Investigational Analysis of the Geothermal Springs of India for Cellulose Degrading Enzymes

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Hot springs host unique microbial communities that flourish in extreme environment, adapting to factors like high temperatures and pH fluctuations (Dodds and Whiles, 2010; Verma et al., 2022). Microorganisms in these environments produce extremozymes which are adapted to high temperatures and exhibit exceptional thermal stability. The diverse microbial communities in hot springs contribute to enzymatic diversity, and these extremozymes find applications in various industries (Akanbi et al., 2019). Recently, thermophilic cellulases have gained renewed interest in generating second-generation biofuels derived from lignocellulosic biomass (Goswami et al., 2022). Lignocellulosic biomass includes agricultural residues, forestry waste, and dedicated energy crops. They are abundant and represent a sustainable feedstock for biofuel production. However, they are "recalcitrant" in nature. The recalcitrance of lignocellulosic biomass arises from the complex and rigid structure of the plant cell wall, which comprises cellulose microfibrils embedded in a matrix of hemicellulose and lignin (Zoghlami & Paës, 2019). Lignin acts as a barrier, making it difficult for enzymes to access and break down cellulose and hemicellulose into fermentable sugars (Saritha et al., 2012). This recalcitrance poses a significant obstacle to the efficient and cost-effective conversion of lignocellulosic biomass into biofuels.

Cellulase is an enzyme mixture containing various cellulases and plays a crucial role in addressing the recalcitrance of lignocellulosic biomass. Cellulases comprise endo-glucanases, exo-glucanases, and β-glucosidases that hydrolyse cellulose into glucose (Behera et al., 2017; Ejaz et al., 2021). By breaking down the cellulose component, cellulases make it easier for microorganisms to ferment the released sugars into biofuels such as ethanol. The application of cellulase in biofuel production involves the pretreatment of lignocellulosic biomass to reduce its recalcitrance (Ilić et al., 2023). Various pretreatment methods, such as steam explosion, acid hydrolysis, and enzymatic pretreatment, are employed to disrupt the structure of the plant cell wall, making cellulose more accessible to cellulase enzymes during the hydrolysis step (Preethi et al., 2021). These cellulases play a vital role in overcoming the

recalcitrance of lignocellulosic biomass by breaking down cellulose into fermentable sugars, which can then be converted into biofuels (Mujtaba et al., 2023). The efficient utilization of cellulase in biofuel production is crucial for improving lignocellulosic biomass-based biofuel production's overall yield and economic feasibility.

Cellulases are essential enzymes that have various applications in multiple sectors. Besides biofuel production, they can enhance fabric properties like softness and dye uptake and aid in stain removal for textile and laundry industries. They also assist in starch processing for food and beverage industries, improve pulp bleaching and paper quality for paper and pulp industries, and contribute to organic waste treatment for bioremediation (Jayasekara & Ratnayake, 2021). Thermostable cellulases offer stability, substrate specificity, and versatility for diverse industrial applications.

Recent years have seen cellulases being widely utilized in coffee processing during drying (Nguyen et al., 2022) and playing a crucial role in winemaking by breaking down grape skin, removing tannins, and eliminating unpleasant aromas (Claus & Mojsov, 2018; Gao et al., 2019) Its diverse applications extend to fruit juice production and the formulation of laundry detergents, positively impacting the cellulase market. The steady growth of the food and beverages industry is expected to drive the cellulase market at a considerable Compound Annual Growth Rate (CAGR). Cellulase finds increasing use in industrial sectors such as paper, pulp, bioethanol, and agriculture. In the paper and pulp industry, cellulase adoption enhances paper quality, reduces energy consumption during refining, and improves characteristics like freeness, cleanliness, and brightness. Additionally, cellulase demand is rising in agriculture to enhance soil fertility, promote plant growth, and control plant diseases.

However, the cost-effectiveness of cellulase processing and its use in biomass saccharification pose challenges to market growth. Biomass bio-refinery commercialization is restricted due to cellulose use, hindering cellulase demand (Balan, 2014; Ellilä et al., 2017). Despite these challenges, North America holds a significant market share of 34.4%, with the USA leading the cellulase market. The region is expected to maintain its dominance, driven by increased biofuel production and demand. The USA Energy Information Administration reported a substantial rise in biofuel production, further fueling cellulase market growth. The global cellulase market was valued at US$1.7B in 2023 and is projected to hit US$3.3B by 2033, driven by a 6.8% CAGR. The paper industry is the primary driver of cellulase demand, with the mechanical pulping process boosting pulp characteristics. The top five market players are AB Enzymes, Amano Enzyme, DSM, Genencor (DuPont), and Novozymes. Europe leads with a 30% market share, followed by Asia-Pacific and North America with over 25%.

Comparing cellulases from various microbes is challenging due to different measuring conditions and these enzymes' complex, variable nature. However, thermostable cellulases provide more advantages than their mesophilic counterparts. In a study by Mingardon et al., (2011), the thermostabilities and activities of three homologous GH9-CBM3c cellulases were compared under consistent experimental conditions. Results showed similar activities at low temperatures, increasing significantly with temperature until a point (temperature optimum) where enzymes lost function. Their study suggests that although mesophilic cellulolytic bacteria can grow on cellulose, their efficiency is lower at lower temperatures, possibly limiting microbial cellulose utilization rates (Mingardon et al., 2011).

The search for novel thermophilic cellulases is motivated by their ability to operate at high temperatures, offering advantages such as increased reactivity, reduced contamination risks, synergy with thermal pretreatment, integration with thermophilic microorganisms, cost savings, expanded feedstock options, and environmental adaptability (Mingardon et al., 2011; Acharya and Chaudhary, 2012; Escuder-Rodríguez et al., 2018; Ajeje et al., 2021). These factors collectively enhance the efficiency and sustainability of biofuel production from lignocellulosic biomass.

Keeping the above in view, the present work, entitled "**Investigational Analysis of the Geothermal Springs of India for Cellulose Degrading Enzymes",** has been undertaken to isolate a thermophilic cellulolytic organism which possesses the ability to utilise lignocellulosic wastes and their saccharification. The cellulase production condition optimisation, gene cloning, heterologous expression, and purification of this enzyme have been carried out.

The objectives of this research work were:

1. To isolate and screen thermophilic microorganisms for cellulose-degrading activity.
2. To optimize culture conditions using the design of experiment and reaction conditions for the cellulase activity
3. Cloning of the cellulase gene and sequence analysis of the gene/enzyme (cellulase) using bioinformatics tools.
4. Heterologous expression, purification, and characterization of the cellulase enzyme.

The salient features of this study are summarized in the subsequent sections.

1. To isolate and screen thermophilic microorganisms for cellulose-degrading activity.

Ten different bacterial strains were isolated from hot springs in India using carboxyl methyl cellulose (CMC) enrichment at 50 °C and 180 rpm. Out of these strains, two strains (TP-1 and TP-3) showed higher cellulase activity, and TP-3 was chosen for further analysis because it exhibited the maximum hydrolysis zone. TP-3 was identified as a thermophilic bacterium. Its morphology and growth characteristics showed that TP-3 had slimy, spherical, off-white, transparent colonies with flat, undulating margins. Gram staining revealed that it was Gram-positive, and microscopy demonstrated that it had long rod-shaped bacteria. The enzyme activity of TP-3 was found to be 0.692 μM/min/mL. To identify the TP-3 strain at the molecular level, a 1.5 kb fragment of the 16S rRNA gene was amplified and sequenced. The sequence revealed that TP-3 belongs to the *Geobacillus* genus, closely related to *Geobacillus stearothermophilus* strains. The 16S rRNA gene sequence of TP-3 has been deposited in GenBank with accession number OP962434. Phylogenetic analysis confirmed TP-3's similarity to *Geobacillus stearothermophilus* strain S YE6-1017-022.

2. To optimize culture conditions using the design of experiment and reaction conditions for the cellulase activity

The study aimed to improve the conditions for cellulase enzyme production by *Geobacillus* sp. TP-3. The six different media were screened, and the best results were obtained using a medium reported by Sakthivel et al., (2015). A methodology called One-Factor-At-A-

Time (OFAT) was used to optimize the production process further. It was found that combining glucose and carboxymethyl cellulose (CMC) as carbon sources and yeast extract as a nitrogen source led to the highest cellulase activity. An inoculum size of 6%, a pH of 8, an incubation temperature of 50 °C, and an incubation time of 24 hours maximized cellulase activity. After OFAT optimization, cellulase activity significantly increased from 0.692 µM/min/mL to 1.06 µM/min/mL.

Plackett Burman's statistical analysis identified seven crucial factors affecting cellulase production, with glucose, CMC, and yeast extract having significant impacts. The model's significance was confirmed by ANOVA, with a p-value of 0.0371, and yeast extract was identified as the most crucial factor. The study demonstrated the effectiveness of the PB approach in enhancing cellulase production.

The reaction conditions of *Geobacillus* sp. TP-3 cellulase enzyme was characterized, and it was found to show maximum activity at pH 8 and 50 °C and high thermostability at elevated temperatures. The different agricultural waste products were explored as carbon sources, and cane sugar molasses exhibited the highest cellulase production (~9.60 µM/min/mL). They also studied pretreatment methods for wood sawdust, including biological and alkali treatments, and found that these methods significantly altered biomass composition and improved cellulose content. Scanning electron microscope (SEM) analysis revealed structural changes in the sawdust, enhancing exposure to cellulase enzymes. Alkali-treated sawdust exhibited significantly higher saccharification efficiency (49.71%) than untreated sawdust (1.08%). The hydrolysis rate of alkali-treated sawdust was about 48 times higher. The presence of high lignin content in untreated sawdust hindered enzyme penetration. The highest sugar yield was achieved with alkali-treated sawdust in citrate buffer (pH 5.5). Alkaline and biological treatments also showed notable saccharification rates.

Overall, the study demonstrated the successful optimization of cellulase production conditions, the impact of various factors, and the potential use of low-cost waste substrates for cellulase production. The findings also highlighted the effectiveness of pretreatment methods in enhancing biomass composition and saccharification efficiency, which are crucial for efficiently converting lignocellulosic materials into sugars.

3. Cloning of the cellulase gene and sequence analysis of the gene/enzyme (cellulase) using bioinformatics tools.

In this investigation, genomic DNA from *Geobacillus* sp. TP-3 was isolated from 24-hour-old cultures, and the cellulase gene was amplified using degenerate primer-based PCR. Primers were designed using conserved sequences from known thermophilic cellulase genes and pre- and post-sequences of the *Geobacillus* sp. cellulase gene. The resulting 1.2 kb cellulase gene fragment was cloned into a pGEMT vector (3.0 kb). Following vector introduction into *E. coli* DH5α, 18 transformed colonies were selected for plasmid isolation. Six colonies were randomly chosen from these, and two were confirmed positive via PCR for subsequent plasmid preparation. Plasmid analysis through agarose gel electrophoresis demonstrated the presence of the 4.2 kb recombinant vector, and restriction digestion yielded a clean 1.2 kb insert and a 3 kb empty vector.

The plasmid was further sequencing and the 1229 bp data from cellulase gene fragment sequencing was used for sequence analysis using various bioinformatics tools. BLASTn analysis revealed a substantial nucleotide similarity of 90.77% with *Geobacillus stearothermophilus.* The Open Reading Frame (ORF) finder identified the longest ORF for six-frame translation, and the resulting amino acid sequence underwent BLASTp analysis. The BLASTp analysis indicated similarity with endoglucanases M42 family and endocellulases, with specific matches to known cellulase proteins from other *Geobacillus* species (*Geobacillus* sp. WSUCF1, *Geobacillus kaustophilus* GBlys, *Geobacillus thermodenitrificans*). Maximum similarity (96.41%) was observed with the Cel 9 endocellulase of *Geobacillus thermodenitrificans* (95% query coverage). The translated protein, consisting of 362 amino acids, exhibited a molecular weight of approximately 39.266 kDa, slight hydrophilicity (GRAVY value of -0.183), and stability suggested by the instability index. The aliphatic index indicated thermal stability across a broad temperature range, and a homology model revealed sequence identity with the aminopeptidase/glucanase homolog and endoglucanase of *Thermotoga maritima* by the Swiss model.

4. Heterologous expression, purification, and characterization of the cellulase enzyme.

A recombinant vector, *pET-Cel3*, was constructed using the pET-28a (+) expression vector with *BamHI* and *XhoI* sites for directional cloning. The cellulase gene was amplified from the pGEMT clone using restriction site (*BamHI* and *XhoI*) containing primers. The resulting cellulase coding fragment and pET-28a (+) were double-digested with *BamHI* and *XhoI*. After purification, the fragments were ligated and transformed into competent *E. coli* BL21 (DE3) hosts. Positive clones were confirmed through colony PCR and double digestion. Plasmids were isolated, and the presence of the cellulase gene was established by restriction double digestion and sequencing.

Fermentation conditions were optimized for the *E. coli* BL21 (DE3) host cells containing pET-Cel3. It showed maximum *rCel_TP* enzyme expression at 25–30 °C with 0.5 mM IPTG induction. The *rCel_TP* enzyme, lacking a secretary signal, was obtained as an intracellular solubilized fraction.

The purified *rCel_TP* was obtained through Ni^{2+}-NTA affinity chromatography. It showed a single band of approximately 40.2 kDa on SDS-PAGE and was confirmed by a zymogram, indicating cellulase activity. The characterized *rCel_TP* enzyme demonstrated optimum activity at pH 8.0, retained 90% activity for up to 1 hour, and maintained activity in the pH range of 5.0–9.0. Optimal temperature was observed at 50 °C, with 80% activity retention for an hour, but decreased significantly at higher temperatures.

Various metal ions, detergents, solvents, and inhibitors influenced the enzyme's stability and activity. Cations like Hg^{2+}, Cu^{2+}, and Co^{2+} improved cellulase activity, while high Ca^{2+}, NH_4^{2+}, Fe^{3+}, and Mg^{2+} concentrations inhibited it. Surfactants exhibited varied effects, with SDS reducing activity by 30% at 0.5%, while Tween 20 and Triton X-100 showed differential impacts. Inhibitors such as EDTA, β-mercaptoethanol, and DTT also affected enzyme activity.

The *rCel_TP* enzyme exhibited specificity against the CMC substrate. The CMC's kinetic parameters (K_M and V_{Max}) were determined at pH 8 and 50 °C. These findings provide insights into the successful production, purification, and characterization of the recombinant cellulase enzyme, *rCel_TP*, with potential applications in various biotechnological processes. At pH 8 and 50 °C, the K_M and V_{Max} kinetic parameters for *rCel_TP* for CMC substrate were 116.78 mg/mL and 44.05 µmol/mg/min, respectively.

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