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Application of Statistical Approach for Optimizing CMCase Production by *Bacillus tequilensis* S28 Strain via Submerged Fermentation Using Wheat Bran as Carbon Source

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***Abstract*—**Biofuels production has come forth as a future technology to combat the problem of depleting fossil fuels. Bio-based ethanol production from enzymatic lignocellulosic biomass degradation serves an efficient method and catching the eye of scientific community. High cost of the enzyme is the major obstacle in preventing the commercialization of this process. Thus main objective of the present study was to optimize composition of medium components for enhancing cellulase production by newly isolated strain of *Bacillus tequilensis*. Nineteen factors were taken into account using statistical Plackett-Burman Design. The significant variables influencing the cellulose production were further employed in statistical Response Surface Methodology using Central Composite Design for maximizing cellulase production. The optimum medium composition for cellulase production was: peptone (4.94 g/L), ammonium chloride (4.99 g/L), yeast extract (2.00 g/L), Tween-20 (0.53 g/L), calcium chloride (0.20 g/L) and cobalt chloride (0.60 g/L) with pH 7, agitation speed 150 rpm and 72 h incubation at 37^{o}C. Analysis of variance (ANOVA) revealed high coefficient of determination (R^2) of 0.99. Maximum cellulase productivity of 11.5 IU/ml was observed against the model predicted value of 13 IU/ml. This was found to be optimally active at 60^{o}C and pH 5.5.

Keywords*—Bacillus tequilensis*, CMCase, Submerged Fermentation, Optimization, Plackett-Burman Design, Response Surface Methodology.

I. Introduction

WITH the growing inevitable demand for alternate bio-based fuels worldwide, degradation of lignocellulosic biomass by enzymes is gaining immense attention in research. Lignocellulosic biomass is considered as the most abundant, inexpensive and renewable source on earth which can be utilized to get industrially relevant compounds by microbial conversion. Lignocellulosic biomass is composed of three major components: cellulose, hemicellulose and lignin; out of which cellulose comprises the largest fraction i.e. 30-50% of total biomass. Cellulase market is accelerating dramatically due to their wide range of application in textile industry, paper and pulp industry, food and feed industry. They are successfully showing promising results in waste management as well as judicial utilization of these wastes to produce commercially important products [1]-[3].

The economics of bioethanol production is primarily affected by the production cost of enzyme. But the high cost of cellulase production serves as a bottleneck in the success of bioprocess development. Efforts for minimizing this cost have shifted the focus of scientists towards better strain development, exploring newer and richer habitats, optimizing production conditions and genetic manipulation. Though fungi are the major producer of cellulases [4]-[6], role of bacterial cellulases is gaining adequate importance [7]-[9]. This is because of enormous natural diversity and higher growth rate of bacteria as compared to fungi [1], [10]. Bacteria produce stable enzymes complement which are less affected by feedback inhibition and can be manipulated by genetic engineering with greater ease [1].

Enzymatic degradation of cellulose involves the synergistic action of three enzymes: (i) Endoglucanases (EC 3.2.1.4), (ii) Exoglucanases including cellodextrinases (EC 3.2.1.74) and cellobiohydrolases (EC 3.2.1.91), and (iii) β-glucosidases (EC 3.2.1.21). Out of these enzymes, endoglucanases are the most important enzymes involved in cellulose degradation. The yield of cellulases produced by different microorganisms is remarkably affected by various parameters like pH, temperature, aeration, incubation time and growth nutrients [8], [11], [12]. Therefore, evaluation of nutritional and environmental factors for enzyme production plays a pivotal role in success of a process technology. Optimization strategies involving one-factor-at-a-time approach are not competent enough as they overlook the multiple factor interactions and may lead to misinterpretations. Statistical approach provides better understanding of effects of interacting factors and thus aid in minimizing errors in a significant manner.

Perhaps, genetic engineering is a leading field in developing efficient cellulose degrading microorganisms but role of traditional isolation technique cannot be denied. New isolates with better cellulose degrading potential lay the foundation for further improvement through molecular engineering. Therefore research should be focused primarily on isolating novel microorganisms from diverse natural habitats.

The main aim of the present investigation was to explore the best conditions for the maximum production of extracellular carboxymethylcellulase by isolated soil

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microorganism. Statistical optimization of cellulase production was done by employing statistical tools like Plackett-Burman Design (PBD) [13] and Response Surface Methodology (RSM). The approach involved the following steps: (1) screening of the most relevant variables which affects cellulase production (2) optimization of the these important parameters thereby generating a mathematical model by employing Central composite design for the evaluation of relationship between optimized factors and cellulase (3) verification of the model. Plackett-Burman design was applied to screen out significant variables for enhanced cellulase production. Further, Central Composite Design was applied to evaluate relationship between concentration of the variables and cellulase production.

II. Materials and Methods

A. Isolation of Cellulose Degrading Bacteria

The soil samples were collected from different agricultural fields and serially diluted with sterile distilled water. Isolation of bacterial strains was done by serial dilution method and pour plate technique. 100 microlitre of each dilution was plated on agar plates and incubated at 37ºC for 24 hrs. Isolates were purified by streaking repeatedly and preserved for further screening and identification.

B. Screening for Cellulose Degrading Bacteria

Purified isolates were spot inoculated on carboxymethyl cellulose (CMC) agar plates and incubated for 48 hrs at 37ºC. Plates were flooded with aqueous solution of 1% Congo red for 15 min at room temperature and thoroughly washed with 1N NaCl for counterstaining the plates. Clear hydrolysis zone observed around colonies qualitatively indicated the cellulase activity [14]. Hydrolysis capacity (HC) ratio for each isolate was calculated by dividing the zone diameter by colony diameter (data not shown). Isolates with highest HC ratio were selected for identification and quantitative determination of CMCase activity.

C. CMCase Production by Submerged Fermentation

Primary culture preparation was done by inoculating a single colony of the selected isolate into 100 ml Erlenmeyer flask containing 20 ml nutrient broth media and incubated at 37°C, 150 rpm for 24 hrs. 100 ml production media (nutrient broth supplemented with 1% carboxymethylcellulose as carbon source) in 250mL Erlenmeyer flask was prepared and inoculated with 1% primary culture followed by incubation at 37°C for 48 hours with agitation speed of 150 rpm in an orbital Incubator-shaker. Centrifugation of the culture was done at 4°C, 8944×g for 20 minutes to pellet down bacterial cells and other unwanted materials. The clear supernatant obtained served as crude enzyme source and was stored at -20°C until analysis.

D. Enzyme Assay

The crude enzyme extract was assayed for CMCase activity by DNS method [15]. The endoglucanase activity was determined as per the method given by [16] in which the reaction mixture with 0.5 ml crude enzyme and 0.5 ml substrate was incubated at 50°C for 30 minutes. Substrate was prepared by dissolving 1% (w/v) carboxymethylcellulose (High viscosity, HiMedia) in 0.1 M phosphate buffer (pH 6.4). The reaction was terminated by adding 1.5 ml DNS followed by boiling at 100°C for 15 minutes and cooled by adding water for color stabilization. The amount of reducing sugars released was determined by measuring absorbance at 560 nm using spectrophotometer (Labindia). Enzyme activity was calculated by using glucose calibration curve. One unit (IU) of CMCase activity was described as the amount of CMCase needed to release 1 micromole of reducing sugars per ml per minute under the given assay conditions.

E. Bacterial Identification by 16S rRNA Gene Sequencing

Genomic DNA was extracted from pure cultures using HiPurA^{TM} Plant Genomic DNA Miniprep Purification Spin Kit (HiMedia) according to the manufacturer's instructions. The amplification of 16S rRNA gene was achieved using the following primers: 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (3'-ACG GCT ACC TTG TTA CGA CTT-5') [17]. Sequencing of the purified 16S rRNA gene was done using four sequencing primers and Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA).

F. Sequence Assembly and Phylogenetic Analysis

The sequence data obtained were assembled and analyzed using DNA sequence assembling software SEQUENCHER™ 4.10.1 (Gene Codes Corporation, MI, USA). The sequences were used to blast as query in nucleotide database of NCBI (http://www.ncbi.nlm.nib.gov/) to find the similar sequences. Phylogenetic tree was constructed using ClustalW software [18] by aligning all acquired and related sequences. The neighbor-joining method was used to measure the evolutionary distances and operated in MEGA 4 and the Kimura 2 parameter model while bootstrapping was done with 1000 replications [19].

G. Optimization of Conditions for Cellulase Production

Growth conditions like incubation time, temperature, carbon source, and substrate concentration were optimized by taking one-factor-at-a-time approach. A single colony of the bacterial isolate was inoculated into 100 ml Erlenmeyer flask containing 20 ml nutrient broth and incubated at 37°C for 24 h. This culture served as the source of primary inoculum for further secondary inoculation. For optimizing incubation time for cellulase production, 100 ml nutrient broth media in a 250 ml Erlenmeyer flask was prepared and supplemented with 1% (w/v) wheat bran as the carbon source. Secondary inoculation of the media was done with 1% (v/v) primary culture and incubated in an incubator-shaker at 37°C. Enzyme extraction was done by centrifugation at regular time intervals (6, 12, 24, 48, 72, 96 and 120 hours) and enzyme assays were performed. To identify a carbon source optimum for cellulase production, Erlenmeyer flasks with 100 ml nutrient broth medium was prepared supplemented with 1% of the following carbon sources: glucose, lactose, sucrose, carboxymethylcellulose (CMC), rice bran and wheat bran. All the media combinations

were inoculated with 1ml of primary culture and incubated further at 37°C, 150 rpm for 72 hours in an incubator-shaker. To study the effect of temperature on cellulose production, the production medium was incubated at different temperatures including 28, 30, 37 and 45°C. 100 ml nutrient broth medium with 1% wheat bran was inoculated and incubated at these temperatures for 72 hrs with agitation at 150 rpm. Different concentration of wheat bran like 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% were used in 100ml production media (nutrient broth) and incubated at 37°C, 150 rpm for 72 hrs. Enzyme assays were performed by using DNS method to determine effect of these conditions on cellulase production.

H.Statistical Experimental Designs

Cellulase production enhancement by different media components was studied using statistical tools like Plackett-Burman Design and Response Surface Methodology. First, significant factors affecting cellulase production were identified using Plackett-Burman Design represented as:

$$Y = \beta o + \sum \beta i Xi \quad (1)$$

Y= response; βo= model intercept; βi= linear coefficient; Xi= level of the independent variable.

19 variables at high and low levels were taken into account in a 20 run PBD to study the effect on cellulase production. In this 20 run PBD experimental matrix, each column represented a variable and each row represented an experimental run (Table I). Each experimental run represented 100 ml production media in a 250 ml Erlenmeyer flask, supplemented with 1% wheat bran as sole carbon source and pH was maintained to be 7. All the flasks with different media composition were incubated at 37°C in an incubator shaker for 72 hours with an agitation speed of 150 rpm.

Once the significant factors were selected on the basis of PBD, RSM was further employed using Central Composite Design (CCD). The regression equation was developed using RSM, allowing for the analysis of interacting factors by identifying which significant factors contribute to the regression model, and determining the optimal values of the most significant independent variables. A 2^4 factorial central composite experimental design resulting in 30 experimental runs was generated. The modeling and statistical analysis was done with Design Expert software Version 8.0, Stat-Ease Inc., Minneapolis, MN. The relation between coded and actual values is described according to:

$$xi = (Xi - X^0 i) / \Delta Xi \quad (2)$$

$$i = 1,2,3\ldots j$$

where xi = coded (dimensionless) value of the variable Xi, Xi = actual value of the i^{th} variable, X_0 = the value of Xi at the center point, ΔX = the step change value.

The behavior of the system was explained by the following second order polynomial equation:

$$Y = bo + \sum bi\, xi + \sum\sum bij\, xi\, xj + \sum bii\, x^2 i + e. \quad (3)$$

where Y is the measured response; bo, bi, bij, bii are constant and regression coefficients of model; xi and xj are levels (codes values) of independent variables; e is random error. The Design Expert was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. 3D and Contour plots were also obtained by using Design Expert software to illustrate the relationship between the variables. Accuracy and general ability of polynomial model was evaluated by coefficient of determination (R^2).

A quadratic model was build containing factorial and axial runs which was used to analyze the quadratic effects of variables and centre points to study the process variability of the cellulase production as response. The significant factors identified were cobalt chloride, ammonium chloride, peptone, Tween-20, yeast extract, calcium chloride and their main effect on CMCase production. Out of these, calcium chloride and yeast extract were studied at fixed concentrations and other variables were studied at five different concentration levels. The experimental matrix of CCD contained 30 runs including 24 non-centre experimental trials and 6 centre point trials. Each experimental run represented a 250 ml Erlenmeyer flask containing 100 ml production media (pH 7) supplemented with 1% (w/v) wheat bran as carbon source. Different combinations in the design were allocated into blocks. First block and second block contained factorial trials as well as two center trials. Third block contained axial trials and two center trials. The optimization of cellulase production was done with fixed composition of 40mg cobalt chloride, 300 mg peptone, 300 mg ammonium chloride, 75 mg Tween-20, 200 mg yeast extract and 20 mg calcium chloride in 100ml media in the central point experimental runs. The overall experimental design matrix with actual values of different variables was given in Table II. All the experimental runs were incubated at 37°C for 72 hours with an agitation speed of 150 rpm.

Statistical analysis using RSM was used to evaluate the relationship between cellulase production and different media ingredients. Analysis of variance (ANOVA) was calculated to evaluate the relevance of model and goodness of fit. Predicted and observed values of cellulase production were analyzed to explain the fitness of the model. Coefficient of variance, analysis of variance and coefficient of determination were represented in Table III. Three dimensional surface plots were used to evaluate the interaction between the variables and the effect of these independent variables on the dependent variable i.e. cellulase production. The optimal concentration of significant variables for the cellulase production was illustrated form these 3D plots. The validation of the model was done by repeating the experimental run resulting in maximum response. The accuracy of model was verified by comparing the response of un-optimized and optimized media.

I. Validation of the Experiments

Two experiments were performed to test the accuracy and fitness of the model. The following composition of independent variables was used in the first run: cobaltous chloride 0.60 g/L, ammonium chloride 4.92 g/L, yeast extract

2.00 g/L, calcium chloride 0.20 g/L, peptone 4.98 g/L and Tween-20 0.87 g/L. In the second run, peptone and Tween-20 was changed to 4.94g/L and 0.53 g/L respectively without changing the rest composition. These two experimental runs were repeated to determine the validity of the model. Wheat bran (1% w/v) was added to all the different combinations and pH was maintained to be 7.

J. Effect of pH, Temperature, Metal Ions and EDTA on CMCase Activity

The enzyme activity versus pH was studied by assaying the enzyme activities at different pH ranging from 4.0-10.0 using three buffer systems: 0.1 M sodium acetate (4-6), 0.1 M Phosphate buffer (6-8) and 0.1 M Tris buffer (8-10). Optimum temperature for CMCase activity was evaluated by carrying the enzyme assay at different temperatures (30-100ºC). The effect of metal ions and EDTA on the enzyme activity was determined by pre-incubating the crude enzyme with 5mM metal ion solution [Co^{2+} ($CoCl_2$), Ni^{2+} ($NiSO_4$), Ca^{2+} ($CaCl_2$), Fe^{2+} ($FeSO_4$), Mg^{2+} ($MgCl_2$), Mn^{2+} ($MnCl_2$), Cu^{2+} ($CuSO_4$)] and 5mM EDTA before carrying out the enzyme assay at optimal conditions.

III. Results

A. Isolation and Identification of Cellulose Degrading Bacteria

Till date numerous enzymes have been identified and established themselves as potential industrial candidates. But still the present enzyme toolbox is not enough to fulfill all the industrial requirements. The high cost of enzyme is the major bottleneck in the path of its commercialization. Enzymes with high activity and stability over broad range of conditions are in demand to find their role in biotechnological applications. Cellulases are biotechnological potential candidates to be exploited in industrial applications and synthesis of bio-based fuels. Considering the increasing demand for cellulases commercially, this study has been done to optimize conditions for enhanced production of CMCase from a soil isolate. Seventy bacterial isolates in total were isolated from the various soil samples which were collected from different agricultural fields. Out of these, 17 were found to possess cellulose degrading ability screened on the basis of Congo red plate assay. Maximum clearance zone after the Congo red assay was shown by bacterial isolate 28 (data not shown).

Morphological examination of the bacterial strain revealed creamish white, round, rough, glistening, opaque and raised colonies. Bacterial strain was Gram positive and isolated rods were seen microscopically after Gram's reaction. 16S rRNA gene sequencing analysis of the strain showed 99.6% identity with *Bacillus tequilensis* 10bT (HQ223107). The nucleotide sequence obtained after sequencing was submitted in GENBANK under the Accession no. KJ830747. Phylogenetic analysis was done and tree was constructed by Neighbor joining method (Fig. 1).

B. Optimization of the Cultural Conditions for Bacterial Isolate

Bacillus tequilensis S28 was subjected to submerged fermentation and CMCase activity increased with time resulting in maximum production (1.4 IU/ml) after 72hrs of incubation (Fig. 2 (a)). Effect of temperature on CMCase activity was studied and optimum temperature for maximal production of CMCase (1.4 IU/ml) was found to be 37^0C (Fig. 2 (b)). A study related to the variation in enzyme yield due to change in carbon source was shown in Fig. 2 (c). Maximum CMCase activity (1.4 IU/ml) was observed with wheat bran as carbon source. CMCase activity with different concentration of wheat bran was determined and maximum activity (1.6 IU/ml) was observed with 3% substrate concentration as depicted in Fig. 2 (d).

Most influential and relevant variables for cellulase production were identified by employing the statistical tool Plackett-Burman Design. It is a statistical multifactorial design which is well established in computing the linear effect of all the factors involved for given number of observations [20], [21]. Cellulase activity for each experimental run was calculated and displayed in the Table I. The final model equation using Plackett-Burman Design for CMCase activity in terms of coded factors may be represented as:

$$\text{CMCase}=+1.55-0.034\times A-0.085\times B+0.26\times C-0.068\times D+0.14\times E-0.13\times F-0.17\times G-0.16\times H+0.19\times J-0.13\times K-0.12\times L+0.041\times M-0.030\times N-0.031\times O+0.57\times P-0.084\times Q-.030\times S+0.11\times T \quad (4)$$

Here A, B, C, D, F, G, H, J, K, L, M, N, O, P, Q, S and T are urea, ammonium sulphate, peptone, tryptone, yeast extract, beef extract, soybean meal, sodium nitrate, ammonium chloride, ammonium nitrate, potassium hydrogen phosphate, calcium chloride, magnesium sulphate, manganese chloride, cobalt chloride, ferrous sulphate, sodium chloride and Tween-20 respectively.

The data in the table revealed that 7^{th} experimental run has maximum cellulase activity (2.84 IU/ml) and 2^{nd} experimental run has minimum cellulase activity (0.193 IU/ml). The Model F-value was observed to be 1923.71 which clearly imply that this model is significant. This value also indicated that the chance of occurrence of "Model F-Value" this large due to noise is only 1.79%. Model terms are significant as Values of "Prob > F" obtained were found to be less than 0.0500. In this model B, C, D, E, F, G, H, J, K, L, P, Q, T are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. It was evaluated from PB Design that out of 19 factors (designated as A, B, C…….T) cobalt chloride (P), peptone (C), calcium chloride (M), Tween-20 (T) and ammonium chloride (J) had a positive effect whereas soyabean meal (G), sodium nitrate (H), ammonium nitrate (K), beef extract (F), potassium dihydrogen phosphate (L) had a negative effect on cellulase production as depicted in Fig. 3.

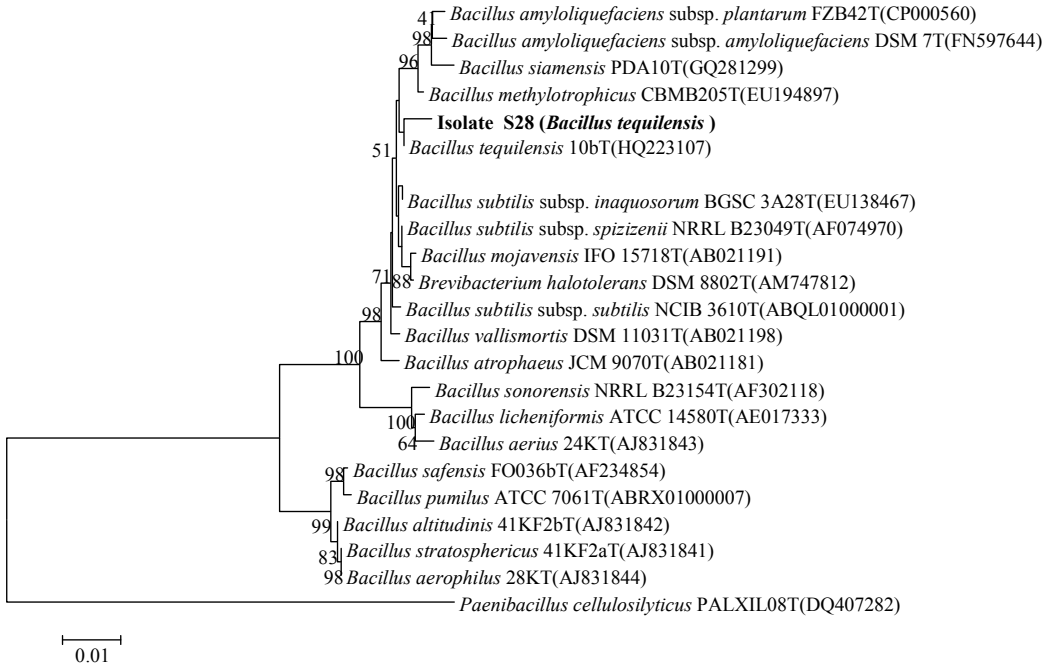

Fig. 1 Phylogenetic tree constructed using the Neighbor-Joining method based on 16S rDNA sequences of bacteria. The bootstrap test (1000 replicates) analysis was done and the associated taxa were clustered together in accordance with percentage of replicate trees which was displayed next to the branches. The evolutionary distances were measured utilizing the Kimura 2-parameter method and were operated further in MEGA4. Bar 0.01 indicated substitution per nucleotide position

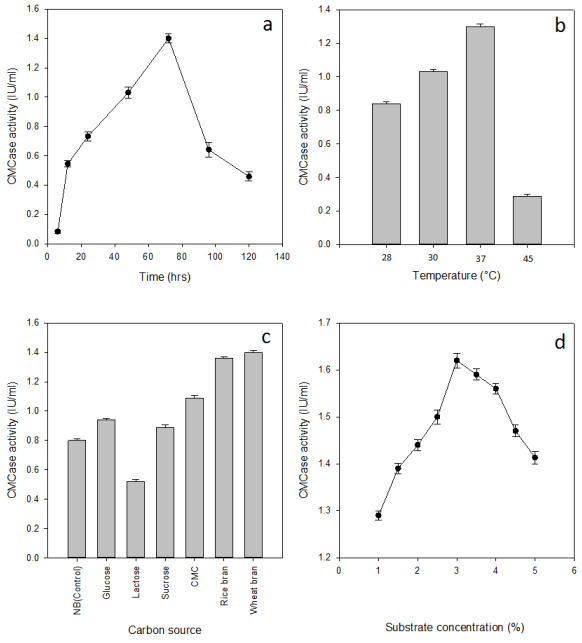

Fig. 2 Effect of (a) incubation time (b) incubation temperature (c) different carbon sources (d) substrate concentration on CMCase activity of *Bacillus tequilensis* S28. Values were expressed as mean ±S.D. of three individual values

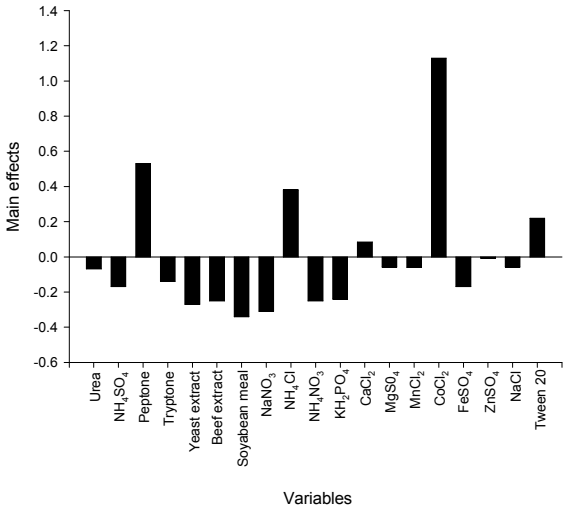

Fig. 3 Main effects of independent variables on CMCase production

It is already reported that yeast extract as well as peptone affect cellulase production up to a significant level [22]. Media components selected on the basis of Pareto chart were further employed as variables in experimental design matrix of Response Surface Methodology. Response Surface Methodology is a statistical tool which involves the use of a group of empirical techniques to evaluate a relationship between number of controlled experimental factors and measured response [20], [23], [24]. Cobalt chloride, peptone, ammonium chloride, Tween-20, yeast extract and calcium chloride were taken into account for designing experimental matrix of Central Composite Design using RSM to study their levels and effect of their interactions on cellulase production. Each variable was studied at five different levels and response observations in accordance with CCD experimental design matrix were displayed in Table II. The following second order polynomial equation explained the CMCase production by taking into account the significant factors and is represented below:

$$\text{CMCase}=+5.76-1.14\times A+0.88\times B-0.17\times C-0.34\times D-0.15\times A\times C+0.24\times A\times D+0.11\times B\times C-0.29\times B\times D+0.16\times C\times D+0.31\times A^2+0.97\times B^2+0.77\times C^2+0.45\times D^2 \quad (5)$$

where A, B, C, D are cobalt chloride, peptone, ammonium chloride and Tween-20 respectively.

Analysis of Variance and coefficients of variance were calculated to verify the significance of the model and shown in Table III. The Model F-value came out to be 238.98 which implied that this model was significant. The chance of occurrence of "Model F-Value" this large due to noise is only 0.01%. Values of "Prob > F" were observed to be less than 0.0500 indicating significant model terms.

In this case A, B, C, D, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are found to be significant model values. The "Lack of Fit F-value" of 1.09 was observed which clearly implied that Lack of Fit value was not significant when compared to the pure error. The chance of observance of "Lack of Fit F-value" this large due to noise is 53.4 % only. The "Pred R-Squared" of 0.9761 was in reasonable agreement with the "Adj R-Squared" of 0.9920.

"Adeq Precision" is a measure of ratio of signal to noise. Usually a ratio greater than 4 is desirable for significant model. Ratio of 52.453 indicated an appropriate signal. The regression based coefficient of determination (R^2) was observed to be 0.99 which is very close to 1 indicating that this model could explain 99% variability of the response. The actual values were in close agreement with the model predicted values of reponse.

3-D response surface plots were drawn to describe relationship between independent variables and the response variable. 3D response surface plots revealed the interactive effect of two independent variables on cellulase production. whereas third variable was kept at basal level (0).

Fig. 4 (a) showed the effect of cobalt chloride and peptone on cellulase production at fixed concentration of ammonium chloride (3 g/L) and Tween-20 (0.75 g/L). Cellulase activity was increased with increase in the concentration of peptone and similar results were observed in Figs. 4 (d) and (e).

TABLE I
RANDOMIZED PLACKETT-BURMAN EXPERIMENTAL DESIGN FOR EVALUATING FACTORS INFLUENCING CMCASE PRODUCTION

Run	A g/L	B g/L	C g/L	D g/L	E g/L	F g/L	G g/L	H g/L	J g/L	K g/L	L g/L	M g/L	N g/L	O g/L	P g/L	Q g/L	R g/L	S g/L	T g/L	Response IU/ml
1	2	2	0	2	0	2	0	0	0	0	1	0.2	0.0	0.1	0.2	0.00	0.00	1	0.5	1.76
2	2	2	0	0	2	2	2	2	0	2	0	0.2	0.0	0.0	0.0	0.00	0.05	1	0.0	0.19
3	2	2	0	2	2	0	0	2	2	2	1	0.0	0.2	0.0	0.2	0.00	0.00	0	0.0	1.85
4	0	2	0	2	0	0	0	0	2	2	0	0.2	0.2	0.0	0.0	0.05	0.05	1	0.5	1.13
5	2	2	2	2	0	2	0	2	0	0	0	0.0	0.2	0.1	0.0	0.05	0.05	0	0.0	0.59
6	2	0	2	2	0	0	2	2	2	2	0	0.2	0.0	0.1	0.0	0.00	0.00	0	0.5	1.34
7	0	2	2	2	2	0	2	0	2	0	0	0.0	0.0	0.1	0.2	0.00	0.05	1	0.0	2.84
8	0	0	0	0	2	2	0	2	2	0	0	0.2	0.2	0.1	0.2	0.00	0.05	0	0.5	2.70
9	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.00	0.00	0	0.0	1.29
10	2	0	2	0	0	0	0	2	2	0	1	0.2	0.0	0.0	0.2	0.05	0.05	1	0.0	2.57
11	2	0	2	0	2	0	0	0	0	2	1	0.0	0.2	0.1	0.0	0.00	0.05	1	0.5	1.58
12	0	0	0	2	2	0	2	2	0	0	1	0.2	0.2	0.1	0.0	0.05	0.00	1	0.0	0.29
13	0	2	2	0	0	2	2	2	2	0	1	0.0	0.2	0.0	0.0	0.00	0.00	1	0.5	1.00
14	2	0	0	2	2	2	2	0	2	0	1	0.0	0.0	0.0	0.0	0.05	0.05	0	0.5	0.97
15	0	0	2	2	2	2	0	2	0	2	0	0.0	0.0	0.0	0.2	0.05	0.00	1	0.5	2.28
16	2	2	2	0	2	0	2	0	0	0	0	0.2	0.2	0.0	0.2	0.05	0.00	0	0.5	2.74
17	0	2	2	0	2	2	0	0	2	2	1	0.2	0.0	0.1	0.0	0.05	0.00	0	0.0	1.42
18	0	2	0	0	0	0	2	2	0	2	1	0.0	0.0	0.1	0.2	0.05	0.05	0	0.5	1.11
19	0	0	2	2	0	2	2	0	0	2	1	0.2	0.2	0.0	0.2	0.00	0.05	0	0.0	1.76
20	2	0	0	0	0	2	2	0	2	2	0	0.0	0.2	0.1	0.2	0.05	0.00	1	0.0	1.55

A: Urea, B: Ammonium sulphate, C: Peptone, D: Tryptone, E: Yeast extract, F: Beef extract, G: Soyabean meal, H: Sodium nitrate, J: Ammonium chloride, K: Ammonium nitrate, L: Potassium dihydrogen phosphate, M: Calcium chloride, N: Magnesium sulphate, O: Manganese chloride, P: Cobalt chloride, Q: Ferrous sulphate, R: Zinc sulphate, S: Sodium chloride, T: Tween-20.

TABLE II
CENTRAL COMPOSITE DESIGN MATRIX WITH EXPERIMENTAL VALUES FOR CMCASE PRODUCTION BY *BACILLUS TEQUILENSIS* S28

	RUN	Type	Cobalt chloride (g/L)	Peptone (g/L)	Ammonium Chloride (g/L)	Tween-20 (g/L)	Response (IU/ml)
BLOCK 1	1	Factorial	0.6	2	2	0.50	06.57
	2	Factorial	0.2	2	2	1.00	08.23
	3	Factorial	0.6	2	4	1.00	06.34
	4	Factorial	0.6	4	2	1.00	07.58
	5	Factorial	0.2	2	4	0.50	08.62
	6	Factorial	0.2	4	2	0.50	11.30
	7	Factorial	0.2	4	4	1.00	10.00
	8	Center	0.4	3	3	0.75	05.55
	9	Center	0.4	3	3	0.75	05.93
	10	Factorial	0.6	4	4	0.50	08.00
BLOCK 2	1	Factorial	0.6	2	4	0.50	05.51
	2	Factorial	0.6	4	2	0.50	08.73
	3	Center	0.4	3	3	0.75	05.75
	4	Factorial	0.6	4	4	1.00	07.29
	5	Center	0.4	3	3	0.75	05.87
	6	Factorial	0.6	2	2	1.00	06.85
	7	Factorial	0.2	2	2	0.50	09.10
	8	Factorial	0.2	2	4	1.00	08.00
	9	Factorial	0.2	4	4	0.50	10.90
	10	Factorial	0.2	4	2	1.00	09.10
BLOCK 3	1	Axial	0.0	3	3	0.75	09.27
	2	Axial	0.4	3	5	0.75	08.56
	3	Axial	0.4	5	3	0.75	11.49
	4	Center	0.4	3	3	0.75	05.72
	5	Axial	0.4	1	3	0.75	07.81
	6	Axial	0.4	3	1	0.75	09.19
	7	Axial	0.8	3	3	0.75	04.79
	8	Center	0.4	3	3	0.75	05.75
	9	Axial	0.4	3	3	0.25	08.27
	10	Axial	0.4	3	3	1.25	06.89

TABLE III
ANOVA RESULTS FOR CMCASE PRODUCTION UNDER RESPONSE SURFACE QUADRATIC MODEL AND MODEL COEFFICIENTS ESTIMATED BY MULTIPLE LINEAR REGRESSION

Source	Sum of Squares	DF	Mean square	F-value	p-value (prob>F)
Model	94.95	14	06.78	0238.98	< 0.0001
A-Cobalt chloride	31.15	01	31.15	1097.63	< 0.0001
B-Peptone	18.44	01	18.44	0649.84	< 0.0001
C-Ammonium chloride	00.69	01	00.69	0024.18	0.0003
D-Tween-20	02.74	01	02.74	0096.38	< 0.0001
AB	00.06	01	0.065	0002.28	0.1548
AC	00.35	01	00.35	0012.50	0.0037
AD	00.92	01	00.92	0032.51	< 0.0001
BC	00.19	01	00.19	0006.81	0.0216
BD	01.31	01	01.31	0046.16	< 0.0001
CD	00.40	01	00.40	0014.19	0.0024
A2	02.68	01	02.68	0094.58	< 0.0001
B2	25.69	01	25.69	0905.30	< 0.0001
C2	16.43	01	16.43	0579.11	< 0.0001
D2	05.56	01	05.56	0195.99	< 0.0001
Residual	00.37	13	0.028		
Lack of Fit	00.29	10	0.029	0001.09	0.5340

Std. Dev.=0.17, R^2=0.9961, Mean=7.77, Adj R^2=0.9920, C.V. % =2.17, Pred R^2=0.9761, PRESS=2.28, Adeq Precision =52.453

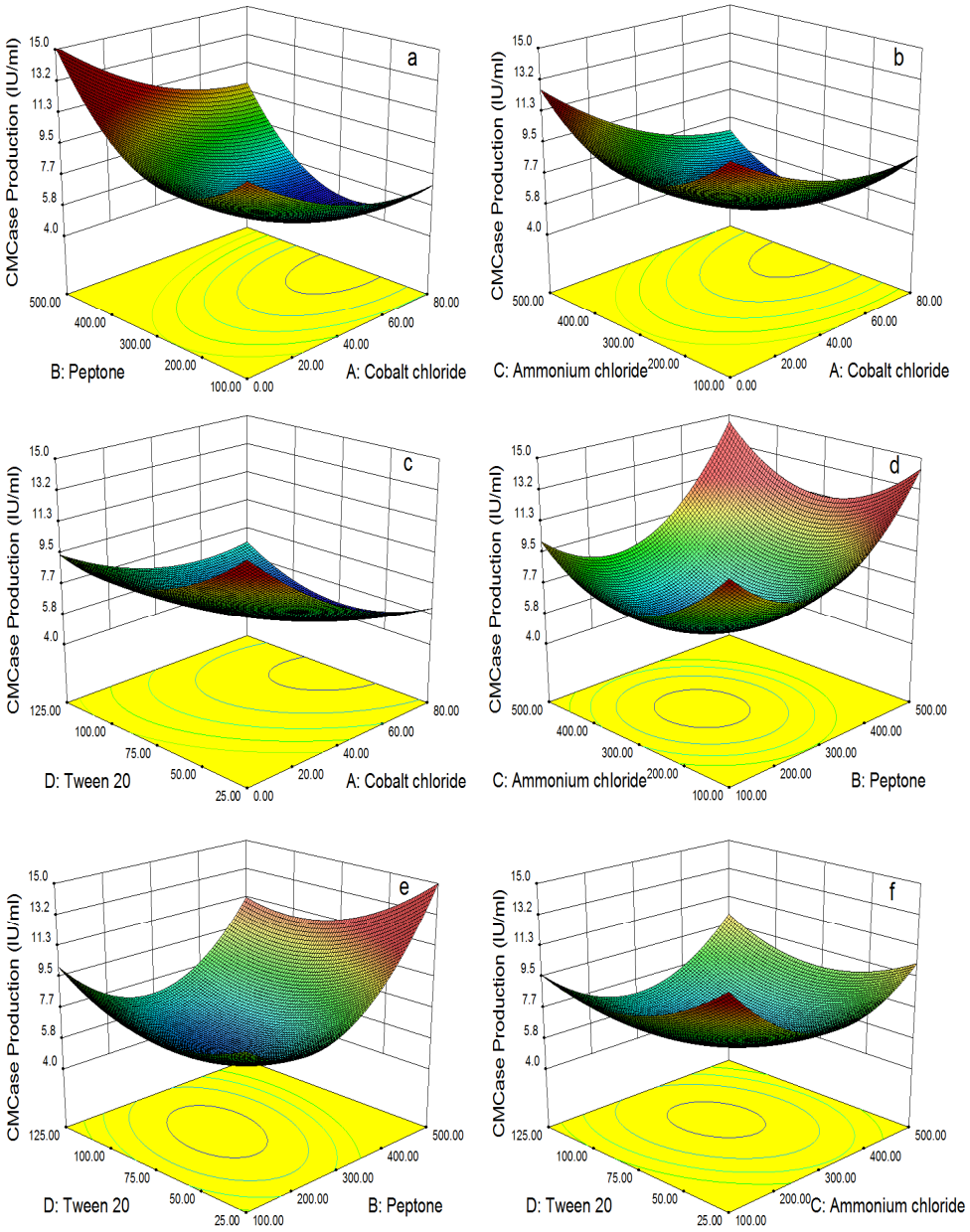

Fig. 4 3-D Surface plots representing CMCase yield from submerged culture of *Bacillus tequilensis* S28 as affected by cultural conditions (a) cobalt chloride and peptone (b) cobalt chloride and ammonium chloride (c) cobalt chloride and Tween-20 (d) ammonium chloride and peptone (e) peptone and Tween-20 (f) ammonium chloride and Tween-20

Dependency of cellulase activity on cobalt chloride and ammonium chloride with fixed concentration of peptone (3.00 g/L) and Tween-20 (0.75g/L) was displayed in Fig. 4 (b). Higher ammonium chloride concentration had a positive effect on cellulase production as observed in Figs. 4 (b) and (d) also. In Fig. 4 (c), effect of cobalt chloride and Tween-20 on cellulase production was depicted with peptone (3.00 g/L) and ammonium chloride (3.00 g/L) as fixed variables. Higher values of cobalt chloride had a negative effect on cellulase production and cellulase activity showed a decrease with increase in concentration of cobalt chloride as depicted in Figs. 4 (a) and (b) also. Effect of peptone and Tween-20 on cellulase production at fixed concentration of ammonium chloride (3.00 g/L) and cobalt chloride (0.40 g/L) was represented in Fig. 4 (e). With increase in Tween-20 concentration, there was increase in cellulase production and similar results were also found in 3D graphs shown in Figs. 4 (c) and (f).

Based on the statistical experimental results, maximum cellulase production was achieved with following media composition: cobalt chloride (0.40 g/L), ammonium chloride (3.00 g/L), peptone (5.00 g/L), Tween-20 (0.75 g/L), calcium chloride (0.20 g/L), yeast extract (2.00 g/L) and 1% (w/v) wheat bran at pH 7.

Temperature is very crucial factor which significantly affect the enzyme production. Similar results were reported for *Bacillus pumilus* EB3 and *B. amyloliquefaciens* DL-3, where 37°C was found to be the optimum temperature for cellulase production [6], [25]. In the present study, pH affected the enzyme production up to significant levels and optimum pH for CMCase production was found to be 7. Rastogi et al. [26] and Deka et al. [27] also reported similar results for cellulase production where pH optimum was found to be 7.2 for cellulase production.

Verification experiments were done to confirm the accuracy of the model. These experiments revealed an increase of CMCase activity from 1.4 U/ml in unoptimized medium or basal medium to 11.5 U/ml in optimized medium. CMCase activity (11.5 IU/ml) under optimized conditions showed an 8.2-fold increase from basal level production (1.4 IU/ml) which was very close to the predicted response value 13 U/ml. The optimized CMCase values obtained in the present study was found to be much higher than most of the bacterial strains already studied [8], [21], [24]-[32]. The optimized media composition for the maximal production of cellulase by this isolate under study comprised of following components: peptone (4.94 g/L), ammonium chloride (4.99 g/L), yeast extract (2.00 g/L), Tween-20 (0.53 g/L), calcium chloride (0.20 g/L) and cobalt chloride (0.60 g/L) with pH 7 at 37°C, agitation speed 150 rpm.

Optimal cellulase activities of different isolates like *Clostridium thermocellum* (5.32 IU/ml), *Bacillus circulans* (4.80 IU/ml), *Bacillus subtilis* (4.64 IU/ml), *Pseudomonas aeruginosa* (3.61 IU/ml), *Clostridium cellobioparum* (3.17 IU/ml), *Serratia* spp. (1.64 IU/ml) and *Erwinia* spp. (1.23 IU/ml) were well reported at pH 6 after incubation at 40°C [12]. Ali et al. [21] had utilized Plackett-Burman Design and RSM for optimizing cellulase production by *Cellulomonas flavigena* and found cellulase activity up to 1.16 U/ml under optimized conditions. RSM based on CCD at two level pattern involving three variables (galactose, malt extract and incubation time) was employed for optimizing cellulase production by *Bacillus pumulis* EWBCM1 by Shankar and Isaiarasu [24]. Maximal cellulase production up to 0.575 IU/ml was obtained under these optimized conditions. Enhancement in cellulase production from basal level 0.425 IU/ml to optimized 0.8 IU/ml by *Geobacillus* sp. was observed by addition of ammonium sulphate and yeast extract utilizing Plackett-Burman factorial design [28]. Khan *et al*. [31] obtained similar findings and media after incubation at 37°C. *Psuedomonas* sp. HP207 was found to possess cellulolytic activity of 1.432 U/ml with yeast extract, bean flour and ammonium chloride observed as favorable media components for its production [33].

C. Effect of pH, Temperature, Metal Ions and EDTA on CMCase Activity

The maximum CMCase activity was observed at pH 5.5 while the enzyme was active at a broad pH range of 5-8 (Fig. 5a). CMCase retained 50% of its maximum activity when pH was dropped to 4. As the pH increased from 5.5, activity decreased further while maintaining more than 60% activity in pH range 5-8. Rastogi et al. [26] reported similar findings for endoglucanase of *Brevibacillus* which was found optimally active at pH 5.5. Several workers have well documented the broad pH range activity of endoglucanases of *Bacillus* sp. [26], [35].

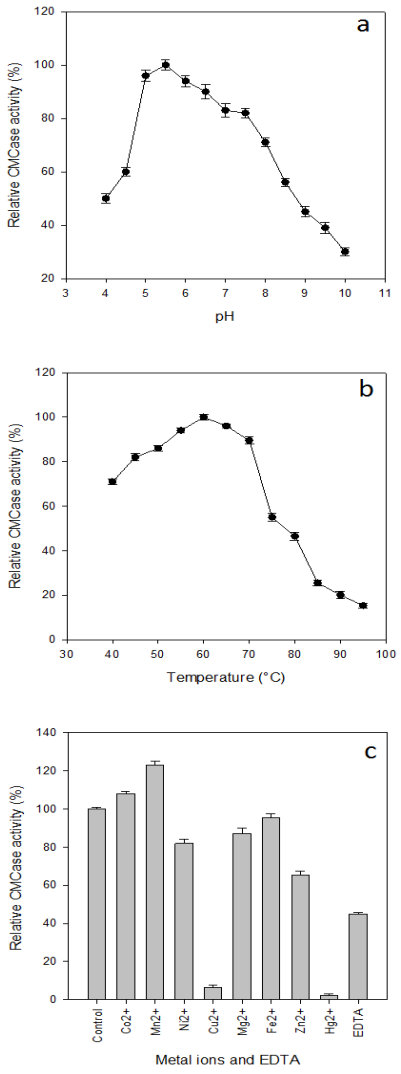

Fig. 5 Activity of crude CMCase of *Bacillus tequilensis* S28 with (a) change in pH (b) change in temperature (c) different additives (metal ions and EDTA with 5mM concentration); 100% activity was expressed as the maximum activity of CMCase observed in pH 5.5 (0.1M Acetate Buffer) at 60°C without additives

The temperature favorable for obtaining maximum CMCase activity was observed to be 60°C. Activity of the enzyme showed a decrease on both the sides when temperature is changed from 60°C. The relative activity of the enzyme at different temperatures was displayed in Fig. 5b. Our results were in compliance with the earlier reports regarding temperature optima for endoglucanases [8], [26], [30], [36].

Relative activities of the enzyme at temperature 70ºC, 80ºC and 90ºC were 80%, 46% and 20% respectively.

Bacillus sp. cellulases generally show different inhibition and activation results of enzyme activity on addition of various metal ions [30], [35]. The effect of different divalent ions and EDTA was observed after incubating the crude enzyme for 30 min and relative activity was calculated. Relative CMCase activity with Mn^{2+}, Mg^{2+}, Co^{2+} Cu^{2+}, Ni^{2+}, Hg^{2+} and EDTA were 123%, 87%, 108%, 6%, 82%, 2% and 45% respectively. Metal ions Mn^{2+} and Co^{2+} had an enhancing effect on the enzyme activity whereas Cu^{2+}, Hg^{2+} and EDTA had strongly inhibited CMCase action (Fig. 5c). Our results were in accordance with the reported enhancing effect of Mn^{2+} and Co^{2+} on the activity of endoglucanase [30], [35]. Hg^{2+} and Cu^{2+} strongly inhibited CMCase activity as observed for *Cellulomonas* sp. ASN2 [30] and *Bacillus* sp. [35].

Though cellulase activity of this isolate is less than fungal counterparts but recently bacteria are explored much for cellulase production as they are present in wide variety of natural habitats and thus posses the ability to withstand environmental stresses. Bacteria have higher growth rate and produce enzyme complements which are stable at extreme temperature and pH. They are less affected by the end product accumulation i.e. feedback inhibition during the cellulose hydrolysis. They can be genetically engineered with greater ease in comparison to fungal cellulases [10], [20], [27]. Literature search clearly indicate that *Bacillus* spp. are good producers of extracellular enzymes [1], [3]. Cellulolytic activity (10 IU/ml) of *Bacillus tequilensis* NRRL B-41771 under submerged fermentation for 7 days was reported by Kamble and Jadhav [34]. *Bacillus tequilensis* S28 strain isolated in our study was able to produce CMCase with 11.5 IU/ml activity after incubation for less time period *i.e*. 72 hours, thus efficient enough to prove its potential in degrading lignocellulosic biomass efficiently.

IV. Conclusion

In this study cellulose degrading bacteria *Bacillus tequilensis* S28 was successfully isolated from soil and optimization of the cultural conditions was done to enhance CMCase production. Optimization was done by employing statistical approach using statistical tools Plackett Burman and Response Surface Methodology. Using these statistical designs, maximum CMCase activity was observed to be 11.5 IU/ml which was 8.2 fold higher than CMCase activity in unoptimized media. The crude CMCase was characterized by observing effect of pH, temperature and metal ions on activity and stability of the CMCase. The enzyme was found to be most active at pH 5.5 and temperature 60ºC. Mn^{2+} was found to be strong activator whereas Hg^{2+} was found to be strong inhibitor for CMCase activity. Therefore present study reveals that *Bacillus tequilensis* S28 is a promising candidate of industrial importance which can be exploited for commercial cellulase production.

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