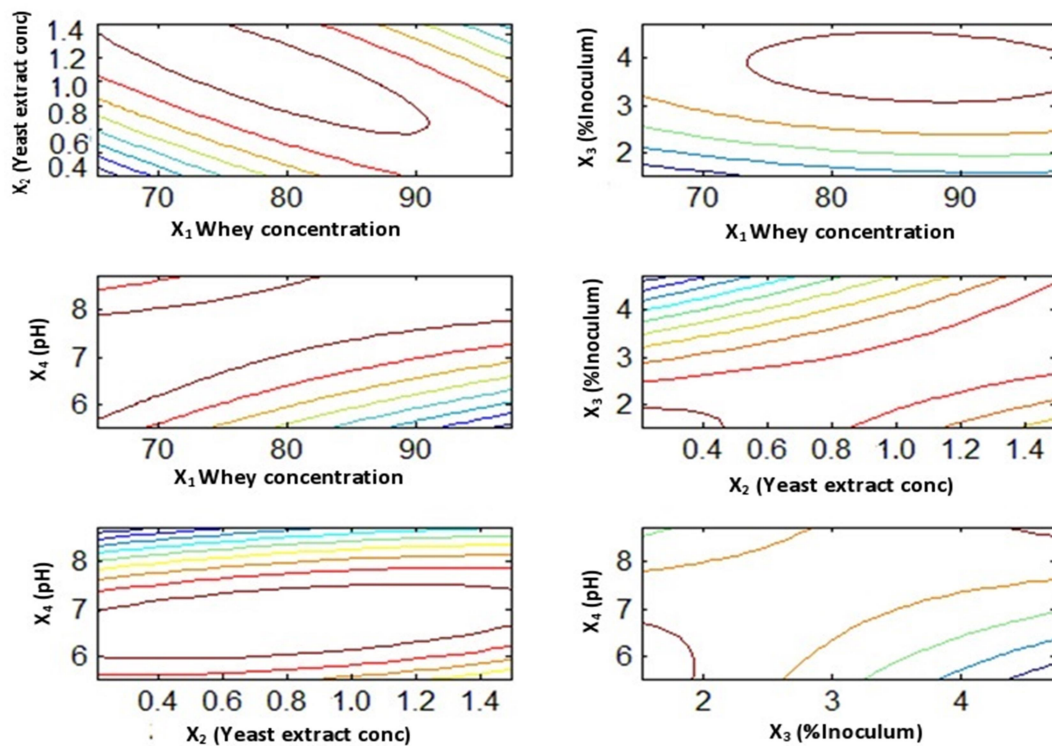


Figure 1: Contour plots showing the interaction effect among the four process variables

Saddle contours were observed among all the variables (X_1 versus X_4 ; X_2 versus X_3 ; X_3 versus X_4) and hence unique optimum cannot be derived from quadratic response surface function represented by Eq. 12. Application of Eq. 6 with estimated coefficients resulted in a maximum lactic acid yield of 31.84 g/L which was much lower than the 15th run of Table 5 and hence obviously it cannot be considered to be experimentally attainable maximum. So ridge analysis was employed to obtain reliable optimal settings. MATLAB program 'fmincon' was used for optimizing the Eq. 12 with constraints imposed by Eq. 10. The optimum values of the process variables derived via ridge analysis were found to be 76.9584% (v/v) whey concentration, 1.16344% (w/v) yeast extract concentration, 4.4697% (v/v) inoculum percentage, and pH of 6.80371, with the optimum lactic acid production being 36.0916 g/L which was substantially higher than that obtained from the preliminary studies or by the 15th run of hybrid design response value (33 g/L).

Lactic acid production from whey was reported in literature mainly derived from *Lactobacillus helveticus* (Roy et al. 1986; Chiarini et al. 1992). Studies on the lactic acid production from whey using *Lactobacillus plantarum* are meager in literature. In the present study, *Lactobacillus plantarum* JX183220 produced 36.0916 g/L of lactic acid from 42.59g/L of lactose present in 76.9% (v/v) whey with a yield of 85%. These results were in close agreement with lactic acid production from glucose present in MRS medium by LAB (Sheeladevi and Ramanathan 2011).

Conclusion

Submerged state fermentation has been performed for the production of lactic acid from the isolated species of *Lactobacillus plantarum* JX183220 using whey as the substrate. The effect of four variables, namely, whey concentration, yeast extract concentration, inoculum percentage, pH have been studied for optimized production of lactic acid using hybrid design of RSM. Even though the second-order model obtained by hybrid design was adequate in data fitting with a very high value of R^2 (0.9882), saddle response surface was obtained and hence ridge analysis was employed to estimate reliable optimum conditions. The study proved the utility of hybrid design (with a minimum of 18 runs) coupled with ridge analysis in arriving at the optimum conditions for the production of lactic acid from a low value dairy waste, i.e., cheese whey.

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