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Statistical analysis of cellulase production in *Bacillus amyloliquefaciens* UNPDV-22

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Abstract. The production of cellulase in *Bacillus amyloliquefaciens* UNPDV-22 was optimized using response surface methodology (RSM). Central composite design (CCD) was used to study the interactive effect of fermentation medium components (wheat bran, soybean meal, and malt dextrin) on cellulase activity. Results suggested that wheat bran, soybean meal, and malt dextrin all have significant impact on cellulase production. The use of RSM resulted in a 70% increase in the cellulase activity over the control of non-optimized basal medium. Optimum cellulase production of 11.23 U/mL was obtained in a fermentation medium containing wheat bran (1.03%, w/v), soybean meal (2.43%, w/v), and malt dextrin (2.95%, w/v).
Key Words: Cellulase production, *Bacillus amyloliquefaciens*, optimization, response surface methodology, central composite design

Rezumat. Producerea de celulază în *Bacillus amyloliquefaciens* UNPDV-22 a fost optimizată folosind metodologia suprafeţei de răspuns. Design-ul combinat central a fost folosit pentru a studia efectul interactiv al componentelor de fermentare din mediu (tărâţe de grîu, făină de soia şi dextrină din malţ) asupra activităţii celulazei. Rezultatele sugerează că tărâţele de grâu, făina de soia şi dextrină din malţ au toate un impact semnificativ asupra producţiei de celulază. Folosirea metodologiei suprafeţei de răspuns a determinat o creştere cu 70% a activităţii celulazei faţă de mediul bazal neoptimizat folosit ca şi control. Producţia optimă de celulază de 11.23 U/mL a fost obţinută într-un mediu de fermentaţie conţinând tărâţe de grâu (1.03%, greutate/volum), făină de soia (2.43%, greutate/volum) şi dextrină din malţ (2.95%, greutate/volum).
Cuvinte cheie: producerea de celulază, *Bacillus amyloliquefaciens*, optimizare, metodologia suprafeţei de răspuns, design-ul combinat central.

Introduction. Cellulose, the most abundant organic biopolymer on earth (Aygan & Arikan 2008), is commonly degraded by microorganisms through their cellulolytic enzyme system. Cellulases are produced by a number of microorganisms such as fungi and bacteria (Ng et al 2009; Paripok et al 2010) and find a wide application in the textile, food and animal feed industries, and more recently in the production of biofuels (Sukumaran et al 2005). Cellulase production depends on several process variables such as pH, temperature, carbon sources, nitrogen sources, substrate concentration, inoculum level, inducer sources, concentration, and aeration (Immanuel et al 2006). It is well known that the enzyme cost is one of the primary factors that determine the economics of an industrial process (Galbe et al 2007). Reduction of the enzyme production costs for the establishment of a viable industrial process can be achieved through optimization of the cultivation medium and fermentation conditions (Gigras et al 2002).

The conventional approach for determination of the optimal conditions for enzyme production is based on varying one parameter while keeping the others at constant levels. The major disadvantages of the single-variable optimization approach are: 1) it does not factor in the interaction effects among the variables; 2) it does not depict the net effect of the various medium constituents on the enzyme activity; and 3) it is time consuming and requires a number of experiments to determine the optimum levels. Therefore, for the reasons mentioned above, this method does not guarantee an accurate determination of optimal conditions. However, the above limitations can be overcome by

using statistical experimental design. While statistical analysis offers many tools for optimizing medium components, response surface methodology (RSM) is probably the most extensively used (Gao & Jiang 2005; Jeya et al 2009).

RSM is a collection of mathematical and statistical techniques for designing experiments, building models, searching optimum conditions of factors for desirable responses, and evaluating the relative significance of several affecting factors in the presence of complex interactions (Xu et al 2010). RSM can be used to determine the optimal production conditions and range of controllable variables, to generate a polynomial equation, and to estimate the relationships between controllable variables and observed results (Min et al 2007). For instance, RSM has been recently used for modeling and optimization of: fermentation media (Ruchi et al 2008); process conditions (Gao & Jiang 2005); enzyme-catalyzed reaction conditions (Ferella et al 2010); and production of biohydrogen (Ghosh & Hallenbeck 2010), lipase (Ruchi et al 2008), glucosidase (Kang et al 1999), γ- polyglutamic acid (Shi et al 2006), and extracellular polysaccharides (Hsieh et al 2005). Using RSM, the cellulolytic enzyme production from various microorganisms such as *Scytalidium thermophilum* (Jatinder et al 2006), *Trichoderma reesei* (Hao et al 2006), *Penicillium waksmanii* (Han et al 2009); *Aspergillus heteromorphus* (Singh et al 2009)*, Debaryomyces pseudopolymorphus* (Barbosa et al 2010) has been studied and optimized. Here we report on the optimization of cellulase production in *Bacillus amyloliquefaciens* UNPDV-22 using RSM. Although numerous strains of *Bacillus* species producing cellulase activity have been described in literature (Crispen et al 2000; Femi-Ola & Aderibigbe 2008; Odeniyi et al 2009; Tabao & Monsalud 2010), to date there appear to have been no prior literature studies on the use RSM for optimization of cellulase production in *Bacillus*.

Here we report on the RSM-aided optimization of selected medium components (wheat bran, soybean meal, and malt dextrin) during cellulase production in *B. amyloliquefaciens* UNPDV-22.

Materials and Methods. *Microorganism, media and culture conditions, cellulase production. Bacillus amyloliquefaciens* UNPDV-22 was isolated from water samples collected from a natural hot spring in Unapdev, India. Screening of this isolate for cellulase production was carried out on selective agar plates containing (w/v): 0.2% carboxymethyl cellulose (CMC), 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.02% peptone, and 3% agar. The agar plates were inoculated with pure cultures, isolated from the hot spring water samples, and incubated at 40^0C for 48 h. After incubation, the plates were flooded with 0.1% Congo red solution, kept for 10 min at room temperature, and then washed with 0.1M NaCl. Isolates showing larger clearing zone around the grown cells were selected for further identification and optimization studies. The UNPDV-22 isolate was characterized as a gram positive bacterium and identified as *B. amylolequifaciens* on the basis of its 16S ribosomal DNA sequencing. A comparison of the DNA sequence with sequences in the National Center for Biotechnology Information (NCBI) database with BLAST software (Altschul et al 1997) showed a 98% sequence identity with the 16S rRNA sequences of *B. amylolequifaciens* BFE 5359 (NCBI GenBank database, accession number GU250449). The 16S ribosomal DNA sequence of the isolate was deposited in GenBank database with accession number HQ005305. The stock culture of the isolate was maintained on nutrient agar at 4^0C.

A seed culture of *B. amyloliquefaciens* UNPDV-22 was developed in a basal medium containing 1% wheat bran, 0.5% soybean meal, 1.5% malt dextrin, and 5% (v/v) trace element solution (0.5% $FeSO_4$, 0.01% $MgSO_4$, and 0.05% $ZnSO_4$). Wheat bran was collected from a local market in Nashik, India whereas soybean meal, malt dextrin, and dinitrosalicylic acid were procured from Sigma Chemicals (St. Louis, MO, USA). Incubations to produce seed culture inoculum of *B. amyloliquefaciens* were carried out in shake flaks at 40^0C, pH 6 and 250 rpm for 24 h. Cellulase production in *B. amylolequifaciens* UNPDV-22 was carried out with 1% inoculum for 48 h using the basal medium and conditions described above. Following fermentation, samples were taken from the fermentation broth and analyzed for cellulase activity.

Cellulase assay. Cellulase activity was assayed in a 1.5 ml reaction mixture containing 0.5 ml of diluted enzyme solution and 1 ml of 2% CMC suspension in 0.05 M citrate buffer (pH 6). The reaction mixture was incubated at 45^0C for 20 min. Following incubation, the reaction was stopped by addition of 3 ml of dinitrosalicylic acid and boiling for 10 min. After cooling, the reaction mixture was diluted with 10 ml of distilled water and the optical density was measured spectrophotometrically at 540 nm using glucose as standard (Miller 1959). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per min under the assay conditions. Each value represented the average of triplicate determinations ± standard deviation (SD).

Table 1

Experimental variables used for optimization of cellulase production in *B. amyloliquefaciens* UNPDV-22 with response surface methodology

Variables	Levels (Coded and Actual)					
	Code	-1.68	-1	0	1	1.68
Wheat bran (%)	X_1	0.32	1.00	2.00	3.00	3.68
Soybean meal (%)	X_2	0.32	1.00	2.00	3.00	3.68
Malt dextrin (%)	X_3	0.32	1.00	2.00	3.00	3.68

Table 2

Central composite design with experimental and predicted values for cellulase production in *B. amyloliquefaciens* UNPDV-22

Run No.	Variables			Cellulase activity (U/ml)	
	[a]X_1	[b]X_2	[c]X_3	Experimental	Predicted
1	-1	-1	-1	10.25	10.4
2	1	-1	-1	9.82	9.76
3	-1	1	-1	10.80	10.89
4	1	1	-1	10.24	10.37
5	-1	-1	1	10.77	10.79
6	1	-1	1	10.03	10.09
7	-1	1	1	10.87	11.08
8	1	1	1	10.50	10.50
9	-1.68	0	0	11.23	11.02
10	1.68	0	0	10.00	10.00
11	0	-1.68	0	9.96	9.93
12	0	1.68	0	10.86	10.68
13	0	0	-1.68	10.62	10.50
14	0	0	1.68	11.02	10.93
15	0	0	0	10.90	10.91
16	0	0	0	10.85	10.91
17	0	0	0	10.88	10.91
18	0	0	0	10.93	10.91
19	0	0	0	10.91	10.91
20	0	0	0	10.95	10.91

[a]coded value of wheat bran; [b]coded value of soybean meal; [c]coded value of malt dextrin

Experimental design. RSM was used (Anderson & Whitcomb 2007) to optimize selected parameters (experimental variables; Table 1) influencing cellulase production in *B. amyloliquefaciens*: carbon/nitrogen source (wheat bran, soybean meal, and malt dextrin). The experimental variables were used at different levels for a total of 20 runs applying central composite design (CCD; Table 2).

The experimental design was carried out using Design Expert 7.1.5 (Stat Ease, MN, USA). The CCD was used to identify the optimum operating conditions in order to obtain maximum cellulase production (*y*) as response. The collection of experiments provides an effective means for optimization through process variables. The CCD permits the estimation of all main and interaction effects whereas the purpose of the center points is to estimate the pure error and curvature (Techapun et al 2002). A second-order quadratic polynomial model for three factors can be used to represent the function in the range of interest as shown in Eq. 1.

$$y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} \sum_{j=i}^{3} a_i X_{ij} \quad \text{.....(Eq. 1)}$$

where *y* is the predicted response (cellulase production) used as a dependent variable; *Xi* (*i*=1,2 and 3) are the input predictors or controlling variables; and a_0, a_i (*i*=1,2,3) and a_{ij} (*i*=1,2,3; *j*=*i*, ….., 3) are the model coefficient parameters. The coefficients were estimated by multiple linear regression analysis using the method of least squares (Tanyildizi et al 2005). The experimental data were empirically fitted using polynomial regression based on analysis of variance (ANOVA) (Min et al 2007) to create an empirical model that relates the measured response to the independent variables of the experiment (Rashid et al 2009).

Results and Discussion. Under the non-optimized medium described in "Materials and methods", *B. amylolequifaciens* UNPDV-22 produced 6.62 U/ml of cellulase activity after 48 h of incubation (data not shown). Preliminary experiments with *B. amylolequifaciens* UNPDV-22 indicated that wheat bran, soybean meal, and malt dextrin as the carbon/nitrogen source in the basal médium had the most profound impact on cellulase activity (data not shown). Hence, these factors were selected as independent variables and their effects on cellulase production were studied using CCD of RSM. The results from the CCD experiments on the effects of the independent variables along with the predicted and observed responses of cellulase activity are shown in Table 2. The response equation obtained (Eq. 2) for the critical medium components were as follows:

$Y = 10.91 - 0.31X_1 + 0.22X_2 + 0.13X_3 + 0.03X_{12} - 0.015X_{13} - 0.05X_{23} - 0.14X_{11} - 0.21X_{22} - 0.06X_{33}$ …….Eq. 2)

where *Y* is the predicted cellulase activity for critical medium components; X_1 - the coded value of wheat bran; X_2 - the coded value of soybean meal; and X_3 - the coded value of malt dextrin. The statistical significance of Eq. (2) was controlled by the F- and p-values (Table 3).

Table 3

Analysis of variance (ANOVA) for the response surface quadratic model of selected fermentation medium components (wheat bran, soybean meal, and malt dextrin) used in optimization of cellulase production in *B. amyloliquefaciens* UNPDV-22

Source	Coefficient	F-value	P-value (Prob>F)
Model	10.91	16.82	< 0.001*
X_1	-0.31	62.24	< 0.001*
X_2	0.22	33.40	< 0.001*
X_3	0.13	10.75	0.008*
X_{12}	0.03	0.35	0.566
X_{13}	-0.01	0.08	0.772
X_{23}	-0.05	0.98	0.346
X_1^2	-0.14	14.12	0.003*
X_2^2	-0.21	32.28	< 0.001*
X_3^2	-0.06	3.36	0.096

*Significant variable; Determination coefficient (R^2), 0.93; Adjusted determination coefficient (R^2_{Adj}), 0.88; Coefficient of variation (CV), 1.35%; Adequate precision ratio, 12.97

The significant response for coefficients was found to be mainly dependant on the F-value and the resultant low p-value. Therefore, the greater the F-value and lower the p-value,

the more significant the corresponding coefficient. The Fisher F-test (Long et al 2009) with a very low probability value demonstrated a very high significance for the regression model. The goodness of model fit was verified by the determination coefficient (R^2= 0.93) which indicated that only 7% of the total variations could not be explained by the model. The value of the adjusted determination coefficient was also high (R^2_{Adj} = 0.88), thus suggesting a high significance (p-value < 0.01) of the model. The improved precision and reliability of the conducted experiments was evident from the relatively low value of the coefficient of variation (CV=1.35%; Table 3). Furthermore, adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable (Lu et al 2009). In this work, a ratio of 12.97 was obtained, which indicates an adequate signal (Table 3). Therefore, our model can be used to navigate the design space.

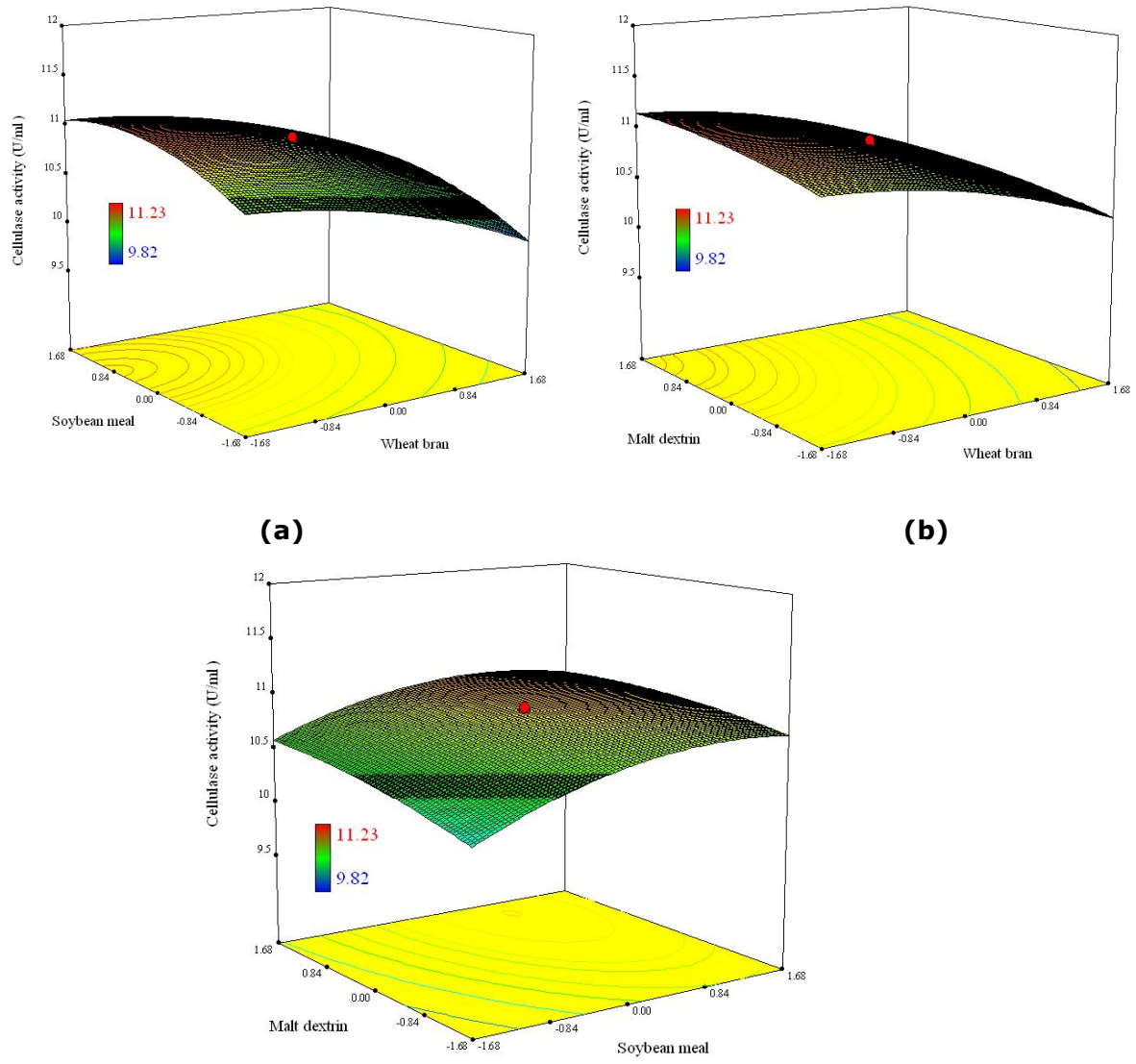

Figure 1. Response surface plots, described by Eq. 2, representing the effect of wheat bran and soybean meal concentrations (a), wheat bran and malt dextrin concentrations (b), soybean meal and malt dextrin concentrations (c), and their mutual effects on cellulase activity of *B. amyloliquefaciens* UNPDV-22.

The counter plot for response surfaces are plotted in Figures 1a-c which depicts the combined effects of wheat bran and soybean meal, wheat bran and malt dextrin, soybean meal and malt dextrin, respectively. The response surfaces obtained suggested that cellulase production could be enhanced by lowering the wheat bran concentration

(Figures 1a,b) and increasing the concentrations of soybean meal (Figure 1a) and malt dextrin (Figure 1b). Figure 1c revealed that soybean meal, at a concentration of 2.43%, and malt dextrin, at 2.95%, produced a significant effect on the cellulase activity. Hence, based on the RSM model from Eq. 2, the recommended concentrations of the fermentation medium components to attain a predicted optimum cellulase activity of 11.23 U/ml were: 1.03% wheat bran; 2.43% soybean meal; and 2.95% malt dextrin (Figure 1). At the same time, the central points at zero level (2% wheat bran; 2% soybean meal; 2% malt dextrin) indicated a cellulase activity of 10.91 U/ml (Table 2), which represents a deviation of only 3% from the predicted optimum cellulase activity (11.23 U/ml). Thus, the RSM model showed a satisfactory performance and offered a stable response in predicting the combined interactions of the three independent variables (wheat bran, soybean meal, and malt dextrin) with respect to the levels of extracellular cellulase production attained.

Conclusions. In this work, it was demonstrated for first time that cellulase production in *Bacillus* sp. can be successfully optimized using statistical methods such as RSM. A reliable RSM model for predicting the optimum cellulase activity was obtained which deviated only 1-3% from the activity at the zero level central points of selected fermentation medium (wheat bran, soybean meal, malt dextrin) parameters. The optimization of these parameters with RSM led to a 70% increase in the cellulase production. In comparison to the single-variable optimization approach, the use of RSM provides a more accurate determination of the parameters under optimization and reduces the time and number of experiments required.

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