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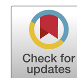
Original Article

Augmented cellulase production by *Bacillus subtilis* strain MU S1 using different statistical experimental designs



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ABSTRACT

The production of cellulase by *Bacillus subtilis* MU S1, a strain isolated from Eravikulam National Park, was optimized using one-factor-at-a-time (OFAT) and statistical methods. Physical parameters like incubation temperature and agitation speed were optimized using OFAT and found to be 40 °C and 150 rpm, respectively, whereas, medium was optimized by statistical tools. Plackett-Burman design (PBD) was employed to screen the significant variables that highly influence cellulase production. The design showed carboxymethyl cellulose (CMC), yeast extract, NaCl, pH, $MgSO_4$ and $NaNO_3$ as the most significant components that affect cellulase production. Among these CMC, yeast extract, NaCl and pH showed positive effect whereas $MgSO_4$ and $NaNO_3$ were found to be significant at their lower levels. The optimum levels of the components that positively affect enzyme production were determined using response surface methodology (RSM) based on central composite design (CCD). Three factors namely CMC, yeast extract and NaCl were studied at five levels whilst pH of the medium was kept constant at 7. The optimal levels of the components were CMC (13.46 g/l), yeast extract (8.38 g/l) and NaCl (6.31 g/l) at pH 7. The maximum cellulase activity in optimized medium was 566.66 U/ml which was close to the predicted activity of 541.05 U/ml. Optimization of physical parameters and medium components showed an overall 3.2-fold increase in activity compared to unoptimized condition (179.06 U/ml).
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1. Introduction

Cellulose, the most abundant and renewable material on earth, is a biopolymer of D-glucose units connected by β-1, 4 glycosidic linkages. The complete hydrolysis of cellulose into glucose involves the enzyme cellulase. Cellulase is a multi-enzyme system comprising of endo-β-1, 4-glucanases [EC 3. 2. 1. 4], exo-β-1,4-glucanases or cellobiohydrolases [EC 3. 2. 1. 91] and β-1,4-glucosidases [EC 3. 2. 1. 21]. These enzymes have proven their potential application in wide variety of industries like food and animal feed, laundry and detergents, pulp and paper, textiles, brewing and wine making, and biofuel. Besides this they find application in medical/pharmaceutical industry, protoplast production, genetic engineering, pollution treatment and waste management [1]. Cellulases accounted for approximately 20% of world enzyme market between 2005 to 2010 [2] and its demand is thought to increase drastically due to its application in second generation bioethanol production.

Cellulases are produced by bacteria, fungi, protozoans, plants and animals [3]. However, enzymes of microbial origin are more widespread due to their broad biochemical diversity, feasibility of mass culture, and ease of genetic manipulation. Moreover, they possess high degree of stability under extreme conditions [4]. Currently most of the commercial cellulases are obtained from fungi mainly *Trichoderma, Humicola, Aspergillus,* and *Penicillium* [5]. However, bacterial cellulases are gaining attention because of their high natural diversity, higher growth rate, easier product recovery and ability to produce enzymes that withstand harsh environmental conditions [6,7]. The cellulolytic potentials of bacteria belonging to different genera such as *Acetivibrio, Bacillus, Bacteroides, Cellulomonas, Clostridium, Erwinia, Ruminococcus,* and *Thermomonospora* have been well studied. Among them *Bacillus* spp. are known to produce and secrete large quantities of extracellular enzymes and hence dominate the bacterial workhorses [8]. Moreover, the endospore forming ability and production of secondary metabolites give them an additional advantage over competitors under conditions of slow growth on cellulosic substrates [9]. The strains of *B. sphaericus* and *B. subtilis* are excellent cellulase producers [10,11].

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The cost of enzymes is one major factor determining the economics of a biocatalytic reaction and it can be reduced by finding the optimum conditions for their production, isolation of hyperproducers and production of efficient strains by genetic engineering [12]. Cost reduction by media optimization is the basic research for industrial application. Various factors are known to influence the extracellular enzyme production. Some of these are temperature, pH, aeration [13], and medium constituents [14]. The Plackett-Burman design is a two level fractional factorial design, which allows screening and selection of most significant variables from among large number of variables. However, it does not consider the interactions between the variables [15]. The selected variables can be further optimized by using statistical and mathematical optimization tools such as Response Surface Methodology [16]. This technique enables to evaluate the optimal level of each variable, their interactions with other variables and their effect on product yield [17]. Recently, PBD and RSM have been successfully used to optimize many fermentation media [6].

The aim of the present study was to increase the cellulase production by optimizing fermentation conditions. Initially the physical parameters were optimized using OFAT method. Later, Plackett-Burman Design was used for selecting relevant medium components which were further optimized by central composite design of RSM and the model was verified.

2. Materials and methods

2.1. Microorganism and culture condition

A cellulolytic bacterium *Bacillus subtilis* MU S1 (accession No. KT715518) isolated from Eravikulam National Park was used for the study [18]. The culture was maintained on nutrient agar slant at 4 °C and subcultured regularly. The seed culture was produced by inoculating a loopful of the culture into 50 ml unoptimized medium [18] containing following components in g/l: CMC (10.0), NaCl (6.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.1), $CaCl_2$ (0.1), $NaNO_3$ (0.1) and yeast extract (1.0). The pH of the medium was adjusted to 7.0. The culture was grown at 37 °C in shaking incubator. One percent of the overnight grown culture (adjusted to a McFarland standard of 1.0) was used as seed for extracellular cellulase production using the unoptimized medium and conditions described above. After 24 h incubation, the culture was centrifuged at 10,000*g* for 10 min and the supernatant was used to analyze enzyme activity.

2.2. Cellulase assay

Cellulase assay was performed according to Ghose et al. [19] using CMC as the substrate. One milliliter of the enzyme was incubated with same volume of substrate in 0.05 M sodium citrate buffer (pH 5.0) for 30 min at 40 °C. The reducing sugar released was estimated using DNS method [20]. All experiments were performed in duplicates and the enzyme activity was determined using calibration curve of glucose. One unit of cellulase activity is defined as the amount of enzyme required to liberate 1 μmol of glucose per minute under the assay conditions.

2.3. Optimization of physical parameters by OFAT design

The temperature and agitation speed for cellulase production were optimized by OFAT method. For optimizing temperature the unoptimized medium was inoculated with seed culture and incubated at varying temperatures (30 °C, 40 °C and 50 °C) in shaking incubator. After 24 h the culture was centrifuged and the cell-free supernatant was used as crude enzyme for cellulase assay. The optimal agitation speed was determined by inoculating and incubating medium at different agitation speeds (50, 100, 150 and 200 rpm) at optimized temperature. Cellulase assay was performed in duplicate with crude enzyme obtained from 24 h culture. The medium components were optimized by growing the organism under the optimized physical conditions.

2.4. Plackett-Burman design for screening of significant medium components

The most important variables that significantly influence cellulase production were selected by PBD using the statistical software package MINITAB (Release 16, PA, USA). A total of ten parameters were screened and each parameter was examined at its low level (−1) and high level (+1) (Table 1). PBD is based on the first order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \qquad (1)$$

where Y is the response (cellulase activity), β_0 is the models intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. This design does not consider the interaction among variables and a linear approach is considered to be sufficient for screening. The main effects of such a design are calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements at the low level (−1) [15].

The ten variables were screened in 20 experimental runs. Averages of cellulase activity obtained from duplicate experiments were taken as the response (Table 2). Regression analysis was performed to determine the factors that influence enzyme production. The factors which were significant at or above 95% level ($p < 0.05$) were selected and later optimized by central composite design.

2.5. Central composite design

After identifying the significant variables for cellulase production by PBD, response surface methodology using CCD was employed to determine the optimal levels of these variables. A 3-factor-5-level design was used and five coded levels (−α, −1, 0, +1, +α) were assigned to each factor (Table 3). Alpha is the extended level with value of $(2)^{3/4} = 1.682$. A 2^3 full-factorial CCD experimental design containing three significant medium components (CMC, yeast extract and NaCl) at five coded levels was generated using the statistical software package "Design Expert 7" (Stat Ease Inc., Minneapolis, USA). The experimental design comprised of 20 runs ($=2^k + 2k + n_0$), where '*k*' is the number of independent variables and n_0 is the number of replicate runs at center point of the variables. All experiments were carried out in duplicate and the averages of the cellulase activity were taken as the response (Table 4).

Table 1
Medium components and their variables used in Plackett- Burman design.

Nutrient code	Nutrients (g/l)	Low (−1)	High (+1)
A	CMC	2	18
B	Yeast extract	0.5	10.5
C	NaCl	2	14
D	$(NH_4)_2SO_4$	0.5	2.5
E	KH_2PO_4	0.05	2.05
F	K_2HPO_4	0.05	0.45
G	$MgSO_4$	0.01	0.21
H	$CaCl_2$	0.005	0.405
I	$NaNO_3$	0.005	0.805
J	pH	5	7

Table 2
Plackett-Burman design generated for ten variables.

Run	CMC	Yeast extract	NaCl	$(NH_4)_2SO_4$	KH_2PO_4	K_2HPO_4	$MgSO_4$	$CaCl_2$	$NaNO_3$	pH	Cellulase activity (U/ml)
1	1	−1	−1	−1	−1	1	−1	1	−1	1	190.48
2	1	−1	−1	1	1	−1	1	1	−1	−1	8.30
3	1	1	−1	1	1	1	−1	−1	−1	1	500.80
4	1	1	1	−1	−1	1	1	−1	1	1	440.32
5	−1	−1	1	−1	1	−1	1	1	1	1	10.81
6	−1	−1	−1	1	−1	1	−1	1	1	1	15.30
7	1	−1	1	−1	1	1	1	1	−1	−1	150.83
8	1	−1	1	1	1	1	−1	−1	1	1	170.69
9	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	10.53
10	1	−1	1	1	−1	−1	−1	−1	1	−1	130.40
11	−1	−1	1	1	−1	1	1	−1	−1	−1	16.34
12	−1	1	1	1	1	−1	−1	1	1	−1	320.16
13	1	1	−1	−1	1	1	−1	1	1	−1	300.64
14	1	1	1	1	−1	−1	1	1	−1	1	444.50
15	−1	1	−1	1	−1	1	1	1	1	−1	100.40
16	−1	−1	−1	−1	1	−1	1	−1	1	1	9.86
17	1	1	−1	−1	−1	−1	1	−1	1	−1	330.42
18	−1	1	1	−1	−1	−1	−1	1	−1	1	370.62
19	−1	1	1	−1	1	1	−1	−1	−1	−1	382.63
20	−1	1	−1	1	1	1	1	−1	−1	1	278.21

Table 3
Experimental range and levels of independent variables used for central composite design.

Variables	Components	−α	−1	0	+1	+α
A	CMC	2	5.24	10	14.76	18
B	Yeast extract	0.5	2.53	5.5	8.47	10.5
C	NaCl	2	4.43	8	11.57	14

Table 4
Full factorial central composite design matrix with actual and coded value of variable and the observed and predicted response.

Run	CMC		Yeast extract		NaCl		Cellulase activity (U/ml)	
	Actual	Coded	Actual	Coded	Actual	Coded	Observed	Predicted
1	14.76	+1	2.53	−1	4.43	−1	350.26	337.44
2	10.00	0	5.50	0	2.00	−α	492.13	486.10
3	5.24	−1	8.47	+1	4.43	−1	324.28	341.22
4	5.24	−1	2.53	−1	11.57	+1	268.31	257.74
5	10.00	0	5.50	0	8.00	0	479.33	463.25
6	14.76	+1	8.47	+1	11.57	+1	506.40	514.18
7	14.76	+1	8.47	+1	4.43	−1	536.32	549.79
8	14.76	+1	2.53	−1	11.57	+1	232.50	218.46
9	2.00	−α	5.50	0	8.00	0	268.03	259.19
10	18.00	+α	5.50	0	8.00	0	396.63	401.46
11	10.00	0	5.50	0	8.00	0	465.26	463.25
12	5.24	−1	8.47	+1	11.57	+1	343.48	359.24
13	10.00	0	5.50	0	8.00	0	471.32	463.25
14	5.24	−1	2.53	−1	4.43	−1	328.06	323.08
15	10.00	0	5.50	0	8.00	0	456.53	463.25
16	10.00	0	5.50	0	14.00	+α	399.26	401.27
17	10.00	0	5.50	0	8.00	0	434.32	463.25
18	10.00	0	5.50	0	8.00	0	472.04	463.25
19	10.00	0	0.50	−α	8.00	0	172.19	199.02
20	10.00	0	10.50	+α	8.00	0	494.06	463.21

The coded values of independent variables were calculated using following equation

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3, \ldots k \tag{2}$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change. The experimental data was analyzed using multiple regression analysis procedure. The following second-order polynomial equation was applied to determine the relationships and interrelationships of the variables

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC \tag{3}$$

where Y is the predicted response, β_0 is intercept, β_1, β_2, β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are squared coefficients, and β_{12}, β_{23}, β_{13} are the interaction coefficients, A, B and C are the independent variables studied.

In order to determine the significance of each term in the equations fitted and to estimate the goodness of fit, ANOVA (analysis of variance) test was performed using Design Expert 7. The fitted polynomial equation was then expressed in the form of

three-dimensional response surface plots to demonstrate the main and interactive effects of the independent variables on the dependent ones.

2.6. Validation of model

The statistical model for the production of cellulase was validated by performing experiments under predicted set of conditions. The fold increase in cellulase activity after optimization was determined by comparing the activity with unoptimized medium. The cellulase activity in optimized medium containing $MgSO_4$ (0.01 g/l) and $NaNO_3$ (0.005 g/l) was determined.

2.7. Growth curve analysis

To determine the relationship between the time course of bacterial growth and the cellulase production in optimized medium, a growth curve analysis was performed. Bacterial growth and activity was measured after every 2 h.

3. Results and discussions

In the present scenario the demand for low cost, highly efficient and eco-friendly enzymes are increasing day by day. There is no general medium for enzyme production by different microbial strains. Cell growth and enzyme production are greatly influenced by the chemical composition of culture medium and environmental factors [21]. Moreover, each strain has its own idiosyncratic physicochemical and nutritional requirements for growth and secretion of enzyme [22]. A better understanding of the essential medium components and their optimal levels may be used to enhance the enzyme production. In the present study, cellulase was produced from *Bacillus subtilis* MU S1 by fermentation in unoptimized medium at 37 °C in shaking incubator. After 24 h incubation an activity of 179.06 U/ml was obtained. In order to improve the enzyme production the physical parameters and medium components were optimized. Lastly, the model obtained after statistical optimization was validated.

3.1. Optimization of physical parameters for cellulase production

Temperature is a critical parameter for success of fermentation reaction. It controls growth and production of metabolites by microorganisms and usually differs from one organism to other [23]. The temperature influences extracellular enzyme production by changing the physical properties of cell membrane [24]. In case of *Bacillus subtilis* MU S1 the maximum cellulase production (187.08 ± 2.8 U/ml) was observed at 40 °C. The production decreased at 30 °C followed by 50 °C (Fig. 1a). The studies of Immanuel et al. [13] showed that the microorganisms belonging to *Cellulomonas*, *Bacillus*, and *Micrococcus* sp. produced maximum endoglucanase at 40 °C and neutral pH. Ray et al. [25] also observed maximum cellulase yield at 40 °C by *Bacillus subtilis* and *Bacillus circulans*. Agitation speed is another important culture parameter that maintains homogenous condition and disperses dissolved oxygen. This increases the interfacial area and oxygen mass transfer rate for enhancing both substrate utilization and microbial activity [26]. From Fig. 1b it is evident that the optimum agitation speed for strain MU S1 was 150 rpm (191.44 ± 3.8 U/ml). Further increase in agitation rate decreased the cellulase production. Dash et al. [27] also recorded an optimum agitation speed of 150 rpm for *Bacillus subtilis* BI19. The optimum temperature and agitation speed for further experiments were fixed based on OFAT results.

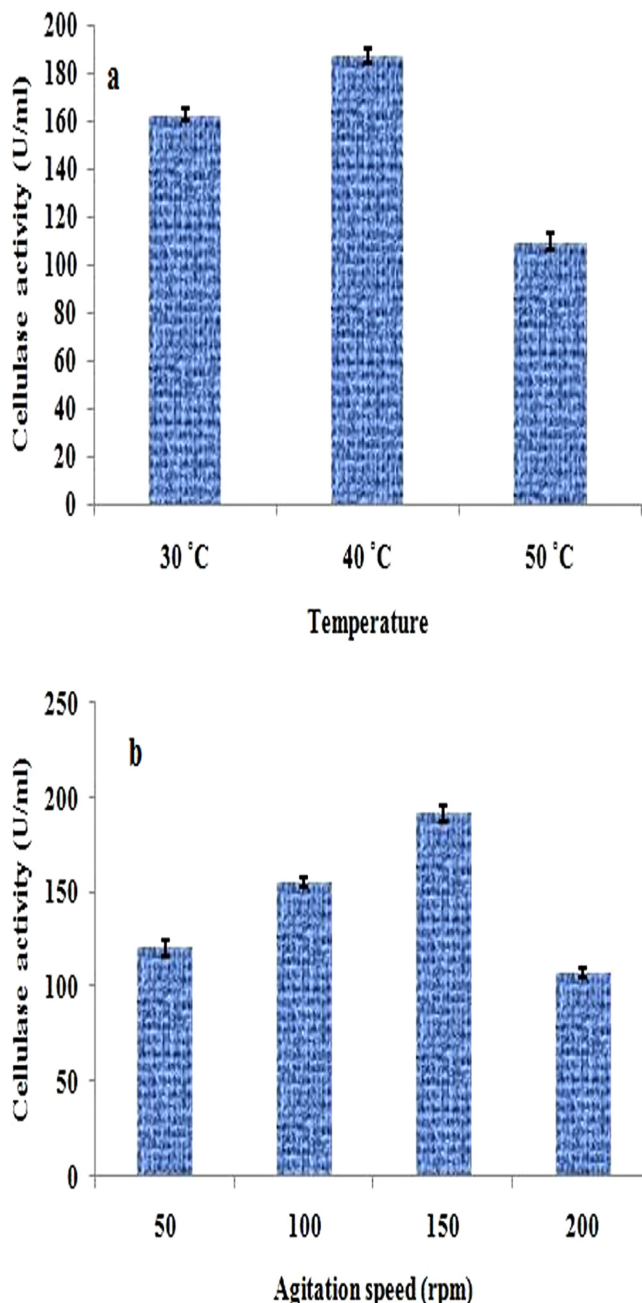

Fig. 1. Optimization of physical parameters (a) temperature (b) agitation speed.

3.2. Screening the significant medium components using Plackett-Burman design

Using PBD the most significant factors were screened from among ten components in 20 experimental runs. As indicated in Table 2, the cellulase activity of *Bacillus subtilis* MU S1 showed wide variation from 8.30 to 500.80 U/ml. The values of first order model coefficient, *t*-value, *p*-value and confidence levels of all ten variables are represented in Table 5. The important variables influencing cellulase production were selected based on p value <0.05 and confidence level ≥95%. The components CMC, yeast extract, NaCl, pH, $MgSO_4$ and $NaNO_3$ showed p <0 .05. Among them CMC (p = 0.00), yeast extract (p = 0.00), NaCl (p = 0.003) and pH (p = 0.003) showed significant positive effect whereas $MgSO_4$ (p = 0.006) and $NaNO_3$ (p = 0.012) showed negative effect. The confidence level

Table 5
Statistical analysis of Plackett-Burman design for ten variables.

Term	Coef	T-Value	P-Value	Confidence level (%)
Constant	209.11	24.92	0.000	100
CMC	57.63	6.87	0.000	100
Yeast extract	137.76	16.42	0.000	100
NaCl	34.62	4.13	0.003	99.7
$(NH_4)_2SO_4$	−10.60	−1.26	0.238	76.2
KH_2PO_4	4.18	0.50	0.630	37.0
K_2HPO_4	−4.53	−0.54	0.603	39.7
$MgSO_4$	−30.11	−3.59	0.006	99.4
$CaCl_2$	−17.91	−2.13	0.062	93.8
$NaNO_3$	−26.21	−3.12	0.012	98.8
pH	34.05	4.06	0.003	99.7

was approximately 100 for all the five factors. The *t* value limit is another measure of significance. The *t* value limit for the analysis is 2.26 and all the five variables have higher *t* value than the limit which indicates their significance. The overall regression coefficient for Plackett-Burman design is R^2 = 97.68% with adjusted R^2 = 95.11% and predicted R^2 = 88.56%.

Among the selected components CMC acts as the sole carbon source. Carboxymethyl cellulose is reported as the best carbon source for cellulase production by many *Bacillus* spp. [28,29]. Niranjane et al. [30] also found that CMC was a superior carbon source for cellulase production when compared with cellulose. Among the nitrogen sources yeast extract was shown to have an immense influence on enzyme production. The presence of external nitrogen source is essential in fermentation media during extracellular enzyme production for effective utilization of carbohydrates. Organic nitrogen sources are preferred for cellulase production compared to inorganic sources. In our medium yeast extract, $(NH_4)_2SO_4$ and $NaNO_3$ acted as nitrogen sources and among them yeast extract was found to have an immense influence on enzyme production. Abou-Taleb et al. [29] has also reported yeast extract to be the best nitrogen source. Sodium nitrate was also selected but was found to be significant at lower level. The results suggest that organic nitrogen was more suitable for cellulase production than inorganic source. This was in accordance with the results of Ray et al. [25] who reported that *Bacillus subtilis* and *Bacillus circulans* preferred organic nitrogen sources. Higher enzyme production in the presence of organic nitrogen may be attributed to the vitamins and growth precursors present in it [31]. Acharya and Chaudhary [32] reported that *Bacillus licheniformis* WBS1 and *Bacillus* sp. WBS3 did not show any detectable cellulase activity when $(NH_4)_2SO_4$ and $NaNO_3$ was used as the sole source of nitrogen.

Sodium chloride and $MgSO_4$ were also found to influence cellulase production; NaCl was positively significant whereas $MgSO_4$ showed significance at its lower concentration. Presence of both these components in the medium are thought to play crucial role in initial cell growth. Researchers have demonstrated a positive effect of NaCl on cellulase production by different *Bacillus* species [33,34]. Whereas, in case of $MgSO_4$ there are contrasting reports. Singh et al. [35] recorded a negative impact of $MgSO_4$ on cellulase activity whereas, Thakkar and Saraf [36] recorded a positive effect with confidence level >95%. The production of cellulase by *Bacillus* sp. JS14 was enhanced by addition of NaCl and $MgSO_4$ [37]. Another factor that was selected was initial pH of the medium. The pH of the growth medium strongly influences many enzymatic reactions by affecting the transport of chemical products and enzymes across the cell membrane [38]. *Bacillus subtilis* MU S1 showed high cellulase production with initial pH 7. Optimum growth pH near neutrality has been reported earlier for *Bacillus subtilis* KG10 [34], *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 [25]. Amongst the remaining components KH_2PO_4 had positive *t* value while $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$ showed a negative value, but none of them were found to significantly affect cellulase production by *Bacillus subtilis* MU S1.

3.3. Optimization by central composite experimental design

Among the six factors selected in PBD, the factors which showed negative effect viz $MgSO_4$ and $NaNO_3$ were eliminated from further optimization, and CCD was carried out with CMC, yeast extract and NaCl maintaining pH at neutral, at which the strain exhibited maximum cellulase production. A full factorial CCD was performed with these 3 factors and the results were

Table 6
Analysis of variance (ANOVA) of response surface quadratic model for the production of cellulase by *Bacillus subtilis* MU S1.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	1.988E+005	9	22083.76	50.29	<0.0001	significant
A-CMC	24431.30	1	24431.30	55.63	<0.0001	
B-Yeast extract	84252.06	1	84252.06	191.85	<0.0001	
C-NaCl	8686.03	1	8686.03	19.78	0.0012	
AB	18873.33	1	18873.33	42.98	<0.0001	
AC	1434.60	1	1434.60	3.27	0.1008	
BC	3477.36	1	3477.36	7.92	0.0184	
A^2	318^{2}9.57	1	31829.57	72.48	<0.0001	
B^2	31449.98	1	31449.98	71.61	<0.0001	
C^2	689.26	1	689.26	1.57	0.2388	
Residual	4391.58	10	439.16			
Lack of Fit	3104.57	5	620.91	2.41	0.1780	not significant
Pure Error	1287.02	5	257.40			
Cor Total	2.031E+005	19				

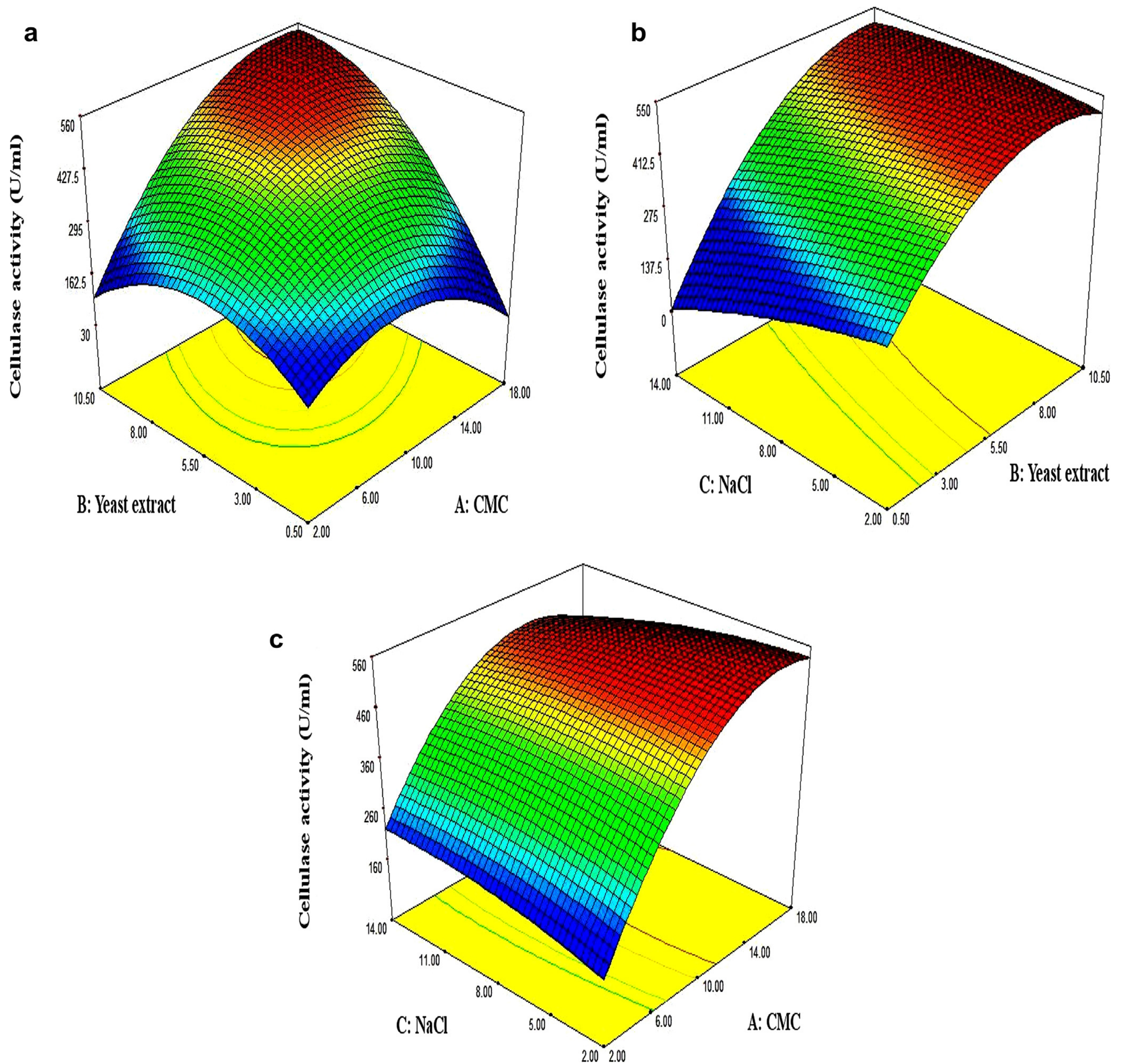

Fig. 2. Response surface graph showing interaction effects between concentrations of (a) CMC and yeast extract; (b) NaCl and yeast extract; (c) CMC and NaCl.

recorded in Table 4. The predicted cellulase activity was calculated using the second order polynomial equation

$$\begin{aligned}\text{Cellulase activity}\,(\text{U/ml}) = &+95.48885 + 37.85381 * A \\ &+ 34.48640 * B - 1.29548 * C \\ &+ 3.43451 * A * B - 0.78909 * A * C \\ &+ 1.96564 * B * C - 2.07696 * A^2 \\ &- 5.28523 * B^2 - 0.54335 * C^2 \qquad (4)\end{aligned}$$

where A, B and C are concentrations of CMC, yeast extract and NaCl in g/l. The statistical significance of the second order polynomial equation was evaluated by the *F* test and the result of ANOVA was recorded in Table 6. The significance of the model was established by "model *F*-value" of 50.29. There is very less chance that this large "model *F*-value" could occur due to noise. Values of "Prob > *F*" less than 0.05, specify that the model terms are significant. Whereas values above 0.1 indicate the model terms are not significant. In the present work, linear terms A, B, C the square effect of A, B and combinations of AB and BC were found to be significant for cellulase activity. The "lack of fit *F*-value" was 2.41 which implies that the lack of fit is not significant relative to the pure error. There is a 17.80% chance that a "lack of fit *F*-value" this large could occur due to noise. Non-significant lack of fit is good for the model to fit. The regression equation obtained from ANOVA with the R^2 value (multiple correlation coefficients) of 0.9784 revealed that the model could explain 97.84% variation in the response. Normally the R^2 value is between 0 and 1, and closer the value to 1 stronger the model and better it predicts the response. The predicted R^2 of 0.8747 is also in reasonable agreement with adjusted R^2 of 0.9589. This fundamentally indicates that the model fits very well to the experimental data. This

Table 7
Validation of model.

Run	CMC	Yeast extract	NaCl	Cellulase activity (U/ml)	
				Observed	Predicted
1	13.46	8.38	6.31	566.62	541.05
2	13.17	8.32	5.15	551.23	540.81
3	13.98	8.45	7.72	528.54	539.21
4	14.02	7.55	5.23	546.08	538.71
5	13.26	7.58	4.92	519.78	537.46

was confirmed by the predicted cellulase activity which match well with the observed activity. Another index for the fitness of model is Adeq Precision that measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio obtained for the present analysis is 23.672 which indicates an adequate signal. Thus this model can be used to navigate the design space.

The effect of interaction of variables on cellulase production was studied by three dimensional surface curves against two independent variables while the other independent variable was maintained at its constant level. These surface plots can be used to predict the optimal value for different variables. Three response surface plots were obtained by considering all the possible combinations (Fig. 2). The 3D response surface plot shown in Fig. 2a depicts the interaction between CMC and yeast extract. The cellulase activity increases with increasing concentration of both the components, however when the concentrations reach beyond the middle value the activity tends to decline. The plot shows strong interaction between the components which is confirmed by *p* value <0.0001. Similar observations were made by Deka et al. [6] and Goyal et al. [28]. Cellulases are inducible enzymes and CMC is known to have an inducing effect on cellulase production. Sadhu et al. [39] has also reported the importance of CMC as substrate for cellulase production by *Bacillus* sp. These facts can be justified by the report that enzymes involved in substrate degradation are generally inducible and produced only when the corresponding substrate is present in the nutrient media. Fig. 2b shows the interaction between yeast extract and NaCl. In case of yeast extract the surface curvature is ascending indicating increase in activity but beyond about 8 g/l the activity decreases. One mole of CMCase production requires large quantity of nitrogen, therefore nitrogen sources like yeast extract play vital role in cellulase production. High yeast extract concentrations were found to be optimal for cellulase production by many *Bacillus* spp. [28]. For NaCl no much surface curvature was seen but the activity tends to decrease with higher concentration. Fig. 2c describes the interaction between CMC and NaCl. From the graph it is evident that there is no or little interaction between the two components. High level of CMC and almost middle level of NaCl was most optimum for high cellulase production.

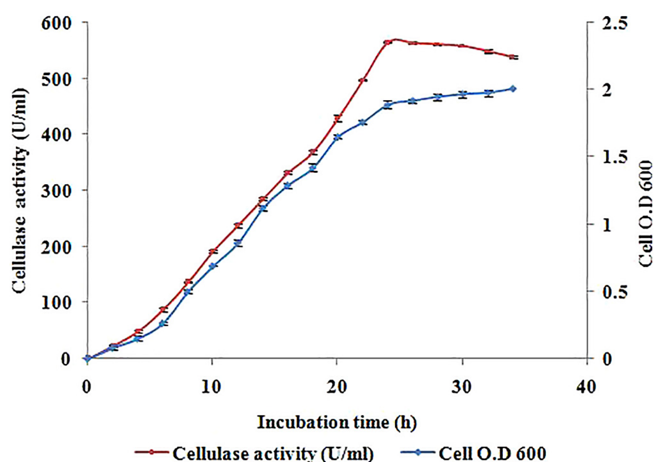

Fig. 3. Cellulase production profile and growth curve of strain MU S1 in optimized medium.

3.4. Validation of the model

In order to verify the adequacy of statistical analysis and quadratic model a set of experiments was performed in duplicate using the combinations suggested by the software (Table 7). The maximum cellulase activity predicted by the model using the optimum concentration (13.46 g/l CMC, 8.38 g/l yeast extract and 6.31 g/l NaCl at pH 7) was 541.05 U/ml. This was in agreement with the experimental yield of 566.62 U/ml, which verifies the validity of the model and existence of optimal points. Statistical optimization was thus an efficient tool for identification of significant variables and optimization of medium using minimum number of experiments. The presence of $MgSO_4$ and $NaNO_3$ in the optimized medium decreased the activity to 516.23 U/ml.

3.5. Growth curve analysis

The growth curve analysis showed that growth and cellulase activity were related to each other. Highest activity was observed in the late log phase, thereafter the activity reduced slightly (Fig. 3).

4. Conclusion

In the present study cellulase production from newly isolated *Bacillus subtilis* MU S1 was enhanced by optimizing the physical parameters and medium components. The cultivation of strain MU S1 in medium containing CMC, yeast extract and NaCl at pH 7, temperature 40 °C and agitation speed of 150 rpm significantly improved enzyme production. The optimal levels of the components were CMC 13.46 g/l, yeast extract 8.38 g/l and NaCl 6.31 g/l. The cellulase activity under unoptimized condition was 179.06 U/ml, however after optimization the overall activity increased by 3.2-fold as compared to the unoptimized condition. Statistical approach for optimization reduced the number of parameters and experiments required for the production of enzymes thus reducing the production cost. The data reveal that *B. subtilis* MU S1 can produce high levels of cellulase with minimum media components and its use in industries could result in considerable reduction in production cost and thereby savings on industrial scale.

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