

*Research Paper***Optimization of Culture Conditions for Production of Amylase by *Bacillus amyloliquifaciens* SH8 Using Response Surface Methodology**

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(Received on 21 April 2016; Revised on 20 July 2016; Accepted on 28 September 2016)

In the present study Response surface methodology (RSM) was used to investigate the combined effect of relevant process variables to enhance the production of amylase under submerged fermentation by *Bacillus amyloliquifaciens* SH8. The process variables included pH, temperature, inoculum size, incubation days and substrate concentration. A 54 factorial central composite design (CCD) using response surface methodology (RSM) was employed to obtain interaction between the process variables and optimizing amylase titers. Total 54 experiments were carried out in shake flask and a three dimensional response surface was generated to determine the effect of process variables on amylase production. The optimal calculated values of tested variables for maximal production of amylase were: pH 5, temperature 45°C, inoculum size 5%, incubation day of 5 and substrate concentration of 0.60% with 16.07 IU/ml of amylase activity

**Keywords:** Amylase; *Bacillus amyloliquifaciens* SH8; Submerged Fermentation; Response Surface Methodology

**Introduction**

Starch, a homo-polysaccharide is an important and abundant food reserve and energy source in plants. It is found in seeds, fruits, leaves, bulbs and tubers (Bozic *et al.*, 2011). Structurally it is a composite polymer of amylose and amylopectin which varies in proportion depending on its source. The  $\alpha$ -amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate being glucose residues linked through a  $\alpha$ -1-1,  $\alpha$ -1-4,  $\alpha$ -1-6, glycosidic bonds. Members of this family share a number of common characteristics (Van der Maarel *et al.*, 2002). Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non-reducing end successively resulting in short end products (Gupta *et al.*, 2003). Although they can be derived from a variety of sources such as plants, animals and microorganisms, microbial enzymes are generally used to meet the industrial demands. The

performance, economics and feasibility of each  $\alpha$ -amylase applications is influenced by important enzyme characteristics, including specificity, stability, optimum temperature and pH performance (Gupta *et al.*, 2003). Therefore, selection of the suitable microbial strains which produce enzymes that's how good activity and stability at industrially desirable conditions will be valuable. Generally, the microbial production of  $\alpha$ -amylase is greatly influenced by the components of the culture medium, especially the carbon and nitrogen sources and physical conditions, such as the pH, temperature, agitation, level of dissolved oxygen and the inoculum concentration (Rezaei *et al.*, 2010).

Several models for amylase action pattern have been proposed, such as the random action and the multiple attack action. Random action has also been referred to as a single attack or multi-chain attack action (Azhari & Lotan, 1991). In short, it can clearly be seen that the multiple attack action is generally an accepted concept to explain the differences in action pattern of amylases (Kramhøft *et al.* 2005; Svensson *et al.*, 2002; Bijttebier *et al.* (2008) showed that the

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level of multiple attack of several end amylases increased with temperature to a degree depending on the amylase. Submerged fermentation is the cultivation of the microorganism in a liquid medium containing soluble carbon source and nutrients maintained under agitation. The use of the submerged culture is advantageous because of the ease of sterilization and process control rendering it easier to engineer in these systems. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible in the manner of production the latter, however, is a preferable method for enzyme production from bacterial fermentations (Vidhayalakshni *et al.*, 2009).

In the present study optimization of various process parameters were done under submerged fermentation by response surface methodology for enhanced amylase production.

## Materials and Methods

### *Microorganism and Culture Condition*

*B. amyloliquifaciens* SH8 was isolated from the compost sample. It was maintained on nutrient agar medium and stored at 4°C. Seed culture was developed by inoculating nutrient broth of *B. amyloliquifaciens* SH8 into 50 ml of production medium Starch 4g, (NH<sub>4</sub>)<sub>2</sub>HSO<sub>4</sub> 0.5g, Yeast extract 0.5g; sodium citrate 0.2; Mg<sub>2</sub>SO<sub>4</sub> 0.05; CaCl<sub>2</sub> 0.08g; DW 1000ml of same composition were inoculated with 2 ml of seed culture and grown for 5 days. Samples were withdrawn after 5 days and centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was used for analysis of starch activity.

### *Enzyme Assay*

**Quantitative Assay:** Starch Hydrolysis (Shaw *et al.*, 1995): Starch hydrolysis test was performed on amylase medium and incubated at 45°C for 72h. The size of zone of hydrolysis was measured.

**Qualitative Assay:** The enzyme assay was performed to quantify amylase (Xiao *et al.*, 2006).

0.5 ml of enzyme solution was incubated with 0.2% starch at 37 °C for 15 min. 3 ml of DNSA reagent was added to it and the mixture was heated on boiling water bath for 15 min. After cooling down to room temperature, absorbance of reaction mixture was read at 540 nm. The standard curve was made from the

stock solution of glucose (0.4 mg/ml). One International unit of enzyme activity was defined as the amount of enzyme, releasing 1 mol of reducing group per minute per ml.

### *Optimization of Amylase Production Using Response Surface Methodology*

Four experimental factors (pH temperature, inoculum size, incubation days, substrate concentration) were found to have significant effect on amylase production. RSM using a three level central composite design was applied to optimize the response of four variables. A 54 factorial design was used in order to study the effect of pH, temperature, inoculum size (%), incubation day (days) and substrate concentration (%). Preliminary experiments revealed that optimum incubation time for amylase production was 5 days. Therefore, enzyme activity after 5 days of production was measured as responses. The statistical analysis of the results was performed using Design Expert ver. 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN). Amylase activity was analyzed using the analysis of variance (ANOVA) combined with the Fischer test to evaluate if a given term has a significant effect ( $\geq 0.05$ ). The optimum levels of the variables were obtained by graphical and numerical analysis using Design Expert program.

## Result and Discussion

In the present work the isolate used was isolated from the compost and showed potential amylase production on starch agar plate with a zone of hydrolysis. The clear zone of hydrolysis around the bacterial colony is due to hydrolysis of starch by amylolytic enzyme (Fig. 1). It was identified by 16s rRNA as *B. Amylolyticifaciens* SH8 with accession number from NCBI |JX129360.1|.

### *Optimization of Culture Conditions by Central Composite Design*

Temperature, pH, agitation speed, and substrate concentrations were chosen as the process parameters to optimize the conditions for maximum amylase production by a statistical design (CCD and RSM). A CCD with three coded levels for all the four factors pH (A), temperature (B), agitation speed (C) and starch concentration (D) were used for this purpose. The levels of parameters for the CCD were



Fig. 1: Amylase production on starch agar plate by *B. amyloliquifaciens* SH8 after flooding with iodine

Table 1:

Factors	Unit	Symbols	Actual levels of coded factors	
			-1	+1
pH	-	A	5	8
Temperature	°C	B	30	45
Inoculum size	%	C	5	15
Incubation days	Days	D	3	7
Substrate concentration	%	E	0.2	0.6

Table 2:

Run	pH	Temperature	Inoculum size	Incubation days	Substrate concentration	Amylase activity (IU/ml)
1	6.5	37.50	10	5	0.40	8.66
2	8.00	45	15	3	0.60	6.11
3	5.00	45	5	7	0.20	10.43
4	6.50	37.50	10	5	0.40	8.77
5	6.50	37.50	10	5	0.40	7.66
6	8.00	30	5	7	0.20	5.66
7	8.00	45	5	7	0.60	6.73
8	5.00	30	5	3	0.20	9.82
9	5.00	30	5	7	0.60	10.2
10	8.00	45	5	3	0.20	6.12
11	5.00	45	5	3	0.60	11

12	5.00	30	15	7	0.60	9.11
13	8.00	45	15	3	0.20	5.77
14	8.00	30	5	3	0.60	5.85
15	5.00	45	15	7	0.60	10.18
16	6.50	37.50	10	5	0.40	7.99
17	8.00	30	15	3	0.20	5.22
18	6.50	30	10	7.00	0.60	5.83
19	8	30	15	3.00	0.20	10.03
20	5	45	15	7.00	0.60	10.01
21	5	30	15	3.00	0.20	6.43
22	5	45	5	7.00	0.20	4.09
23	8	45	5	7.00	0.20	10.32
24	5	45	15	5.00	0.40	8.22
25	6.50	37.50	10	7.00	0.60	6.55
26	8.00	30	5	3.00	0.60	9.5
27	5.00	45	15	3.00	0.60	4.32
28	8.00	30	15	7.00	0.60	10.21
29	5.00	30	5	3.00	0.60	6.99
30	8.00	45	15	3.00	0.60	6.12
31	8.00	45	10	5.00	0.40	8.97
32	6.50	37.50	15	3.00	0.20	9.93
33	5.00	30	5	3.00	0.60	10.50
34	5.00	30	5	7.00	0.60	6.41
35	8.00	45	15	5.00	0.40	8.7
36	<b>5.00</b>	<b>45.50</b>	<b>5</b>	<b>5.00</b>	<b>0.60</b>	<b>16.07</b>
37	5.00	37.50	15	5.00	0.40	8.94
38	6.50	37.50	10	5	0.40	8.94
39	8.00	37.50	10	5	0.60	12.92
40	8.00	30	5	3	0.40	8.94
41	6.50	30	15	7	0.20	5.44
42	6.50	37.50	21.89	5	0.20	5.22
43	6.50	55.34	10	5	0.40	7.48
44	6.50	37.50	10	9.76	0.40	8.17
45	6.50	37.50	10	5	0.40	8.28
46	10.0	37.50	10	5	0.40	8.28
47	6.50	37.50	10	5	0.40	7.69
48	6.50	37.50	10	5	0.40	2.11
49	6.50	19.66	10	5	-0.08	6.71
50	6.50	37.50	10	5	0.40	6.55
51	6.50	37.50	-1.89	5	0.88	7.66
52	6.50	37.50	10	0.24	0.40	7.1
53	2.93	37.50	10	5	0.40	11.94
54	6.50	37.50	10	5	0.40	8.90

based on preliminary experimental results and are represented in Table 1.

The central composite design and the results of the CCD obtained for amylase production are presented in Table 2.

### Validation of the Experimental Model

Validation of the predicted results was accomplished by performing additional experiments in triplicate with the parameters suggested by the numerical modeling (suggested solution). These three sets of experiments yielded an average enzyme production of 16.07 IU/ml at pH 5, temperature 45°C, inoculum size 5%, after 5 days of incubation days and 0.60 % of substrate concentration i.e. starch. Good agreement between the predicted and experimental results confirmed the experimental adequacy of the model and the existence of the optimal point. The regression model was drawn to illustrate the combined effects of the independent variables and combined effects of each independent

variable upon the response variable. The response surface describing the quadratic effect of pH and temperature on xylanase production by *Bacillus amyloliquifaciens* SH8 is shown in Fig. 1a,b,c,d,e,f,g,h.

In case of amylase production from *B. amyloliquifaciens*, of all the parameters pH and incubation time exerted the most significant effect. The amylase model showed F value corresponding to 1.19 indicating the significance of the model with p value of <0.05 (Table 3). The lower value of CV corresponding to 0.991 indicated a greater reliability of the experiment performed. The analysis showed that the form of the model chosen to explain the relationship between the factors and the response is correct. Further the Adj R-squared value of 0.968 was found to be very close to pre R-squared value of 0.970 and the corresponding R<sup>2</sup> value of 0.907 indicated that the model was appropriate and can be used for the quantitative of amylase production.

**Table 3: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental designs**

Source	Sum of square	df	Mean square	F value	Prob>F	
Block	2.37	2	1.19			
Model	216.76	15	14.45	23.51	<0.0001	Significance
A	195.50	1	195.50	318.09	<0.0001	
B	1.15	1	1.15	1.88	0.1792	
C	0.11	1	0.11	0.19	0.669	
D	2.97	1	2.97	4.84	0.034	
E	4.24	1	4.24	6.89	0.0126	
AB	0.41	1	0.41	0.66	0.4223	
AC	4.513E-003	1	4.513E-003	7.342E-003	0.9322	
AD	2.17	1	2.17	3.54	0.0681	
AE	0.016	1	0.016	0.026	0.8719	
BC	0.46	1	0.46	0.74	0.3947	
BD	0.014	1	0.014	0.022	0.8825	
BE	3.23	1	3.23	5.25	0.0279	
CD	0.022	1	0.022	0.036	0.8508	
CE	6.28	1	6.28	10.22	0.0029	
DE	0.19	1	0.19	0.31	0.5825	
Residual	22.13	36	0.61			
Lack of Fit	19.88	27	0.74	2.95	0.043	Significant
Pure Error	2.24	9	0.25			
Core Total	241.26	53				

Std Dev 0.78; R-squared 0.907; Adj-squared 0.968; CV 0.991; pred r squared 0.970

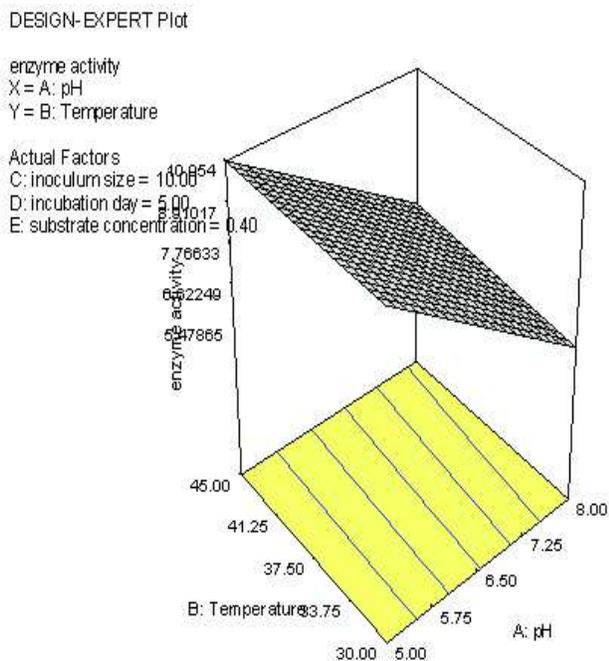


Fig. 2A: Effect of pH and Temperature on amylase production using *B. amyloliquifaciens* SH8

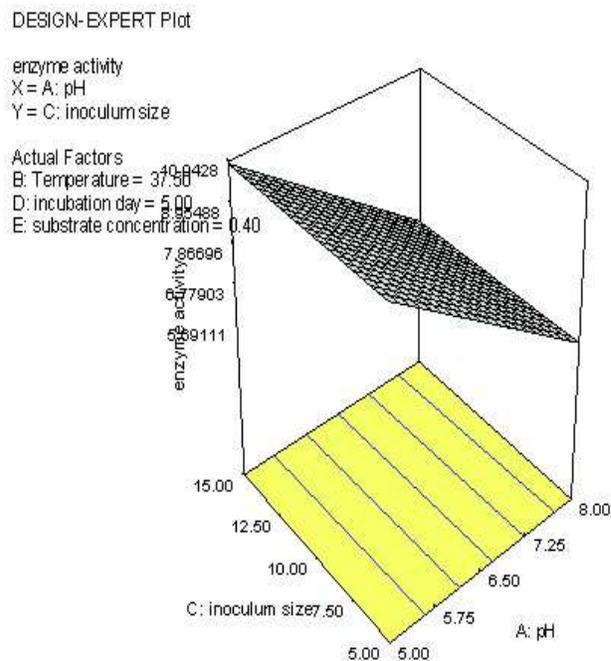


Fig. 2B: Effect of pH and inoculum size on amylase production using *B. amyloliquifaciens* SH8

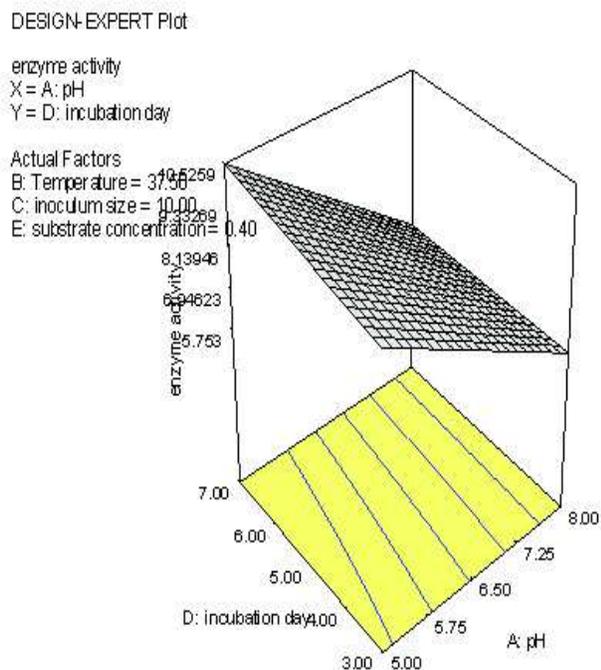


Fig. 2C: Effect of pH and incubation days concentration on amylase production using *B. amyloliquifaciens* SH8

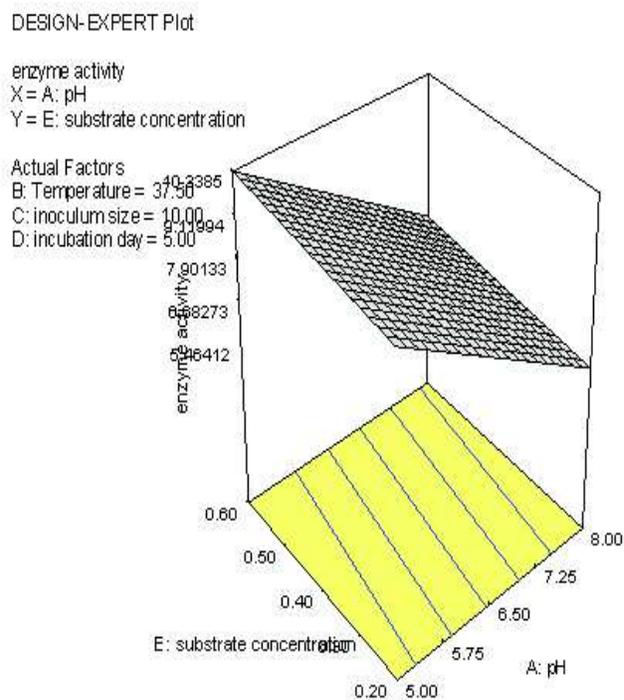


Fig. 2D: Effect of pH and substrate on amylase production using *B. amyloliquifaciens* SH8

DESIGN-EXPERT Plot

enzyme activity  
 X = B: Temperature  
 Y = E: substrate concentration

Actual Factors

A: pH = 6.50  
 C: inoculum size = 10.00  
 D: incubation day = 5.00

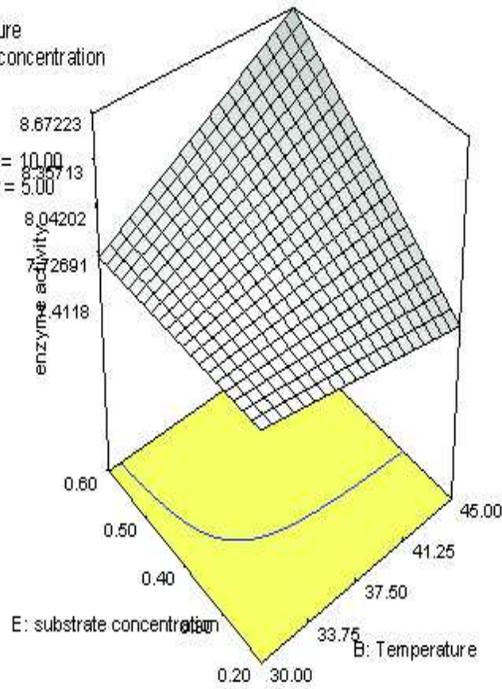


Fig. 2E: Effect of inoculum size and incubation days on amylase production using *B. amyloliquifaciens* SH8

DESIGN-EXPERT Plot

enzyme activity  
 X = C: inoculum size  
 Y = E: substrate concentration

Actual Factors

A: pH = 6.50  
 B: Temperature = 37.50  
 D: incubation day = 5.00

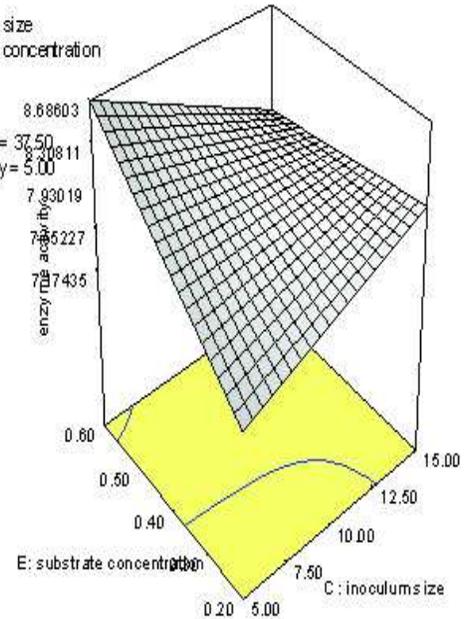


Fig. 2F: Effect of inoculum size and substrate concentration on amylase production by *B. amyloliquifaciens* SH8

DESIGN-EXPERT Plot

enzyme activity  
 X = D: incubation day  
 Y = E: substrate concentration

Actual Factors

A: pH = 6.50  
 B: Temperature = 37.50  
 C: inoculum size = 10.00

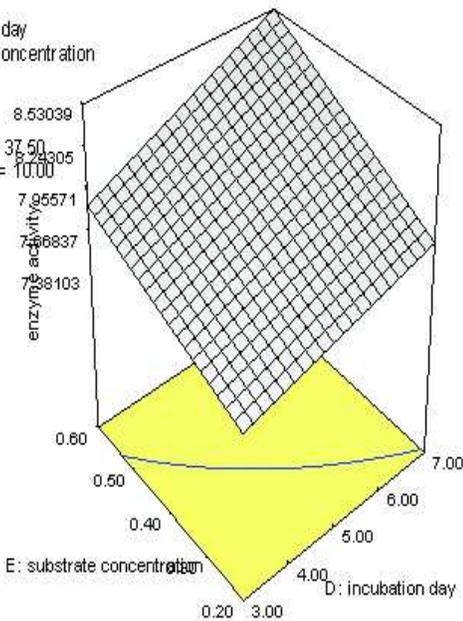


Fig. 2G: Effect of incubation days and substrate concentration on amylase production using *B. amyloliquifaciens* SH8

DESIGN-EXPERT Plot

enzyme activity  
 X = C: inoculum size  
 Y = E: substrate concentration

Actual Factors

A: pH = 6.50  
 B: Temperature = 37.50  
 D: incubation day = 5.00

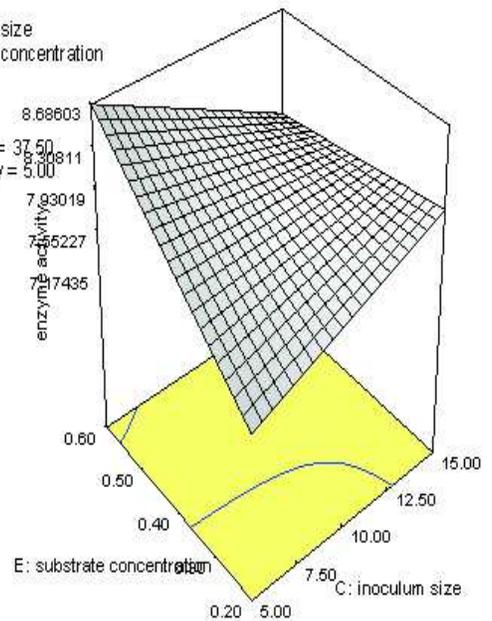


Fig. 2H: Effect of inoculum size and substrate concentration on amylase production using *B. amyloliquifaciens* SH8

$$\begin{aligned} \text{Enzyme activity} = & +7.88 - 2.1 * A + 0.16 * B - \\ & 0.051 * C + 0.2 * D + 0.31 * E + 0.11 * A * B - 0.012 \\ & * A * C - 0.26 * A * D - 0.02 * A * E + 0.12 * B * C \\ & + 0.021 * B * D + 0.32 * B * E - 0.026 * C * D - \\ & 0.44 * C * E + 0.07 * D * E \end{aligned}$$

Table 1 gives the CCD matrix with experimental and predicted values for cellulase activity. The regression equation showed the cellulase activity as an empirical function in terms of coded factors, where  $Y_i$  is the predicted cellulase activity in U/ml. ANOVA for response surface quadratic model gave F value = 23.51 with p values of all the coefficients ( $p < 0.0001$ ), implying the significance of the model. The coefficient of variation of the model was (C.V = The goodness of fit of the model was examined by determination coefficient ( $R^2=0.999$ ) which implied that sample variation of more than 99.9% was attributed to the variables and only 0.1% of total variance could not be explained by the model (Haaland, 1989). The adjusted determination coefficient (Adj  $R^2 = 0.999$ ) was also satisfactory to confirm the significance of the model.

The “Pred R-Squared” of 0.741 was in reasonable agreement with the “Adj RSquared” 0.869 of. Adeq precision measured the signal to noise ratio. A ratio greater than 4 is desirable. The results of the response surface quadratic model in the form of analysis of variance (ANOVA) with significance of each coefficient, determined by student’s t- test are listed in Table 2.

Response surface methodology (RSM) was used to investigate the combined effect of relevant process variables to maximize the production of xylanase in submerged fermentation by *Penicillium citrinum* MTCC 2553. The process variables include pH (6.5, 7.0 and 7.5); temperature (25, 30, and 35°C); agitation speed (190, 200, and 210 rpm); and, substrate (xylan) concentration (0.70%, 0.75%, and 0.80%).

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The optimal calculated values of tested variables for maximal production of xylanase were: pH 7.49, temperature 29°C, agitation speed 209 rpm, and substrate concentration of 0.75% (Ghoshal *et al.*, 2011).

Gangadharan *et al.*, 2007) used RSM of synthesis of alpha amylase by *B. amyloliquefaciens* under submerged fermentation using Plackett-Burman design which revealed the following i.e. substrate concentration (12.5%), incubation period (42h) and  $\text{CaCl}_2$  (0.0275 M).

## Conclusion

The present study using central composite design (CCD) of RSM is a collection of statistical techniques for designing experiments building models, evaluating the effect of factors and obtaining optimum conditions for desirable responses. Amylase activity obtained by optimizing the medium contents was found to be significantly affected by the interaction of *B. amyloliquifaciens* SH8 with the designed medium. Final optimized conditions are obtained by solving inverse matrix from the equation and through statistical analysis of the constraints. The optimum values of the tested variables were obtained using both the means with pH 5, temperature 45°C, inoculum size 5%, after 5 days of incubation days and 0.60 % of substrate concentration i.e. starch at these optimized conditions the model predicted 16.07 U/ml of amylase activity.

## Acknowledgements

The financial support received from Department of Biotechnology, Govt. of India, New Delhi, India is acknowledged.

## Conflict of Interest

The authors have declared that no conflict of interest exists.

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