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Bacterial Cellulase: Isolation, Characterization, And Production Optimization

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Abstract

Cellulose degrading bacteria were isolated from the soil. The colonies were isolated using CMC media and serial dilution. Biochemical tests, plate assays, and molecular methods such as PCR were used in the screening process and identification. The production of cellulase increased by ooptimizing the culture conditions for pH, temperature, incubation time and nutrition sources. The enzyme was partially purified using acetone, ammonium sulphate precipitation and dialysis. The bacterial strain that showed clear transparent zone on CMC agar plate, was identified as cellulase producing bacteria. The strain showed high potentiality for maximum cellulase production at pH 7.0 after 72 hours incubation at 37°C in a medium containing 3% CMC and yeast extract as nitrogen source. Through morphological and biochemical characteristics followed by 16S rRNA, the strain was identified as *Bacillus cereus.* The specific activity, recovery and purification fold of enzyme were found to be 2.64 U/mg, a yield of 71.9%, and a 3.51 respectively by ammonium sulphate precipitation.

Keywords
Cellulase, Bacteria, Enzyme isolation, optimization.

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1. INTRODUCTION

Cellulose, the most abundant biopolymer on earth, serves as a major renewable carbon source for various microorganisms [1]. Celluloses are responsible for cellulose degradation by hydrolyzing the β-1,4-glycosidic bonds [2]. Cellulases are one of the widely used industrial enzymes which are commercially available for more than 30 years [3]. The cellulolytic enzymes are produced both by bacteria and fungi. However, at the industrial scale the bacterial species have been used predominantly, wherein the *Bacillus* genus is a known for secreting a collection of hydrolytic enzymes [4].

Cellulases possess diverse application in textile, detergent, leather, food, feed and paper industries. They are also part of biomass fermentation, fiber modification, hence application of these enzymes in such industries demands the identification of stable enzymes that can active at high pH and increased temperature . The growth rate of bacteria is easier and faster than fungi and hence considered for cellulase production[5].

Cellulases are secreted extracellularly by many bacterial species in abundant amounts, resulting in easy extraction and purification processes. The cellulolytic enzymes from *B. subtilis* have been studied thoroughly for their enough production and application for biomass valorization [6]. Bacterial species, such as *B. subtilis subsp. subtilis* JBS250[5], *B. subtilis* strain LFS3[7], *B.*

subtilis Strain CBS31 [8], *Bacillus halodurans* IND 18 [9], *B. subtilis* BC1 [10], *B. subtilis* BY-4 [11], and *B. subtilis* CD001 [12] have been recently reported for cellulase production.

The present study aims to isolate cellulase-producing bacteria from a natural environment, characterize the most potent strain, and evaluate its enzymatic properties under various physicochemical conditions.

2. MATERIALS AND METHODS

2.1. Sample Collection

Soil samples were collected from various locations in Kasaragod district, Karnataka, India. The collected samples were taken from the rhizosphere region at a depth of 5–10 cm using sterile tools. The samples were stored in sterile containers and transported to the laboratory for further microbiological analysis.

2.2. Screening and Isolation of Cellulase-Producing Bacteria

Soil samples collected from various locations were directly inoculated onto Carboxymethyl Cellulose (CMC) agar plates and incubated at 37°C for 24 hours. Following incubation, five plates showed the growth of multiple colonies with diverse morphological features. Distinct colonies were selectively isolated and sub-cultured on fresh CMC agar plates to obtain pure cultures for preliminary screening. The CMC agar medium consisted of the following components: CMC – 10.0 g, Peptone – 10.0 g, K_2HPO_4 – 2.0 g, $MgSO_4$ – 0.03 g, Gelatin – 2.0 g, Ammonium sulfate – 2.5 g, Agar – 15.0 g, Distilled water – 1000 mL, The medium pH was adjusted to 7.2 before sterilization.

For secondary screening, the selected isolates were re-inoculated onto fresh CMC agar plates and incubated at 37°C for 24 hours. After incubation, the plates were flooded with 1% Congo red solution and allowed to stain for 15 minutes, followed by destaining with 1M NaCl for 20 minutes [13]. The formation of clear hydrolysis zones around the colonies confirmed cellulase-producing activity.

2.3. Enzyme Activity determination

Colonies exhibiting positive cellulase activity were cultured in CMC broth and incubated at 37°C for 72 hours in a shaker incubator. After incubation, the cultures were centrifuged at 8000 rpm for 15 minutes at 4°C, and the resulting supernatant was collected as the crude enzyme extract.

Cellulase activity was determined using the Dinitrosalicylic Acid (DNS) method, which measures the amount of reducing sugars released during enzymatic hydrolysis [14]. For the assay, 1 mL of crude enzyme was mixed with 1 mL of CMC substrate, and citrate buffer (pH 5) was added to bring the total volume to 3 mL. The mixture was incubated at room temperature for 10 minutes, followed by the addition of 3 mL of DNS reagent. The tubes were then heated in a boiling water bath for 10 minutes, cooled, and the absorbance was measured at 540 nm against an appropriate blank. A glucose standard curve was used to estimate the concentration of reducing sugars released.

2.4. Morphological and Biochemical Identification

The bacterial isolate exhibiting the highest cellulase activity was selected for further morphological and biochemical characterization. Gram staining was performed to determine the cellular morphology and Gram reaction [15]. Biochemical tests, including catalase, oxidase, and carbohydrate utilization assays, were carried out following the standard procedures described in *Bergey's Manual of Systematic Bacteriology* [16].

2.5. Molecular Identification of Bacteria

Molecular identification of the potent cellulolytic strain was performed by Anderson Diagnostic Services Private Limited, a research services provider based in Bangalore. Genomic DNA was extracted and quantified using a Nano Drop spectrophotometer, and the integrity of the DNA was evaluated through 0.8% agarose gel electrophoresis. The 16S rRNA gene was amplified using universal primers [17].

2.6. Optimization of Culture Conditions

Optimization of cellulase production was performed by varying the concentrations of CMC (0.5%, 1%, 1.5% 2%, 2.5% and 3%) and using different nitrogen sources, including peptone, yeast extract, and ammonium sulfate. The influence of pH on enzyme production was evaluated at different levels: pH 4 pH 5, p H 6, pH 7, pH 8 and pH 9. Cultures were incubated at 37°C for 72 hours in a shaking incubator. After incubation, the cultures were centrifuged at 8000 rpm for 15 minutes at 4°C, and the resulting supernatant was collected as the crude enzyme extract. Cellulase activity was subsequently determined using the DNS method, as previously described.

2.7 Partial Purification of Cellulase Enzyme

2.7.1. Acetone and Ammonium sulphate precipitation

The crude enzyme extract was subjected to cold acetone precipitation by adding three volumes of chilled acetone to one volume of enzyme solution. The mixture was incubated at 4°C for 24 hours to facilitate protein precipitation. Following incubation, the precipitate was collected by centrifugation at 10,000 rpm for 15 minutes at 4°C. The resulting pellet was air-dried overnight and subsequently redissolved in a minimal volume of 1 M citrate buffer. The protein concentration and cellulase activity were

then determined, and the specific activity was calculated.

Ammonium sulfate was gradually added to 100 mL of crude enzyme extract to achieve 90% saturation (w/v). The mixture was centrifuged at 10,000 rpm for 15 minutes at 4°C, and the resulting pellet was dissolved in 1 M citrate buffer. The enzyme solution was then dialyzed against 0.001 M citrate buffer to remove residual ammonium sulfate. Following dialysis, the protein concentration and cellulase activity of the enzyme solution were determined, and the specific activity was calculated.

3. RESULTS AND DISCUSSION

The present study focused on the isolation, purification, and optimization of cellulase production from a novel bacterial strain belonging to the genus *Bacillus*, which is recognized for its industrial and economic significance. Cellulose, a major component of agricultural residues, is among the most abundant natural biopolymers on Earth. This cellulosic biomass has considerable industrial relevance due to the valuable bioproducts generated through its degradation, with cellulase enzymes playing a pivotal role. Cellulases produced by cellulolytic bacteria efficiently hydrolyze cellulose into fermentable sugars. Since their initial discovery and isolation, cellulases have become an essential class of industrial enzymes because of their diverse applications[18] .

3.1 Isolation of Cellulolytic Bacteria

Following 24 hours of incubation at 37°C, soil sample yielded a diverse array of bacterial colonies on CMC agar plates. The colonies exhibited considerable variation in size, shape, texture, and pigmentation. A total of five plates produced numerous distinct colonies, from which individual isolates were purified for subsequent analyses.

The observed morphological diversity suggests the presence of a rich bacterial population in soil capable of cellulose degradation. This observation aligns with previous reports highlighting high microbial diversity in lignocellulosic environments [5].

3.2 Secondary Screening and Cellulase Activity Assay

The purified colonies were subjected to Congo red staining on CMC agar for qualitative screening of cellulase activity. The formation of clear hydrolysis zones around several colonies confirmed their cellulose-degrading ability. The diameters of these zones varied among isolates, indicating differences in cellulase production levels.

Quantitative analysis using the DNS method revealed that one strain (S2), exhibited the highest cellulase Activity, producing reducing sugars. These findings demonstrate the potential of these strains for industrial-scale cellulase production.

3.3 Morphological, Biochemical, and Molecular Identification

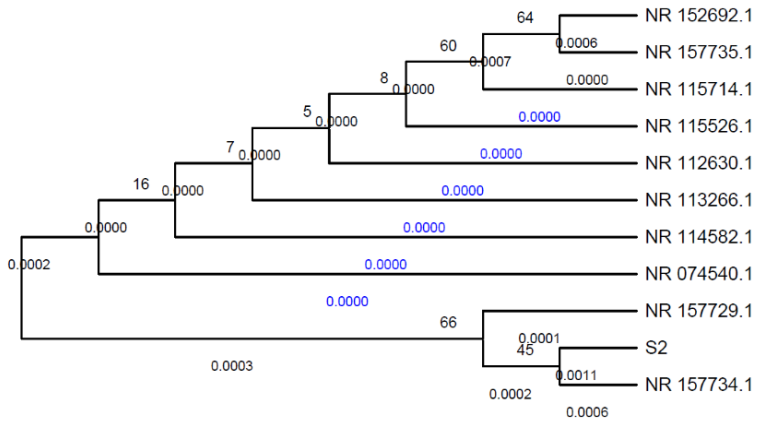
The strain which exhibited the highest cellulase activity, was identified morphologically as a Gram-positive, rod-shaped bacterium (Table I). Biochemical tests confirmed its metabolic characteristics consistent with members of the genus *Bacillus*. Molecular identification via 16S rRNA sequencing revealed 100% similarity of strain S2 with *Bacillus,* confirming its taxonomic assignment. Phylogenetic analysis using MEGAXI placed the isolate within a clade of known cellulolytic *Bacillus* species.

Table 1: Results of morphological and Biochemical tests

TEST	RESULT
Gram staining	Gram positive rods
Catalase test	Positive [Bubble formation]
KOH test	Negative [no viscous formation]
Starch hydrolysis	Positive [zone of clearance]

Consensus Sequence of Cellulase producing bacteria

GTNCATATACATGCAGTCGAGCGAATGGATTRAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGN
GGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC
GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCAC
CAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGG
TCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCA
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCG
CAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGG



3.4 Optimization of Culture Conditions

Cellulase production was influenced significantly by substrate concentration, nitrogen source, and pH. Maximum enzyme activity was observed at 3 % CMC concentration, peptone as the nitrogen source, and pH 7.0 (fig. 1), with activities reaching up to 0.76 U/mL. These results align with previous reports indicating optimal cellulase production under neutral pH and moderate substrate concentration conditions [18, 5].

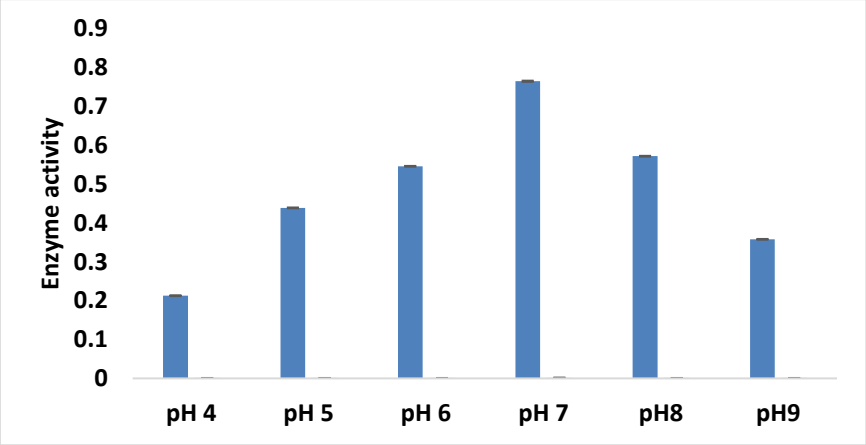


Figure 1: Effect of various pH on enzyme activity

3.5 Partial Purification of enzyme

Partial purification using acetone and ammonium sulfate precipitation resulted in increased specific activity of the enzyme, indicating successful enrichment of cellulase from the crude extract. Acetone precipitation yielded a 1.90-fold purification, whereas ammonium sulfate precipitation provided a 3.51-fold increase in specific activity (Table II). These methods provide a simple and cost-effective means to concentrate cellulase for further applications.

Table 2: Purification summary showing yield and specific Activity of Cellulase

SL No	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity(U/mg)	%yield	Fold purification
Crude	300	13.8	10.5	0.760	100	1.00
Ammonium sulphate	25	2.85	7.55	2.64	71.9	3.51
Acetone precipitation	25	0.275	0.4	1.45	3.80	1.90

4.0 CONCLUSION

This study successfully isolated and characterized a novel cellulolytic bacterium from soil collected from Kasargod. The isolate was identified as *Bacillus cereus* through 16S rRNA gene sequencing, biochemical tests, and morphological analysis.

Optimization of culture conditions revealed that maximum bacterial growth and cellulase production occurred using 3% CMC as the carbon source, yeast extract as the nitrogen source, and at neutral pH (7.0). Partial purification of the extracellular cellulase enzyme using ammonium sulfate precipitation resulted in a specific activity of 2.64 U/mg, a yield of 71.9%, and a 3.51-fold purification.

Future work will focus on detailed characterization of the enzyme, including its stability, substrate specificity, and potential applications in industrial processes such as biofuel production and waste management.

5.0 AUTHOR CONTRIBUTIONS

Vidya A S and Karigar S**:** Conceptualization of the study, supervision of enzyme isolation procedures, data analysis, and writing – original draft.Nandhini K and Chandan N **:** Performed enzyme extraction and purification experiments, maintained laboratory records, and assisted in data interpretation. Vitha Ramesh, Bhavya J and Sunil S More**:** Conducted biochemical characterization assays, analyzed enzyme activity data, and contributed to writing – review & editing.

6.0 CONFLICT OF INTEREST

Authors declare that they have no conflict of interest

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