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RESEARCH

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Cloning, heterologous expression and purification of the novel thermo-alkalistable cellulase from *Geobacillus* sp. TP-3 and its molecular characterisation

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Abstract

Background Thermophilic cellulases are essential for effectively degrading cellulose, which is a significant part of lignocellulosic waste. In this study, we focused on a cellulase gene (~ 1.2 kb) obtained from *Geobacillus* sp. TP-3, a thermo-alkalophilic bacterium isolated from the hot springs of Tapovan (Uttarakhand, India). Cellulase gene (~ 1.2 kb) was amplified via PCR, cloned into pET-28a (+) vector, transferred to *Escherichia coli* DH5α cells and expressed in *Escherichia* coli BL21 (DE3). The recombinant cellulase (*rCel_TP*) was purified using Ni^{2+}-NTA affinity chromatography.

Results The purified *rCel_TP* enzyme exhibited optimal activity at 50 ºC and pH 8, displaying stability even after 3 h of incubation at 50 ºC. The molecular weight of the purified 6 × His-tagged *rCel_TP* was determined to be ~ 40.2 kDa. Under conditions of 50 ºC and pH 8, the kinetic parameters of the purified enzyme were determined, with K_m and V_{max} values of 116.78 mg/mL and 44.05 µmolmg^{-1} min^{-1}, respectively. The activity of the *rCel_TP* cellulase was significantly improved by Hg^{2+}, Cu^{2+} and Co^{2+}. However, it was suppressed by dithiothreitol and β-mercaptoethanol. Ethylenediaminetetraacetic acid and solvents also had a slight inhibitory effect.

Conclusion These results suggest the potential applications of the recombinant cellulase in biomass conversion processes for the production of fuels and other industrial operations. The study contributes valuable insights into the properties and applicability of cellulases derived from extremophilic microorganisms.

Keywords Cloning, Heterologous expression, *Geobacillus*, Ni^{2+}NTA affinity chromatography, Thermo-alkalistable cellulase

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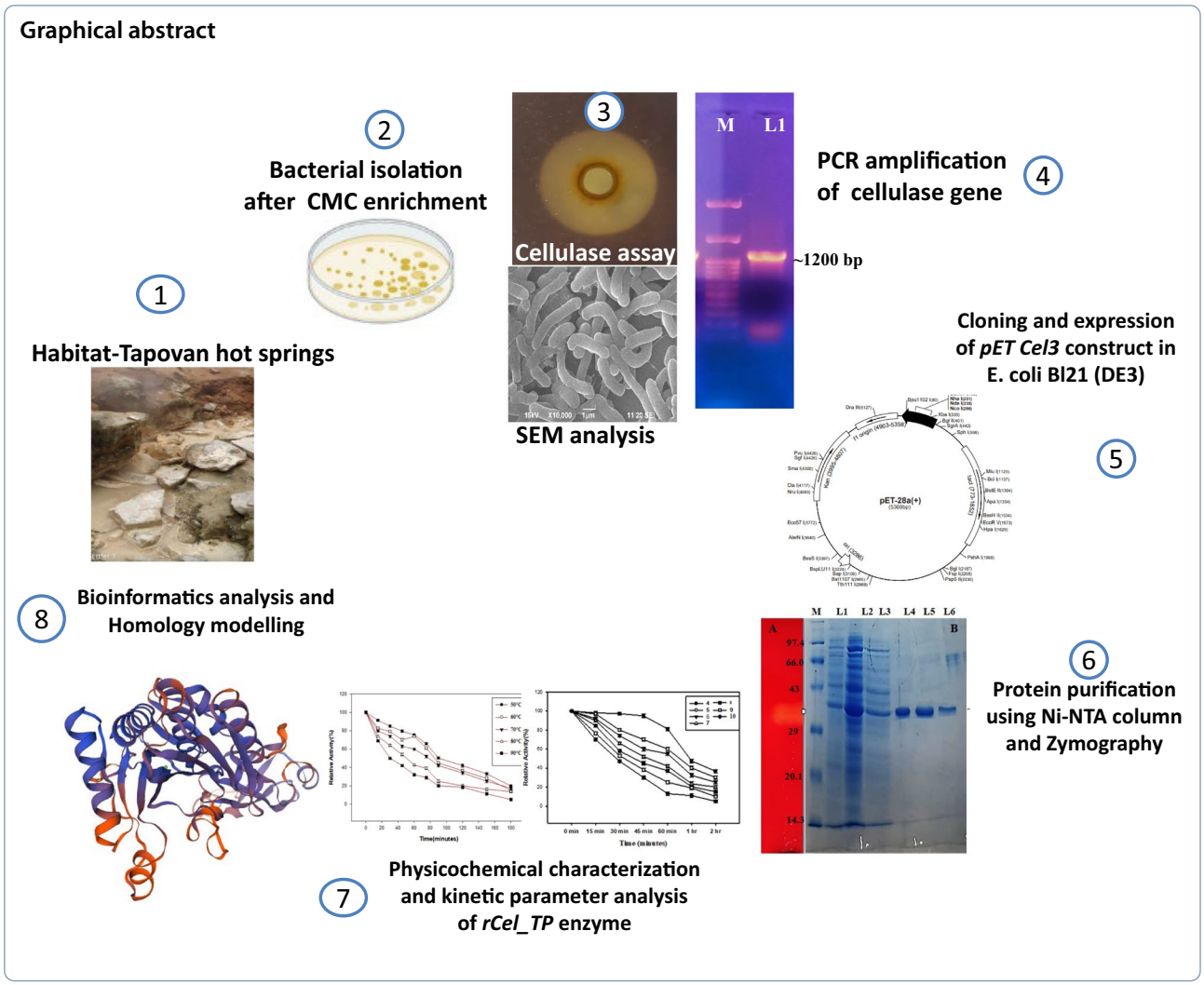

1 Background

The world is facing several challenges like energy crisis, resource shortages and pollution. One promising solution to some of these issues is using lignocellulosic agro-waste material. These materials can be converted into high-value compounds, which not only help us get rid of them from the environment but also can be used as a substrate for bioethanol production [1–3]. Bioethanol derived from lignocellulosic biomass presents a promising alternative as a renewable fuel source. This has the potential to address the current energy crisis and contribute to the mitigation of greenhouse gas emissions [4–6]. However, converting lignocellulosic biomass into any product is challenging due to its recalcitrant nature. Many use chemical treatments like solvents, acids and bases, which lead to low product yield and harmful by-products, making the process extremely costly [7–9]. A method that shows promise is microbial enzyme-based hydrolysis because of its affordability, eco-friendliness, gentle operating conditions and selectivity and specificity [10, 11]. Cellulases and xylanases are used in combination with laccases to degrade lignocellulosic waste [12, 13]. Cellulases are the key enzymes in cellulose degradation. Cellulase enzyme is classified into three groups based on their specific hydrolytic site: endoglucanase, exoglucanase and β-glucosidase. Cellobiohydrolase, endoglucanase, carboxymethyl cellulase (CMCase) and β-glucosidases are necessary for cellulosic matters degradation [14]. Natural cellulosic materials are consumed in huge quantities, yet agro-waste raw materials containing cellulose are still underutilised [10, 15].

Diverse microorganisms (actinomycetes, bacteria, or fungi) harbour cellulase enzymes for degradation of the cellulosic material and utilisation of product as substrates [16]. Fungi produce extracellular cellulases; however, they are not used in industry because of the sluggish growth

rate of fungi on lignocellulosic waste and the use of pure cellulose as a cellulase inducer, which makes the whole process expensive [17, 18]. The mesophilic microbes cannot be employed in industries due to their moderate thermal and pH stability and less robustness in industrial settings [19]. Therefore, researchers have continuously tried to search for robust enzymes from different environments. Thermophilic cellulase-based bioprocessing of cellulosic biomass into biofuels has recently gained much attention. This method involves using cellulase enzymes at high temperatures, which can provide several benefits. These benefits include better hydrolysis of the cellulosic substrate, improved mass transfer rates leading to better substrate solubility, reduced risk of contamination and increased flexibility in process design. All of these factors combine to enhance the overall economics of the process [20–22]. Thermophilic bacteria are a promising source of enzymes that can break down cellulose, offering greater stability, higher specific activity and easier mass transfer [21–24]. Thermo-alkalistable cellulases are enzymes that have potential in various industrial processes, especially in harsh conditions. However, we still need to fully understand their stability and activity under extreme conditions and the genetic determinants that govern their expression. Investigating their genetic and biochemical basis is essential for optimising them for industrial use. Closing these knowledge gaps will advance our understanding of enzyme structure–function relationships and facilitate more efficient and economically viable processes in the biotechnology and bioenergy sectors.

Cellulase can be produced using naturally occurring microorganisms or through recombinant expression in either prokaryotic or eukaryotic host systems [25]. Naturally occurring microbes secrete cellulases in the presence of cellulose inducer, which makes the process economically unviable. Additionally, these microbes cannot grow easily on complex lignocellulosic agricultural waste due to recalcitrant nature of agro-waste.

Cellulase production faces several challenges, including a lack of understanding of the genetic controls required for optimisation, which previous studies have yet to fully elucidate [26]. Despite advancements in commercial enzymes, operational costs remain high, making widespread industrial use difficult [27, 28]. Fermentation using natural microorganisms is hindered by high substrate costs and difficulties in maintaining optimal conditions for production [29]. While recombinant systems hold promise, further enhancements and optimisation are needed to compete with commercial cellulases [27, 29]. Challenges persist in heterologous expression, with difficulties achieving high yields and full-length protein expression in bacterial and plant systems [30]. Transporting proteins across cell walls presents additional hurdles, leading to enzyme truncation and expression failures in certain host organisms. The host-specific features, including variations in cell wall structures and subcellular compartments, further complicate efficient cellulase expression [30, 31]. Researchers are focusing on using the *Escherichia coli* expression system to create recombinant cellulase. *E. coli* is a good choice because it proliferates quickly, does not require any specific medium and can be induced using inexpensive substrates [32].

This paper describes the cloning and expression of the cellulase gene from *Geobacillus* sp. TP-3 which was obtained from the Tapovan hot spring (Uttarakhand) in India. The fermentation conditions were optimised for soluble recombinant enzyme production, purified the enzyme using affinity chromatography and characterised its kinetic parameters.

Table 1 Degenerate PCR oligo-primers designed for thermophilic cellulase genes based on literature

Forward primer	5′ATCCTCGAGATG GCRAARTTNGAYG-MAACRTTRAC3′ Xho1
Reverse primer	5′TGCGGATCC TTATTMGTCAAA BGTMAKTTS-BTTCAC3′ BamH1
R	A, G
N	A, C, G, T
Y	C, T
M	A, C
B	C, G, T
K	G, T
S	C, G

2 Methods

2.1 Strains and culture conditions

Geobacillus sp. TP-3 is a thermo-alkalophilic bacterium isolated from the Tapovan hot spring in Uttarakhand. *Geobacillus* sp. TP-3 was grown routinely on media [g/L glucose-5.0, KH_2PO_4-1.0, K_2HPO_4-11.5, $MgSO_4$-0.05, yeast extract-5.0, $FeSO_4$-0.00125, carboxymethyl cellulose-10.0] at 50 °C. *Escherichia coli* DH5α (Himedia, India) was chosen for gene cloning and vector construction due to its high transformation efficiency. It is grown in LB broth/agar at 37 °C. *Escherichia coli* BL-21 (DE3) (NEB, Massachusetts, USA), known for its robust protein expression, was used for heterologous expression and is grown in/on LB-kanamycin (50 µg/mL) broth/agar at 37 °C.

2.2 Chemicals

The pET-28a (+) vector was bought from Merck (New Jersey, USA). Restriction endonuclease enzymes and ligase enzymes were procured from Genei (India). The protein medium range marker and the DNA Ladder were procured from Genei and Himedia India. PCR primers were prepared by Integrated DNA Technologies Inc. (USA). The Ni–NTA agarose resin, PCR cleanup kit and gel extraction kit were bought from ThermoFisher Scientific (Massachusetts, USA). Plasmid extraction kits were bought from Helix Company. Imidazole was procured from Merck (New Jersey, USA). All other chemicals utilised during the process were of analytical grade, ensuring their high quality.

2.3 Analysis of cellulase gene/enzyme using bioinformatics tools

Open Reading Frame Finder (ORF) tool and Blast analysis of NCBI were used for initial bioinformatics analysis (https:// www. ncbi. nlm. nih. gov/). The ProtParam tool of Expasy was used for the prediction of theoretical parameters of the translated protein (https://web.expasy.org/protparam/). The Signal P online website was utilised to predict the signal peptide in the cellulase enzyme (https://services.healthtech.dtu.dk/services/SignalP-5.0/). The Mega 11 software was employed for phylogenetic studies (https://www.megasoftware.net/) [26, 33]. The SWISS-MODEL and PyMOL software was used to predict the tertiary structure of the cellulase enzyme (https://swissmodel.expasy.org/). ProteinTools a web server toolkit was used for protein structure analysis, e.g. hydrogen bond networks, hydrophobic clusters, contact maps and salt bridges (https://proteintools.uni-bayreuth.de) [34].

2.4 Genomic DNA isolation and PCR amplification of the cellulase gene

Geobacillus sp. TP-3 was grown aerobically in 50 mL of Luria broth medium at 50 °C overnight. DNA was isolated using a conventional phenol–chloroform extraction method. After purification, the genomic DNA was suspended in 50 µL of MilliQ water and used as a template. Degenerate primers were designed based on the literature on thermophilic cellulase gene sequences retrieved from NCBI (Table 1). The polymerase chain reaction (PCR) was conducted with the following parameters: 94 °C for 5 min (initial denaturation); 94 °C for 60 s (denaturation); 58 °C for 30 s (annealing), 72 °C for 80 s (extension) for 30 cycles, followed by incubation at 72 °C for 10 min (final extension step). The presence of amplicon was confirmed using agarose gel electrophoresis (1.2% w/v). Finally, the gene sequence was confirmed by Sanger's sequencing to verify the presence of the cellulase gene (Additional files 1: Fig. S1; Additional File 2).The nucleotide sequence encoding cellulase gene was submitted to the GenBank database under accession no. WET54884.1.

2.5 Construction and expression of recombinant vector*pET-Cel3*

A recombinant vector named pET-*Cel3* was created using the pET-28a (+) expression vector. The primers used in the process incorporated *BamHI* and *XhoI* sites, which were determined by analysing the cellulase gene using the NEB cutter tool (https:// nc3. neb. com/NEBcutter/) (Additional files 1: Fig. S2). This was done to enable directional cloning in the pET-28a (+) vector. The amplified cellulase coding fragment and pET-28a(+) were double-digested using *BamHI* and *XhoI* and then purified using a gel extraction kit. They were then combined in appropriate concentrations with the ligase enzyme and left overnight at 16 °C. The competent *E. coli* DH5α hosts were transformed with recombinant *pET-Cel3* using the heat shock method at 42 °C for 45 s in a water bath. Random clones were selected from a kanamycin (50 µg/mL) LB-agar plate and confirmed by colony PCR using cellulase gene primers. Positive clones were cultured in LB-kanamycin media, followed by plasmid preparation. The presence of the cellulase fragment in the recombinant vector *pET-Cel 3* was confirmed by double digestion with *BamHI* and *XhoI* followed by sequencing.

E. coli BL21 (DE3) cells were transformed with ~ 10 ng of recombinant construct vector. The clone expressing *rCel_TP* was then grown overnight in LB-agar kanamycin (50 µg/mL) broth until it OD_{600} (optical density) reached 0.5 – 0.7. Once the desired optical density was achieved, induction of the cellulase gene expression was done with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After approximately 12 h of incubation at 37 °C and 150 rpm, the culture was centrifugation (4000 rpm at 4 °C). While the culture was growing, 1 mL aliquots were collected every two hours to monitor its growth and protein profile. The expression of *rCel_TP* enzyme was determined by analysing the protein profile on a 12% (w/v) SDS-PAGE stained with Coomassie staining solution.

2.6 Fermentation conditions optimisation for *rCel_TP* cellulase enzyme production

To optimise the production of cellulase enzyme, the positive clone of *E. coli* BL21 (DE3) containing *pET-Cel3* construct was cultured in different temperatures (18 °C to 37 °C) and harvested at different times, ranging from 4 to 12 h after induction. The induction was done with various IPTG concentrations ranging from 0.5 mM to 3 mM.

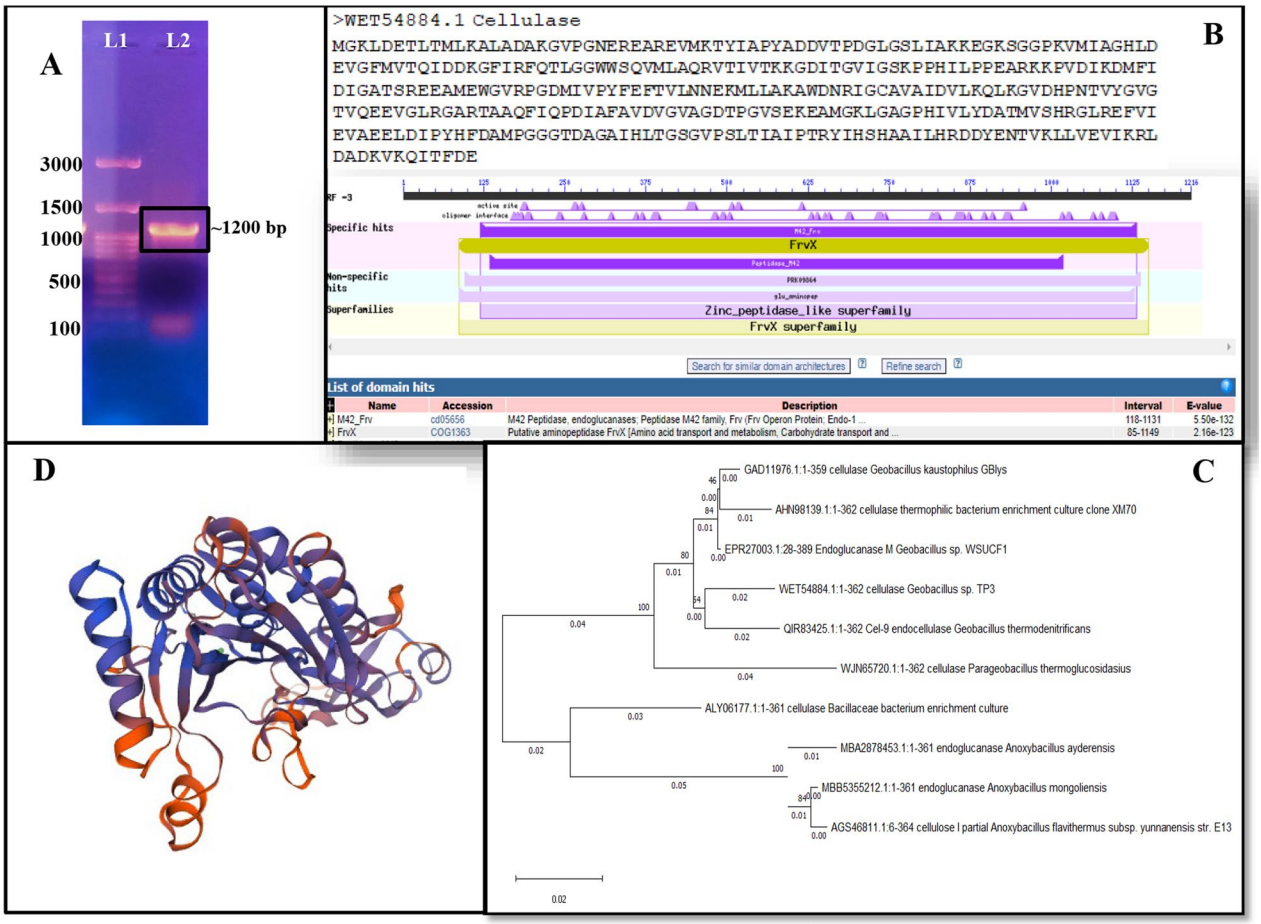

Fig. 1 **A** PCR amplification of cellulase gene using degenerate primers and genomic DNA of *Geobacillus* sp. TP3 as template; **B** Translated amino acid sequence of cellulase gene (accession no. WET54884.1) and BLASTp analysis of translated sequence showed homology with peptidase/endoglucanase M42 family; **C** The phylogenetic tree showed a close relatedness of cellulase with endocellulase of *Geobacillus thermodenitrificans* and cellulases/endoglucanases of other bacterial sources; **D** three-dimensional structure model of cellulase enzyme designed based on Endoglucanase (TM1050) from *Thermotoga maritima* using SWISS-MODEL software

2.7 Purification of *rCel_TP* cellulase enzyme

Ni^{2+}-NTA resin-based affinity chromatography was used for purification. *E. coli* BL21 (DE3) cells expressing *rCel_TP* were cultured in kanamycin-supplemented LB medium at 25 °C and 150 rpm. The cells were induced with 0.5 mM IPTG and again grown for 6 h and then harvested by centrifugation. The resultant pellet was suspended in a lysis buffer containing 30 mM phosphate buffer (pH 8.0), 0.3 M NaCl and 10 mM imidazole. The suspension was sonicated for 15 cycles of 60 s on and 90 s off in an ice bucket. This was followed by centrifugation at 7000 rpm for 20 min. The resulting cell-free extract (CFE) was sieved using a 0.22 µm membrane filter to facilitate protein purification. The filtered CFE was then introduced onto a precalibrated column of Ni^{2+}-NTA, which was equipped with two buffers—buffer A [6 M urea in 100 ml of 0.1 M phosphate buffer (pH 8.0) and 5 ml of β-mercaptoethanol] and buffer B [buffer A supplemented with 10 ml of Tris–HCl at pH 8]. The *rCel_TP* enzyme was allowed to bind to the resin for 50–60 min at room temperature followed by washing with buffer C (buffer B with 20 mM imidazole buffer) to remove weakly bound heteroproteins and non-specific His-tagged proteins. The bound protein was then collected in 1 mL fractions using elution buffer D (0.1 M phosphate buffer pH 8, 10 mM Tris–HCl pH 8, 5 mM β-mercaptoethanol, 20 mM imidazole and 10% v/v glycerol) in a gradient manner, followed by washing with buffer E (buffer D with 100 mM to 500 mM imidazole). The purified recombinant protein *rCel_TP* was checked for the degree of protein purity and the protein expression profile of the eluted fractions on 12% w/v SDS-PAGE. Bradford method was used for estimation of the protein concentration with bovine serum albumin (BSA) as a reference [35].

2.7.1 Zymography

Zymography was used to assess cellulase activity in a 12% SDS-PAGE gel with carboxymethyl cellulose (0.2% w/v) at 80 V for three hours. After electrophoresis, the gel was rinsed with phosphate buffer (0.1 mM, pH 8) having 2.5% Triton X-100 for 30 min, so that enzyme can refold. Then, the gel was incubated in the buffer for one hour at 50 °C. Finally, staining of the gel was done with Congo red (0.2% w/v) for 25 min at room temperature and decolorised with sodium chloride (1.0 M) [36].

2.8 Quantitative enzymatic assay of *rCel_TP*

The *rCel_TP* activity was determined by the DNS method [29, 37]. For the same, 50µL of *rCel_TP* was mixed with 1% CMC and glycine NaOH buffer (pH 8) and incubated for 10 min at 50 °C. DNS reagent (3 mL) was added to stop the reaction and boiled for 10 min, and the reducing sugars present in the supernatant were measured at 545 nm. Enzyme activity was expressed as µmol of reducing sugar equivalent to glucose released per minute per mg of protein under standard assay conditions. Enzyme activity was measured in triplicate reaction.

2.9 *rCel_TP* enzyme characterisation

The standard assay method was used to determine the optimum pH for *rCel_TP* activity. The assay was conducted at different pH levels using three different buffers: 50 mM sodium acetate for pH 4.0–5.0, 50 mM sodium phosphate for pH 6.0–7.0 and 50 mM glycine NaOH for pH 8.0–12. After incubating it in the appropriate buffers for 24 h at 4 °C, the stability of *rCel_TP* at various pH levels was examined. The residual activity was calculated using the conventional assay. The standard cellulase experiment was conducted at several temperatures within the 40–90 °C range to determine the ideal temperature for *rCel_TP*. The enzyme was incubated at different temperatures (50–90 °C) for varying durations (15–210 min) to ascertain thermal stability. The standard test was then used to detect the residual activity level. The impact of metal ions and additives on the activity of purified *rCel_TP* was assessed by incubating enzyme in the presence of various metal ions and inhibitors (β-mercaptoethanol, dithiothreitol and ethylenediaminetetraacetic acid) at three concentrations (1 mM, 5 mM and 10 mM). The impact of detergents (Triton X, Tween-20, Tween-80 and sodium dodecyl sulphate) as well as solvents (toluene, acetone, chloroform, ethanol, methanol, butanol and propanol) was also assessed at 0.5%, 1% and 10% concentration. Following a half-hour incubation period with these substances, the enzyme activity was performed under standard assay procedure [35]. After every treatment, the quantity of cellulase activity that remained was assessed. Cellulase, *rCel_TP,* was tested under standard conditions (0.1 mM glycine NaOH buffer, pH 8, 50 °C) with varying concentrations of carboxymethyl cellulose (1–18 mg/mL%) as a substrate to ascertain its substrate specificity and enzyme kinetics. A Lineweaver–Burk plot was used to measure the rate of CMC hydrolysis to calculate the maximum velocity (V_{max}) and Michaelis–Menten constants (K_m) of the *rCel_TP* enzyme.

2.9.1 Data analysis

All reactions were conducted in triplicate to assess the variability within each set of triplicates, and the values were reported as mean ± S.D. SigmaPlot was used for data analysis and graphical illustrations.

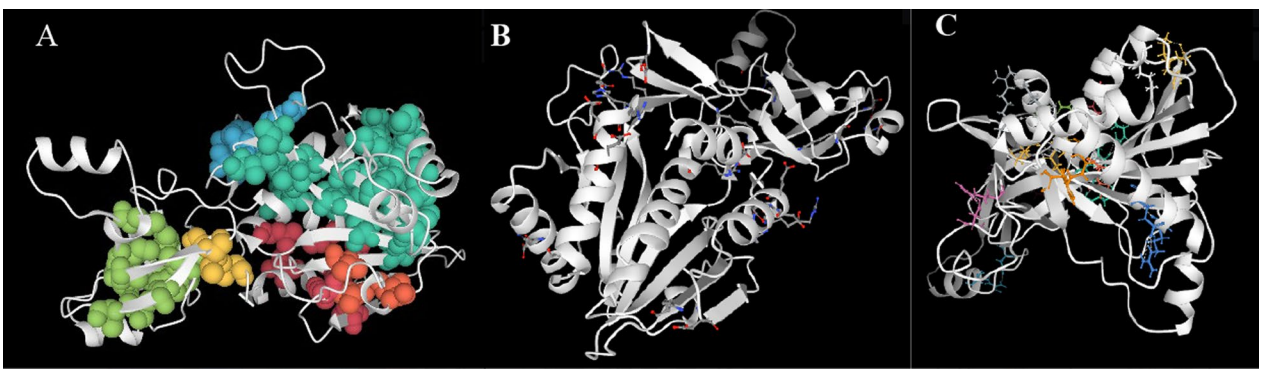

Fig. 2 Analysis of the cellulase enzyme model using Protein toolkit. **A** Presence of six of hydrophobic cluster **B** Salt bridges (10) present in the cellulase protein using fraction of Charged Residues (FCR) of 0.24 and Kappa value (κ) 0.15; **C** The hydrogen bonding interaction (13) in the cellulase enzyme

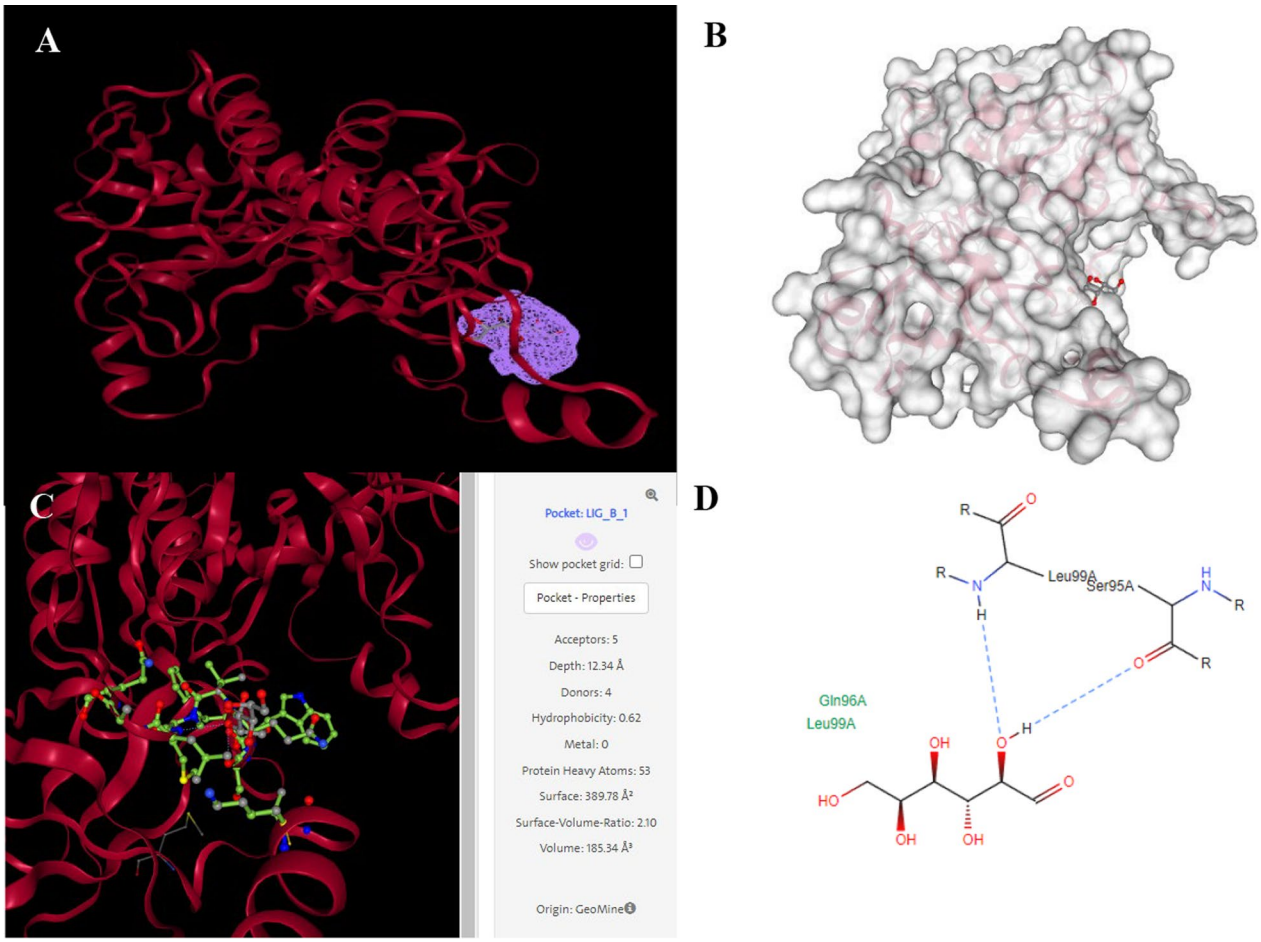

Fig. 3 Active site prediction studies. **A** Prediction of the potential binding site of carboxymethyl cellulose using grid-based method (DoGSite3). **B** The space filled model of the protein showing the binding site of the cellulose molecule **C** Binding pocket properties **D**. 2-D interaction of the substrate residues with the amino acid at active site

3 Results

3.1 Genomic DNA isolation and PCR amplification of the cellulase gene

The genome DNA of *Geobacillus* sp. TP-3 was used a template and ~ 1.2 kb long cellulase DNA gene was amplified using PCR (Fig. 1A). The cellulase gene sequence analysis revealed the higher presence of G + C content, which may be the reason of higher thermostability of the cellulase gene. The composition of the 1149 bp fragment was as follows: A 18% (211), T24% (268), G27% (311) and C 31% (359). After sequencing, the sequencing data were analysed using ORF finder tool, and the longest ORF was subjected to Blast P analysis. The BLASTp analysis showed similarity with endoglucanases and endocellulases (Fig. 1B). Translated protein showed 97.24% similarity with endoglucanase M of *Geobacillus* sp. WSUCF1 (89% query coverage), 97.21% similarity to cellulase of *Geobacillus kaustophilus* GBlys (94% query coverage) and 96.41% similarity to Cel 9 endocellulase of *Geobacillus thermodenitrificans* (95% query coverage) (Additional files 1: Fig. S3). The encoded fragment comprised 362 amino acids with approximately 39.266 kDa molecular weight with *pI* value of 5.44. Notably, the computed instability index was 20.12, signifying stability, and the aliphatic index was determined as 93.45. A high aliphatic index suggests that the translated protein is thermally stable across a broad temperature range [38]. The Grand Average of Hydropathy (GRAVY) for protein was -0.183, meaning the proposed protein was slightly hydrophilic. The Signal P tool detected no presence of signal peptide in the sequence (see Additional files 1: Fig. S4). Multiple sequence alignment (Additional files 1: Fig. S5.) and phylogenetic analysis was done utilising the Mega 11 software using neighbour joining method and it was deduced that the cellulase enzyme is phylogenetically related to the *Geobacillus thermodenitrificans* endocellulase enzyme (Fig. 1C). The translated protein belongs to peptidase/endoglucanases M42 family, and the homology

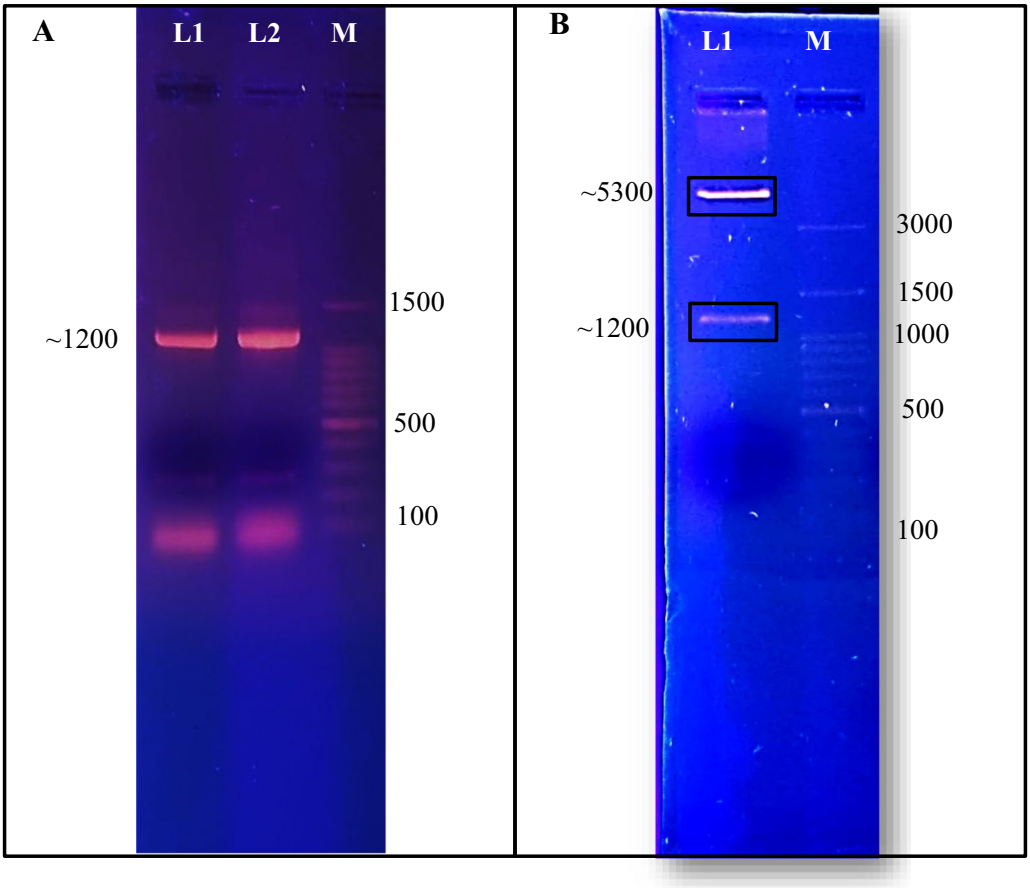

Fig. 4 Confirmation of *pET-Cel3* construct in the transformed *E. coli* DH5α host. **A** Colony PCR analysis of cellulase gene from two clones. L1 and L2:1200 bp PCR product amplified using cellulase primers from two random colonies; M Marker; **B** Restriction digestion of pET-Cel3 recombinant expression vector. L1, Fragments ~ 5300 corresponding to pET 28 (+) vector and ~ 1200 bp fragment of cellulase gene resulted double digestion of expression with *XhoI* and *BamHI* enzymes; M- 100 bp DNA Marker. L denotes lane here

model (Fig. 1D) created by SWISS-MODEL showed it sequence identity (77.44%) with aminopeptidase/glucanase homolog (SMLT id: 1.vhe.1) [39] as well as with endoglucanase (36.36%) of *Thermotoga maritima* (SMLT id: 3isx.1).

To understand the thermal stability of the protein, amino acid composition analysis was done using ProtParam software (Additional files 1: Fig. S6). The analysis of translated protein amino acids sequence showed that the enzyme contains fewer thermolabile amino acids like Asn (6), Gln (9), Met (13) and Cys (1). These amino acids make the protein structure unstable at high temperatures as they undergo deamidation (Asn and Gln) or oxidation (Met and Cys) [40]. Such amino acids are less common in thermophilic proteins; when they occur, they are usually buried [41]. The enzyme also showed a higher prevalence of Gly (38), Lys (25) and Ile (27), which are preferred in thermophilic proteins [42]. The cellulase protein also has more charged residues, such as Lys (25) and Glu (23), common in other thermophilic proteins. Furthermore, it has fewer Gln (9), Ala (33) and His (10) residues on the surface [43]. Cellulase enzymes also have a higher concentration of seven amino acids -IVYWREL-(137), which is a universal predictor of optimal growth temperature in prokaryotes. The enzyme also have a high purine (A + G) content, which results in better protein thermal adaptation [44].

The modelled protein revealed the presence of 6 hydrophobic clusters, ten salt bridge formations and thirteen hydrogen bonding patterns (Fig. 2). A grid-based method (DoGSite3) based on the difference of the Gaussian filter of the Protein toolkit was used to detect potential binding pockets. The pocket analysis showed the presence of five acceptors, four donors, hydrophobicity of 0.62, active site depth of 12.34 Å and volume of 185.34 Å (Fig. 3). Enzyme exhibited substrate interaction with Leu 99 and Ser 95 amino acid residues of the enzyme (Fig. 3D). However, the exact mechanism is yet to be elucidated. The Ramachandran plot of the model showed that 93.53% of the residues were Ramachandran favoured, 1.47%

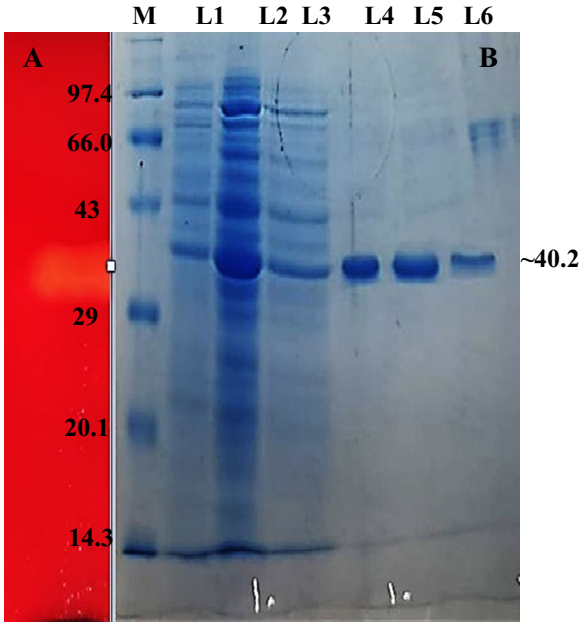

Fig. 5 Protein purification of *rCel_TP*. **A** Zymogram analysis of purified *rCel_TP* cellulase enzyme; **B** A SDS-PAGE (12% w/v) analysis of His-tagged *rCel_TP* cellulase enzyme purified using immobilised metal affinity chromatography (Ni–NTA). M- Protein molecular weight marker ((14.3–97.4 kDa); L1 Cell-free extract of uninduced cells; L2 Cell-free extract of IPTG (0.5 mM) induced cells; L3 Proteins eluted with buffer containing 100 mM imidazole; L4-6 Eluted purified *rCel_TP* proteins at 200 mM, 250 mM and 500 mM imidazole, respectively. L denotes lane here

Ramachandran outliers and 1.10% rotamer outliers (Additional files 1: Fig. S6).

3.2 Construction of recombinant expression plasmid

The positive clones expressing *pET-Cel3* vectors were confirmed by colony PCR (see Fig. 4A) as well as double digestion with *BamHI* and *XhoI* restriction endonucleases. The recombinant plasmid smoothly released the insert (cellulase gene) of the size ~ 1.2 kb fragment and ~ 5300 kb size of linearised empty pET-28a (+) vector (see Fig. 4B). The *E. coli* BL21 (DE3) host cells containing the *pET-Cel3* vector were cultured in LB-kanamycin at different temperatures. However, they showed negligible expression of the *rCel_TP* enzyme at 35 °C and 37 °C. The band of *rCel_TP* showed a denser band in 25 °C to 30 °C. At 25 °C, the thickest band was observed, indicating the highest level of gene expression. The *pET-Cel3* expression was induced with various concentrations of IPTG and a high titre of cellulase was attained at 0.5 mM IPTG after 6 h post-induction at 25 °C. *rCel_TP* was obtained as an intracellular solubilised fractions which lacked secretary signal.

3.3 Purification of *rCel_TP*

The protein *rCel_TP* was purified using a Ni^{2+}-NTA affinity column with 100-200 mM imidazole concentration. A single band of ~ 40.2 kDa was observed on a 12% (w/v) SDS-PAGE at 200 mM, 250 mM and 500 mM imidazole eluted fractions. The recombinant protein has been purified successfully using a single-step affinity chromatography method. Protein profiling of induced *E. coli* cells and eluted fractions showed a single thick band at ~ 40.2 kDa, matching the calculated molecular mass of ~ 40.1 kDa six His-tagged *rCel_TP* (the theoretical molecular weight is 39.266 kDa, with an additional 0.8 kDa for the 6xHis-tag) using SDS-PAGE. No similar-sized and intensity band was found in uninduced cells. The cellulase activity was confirmed through a zymogram, which showed a yellowish-orange halo corresponding to the ~ 40.2 kDa band (Fig. 5A, 5B).

3.4 Recombinant cellulase *rCel_TP* enzyme characterisation

The *rCel_TP* enzyme exhibited optimum activity at pH 8.0 in glycine NaOH buffer and retained 90% activity for up to 1 h. It stayed active in 5.0–9.0 pH range, maintaining 70–75% activity at pH 6.0–9.0 for up to an hour (Fig. 6A and 6B). However, at higher temperatures, the enzyme lost its activity more quickly. The enzyme had optimal activity at 50 °C, with 80% activity retention for an hour. It retained 75%-60% activity between 50 °C and 90 °C for an hour, but decreased significantly afterwards (Fig. 6C and 6D).When incubated for more extended periods, the enzyme's activity declined more sharply.

The impacts of diverse metal ions, detergents, solvents and inhibitors on *rCel_TP* have been summarised and presented in Table 2. The presence of cations such as Hg^{2+}, Cu^{2+} and Co^{2+} improved cellulase activity. However, high concentrations of Ca^{2+}, NH_4^{2+}, Fe^{3+} and Mg^{2+} inhibited cellulase activity. The purified *rCel_TP* showed weaker stability in surfactants. In the presence of anionic surfactant SDS (0.5%), the recombinant enzyme retained 70% activity, and with non-ionic surfactant Tween 20 (1%), it retained 80% activity. However, it showed reduced activity of 70% in Tween 80 and approximately 50% in Triton X. At a concentration of 0.5%, Triton X-100 reduced cellulase activity by 70%. Surfactants interact with enzymes using hydrophobic interaction as well as ionic interactions. These interactions may result in conformational changes in the enzyme and cause reduced / loss of enzyme activity [45]. Additionally, at a 10% concentration, EDTA inhibited cellulase activity by 50%, demonstrating the role of divalent cations in cellulase

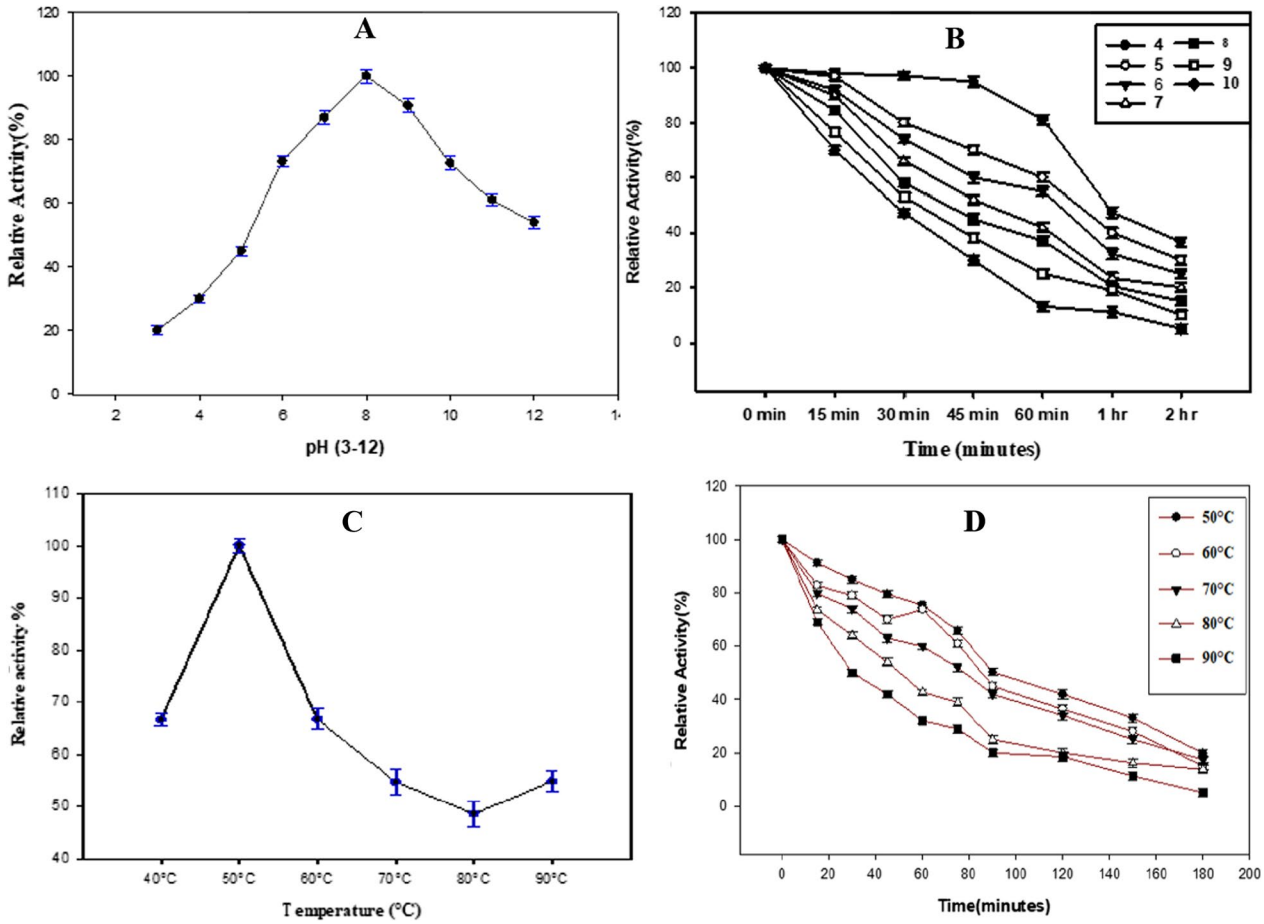

Fig. 6 Characterisation of purified *rCel_TP* enzyme. **A** Optimum pH; **B** pH stability of *rCel_TP* enzyme; **C** Optimum temperature; **D**. Thermostability of the *rCel_TP* cellulase enzymes under varying temperatures (all reactions were performed in triplicate)

activity. It was also evident from enhanced cellulase activity in the presence of metal cations (Hg^{2+}, Cu^{2+} and Co^{2+}). Chelation of the metal cofactor by EDTA might have resulted in reduced activity. Bioinformatics domain analysis also revealed that the protein belongs to the metalloprotein M42 family. The *rCel_TP* enzyme was inhibited by β-mercaptoethanol and DTT, and its activity was reduced by 80% in the presence of DTT. The reduced activity may be due to breaking protein disulphide bonds, which ultimately results in protein unfolding by these thiol-containing reagents [46]. The recombinant cellulase enzyme showed the highest activity against CMC substrate, with an activity of 100%. However, no activity was observed when beechwood xylene, starch and pectin were used as substrates. The K_m and V_{max} kinetic parameters for *rCel_TP* for CMC substrate were 116.78 mg/mL and 44.05 μmol^{-1} mg^{-1} min, respectively, at pH 8 and 50 °C.

4 Discussion

Cellulases are biocatalysts that have significant industrial importance and are widely used in various processes such as paper and pulp industry, detergent, juice extraction and feed additives [18]. They are also gaining interest in agriculture, biotechnology and bioenergy sectors for utilising cellulosic biomass to produce ethanol, butanol, or other fermented products [47]. Cellulases have the prospect to become the major industrial player worldwide due to their diverse applications.

There are numerous research papers available on the recombinant expression of these enzymes. The cellulase gene from *Geobacillus* sp TP-3 was amplified using a

Table 2 Effect of metal ions, inhibitors, detergents and solvents on *rCel_TP* enzyme

	1 mM	5 mM	10 mM
Metal ions			
$MgCl_2$	82.16 ± 1.23	80.11 ± 2.12	75.11 ± 0.54
$MnCl_2$	90.03 ± 0.23	85.05 ± 2.12	80.52 ± 1.23
$CaCl_2$	79.53 ± 1.25	78.22 ± 2.32	70.45 ± 1.98
$HgCl_2$	133.85 ± 1.30	130.23 ± 1.74	125.42 ± 2.45
KCl	114.70 ± 2.25	105.77 ± 3.32	103.68 ± 2.10
NH_4Cl_2	62.74 ± 2.21	54.69 ± 0.87	50.65 ± 1.63
$CuCl_2$	132.80 ± 1.85	125.96 ± 1.74	120.93 ± 1.41
$CoCl_2$	113.64 ± 0.98	110.05 ± 0.64	98.98 ± 0.73
NaCl	93.44 ± 0.54	92.66 ± 0.74	85.36 ± 3.63
$FeCl_3$	73.76 ± 1.45	66.41 ± 2.74	52.32 ± 1.09
Inhibitors			
EDTA	79.49 ± 1.63	65.82 ± 2.23	52.06 ± 2.14
β-mercaptoethanol	57.28 ± 3.54	37.91 ± 2.74	27.94 ± 1.94
DTT	27.37 ± 1.13	21.67 ± 1.20	20.01 ± 0.87
Detergents	0.5%	1%	10%
SDS	64.37 ± 1.2	56.92 ± 0.56	52.77 ± 1.32
Tween 20	74.87 ± 2.32	70.17 ± 1.47	66.58 ± 1.54
Tween 80	63.82 ± 2.05	61.06 ± 1.04	55.54 ± 1.45
Triton × 100	48.63 ± 1.45	44.21 ± 2.12	30.96 ± 2.36
Solvent	0.5%	1%	10%
Chloroform	85.80 ± 1.03	70.60 ± 1.36	55.13 ± 1.45
Acetone	81.73 ± 0.96	51.87 ± 1.87	47.80 ± 1.50
Isopropanol	68.16 ± 2.30	64.08 ± 2.10	52.14 ± 3.63
DMF	50.24 ± 1.50	25.81 ± 1.33	24.08 ± 1.20
Glycerol	80.88 ± 3.32	78.01 ± 3.30	60.02 ± 2.97
Methanol	68.16 ± 0.90	60.08 ± 1.15	41.56 ± 1.10
Ethanol	78.64 ± 2.20	69.97 ± 2.42	67.35 ± 2.60

Enzyme activity without addition of any inhibitor is taken as 100% relative activity

primer containing *BamHI* and *XhoI* sites. The resultant amplicon of ~ 1200 bp was subsequently inserted into the pET-28a (+) vector, with *E. coli* DH5α as the cloning host and *E. coli* BL21 as the expression host. Similarly, the cellulase gene of 1500 kb from the *B. subtilis* strain was cloned in the pET-21a expression vector, and the cellulase enzyme formed inclusion bodies in *E. coli* BL21(DE3) host cells [29]. In another work, a cellulase coding gene CelC307 was treated with *NdeI* and *XhoI* and further cloned in the pET-26b(+) vector and expressed as fusion protein containing His-tag in BL21 host cells [48]. Ma et al. (2020) cloned and expressed the cellulase gene from *G. thermodenitrificans* Y7 in *E. coli* BL21 using pET-28a (+) vector. In the present study, we employed the gradual protein induction method, inducing at 25 °C for 16 h using 0.5 mM IPTG. The cellulase gene expression of *G. thermodenitrificans* Y7 was induced with 0.4 mM IPTG [49]. A successful in-frame gene insertion for expression studies was achieved by using two different restriction enzymes sites at the 5′ and 3′ ends of the gene's coding region of interest, as previously reported [50].

Using a Ni^{2+}-NTA column and the 6 × His-tag fused *rCel_TP* enzyme was purified, and SDS-PAGE analysis revealed that its molecular weight was approximately 40.2 kDa. The predicted molecular weight of *rCel_TP* without histidine tag is 39.266 kDa, and the molecular weight of the 6x-His tag is 0.8 kDa. The cumulative molecular weight comes to be around 40.1 kDa, closer to the molecular weight (MW) determined by plotting relative front (Rf) vs log MW for ladder in the SDS-PAGE. The purified cellulase of other cellulase purified from thermophilic cellulolytic *Geobacillus* sp. HTA426 bacterium and *G. thermodenitrificans* Y7 were also in same molecular weight range [49, 51]. Some other studies have reported smaller-sized thermostable alkaline cellulases (~ 38kDA) from marine bacterium *Bacillus licheniformis* AU01 and *B. licheniformis* [52], while others have reported cellulases in a higher molecular weight range (47–439 kDa) from various strains of *Bacillus, Geobacillus* and *Cohnella* [32, 48, 53–56]. The SDS-PAGE profile of pure cellulase revealed no additional subunits, indicating that it is most likely a monomer. This is consistent with the findings that most bacterial cellulases, unlike fungal cellulases, are monomers [57]. The zymogram revealed a discrete band of CMCase activity, which closely matched the molecular weight values reported on SDS-PAGE. A comparative analysis of cellulases isolated from various *Geobacillus* strains as well as other microorganisms is given in Table 3. The effect of temperature and pH on *rCel_TP* cellulase activity was studied in different temperature and pH range. The cellulase produced has a thermophilic nature, with maximum activity observed at pH 8 and 50 °C. The enzyme retained approximately 60% of its cellulase activity when exposed to temperatures ranging from 50 to 80 °C and was found to be heat-stable. *Geobacillus* sp. HTA426 cellulase retained over 80% CMCase activity when pre-incubated for 1 h at pH 7 and 50–70 °C [51]. For instance, *Geobacillus* sp. T1 cellulase enzyme maintained ~ 100% activity between 40–60 °C, but at 70 and 80 °C, activity decreases to 86% and 59.77%, respectively [55]. *Geobacillus* sp. WSUCFI's CMCase retained 70% of initial activity after one day at 70 °C [23]. *Bacillus* cellulases are less thermally stable, with *Bacillus subtilis* DR cellulase retaining 70% of its maximum activity after 30 min at 75 °C [58]. The findings align with the results documented in the literature for other cellulases that thrive in high-temperature environments [21, 59].

Table 3 Comparison of physicochemical and kinetic parameters of *rCel_TP* enzyme with other cellulases

Microorganism	Optimum pH and temperature	Kinetic parameters (Specific activity, K_m, V_{max}, K_{cat})	Thermostability/half life	Molecular mass	Activity Enhancer
Bacillus licheniformis A5 [67]	pH 6, 60 °C	ND	Residual activity 82% at 80 °C for 120 min	ND	ND
Bacillus licheniformis PANG L [68]	pH 5, 70 °C	K_m -1.8 mg/ml, V_{max} 10.92 µg/ml/min	Residual activity 67% at 55 °C for 1 h	ND	Cd^{2+}, Pb^{2+} and Ba^{2+}
Bacillus sp DUSELR13[23]	pH 5, 75 °C	K_m 3.11 mg/ml; V_{max} 0.56 U/ml	Residual activity 78% at 70 °C for 1 day	***ND***	ND
Cohnella sp. A01 [48]	pH 7 and 50 °C	K_m 0.46 mM, K_{cat} 104.30×10^{-3} (S^{-1}) and K_{cat}/K_m 226.73 (M^{-1} S^{-1})	Residual activity of 95% and 75% at 60 and 70 °C after 1 h	56 kDa	Na^+, Li^+, Ca^{2+}
Geobacillus sp. TP-3 (This study)	pH 8, 50 °C	K_m116.78 mg/mL V_{max} 44.05 µmol mg^{-1} min^{-1}	Residual activity ~ 50% within 50 to 70 °C for 1.5 h	~ 40.2 kDa	Hg^{2+}, Cu^{2+} and Co^{2+}
Geobacillus sp. HTA 486 [51]	pH7, 60 °C	CMCase activity 103.67 U/mL	Stable at 50–70 °C for 5 h	40 kDa	Ca^{2+}, Na^+ and K^+
*Geobacillus*sp. T1 [55]	pH 6.5, 70 °C	Specific activity 5.79 U/mg	Stable at 60 °C for 1 h	54 kDa	ND
Geobacillus thermodenitrificans Y7 [49]	pH 8.6, 50 °C	K_m 0.310 mg/mL V_{max} 0.024 µmol/mg/min	Retained 53.79% activity at70oC after 30 min	40.4 kDa,	Mn2 +, Fe2 + and Co2 +
Geobacillus sp. KP43 [54]	pH 7, 70 °C	CMCase activity 0.018U/ml	Maximum residual activity at 70 °C for 1 h	66 kDa	Fe^{2+}
Geobacillus sp. WSUCF1 [23]	pH5, 70 °C	K_m1.08 mg/ml,V_{max} 1.27 U/ml,	Residual activity 89% at 70 °C for 1 day	***ND***	ND
Parageobacillus thermoglucosidasius NBCB1 [69]	pH 5.5, 60 °C,	CMCase activity crystalline cellulose 305 IU/mg and amorphous cellulose 184 IU/mg	Residual activity 100% at 60 °C for 1 h in	≈33 kDa	Zn^{2+}
Thermotoga maritime [70]	pH 7.0., 70 °C	K_m 5.1 mg/mL,V_{max}682 U/mg,	> 50% of its activity after pre-incubation at 80 °C for 5 h	79 kDa	Mn^{2+} and Ca^{2+}

Various microorganisms appear to respond differently to metal ions in terms of how they affect enzyme function. The metal ions either increase or decrease the rate of enzyme activity when they attach to the carboxylic acid or amine groups in amino acids. Ionic radius size, in addition to ionic charges, significantly impacts the enzyme stability [60]. Although the precise mechanism by which metal ions affect cellulase activity is unknown, it is possible that they do so through redox reactions with amino acids, which can either raise or lower the enzyme's activity [61]. The *rCel_TP* exhibited improved activity in the presence of Hg^{2+}, Cu^{2+} and Co^{2+}, while Fe^{3+}, $NH_4{}^{2+}$, Ca^{2+} and Mg^{2+} repressed the cellulase activity at high concentrations. It is interesting to note that Hg^{2+} increased enzyme activity by about 33% at 1 mM concentration. According to Sharma et al. (2015), the cellulase of *Geobacillus toebii* PW12 also showed slightly improved cellulolytic activity in the presence of Hg^{2+} (1 mM) and Cd^{2+} (5 Mm) [62]. There are reports of enhanced cellulase activity in the presence of Cu^{2+} and Co^{2+} ions [63, 64]. Purified *rCel_TP* was shown to have reduced stability in the presence of surfactants. SDS reduced enzyme activity by 35%, even at low concentrations (0.5%), which were also observed in other cellulases in the glycoside hydrolase family (GH5 family) [65]. The recombinant cellulases of *Geobacillus* sp. TP-3 retained 70–80% activity towards SDS and Tween-20, Tween-80, but lost about 50% activity in the presence of Triton X. Yin et al. (2010) reported a thermo-alkali stable cellulase which retained 95% of its activity after 1-hour incubation with SDS. Sadhu et al. (2013) found that SDS and Tween-80 inhibit cellulase activity [66]. Therefore, *rCel_TP* is stable with SDS suggests that the enzyme can be used as an effective additive in detergents. All inhibitors inhibited the *rCel_TP* enzyme, and steep deceased in β-mercaptoethanol and DTT may be attributed to disruption of the disulphide linkages maintaining protein folding. At lower doses, EDTA inhibited *rCel_TP* activity, demonstrating the necessity of divalent cations for enzyme function. The application of *Geobacillus* sp. TP-3 thermo-alkali stable cellulase in saccharification was explored. It was found that biological pretreatment of wood sawdust with the strain increased sawdust cellulose content from 48 to 65%. The optimal conditions for saccharification were achieved through alkali-treated sawdust in citrate buffer pH 5.5 at 50 °C, resulting in a maximum rate of 49.71% (unpublished data).

The primary constraint encountered in this research was the inability to achieve hyper-induction of the cellulase enzyme despite successful recombinant heterologous expression of *rCel_TP* in *Escherichia coli* BL21. Additionally, the recombinant enzyme exhibited limited thermostability over an extended period. Future investigations could explore alternative expression vectors that respond to cost-effective inducers, enabling overexpression of the enzyme. Furthermore, protein engineering approaches could be explored to enhance the thermostability of the *rCel_TP* enzyme for prolonged functionality. Leveraging the potential of the cellulase enzyme to improve bioethanol production warrants further exploration at both bench and pilot scales.

5 Conclusion

Cellulase gene from *Geobacillus* sp. TP-3 was inserted into pET-28a (+) vector. The recombinant construct *pET-Cel3* was expressed in *E. coli* BL21 (DE3) host cells using 0.5 mM IPTG as inducer. After approximately 6 h of induction at pH 8, 0.5 mM IPTG and an incubation temperature of 25 °C, cellulase production was achieved. The molecular weight of the recombinant cellulase enzyme is ~ 40.2 kDa. The enzyme showed the highest stability and activity when exposed to a temperature of 50 °C, pH 8 and metal ions such as $HgCl_2$, $CuCl_2$, $CoCl_2$ and KCl. However, the presence of metal ions like $FeCl_3$, NH_4Cl, $CaCl_2$ and inhibitors such as EDTA, β-mercaptoethanol and DTT was found to reduce the cellulase activity. In conclusion, the enzyme's stability under harsh conditions increases its potential for use in industries such as feed, textile, beverage and detergent. Moreover, further experiments on a larger scale will be useful to get the maximum cellulase enzyme.

Abbreviations

BSA	Bovine serum albumin
CFE	Cell-free extract
CMC	Carboxymethyl cellulose
CMCase	Carboxymethyl cellulase
DNS	Dinitrosalicylic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GH5	Glycoside hydrolase family 5
GRAVY	Grand Average of Hydropathy
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
K_M	Michaelis Menten constant
LB	Luria–Bertani
MW	Molecular weight
Ni^{2+}-NTA	Nickel nitrilotriacetic acid
OD_{600}	Optical density at 600 nm
ORF	Open Reading Frame Finder
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
rCel_TP	Recombinant cellulase enzyme
Rf	Relative front
Rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
V_{max}	Maximum velocity

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43088-024-00495-9.

Additional file 1: **Fig. S1**. Sanger's sequencing data of PCR amplicon amplified using Geobacillus sp. TP3 as template and degenerate primers based on thermophilic cellulases. **Fig. S2**. Restriction mapping of sequenced cellulase gene data showing no site for BamHI and XhoI restriction enzyme sites. **Fig. S3**. BLAST p analysis of the translated proteins. Pairwise alignment of query protein with endoglucanase M of Geobacillus sp. WSUCF1 and Cel-9 endocellulase of Geobacillus thermodenitrificans. **Fig. S4**. Analysis of translated protein for the presence of signal peptide for secretary pathway. **Fig. S5**. Multiple sequence alignment of the proteins showing homology with rCel_TP protein. **Fig. S6**. ProtParam analysis of the translated ORF coding for cellulase gene. **Fig. S7**. Ramachandran plot of the model of cellulase generated through homology modelling.

Additional file 2. Sequencing Chromatogram.

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Author contributions

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Data availability

The gene sequence information can be retrieved accession no. WET54884.1 from NCBI site. All data generated or analysed during this study are included in this article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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