



Research article

Isolation, Identification and Characterization of Cellulolytic Bacteria from Agricultural Waste Residues

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ABSTRACT

Cellulases, the enzymes that catalyze the breakdown of cellulose, have emerged as critical players in the quest for sustainable energy solutions and the bioconversion of lignocellulosic biomass into valuable products. This study investigates the potential of novel, cellulase-producing bacteria isolated from soils amended with agricultural residues with the goal of improving biorefinery efficiency and reducing biofuel production costs. Through the selective screening of cellulose-degrading bacteria on carboxymethyl cellulose (CMC) agar, we identified a range of facultative anaerobic, Gram-negative, motile rod-shaped isolates. Morphological, cultural, and biochemical analyses suggested affiliations with the genera *Enterobacter* and *Pseudomonas*. We further elucidated the taxonomic identities of these isolates by amplifying and sequencing the 16S ribosomal ribonucleic acid (16S rRNA gene) using universal primers (27F and 1492R). The resulting 1465 bp nucleotide sequences were subjected to a comprehensive comparison with the NCBI nucleotide database, leading to the identification of *Enterobacter cancerogenus*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*. Phylogenetic analysis via the maximum likelihood method Phylogenetic analysis was conducted using the Maximum Likelihood method based on the Tamura-Nei model in MEGA 7.0 confirmed the evolutionary relationships among the isolates. Our findings demonstrate the cellulose-degrading potential of these strains based on qualitative screening assays. However, quantitative cellulase activity assays are required to fully evaluate their industrial applicability and enzyme production capacity.

Introduction

The urgent imperative to transition from fossil fuels to sustainable energy sources has catalyzed an increasing focus on identifying abundant and economically viable biofuel alternatives. Among these, second-generation bioethanol sourced from lignocellulosic byproducts of agriculture, forestry, industry, and urban waste emerges as a highly promising solution. Cellulose, recognized as the most prevalent biomass on our planet, is not only renewable but also serves as a critical feedstock for bioconversion into high-value products like second-generation bioethanol can reach yields of up to 0.45 g ethanol/g cellulose under optimized conditions (Ilić et al., 2023; Jayakumar et al., 2023). This biological conversion of the cellulosic biomass to fuels and chemicals offers a high yield of products vital to economic success and

potential for very low cost (Benatti & Polizeli, 2023; Nargotra et al., 2023). Cellulase is responsible for the bioconversion of cellulosic and lignocellulosic residues. It hydrolyses β -1,4-glycosidic bonds in cellulose. Agricultural waste, in particular, presents a rich reservoir of cellulose, which can be naturally degraded by microorganisms, primarily through the action of cellulases that hydrolyze β -1,4-glycosidic bonds in plant materials (Bayer et al., 1998).

Cellulases are inducible enzymes synthesized by a diverse array of microorganisms (Bautista-Cruz et al., 2024; Dobrzyński et al., 2023; Huang et al., 2023). While fungi have long been recognized for their efficient cellulase production, there is a growing interest in bacterial cellulase synthesis due to the rapid growth rates of bacteria compared to fungi (Bhatia et al., 2024). The

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cellulolytic efficiency of bacterial strains is influenced by their environmental conditions, highlighting the significance of localized microbial diversity. In Bangladesh, the surge in urbanization has resulted in an increase in solid waste, much of which is rich in cellulosic material, offering a viable avenue for biological conversion to alleviate environmental pollution (Bahatkar et al., 2023). Furthermore, the textile industry in Bangladesh has a substantial demand for cellulases, and local production of these enzymes could significantly reduce reliance on imports and associated costs.

Advancements in biotechnology hold the potential to enhance enzyme characteristics, improving their activity, stability, and synergistic interactions while simultaneously lowering production costs (Basera et al., 2024; Nargotra et al., 2023). Although cellulolytic bacteria have previously been isolated from diverse environmental sources, information regarding cellulolytic bacterial communities associated with partially decomposed agricultural residues in the Khulna region of Bangladesh remains limited. The present study contributes region-specific data on cellulolytic bacterial diversity and combines phenotypic characterization with molecular identification of promising isolates obtained from locally available agricultural waste residues. Such information may support future development of indigenous microbial resources for biomass conversion and enzyme production.

Materials and Methods

Sample collection and preparation

Agricultural waste residues, partially decomposed in soil including jute sticks, rice straw, and sugarcane bagasse were collected from the Gollamari region of Khulna district. The 3 samples collected were transferred to sterile containers and maintained at 4°C until further analysis. For sample preparation, a serial dilution process was employed, mixing the degrading agricultural residues with soil to facilitate the isolation of microorganisms. This method is essential for accurately assessing the microbial diversity and cellulolytic activity present in the collected materials.

Screening and Isolation

One milliliter sample from the test tubes labeled 10-4, 10-5, and 10-6 were spread plated onto Nutrient Agar (NA) plates, which were subsequently incubated at 37°C for 24 hours where n=3 biological replicates per sample type were maintained. Plates exhibiting discrete colonies were selected for further analysis. Using sterile needles, colonies were transferred to carboxymethyl cellulose (CMC) media composed of 2 g CMC, 1 g NaNO₃, 1 g K₂HPO₄, 1 g KCl, 0.5 g MgSO₄, 0.01 g FeSO₄, 5 g yeast extract, 15 g agar, and 1000 ml distilled water (Irfan et al., 2012; Vijayaraghavan et al., 2012). After an incubation period of 48 hours at 37°C, all CMC agar plates were stained with 1% (w/v) Congo Red solution for 15 minutes and then decolorized with 1 M NaCl for an additional 15 minutes (Teather et al., 1982). The appearance of degradation zones around bacterial colonies indicated the ability of the strains to hydrolyze CMC (Ahmad et al., 2013; Gaur et al., 2015). Colonies exhibiting the largest clear zone ratios (zone

diameter/colony diameter) were selected for isolation and pure culture, subsequently streaked onto Nutrient Agar medium and incubated at 37°C for 24 hours before being stored at 4°C for future use (Immanuel et al., 2006). For preservation, a 20% glycerol broth was employed. A loop-full of the bacterial inoculum was transferred to 5 ml of nutrient broth medium and incubated at 37°C for 24 hours. Following incubation, 800 µl of the bacterial suspension was mixed with 200 µl of autoclaved glycerol in 1.5 ml autoclaved centrifuge tubes, which were then sealed with parafilm and stored at -20°C.

Identification of the bacteria

Employing sterile techniques, a Nutrient Agar (NA) plate was streaked to obtain isolated discrete colonies, which were then incubated at 37°C for 24 hours. Post-incubation, the bacterial colonies were assessed for size, pigmentation, form, margin, elevation, and texture (Cappuccino & Sherman, 2005). Potential bacterial strains were examined microscopically to evaluate their morphological characteristics, followed by Gram staining to ascertain their Gram reaction.

Biochemical Characterization

A series of biochemical tests were conducted to presumptively identify the selected bacterial isolates, following established protocols from the microbiology laboratory manual (Cappuccino & Sherman, 2005). Each isolate underwent characterization through various assays, including oxidation reactions, catalase test, citrate utilization test, indole test, triple sugar iron (TSI) test, methyl red test, Voges-Proskauer test (A positive result was indicated by a pink to red color developing within 15–30 minutes due to acetoin production), urease test (A color change from yellow/orange to bright pink in urea broth after incubation indicated a positive result), and starch hydrolysis test (After incubation, plates were flooded with iodine solution. A clear zone around the bacterial growth against a blue-black background indicated positive starch hydrolysis). To ensure reliability and accuracy, all biochemical tests were performed in triplicate, minimizing the risk of false-positive or false-negative results.

Genotypic characterization

Genomic DNA from the selected bacterial isolates was extracted using the Maxwell Blood DNA Kit (Model: AS1010, Promega, USA) and stored at 2 to 8°C until further analysis. The 16S rRNA gene was amplified via Polymerase Chain Reaction (PCR) with universal bacterial primers (27F: AGAGTTTGATCCTGGCTCAG and 1492R: GGT TACCTTGTTACGACTT), yielding a product size of approximately 1465 base pairs (Rimi, A. A., et al., 2026) PCR reactions were conducted in an Applied Biosystems thermal cycler (USA). Post-amplification, the PCR products were verified using horizontal electrophoresis on a 1.0% agarose gel in Tris-acetate EDTA (TAE) buffer, with a 1 Kb Plus DNA marker (Invitrogen, USA) employed to ascertain amplicon size. Stained DNA bands were visualized using an Alpha Imager (Protein Simple, USA). Amplified products were purified with the SV Gel and PCR Clean-

Up System, and DNA concentration was assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA). Purified PCR products underwent Sanger sequencing via ABI Genetic Analyzer (Model: 3700) at 1st BASE Laboratories, Malaysia. Non-essential vector sequences were removed from the forward and reverse sequences using alignment software, and consensus sequences were submitted to GenBank. To identify homologous sequences, multiple BLASTn searches were performed at the National Center for Biotechnology Information. Initially, Clustal Omega facilitated multiple sequence alignment, followed by trimming with the trimAI tool (Singh et al., 2015). A total of ten divergent species and their respective sequences were retrieved in FASTA format. These sequences, along with the query sequence, were subjected to multiple sequence alignment using MEGA 7 software to identify evolutionarily conserved regions of the 16S ribosomal RNA gene (Singh et al., 2015). Phylogenetic analysis was conducted using the Maximum Likelihood method based on the Tamura–Nei model implemented in MEGA 7.0. The robustness of the phylogenetic tree topology was evaluated using 1000 bootstrap replicates, and bootstrap support values greater than 50% were displayed at the corresponding nodes.

Statistical Analysis

All screening and biochemical experiments were conducted in triplicate. Data are presented as mean ± standard deviation (SD). Due to the qualitative nature of the screening assay and the limited number of selected isolates, statistical comparisons were not performed in the present study. Future quantitative cellulase activity analyses will include appropriate statistical testing such as one-way analysis of variance (ANOVA).

Results

Screening and Isolation

Among 15 isolates, 3 bacterial colonies (isolate 5, 7 and 11) were taken according to their ability to produce cellulase determined by screening on Carboxy Methyl Cellulose (CMC) agar showing the best zone of hydrolysis (Figure 1). The well isolated colonies on the nutrient agar plates were evaluated, and their cultural and morphological characteristics were found similar with the rod-shaped opaque colony forming bacteria (Figure 2 and

Table 1). Under light microscope the bacterial cells (all three isolates) were found to be gram negative (Table 2).

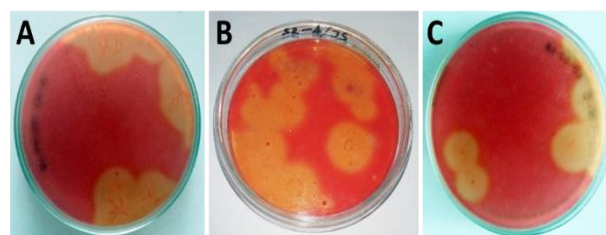


Figure 1: Zone of hydrolysis of isolate 5, 7 and 11 (A, B and C respectively)

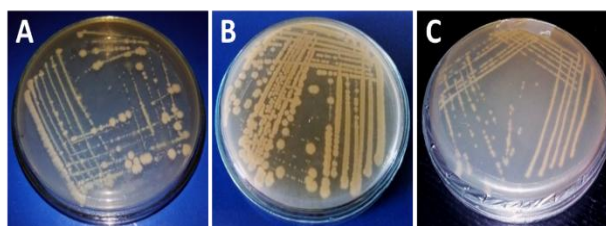


Figure 2: Discrete isolated colonies of isolate 5, 7 and 11(A, B and C respectively) on Nutrient agar plate

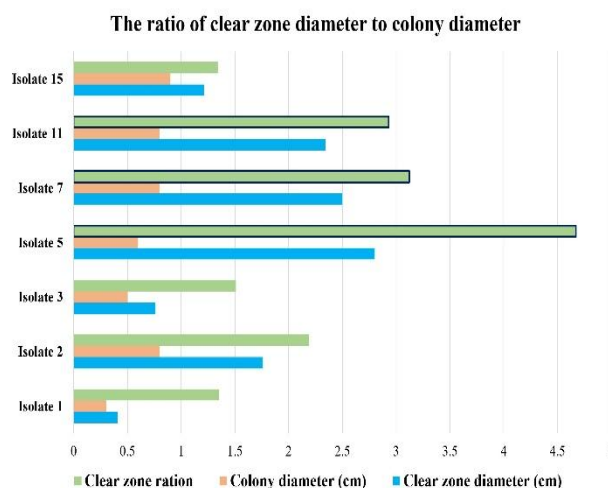


Figure 3: The ratio of clear zone diameter to colony diameter of the selected isolates

Table 1: Colony morphology of the isolates

Parameters	Isolate 5	Isolate 7	Isolate 11
Size	Moderate	Moderate	Moderate
Pigmentation	Opaque and white	Opaque and white	Translucent opaque
Form	Irregularly edged circular	Irregularly edged circular	Circular
Margin	Undulate (wavy indentations)	Undulate (wavy indentations)	Smooth
Elevation	Convex	Low convex	Flat
Texture	Mucoid	Mucoid	Smooth

Biochemical characterization

Biochemical tests gave a presumptive idea to identify the isolates. The results of the tests were compared and found to be the same for isolate 5 and 11 whereas, the third one

was a little bit different in performing the reactions against the ingredients induced (Table 3).

Table 2: Gram staining properties of the selected isolates

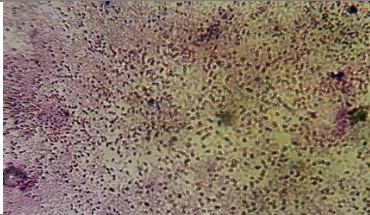
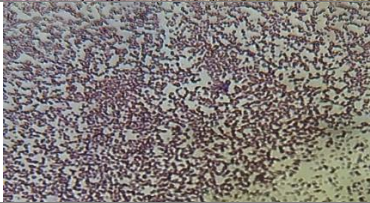
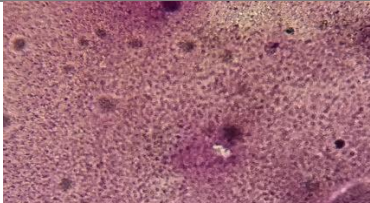
Isolates	Microscopic picture	Parameters	Observation
Isolate 5		Shape	Rod shaped
		Arrangement	Single
		Motility	Motile
		Gram Reaction	Negative
Isolate 7		Shape	Rod shaped
		Arrangement	Single & paired
		Motility	Actively Motile
		Gram Reaction	Negative
Isolate 11		Shape	Rod shaped
		Arrangement	Single
		Motility	Motile
		Gram Reaction	Negative

Table 3: Biochemical test results

Biochemical properties	Isolate 5	Isolate 7	Isolate 11
Oxidase	+	+	+
Catalase	+	+	+
Indole	-	-	-
Citrate	+	+	+
Methyl red	-	-	-
Voges-Proskauer	+	-	+
Glucose	+	+	+
Lactose	-	-	-
Sucrose	-	-	-
Gas	+	+	+
H ₂ S	-	-	-
Urease	-	-	-
Starch hydrolysis	-	-	-

Genotypic characterization

with two universal primers and the product size was found to be around 1465bp by agarose gel electrophoresis (Figure 4) and the found concentration of DNA and purity were well enough to carry out sequencing process. The results from the NCBI database presented in the table 4 provide the identifying key and the names of the

bacteria which were isolated from the soil sample with the help of BLAST sequence matching. From the similarity values or the distances estimated after pair wise alignment, we've got a phylogenetic tree representing the relationship among the query sequence of isolate number 5, 7 and 11.

Table 4: Sequencing results from NCBI with higher percentage of identity (99%) and 0.0 E value

Isolates	Name of the Bacteria	Genbank Accession Number	BLAST match Sequence		
			Reference Accession NO	Query Length	Max Iden
5	<i>Enterobacter cancerogenus</i>	MH844557	<i>Enterobacter cancerogenus</i> CP025225.1	1417	99%
11	<i>Enterobacter cloacae</i>	MH844562	<i>Enterobacter cloacae</i> CP021851.1	1263	99%
7	<i>Pseudomonas aeruginosa</i>	MH844577	<i>Pseudomonas aeruginosa</i> KY549647.1	1271	99%

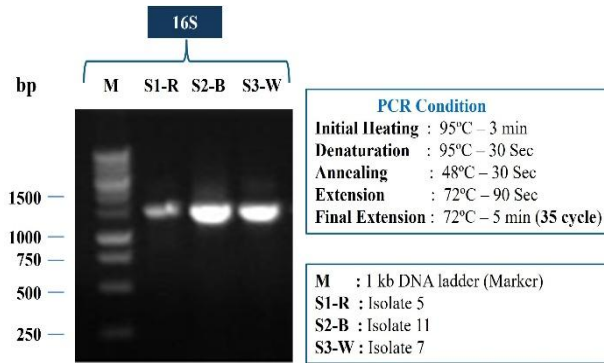


Figure 4: Electrophoresis result of the amplification of 16S rRNA gene of the isolates

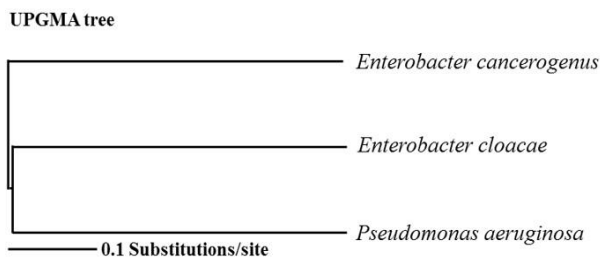


Figure 5: Phylogenetic Tree

Discussion

Screening of the strains

Of the colonies obtained from enriched soils, 15 showed cellulolytic activity, and three top performers (isolates 5, 7, 11) were pursued further. The ratios (isolate 5 (4.67), isolate 7 (3.12), and isolate 11 (2.93)) were significantly higher than the mean clear zone ratios observed in isolates from farming and forest soils (Hatami et al., 2008; Lu et al., 2005) because the samples like jute sticks are rich in phenolic compounds, lignin derivatives, and simple sugars, may contribute to the enrichment of cellulolytic microbial populations, although this hypothesis was not directly tested in the present study. The enhanced clear zone observed can be attributed to the diverse microbial community present in the samples, along with the specific conditions of organic matter (Tabao et al., 2010; Gupta et al., 2012). Therefore, the degrading samples mixed with soil indicate the presence of superior strains with heightened survival capabilities and cellulase production potential (Rowińska et al., 2024).

The percentage of the types of bacteria in the samples

The agricultural waste residues utilized in this study, specifically jute sticks, rice straw, and sugarcane bagasse, were collected from Gollamari, Khulna. The analysis revealed that the prevalence of cellulolytic bacteria in these samples was 47%. Previous studies reported varying percentages of cellulolytic bacteria, with 34.7% in farming soil, 52.4% in forest soil (Hatami et al., 2008), 42.86% in cow dung (Bai et al., 2012), 46.15% in retting ponds and estuaries (Shenkani & Sundara, 2015), and 32.35% in various environments, including soil, wood furnishing regions, sugarcane farms, and water samples from paper industry and municipal waste (Gocheva et al., 2023). In comparison to these findings, the soil samples

in this study demonstrated a notable potential as a source of cellulose-producing bacteria, slightly surpassing the results observed in cow dung studies by Bai et al. (2012) and Sadhu et al. (2013).

Biochemical characterization

In this study, a series of biochemical tests were conducted to characterize the isolates and preliminarily identify cellulase-producing bacteria. The oxidase test, which detects cytochrome oxidase activity, is instrumental in differentiating among bacterial genera; it yielded positive results for *Pseudomonas*, *Vibrio*, *Neisseria*, *Brucella*, and *Pasteurella*, while *Enterobacteriaceae* displayed a negative result.

All three isolates were catalase positive, indicating their capability to respire using oxygen as a terminal electron acceptor, a characteristic common among strict aerobes and facultative anaerobes. Conversely, catalase-negative bacteria may either be anaerobes or facultative anaerobes that exclusively ferment (Reiner, 2010). Citrate agar testing confirmed the ability of the isolates to utilize citrate as an energy source, with all isolates demonstrating citrate positivity. In the Triple Sugar Iron (TSI) test, the isolates exhibited a red slant and yellow butt with gas production, indicative of fermentation of lactose or sucrose. TSI agar, containing lactose, sucrose, glucose, and iron, facilitates the assessment of carbohydrate fermentation and gas production (Cappuccino & Sherman, 2005). Indole production, tested using Kovac's Reagent, yielded negative results for all isolates. The Methyl Red test showed negative results, while the Voges-Proskauer test was positive, suggesting mixed acid fermentation capabilities (Cappuccino & Sherman, 2005; McDevitt, 2009). The urease test revealed that all isolates were urease negative, indicating an inability to hydrolyze urea. The starch hydrolysis test, essential for identifying bacteria capable of hydrolyzing starch through the secretion of α -amylase and oligo-1,6-glucosidase, yielded negative results for all three isolates. The absence of a clear zone after iodine application indicated a lack of starch degradation.

Based on the biochemical profiles obtained, the isolates are tentatively classified within the genera *Enterobacter* and *Pseudomonas*, pending further molecular analysis.

Genetic analysis

The molecular identification of the selected bacterial isolates was successfully achieved through 16S rRNA gene amplification using universal primers, giving a product size of approximately 1465 base pairs, matching the target amplicon size for bacterial 16S rRNA genes. The clarity and intensity of the bands on agarose gel electrophoresis (Figure 4) and the satisfactory DNA concentration and purity confirmed the suitability of the samples for subsequent sequencing analysis.

Sequence analysis via BLAST against the NCBI GenBank database showed high sequence identity (99%) and a perfect E-value (0.0), indicating highly valid species-level identification. Isolate 5 showed closest homology to *Enterobacter cancerogenus* (Accession No. CP025225.1), isolate 11 matched with *Enterobacter*

cloacae (CP021851.1), and isolate 7 aligned with *Pseudomonas aeruginosa* (KY549647.1), as shown in Table 4. However, 16S alone cannot confirm species identity at >99% resolution and further whole-genome sequencing can facilitate full confirmation of the identity.

These bacteria are generally found in soil environments and are known for their various metabolic capabilities. Members of the *Enterobacter* genus are facultative anaerobes and are often associated with nitrogen cycling, while *Pseudomonas aeruginosa* is a flexible, Gram-negative bacterium capable of degrading a wide range of organic compounds, previously reported to possess traits relevant to bioremediation and industrial biotechnology.

The phylogenetic tree constructed from the synchronized sequences further supports the relatedness of the isolates and their precise classification. The close clustering of isolates with their reference strains confirms the validity of the molecular approach used. These results not only validate phenotypic identification but also highlight the presence of potentially beneficial and metabolically active microbial species in the sampled soil environment.

A limitation of the present study is that cellulolytic potential was evaluated using Congo red-based CMC hydrolysis assays only. Although the hydrolysis zone provides a useful preliminary indication of cellulase production, quantitative enzyme activity measurements such as CMCase, FPase, and β -glucosidase assays are required to accurately compare cellulase productivity among isolates. Future studies will therefore focus on enzyme quantification under different growth conditions and determination of specific cellulase activity.

Although the identified isolates demonstrated promising cellulolytic potential, biosafety considerations must be addressed before industrial application. *Pseudomonas aeruginosa* is recognized as an opportunistic human pathogen, while members of the *Enterobacter cloacae* complex and *Enterobacter cancerogenus* have been associated with opportunistic infections in immunocompromised individuals. Therefore, future development of these strains for industrial use should include comprehensive biosafety assessment, pathogenicity evaluation, and compliance with relevant biosafety regulations.

Conclusion

In this study, species were selected based on their superior cellulase-degrading capabilities, as indicated by the hydrolysis zone ratios, followed by species identification. This identification is crucial for optimizing cellulolytic fermentation tailored to specific organisms. Moreover, understanding the genomes of the identified species facilitates genetic manipulation, such as site-

directed mutagenesis, to enhance strain development and improve industrial performance.

A comprehensive investigation into the factors influencing cellulase-cellulose interactions—including substrate adsorption, accessibility, processability, synergism, and the mechanistic details of cellulose depolymerization—presents numerous opportunities for enzyme utilization in targeted applications (Zhang et al., 2024; Review on Progress in Cellulase Catalyzed Saccharification, 2024). In addition to cellulase production, it is essential to deepen our understanding of microbial physiology, pretreatment methods for cellulosic biomass to enhance microbial accessibility, and cost-effective cellulase production processes. Treatments that convert biomass into hydrolytic products, which can serve as substrates for downstream fermentation to produce various essential metabolites, are critical. Furthermore, applying principles of metabolic and protein engineering is vital for advancing these processes.

Ultimately, a detailed quantitative analysis of the cellulase-producing strains *Enterobacter cancerogenus*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* will substantiate their potential for applications in biofuel production and other cellulase-based industries and for this, we have a plan to measure specific activity (U/mg) of crude cellulase extracts at pH 5–8 and 30–60 °C to determine optimal conditions.

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Competing Interest

The authors report that there are no competing interests to declare.

Credit Author Statement

H. M. Rakib Hossain: Methodology, Investigation, Data Analysis, Writing- the first draft; Md. Morsaline Billah: Supervision, Conceptualization; Tauhidur Rahman Nurunnabi: Methodology, Physical and Chemical Analyses; S. M. Mahbubur Rahman & Md Emdadul Islam Co-supervision, Data Analysis; Kazi Mohammed Didarul Islam & Anti Islam: Writing – final draft, review & editing.

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