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Original Article

Production and Properties of Bacterial Cellulase from Arecanut Waste

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ABSTRACT

Background: Cellulases are the second most popular industrial enzyme because of their many uses, especially in turning cellulosic biomass into sugars for further processing. **Objectives:** The current study focused on the isolation, screening, molecular characterization, optimization of culture conditions and partial purification of the cellulase producing bacteria. **Materials and Methods:** Cellulose degrading bacteria were isolated from soil surrounding arecanut waste. Single colonies were isolated using selective media and serial dilution. Biochemical tests, plate assays, spectrophotometric measures, and molecular methods such as PCR were used in the screening process and identification. The production of cellulase increased by optimizing the culture conditions for pH, temperature, incubation time and nutrition sources. The enzyme was partially purified using acetone, ammonium sulphate precipitation and dialysis. The molar mass was determined by SDS-PAGE. **Results:** Three bacterial strains B1, B2 and B3 produced clear transparent zone on CMC agar plate, were identified as cellulase producing bacteria. The B2 strain showed high potentiality for maximum cellulase production at pH 7.0 after 72 hr incubation at 37ºC 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 thuringiensis* JN194. The specific activity, recovery and purification fold of enzyme were 2.64 mg/mL, 57.5% and 3.47 respectively. **Conclusion:** A novel strain from genus Bacillus has been isolated producing the enzyme cellulase which has application in both agriculture and industry.

Keywords: Cellulase, CMC, Arecanut Soil, Isolation, Screening.

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INTRODUCTION

Cellulose is the most abundant organic polymer on Earth and forms a major structural component of plant biomass. It is composed primarily of glucose molecules linked by β-1,4-glycosidic bonds, making it a key constituent of lignocellulosic biomass, along with hemicellulose and lignin.[1] Due to its abundance, cellulose represents a vast and renewable resource for the production of biofuels, biochemicals, and other value-added products. Approximately half of the dry weight of plant material is cellulose, making it a critical target for bioconversion technologies.[2]

Cellulases are a group of hydrolytic enzymes that catalyze the breakdown of cellulose into simple sugars. This enzyme system includes three major types: endoglucanases (EC 3.2.1.4), which randomly cleave internal bonds within the cellulose chain; exoglucanases or cellobiohydrolases (EC 3.2.1.91), which act on the ends of cellulose chains to release cellobiose; and β-glucosidases (EC 3.2.1.21), which convert cellobiose and other oligosaccharides into glucose.[3,4] These enzymes work synergistically to achieve complete hydrolysis of cellulose, a process central to the production of second-generation biofuels.

Bacteria have recently attracted considerable attention as sources of cellulase enzymes due to their high specific activity, rapid growth rates, and ease of genetic manipulation for heterologous expression. Microorganisms are responsible for the degradation of roughly 70% of organic matter globally, with the remainder decomposed by plants and animals.[2] Among microbial sources, bacteria offer advantages in terms of enzyme production cost and process scalability, making them promising candidates for industrial applications.

Lignocellulosic waste materials such as sawdust, rice straw, corn fiber, paper mill sludge, and sugarcane bagasse are available at low cost and in large quantities, offering abundant feedstocks for

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cellulase-based bioconversion. Moreover, research into dedicated energy crops like switchgrass and miscanthus has further supported the use of cellulases in biofuel production.[5]

The global cellulase market is growing rapidly, fueled by applications in bioethanol production and industries such as textiles, detergents, and animal feed.[6] Biotechnology firms like Genencor International and Novozymes Biotech have made significant advances in lowering cellulase production costs and enhancing enzyme efficiency.[7] These enzymes are not only critical for sustainable energy solutions but also find applications in improving fabric quality in textiles and enhancing digestibility in animal feed, reflecting their versatility.[8]

Given this context, the present study was designed to isolate cellulolytic bacteria from soil samples and evaluate their potential for cellulase production. The work also focuses on optimizing various environmental and nutritional parameters to enhance enzyme yield, followed by partial purification and characterization of the enzyme for potential industrial applications.

MATERIALS AND METHODS

Source and Sample Collection

Soil samples were collected from the base of Arecanut plants from Kasargod district, Karnataka India. The samples were taken from various parts of the plant environment, including roots, flower covers, leaf stems, nut shells, tuft coats, husks, and leaves, to ensure a comprehensive microbial profile. The goal was to identify potential cellulase-producing microorganisms present in the rhizosphere and surrounding plant litter.

Isolation and Screening of Cellulase-Producing Bacteria

The collected soil samples were directly inoculated onto Carboxymethyl Cellulose (CMC) agar plates and incubated at 37°C for 24 hr. Twelve different plates yielded numerous colonies exhibiting varied morphological characteristics. Individual, distinct colonies were carefully isolated and sub-cultured onto fresh CMC agar plates 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, selected colonies were re-inoculated onto fresh CMC agar plates and incubated at 37°C for 24 hr. The plates were then stained with 1% Congo red solution for 15 min, followed by destaining with 1M NaCl for 20 min.[9] The presence of a clear hydrolysis zone around the colonies indicated cellulase production.

Determination of Cellulase Enzyme Activity

Colonies showing positive results for cellulase production were cultured in CMC broth and incubated at 37°C for 72 hr in a shaking incubator. After incubation, the cultures were centrifuged at 8000 rpm for 15 min at 4°C. The supernatant was collected and used as the crude enzyme extract.

Cellulase activity was measured using the Dinitrosalicylic Acid (DNS) method, which quantifies the reducing sugars released during enzymatic hydrolysis.[12] For the assay 1 mL of crude enzyme (from strains B1, B2, and B3), 1 mL of CMC substrate. Citrate buffer (pH 5) was added to bring the volume to 3 mL. The mixture was incubated at room temperature for 10 min. Then, 3 mL of DNS reagent was added, and the tubes were incubated in a boiling water bath for another 10 min. Absorbance was recorded at 540 nm against an appropriate blank. A glucose standard curve was used to determine the concentration of reducing sugars released.

Morphological and Biochemical Identification

The bacterial strain exhibiting the highest cellulase activity was selected for further morphological and biochemical characterization. Gram staining was performed to determine cell morphology.[10] Biochemical tests, including catalase, oxidase, and carbohydrate utilization assays, were carried out following protocols described in Bergey's Manual of Systematic Bacteriology.[11]

Molecular Identification of Bacteria

Molecular identification of the potent cellulolytic strain was carried out by MEDAUXIN, a research services provider based in Bangalore. Genomic DNA was extracted, quantified using a NanoDrop spectrophotometer, and assessed for quality using 0.8% agarose gel electrophoresis.

The 16S rRNA gene was amplified using universal primers:

27F: 5′-AGAGTTTGATCCTGGCTCAG-3′

1492R: 5′-TACGGYTACCTTGTTACGACTT-3′

PCR amplicons were sequenced using BDT v3 forward and reverse primers on the ABI 3730xl Genetic Analyzer. The sequences were analyzed using the NCBI BLAST tool to identify the closest homologs. The top ten sequences with the highest similarity were aligned using ClustalW. A distance matrix was generated, and a phylogenetic tree was constructed using MEGAXI software.[12]

Optimization of Culture Conditions

Optimization of cellulase production was carried out using various concentrations of CMC (0.5%, 1%, 2%, and 3%) and different nitrogen sources (peptone, yeast extract, ammonium sulfate). The effect of pH was also evaluated at three levels: pH 5, pH 7, and pH 9. Cultures were incubated at 37°C for 72 hr

in a shaker incubator. Following incubation, cultures were centrifuged at 8000 rpm for 15 min at 4°C, and the crude enzyme extract was collected. Enzyme activity was determined using the DNS method as previously described.

Partial Purification of Cellulase Enzyme

Acetone Precipitation

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 hr. After incubation, the precipitate was recovered by centrifugation at 10,000 rpm for 15 min at 4°C. The pellet was air-dried overnight and redissolved in a minimal volume of 1 M citrate buffer. Protein concentration and cellulase activity were assessed, and specific activity was calculated.[13]

Ammonium Sulfate Precipitation

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 min at 4°C. The resulting pellet was dissolved into 1 M citrate buffer and subjected to dialysis against 0.001 M citrate buffer to remove residual ammonium sulfate. The dialyzed enzyme solution was evaluated for protein content and cellulase activity, and the specific activity was determined.

RESULTS

The present work focused on the isolation, purification, and optimization of cellulase production from a novel strain belonging to the genus Bacillus, recognized for its industrial and economic importance.

Following 24 hr of incubation at 37°C, the soil and Arecanut plant-associated samples yielded a diverse array of bacterial colonies on CMC agar plates. Colonies exhibited considerable morphological variation in terms of size, shape, texture, and pigmentation (Figure 1). A total of twelve plates produced numerous distinct colonies, from which individual isolates were purified for further analysis.

Secondary Screening and Cellulase Activity Assay

The isolated colonies were subjected to Congo red staining on CMC agar to qualitatively screen for cellulase production. Clear hydrolysis zones around colonies confirmed the ability of several isolates to degrade cellulose (Figure 2). The diameter of the clear zones varied among isolates, indicating differences in cellulase production levels.

Quantitative enzyme assays using the DNS method revealed that three strains, designated B1, B2, and B3, exhibited the highest cellulase activities, producing reducing sugars at concentrations of mg/mL glucose equivalents respectively (Figure 3). These results demonstrate the potential of these strains for industrial cellulase production.

Morphological, Biochemical, and Molecular Identification

The strain B2, showing the highest cellulase activity, was identified morphologically as a Gram-positive rod-shaped bacterium (Table 1). Biochemical tests confirmed its metabolic characteristics consistent with members of the genus Bacillus (Figure 4). Molecular identification through 16S rRNA sequencing revealed that strain B2 shared 100% similarity with *Bacillus thuringiensis* Strain JN194, supporting its assignment to this species. The phylogenetic tree constructed using MEGAXI placed B2 in a clade with known cellulolytic Bacillus species.

Consensus Sequence of Cellulase producing bacteria

AACACTTAGCACTCATCGTTTACGGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCG
CGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCC
TTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTC
ACCGCTACACATGGAATTCCACTTTCCTCTTCTGCACT
CAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGA
GCCGTGGGCTTTCACATCAGACTTAAGAAACCACCT
GCGCGCGCTTTACGCCCAATAATTCCGGATAACGCTT
GCCACCTACGTATTACCGCGGCTGCTGGCACGTAGT
TAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCA
GCTTATTCAACTAGCACTTACTCCGCCTGGGGAGTA
CGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTTACCAGGTCTTGACATCCTCT
GAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAG
AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCT
AAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGACCT.

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 (Figure 5), with activities reaching up to 0.078 U/mL.

Table 1: Morphological and biochemical characteristics of bacterial isolates.

Test	Result
Gram staining	Gram positive rods
Catalase test	Positive [Bubble formation]
KOH test	Negative [No viscous formation]
Starch hydrolysis	Positive [Zone of clearance]

Partial Purification

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 1.93 fold purification, whereas ammonium sulfate precipitation provided a 3.47 fold increase in specific activity (Table 2). These methods provide a simple and cost-effective means to concentrate cellulase for further applications.

DISCUSSION

Cellulose, a major component of agricultural waste, is one of the most abundant natural biopolymers on Earth. This cellulosic biomass holds industrial application because of its valuable bioproducts, with cellulase enzymes playing a key role in its degradation. Cellulases produced by cellulolytic bacteria efficiently hydrolyze cellulose into fermentable sugars. Since their initial identification and isolation in cellulases have become an essential class of industrial enzymes due to their wide-ranging applications.[14]

The variation in the morphology of colonies isolated suggests the presence of abundant bacterial species in the soil and associated plant debris, capable of cellulose degradation. This finding is consistent with previous studies reporting high microbial diversity in lignocellulosic environments.[6]

The substrate CMC, soluble derivative of cellulose is used to evaluate endoglucanase activity. The enzyme showed significant hydrolytic activity on it, which acts by cleaving internal β-1,4-glycosidic linkages of cellulose chains.

The enzyme exhibited an optimal activity at pH 7.0, indicating its suitability for processes operating under neutral conditions. The optimization results align with previous reports indicating optimal cellulase production under neutral pH and moderate substrate concentration conditions.[6,14] This propery aligns with earlier findings where bacterial cellulases from Bacillus and Cellulomonas species have shown maximum activity at neutral pH.[15,16] Such properties are highly desirable in applications like animal feed formulation, wastewater treatment, and biofuel

Figure 1: Morphological characteristics of bacterial colonies isolated from soil sample.

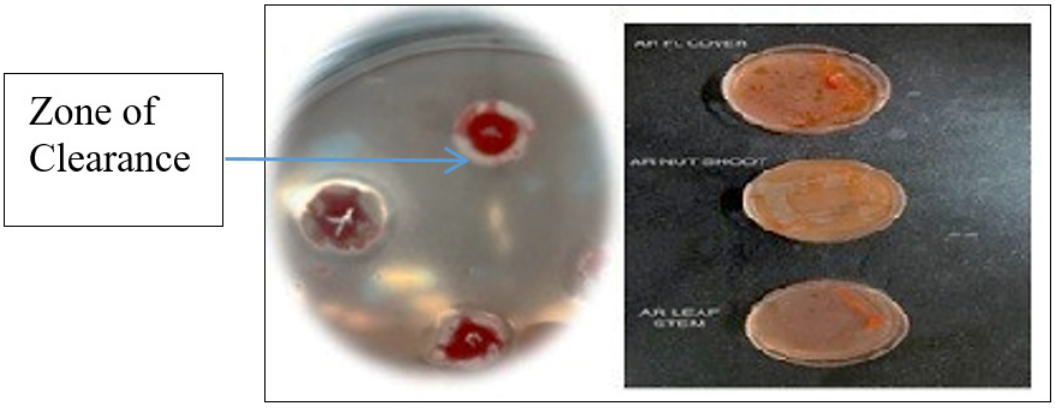

Figure 2: Secondary screening and cellulase activity assay of soil isolates.

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	450	20.7	15.75	0.760	100	1.00
Ammonium sulphate	30	3.42	9.06	2.64	57.5	3.47
Acetone precipitation	30	0.33	0.48	1.47	3.09	1.93

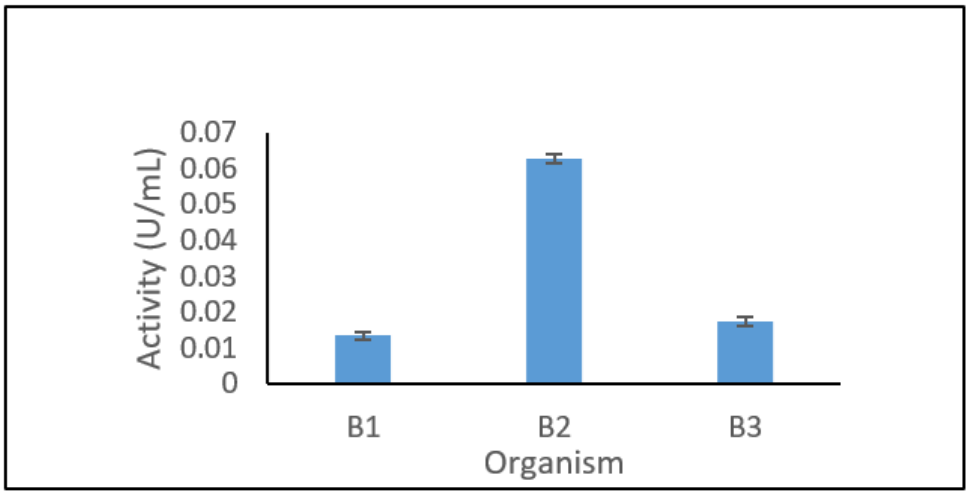

Figure 3: Comparative cellulase activity levels of three microbial isolates.

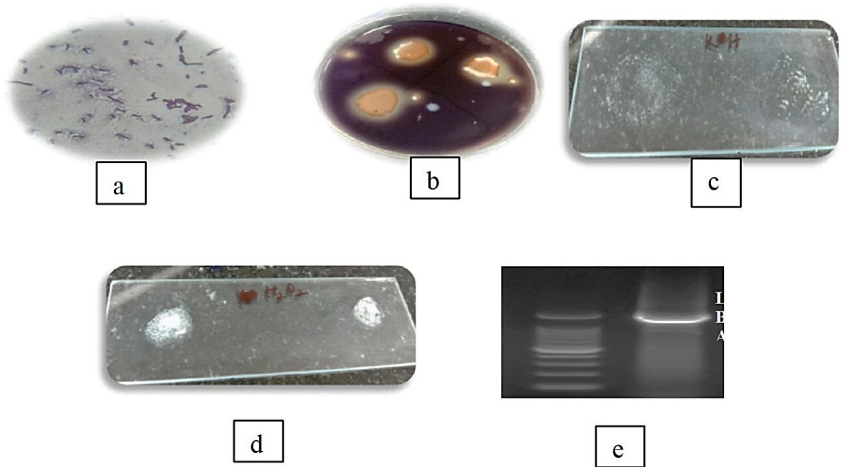

Figure 4: a. Zone of hydrolysis, b. Microscopic view of Gram positive Rods, c. Bubble formation, d. No viscous solution formation and e. A single band of intact, clear, high-molecular weight DNA was observed.

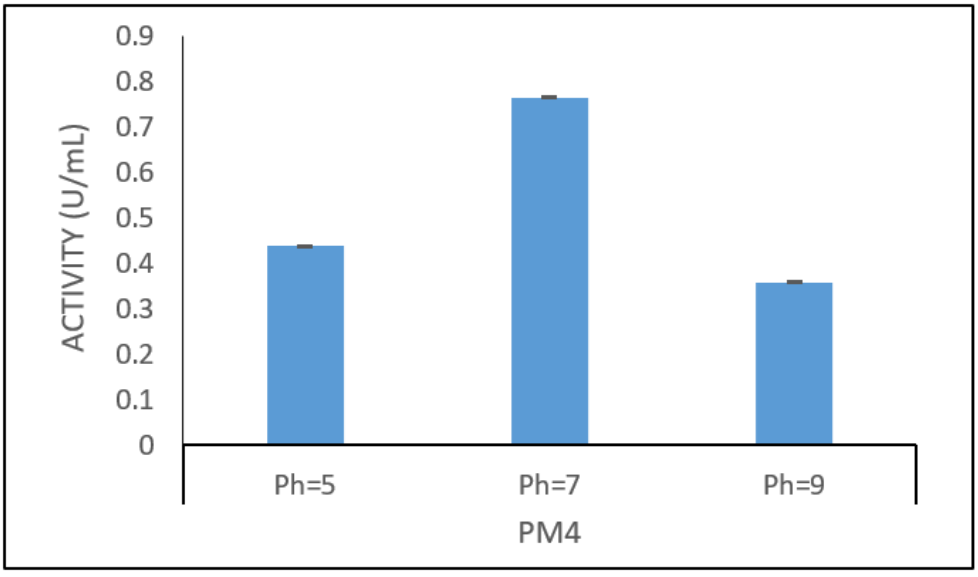

Figure 5: Effect of various pH on enzyme activity.

production, where extreme pH conditions are either unfavorable or cost-intensive to maintain.

The fold purification by ammonium sulphate indicates effective enrichment of the cellulase protein from the crude extract. This indicates a substantial removal of non-cellulolytic proteins and other impurities, which is critical for downstream applications and characterization. Comparable studies have reported similar fold purifications using ammonium sulfate, followed by dialysis or chromatographic methods for further refinement.[17]

CONCLUSION

The additional purification by ion-exchange chromatography or gel filtration could be employed in future work to achieve higher purity and specific activity for industrial applications or structural studies.

ABBREVIATIONS

CMC: Carboxymethyl Cellulose; **SDS:** PAGE-Sodium dodecyl sulphate poly acrylamide gel; **w/v:** Weight by volume; **K_2HPO_4:** Dipotassium hydrogen sulphate; **NaCl:** Sodium chloride; **$MgSO_4$:** Magnesium Sulphate; **DNS:** Dinitrosalicylic acid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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 and editing.

SUMMARY

This study successfully isolated and characterized a novel cellulolytic bacterium from soil associated with the Arecanut plant. The isolate was identified as Bacillus thuringiensis JN194 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 57.5%, and a 3.47-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.

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