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Optimization of Culture Conditions for Production of Cellulase by a Thermophilic *Bacillus* Strain

Zambare Vasudeo* and Christopher Lew

Center for Bioprocessing Research and Development, South Dakota School of Mines and Technology, Rapid City SD 57701, USA

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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 culture conditions (temperature, pH, and inoculum) on cellulase activity. Results suggested that temperature and pH all have significant impact on cellulase production. The use of RSM resulted in a 96% increase in the cellulase activity over the control of non-optimized basal medium. Optimum cellulase production of 13 U/mL was obtained at a temperature of 42.24 °C, pH of 5.25, and inoculum size of 4.95% (v/v) in a fermentation medium containing wheat bran, soybean meal and malt dextrin as major nutritional factors.

Key words: Cellulase production, *Bacillus amyloliquefaciens*, culture conditions, response surface methodology, central composite design.

1. Introduction

Cellulose, the most important complex polysaccharide produced by plants and abundantly available on earth [1], is commonly degraded by microorganisms through their cellulolytic enzyme system. Cellulases are produced by a number of microorganisms such as fungi and bacteria [2] and find a wide application in the textile, food and animal feed industries, and more recently in the production of biofuels [3]. Cellulase production depends on several process variables such as pH, temperature, carbon sources, nitrogen sources, substrate concentration, inoculum level, inducer sources, concentration, and aeration [4]. It is well known that the enzyme cost is one of the primary factors that determine the economics of an industrial process [5]. 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 [6].

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 [7, 8].

RSM is a collection of mathematical and statistical techniques for designing experiments, building models, searching optimum conditions of factors for

*Corresponding author: Zambare Vasudeo, research scientist, PhD, research fields: extremophiles, biofuels, microbial enzymes. E-mail:Vasudeo.Zambare@sdsmt.edu.

desirable responses, and evaluating the relative significance of several affecting factors in the presence of complex interactions [9]. 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 [10]. For instance, RSM has been recently used for modeling and optimization of: process conditions [11]; enzyme-catalyzed reaction conditions [12]; adsorbents-aided dye removal from waste waters [13]; and production of enzymes like lipase [14], glucosidase [15], and extracellular polysaccharides [16]. Using RSM, the cellulolytic enzyme production from various microorganisms such as *Scytalidium thermophilum* [17], *Aspergillus heteromorphus* [18], *Debaryomyces pseudopolymorphus* [19] 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 Refs. [20-22], 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 culture conditions (temperature, pH, and inoculum) during cellulase production in *B. amyloliquefaciens* UNPDV-22.

2. Experiment

2.1 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. The stock culture of the isolate was maintained on nutrient agar at 4 °C. 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 °C, pH 6 and 250 rpm for 24 h. Furthermore major nutritional parameters were optimized and the above basal medium with different concentration of wheat bran (1.03%, w/v), soybean meal (2.43%, w/v), and malt dextrin (2.95%, w/v) were used. Cellulase production in *B. amylolequifaciens* UNPDV-22 was carried out with 1% inoculum for 72 h using the basal medium and conditions described above. Following fermentation, samples were taken from the fermentation broth and analyzed for cellulase activity.

2.2 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 °C 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 [23]. 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).

2.3 Experimental Design

RSM was used [24] to optimize selected cultural parameters (experimental variables; Table 1) influencing cellulase production in *B. amyloliquefaciens*. The experimental variables were used at different levels for a total of 20 runs applying central composite design (CCD; Table 2).

Table 1 Experimental variables used for optimization of cellulase production in *B. amyloliquefaciens* UNPDV-22 with response surface methodology (central composite design).

Variables	Code	Levels (Coded and Actual)				
		-1.68	-1	0	1	1.68
Temperature (°C)	X_1	33.18	40.00	50.00	60.00	66.82
pH	X_2	3.99	4.50	5.25	6.00	6.51
Inoculum (%)	X_3	0.98	2.00	3.00	5.00	6.02

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	12.41	12.61
2	1	-1	-1	11.66	11.91
3	-1	1	-1	9.51	10.35
4	1	1	-1	10.01	10.36
5	-1	-1	1	12.31	12.42
6	1	-1	1	10.76	10.37
7	-1	1	1	12.31	12.51
8	1	1	1	10.91	11.17
9	-1.68	0	0	12.51	11.92
10	1.68	0	0	10.26	10.20
11	0	-1.68	0	11.76	11.88
12	0	1.68	0	11.41	10.65
13	0	0	-1.68	12.76	12.00
14	0	0	1.68	12.41	12.52
15	0	0	0	12.61	12.63
16	0	0	0	12.61	12.63
17	0	0	0	12.61	12.63
18	0	0	0	12.61	12.63
19	0	0	0	12.61	12.63
20	0	0	`0	12.61	12.63

[a]coded value of temperature; [b]coded value of pH; [c]coded value of inoculums.

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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 [25]. 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.

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

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 X_{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 [26]. The experimental data were

empirically fitted using polynomial regression based on analysis of variance (ANOVA) [10] to create an empirical model that relates the measured response to the independent variables of the experiment [27].

3. Results and Discussion

Optimization of Fermentation Culture Conditions

Under the non-optimized medium and process conditions described in "Materials and methods", *B. amylolequifaciens* UNPDV-22 produced 8.62 U/mL of cellulase activity after 48 h of incubation (data not shown). The response equation for the culture conditions (pH, temperature, and inoculums) obtained (Eq. 2) was as follows:

$$Y = 12.63 - 0.5X_1 - 0.37X_2 + 0.15X_3 + 0.18X_{12} - 0.34X_{13} + 0.59X_{23} - 0.55X_{11} - 0.48X_{22} - 0.13X_{33} \quad (2)$$

where Y is the predicted cellulase activity for process parameters, X_1 - the coded value of temperature; X_2 - the coded value of pH; and X_3 - the coded value of inoculum. The statistical significance of Eq. 2 was controlled by the F-value (6.80) and p-value of 0.003 (Table 3). The Fisher F-test with a very low probability value ($P_{model} > F = 0.003$) demonstrates a very high significance for the regression model. As previously observed in the optimization of the fermentation medium, the increase in the magnitude of the F-value and the decrease in the p-value resulted in a more significant corresponding coefficient. The CCD of the response surface method was used to obtain data that fit a full second order quadratic model. The Fisher F-test with a very low probability value demonstrated a very high significance for the regression model. The goodness of fit of the model was confirmed by the determination coefficient (R^2). In this case, the value of the coefficient of determination ($R^2 = 0.85$) indicated that only 15% of the total variations could not explained by the model. The value of the adjusted coefficient of determination ($R^2_{Adj} = 0.73$) was also high and thus indicative of the high significance (p-value<0.01) of the model. The relatively lower value of the coefficient of variation (CV= 4.42%) suggests improved precision and reliability of the conducted experiments whereas the adequate precision ratio of 6.55 indicates an adequate signal (Table 3).

Table 3 Analysis of variance (ANOVA) for the response surface quadratic model of the selected fermentation process parameters (temperature, pH, and inoculums) used in optimization of cellulase production in *B. amyloliquefaciens* UNPDV-22.

Source	Coefficient	F-value	P-value (Prob>F)
Model	12.63	6.80	0.003*
X'_1	-0.51	13.05	0.004*
X'_2	-0.37	6.66	0.027*
X'_3	0.15	1.19	0.300
X'_{12}	0.18	0.90	0.366
X'_{13}	-0.34	3.33	0.098
X'_{23}	0.59	10.09	0.009*
X'^2_1	-0.55	16.14	0.002*
X'^2_2	-0.48	12.28	0.005*
X'^2_3	-0.13	0.88	0.370

*Significant variable; Coefficient of determination (R^2), 0.85; Adjusted determination coefficient (R^2_{Adj}), 0.73; Coefficient of variation (CV), 4.42%; Adequate precision ratio, 6.55.

The counter plot for response surfaces, displayed in Figs.1a-c, represented the combined effects of: temperature and pH (Fig. 1a); temperature and inoculum (Fig. 1b); pH and inoculum (Fig. 1c). The response surfaces obtained suggested that optimum cellulase production by *B. amylolequifaciens* UNPDV-22 would be obtained at a temperature of 42.24 °C, pH of 5.25, and 4.95% inoculum concentration. A significant variation in the cellulase activity at pH higher than 5.25 and inoculum concentration lower than 4.95% is evident from Fig. 1c. The RSM model (Eq. 2) predicted an optimum cellulase activity of 12.76 U/mL under the following fermentation parameters: temperature of 42.24 °C; pH of 5.25; and inoculum of 4.95%. The predicted cellulase activity from the RSM model deviated only 1% from the predicted cellulase activity of 12.61 U/mL from the central points at zero level (50 °C; pH 5.25; 3 % inoculum). This verifies the fitness of the RSM model in predicting the combined interactions of the three independent variables (temperature, pH, and inoculum) on the cellulase activity.

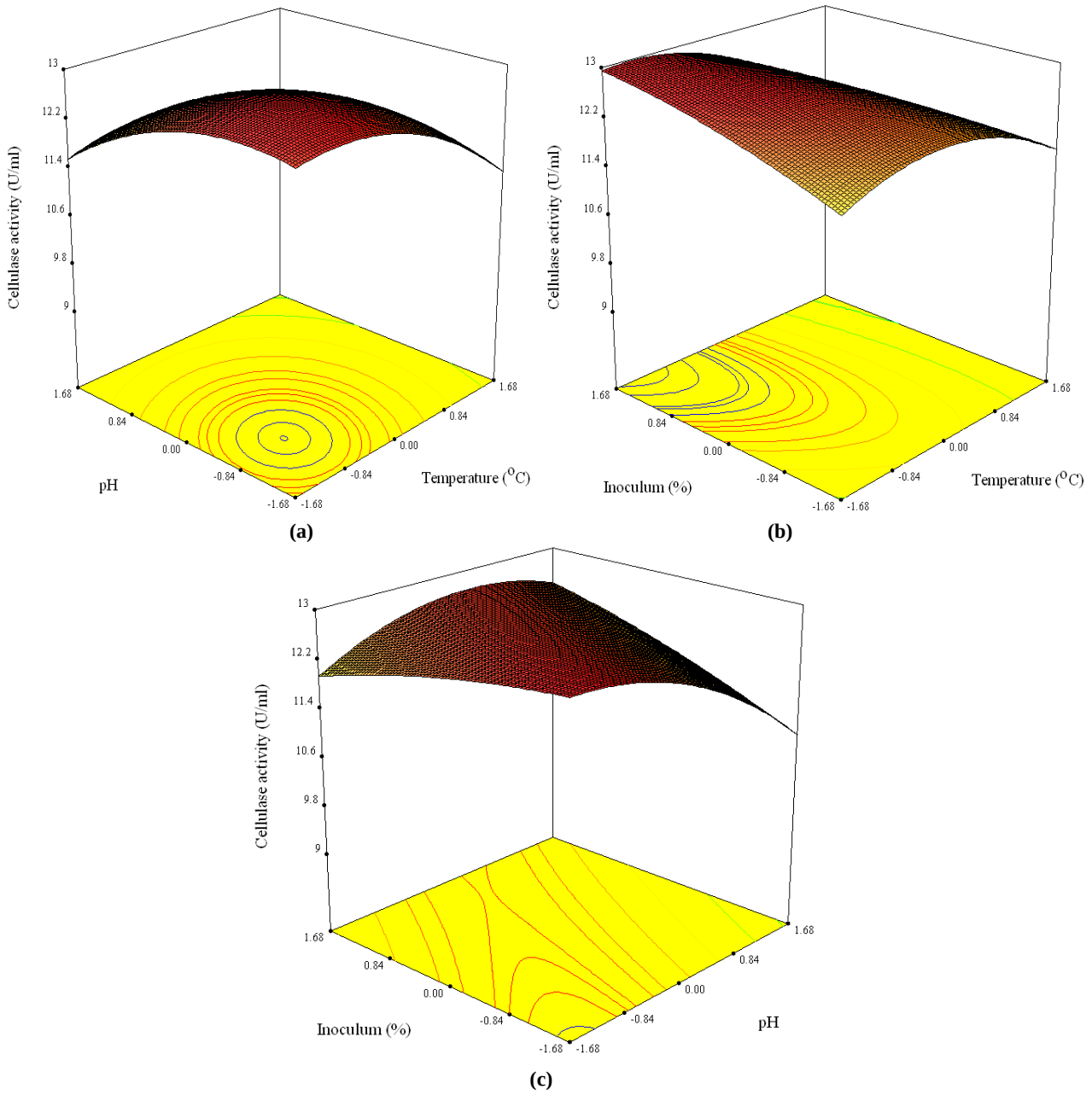

Fig. 1 Response surface plots, described by Eq. 2, representing the effect of temperature and pH (a), temperature and inoculum (b), pH and inoculum (c), and their mutual effects on cellulase activity of *B. amyloliquefaciens* UNPDV-22.

The cellulase production in *B. amyloliquefaciens* UNPDV-22 before and after optimization using RSM is illustrated in Fig. 2. This comparison reveals that after optimization, the cellulase production on the basal medium (6.62 U/mL) increased 96% to reach 13 U/mL. To the best our knowledge, the data presented here are the first report on statistical optimization of cellulase production in *Bacillus* sp.

4. Conclusion

Response surface methodology was applied for the optimization of culture conditions for the production of cellulase. The model developed for CCD had R^2 values of 0.85 for cellulase production. The optimum values obtained by substituting the respective coded values of variables are: 42.24 °C-temperature, 5.25-pH, and 4.95%-inoculum. The regression model fitted for the

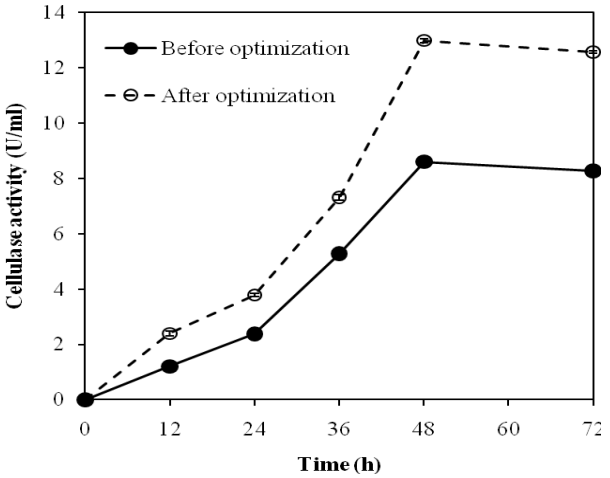

Fig. 2 Cellulase production in *B. amyloliquefaciens* UNPDV-22 before and after optimization of selected fermentation medium components (wheat bran, soybean meal, and malt dextrin) and process parameters (temperature, pH, and inoculum) using response surface methodology. Data represent the average of triplicate determinations ± standard deviation (SD).

present CCD predicts that the maximum concentration of cellulase can be obtained using the optimal concentrations of three test variables calculated previously is 6.62U/mL. The analysis of the data shows that optimized values of culture conditions give 96% more cellulase production (13 U/mL) in comparison with the unoptimized conditions.

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