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RESEARCH ARTICLE OPEN ACCESS

Statistical Optimization of Cellulase Production by A Novel Thermophilic Lignocellulolytic Bacterium *Geobacillus stearothermophilus* TP-3 from Tapovan Hot Spring in India

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Abstract

A novel thermophilic lignocellulolytic bacterium, *Geobacillus stearothermophilus* TP-3, was isolated and characterized from the Tapovan hot spring in India. The cellulase production of TP-3 was optimized using a One-Factor-at-a-Time (OFAT) approach followed by a Plackett-Burman design, leading to a three-fold enhancement in enzyme yield. Phylogenetic analysis based on 16S rRNA sequencing revealed high sequence similarity with *Geobacillus* sp. H6a. The cellulase enzyme exhibited optimal activity at 50 °C under alkaline conditions (pH 8.0) and retained ~68% of its activity across a broad temperature range (40-70 °C) for up to three hours, demonstrating remarkable thermo-alkali stability. The ANOVA revealed that three factors-glucose, carboxymethyl cellulose (CMC), and yeast extract-significantly affected cellulase production, with yeast extract emerging as the most influential factor. Notably, TP-3 efficiently degraded agronomic residues, including wheat bran and sugarcane molasses, highlighting its potential for sustainable agricultural waste valorization and bioethanol production. The exceptional thermostability and lignocellulolytic potential of *G. stearothermophilus* TP-3 position it as a promising candidate for industrial bioconversion processes.

Keywords: Lignocellulosic, Thermophilic Cellulase, *Geobacillus*, Agro-waste Management, Saccharification

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INTRODUCTION

Lignocellulosic materials are highly abundant, biodegradable, renewable, biocompatible polysaccharides with immense industrial potential. Their diverse sugar composition makes them an attractive resource for sustainable development, particularly in bioethanol production-a promising alternative to fossil fuel dependency.[1] Agricultural residues-including sugarcane molasses, bagasse, rice hulls, woody crops, corn stover, and forest waste-constitute a substantial portion of lignocellulosic materials and can serve as potential feedstock for bio-based industries.[2,3] However, the efficient utilization of lignocellulosic biomass remains a challenge due to its low solubility and recalcitrant structure, thus making them less effective for their actual applications.[4] Therefore, prior treatment of the lignocellulosic biomass is the prerequisite for its subsequent employment as an energy source. Enzymatic hydrolysis offers a sustainable and eco-friendly solution by breaking down lignocellulosic biomass into valuable compounds such as acetone and ethanol.[5,6]

Hot springs are natural reservoirs of novel microbial communities harbouring glycosyl hydrolases for cellulose and hemicellulose degradation.[7] A few thermostable cellulases isolated from geothermal springs are CelDZ1,[8] *Bacillus velezensis* strain MRC 5958,[9] *Geobacillus* sp. TP-1[10] and *Bacillus licheniformis* PANG L.[11] Alkali-thermostable cellulases are valuable for their resilience in harsh industrial conditions, making them ideal for applications where conventional cellulases fail. Key uses include bio-stoning, fabric biopolishing, bio-bleaching, deinking, laundry detergents, industrial cleaning, dark fermentation, bioethanol production, and waste treatment.[12-14] Their effectiveness under extreme conditions reduces chemical use and costs and enhances sustainability, making them highly desirable for lignocellulosic biomass valorisation.[15]

Statistical optimisation of production conditions (pH, temperature, carbon and nitrogen sources, and incubation time) is essential for enhancing microbial enzyme production's efficiency, yield, and cost-effectiveness. By identifying the ideal combinations of these parameters, statistical optimisation reveals interactions that maximise enzyme yield and activity.[16] This approach ensures a more predictable and reproducible process, which is crucial for consistency in industrial-scale production.

Traditional one-variable-at-a-time (OVAT) optimisation is labour-intensive and inefficient. Statistical methods like Response Surface Methodology (RSM) and Design of Experiments (DoE) enable simultaneous evaluation of multiple variables, cutting down time and costs.[17] Key techniques include RSM, Plackett-Burman Design (PBD), Box-Behnken Design (BBD), and Central Composite Design (CCD). PBD is particularly useful for screening numerous variables to identify the most significant ones affecting enzyme production. It requires fewer experimental runs, making it cost-effective and ideal for initial screenings. However, PBD only estimates the main effects and doesn't consider interactions. RSM and CCD are better for detailed optimisation but require more runs as they assume all factors are important.[16] Thus, a combined approach-PBD for screening and RSM/CCD for optimisation, is often best.

The present study focuses on the isolation and characterisation of a thermostable cellulase from the thermophilic bacterium *Geobacillus stearothermophilus* TP-3, obtained from the Tapovan hot spring in India. The study optimizes growth conditions for enhanced cellulase production using a combination of One-Factor-at-a-Time (OFAT) and Plackett-Burman Design (PBD), allowing for the identification of key factors influencing enzyme yield. Additionally, the potential of lignocellulosic waste as a carbon source for cellulase production was investigated, providing insights into its feasibility for enzyme biosynthesis. Given the enzyme's thermostability and activity under alkaline conditions, *G. stearothermophilus* TP-3 bacteria, this work contributes to advancing green technologies for efficient lignocellulose degradation, bioethanol production, and industrial waste management.

MATERIALS AND METHODS

Chemicals

Luria Bertani broth, Zinc chloride, Ammonium chloride, Sodium acetate, Glycine, Sodium chloride, Peptone, Yeast extract & Magnesium sulphate were purchased from

Himedia, India. Carboxymethyl cellulose, Glucose, Beef extract, Ammonium sulphate, Sodium nitrate, Maltose, Lactose, Sucrose, Sodium succinate, Sodium citrate was purchased from SRL (India). DNSA (3,5-dinitrosalicylic acid), Urea, Sodium hydroxide, and HCl were purchased from Sigma Aldrich.

Sample collection and sampling procedure

Soil samples were collected from the Tapovan hot springs (30.490897°N and 79.646662°E) of India (Figure 1).[18] They were collected using a sterile spatula from 4-5 cm below the surface soil and stored in sterile polybags. The samples were brought to the laboratory and maintained at 4 °C in a refrigerator until further processing. The soil temperature was recorded during sampling using a thermometer, and it was ~80 °C. During laboratory analysis, the sample exhibited a pH of 8.0.

Isolation of thermophilic cellulase-producing bacteria

To isolate bacteria, 50 mL of enrichment medium and 5 g of soil were mixed in a 250 mL Erlenmeyer flask. Here, the enrichment medium was used where 1% carboxymethyl cellulose (CMC) was added to the medium as an additional carbon source and as an inducer.[19] For 24 hours, the flask was shaken at 50 °C and 150 rpm in a shaker incubator. To obtain the thermophilic cellulase-producing bacteria, 100 µL of the overnight culture was spread on a CMC agar plate and cultured for 24 hours at 60 °C. The colonies showing distinct morphology were streaked on a nutrient agar plate, and pure colonies were obtained by using multiple streaking under similar cultivation conditions. The pure isolates of bacteria were maintained on slants for short-term use. The glycerol stocks were prepared in a 1:1 ratio using 50% (v/v) glycerol and kept at -20 °C.

Figure 1. Sampling site of Tapovan hot spring (30.490897°N and 79.646662°E) in Uttarakhand State, India (Source: Google map)

Primary screening of cellulolytic bacterial isolates

The isolates were inoculated in 10 mL of minimal salt media supplemented with CMC to produce cellulase at 50 °C for 24 hours. Bacterial cells were separated from the fermentation broth by centrifugation. The supernatant was then used as a crude enzyme to test the thermophilic isolates for cellulase activity using the well diffusion method. The enzyme was added to the well of CMC-agar plates and then incubated at 50 °C for 48 hours to allow the enzyme to diffuse. The cellulase activity was confirmed by observing a clear zone of hydrolysis. For this, the plates were flooded with 1% (w/v) Congo red solution after complete diffusion and then repeatedly destained with 1 M NaCl solution.[20] The potent cellulase-producing isolates were further processed.

Cellulase activity assay

The 3,5-dinitrosalicylic acid (DNSA) technique was used to test the cellulase activity in triplicates.[21] Glycine-NaOH buffer-50 mM (pH 8) was used to prepare the substrate by suspending 1% (w/v) CMC. Equal quantities of crude enzyme and substrate were mixed to form the reaction, which was kept at 50 °C for 10 min. The process was stopped by adding an equal volume of DNSA reagent and boiling at 100 °C for 10 minutes. In the final reaction, 400 µL of 33% (w/v) sodium potassium tartrate was added and cooled. The released glucose was quantified by using a spectrophotometric method at the wavelength of 540 nm. A bacterium was selected for further studies based on the highest cellulase activity. The Bradford protein assay was used to estimate the protein content in the fermentation broth.[22]

One-unit cellulase activity can be defined as the amount of enzyme that releases 1 µmole of glucose per minute per mL under assay conditions.

Table 1. Plackett-Burman design with experimental levels of independent variables for cellulase production from *Geobacillus stearothermophilus* TP-3

Factor	Production parameter	Low level (-1)	High Level (+1)
A	Glucose (%)	0.4	0.6
B	Carboxy methyl cellulose (%)	1.0	1.5
C	Yeast Extract (%)	0.4	0.6
D	pH	7	9
E	Temperature (°C)	30	50
F	Inoculum Size (%)	4	8
G	Incubation Time (hrs.)	20	28

Morphological and growth characteristics

After being cultured on a nutrient agar plate for 20 hours at 50 °C, the bacterial strain's phenotypic traits, colour, and margin were examined for their morphological characteristics. The cell morphology was examined using a compound light microscope (Olympus model CX41) and scanning electron microscope (SEM Model: JSM 6490 LV, JEOL, Japan).

Identification of bacterial isolate

The bacterial-specific 16S rRNA gene was amplified using genomic DNA from the chosen bacterial strain. The PCR condition was set as initial denaturation (pre-PCR) for 5 min at 95 °C followed by 30 cycles (denaturation 90

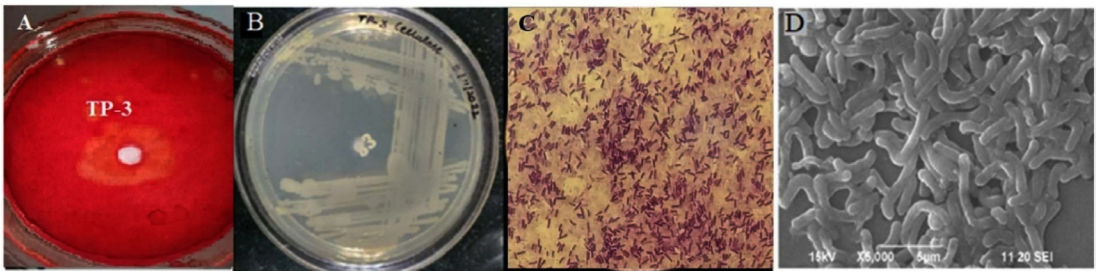

Figure 2. Cellulase enzyme screening and morphological features of isolate. (A) Zone of hydrolysis on CMC-containing agar plates after Congo red staining; (B) TP-3 isolate grown on the nutrient agar plate showing slimy off-white coloured, translucent, circular shaped with flat elevation with slightly undulating margins colonies; (C) Rod-shaped Gram-positive purple cells at 100× magnification using a compound light microscope; (D) Rod-shaped cells visualized using SEM (Scanning Electron Microscope).

sec at 95 °C, 30 sec at 58 °C, 90 sec at 72 °C) and final extension (post-PCR) for 10 min at 72 °C. The bacterial 16S rRNA gene universal forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene.[23] The amplified gene was sequenced using Sanger sequencing, and the obtained sequence was compared to that of other closely related bacterial species using BLASTn at NCBI. Phylogenetic analyses were conducted in the T-Coffee multiple sequence alignment program, and the Neighbour-Joining (NJ) method was applied to infer evolutionary relatedness.

Selection of media for cellulase production and OFAT analysis

A few media compositions cited in the literature were used for cellulase production (Supplementary Table). A medium was chosen for further analysis based on the highest cellulase production. One-Factor-at-a-Time (OFAT) analysis was performed to optimize the carbon source, nitrogen source, pH, temperature, and inoculum size to enhance cellulase production.

Plackett-Burman design for statistical optimization of cellulase production

Our study used the Plackett-Burman (PB) factorial design (PBD) to pinpoint the key medium elements affecting cellulase production. The parameters from OFAT analysis were used as a reference for PB designing for cellulase enzyme production. The PB analysis was used to identify the most significant culture variables and medium components that might greatly improve cellulase outputs.[24] As shown in Table 1, various independent factors were selected for this study on two different levels, i.e., low (-1) level and high (+1). Seven variables that influenced cellulase production, namely carbon source (Glucose), inducer (CMC), nitrogen source (Yeast extract concentration), pH, temperature, inoculum size, and incubation time, were taken and varied according to PB design. Design Expert Software 6.1.10 was used to examine predicted responses. Each experiment was performed in triplicates, and the conclusion was determined by averaging the outcomes. The experimental outcome was the estimated mean of cellulase production (dependent variable). The experimental design is based on the first-order model shown in Equation 1.[25]

$$Y = b_0 + \Sigma b_i x_i \quad \ldots 1$$

Y represents the cellulase enzyme activity response, b_0 the model intercept, b_i the linear variable coefficient, i is the variable number, and x_i is the independent variable. The DNSA test was used to determine particular enzyme activity by quantifying the quantity of glucose generated using a standard curve.

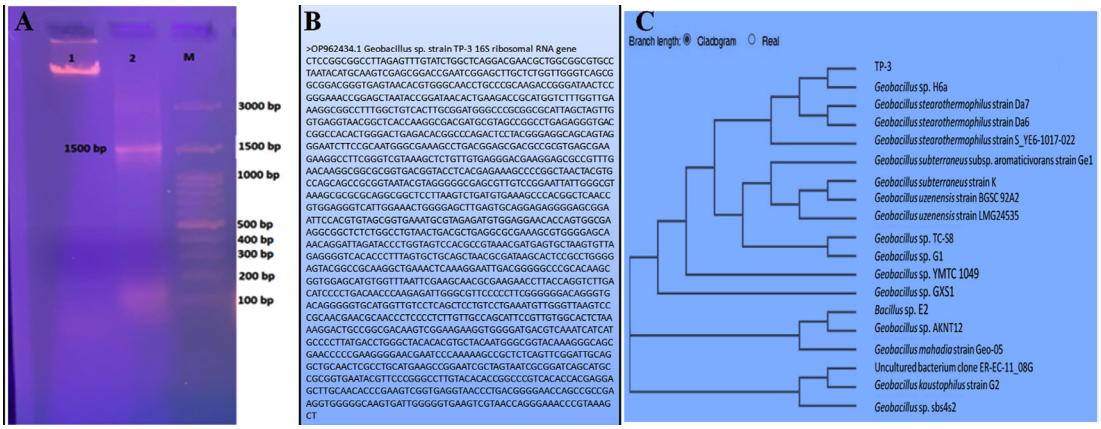

Figure 3. Identification of the isolate using 16S rRNA sequencing: (A) PCR amplicon of 16S rRNA gene of *Geobacillus* sp. strain TP-3 using universal primers; (B) NCBI submitted sequencing data of 16S ribosomal RNA gene of *Geobacillus* sp. strain TP-3; (C) Phylogenetic analysis to infer relatedness amongst *Geobacillus* strains and other *Bacillus* species after BLASTn and for determining identity of the bacterium using T-Coffee method

Low-cost wastes as the substrate for cellulase production

The bacterial isolate was inoculated in optimized media from the PB experiment for cellulase production, where inexpensive agricultural waste materials replaced the carbon source. A range of low-cost agricultural waste products, including sugar cane molasses, rice straw, wheat bran, and corn cob, were employed as the only carbon source to determine the optimal substrates for cellulase production. These wastes were dried, crushed, and used at 2% w/v (for

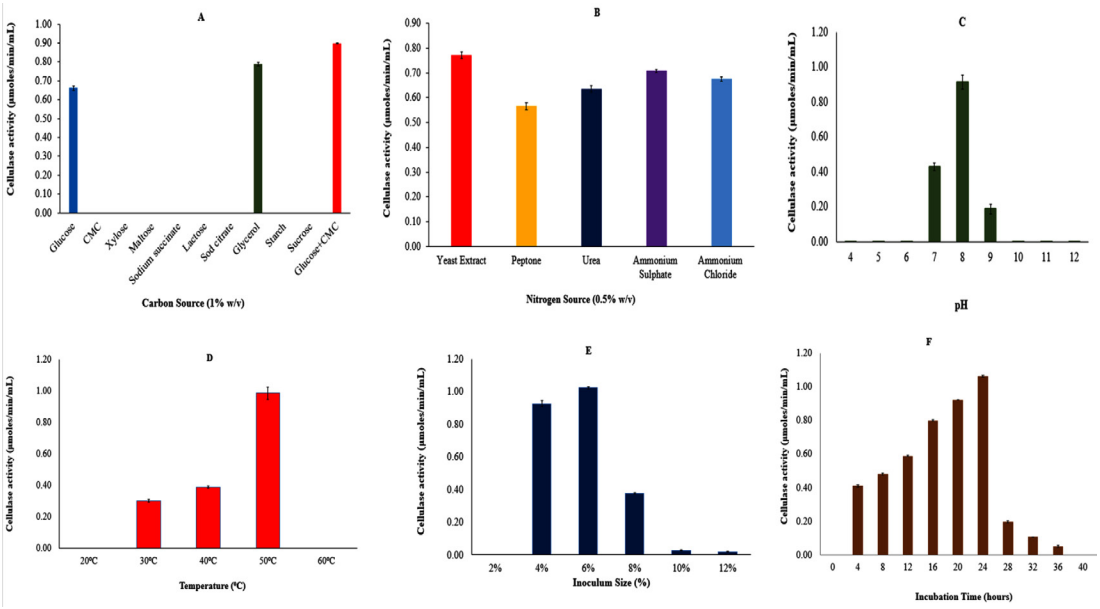

Figure 4. Optimization of various factors on cellulase production by using OFAT method (A) Effect of different carbon sources; (B) Effect of different nitrogen sources; (C) Effect of pH; (D) Effect of Temperature; (E) Effect of Inoculum size and (F) Effect of incubation time

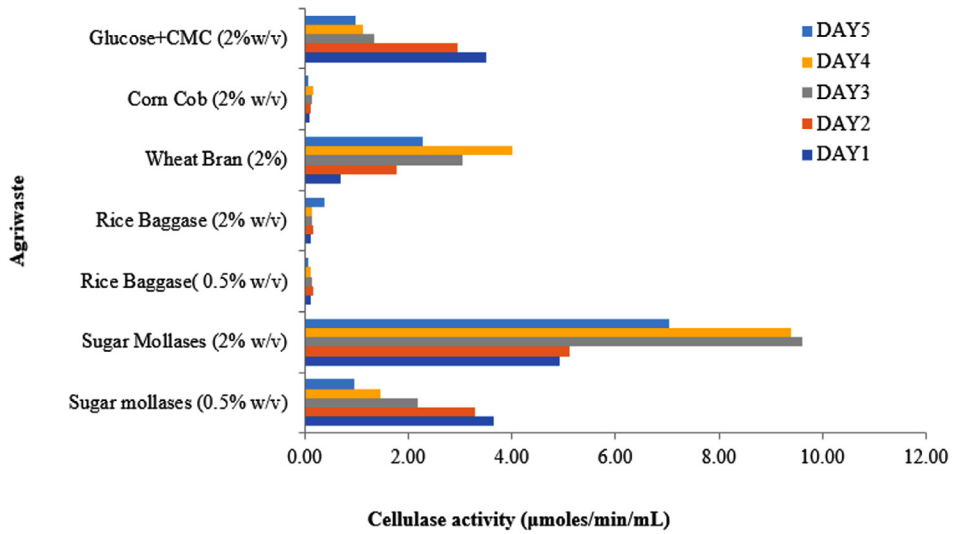

Figure 5. Effect of different lignocellulosic waste material (as a carbon source) on cellulase production

Table 2. Plackett-Burman experimental trial table with cellulase activity response

Trial	A: Glucose (gm/L)	B: CMC (gm/L)	C: Yeast Extract (gm/L)	D: pH	E: T (° C)	F: Inoculum Size (%)	G: Incubation Time (hrs.)	H: Dummy 1	I: Dummy 2	J: Dummy 3	K: Dummy 4	Experimental U/mL
1	-1.0 (0.4)	+1.0 (1.5)	+1.0 (0.6)	+1.0 (9.0)	-1.0 (30)	+1.0 (8.0)	+1.0 (28)	-1.0	+1.0	-1.0	-1.0	0.078
2	+1.0 (0.6)	-1.0 (1.0)	+1.0 (0.6)	-1.0 (7.0)	-1.0 (30)	-1.0 (4.0)	+1.0 (28)	+1.0	+1.0	-1.0	+1.0	0.557
3	-1.0 (0.4)	-1.0 (1.0)	-1.0 (0.4)	-1.0 (7.0)	-1.0 (30)	-1.0 (4.0)	-1.0 (20)	-1.0	-1.0	-1.0	-1.0	0.563
4	+1.0 (0.6)	+1.0 (1.5)	+1.0 (0.6)	-1.0 (7.0)	+1.0 (50)	+1.0 (8.0)	-1.0 (20)	+1.0	-1.0	-1.0	-1.0	0.273
5	+1.0 (0.6)	+1.0 (1.5)	-1.0 (0.4)	+1.0 (9.0)	+1.0 (50)	-1.0 (4.0)	+1.0 (28)	-1.0	-1.0	-1.0	+1.0	0.624
6	-1.0 (0.4)	+1.0 (1.5)	+1.0 (0.6)	-1.0 (7.0)	+1.0 (50)	-1.0 (4.0)	-1.0 (20)	-1.0	+1.0	+1.0	+1.0	0.072
7	-1.0 (0.4)	+1.0 (1.5)	-1.0 (0.4)	-1.0 (7.0)	-1.0 (30)	+1.0 (8.0)	+1.0 (28)	+1.0	-1.0	+1.0	+1.0	0.051
8	+1.0 (0.6)	-1.0 (1.0)	-1.0 (0.4)	-1.0 (7.0)	+1.0 (50)	+1.0 (8.0)	+1.0 (28)	-1.0	+1.0	+1.0	-1.0	0.925
9	-1.0 (0.4)	-1.0 (1.0)	-1.0 (0.4)	+1.0 (9.0)	+1.0 (50)	+1.0 (8.0)	-1.0 (20)	+1.0	+1.0	-1.0	+1.0	0.541
10	+1.0 (0.6)	+1.0 (1.5)	-1.0 (0.4)	+1.0 (9.0)	-1.0 (30)	-1.0 (4.0)	-1.0 (20)	+1.0	+1.0	+1.0	-1.0	0.100
11	-1.0 (0.4)	-1.0 (1.0)	+1.0 (0.6)	+1.0 (9.0)	+1.0 (50)	-1.0 (4.0)	+1.0 (28)	+1.0	-.1.0	+1.0	-1.0	0.090
12	+1.0 (0.6)	-1.0 (1.0)	+1.0 (0.6)	+1.0 (9.0)	-1.0 (30)	+1.0 (8.0)	-1.0 (20)	-1.0	-1.0	+1.0	+1.0	0.157

Independent variables: High level +1 and low level -1. The mean of triplicate culture trials is used to calculate response values

solids) and 2% v/v (for liquids) concentrations. The enzyme production was measured by using the DNSA method.

Characterisation of cellulase

The cellulase activity of strain *Geobacillus stearothermophilus* TP-3 was measured at different pH (5-9) and temperatures (40-70 °C). The thermostability of the cellulose enzyme was assessed at various temperatures from 40 °C to 70 °C by varying the incubation time from 10 min up to 3 hours using the DNSA method.

RESULTS

Isolation and selection of thermophilic cellulase-producing bacteria

A total of ten bacterial isolates were obtained on CMC-containing enrichment media plates from Tapovan soil samples. Two isolates (TP-1 and TP-3) showed comparatively higher cellulase activity based on the zone of hydrolysis (diameter in mm) on agar plates containing 1% Carboxyl Methyl Cellulose (CMC) medium. Of these two isolates, TP-3 was selected for further analysis due to the maximum zone of clearance during the plate diffusion assay (Figure 2A). Therefore, TP-3 was selected for further optimizing the cellulase production. The isolate exhibited optimum growth at 50 °C; hence, it is considered a thermophilic bacterium.

Morphological and growth characteristics

Bacterial colonies were grown on the nutrient agar plate and subjected to phenotypic characterization. It had a spherical colony, off-

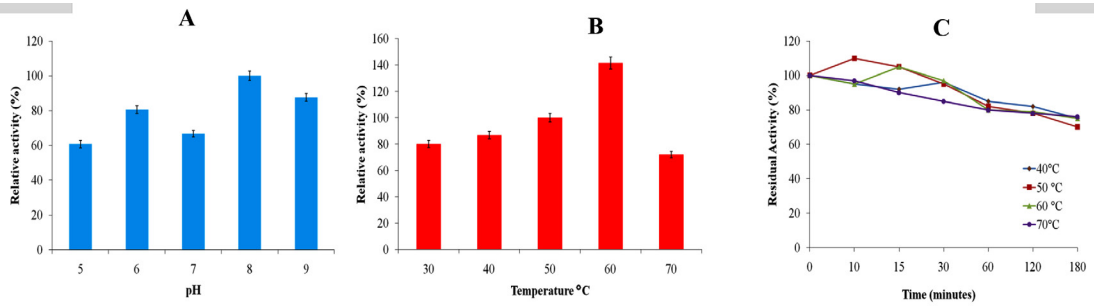

Figure 6. Characterization of cellulase enzyme (A) Effect of reaction buffer pH on cellulase activity (100% = 9.2 µmol/min/mL); (B) Effect of reaction temperature on cellulase activity (100% = 9.45 µmol/min/mL); (C) Thermal stability profile of cellulase enzyme of *Geobacillus* sp. TP-3 (100% = 9.68 µmol/min/mL)

Table 3. Plackett-Burman design ANOVA analysis using the partial sum of squares for *Geobacillus* sp. TP-3 cellulase production

Source	Sum of Squares	Degree of Freedom	Mean Square	F-Value	p-value Prob > F	Inference
Model	1.064E+006	8	1.330E+005	10.98	0.0371	Significant
A: Glucose	1.0254E+005	1	1.254E+005	10.35	0.0487	Significant
B: CMC	1.728E+005	1	1.728E+005	14.26	0.0325	Significant
C: Yeast Extract	2.621E+005	1	2.621E+005	21.64	0.0187	Significant
D: pH	91564.60	1	91564.60	7.56	0.0708	Not Significant
E: T (°C)	1.078E+005	1	1.078E+005	8.90	0.0584	Not Significant
H: Dummy	52219.38	1	52219.38	4.31	0.1294	Not significant
J: Dummy	55053.66	1	55053.66	4.55	0.1228	Not significant
K: Dummy	1.971E+005	1	1.971E+005	16.28	0.0274	Significant
Residual	36334.24	3	12111.41			
Cor Total	1.100E+006	11				

Std Dev 110.05, mean 311.05, C.V. 35.38, Press 5.813E+005; R squared 0.9670; Adj R-Squared 0.8789; Pred R squared 0.4717; Adeq Precisior 9.885 [*p-value < 0.01 highly significant; 0.01 < P < 0.05 significant]

Table 4. Comparison of few thermophilic organisms reported in literature for cellulase production

Strain Name	Substrate used as Carbon source	pH	Temp. (°C)	Production time (h)	Enzyme Activity
B. licheniformis KBFB3[56]	1% CMC	7	50	72	4.06 IU/mL
B. licheniformis NIBE23[57]	1% CMC	7	55	24	20.3 U/mL
Bacillus sp. PCH94[52]	1% CMC	7	50	12	3.55 IU/mg
Bacillus licheniformis TLW-3[58]	1% CMC	7	50	72	452.57IU/mL/min
Bacillus smithi NIBE10[57]	1% glucose	-	55	22 h	14. 57 U/mL
Bacillus sp. CX6[50]	Wheat straw	7	30	18 h	524.66 U/mL
Bacillus subtilis MC4[51]	CMC (1%)	7	60	NA	10.37 U/mL
Bacillus subtilis K-18[59]	2% potato peel pH 5	-	50	24	350 IU/mL/min
Brevibacillus borstelensis MX18[51]	CMC (1%)	6	50	NA	9.69 U/mL
Geobacillus sp. KP43[60]	CMC (1%)	6.5	55	18	0.186 U/mg
Geobacillus sp. TP-1[10]	Sugar Molasses (2%)	8	50	20	1263 (U/L)
Geobacillus stearothermophilus TP-3 (current study)	Sugar Molasses (2%)	8	50	24	9.60 (U/mL)
Talaromyces thermophilus[61]	Kans grass (2%)	6	50	72	~1.8 U/mL
Geobacillus sp. T1[62]	Barley straw (0.5%)	6.5	50	24	143.5 U/mL
Geobacillus sp. HTA426[63]	Sugarcane bagasse (1%)	NA	60	144	103.67 U/mL

white, transparent, and slimy appearance with flat, slightly undulating margins (Figure 2B). Gram staining identified it as a Gram-positive bacterium. A compound light microscope at 100X magnification revealed long rod-shaped bacteria (Figure 2C), and SEM corroborated similar findings (Figure 2D). Gram-positive bacterial cells with terminal and sub-terminal endospores were observed. The TP-3 strain exhibited enzyme activity of 0.692 µmoles/min/mL using the DNSA assay of glucose estimation.

Identification of TP-3 cellulose-degrading bacterial strain

A ~1.5 kb fragment of the 16S rRNA gene was successfully amplified and sequenced to identify the cellulolytic bacterial isolate (Figure 3A). Phylogenetic analysis based on the sequence data confirmed that the isolate, designated as TP-3, belongs to the genus *Geobacillus*. The 16S rRNA gene of TP-3 exhibited the highest sequence similarity with multiple *Geobacillus stearothermophilus* strains, including *G. stearothermophilus* YE-6 (98.53%), *G. subterraneus* E-55 I (98.03%), *G. stearothermophilus* D6a (98.02%), *Geobacillus* sp. TC-S8 (97.64%), *Geobacillus* sp. G1 (97.57%), *G. kaustophilus* HTA426 (97.44%), and *G. thermoleovorans* SURF-48B (97.37%). The 16S rRNA gene sequence has been deposited in GenBank under accession number OP962434 (Figure 3B).

Using the Neighbor-Joining (NJ) method with the T-Coffee multiple sequence alignment tool, phylogenetic analysis revealed that TP-3 shares the closest evolutionary relationship with *Geobacillus* sp. H6a (Figure 3C). These findings suggest that TP-3 is a novel strain within the *Geobacillus* genus, exhibiting significant cellulolytic potential. Its close phylogenetic relationship with *G. stearothermophilus* and other thermophilic species underscores its adaptation to high-temperature environments, making it a promising candidate for industrial applications involving lignocellulose degradation.

Selection of media for cellulase production and OFAT analysis

The maximum cellulase production was observed in a medium reported by Sakthivel *et al*.[26] of the six different media. The selected medium was optimised using a One-Factor-at-a-Time (OFAT) approach to enhance cellulase production

by *Geobacillus stearothermophilus*. This method systematically assessed the effect of individual parameters, with results presented in Figure 3.

Screening of various carbon sources, including glucose, carboxymethyl cellulose (CMC), xylose, and maltose, identified glucose and CMC as the most effective combination for maximizing cellulase activity (Figure 4A). Similarly, among the nitrogen sources tested, yeast extract yielded the highest cellulase activity (Figure 4B).

The effect of pH on enzyme production was evaluated by adjusting the pH of the production medium (M5) from 4 to 12. Optimal growth was observed within the pH range of 7-9, with peak cellulase production occurring at pH 8 (Figure 4C). Temperature optimization, conducted over a range of 20 °C to 60 °C, revealed that maximum cellulase production occurred at 50 °C (Figure 4D).

The impact of inoculum size was also assessed, with variations tested to determine the ideal concentration. A 6% inoculum size resulted in the highest cellulase activity (Figure 4E). Additionally, cellulase production was monitored at different incubation times (0-60 hours), showing a steady increase up to 24 hours, followed by a gradual decline (Figure 4F). Following OFAT optimization, enzyme activity significantly improved, increasing approximately 1.5-fold from 0.692 µmol/min/mL to 1.06 µmol/min/mL. This enhancement highlights the effectiveness of systematic parameter optimization in boosting cellulase production for potential industrial applications.

Plackett-Burman design for statistical optimization of cellulase production

Seven factors were examined to identify the crucial variables appropriate for cellulase production. The model produced a design for 12 trials (Table 2), where each column corresponds to a variable and each row to an experiment. *Geobacillus* sp. TP-3 produced cellulase in varying amounts, and these differences are shown in Table 2, where they highlight the significance of factor optimization. The eighth experimental run had the highest cellulase activity (0.925 µmoles/min/mL), whereas the seventh run showed the lowest (0.051 µmoles/min/mL). The data in Table 2 were subjected to multiple linear regression analysis based on the PBD to estimate the F-value and p-value of each component. The first-order linear model determines how independent variables affect cellulase production, and it provides these results in equations 2 (coded factors) and 3. (actual factors).

Final Equation in Terms of Coded Factors

$$Y \text{ (cellulase activity)} = +102.23*A-119.99*B-147.79*C-87.35*D+94.79*E-65.97*H+67.73*J-128.17*K \quad ...(2)$$

Final Equation in Terms of Actual Factors

$$Y \text{ (cellulase activity)} = 1022.29171*\text{Glucose}-479.95132*\text{CMC}-1477.88043*\text{Yeast Extract}-87.35206*\text{pH}+9.47902*\text{Temperature}-65.96677*\text{dummy1}+67.73334*\text{dummy2}-128.16932*\text{dummy3} \quad ...(3)$$

$Y = b_0 + \Sigma b_i + x_i$;

where Y represents the response of the cellulase enzyme activity, b_0 the model intercept, b_i the linear variable coefficient, i the variable number, and x_i the level independent variable. Design Expert 6.1.10 software.

The factors affecting the generation of cellulase by *Geobacillus stearothermophilus* TP-3 were examined using an analysis of variance (ANOVA), and the findings were presented in Table 3. The p-value of 0.0371 in the statistical design made it clear that the model is significant. Three factors (glucose, CMC, and yeast extract) were determined by p-value analysis to impact cellulase production significantly. However, the yeast extract was the most important factor, with a p-value of 0.0187. A p-value greater than 0.05 indicates that the studied component was not statistically significant; however, it did not play a specific function in enzyme synthesis. The F-value of 10.98 for the model suggests that it is significant. A "Model F-value" this large might happen due to noise with a mere 3.71% probability. Because the "Pred R-Squared" of 0.4717 is not as close to the "Adj R-Squared" of 0.8789, the coefficient (R^2) revealed that the model has a block effect. By the criterion known as "Adeq Precision," a measure of signal-to-noise ratio that can be used to explore the design space, our model's ratio value of 9.885 shows that it has a satisfactory signal-to-noise ratio.

Low-cost wastes as the substrate for cellulase production

Various agricultural waste products were evaluated as carbon sources to assess their impact on cellulase enzyme induction over five days. Among the tested substrates, cane sugar molasses supported the highest cellulase production (~9.60 µmol/min/mL), reaching its peak on the third day before experiencing a slight decline on the fourth day. In contrast, wheat bran yielded approximately 2.4 times lower cellulase production, with maximum enzyme activity (~4 µmol/min/mL) observed on the fourth day, followed by a significant decline on the fifth.

Untreated rice bagasse, a more complex lignocellulosic substrate, exhibited initial resistance to degradation. However, cellulase activity gradually increased, reaching its peak (~0.37 µmol/min/mL) on the fifth day, suggesting a slower enzymatic response likely due to its recalcitrant structure. Similarly, corn cob demonstrated minimal cellulase production (~0.19 µmol/min/mL) until the fourth day, with enzyme activity dropping to half its initial value by the fifth day (Figure 5).

These findings indicate that readily available and less structurally complex carbon sources, such as cane sugar molasses and wheat bran, facilitate higher cellulase production in shorter timeframes. In contrast, complex lignocellulosic materials like rice bagasse and corn cob require extended incubation periods for enzyme induction, highlighting the necessity of pretreatment strategies for efficient bioconversion. This study underscores the potential of agro-waste valorization for sustainable cellulase production, particularly from substrates with high fermentable sugar content.

Characterization of cellulase enzyme

The cellulase activity of *Geobacillus stearothermophilus* TP-3 was measured at different pH (5-9) and temperatures (40-70 °C). Optimum cellulase activity was observed at pH 8.0 and 50 °C (Figure 6A, 6B). Moreover, the cellulase enzyme was active at 40-70 °C. Up to 15 minutes, a little increase in cellulase activity was seen at 50 °C and 60 °C. The cellulase enzyme retained ~68% of its original activity at different temperature ranges, 40-70 °C after 3 hours of incubation (Figure 6C). From these results, the cellulase seems to have considerably high thermostability at higher temperatures for a long incubation period.

DISCUSSION

The agricultural and industrial sectors together generate approximately 200 billion tons of lignocellulosic biomass annually, making lignocellulosic waste management a critical challenge in reducing environmental pollution, from air to soil.[27] The abundance of cellulose in these materials has drawn attention for their potential conversion into valuable products. However, lignocellulosic biomass is highly recalcitrant, and its depolymerization remains a complex task for extracting fermentable sugars.[28] While various physical and chemical methods can break down lignocellulose, these processes can be energy-intensive and environmentally unsustainable. As a result, lignocellulolytic enzymes offer a more eco-friendly alternative for the degradation of lignocellulosic biomass into desired sugars.[29]

Several microorganisms, including bacteria and fungi, have been identified to produce lignocellulolytic enzymes, which are essential for efficiently breaking down lignocellulosic materials.[30] Among these enzymes, cellulases are particularly significant. They specifically target the β 1, 4- glycosidic bonds in cellulose fibers, releasing reducing sugars such as glucose. Thermophilic cellulases are of particular interest, as they can enhance the efficiency of combined bioprocessing methods and accelerate reaction rates during hydrolysis. In this context, the study isolated a novel indigenous thermophilic bacterium, *Geobacillus* sp. TP-3, which demonstrates potential for cellulase production at elevated temperatures. A comparison of *Geobacillus* sp. TP-3 cellulase with other cellulases is presented in Table 4.

Geobacillus species are known for their ability to thrive at temperatures exceeding 45 °C, classifying them as thermophilic bacteria.[31] The *Geobacillus* sp. TP-3 strain secretes cellulase when supplemented with 1% (w/v) carboxymethyl cellulose (CMC) as an inducer, indicating that it produces inducible enzymes. Similar substrate

affinities for 1% (w/v) CMC have been reported in other strains, such as *Cohnella* sp. A01[32] and *Geobacillus* sp. KP43.[33] In this study, optimal cellulase production was observed at 50 °C, which aligns with previous reports on *Bacillus subtilis* K-18.[34] This high-temperature tolerance enhances the strain's applicability in industrial processes, particularly those requiring thermostable enzymes.

The ability of *Geobacillus* sp. TP-3 to function optimally at higher temperatures is an advantage, as many mesophilic enzymes denature under such conditions. This property makes it a promising candidate for industrial biotechnological applications.[35] Furthermore, bacterial cellulases hold great potential for biofuel production from lignocellulosic biomass, offering a more sustainable and cost-effective alternative to traditional chemical methods. The culture medium for this isolate was optimized with a pH of 8.0, leading to maximum cellulase activity, and the enzyme's performance under alkaline conditions enhances its potential for applications in industries like food, brewing, and wine production.

In terms of enzyme optimization, traditional methods such as the One-Factor-at-a-Time (OFAT) approach have been commonly used to optimize nutrient conditions for enhanced enzyme production.[36-41] However, more sophisticated statistical methods like Plackett-Burman (PB) design are increasingly used to identify the most influential factors in enzyme production.[42-44] In this study, PB design led to a five-fold increase in cellulase production. Similar approaches have been used for optimizing the production of other thermostable enzymes, such as recombinant xylanases[40] and cellulases. Additionally, the use of alternative carbon sources, such as sugar cane molasses and wheat bran, showed significant improvements in cellulase production compared to purified carbon sources, offering a cost-effective solution for large-scale fermentation processes.[44,45]

Several studies have explored the use of agricultural waste as an alternative carbon source for cellulase productions.[45,46] While untreated lignocellulosic waste materials, such as rice straw and corn cob, have demonstrated low cellulase production, treatment methods (acid/alkali/steam) can enhance enzyme production by inducing cellulase enzyme gene expression.[47-49] For instance, Ahmad *et al*. reported a *Bacillus* sp. CX6 showed the highest ability to saccharify wheat straw.[50] *Brevibacillus borstelensis* and *Bacillus subtilis* have been shown to achieve significant saccharification efficiencies using corncob as a substrate.[51] In this study, *Geobacillus* sp. TP-3 exhibited optimal cellulase activity at 50 °C and pH 8.0, and its enzyme retained considerable stability, with 68% activity after 3 hours at elevated temperatures. The structural stability of cellulases is a key factor in their industrial application. Studies have shown that thermostable cellulases, such as those from *Thermobifida fusca,*[52] *Geobacillus* sp. 70PC53[53] and *Dictyoglomus turgidum*,[54] maintain their activity even at elevated temperatures. This thermostability is essential for applications in industries that require high-temperature processing, such as textile, detergent, paper, and biofuel production.

The potential of *Geobacillus* sp. TP-3 was evaluated for saccharification of pretreated sawdust, with promising results. Approximately 50% saccharification was achieved using alkali-pretreated sawdust under optimized conditions of pH 5.5 and 50 °C.[55] This indicates that *Geobacillus* sp. TP-3 is capable of efficiently breaking down the complex lignocellulosic structure of sawdust, particularly when the material undergoes alkali pretreatment, which enhances the accessibility of cellulose for enzymatic hydrolysis. The observed saccharification efficiency suggests that *Geobacillus* sp. TP-3 could be a viable candidate for industrial applications in biofuel production, particularly in processes involving lignocellulosic biomass as a feedstock. Additionally, the enzyme's activity under mild acidic conditions (pH 5.5) and at relatively high temperatures (50 °C) highlights its robustness and potential for use in a variety of industrial processes where such conditions are common. These findings further emphasize the importance of optimizing pretreatment methods to enhance enzyme-substrate interactions and improve the overall efficiency of lignocellulosic biomass conversion.

CONCLUSION

Geobacillus stearothermophilus TP-3, isolated from the Tapovan hot spring, is a novel thermophilic lignocellulolytic bacterium with significant industrial potential due to its strong

cellulolytic activity. Optimization strategies achieved a three-fold increase in enzyme yield, with the cellulase demonstrating exceptional thermo-alkali stability and effective degradation of agricultural residues. TP-3 is a promising biocatalyst for sustainable biomass valorization and bioethanol production.

Future research should focus on genetic engineering and optimizing metabolic pathways to enhance cellulase production. Additionally, scaling up fermentation processes and integrating TP-3-derived enzymes into industrial applications could boost bioethanol efficiency. Exploring co-culturing strategies and conducting detailed studies on TP-3 cellulases will facilitate its broader use in biofuel production and other industrial sectors requiring effective biomass degradation.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.19.2.11

Additional file: Additional Table.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

DV and MS conceptualized and designed the study. MA and GC collected the sample, and carried out the experimental work. TF and AK assisted in experimental work. MA wrote the original draft. MS performed supervision. All authors reviewed, edited and approved the final manuscript for publication.

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DATA AVAILABILITY

The 16S rRNA gene sequence information is available in the NCBI database repository, with Accession No. OP962434.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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