J. Microbiol. Biotechnol. (2006), **16**(9), 1410–1415

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Application of Response Surface Methodology for Optimization of Lactic Acid Production Using Date Juice

CHAUHAN, KISHOR, UJJVAL TRIVEDI, AND K. C. PATEL*

Department of Biosciences, Sardar Patel University, VallabhVidyanagar-388 120, Gujarat, India

Received: March 7, 2006
Accepted: May 4, 2006

Abstract Media components, including date juice, sodium acetate, peptone, and K_2HPO_4, which were screened by Plackett-Burman fractional factorial design, were optimized for lactic acid production from date juice using the response surface method (RSM). Sodium acetate, peptone (p<0.0001), and K_2HPO_4 (p=0.0029) were highly significant in influencing the lactic acid production. Close correlationship between predicted and experimental values was observed. When the optimum values of the parameters obtained through RSM (25.0 g/l date sugar, 15.0 g/l sodium acetate, 19.1 g/l peptone, and 4.7 g/l K_2HPO_4) were applied, lactic acid production (22.7 g/l) increased by 50.33%, compared with unoptimized media (15.1 g/l). The subsequent validation experiments confirmed the validity of the statistical model.

Key words: Date juice, lactic acid, *Lactobacillus* sp., RSM

Lactic acid, a valuable industrial chemical, is used as an acidulant, preservative for beverages and in foodstuffs applications. Crude lactic acid is used for deliming of hides in the leather industry and for fabric treatment in the textiles and laundry industries. Calcium lactate is employed in baking powders, as an animal feed supplement and a means to provide Ca source in pharmaceutical preparations. Lactic acid can be obtained on an industrial scale either by fermentation or chemical synthesis. Since lactic acid can be readily polymerized, it has become the most demanding monomer for the synthesis of the biodegradable polymer polylactide. Polylactide is used for the preparation of scaffolds for biocompatible artificial organs, self-dissolving sutures and as a means to sustain drug release [4].

*Corresponding author
Phone: 91-2692-222297; Fax: 91-2692-226865;
E-mail: comless@yahoo.com

Productivity of microbial metabolites can be increased by manipulating nutritional requirements, physical parameters and the genetic makeup of the producing organism [8]. The conventional method of media optimization, changing one parameter at a time and keeping the others constant, provides information related to that parameter only. Moreover, such methods are time consuming and can not take mutual interactions of the parameters into the desired outcome.

Statistical methods provide an alternative methodology to optimize a particular process by considering the mutual interactions among the variables and give an estimate of combined effect of these variables on final results. Response surface methodology (RSM) is one such technique based on the fundamental principles of statistics, such as randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. This is generally known as full factorial design [14]. Application of factorial designs and RSM is a common practice in biotechnology for the optimization of media components and culture conditions. These optimization methods involve three major steps; performing statistically designed experiments, estimating coefficients in a mathematical model, predicting the response, and checking the adequacy of the model. Availability of user-friendly software packages has made this technique increasingly popular for media optimization [4, 14, 17, 21].

In the present study, media components such as date juice, peptone, sodium acetate and K_2HPO_4 were evaluated for increasing lactic acid production and statistically optimized using central composite design. Media components have been selected as variables after screening fifteen other ingredients by Plackett-Burman design in our previous study [3]. The role of each variable, their interactions and statistical analysis for lactic acid production were explained by applying the second-order polynomial model. The analysis was done using Design-Expert version 7.0.

MATERIAL AND METHODS

Organism

A homofermentative bacterial isolate, *Lactobacillus* sp. KCP01, has been employed for lactic acid production using date juice in the present study [3]. The organism was grown on MRS medium and maintained in 50% glycerol as a pure culture and stored below 0°C.

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 40°C for 24 h. Cells were harvested in a sterile centrifuge tube by centrifugation at 9,000 rpm for 10 min. 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 Preparation and Lactic Acid Production

Forty g of thoroughly washed seedless dates were minced in distilled water, and the final volume was made up to 300 ml and boiled for 10 min. This was followed by filtration to obtain clear date juice [16, 22]. Crude juice of dates was further diluted with distilled water to obtain a final concentration of 5% reducing sugar. The juice was used as the production medium along with the significant components (Na-acetate, peptone and K_2HPO_4). The medium was adjusted to pH 7.0 and sterilized by autoclaving at 121°C for 15 min. After inoculation, the flasks were incubated at 40°C 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.

Analysis of Media Constituents and Estimation of Lactic Acid

Reducing sugar from date juice was estimated by the dinitrosalicylic acid method [13]. Lactic acid production was confirmed by paper chromatography, HPLC, and enzymatic tests [16], and routine estimations were carried out by a colorimetric method [10].

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 date juice, sodium acetate, peptone and K_2HPO_4 were studied at five different levels (Table 1).

Table 1. Range of values for the response surface method.

Independent variables[a]	Levels				
	−α	−1	0	+1	+α
Date juice (sugar)	12.5	25.0	37.5	50.0	62.5
Sodium acetate	0.0	5.0	10.0	15.0	20.0
Peptone	5.0	10.0	15.0	20.0	25.0
K_2HPO_4	0.0	2.0	4.0	6.0	8.0

[a]Values are expressed as g/l.

A set of 36 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)].

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_1\beta_2AB+\beta_1\beta_3AC+\beta_1\beta_4AD+\beta_2\beta_3BC+\beta_2\beta_4BD+\beta_3\beta_4CD+\beta_1\beta_1A^2+\beta_2\beta_2B^2+\beta_3\beta_3C^2+\beta_4\beta_4D^2 \quad (1)$$

where Y is the response variable (dependent variable), β_0 is the intercept (constant), β_1, β_2, β_3, and β_4 are linear coefficients $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_2\beta_3$, $\beta_2\beta_4$, and $\beta_3\beta_4$ are interaction coefficients, $\beta_1\beta_1$, $\beta_2\beta_2$, $\beta_3\beta_3$, and $\beta_4\beta_4$ are squared coefficients, and A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, and D^2 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 (R^2) 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.

RESULTS AND DISCUSSION

Among the fifteen variables (date juice, peptone, beef extract, yeast extract, K_2HPO_4, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, sodium acetate, sodium sulfate, *tri*-sodium citrate, sodium succinate, tween-80, $FeSO_4$ and NaCl), significant components were screened out using fractional factorial Plackett-Burman design [19]. This experiment suggested four components, date juice, peptone, K_2HPO_4, and sodium acetate, as having significance of more than 95% confidance level [3]. In the present study, these variables were selected for further optimization by CCD of response surface 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 2^k where k is the number of variables, center points η_0 ($\eta_0>1$) and two axial points for each variable ($\alpha=2^{k/4}$, =2 for k=4).

Table 2. Experimental design and results of CCD of response surface methodology.

Run	Date juice g/l	Na acetate g/l	Peptone g/l	K_2HPO_4 g/l	Lactic acid g/l	
					Experimental	Predicted
1	−1.00	−1.00	−1.00	−1.00	12.0	12.3
2	1.00	−1.00	−1.00	−1.00	14.9	14.3
3	−1.00	1.00	−1.00	−1.00	16.4	17.7
4	1.00	1.00	−1.00	−1.00	18.3	18.0
5	−1.00	−1.00	1.00	−1.00	16.9	17.5
6	1.00	−1.00	1.00	−1.00	17.7	17.8
7	−1.00	1.00	1.00	−1.00	22.2	22.5
8	1.00	1.00	1.00	−1.00	19.9	21.2
9	−1.00	−1.00	−1.00	1.00	13.7	13.7
10	1.00	−1.00	−1.00	1.00	17.1	16.2
11	−1.00	1.00	−1.00	1.00	17.6	16.9
12	1.00	1.00	−1.00	1.00	17.0	17.7
13	−1.00	−1.00	1.00	1.00	21.2	20.9
14	1.00	−1.00	1.00	1.00	21.6	21.6
15	−1.00	1.00	1.00	1.00	21.9	23.8
16	1.00	1.00	1.00	1.00	23.8	22.9
17	−2.00	0.00	0.00	0.00	20.1	18.8
18	2.00	0.00	0.00	0.00	19.2	19.9
19	0.00	−2.00	0.00	0.00	16.3	17.0
20	0.00	2.00	0.00	0.00	24.9	23.5
21	0.00	0.00	−2.00	0.00	9.20	9.6
22	0.00	0.00	2.00	0.00	21.0	19.9
23	0.00	0.00	0.00	−2.00	18.9	17.8
24	0.00	0.00	0.00	2.00	20.0	20.9
25	0.00	0.00	0.00	0.00	18.9	19.2
26	0.00	0.00	0.00	0.00	19.3	19.2
27	0.00	0.00	0.00	0.00	18.9	19.2
28	0.00	0.00	0.00	0.00	18.7	19.2
29	0.00	0.00	0.00	0.00	19.5	19.2
30	0.00	0.00	0.00	0.00	18.6	19.2
31	0.00	0.00	0.00	0.00	17.3	19.2
32	0.00	0.00	0.00	0.00	18.4	19.2
33	0.00	0.00	0.00	0.00	20.4	19.2
34	0.00	0.00	0.00	0.00	20.2	19.2
35	0.00	0.00	0.00	0.00	20.2	19.2
36	0.00	0.00	0.00	0.00	19.8	19.2

The center points are usually replicated six times to give five degrees of freedom for purely experimental error. In the present study, we selected 12 center points in order to have a better idea of the experimental error. Thus, for the present study with four variables and 12 center points, total design points (experiments) would be

$$N=2^k+2k+\eta_0=36$$

The results showed that, among the four variables tested, sodium acetate, peptone, and K_2HPO_4 were highly significant for lactic acid production, whereas interaction among sodium acetate and K_2HPO_4 and peptone and K_2HPO_4 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 sodium acetate and peptone at higher concentrations resulted in increased production of lactic acid (Figs. 1A, 1B). A similar effect was observed for K_2HPO_4 (Fig. 1C). In all the above cases, the concentration of date juice (date sugars) did not have any significant effect on lactic acid production, whereas higher lactic acid production was achieved at a lower concentration of date juice by increasing the concentrations of peptone, sodium acetate and K_2HPO_4. This can be attributed to the better utilization of date sugar by the organism for lactic acid production in the presence of elevated levels of these nutrients.

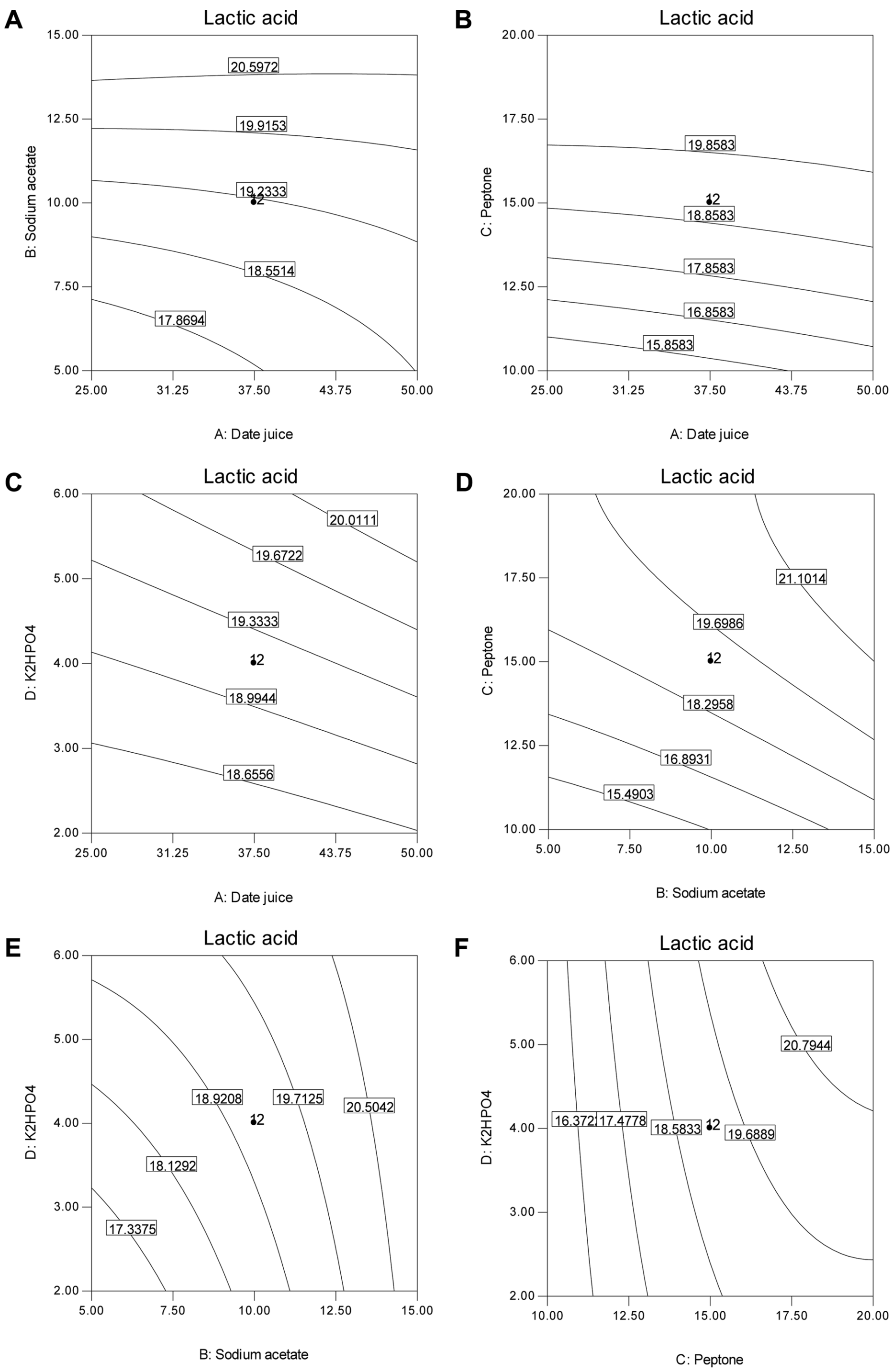

Fig. 1. Contour plots showing the effect of date juice and sodium acetate (**A**), date juice and peptone (**B**), date juice and K_2HPO_4 (**C**), peptone and K_2HPO_4 (**D**), sodium acetate and K_2HPO_4 (**E**) and sodium acetate and peptone (**F**) with other independent variables at 0 level.

Increased concentration of sodium acetate did not have any significant effect, unless peptone was present at concentration above 15.0 g/l (Fig. 1D). Elevated levels of sodium acetate along with increased concentration of K_2HPO_4 resulted in higher lactic acid production (Fig. 1E). The concentration of K_2HPO_4 had no significant effect; however, it resulted in a higher production of lactic acid with increased concentration of peptone (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.

Table 3. ANOVA for response surface design evaluation.

Source	Sum of squares	Df	Mean square	F-Value	*p*-Value
Model	2.95	14	0.21	16.62	<0.0001
A-Date juice	0.018	1	0.018	1.43	0.2449
B-Na-acetate	0.64	1	0.64	50.48	<0.0001
C-Peptone	1.59	1	1.59	125.47	<0.0001
D- K_2HPO_4	0.14	1	0.14	11.36	0.0029
AB	0.027	1	0.027	2.14	0.1577
AC	0.03	1	0.028	2.27	0.1461
AD	0.002	1	0.002	0.15	0.6935
BC	0.00	1	0.00	0.07	0.7925
BD	0.046	1	0.046	3.64	0.0700
CD	0.04	1	0.04	3.15	0.0902
A^2	0.0004	1	0.0004	0.03	0.8605
B^2	0.023	1	0.023	1.87	0.1849
C^2	0.38	1	0.38	30.64	<0.0001
D^2	0.0004	1	0.0004	0.03	0.8605
Lack of fit	0.17	10	0.017	2.17	0.1088
Pure error	0.089	11	0.008		

Adequate precision=26.97.
R^2=0.8856
Adujsted R^2=0.8620
Predicted R^2=0.8018

$$\text{Lactic acid} = +1.92 + 0.028\,A + 0.16\,B + 0.26\,C + 0.078\,D - 0.041\,AB - 0.043\,AC + 0.011\,AD - 0.0075\,BC - 0.054\,BD + 0.050\,CD + 0.0035\,A^2 + 0.027B^2 - 0.11C^2 + 0.0035D^2 \quad (2)$$

where A is date juice (date sugar), B is sodium acetate, C is peptone, and D is K_2HPO_4.

ANOVA test (Table 3) showed that the coefficient of determination (R^2) for production of lactic acid was 0.8856, implying that 88.56% variance can be explained by the model. The R^2 value should be in between 0 and 1. The closer the R^2 is to 1, the stronger the model and the better it predicts the response [9]. Adequate precision measures the signal-to-noise ratio. An adequate precision for lactic acid production was 26.97, and the predicted R^2 of 0.8018 was in reasonable agreement with the adjusted R^2 of 0.8620. This indicated a good agreement between the experimental and predicted values of lactic acid production. The model F-value of 16.62 and value of p of less than 0.0001 indicated that the model was highly significant. The lack-of-fit value of 2.17 and p-value of 0.1088 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 p-value, and the model equation was modified to the reduced fitted model

$$\text{Lactic acid} = +1.94 + 0.16\,B + 0.26\,C + 0.078\,D - 0.054\,BD + 0.050\,CD - 0.11C^2 \quad (3)$$

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 [2, 5]. If inorganic nitrogen sources have to be used in the medium, it must be supplemented with vitamins [16]. Sodium acetate is reported to enhance the cell growth, thereby indirectly increasing the product yield [11, 18]. Inorganic phosphates have an effect on lactic acid production by *Lactobacillus helveticus* [1]. In the present study, we observed that peptone, sodium acetate, and K_2HPO_4 had significant effect on high production of lactic acid, when date juice was used as a sugar source.

Validation of the Model

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: date juice 25.0 g/l; sodium acetate: 15.0 g/l; peptone: 19.1 g/l, and K_2HPO_4: 4.7 g/l. The predicted response for lactic acid production was 23.0 g/l. Hence, the parameters given by the response surface optimization were used for confirmation of the predicted response of 23.0 g/l lactic acid. The organism produced 22.6 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 [17]. It has also been applied for the production of various enzymes, such as cyclodextrin glucanotransferase (CGTase) [6, 12], chitinase [7], α-amylase [21] and pectinase [15], and vitamin riboflavin [20]. RSM can also be useful in optimizing the enzyme reaction conditions [14].

Response surface methodology can be used to determine optimum concentration of significant medium components. In the present study, sodium acetate, peptone, and K_2HPO_4 were identified to be significant, and individual as well as interaction effects of these components were studied. Finally, a higher lactic acid production (50.33%) was achieved by using RSM.

REFERENCES

1. Abdeltif, A. 2000. Effect of inorganic phosphate on lactate production by *Lactobacillus helveticus* grown on supplemented whey permeate. *J. Chem. Technol. Biotechnol.* **75:** 223–228.
2. Chapin, A. 1993. Organization and regulation of genes for amino acid biosynthesis in lactic acid bacteria. *FEMS Microbiol. Rev.* **12:** 21–38.
3. Chauhan, K. H., U. B. Trivedi, and K. C. Patel. 2005. Statistical screening of medium components by Plackett-Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. *Biores. Technol.* (in press), DOI: 10.1016/j.biortech.2005.11.017.
4. Datta, R., S. Tsai, P. Bonsignore, S. H. Moon, and J. R. Frank. 1995. Technological and economic potential of poly (lactic acid) and lactic acid derivatives. *FEMS Microbiol. Rev.* **16:** 221–231.
5. Fitzpatrik, J. J. and U. O'Keeffe. 2001. Influence of whey protein hydrolysate addition to whey permeate batch fermentation for producing lactic acid. *Process Biochem*. **37:** 183–186.
6. Gawande, B. N. and A. Y. Patkar. 1999. Application of factorial design for optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae* AS-22. *Biotechnol. Bioeng*. **64:** 168–172.
7. Gohel, V., D. Jiwan, P. Vyas, and H. S. Chatpar. 2005. Statistical optimization of chitinase production by *Pantoea dispersa* to enhance degradation of crustacean chitin waste. *J. Microbiol. Biotechnol*. **15:** 197–201.
8. Greasham, R. L. 1983. Media for microbial fermentations, pp. 128–139. *In* Rehm, H. J., Read, P. A., and Stagler, P. (eds.). *Bioprocessing*, (Biotechnology, **Vol. 3**). VCH Publisher Inc., New York.
9. Haaland, P.D. 1989. Statistical problem solving, pp. 1–18. *In* Haaland, P. D. (ed.). *Experimental Design in Biotechnology.* Marcel Dekker, Inc., New York and Basel.
10. Kimberley, A. C. and C. Taylor. 1996. A simple colorimetric assay for muramic acid, lactic acid, glyceraldehyde, acetaldehyde and formaldehyde. *Appl. Biochem. Biotechnol.* **56:** 49–58.
11. Krishnan, S., S. G. Prapulla, D. Rajalakshmi, M. C. Mishra, and N. G. Karanth. 1998. Screening and selection of media components for lactic acid production using Plackett-Burman design. *Bioproc. Eng.* **19:** 61–65.
12. Mahat, M. K., R. M. Illias, R. A. Rahman, N. A. A. Rashid, N. A. N. Mahmood, O. Hassan, S. A. Aziz, and K. Kamaruddin. 2004. Production of cyclodextrin glucanotransferase (CGTase) from alkalophilic *Bacillus* sp. TSI-1: Media optimization using experimental design. *Enzyme Microb. Technol.* **35:** 467–473.
13. Miller, G. L. 1951. Use of dinitro salicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426–428.
14. Murthy, M. S. R. C., T. Swaminathan, S. K. Rakshit, and Y. Kosugi. 2000. Statistical optimization of lipase catalyzed hydrolysis of methyl oleate by response surface methodology. *Bioproc. Bioeng.* **22:** 35–39.
15. Nair, S. R. and T. Panda. 1997. Statistical optimization of medium components for improved synthesis of pectinase by *Aspergillus niger. Bioproc. Bioeng.* **16:** 169–173.
16. Nancib, A., N. Nancib, D. Meziane-Cherif, A. Boubendir, M. Fick, and J. Boudrant. 2005. Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by *Lactobacillus casei* subsp. *rhamnosus*. *Biores. Technol*. **96:** 63–67.
17. Naveena, B. J., Md. Altaf, K. Bhadriah, and G. Reddy. 2005. Selection of medium components by Plackett-Burman design for production of L(+) lactic acid by *Lactobacilus amylophilus* GV-6 in SSF using wheat bran. *Biores. Technol*. **96:** 485–490.
18. Peters, V. J. and E. E. Snell. 1954. Peptides and bacterial growth. *J. Bacteriol.* **67:** 69–76.
19. Plackett, R. L. and J. P. Burman. 1944. The design of optimum multifactorial 24 experiments. *Biometrika* **33:** 305–325.
20. Pujari, V. and T. S. Chandra. 2000. Statistical optimization of medium components for enhanced riboflavin production by a UV mutant of *Eremothecium ashbyii*. *Process Biochem.* **36:** 31–37.
21. Rao, J. L. U. M. and T. Satyanarayana. 2003. Statistical optimization of a high maltose-forming, hyper thermostable and Ca^{2+}-independent α-amylase by an extreme thermophile *Geobacillus thermoleovorans* using response surface methodology. *J. Appl. Microbiol.* **95:** 712–718.
22. Samuel, W. A. and Y. Y. Lee. 1980. Lactic acid fermentation of crude sorghum extract. *Biotechnol. Bioeng*. **22:** 757–777.