Valorization of Rice Straw for Optimization of Production of Thermotolerant Cellulase by Parageobacillus Thermoglucosidasius NBCB1 Under Submerged Fermentation

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Research Article

Keywords: Parageobacillus thermoglucosidasius, rice straw, processive endoglucanase, Central Composite Rotatable Design, Artificial Neural Networking, Genetic Algorithm

Posted Date: March 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1450600/v1

Abstract

Rice straw can be a substrate for production of cellulolytic enzymes, a vital step in economical deconstruction of cellulosic biomass in industry. We herein report the optimization of cellulase production by the thermotolerant *Parageobacillus thermoglucosidasius* NBCB1, in rice straw based submerged fermentation. Optimization of process parameters by One Factor At a Time (OFAT) suggested Day 3, medium pH 5.5, 1% (v/v) sorbitol, 1% (v/v) peptone and a cultivation temperature of 60°C as the optimal conditions for cellulase production. Further optimization of effective variables: incubation period, medium pH, sorbitol and peptone concentrations, by Response Surface Methodology (RSM) and Artificial Neural Networking (ANN) improved cellulase yield to 3.106 ± 0.005EU and 3.488 ± 0.004 EU/mg protein, respectively, which were closer to model diagnosed values. OFAT, RSM and ANN improved cellulase production by ~ 6.66, ~ 9.34 and ~ 10 folds, respectively, compared with enzyme production in unoptimized medium, indicating the later two being equally effective in the maximization of cellulase production. Purified cellulase (MW 31 kDa) with pH and temperature optima of 5.5 and 60°C, respectively, retained 100% activity upon pre-incubation at 60°C indicating it's thermostability. It also showed halotolerance and heavy metal tolerance properties. Due to it's versatile nature, the cellulase produced by *P. thermoglucosidasius* NBCB1 could be suitable for industrial degradation of agroresidues.

Introduction

Rice is an important cereal crop, constituting staple food for the majority of global population. Rice straw (RS) is a byproduct of rice cultivation which remains underutilized and accumulates in the environment. It's rapid disposal by burning contributes to air pollution through the emission of green house gases that have hazardous effects on the ecosystem and human health [1]. RS being rich in cellulosic polymers is a source of fermentable sugars for the manufacture of value-added products and it can also be used as substrate for the production of cellulases thus forming a renewable feedstock for valorization [2, 3].

RS is comprised of 30–45% cellulose, 20–25% hemicellulose and 15–20% lignin. The structural organization of cellulose involves collateral and non-collateral alignment of about 30 to 300 linear polymers of glucose through extensive hydrogen bonds and Van der Waals interactions and such structural arrangement greatly influences it's processivity through either chemical or biological techniques [4, 5]. Several bacterial species, such as *Ruminococcus albus*, *Acetivibrio cellulolyticus*, *Erwinia chrysanthemi*, *Thermobifida fusca*, *Caldicellulosiruptor changbaiensis*, *Alicyclobacillus cellulosilyticus*, *Ochrobactrum haematophilum* and *Cellulosimicrobium funkei*, have been reported to depolymerize cellulose by producing cellulase [6]. Cellulase is comprised of three distinct classes of enzymes that synergistically cleave cellulose to monomeric glucose. Endoglucanase (EC 3.2.1.4) cleaves randomly on the internal sites in the cellulose to produce cello-oligosaccharides and the new chain ends thus formed are accessible to cellobiohydrolase or exoglucanase (EC 3.2.1.74). The hydrolysis products of cellobiohydrolase are further broken down to glucose units by β-glucosidase (EC 3.2.1.21) [7–9]. In the cellulolytic enzyme system, β-1,4-endoglucanse and β-1,4-exooglucanse is an important component playing vital role in cellulose degradation [10].

The production of cellulases is a vital step for the economical use of lignocellulosic biomass for various industrial applications. It is highly expensive and contributes to about 50% of the total cellulose depolymerization cost. The lignocellulose residue can be an economic substrate for cellulase production. Moreover, the cellulases functioning at higher temperature are advantageous because of the disorganization of cell wall at higher temperature supporting better penetration of the enzyme, lesser degree of medium viscosity, increased substrate solubility and availability, swift conversion rate and high specific activity [11–13]. Furthermore, optimization of the physicochemical cultural conditions of the microorganisms would enhance the enzyme production, which can be performed in several ways. While the OFAT approach considers a single factor in each step, statistical optimization techniques, such as RSM and ANN consider multiple factors and their interactions, and thus provide the advantage of efficient management of resources and time [14].

RSM is a statistical tool for optimization of complex processes having multiple variables. It is used to design factorial experiments, build experimental models and determine the relative significance of independent variables to a response [15]. ANN is a computational tool that works on the principle of brain neurological system for data learning and training and is known for its resistance to noise, parallel processing capacity and universal function approximation ability [16]. Moreover, ANN based on GA is beneficial as it reduces simulation time and maintains accuracy. Currently, RSM and ANN based on GA have wide applications in biotechnology, including optimization of bioprocesses and enzyme production by microorganisms [17].

Microorganisms thriving in lignocellulose degradation environment have developed cellulolytic enzyme systems that can be exploited for the development of industrially applicable biocatalysts [18]. Vermicomposting is such an environment which includes a caloric active stage for promoting growth of bacterial communities capable of lignocellulosic degradation at elevated temperatures [19]. Present study describes the isolation of an industrially suitable thermophilic cellulolytic bacteria, identified as *Parageobacillus thermoglucosidasius* NBCB1, from a thermotolerant bacterial consortium developed from vermicompost on RS as sole carbon source. The cellulase production by the bacterium growing on RS as substrate was optimized through OFAT, Central Composite Rotatable Design (CCRD) based RSM and GA based ANN approaches.

Materials And Methods

Materials

A thermophilic microbial consortium (RSV) developed from vermicompost using RS as sole carbon source [13] was used for isolation of cellulolytic bacteria. RS was thoroughly washed with distilled water, dried and ground into small (6 ± 2 mm) pieces and stored in zipper lock bags at 4°C until use.

Isolation Of Cellulolytic Bacteria From Rsv Consortium

The consortium was serially diluted (10^{-1} to 10^{-7}) and were spread on Minimal Salt Media-Carboxymethyl cellulose (MSM-CMC) agar plates containing (g/l), Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 5; NH_4Cl, 1; $MgSO_4$, 0.5 and $CaCl_2$, 0.015; and 1% CMC and 2% bacteriological agar. The bacterial colonies obtained after

incubation at 60°C for 24h were sub-cultured weekly in MSM-CMC agar slants and stored at 4°C.

Screening Of Bacterial Isolates For Qualitative And Quantitative Activities

For qualitative assay, the bacterial isolates were spot inoculated in MSM-CMC-agar plate followed by incubation at 60°C for 24 h. The plate was then flooded with 0.1% (w/v) aqueous Congo Red solution. After 30min, the plates were drained and flooded with 0.5M aqueous sodium chloride solution for 30 min [20]. Hydrolytic indices were calculated according to the equation

$$\text{Hydrolyticindex} = \frac{\text{Diameterof(well + clearancezone)}}{\text{Diameterofwell}}$$

(1)

For quantitative determination of activity, the bacterial isolates were individually inoculated in MSM-RS broth in which CMC was replaced by 3% RS and incubated at 60°C and 80 rpm shaking for 24 h. Thereafter, the bacterial culture was centrifuged at 11000xg for 2min at 4°C and the supernatant was used exoglucanase and endoglucanase assay using Avicel and CMC as substrates, respectively [13]. The reaction mixture comprising citrate buffer (pH5.5), 80 mM; Avicel or CMC, 0.1% (w/v) and crude enzyme extract, 0.1ml; was incubated at 60°C under 80 rpm shaking. After 24 h of incubation, 1 ml of dinitrosalicylic acid (DNS) reagent was added followed by incubation in boiling water bath for 10 min. After cooling the reaction mixture at room temperature (RT) the absorbance was measured at 550 nm. One enzyme unit (EU) refers to the quantity of enzyme required to release 1µmole of glucose equivalents per min. under the reaction condition. The total protein (mg/ml) was estimated by the Bradford method using bovine serum albumin (BSA) as standard [21] and was used to calculate the specific activity of the enzyme (EU/mg). One bacterial isolate, NBCB1 was used for further studies on optimization of cellulase production.

Morphological And Molecular Characterization Of Bacterial Isolate Nbcb1

The bacterial isolate NBCB1 was characterized for colony colour, motility, and Gram staining. The morphological features of the isolate were monitored by Scanning Electron Microscopy (SEM). For this the bacterial isolate grown in nutrient broth at 60°C over-night was centrifuged at 2500 x g for 10 min. and the cell pellet was washed with 100 mM phosphate buffer, pH 7.2 (Buffer A). The cells were fixed with 3% glutaraldehyde in Buffer A for 14 h at 4°C followed by centrifugation at 2500 x g for 10 min and washing the cell pellet with Buffer A. The cells were then fixed in 1% osmium tetraoxide for 2-3h at RT. After thorough washing the cells were dehydrated using increasing concentrations of ethanol and then mounted on 0.5cm x 0.5cm cover slips. The sample was sputter coated with gold (Au) nanoparticles in an ion-sputter coater before viewing under SEM (JOEL JSM-IT 100) at 10 mm and 5kV acceleration voltage and 12,000X magnification.

For phylogenetic analysis, genomic DNA was isolated from logarithmic phase of culture using HiPurA™ Bacterial Genomic DNA isolation Kit, following manufacturer's instruction. Universal forward and reverse primers, 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') were used to perform PCR amplification of the 16S rRNA gene (region V3-V4) using the genomic DNA as template. The PCR reaction mixture in a final volume of 25µl contained: 10X buffer, 2.5µl; 50 mM $MgCl_2$, 1µl; 10 mM dNTP mix, 2 µl; and 7 picomole forward and reverse primers, 1 µl each; template DNA, 100 ng; Taq polymerase, 2.5 Units. The reaction condition consisted of initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30 s and extension at 72°C for 1min for every individual cycle and then a final extension at 72°C for 7 min. The PCR amplicon was cloned into pGEMT-Easy vector (Promega, USA) by the manufacturer's protocol and then sequenced using vector specific SP6 and T7 primers. The phylogenetic relationship of the bacterium was determined by comparison of its 16S rDNA sequence with that of closely related neighbor sequences retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI), via BLAST search (https://www.ncbi.nlm.nih.gov/BLAST) [22]. Phylogenetic analysis was done by using the software package MEGA X [23]. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 replicates [24].

Optimization Of Cellulase Production By Ofat

The cellulase production by NBCB1 on MSM-RS was optimized with respect to various process variables, like cultivation time (days), initial medium pH, additional carbon source, nitrogen source, inoculum volume (%) and speed of agitation (rpm), using OFAT approach. Each parameter optimized was incorporated in the subsequent optimization experiments. For all optimization experiments cellulase activity was assayed using Avicel as substrate, and NBCB1 was inoculated at (except for inoculum volume) 1% (v/v).

Incubation period

MSM-RS inoculated with NBCB1 was incubated at 60°C and 80rpm shaking. The culture was withdrawn at one day intervals for five consecutive days, centrifuged at 11000xg for 2min and the enzyme activity was monitored in the culture supernatant.

Initial medium pH

To determine the effect of medium pH on enzyme production the bacterial culture was grown in the MSM-RS of pH values 4.5 to 8.5 at 60°C and 80rpm shaking for 72h. Thereafter, cellulase assay was performed with the culture supernatant.

Carbon source

The bacterial culture was grown in the production medium supplemented with 1% (w/v) of either of the carbon compounds like D-sorbitol, D-mannitol, meso-inositol, native cellulose, Avicel, CMC and trisodium citrate, at 60°C and 80rpm shaking for 72h. Then enzymatic activity was monitored in the culture supernatant. The carbon compound showing the highest enzyme production was varied further to determine its optimum level.

Nitrogen source

To determine the effect of nitrogen source on enzyme production, the bacterium was grown in the production medium supplemented with 1% (w/v) of either of the nitrogen sources, such as beef extract, yeast extract, peptone, casein and ammonium molybdate, followed by the determination of enzyme activity in the culture supernatant. The nitrogen compound showing highest enzyme production was varied further to determine its optimum level.

Inoculum volume

The bacterial inoculum at 0.5–2% (v/v) was added to optimized production medium and grown at 60°C and 80rpm shaking for 72h, after which cellulase assay was performed with the culture supernatant.

Agitation speed

The bacterial culture was grown in the optimized medium at 60°C for 72h at either of the agitation speed, such as 0, 30 and 80rpm. Then enzyme assay was performed with the culture supernatant.

Optimization Of Cellulase Production Through Ccrd Based Rsm

CCRD combined with RSM is a second order design which assesses each parameter at two levels. It increases the number of experimental runs for every additional parameter it analyzes [25]. CCRD has three categories of design points: axial points (2m) which produce quadratic terms, factorial points (2^m) which contain varied ratios of both the high and the low factor levels, and centre points which compute error values that are generated while experimenting. If 'N' number of experiments were conducted with 'm' number of process parameters and 'n' number of repetitions, then the equation

$$N = m^2 + 2m + n$$

(2)

would give the relation between the design points [26]. CCRD model can be represented by the popular quadratic equation

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \sum_{i=1}^{k} \sum_{i \neq y=1}^{k} \beta_{ij} x_i x_j + \epsilon$$

(3)

where Y is the response, 'i' and 'j' are linear quadratic coefficients, ' β_0', ' β_i', ' β_{ii}' and ' β_{ij}' are the constant, linear, interactive and quadratic coefficients, respectively, ' x_i', 'x_{ii}' and ' x_j' are the quantitative variables and 'Σ' is the error value [27].

The production of cellulase was optimized by CCRD-based RSM, which studied the interaction among four effective parameters of OFAT i.e. incubation time (2–4 days), medium pH (4–8), sorbitol concentration (0.5–1.5%) and peptone concentration (0.5–1.5%). Each factor was analyzed at a low (-2) and a high (+ 2) level with a total of 30 runs. The Design Expert Software (Version 8.0.7.1, State-Ease, Minneapolis, MN, USA) was used for statistical analysis by RSM.

Artificial neural networking and global optimization

An ANN consists of an assemblage of input, hidden and output layers and is used to analyze multiple regression models according to a GA. It arbitrarily selects 70% of the data for training, 15% of the data for concurrent validation and the remaining 15% for testing [28]. GA can be performed on non-linear models with bounds (-2 or + 2 levels), to find an optimal solution for a response. It starts with a primary number of chromosomes which are basically matrices containing 'z' numbers of genes or values. Chromosomes give rise to newer values through cyclical customization of genes over 'k' number of generations (s_1^k, s_2^k, s_3^k, ...s_z^k), until a point of convergence is reached where there is minimal difference between the solutions [29]. Obtained data was fed into an ANN for further analysis. The neural network toolbox of the Matlab R2020b (https://in.mathworks.com/products/matlab.html) was used to create a bilayered model with 12 neurons that would functions on a back-propagation algorithm. Weights and biases were adjusted according to the Levenberg-Marquardt (TRAINLM) function which gives a higher degree of error minimization within 1000 epochs. LEARNGDM, mean square error (MSE) and *tansig* were used as the adaption learning function, performance function and the transfer function respectively. Stepwise reduction of insignificant terms was performed for further improvement of the model. Statistics such as R^2, root mean squared error (RMSE), F-value and p-value were obtained through model fitting.

Purification Of Cellulase And Its Characterization

NBCB1 was inoculated into the optimized MSM medium except for replacement of RS by 1% Avicel (Sigma) and cultured at 60°C at 80rpm for 72h. Cellulase from the culture supernatant was purified by ammonium sulphate precipitation, DEAE-Sephacel ion exchange chromatography and gel filtration. The culture supernatant was subjected to 0–75% ammonium sulphate precipitation and the protein pellet obtained after centrifugation was suspended in 2.5ml of 50mM sodium citrate buffer, pH5.5 (Buffer B). The enzyme extract was loaded onto gel filtration column (Biogel P-100, 30 x 1 cm) and eluted with Buffer B into 2ml

fractions. The fractions with cellulase activity were pooled and loaded onto DEAE-Sephacel ion exchange column (7 x 1.5 cm) pre-equilibrated with Buffer B. The bound protein was eluted in step gradient using Buffer B containing 0.15 and 0.30M NaCl into 2ml fractions and all the fractions were analysed for cellulase activity. The crude, ammonium sulphate, and active enzyme fractions of gel filtration and ion-exchange chromatography were analyzed by SDS-PAGE, following the method of Laemmli [30] and the gel was stained by silver staining [31].

The purified enzyme was characterized with respect to pH optima and stability, temperature optima and stability and the effects of metals and denaturants. For the determination of pH optima, cellulase assays was performed at 60°C using different buffers: 100mM citrate buffer (pH3.5, 4.5, 5.5, 6.5) and 100mM Tris buffer (pH7.5, 8.5, 9.5). The pH stability was determined by pre-incubating the enzyme in buffers of indicated pH for 1h followed by monitoring cellulase activity. The temperature optimum was monitored by assaying the enzyme at different temperatures (4°C to 80°C). Temperature stability was determined by pre-incubating the enzyme in 100mM citrate buffer (pH5.5) at different temperatures for 1h followed by assaying the enzyme at optimum pH and temperature.

The effect of metal ions, additives and other metabolites on cellulase activity was tested by carrying out standard enzymatic assay in the presence of 2mM and 5mM of metallic chlorides (NaCl, $FeCl_3$, NH_4Cl, $SnCl_2.2H_2O$, $NiCl_2.6H_2O$, $CoCl_2.6H_2O$, $MnCl_2.4H_2O$, $CuCl_2.2H_2O$, $MgCl_2$, $CaCl_2.2H_2O$, KCl, $ZnCl_2$, $HgCl_2$), 5mM of either of additives, like sodium dodecyl sulphate (SDS), ethylene diamine tetraacetic acid (EDTA), β-mercaptoethanol (β-ME) and hydrogen peroxide (H_2O_2) and 5mM of either of the carbohydrate, like dextrose, cellobiose, xylose and maltose, in the reaction mix.

Statistical analysis

Mean and standard deviations were calculated from the results of triplicate experiments, and comparison of means was performed through one way ANOVA and Tukey's HSD tests with the help of IBM-SPSS (version 28.0.0.0) at a significance level of 95% ($p < 0.05$) [20].

Results

Isolation of cellulolytic bacteria from thermophilic consortium

In this study, the bacterial strains isolated from a vermicompost derived thermophilic consortium (RSV) developed on RS as substrate [13] were assessed for cellulolytic capacity. Among the bacterial isolates with the ability to depolymerize cellulosic substrates, NBCB1 showing qualitative (hydrolytic index = 2.25) (Fig. 1a) and highest quantitative (0.028 EU/ml, 0.331 ± 0.004 EU/mg for Avicel and 0.023 EU/ml, 0.269 ± 0.006 EU/mg for CMC) (Table 1) activities, was selected for further experiments. Since Avicel gave higher cellulase activity as a substrate, it was used as a substrate for further biochemical experiments.

Table 1
Quantitative screening of bacterial isolates for cellulase activity using Avicel and CMC as substrates.

Serial Number	Bacterial isolates	Hydrolytic index (Congo Red assay)	Avicel		CMC	
			Activity (EU/ml)*	Specific activity (EU/mg)*	Activity (EU/ml)*	Specific activity (EU/mg)*
1	CCB1	1.82	0.021 ± 0.002	0.248 ± 0.023	0.016 ± 0.000	0.189 ± 0.004
2	CCB2	2.20	0.024 ± 0.005	0.280 ± 0.067	0.007 ± 0.001	0.081 ± 0.023
3	CCB3	1.89	0.024 ± 0.002	0.278 ± 0.034	0.009 ± 0.000	0.103 ± 0.004
4	NBCB1	2.25	0.028 ± 0.000	0.331 ± 0.004	0.023 ± 0.000	0.269 ± 0.006
5	NBCB2	2.00	0.023 ± 0.002	0.272 ± 0.015	0.020 ± 0.002	0.236 ± 0.014
6	RAC	1.73	0.022 ± 0.005	0.253 ± 0.062	0.019 ± 0.003	0.218 ± 0.028
7	XLN1	2.18	0.022 ± 0.003	0.280 ± 0.061	0.020 ± 0.001	0.250 ± 0.009
8	XLN2	2.00	0.022 ± 0.003	0.266 ± 0.045	0.022 ± 0.000	0.265 ± 0.011
*1 unit (EU) of enzyme activity refers to the quantity of enzyme required to release 1µmole of glucose equivalents per minute under the reaction conditions.						

Morphological And Phylogenetic Characterization Of Isolate Nbcb1

NBCB1 formed tiny, round, opaque, white colonies with concave margins and was Gram positive. In SEM it appeared as medium rods of 4 to 5µM in length and 0.55 to 0.60µM in width (Fig. 1b). Phylogenetic analysis based on comparison of 16SrDNA sequence with that of other closely related neighbors showed that the isolate NBCB1 encompass to the branch containing members of genus *Parageobacillus* and was most closely related with *Parageobacillus thermoglucosidasius* with more than 98% sequence similarity (Fig. 1c). Hence, the bacterial isolate was named as *Parageobacillus thermoglucosidasius* strain NBCB1 (GenBank accession no. MN749529).

Optimization Of Enzyme Production By Ofat

The cultural parameters are known to directly affect bacterial metabolic processes and hence, the cellulase production by *P. thermoglucosidasius* NBCB1 on MSM-RS was optimized by OFAT with respect to various process variables, such as incubation time, initial medium pH, additional carbon source, nitrogen

source, inoculum volume and speed of agitation. The bacterium showed an active enzyme production for five days. The cellulase activity progressively increased from Day1 to Day3 (0.089 ± 0.017EU/ml, 0.998 ± 0.056EU/mg) and subsequently decreased (Fig. 2a). Therefore, Day3 was chosen as optimum incubation time for further analysis of process variables. For studying the effect of pH on enzyme production the bacterial culture was grown on MSM-RS of varying pH (4.5 to 8.5). Maximum enzyme production of (0.107 ± 0.014EU/ml, 1.100 ± 0.165EU/mg) was noted at pH5.5 (Fig. 2b). Although pH4.5 and pH6.5 didn't have significant impact, growing the cells at pH7.5 and 8.5 retarded the enzyme yield. While optimizing the medium for additional carbon compound, the highest enzyme production of 0.146 ± 0.003 EU/ml, 1.530 ± 0.027 EU/mg was obtained on addition of 1% sorbitol (Fig. 2c). Peptone (1%) was the best nitrogen source for enzyme production (Fig. 2d). Altogether, *P. thermoglucosidasius* NBCB1 inoculated at 1% (v/v) into MSM-RS supplemented with 1% peptone and 1% sorbitol, followed by growth at 60°C for 3 days, under 80rpm shaking gave maximum cellulase yield of 0.185 ± 0.004 EU/ml and 2.484 ± 0.104 EU/mg (Fig. 2e, 2f).

Optimization Of Cellulase Production Using Ccrd Based Rsm

Enzyme production was further optimized using CCRD based RSM that studied the interaction between four effective parameters selected from the OFAT approach, namely, the incubation time (Days) (A), initial pH of medium (B), sorbitol concentration (%) (C) and peptone concentration (%) (D). A CCRD with eight axial points, six central points, sixteen factorial points and a total of 30 runs was prepared (Table 2) and Analysis of Variance (ANOVA) was performed (Table 3) on it. The result of ANOVA showed the model to be highly significant ($p < 0.0001$) and can better predict the data. Within the model, A (incubation period), B (medium pH), C (sorbitol concentration), D (peptone concentration), AB (incubation period x pH), AC (incubation period x pH), AD (incubation period x peptone concentration), BC (pH x sorbitol concentration), CD (sorbitol concentration x peptone concentration), A^2 (incubation period^2), B^2 (pH^2), C^2 (sorbitol concentration^2) and D^2 (peptone concentration^2) were considered as significant ($p < 0.0001$) model terms. Using the result of ANOVA, a second order regression equation was generated in terms of coded and actual factors as shown by Eq. 4 and Eq. 5, respectively.

Table 2
Enumeration of predicted and actual responses for both the CCRD-RSM and ANN-GA based models.

Run	Space Type	A*	B*	C*	D*	Response (EU/ml)**	Predicted for RSM (EU/ml)**	Predicted for ANN (EU/ml)**
1	Factorial	4	7	1.5	1.5	0.086	0.088	0.082
2	Center	3	6	1	1	0.264	0.255	0.274
3	Axial	3	4	1	1	0.183	0.182	0.187
4	Axial	3	6	0	1	0.145	0.141	0.146
5	Center	3	6	1	1	0.257	0.255	0.266
6	Factorial	2	7	0.5	1.5	0.092	0.101	0.088
7	Factorial	2	7	1.5	1.5	0.077	0.082	0.072
8	Factorial	2	5	1.5	1.5	0.105	0.110	0.102
9	Factorial	4	5	0.5	1.5	0.242	0.253	0.250
10	Factorial	2	5	0.5	1.5	0.156	0.154	0.158
11	Center	3	6	1	1	0.254	0.255	0.263
12	Axial	3	6	1	0	0.189	0.192	0.193
13	Center	3	6	1	1	0.253	0.255	0.262
14	Axial	3	6	1	2	0.227	0.222	0.234
15	Factorial	4	5	1.5	1.5	0.176	0.168	0.179
16	Axial	3	8	1	1	0.052	0.049	0.045
17	Factorial	4	7	0.5	0.5	0.127	0.128	0.126
18	Factorial	2	7	1.5	0.5	0.077	0.071	0.072
19	Factorial	4	7	1.5	0.5	0.103	0.102	0.100
20	Center	3	6	1	1	0.251	0.255	0.26
21	Axial	1	6	1	1	0.036	0.025	0.028
22	Factorial	4	5	1.5	0.5	0.186	0.182	0.190
23	Factorial	4	5	0.5	0.5	0.24	0.232	0.248
24	Factorial	4	7	0.5	1.5	0.155	0.147	0.156
25	Center	3	6	1	1	0.252	0.255	0.261
26	Factorial	2	5	1.5	0.5	0.094	0.099	0.090
27	Factorial	2	7	0.5	0.5	0.051	0.056	0.044
28	Axial	3	6	2	1	0.071	0.072	0.066
29	Factorial	2	5	0.5	0.5	0.106	0.109	0.103
30	Axial	5	6	1	1	0.148	0.156	0.149

*A: Incubation period (Days), B: medium pH, C: Sorbitol concentration (%), D: Peptone concentration (%)

**1 unit (EU) of enzyme activity refers to the quantity of enzyme required to release 1µmole of glucose equivalents per minute under the reaction conditions.

Table 3
ANOVA for the CCRD model generated through RSM.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value	
Model	0.1586	14	0.0113	199.37	< 0.0001	significant
A-Incubation period	0.0254	1	0.0254	447.32	< 0.0001	
B-pH	0.0266	1	0.0266	468.17	< 0.0001	
C-Sorbitol concentation	0.0071	1	0.0071	125.09	< 0.0001	
D-Peptone concentration	0.0014	1	0.0014	24.03	0.0002	
AB	0.0027	1	0.0027	48.05	< 0.0001	
AC	0.0016	1	0.0016	28.51	< 0.0001	
AD	0.0006	1	0.0006	10.78	0.0050	
BC	0.0006	1	0.0006	11.22	0.0044	
BD	6.250E-08	1	6.250E-08	0.0011	0.9740	
CD	0.0012	1	0.0012	20.65	0.0004	
A^2	0.0463	1	0.0463	815.23	< 0.0001	
B^2	0.0331	1	0.0331	581.91	< 0.0001	
C^2	0.0377	1	0.0377	664.25	< 0.0001	
D^2	0.0040	1	0.0040	70.61	< 0.0001	
Residual	0.0009	15	0.0001			
Lack of Fit	0.0007	10	0.0001	3.21	0.1049	not significant
Pure Error	0.0001	5	0.0000			
Cor Total	0.1594	29				
Predicted R^2 = 0.9723, Adjusted R^2 = 0.9897, Adequate precision = 43.0508						

$$Y = +0.2552 + 0.0325A - 0.0333B - 0.0172C + 0.0075D - 0.0131AB - 0.0101AC - 0.0062AD + 0.0063BC - 0.0001BD - 0.0086CD - 0.($$

(4)

$$\text{Activity(EU/ml)} = -1.73172 + 0.389979*\text{Incubationperiod} + 0.410021*\text{pH} + 0.281208*\text{Sorbitolconcentration} + 0.183958*\text{Peptoneconce}$$

(5)

The fit of the model can be evaluated by using various criteria, like predicted and adjusted R^2, adequate precision, and 'lack of fit'. There was a practicable difference of < 0.2 between the predicted R^2 (0.9723) and the adjusted R^2 (0.9897). An insignificant 'Lack of Fit' (3.21) corresponding to pure error and a low p-value (P < 0.05) determined the compatibility of the model. 'Adequate precision' is the measure of the ratio of signal to noise and a ratio of more than 4 is desirable. A considerable adequate precision value of 43.0508 indicates an adequate signal. Hence, the model can be used to navigate the design space.

Analysis of effects of interaction between independent factors and localization of optimum condition for cellulase production and validation of the models

The polynomial regression equation in terms of actual factors was used to make 3-D plots with the response (cellulase activity in EU/ml) represented on the Z-axis and two factors ranging from low to high levels (-2 to + 2) on the X- and Y-axis, while other two factors were kept constant. Graph AB (Fig. 3a) gave a response of 0.264EU/ml on Day3 at pH5.5 when both sorbitol and peptone concentrations were constant (1% w/v). A further increase in incubation period resulted in decrease in enzyme yield (Fig. 3b). All the considered factors were relevant factors by themselves (p-value < 0.05). There was sufficient interaction between incubation period and peptone concentration (p-value of AD = 0.005) (Fig. 3c) and between pH and sorbitol concentration (p-value of BC = 0.0044) (Fig. 3d). The variables pH and peptone concentration interacted a bit less with each other (p-value = 0.974). Higher pH (= 7) was detrimental to enzyme yield (Fig. 3e) while a slightly acidic pH was considered ideal. Sorbitol (p-value < 0.0001) and peptone (p-value = 0.0002) concentrations were found to influence enzyme activity and they may function as autonomous factors as well (p-value of CD = 0.0004) (Fig. 3f).

Further evaluation of model efficiency was offered by diagnostic plots. Externally studentized residuals are the ratios of residuals, or differences between predicted and actual values, to the standard deviation obtained per run and give a highly sensitive measure of model leverage. The normal plot (Supplementary Fig. 1a) showed the externally studentized residuals aligned along a straight line. Thereby model had a normal distribution pattern. Residuals vs predicted plot (Supplementary Fig. 1b) measures the uniformity of variance. Since the externally studentized residuals were randomly scattered around the mean, they were said to have constant variation. The possibility of false positives was ruled out by the residuals vs run plot (Supplementary Fig. 1c). Obtained

responses were the outcome of the interactions between the selected parameters only and did not arise due to background variables (noise). Also predicted values largely coincided with the actual values (Supplementary Fig. 1d**)**, which is indispensable for an experiment's success.

Numerical validation of the model was performed (Table 4) and the run with the least differences between it's obtained value and suggested value, was chosen as the optimum. Accordingly, a MSM-RS broth culture supplemented with each of 1.095% sorbitol and 1.103% peptone, with a final medium pH of 5.036 were inoculated with fresh 1% (v/v) inoculum and incubated at 60°C, 80rpm for 3.371 days. Response obtained amounted to 0.264 ± 0.008EU/ml, which was close to the predicted value of 0.260EU/ml and corresponded to a specific activity of 3.106 ± 0.005EU/mg.

Table 4
Validation of the CCRD-RSM and ANN-GA models for cellulase production by *P.thermoglucosidasius* NBCB1.

Serial number	A*	B*	C*	D*	Response (EU/ml)**	Predicted for RSM (EU/ml)**	Predicted for ANN (EU/ml)**
56	3.371	5.036	1.095	1.103	0.264 ± 0.008	0.260	0.254
27	3.595	5.643	0.541	0.801	0.263 ± 0.012	0.256	0.253
11	4	5	0.5	1.5	0.257 ± 0.004	0.253	0.256
49	3.376	6.216	0.696	0.863	0.258 ± 0.008	0.246	0.247
20	2.819	5.577	1.142	0.77	0.243 ± 0.010	0.241	0.241
25	2.635	5.008	0.799	1.211	0.245 ± 0.006	0.237	0.240
1	3.331	5.709	1.355	0.871	0.240 ± 0.012	0.234	0.238
29	3.076	6.461	0.868	0.801	0.232 ± 0.009	0.230	0.229
40	3.333	6.403	1.25	1.139	0.216 ± 0.016	0.222	0.212
85	2.416	5.641	1.165	1.074	0.228 ± 0.011	0.219	0.224
*A: Incubation period (Days), B: pH, C: Sorbitol concentration (%), D: Peptone concentration (%)							
**1 unit (EU) of enzyme activity refers to the quantity of enzyme required to release 1µmole of glucose equivalents per minute under the reaction conditions.							

Model Training And Performance Via Ann-ga

The *nntool* program (Matlab R2020b) was used to frame an ANN to examine a data set consisting of four numeric inputs, incubation time (Days) (X1), medium pH (X2), sorbitol concentration (%) (X3) and peptone concentration (%) (X4) and their corresponding outputs (EU/ml). The data set was refined (trained) over consecutive iterations or epochs in order to minimize as much error as possible. Training was halted when the data was at risk of overfitting and the model could not be developed further. Trained data was verified or validated for the fulfillment of all specifications and an error arose if there was a failure. The MSE values are required to be closer to 1.0 for the model to be approved. As shown in Fig. 4a, epoch 2 had the lowest error value ($1.6325e^{-3}$) wherein model training and validation was considered complete and the respective curves were generated.

The error histogram (Fig. 4b) equally divided the total error (ranging from − 0.07562 to 0.0658) into 20 bins, being represented by vertical bars. The regression coefficient (R = 0.99573) was obtained from the training plot (Fig. 4c) which is similar to the predicted vs actual value curve in RSM and it was nearer to 1. Presence of a low RMSE (0.00615), model F-value (1972.008), model *p*-value (< 0.0001) and reasonable $R^2_{unadjusted}$ (0.999773) and $R^2_{adjusted}$ (0.999764) indicate an appropriate Goodness of fit and established the data set to be an efficient model (Fig. 4d).

The global optimizer was run according to the selected toolbox parameters (Supplementary table 1) until there was a constancy in values. GA gave a singular arrangement of solutions corresponding to a singular response during the 20th generation, when the solutions had negligible differences between them (convergence point). According to GA, the optimum conditions consisted of an incubation time of 3.5271 days, an acidic pH of 5.4392, a sorbitol concentration of 0.9879% and a peptone concentration of 1.01645% yielded 0.286 ± 0.002EU/ml of cellulase activity, which was close to the predicted value of 0.273 EU/ml and corresponded to a specific activity of 3.488 ± 0.004 EU/mg (Table 5).

Table 5
Optimum culture parameters for maximum cellulase production as depicted by each of OFAT, RSM and ANN strategies.

Design	A*	B*	C*	D*	Optimum activity		Specific activity (EU/mg)**
					Predicted value (EU/ml)**	Experimental value (EU/ml)**	
OFAT	3	5.5	1	1	-	0.183 ± 0.003	2.415 ± 0.053
RSM	3.37	5.04	1.1	1.10	0.260	0.264 ± 0.008	3.106 ± 0.005
ANN-GA	3.53	5.44	0.99	1.02	0.273	0.286 ± 0.002	3.488 ± 0.004
*A: Incubation period (Days), B: medium pH, C: Sorbitol concentration (%), D: Peptone concentration (%)							
**1 unit (EU) of enzyme activity refers to the quantity of enzyme required to release 1µmole of glucose equivalents per minute under the reaction conditions.							

Purification And Characterization Of Cellulase

Cellulase was purified from the culture supernatant of *P. thermoglucosadius* NBCB1 by ammonium sulphate precipitation, gel filtration and ion exchange chromatography. The enzyme with purification fold and specific activity of 5.22 and 12.86EU/mg, respectively, was used for characterization. SDS-PAGE analysis of the purified enzyme fractions at various purification steps showed the presence of a single band of about 31kDa at both gel-filtration and ion-exchange chromatography steps (Fig. 5a). Cellulase was functional over a range of pH(3.5 to 9.5) with optimum activity at pH5.5 wherein it had a specific activity of 12.87EU/mg. Higher acidity (pH3.5) or alkalinity (pH9.5) were found to be detrimental with about 90 and 70% reduction in enzymatic activity, respectively. Enzyme stability was also highest at pH5.5 wherein it gave 19.45 EU/mg (Fig. 5b). The enzyme showed temperature optima of 60^{o}C (12.87 EU/mg) and the activity decreased at incubation temperature greater and lower than the optimum temperature. Consequently, the activity became 3.433 and 3.719 EU/mg at 4 and 80^{o}C, respectively (Fig. 5c).

The cellulase produced by *P.thermoglucosidasius* strain NBCB1 was found to be resistant to most inhibitors and was only mildly inhibited by galactose (10.87 EU/mg), xylose (9.440 EU/mg) and cellobiose (8.435 EU/mg). Mannose had no effect while dextrose slightly enhanced the activity (13.731EU/mg) (Fig. 5d). Of the additives, SDS and β-ME increased specific activity to 16.877 and to 23.742 EU/mg, respectively while H_2O_2 decreased it to one-third (4.576 EU/mg). EDTA decreased enzymatic activity to 8 EU/mg which established that the enzyme was a metalloprotein and needed metals for proper functioning.

Of the metallic chlorides that were added to the reaction mix, NaCl, $SnCl_2.2H_2O$, $MgCl_2$, $CaCl_2.2H_2O$ and $ZnCl_2$ were found to augment the enzyme specific activity and upon increasing their concentration from 2mM to 5mM, raised the specific activity from 18.307 to 31.180EU/mg, 15.446 to 19.165 EU/mg, 23.742 to 31.180 EU/mg, 28.605 to 33.183 EU/mg and 35.754 to 46.913 EU/mg respectively. NH_4Cl, KCl and $HgCl_2$ on the other hand were found to decrease specific activity from 6.293 to 5.435 EU/mg, 10.011 to 5.435 EU/mg and 8.295 to 6.293 EU/mg respectively (Fig. 5e).

Discussion

Microorganisms in lignocellulolytic environment produce cellulases for biodegradation of natural biomass into fermentable reducing sugars [10]. In our study, a cellulase producing thermophilic bacterium, *Parageobacillus thermoglucosidasius* NBCB1 was isolated from a thermophilic bacterial consortium bred from vermicompost using RS as substrate. *P.thermoglucosidasius* has earlier been isolated from soil [32], marine environment [33], compost [34], wood wastes [35] and hot springs [36]. However, there are limited reports on the production of enzymes by the organism. Although the cellulolytic ability of *P.thermoglucosidasius* NBCB1 was assessed by CMC-based Congo Red assay, it showed *in vitro* cellulase activity with both CMC and avicel as substrates. Endoglucanases capable of dual action on CMC and microcrystalline cellulose were previously reported and are referred to as processive endoglucanases [37]. As in the present study, *Bacillus subtilis* [38, 39] and *Clostridium cellulosi* [40] rapidly degraded cellulose through exoglucanase and endoglucanase activity, however, their genome did not possess any apparent homologues to known exo-glucanases. Similarly, the genome of *P.thermoglucosidasius* has revealed only the homologue to endoglucanase (https://www.ncbi.nlm.nih.gov/protein/?term=parageobacillus+thermoglucosidasius+cellulase) and hence, the simultaneous endoglucanase and exoglucanase activities indicate the expression of processive endoglucanase by the bacterium.

Bacterial production of enzymes is an inducible process and is affected by physicochemical parameters of cultural conditions. Optimization of cultural conditions of *P. thermoglucosidasius* by OFAT approach suggested the bacterium inoculated at 1% (v/v) into MSM-RS (pH 5.5) supplemented with 1% peptone and 1% sorbitol, followed by growth at 60°C for 3 days, under 80 rpm shaking gave maximum cellulase yield of 2.484 ± 0.104 EU/mg which is ~ 6.66 fold increase compared to the unoptimized activity. The starting volume of inoculum is important as lesser volumes slow microbial growth rate and enzyme yield and greater volumes cause intracellular antagonism for nutrients [41]. The medium pH not only influences enzyme production but also required for protonic transfer among the amino acids at the active site, and thus contributes to active site conformations to increase or decrease activity [42]. Agitation speed is another relevant factor due to it's influence on oxygen availability to the cells through bubble formation and a higher speed is desirable due to the increase in contact area between air and broth surface [43]. Sorbitol has been earlier reported to impart osmotolerance, thermotolerance and ethanol tolerance while promoting bacterial cell growth and protein synthesis [44].

The physicochemical variables showing the strongest influence on cellulase production in OFAT were further selected for their statistical optimization by CCRD-based RSM and ANN-GA keeping the level of the other variables same as in OFAT. RSM proved significant interactions between the influencing factors: incubation period (days), medium pH, sorbitol concentration (%) and peptone concentration (%). It stated 3.37 days, pH5.04, 1.1% sorbitol and 1.1% peptone as the optimum culture conditions which gave ~ 9.34 fold the unoptimized yield (0.264 ± 0.008 EU/ml, 3.106 ± 0.005 EU/mg), whereas ANN stated 3.53 days, pH 5.44, 0.99% sorbitol and 1.02% peptone as the optimum and yielded ~ 10 times the unoptimized yield (0.286 ± 0.002EU/ml, 3.488 ± 0.004EU/mg) (Table 5). A comparison of the predicted values generated by both methodologies showed that they had little difference with the actual responses. R^2 coefficients from both RSM and ANN were within reasonable range. Besides ANN gave only a ~ 1.2 fold rise in enzyme production over RSM. Therefore, it was determined that both techniques were efficient and could be used for pattern recognition and data processing.

The cellulase produced by NBCB1 remained active in a wide range of pH (3.5–9.5) and temperature (4-80^{o}C), which is a desirable characteristic for industrial applications [45]. The enzyme had pH and temperature optima of 5.5 and 60^{o}C, respectively, indicating it's acidophilic, thermotolerant nature. The enzyme showed significant enhancement in activity in presence of NaCl, $SnCl_2.2H_2O$, $MgCl_2$, $CaCl_2.2H_2O$ and $ZnCl_2$. Previously alkali metals such as Na, K, and alkaline earth metals such as Mg and Ca are reported to assist in structural modification of proteins via alteration of folding patterns and may enhance or retard their activity [46]. Mg increases enzyme activity through interactions with ligands and active sites and inducing enzyme bridge formation [47] while calcium acts through binding reversibly to specific asymmetrical sites in the enzyme [48]. Heavy metals are known to interact with sulphydryl groups of proteins and cause inhibition [49]. The cellulase produced by NBCB1 showed tolerance to the presence of Co and Ni and even increased it's activity in presence of Sn. Generally, the enzymes tolerant to a particular stress type also show tolerance to other stresses [50] and hence, the halotolerance and heavy metal

tolerance of the enzyme in the present study could be correlated with it's thermotolerant nature. However, the enzyme was slightly inhibited by other divalent cations, such as Hg^{2+}, Mn^{2+} and Cu^{2+}. A previous research work [51] showed the inhibitory effect of Mn^{2+} and Cu^{2+} with 10 and 90% reduction of cellulase activity respectively. Lesser presence of sulphydryl (-SH-) groups was proven by this heavy metal tolerance and the non-inhibtory effect of β-mercaptoethanol while a lesser degree of hydrophobicity was proven by the non-inhibitory effects of SDS [52]. The metalloproteinaceous nature of the enzyme was determined by the drastic inhibitory effect of chelating agents such as EDTA [53]. Cellulase activity was also retained in presence of NH_4 and was resistant to oxidative agents (H_2O_2) and most monosaccharides including dextrose, galactose and mannose. This was in contrast to the findings of Hsieh et al. [54] where dextrose, cellobiose, mannose and galactose had inhibitory effects on both exo- and endo- acting glucanases. However the enzyme was found susceptible to cellobiose and xylose which proved the need for end product removal during downstream saccharification experiments. Future studies include co-culturing this specimen with other bacteria in order to attain maximum saccharification of pre-treated agroresidues.

Conclusion

A thermocellulolytic bacterial strain was isolated from a vermicompost-derived consortium and was designated as *Parageobacillus thermoglucosidasius* NBCB1 based phylogenetic aanalysis. The production of processive endoglucanase by *P.thermoglucosidasius* NBCB1 was optimized through the OFAT approach and through mathematical approaches such as RSM and ANN. OFAT, RSM and ANN gave ~ 6.66, ~ 9.34 and ~ 10 folds higher enzyme production compared with unoptimized culture medium, respectively, thus indicating later two methodologies being equally effective for the maximization of cellulolytic enzyme production by *P.thermoglucosidasius* NBCB1. The extracellular bifunctional cellulase showed activity and stability over a wide pH and temperature range. It had an augment in activity in presence of several monovalent and divalent metal ions and monosaccharides, suggesting the suitability of the enzyme for industrial applications in deconstruction of cellulosic biomass. To our knowledge, this is the first report on the production of a processive cellulase by *P.thermoglucosidasius*.

Declarations

Funding

This work was supported by the Science and Engineering Research Board, Department of Science and Technology (Project No. SB/SO/BB-020/ 2015), Government of India.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Material preparation was performed by Parmeshwar Vitthal Gavande and Nensina Murmu. Data collection and analysis were performed by Shilpi Ghosh and Arijita Basak. The first draft of the manuscript was written by Arijita Basak and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Acknowledgements

Author AB acknowledges the University of North Bengal, Siliguri, West Bengal, India for both *GATE JRF* and *SRF* fellowships.

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Figures

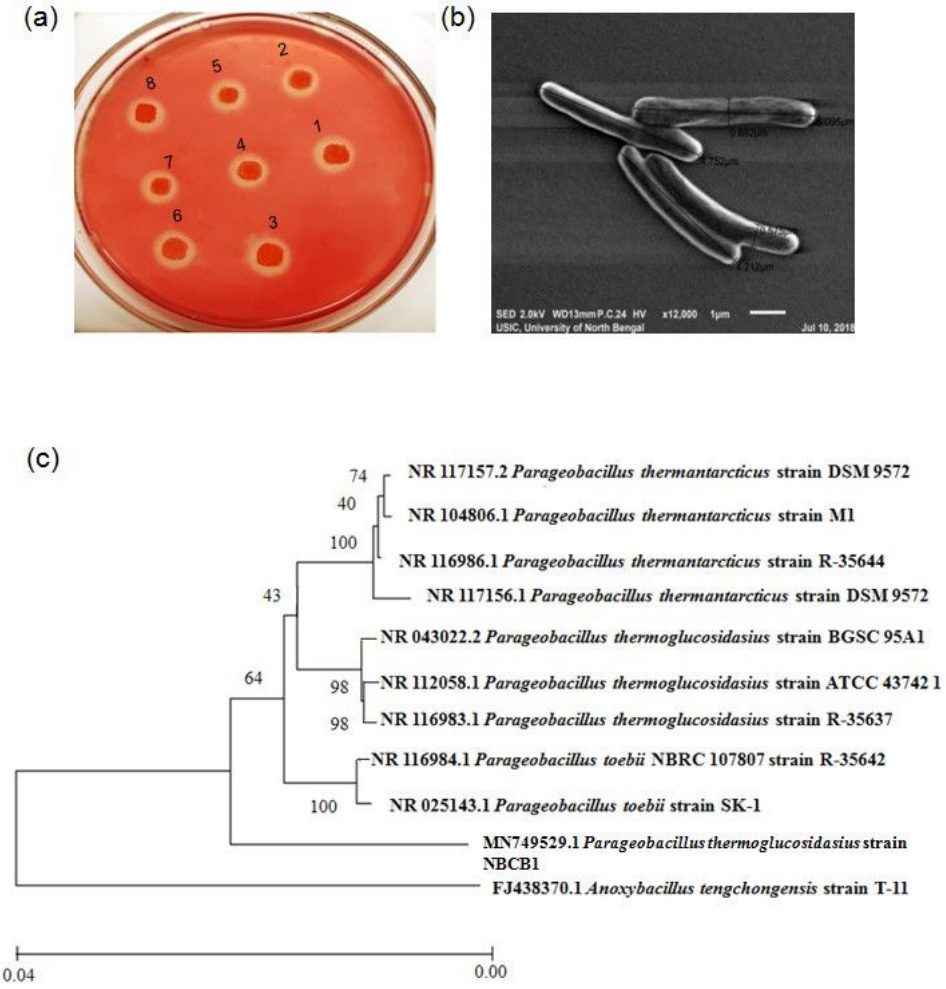

Figure 1

Isolation of thermocellulolytic bacteria from vermicompost-derived consortium (RSV) (**a**) Congo Red assay of isolates for cellulolytic acivity: 1-CCB1, 2-CCB2, 3-CCB3, 4-NBCB1, 5-NBCB2, 6-RAC, 7-XLN1, 8-XLN2 (**b**) SEM image of isolate NBCB1 (**c**) Neighbor-joining tree representing the position of *Parageobacillus thermoglucosidasius* NBCB1 (highlighted) among 16SrRNA sequences of top BLASTn searches. Bootstrap values are represented by the digits at the nodes and Accession numbers are written on the left of each strain

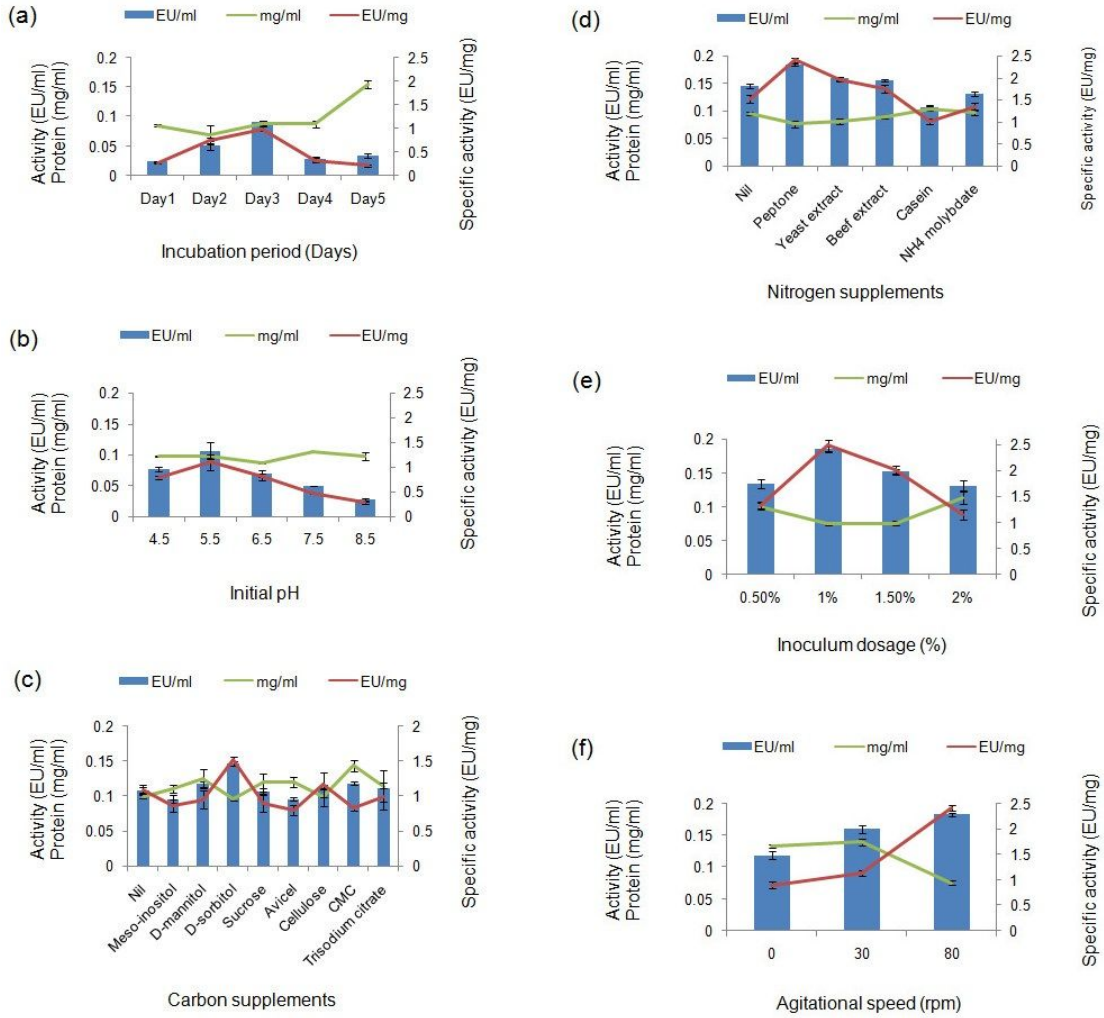

Figure 2

Improvement of culture conditions (**a**) Incubation period (Days) (**b**) Initial pH (**c**) Carbon supplementation through the OFAT approach (**d**) Nitrogen supplementation (**e**) Inoculum dosage (%) (**f**) Agitational speed (rpm) through the OFAT approach

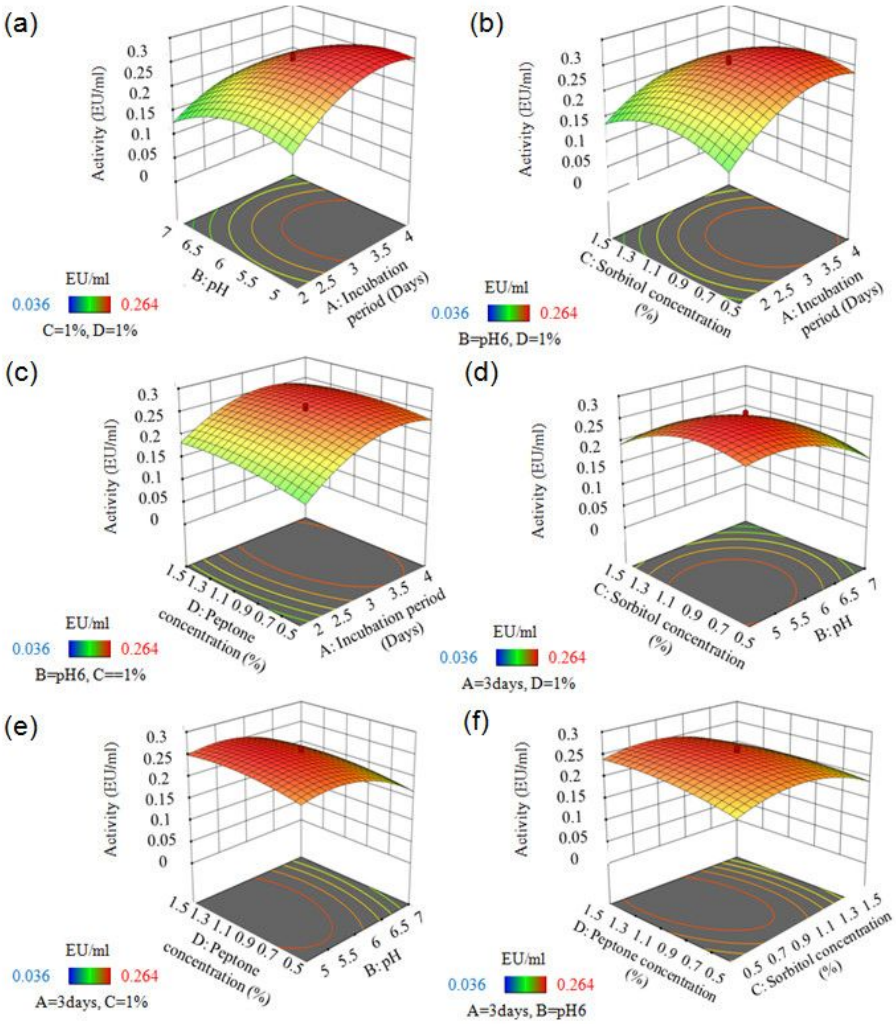

Figure 3

RSM plots for cellulase production by *P.thermoglucosidasius* NBCB1 representing interactions between (**a**) incubation period and pH (AB) (**b**) incubation period and sorbitol concentration (AC) (**c**) incubation period and peptone concentration (AD) (**d**) pH and sorbitol concentration (BC) (**e**) pH and peptone concentration (BD) (**f**) sorbitol concentration and peptone concentration (CD)

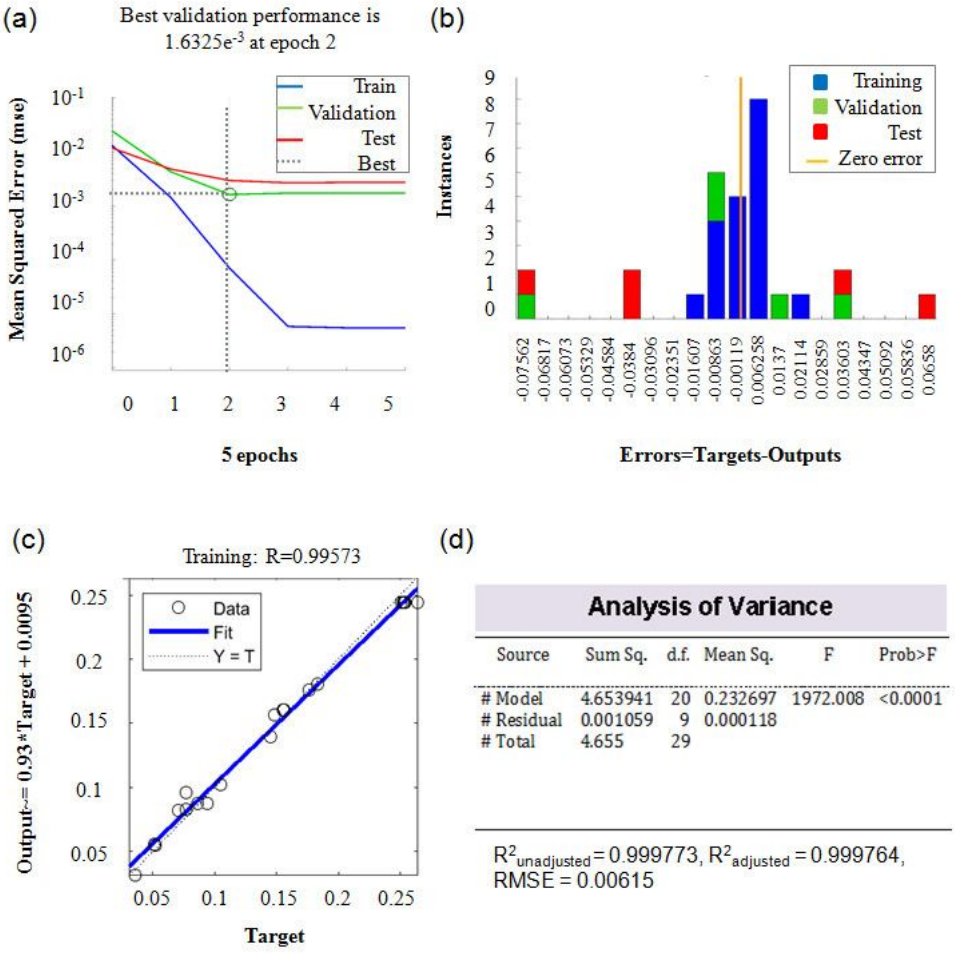

Figure 4

Optimization of cellulase production by *P.thermoglucosidasius* NBCB1 through ANN-GA (**a**) Performance plot for data training, testing and validation (**b**) Error histogram for estimation of data quality (**c**) Training plot for comparing predicted (target) and experimental (output) values (**d**) ANOVA analysis of the ANN model

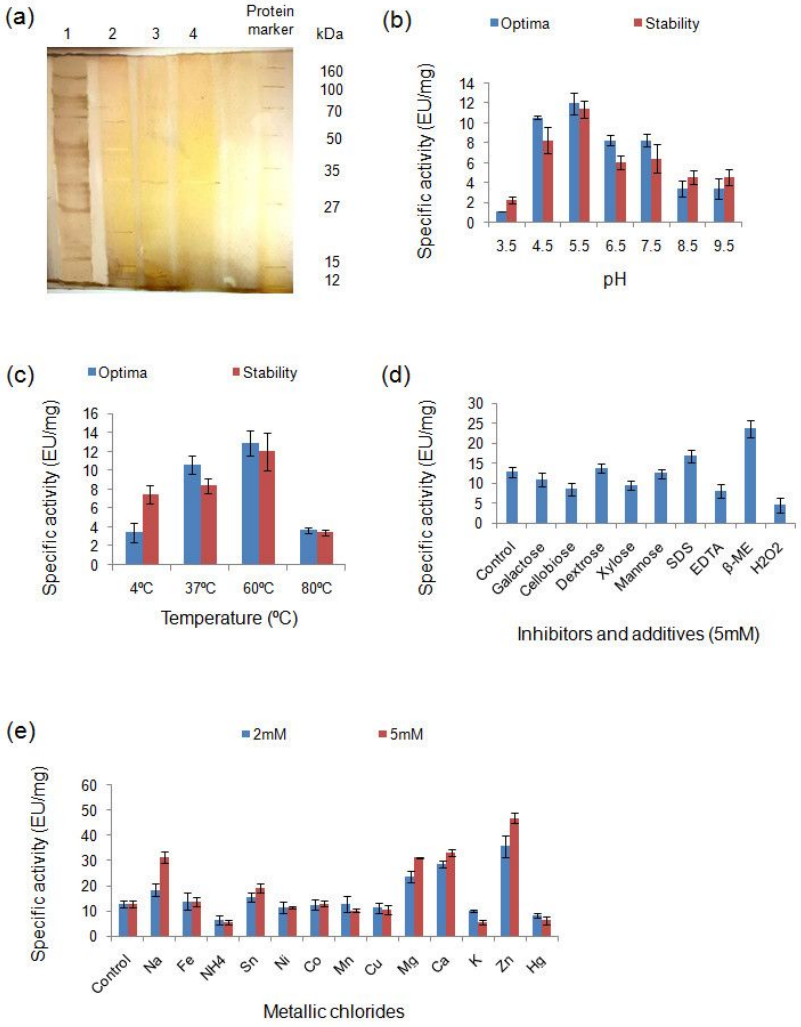

Figure 5

(**a**) SDS-PAGE and biochemical characterization of protein (**b**) pH optima and stability (**c**) temperature optima and stability (**d**) presence of inhibitors and additives (**e**) presence of metallic chlorides

Supplementary Files

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- Supplementarytable1.docx
- SupplemetaryFig.1.docx