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Optimization of Process Parameters for Cellulase Production from *Bacillus sp.* JS14 in Solid Substrate Fermentation Using Response Surface Methodology

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ABSTRACT

The aim of this work was to isolate the potent bacterial strains for the production of cellulose enzyme. A total 30 bacterial isolates showed positive results for the cellulase production but highest enzyme activity was shown by isolate JS 14. From the morphological and biochemical reactions, the isolate was identified as Bacillus sp. Cellulase production was studied by this strain using response surface methodology (RSM). A central composite design (CCD) quadratic response surface was applied to explicate the parameters that significantly affected cellulase production in solid substrate fermentation (SSF). The wheat bran concentration and incubation period were significant factors. The process parameters optimized with response surface methodology was wheat bran concentration 400 g/L; pH, 6.5; temperature, 400C and incubation period 5 days when inoculum 10 % (1x107 cells/ ml) was used for cellulase production in SSF. Supplementation of lactose and CMC to the wheat bran medium favored the enzyme formation.

Key words: Cellulase, response surface methodology, optimization

INTRODUCTION

Cellulosic material is the most abundant renewable carbon source in the world. Cellulose may be hydrolyzed using enzymes to produce glucose, which can be used for the production of ethanol, organic acids and other chemicals. Cellulase (E.C 3.2.1.4) refers to a class of enzyme that catalyze the hydrolysis of 1, 4 β-D glycosidic linkages in cellulose are mainly produced by fungi, bacteria and protozoans (Beguin and Aubert 1994) and have broad range of industrial and commercial applications (Bhat 2000; Adsul et al. 2007; Kaur et al. 2007). An important obstruction in the exploitation of cellulase is expensive production affecting the overall cost of hydrolysis (Chahal et al. 1992; Duff and Murray 1996; Reczey et al. 1996; Nieves et al. 1998). Considerable progress has been made for high cellulase production by optimization of best possible fermentation conditions for the development of economically feasible bioprocess. Optimization of growth and product conditions by classical methods, which involves the change of one variable at a time, is extremely time consuming and expensive. Combinational interactions of medium components for the production of desired compound are numerous and the optimum processes may be developed using an effective experimental design procedure. Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments,

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building models, evaluating the effects of factors and searching for the optimum conditions, has successfully been used in the optimization of bioprocesses (Dey et al. 2001; Wejse et al. 2003; Kristo et al. 2003; Chen et al. 2005). The objective of this work was to isolate an efficient cellulase producing microorganism and produce the enzyme in SSF.

MATERIALS AND METHODS

Isolation screening and identification of microorganisms

Cellulase producing bacteria were isolated from the soils, decomposing logs and composts collected from the Fatehgarh Sahib Punjab (India) by the spread plate techniques using CMC agar media. The plates were incubated at 37^0C for 24 h. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 M NaCl (Ariffin et al. 2008). To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on CMC agar were measured. Besides the cellulase activity of the selected bacterial isolate in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugar liberated by using a dinitrosalicylic acid (DNS) method (Miller 1959). The bacterial isolates with high enzyme production were identified by means of the morphological and biochemical characterizations. The parameters investigated included colonial morphology, Gram staining, catalase production, starch hydrolysis and nitrate reduction test. The results were compared with Bergey's Manual of Determinative Bacteria (Buchanan and Gibbons 1974).

Experimental design and optimization of process parameters for cellulase production

A central composite design (CCD) was used to pick the factors that influence cellulase production significantly with software Design expert 8.01. In CCD, the range and the levels of the variables investigated in this study are given in Table 1. The central values (zero level) chosen for the experimental design were; wheat bran concentration (X1) 35%; pH (X2) 6.25; temperatures (X3) 35^0C and incubation time (X4) 5.5 days. Different combination of variables of wheat bran and pH was adjusted according to the design (Table 2) in the alkaline soya casein medium. Inoculation was carried with 10% (1×10^7 cells/mL) inoculum and incubated at various temperature and time combinations, according to RSM design (Table 2). Low and high factor settings were coded as –1 and 1, the midpoint was coded as 0. The factor settings of trails that ran along the axes drawn from the middle of the cube through the centers of each face of the tube were coded as 1.414 or –1.414. The Design expert 8.0.1 software, was used for regression and graphical analyses of the data obtained. The optimal concentrations of the critical medium components were obtained by the ridge analysis and also by analyzing the contour plots. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The enzyme was extracted by phosphate buffer (0.1M and pH 6.5) from the solid medium on rotary shaker at 200 rpm by filtration and enzyme activity in filtrate was determined.

Table 1 - Factors involved in RSM for optimization of cellulose production.

Factors and Codes value (X)	Value (-1)	Value (0)	Value (+1)
Wheat Bran % (X1)	30	35	40
pH (X2)	5	6.25	7.5
Temperature (°C) (X3)	30	35	40
Incubation Period in days (X4)	2	5.5	9

Effect of inoculum size on cellulase production

Inoculum concentration of *Bacillus* sp. JS14 was varied from 5-20% (1×10^7 cells /mL) in different batches of 200 mL of the sterilized alkaline soya casein medium containing what bran 40% pH 6.5 and Incubation was carried out at 40 ^{0}C for 5 days. Enzyme was harvested by filtration method and enzyme activities was determined

Determination of cellulase activity

CMCase activity assay was carried out according to the methods developed by Mandels, (1985).

Reducing sugar was measured by the DNS method using glucose as the standard (Miller 1959). In this study, one international unit (IU) of enzyme activity was defined as the amount of enzyme that liberated one μmol of glucose per minute under the specified conditions from the appropriate substrate.

RESULTS AND DISCUSSION

Isolation, screening and identification of cellulase producing microorganisms

Total 30 bacterial isolates when applied the Congo red test, showed positive results with clear zone ranging from 1 to 7 mm (data not shown). Upon further quantitative determination of cellulose degrading enzyme, all the isolates displayed activity of cellulase with the highest enzyme activity from the isolate JS14. Although the Congo red test was sensitive enough for primary isolation and screening of cellulytic bacteria, but the clear zone width did not indicate the amount of cellulase activity, Krootdilaganandh, (2000) reported that bacterial isolates CMU4-4 grown on CMC agar, exhibited the highest enzyme activity in the liquid medium whereas its clear zone was smaller than other isolates. The isolate JS14 showed white colonies on CMC agar. A microscopic examination of the isolate revealed that it was a Gram positive bacterium and produced enzyme catalase. Furthermore, the JS14 displayed starch and nitrate reduction test. From these morphological and biochemical characterization, the isolate was identified as *Bacillus* sp.

Optimization of process parameters in SSF by Response surface methodology (RSM) for cellulase production

The concentration of wheat bran (X1 400 g/L), pH (X2 6.5), temperature (X3 40 ^{0}C) and incubation period (X4 5days) in solid substrate were chosen as optimum for cellulase production by SSF. Table 2 shows the design and the results of this experiment.

Table 2 - A central composite designs for cellulase enzyme production using RSM.

S.No	Values of factors (X)				Cellulase activity
	X1	X2	X3	X4	(IU/L)
1.	35	5.50	30	5.50	**540**
2.	35	6.50	30	9	**420**
3.	35	7.50	35	2	**210**
4.	**40**	**6.50**	**40**	**5**	**2040**
5.	35	5.50	40	5.50	**1070**
6.	35	6.50	40	9	**520**
7.	30	6.50	35	2	**130**
8.	30	6.50	30	5.50	**590**
9.	35	7.50	40	5.50	**2010**
10.	40	6.50	35	9	**460**
11.	35	5.50	35	9	**290**
12.	40	5.50	35	5.50	**1030**
13.	30	7.50	35	5.50	**650**
14.	35	6.50	35	5.50	**1330**
15.	35	6.50	30	2	**230**
16.	35	6.50	35	5.50	**1330**
17.	35	6.50	35	5.50	**1330**
18.	30	6.50	40	5.50	**1080**
19.	40	6.50	35	2	**290**
20.	35	6.50	40	2	**250**
21.	35	7.50	35	9	**330**
22.	35	6.50	35	5.50	**1330**
23.	30	6.50	35	9	**280**
24.	40	6.50	30	5.50	**1550**
25.	35	7.50	30	5.50	**200**
26.	35	5.50	35	2	**170**
27.	35	6.50	35	5.50	**1330**
28.	40	7.50	35	5.50	**1490**
29.	30	5.50	35	5.50	**780**
30.	40	7.50	33	5.45	**1250**

Regression analysis was performed to fit the response function with the experimental data. The statistical significance of the second order model equation was checked by an F-test (ANOVA) and the data were shown in Table 3.The regression model for cellulase production was highly significant ($p<0.003$) with a satisfactory value of determination coefficient ($R2=0.95$), indicating that 95 % of the variability in the response could be explained by the second-order model equation given as below:

$$y=284.09494+37.31286X_1+225.23766X_2-43.4723X_3+790.58161X_4+28.95995X_1X_2+0.043204X_1X_3+0.28792X_1X_4+64.21602X_2X_3+0.011021X_2X_4+1.14198X_3X_4-2.47136X_1^2-261.78389X_2^2$$

where y is the measured response, and X1, X2, X3 and X4 are coded independent variables. The Model F-value of 8.16 implied the model was significant (Table 3). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated that the model terms were significant. The "Pred R-Squared" of 0.7664 was as close to the "Adj R-Squared" of 0.7757 as one might normally expect (Table 4). "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 9.309 indicated an adequate signal. This model could be used to navigate the design space.

Table 3 - ANOVA for Response Surface Quadratic Model Analysis of variance table [Partial sum of squares - Type III].

Source*		Sum of Squares	Df	Mean Square	F Value	P value Prob > F
Model		8.405E+006	14	6.004E+005	8.16	0.0001
X1		6.060E+005	1	6.060E+005	8.24	0.0117
X2		3.812E+005	1	3.812E+005	5.18	0.0379
X3		4.427E+005	1	4.s427E+005	6.02	0.0269
X4		67779.73	1	67779.73	0.92	0.3523
X1	***X2***	98643.63	1	98643.63	1.34	0.2650
X1	***X3***	321.72	1	321.72	4.374E-003	0.9481
X1	***X4***	75.80	1	75.80	1.030E-003	0.9748
X2	***X3***	4.225E+005	1	4.225E+005	5.74	0.0300
X2X4		6.007E-003	1	6.007E-003	8.166E-008	0.9998
X3X4		450.17	1	450.17	6.120E-003	0.9387
$X1^2$		23311.90	1	23311.90	0.32	0.5818
$X2^2$		4.621E+005	1	4.621E+005	6.28	0.0242
$X3^2$		11264.03	1	11264.03	0.15	0.7011
$X4^2$		5.386E+006	1	5.386E+006	73.22	< 0.0001
Residual		1.103E+006	15	73559.26		
Lack of Fit		1.103E+006	11	1.003E+005		
Pure Error		0.000	4	0.000		
Cor Total		9.509E+006	29			

*X1 Concentration of wheat bran (%), X2 pH, X3 Temperature (^{0}C) and X4 incubation period (days).

Table 4 - ANOVA results for cellulase production obtained from CCD.

S.NO	Parametr	Value
1.	Std. Dev	271.22
2.	Mean	817.00
3.	Adj R-Squared	0.7757
4.	C.V. %	33.20
5.	R-Squared	0.8840
6.	Pred R-Squared	0.7664
7.	Adeq Precision	9.309

The 3D response surface based on the dependent variables such as wheat bran concentration, pH, temperature and incubation period are shown in Figure 1. The canonical analysis revealed that maximum CMCase activity of 2040 IU/L was achieved at wheat bran concentration 400 g/L; pH 6.5; temperature 40 ^{0}C and incubation period of five days.

Effect of inoculum size on cellulase production

Lower inoculum size requires longer time for the cells to multiply to sufficient number to utilize the substrate and produce enzyme. An increase in the number of cells in the inoculums would ensure a rapid proliferation and biomass synthesis. When inoculum size was increased from 5 to 10% there was increase in enzyme production but after that the activity was decreased (Fig. 2) due to depletion of nutrients by the enhanced biomass, which resulted dwindle in metabolic activity (Kashyap et al. 2002). A balance between the increasing biomass and accessible nutrient would yield an optimal enzyme production (Ramachandran et al. 2004).

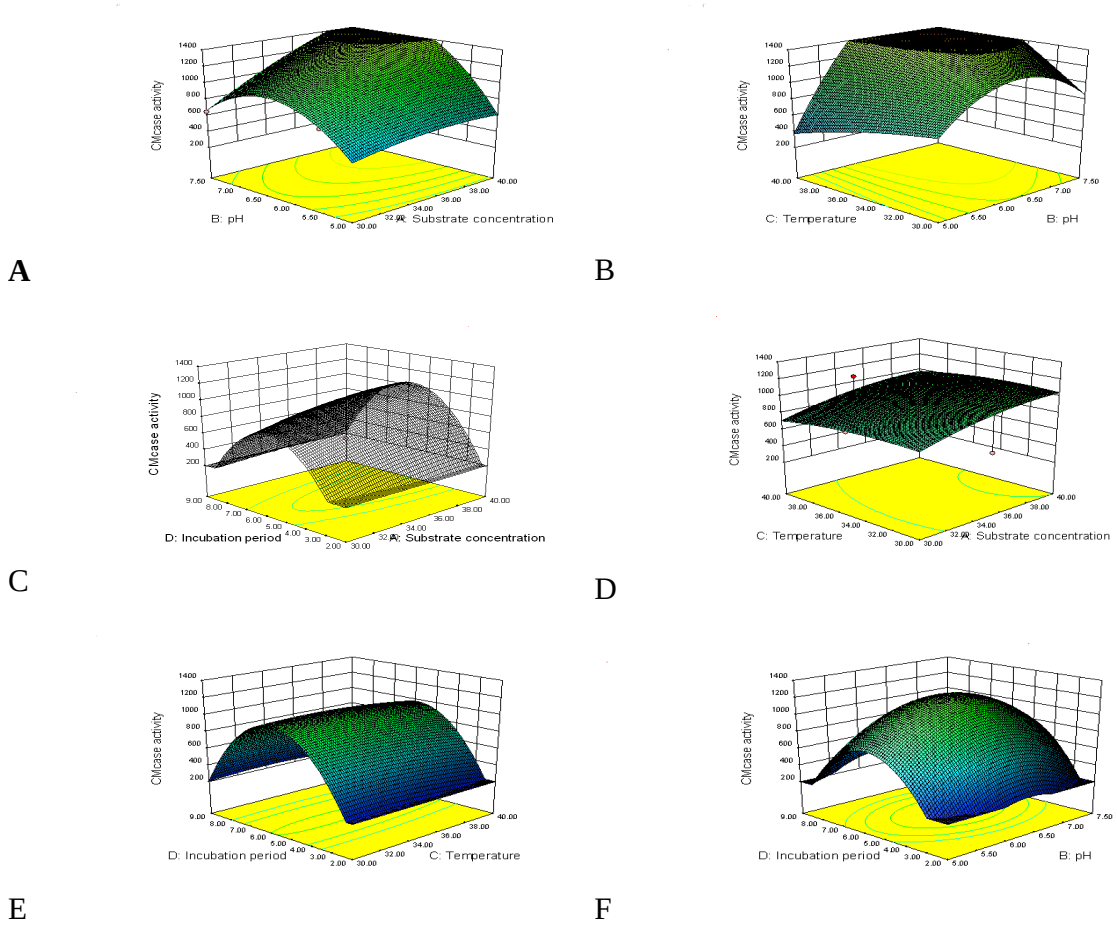

Figure 1 - Surface plot for the effect of (a) wheat bran and pH (b) temp and pH (c) incubation period and substrate conc. and (d) temp and substrate conc. on CMCase activity.

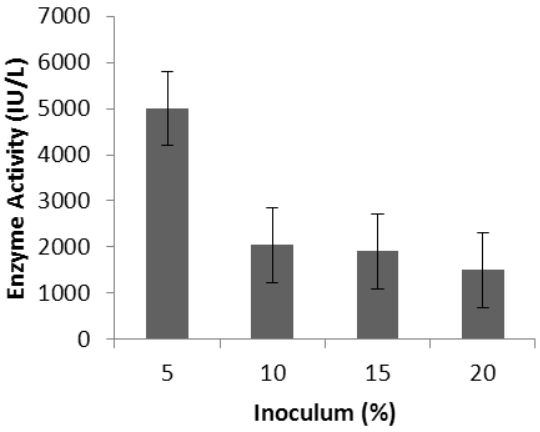

Figure 2 - Effect of inoculum (%) on enzyme production.

Effect of supplementation of what barn with different carbon sources and ions

Although wheat bran supported the growth of *Bacillus* sp. JS14 and cellulase production, but it might not supply sufficient nutrients needed by the organism for maximum enzyme production. Hence, the addition of different carbon sources to the medium was conceded to improve the cell growth and enzyme production. The supplementation of wheat straw, rice husk and baggase had little effect on cellulase production, while lactose and carboxy methyl cellulose enhanced enzyme production. Among them, lactose improved the cellulase production the most, which increased 40% compared to the control (Fig. 3). Lactose was also considered as a good inducer for cellulase production by the Seiboth et al (2005).

The production of cellulase was enhanced by the addition of NaCl and $MgSO_4$ while EDTA reduced the production (Fig.4).

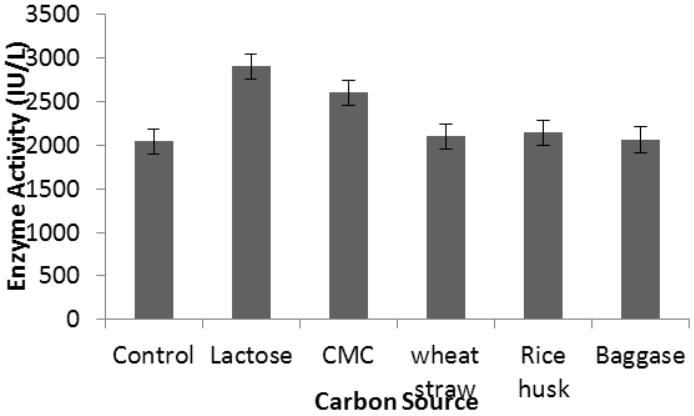

Figure 3 - Effect of different supplement carbon sources on the cellulase production.

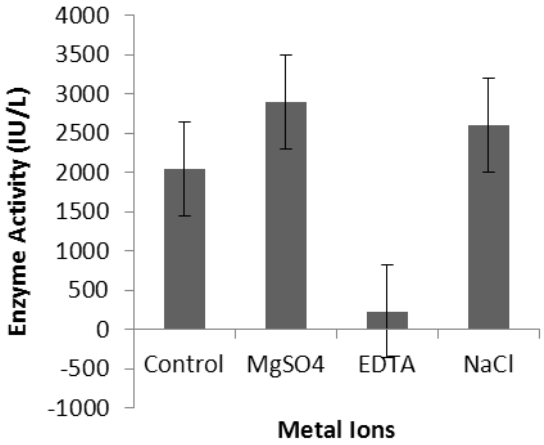

Figure 4 - Effect of different ions on the cellulase production.

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