HAYATI
Journal of Biosciences

Vol. 32 No. 2, March 2025 547-560
DOI:10.4308/hjb.32.2.547-560
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

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Characterization and Enzymatic Assay of Cellulase-Producing Probiotic Bacteria Isolated from Traditional Fermented Bamboo of Bangladesh

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ARTICLE INFO

Article history:
Received August 15, 2024
Received in revised form October 3, 2024
Accepted November 25, 2024

KEYWORDS:
Bacillus cereus,
native,
microorganism,
cellulolytic,
biochemical characterization

ABSTRACT

Fermented bamboo (also known as Ngwopo) is a traditional food item among the indigenous population living in the south-east of Bangladesh. Though fermented foods harbour a significant number of probiotics and enzyme-producing bacteria, there is a lack of research on isolating beneficial bacteria from native fermented food products in Bangladesh. Thus, considering the extensive utilisation of cellulase enzymes and probiotics, our study was conducted with the objective of isolating and characterizing native cellulase-producing bacteria from fermented bamboo and performing crude enzyme activity assay. The sample was collected from Khagrachari hill tracts district of Bangladesh. Three isolated bacteria, namely FB-1, FB-2, and FB-3, screened based on the carboxymethyl cellulose medium, were tested for biochemical and probiotic properties, and the best isolate was genetically identified by molecular characterization through 16S rRNA gene sequencing and evolutionary analysis. All three gram-positive isolates were observed to be positive for starch hydrolysis and fermentation of sucrose, suggesting their ability to breakdown carbohydrates while testing negative for gelatinase, indole, and H_2S test. The isolates demonstrated moderate bile salt and pH tolerance and low to medium autoaggregation. FB-2 had 75% susceptibility to the tested antibiotics, while other isolates were susceptible to 50% antibiotics. All of the bacteria were non-pathogenic, showing γ-haemolysis. FB-2 strain showed an enzyme production capacity of 1.56 units/ml. The best performing isolate was identified as *Bacillus cereus*. Therefore, the study discovered a native bacteria potentially suitable as probiotic and as a source of cellulase for industrial processes, highlighting the enriched indigenous cuisines of Bangladesh.

1. Introduction

The indigenous people in southwest Bangladesh and northeast India ferment bamboo shoots using various preparation methods, as these shoots offer numerous health benefits, possess great medicinal value, and are highly nutritious. The fermentation of bamboo has been extensively practiced in the hilly districts of Rangamati, Bandarban, and Khagrachari in Bangladesh. They are consumed in the form of curry, pickle, and soup, or used as an ingredient in the preparation of other cuisines (Singhal *et al.* 2021). Being called as Ngwopo, fermented bamboo is one of the most delicate and popular fermented products among the indigenous people of Bangladesh. The process of fermenting bamboo shoots not only enhances their taste, smell, texture, and appearance, but also increases their nutritional value and prolongs

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their storage time. This is due to the activity of lactic acid bacteria, which acidify the product and facilitate the digestion of bamboo shoots (Singhal *et al.* 2021). Enzymes or microbes are employed in the production of fermented foods, leading to crucial biochemical transformations and substantial modifications in the food (Ramasubramanian *et al.* 2024). The fermented bamboo contains an extensive range of notable microorganisms, including lactic acid bacteria and yeast (Behera and Balaji 2021). The species identified in the fermented bamboo products were predominantly *Bacillus subtilis, Bacillus cereus, B. pumilus, B. safensis, Lactobacillus brevis, L. plantarum, Carnobacterium* sp., *Enterococcus faecium, Pseudomonas fluorescens* and *Enterobacter* (Jeyaram *et al.* 2010; Kumar *et al.* 2022). Moreover, Bamboo fibre is a regenerated cellulose fibre (Hejazi *et al.* 2012). Therefore, fermented bamboo products are likely to contain cellulase-producing probiotic bacteria that participated in the fermentation process.

Cellulose is a linear polysaccharide that consists of β-1,4 linked D-glucopyranosyl units. It is produced by all higher plants as well as other living beings including bacteria, fungi, protists, and invertebrates. It is regarded as the most plentiful renewable natural biological resource on earth (Afzal *et al.* 2012; Nallusamy *et al.* 2016). Cellulases are enzymes that catalyse the breakdown of cellulosic polymers (Sukumaran *et al.* 2005). Cellulases have been essential biocatalysts for numerous decades. Microbial cellulases are utilised across multiple industries such as textile, paper and pulp, laundry and detergent, agricultural, medical, food and feed industries. Enzyme market research findings indicate that the key areas of application include food and drinks, the textile sector, livestock feed, and biofuels (Jayasekara and Ratnayake 2019). Cellulolytic microbes have been studied comprehensively along with the structure, function, and synergistic activity of different cellulases found in these organisms. Cellulases are synthesised by a diverse range of bacteria and fungi, including both aerobic and anaerobic species, as well as both mesophilic and thermophilic organisms (Lynd *et al.* 2002). Termites and crayfish, among other animal species, have also been documented as producers of cellulase (Afzal *et al.* 2012). While fungi are known for their efficient cellulase activities, there is a growing interest in cellulase production by bacteria due to their fast growth rate and potential for application in cellulase production. The cellulolytic activity exhibited by the bacterial species is observed to be reliant upon its source (Mahjabeen 2016).

Bacillus species have the capacity to produce cellulases. *Bacillus* spp. that are cellulolytic typically release endoglucanases that have the ability to break down carboxymethyl cellulose (Robson and Chambliss 1984). *Bacillus* spp. is commonly utilised in industries due to its non-pathogenic nature, ease of growth and reproduction, absence of foul odours or gases, ability of certain species to thrive in alkaline conditions and high temperatures, secretion of extracellular proteins, and overall safety in terms of health and environmental concerns (Beukes and Pletschke 2006; Nallusamy *et al.* 2016). *Bacillus* strains have been categorised as Generally Recognised as Safe (GRAS) bacteria for their application in human nutrition as meals or dietary supplements, as well as in animal nutrition as feed supplements (Dabiré *et al.* 2022a). Also, certain strains of *Bacillus* are classified as probiotics, meaning they are living microbes that, when consumed in sufficient dosage, provide a health advantage to their recipient (Hill *et al.* 2014). Potential advantages of these substances encompass the ability to regulate the immune system, combat foodborne infections, decrease the risk of cardiovascular disease, lower serum cholesterol levels, and prevent intestinal diseases (Dabiré *et al.* 2022b).

The studies on the enzyme producing or probiotic bacteria isolated from fermented food products in Bangladesh are rare. Exploring the reservoir of microbes in native fermented products and understanding their enzyme production capacity are of immense potential because of the multifaceted applications of the enzymes, enzyme-producing bacteria and probiotics in various industries. Therefore, the current research was undertaken to isolate and characterize probiotic bacteria from traditional fermented food item and assess the enzyme activity of the isolates.

2. Materials and Methods

2.1. Collection of Sample and Stocking of Bacteria

The fermented bamboo samples were collected from hill tracts of Khagrachhari district and packed immediately in polythene bags with sample details which were later placed in icebox. The fermented samples were weighed aseptically and blended in 250 ml sterilized distilled water (DW). After stirring properly, and 10-fold serial dilutions were prepared in sterile DW and inoculated in nutrient broth (NB) medium and incubated at 30°C for 12 hrs. Then, pure colonies were isolated. Pure isolates were labelled and stocked in NB broth

medium (Difco BD, USA) containing 30% (v/v) glycerol and stored at -80°C.

2.2. Screening and Isolation of Cellulase Producing Bacteria

A volume of 5 µL of each broth culture of pure bacterial isolates were transferred in CMC (Carboxymethyl cellulose) agar plates (contains 1.0% peptone, 1.0% carboxymethylcellulose (CMC), 0.2% K_2HPO_4, 1% agar, 0.03% $MgSO_4.7H_2O$, 0.25% $(NH_4)2SO_4$ and 0.2 % gelatine). After incubation at 28°C for 48 hours (hrs), CMC agar plates were flooded with 1% Congo Red and allowed to stand for 15 minutes (min) at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. Colonies showing discoloration of Congo Red indicating cellulose hydrolysis were taken as positive cellulose-degrading bacterial colonies. The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system (Bai *et al.* 2012; Li *et al.* 2016).

2.3. Biochemical Characterization of the Isolates

For selective screening, the isolated bacterial strains were characterized by biochemical tests such as gram test, catalase test, oxidase test, MR-VP test, oxidative-fermentative test, H_2S test, citrate agar test, gelatinase test, urase test, starch hydrolysis test and sugar fermentation test.

2.3.1. Gram Stain Test

Gram stain test was carried out on the bacterial isolates as described by Dussault (1955). A fixed pure bacterial smear on a clean glass slide was flooded with crystal violet for 1 min, rinsed with running tap water, and then flooded with Gram's iodine for 1 min followed by rinsing with running tap water. After that the slide was decolorized with 95% ethanol and washed with tap water. Then, the slide was counter stained with Safranin for 1 min, rinsed with running tap water and allowed to air dry. The dry slide was covered with immersion oil and viewed under a light microscope.

2.3.2. Catalase Test

This test is used to determine the ability of microorganisms to degrade H_2O_2 by producing the enzyme catalase. Overnight bacterial cultures were taken in the marked area of a glass slide and pea shaped smear formed. One to two drops of H_2O_2 were added over the test smear and bubble formation was noted down for catalase positive bacteria (Talaiekhozani 2013).

2.3.3. Oxidase Test

Oxidase was performed according to the description of Shields and Cathcart, (2010). A filter paper was soaked with the substrate N, N, N′, N′-tetramethyl-p-phenylenediamine dihydrochloride, and then it was moistened with sterile DW. The test organism colony was picked and smeared in the filter paper. Finally, the inoculated area of the paper was observed for a colour change to deep blue or purple within 10-30 seconds.

2.3.4. MR-VP Test

In MR (Methyl Red) test, the medium with buffered peptone, dextrose, and di potassium phosphate, was distributed 10 ml in test tubes. The medium was inoculated with isolates separately. 3-4 drops of MR reagent were added in the test tube and incubated at 35-37ºC for 24-48 hrs.

For VP (Voges Proskauer) test, 1 ml culture was transferred into screw cap test tube. Subsequently, 18 drops of Baritt's reagent (5g α naphthol with 100 ml 95% ethanol and 16% potassium hydroxide-KOH) were added (Barritt 1943). The mixture was agitated vigorously for 1-2 min and kept unaltered for 1-2 hrs. A cherry red colour would indicate a positive result, while a yellow-brown colour indicated a negative result (Barry and Feeney 1967).

2.3.5. H_2S Test

The isolates were inoculated into the agar medium with 2.61% peptone, 0.2% ferrous ammonium sulphate and 0.2% sodium thiosulphate in test tube by stab inoculation. Then the strains were incubated at 37°C for 24-48 hrs. Black precipitation after incubation indicated positive for H_2S production (Leber 2016).

2.3.6. Gelatinase Test

Gelatine media was distributed 10 ml in test tubes followed by inoculation of isolated bacteria with loop. The culture was incubated overnight to 48 hrs and the consistency of the media was observed to notice the breakdown of gelatine (Cruz and Torres 2012).

2.3.7. Starch Hydrolysis Test

Chemical for this test was prepared using peptic digest of animal tissue as per Dhawale *et al.* (1982). Using a sterile technique, a single streak inoculation of organism was made to be tested into the centre of labelled plate. After incubation of 48 hrs at 37°C, the surface of the plates was flooded with 1% iodine solution with a dropper for 30 seconds. Then the excess iodine

was poured off. The clear zone was examined around the line of bacterial growth. A blue, purple or black coloration of the medium indicates that the organism failed to hydrolyse starch.

2.3.8. Urase Test

Christensen's Urea Agar was prepared for urase test following the description by Talaiekhozani (2013). A volume of 5 ml agar was poured in each test tube. The entire slant surface with a heavy inoculum of each isolate was streaked and the culture was incubated with loosened cap at 35°C. The slant was observed for a colour change at 6 and 24 hrs. If the organism produced urase enzyme, the colour of the slant would change from light orange to magenta; otherwise, the media retained its original colour.

2.3.9. Citrate Agar Test

Simmon's agar medium was formulated with 0.1% magnesium sulphate, 0.2% sodium citrate and 0.008% bromothymol blue (as an indicator). Overnight freshly cultured bacteria were inoculated in media plate and incubated at 35-37°C for 24-28 hrs. The colour of the slant changed from green to bright blue for positive identification and the colour remained unchanged for negatives (Leber 2016).

2.3.10. Sugar Fermentation Test

The phenol red carbohydrate test media was prepared, and 10 ml was distributed in each test tube. 100 µL of respective bacteria isolates were poured in each test tube. The cultures were incubated at 35-37°C for 18-24 hrs. After incubation when the liquid in the tube turns yellow to red, it indicated a drop in the pH level because of the production of acid by fermentation of carbohydrate in the media. The tube containing media remained red indicating the bacteria could not ferment the carbohydrate source (Reiner 2012).

2.4. Identification of Probiotic Properties

In vitro tests were used to identify several probiotic qualities, such as tolerance to bile, resistance to low pH, auto aggregation, haemolysis, and antibiotic sensitivity.

2.4.1 Bile Salt Tolerance

The tolerance to bile salts was assessed in NB medium containing 0.0 and 0.3% (w/v) of bile salts. Multiple bottles of this broth, which included varying quantities of bile salt, were inoculated with 100 µL of cultured isolates and incubated at 37°C. The growth rate was determined by measuring the optical density using a spectrophotometer (Spectrophotometer C7200S UV Visible Double Beam, Peak, USA) at a wavelength of 600 nm after incubating for 3 h (Balcázar *et al.* 2008; Kim and Austin 2008).

2.4.2. pH Tolerance Test

The acid tolerance of the specified bacterium was examined at various pH levels. Initially, various NB solutions with distinct pH levels of 3, 4, and 7 (as the control), were prepared utilising 1% HCl and 1N NaOH (Samelis *et al.* 1994). The autoclaved broths were inoculated with a sub-culture broth of the chosen strain and then incubated at a temperature of 37°C. The growth rate of bacteria was determined by measuring the optical density (OD) using a spectrophotometer at a wavelength of 600 nm after incubation for 1 and 3 hrs.

2.4.3. Auto Aggregation

The experiment was conducted following the protocol described by Del Re *et al.* (2000), with certain adjustments. An amount of 0.1 ml of the overnight cultures were inoculated into 10 ml of NB media. The mixture was then incubated at 37°C for 24 hrs, while being kept under anaerobic conditions. Next, bacterial cells were collected using centrifugation at 14,000 g for 5 min, rinsed twice with 50 Mm K_2HPO_4 (pH 7.00), and subsequently reconstituted in the same buffer to achieve an absorbance of approximately 0.2 at 600 nm (bacterial suspension). A 5 ml bacterial culture was well mixed by vortexing for 15 seconds (s) and then left to stand at room temperature. The absorbance changes of the cultured media caused by precipitation were determined at 600 nm using a spectrophotometer before (A_0) and after 5 hrs interval (A). The auto aggregation percentage (AAg %) was determined using the following formula:

$$AAg\ \% = \frac{A_0 - A}{A_0} \times 100$$

From AAg %, isolate could be classified into three groups: High auto aggregation (>70% AAg), Medium auto aggregation (20-70% AAg), and Low auto aggregation (<20%).

2.4.4. Hemolytic Activity Test

This test was conducted to evaluate their pathogenic properties. To perform the assay, we streaked the

colony of the selected bacteria onto a blood agar plate containing 5% sheep blood. Subsequently, the outcome was evaluated following 24 hrs incubation period at 37°C. The result was analysed by examining lysis of red blood cells under and around bacterial colonies as α-haemolysis (a partial hydrolysis and brown/green discoloration), β-haemolysis (clearing of red color), and γ-haemolysis (lack of discoloration or clearing/no reaction) (Kavitha *et al.* 2018). Only bacterial isolates with γ-haemolysis were analysed further.

2.4.5. Antibiotic Susceptibility

A disc diffusion technique was used to conduct an antibiotic sensitivity test using the most used antibiotics in aquaculture. Antibiotic disks included Ciprofloxacin (5 µg), Tetracycline (30 µg), Amoxycillin (10 µg) and Doxycycline (30 µg). 50 µL of the sub-culture broth of the isolates was spread on NB agar and, antibiotic discs were subsequently placed on plates after solidification. Afterwards, the plates were subjected to incubation at 37°C for a duration of 24 hrs to visually examine and quantitatively assess the areas of inhibition following the Kirby-Bauer test table (Bauer *et al.* 1966; Kim and Austin 2008).

2.5. Cellulase Enzyme Activity Assay

The measurement of cellulase activity was conducted following the protocol established by Denison and Koehn (1977) using 1% CMC in a 0.1 M citrate buffer solution of pH 5.0 as the substrate. The production media was formulated by combining 0.01% $MgSO_4$, 0.1% yeast extract, 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.05% sodium citrate, and 0.1% CMC. The bacterial isolates were cultured at temperature of 37°C and speed of 200 revolutions per min in a shaker incubator for a duration of 72 hrs. Subsequently, 10 ml aliquot of broth culture was subjected to centrifugation at a speed of 10,500 rpm for 10 m in to get the supernatant containing the crude cellulase enzyme. A total of 0.5 ml substrate solution was added with 0.5 ml culture supernatant (crude enzyme) and incubated at 50°C for 30 min. Then 3 ml dinitro salicylic acid (DNS) reagent was mixed, and the mixture was boiled for 5 min. An amount of 20 ml of distilled water was added, and the measurement of the synthesis of reducing sugar (glucose) from CMC substrate due to cellulolytic activity was conducted at a wavelength of 540 nm, using glucose as the reference. One cellulase unit is defined as the amount of enzyme per ml culture filtrate that released 1 µg glucose per minute. Finaly, the activity of cellulase was calculated using the following formula:

$$\text{Units/ml Enzyme} = \frac{\text{µ mol glucose equivalents released} \times \text{total volume of assay}}{\text{volume of enzyme used} \times \text{time of assay} \times \text{volume used in colorimetric determination}}$$

2.6. Molecular Identification

2.6.1. Identification by MALDI-TOF MS

To identify the selected isolates at the molecular level, we initially employed Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to determine their genus. To conduct MALDI MS analysis, a distinct colony of pure isolates was generated by mixing them with an organic compound matrix solution, which acted as an energy-absorbing material. A laser beam was automatically used to ionize the sample inside the matrix. Subsequently, the ions in their protonated state were propelled forward by a constant electric potential. The charged analytes were subsequently identified and quantified using time of flight (TOF) analysers. The TOF information is used to develop a characteristic spectrum known as peptide mass fingerprint (PMF) for the analytes (Singhal *et al.* 2015).

2.6.2. Identification by Gene Sequencing

The Monarch® Genomic DNA Purification Kit was employed to extract and purify genomic DNA from bacterial cells. A Nano drop spectrophotometer was used to assess the DNA's level of purity. The genomic DNA was then amplified using the polymerase chain reaction (PCR). Reaction volume was prepared using 25.0 µL of PCR master mix, 2.5 µL of forward primer 27F (AGAGTTTGATCCTGGCTCAG), 2.5µL of reverse primer 1492R (CGG TTA CCT TGT TAC GAC TT), 10 µL of DNA template and nuclease free water up to 50 µL. Amplified PCR product was observed on 1% agarose gel through gel electrophoresis and forwarded to gene sequencing. Amplified genomic sequences were uploaded to the dataset of NCBI and NCBI BLAST was used to distinguish the nearest neighbours of the isolates. The amplification conditions initially suggested by Williams *et al.* (1990) were implemented with minor adjustments. The Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993) were utilised to infer the evolutionary history in

the phylogenetic tree. The evolutionary studies were conducted with the MEGA11 program (Tamura *et al.* 2021).

3. Results

3.1. Screening and Isolation

Three bacteria, denoted as FB-1, FB-2 and FB-3, were isolated from fermented bamboo sample collected from Khagrachhari hill tracts district, Bangladesh. The isolates exhibited hydrolytic activity on agar plates using CMC as the primary source of carbon. After staining with Congo Red, the diameters of the hydrolytic zones and colonies were measured and recorded in Table 1.

3.2. Biochemical Characteristics

Different morphological features (shape, colour, and motility) of colonies of pure isolates and their biochemical properties were observed and listed in Table 2. Biochemical characterization of all three isolates including variety of tests such as Gram test, catalase, oxidase test, MR-VP test, H2S test, gelatinase test, starch hydrolysis test, urease test, citrate agar test, sugar fermentation test was carried out for identification. All three motile and gram-positive isolates observed positive for starch hydrolysis and fermentation of sucrose, suggesting their ability to breakdown carbohydrates. All isolated bacteria yielded negative results for gelatinase, indole, and H_2S tests, but tested positive for catalase. Nonetheless, they had mostly similar morphological features with rod shaped structure and, white and yellow colonies.

Table 1. Cellulose (CMC) hydrolysis zones of isolates

Isolate name	Colony diameter (n)(mm)	Zone diameter (z)(mm)	(z/n) (mm)
FB-1	7.5	11.25	1.5
FB-2	7.0	16.8	2.4
FB-3	7.6	15.2	2.0

Table 2. Biochemical features of the isolates

Biochemical test	FB-1	FB-2	FB-3
Gram's	+	+	+
Oxidase	+	+	-
Catalase	+	+	+
MR test	+	-	+
VP test	-	+	-
Gelatinase	+	-	+
Starch hydrolysis	+	+	+
Citrate agar	-	+	-
Sucrose fermentation	+	+	+
Dextrose fermentation	-	-	+
Starch fermentation	-	+	+
Glucose fermentation	-	+	+
H2S test	-	-	-
Motility test	+	+	+
Urase test	-	+	-
Indole test	-	-	-
Morphology	Rod	Rod	Rod
Colony color (Visual test)	Yellow	White	White

3.3. Probiotic Properties

3.3.1. Bile Salt Tolerance

To assess the viability of the bacterial strains (FB-1, FB-2, and FB-3) in the gastrointestinal system of the host, we measured their resistance to the adverse effects of bile salts (0.3% concentration) over 3 hrs incubation period. The control group (0.0% bile) was used as a baseline and all isolates exhibited the highest bacterial growth. In this experiment, all isolates showed substantial growth in 0.3% bile salt concentration (Figure 1). In this study, highest growth was recorded for FB-2 (1.202) while the lowest was recorded for FB-3 (0.789).

3.3.2. pH Tolerance

This study used pH levels of 3 and 4, which are commonly observed in the stomachs of both humans and animals. This experiment aimed to ascertain the ability of bacteria to thrive in acidic environments. Isolates FB-2 and FB-3 exhibited greater tolerance and proliferation (measured by OD) in an acidic environment compared to FB-1 after 3 hrs incubation (Figure 2).

3.3.3. Auto Aggregation

The ability of probiotic strains to aggregate is essential for their attachment to intestinal epithelial cells. The isolates' auto-aggregation capacity is demonstrated in Figure 3. This study revealed out of 3 isolates two FB-1 and FB-2 were ranked as moderately auto-aggregated isolates with 25.30±1.98% and 30.03±2.89% auto aggregation capacity respectively whereas the other isolate (FB-3) had low auto-aggregation ability (2.2±0.51) (Figure 3).

3.3.4. Haemolytic Activity

The haemolytic activity assay is a crucial criterion used to differentiate between pathogenic and non-pathogenic bacteria. In this test, all of three isolates (FB-1, FB-2, and FB-3) showed γ-haemolysis (non-pathogenic) and there was absence of blood cell

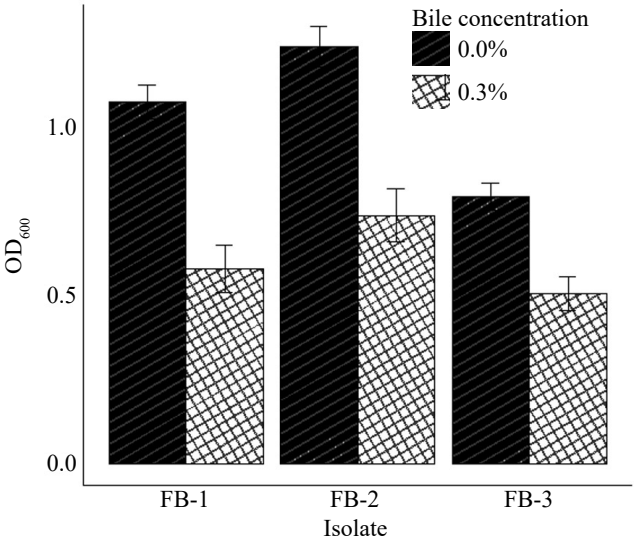

Figure 1. Bile tolerance test of isolated bacteria, with error bars indicating standard deviation (SD)

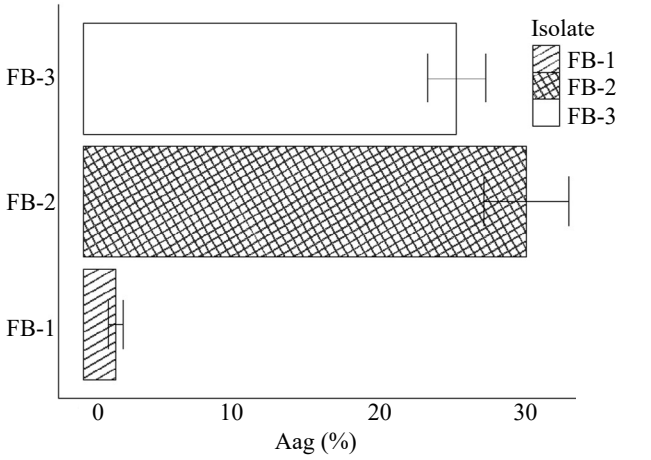

Figure 3. Auto-aggregation ability of the isolates, with error bars indicating standard deviation (SD)

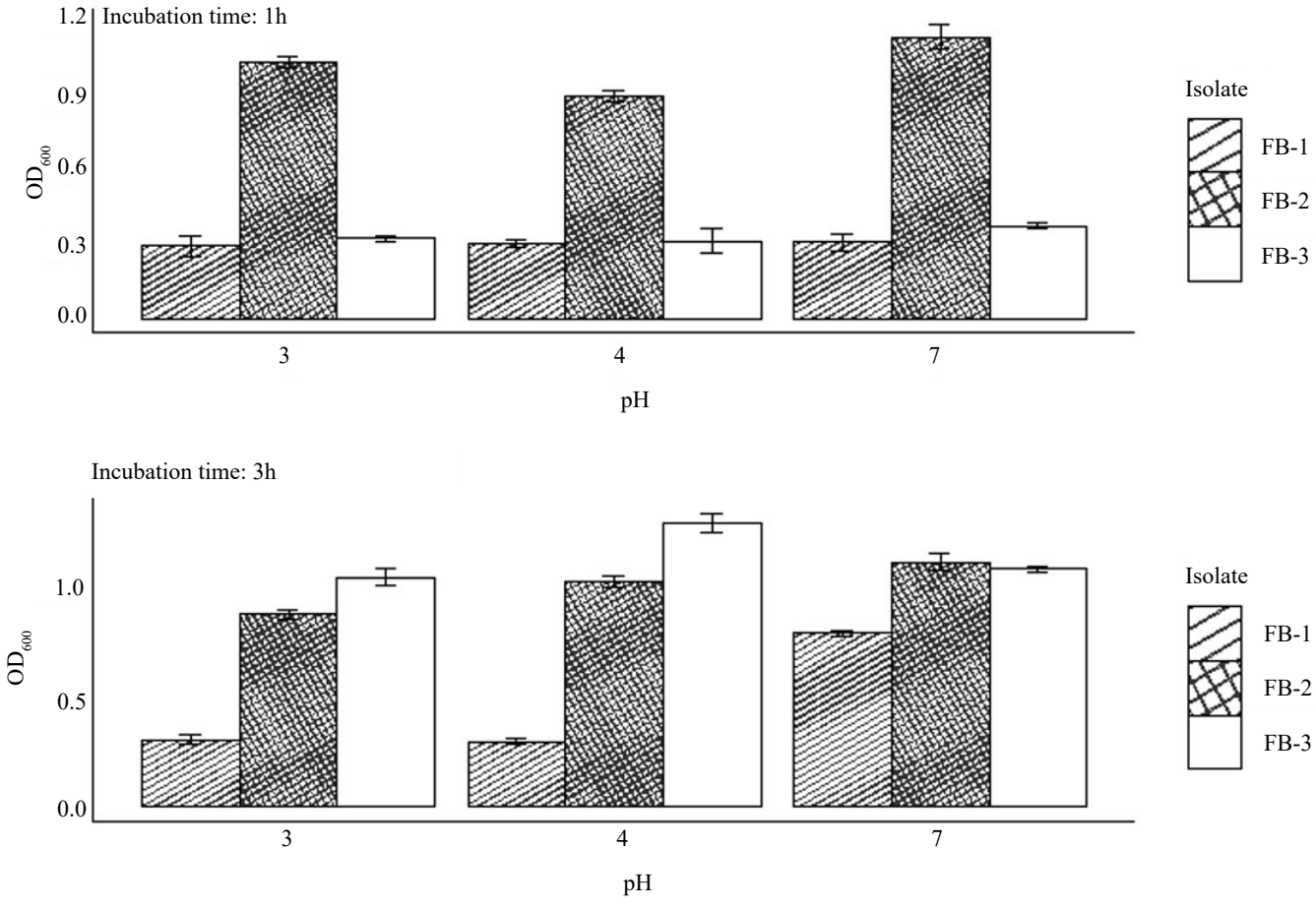

Figure 2. pH tolerance test of isolated bacteria at different incubation hour, with error bars indicating standard deviation (SD)

breakdown (clear or green zone). The observed results were given below in Table 3 .

3.3.5. Antibiotic Susceptibility

Our analysis showed that three isolates exhibited a wide range of susceptibility. FB-1 was highly susceptible to ciprofloxacin and intermediate for amoxicillin, while FB-3 was resistance to doxycycline and amoxicillin, intermediately susceptible to tetracycline and highly susceptible to ciprofloxacin. FB-2 was highly susceptible to all except for amoxicillin. Antibiotics susceptibilities of different isolates and their zone of inhibition are given below in Table 4.

3.4. Enzyme Activity Assay

Three isolates coded by FB-1, FB-2 and FB-3 were tested to produce crude cellulase enzyme. Selected media were prepared, and enzyme produced in shaker incubator. From the observed OD after enzyme production (Figure 4), FB-2 isolate was found to be the highest.

The DNS method is used to identify the reducing sugars that are released when hydrolase enzymes break down carbohydrates. Enzyme produced in 30°C and 72 h incubation was purified through ammonium sulphate precipitation and activity was measured using CMC through DNS method. Before that, a standard curve of glucose was constructed using the DNS method by measuring known concentrations of glucose (Figure 5). Based on the standard curve, FB-2 released the maximum amount of glucose equivalent, measuring 1.27 mg. This was followed by FB-3, which released 1.19 mg, and FB-1, which released 1.17 mg. The cellulase activity was assayed in carboxymethyl cellulose (CMC) solution by DNS method by utilizing data from Figures 4 and 5. The isolate FB-2 demonstrated the most significant cellulase activity, measuring 1.56 units/ml. It was closely followed by FB-3, which displayed a cellulase activity of 1.46 units/ml, and FB-1, which had cellulase activity of 1.44 units/ml.

3.5. Molecular Identification

Colony of the best cellulase producing isolate (FB-2) were identified down to genus through MALDI-TOF analysis. Different genera were identified such as FB-2 as *Bacillus* with 99.45% identification. The genomic DNA of the chosen isolate FB-2 was amplified by PCR and observed using 1% agarose gel electrophoresis under UV light. The contig region of the 16S rRNA gene from the isolate was 945 base pairs long. By utilising the BLAST tool (http://www.ncbi.nlm.nih.gov) to compare these contig areas with entries in the NCBI GenBank, it was determined that the FB-2 isolate has a maximum similarity of 99.46% with the sequences of *Bacillus cereus* strain (NR_114582.1) (Figure 6).

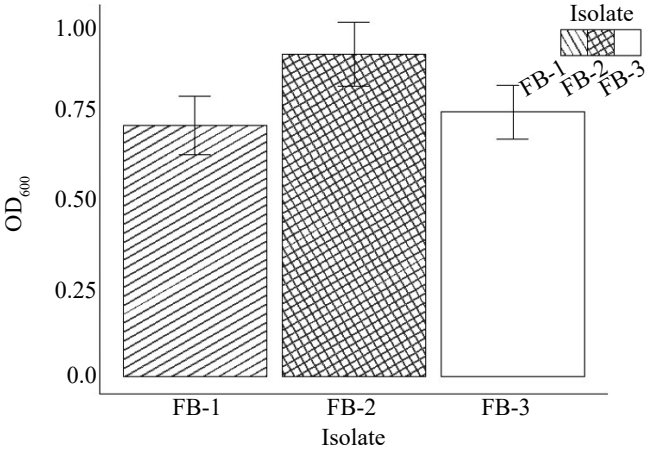

Figure 4. Optical density after enzyme production by the isolates for cellulase activity assay, with error bars indicating standard deviation (SD)

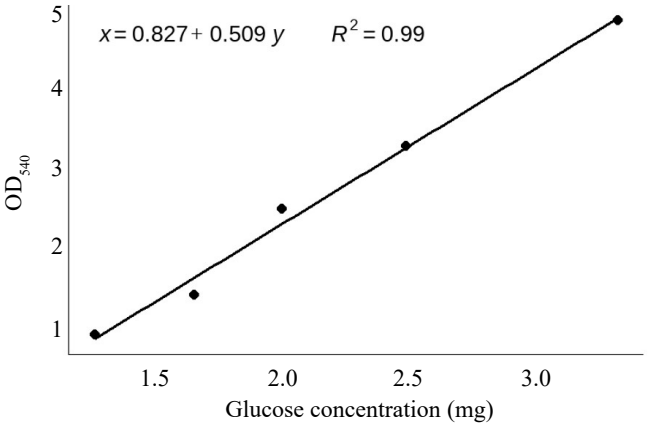

Figure 5. Standard curve of glucose

Table 3. Haemolytic activity of different isolates

Isolates code	Haemolytic activity
FB-1	γ
FB-2	γ
FB-3	γ

Table 4. Antibiotics susceptibilities and inhibition zone for different isolates

Isolates code	DO	TE	AX	CIP
	Zone of inhibition (mm)/antibiotics susceptibilities			
FB-1	14/R	18/R	23/I	35/S
FB-2	19/S	32/S	13/R	29/S
FB-3	9/R	25/I	17/R	21/S

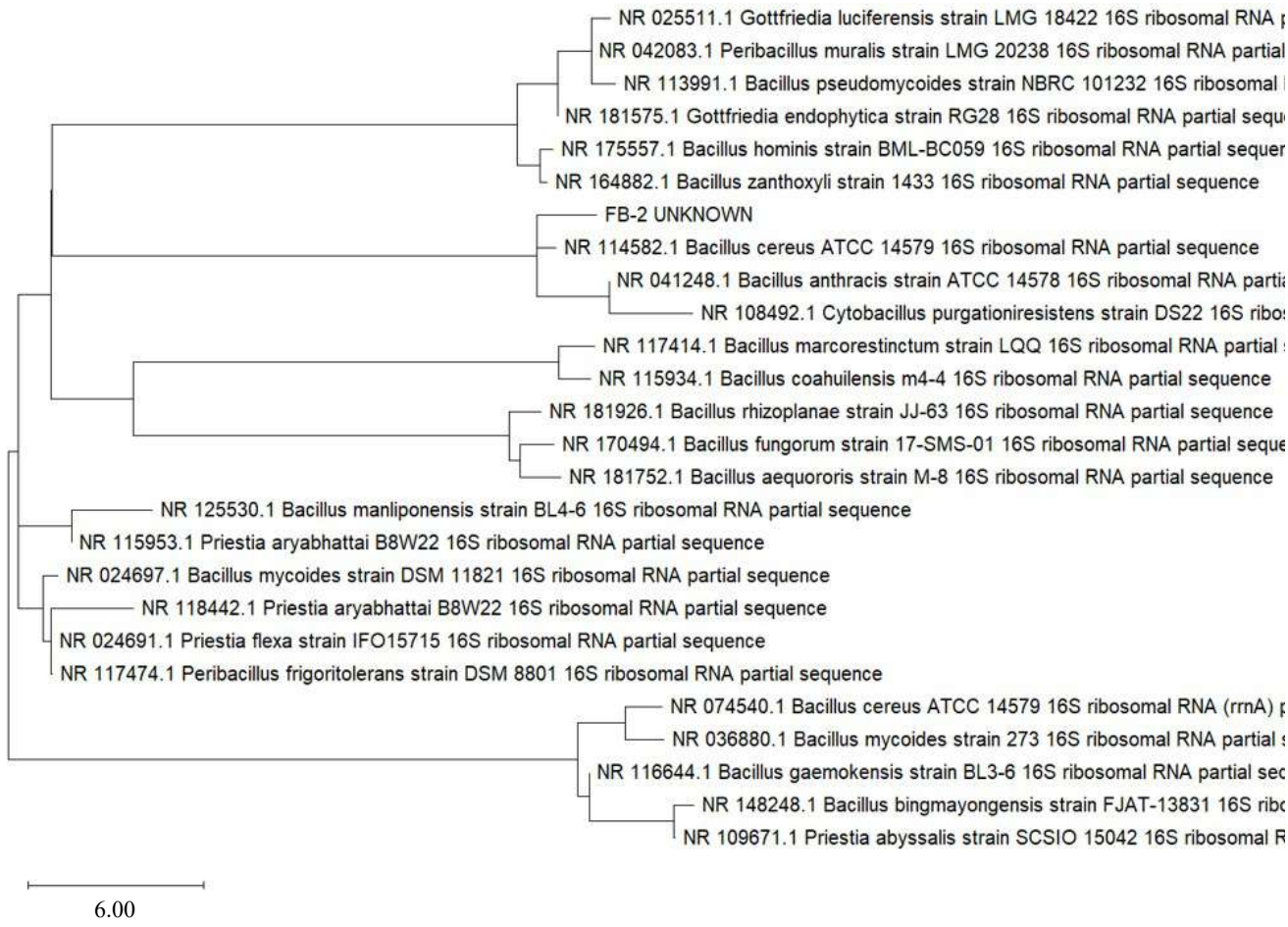

Figure 6. Phylogenetic tree of FB-2 (*Bacillus cereus*) after gene sequencing

4. Discussion

Fermentation is a process that involves the growth and development of a significant number of microbes to produce a desired product (Ramasubramanian *et al.* 2024). Furthermore, fermented bamboo products consist of several types of bacteria such as *Lactobacillus* sp., *Enterococcus* sp., *Bacillus* sp., *Saccharomyces* sp., and others (Behera and Balaji 2021). The bacteria involved in the fermentation process have been linked to numerous health advantages as probiotics, making them the subject of considerable interest (Şanlier *et al.* 2019). Furthermore, microbial enzymes possess the potential to serve as a sustainable resource for industrial processes. So, this study highlighted the identification cellulose-degrading microorganisms in the fermented bamboo and assessed its biochemical and probiotic properties and enzyme production capacity to characterize the bacteria and find the potential ways of application.

In this current work, we initially evaluated the organisms by examining their shape and structure. During the process of examining the isolates for cellulase synthesis, three distinct strains exhibited positive outcomes on the Congo Red plate. The extent of hydrolysis caused by each microorganism was determined and displayed in Table 1. This proves that these bacterial strains could be employed for cellulose degradation. The isolated strain FB-1, FB-2 and FB-3 were identified by morphological characteristics, biochemical tests and molecular identification. Of the three species, FB-2 was determined to be the most effective cellulase producing probiotic bacteria among the isolates. MALDI-TOF assay, DNA sequencing and phylogenetic analysis was used to identify this best isolate as *Bacillus cereus*.

In their study, Karthika *et al.* (2020) observed that out of the nine strains tested, *Bacillus cereus* exhibited a zone clearance value of 5 mm. In contrast, other species of *Bacillus* demonstrated hydrolysed zones ranging from 5 to 10 mm. Gupta *et al.* (2012) conducted a comparable qualitative enzyme investigation. The cellulase activity can be initially quantified by calculating the ratio of the zone diameter to the colony diameter. An effective

cellulase producer should have a ratio of at least 1.5. In a prior study, it was determined that the ratio *B. subtilis* ranged from 1.67 to 3 (Vimal *et al.* 2016). We observed a comparable ratio (1.5-2.4) for the isolates in our study. Nevertheless, the ability to undergo hydrolysis is greater in TSB, NB, and gelatine media, with values ranging from 3.8 to 5.4 for the bacterial samples. This is attributed to alterations in the nutritional composition of the medium (Pandey *et al.* 2019).

Various studies were conducted to determine the morphological and biochemical characteristics of cellulolytic bacteria. All three isolates demonstrated positive results in the fermentation of glucose and sucrose, suggesting that these isolates could be helpful in breaking down carbohydrates. Their biochemical test results have been verified by previously published works on *Bacillus* sp. and were mostly determined to be identical (Table 5).

To harness the advantageous qualities of probiotic microorganisms, it is crucial to examine them. Probiotics must meet certain criteria to be considered safe for both humans and animals. These criteria include the absence of virulence or antibiotic resistance genes, the ability to colonise and replicate within the host, the ability to reach the specific location to act, and the ability to work in actual host (Kesarcodi-Watson *et al.* 2008).

The genus *Bacillus* comprises Gram-positive bacteria that can generate spores and may survive in aerobic or facultative aerobic conditions. Some representatives of this genus, such as *B. subtilis, B. coagulans,* and *B. cereus*, are reported to have probiotic qualities. *Bacillus cereus*, a probiotic bacterium that can break down cellulose, is commonly found in various habitats such as sediment, dust, water, vegetation, and various types of food, including dairy products, meat, and both raw and processed foods and vegetables (Güven and Mutlu 2009). Although certain *B. cereus* strains are human-lethal and highly toxic, many other strains have probiotic properties (Trapecar *et al.* 2011; Zhu *et al.* 2016; Kumar *et al.* 2022).

For probiotics to successfully survive and establish themselves in the gut to provide positive effects, they must be able to tolerate the low pH of the stomach and the high concentration of bile in the intestines (Kuebutornye *et al.* 2020). Prior to choosing a probiotic bacterium for consumption by humans and other animals, it is necessary for it to be able to tolerate a bile concentration of 0.3% (Gilliland *et al.* 1984). According to the findings, all the isolates in this study exhibited resistance and were capable of thriving in a bile salt content of 0.3%. Contrary to other types of probiotics, *Bacillus* species generate spores that exhibit greater resistance to low pH levels and high concentrations of bile. Study showed that spores of *B. subtilis* exhibited significant resistance to both simulated gastric juice and simulated intestinal juice containing 2% bile salt (Barbosa *et al.* 2005). The results in another study indicated that *B. cereus* strain exhibited a high level of tolerance to bile, with 87.91% viability maintained after 3 hrs of incubation in 0.3% (w/v) bile concentration (Dabiré *et al.* 2022a). In a separate investigation, both the *Bacillus* sp. and *B. cereus* strains showed resilience to acidic conditions and a bile concentration of 0.5%

Table 5. Comparison table of biochemical properties of FB-2 (*B. cereus*) with previous literatures on *Bacillus* sp.

Biochemical test	FB-2 (*B. cereus*)	Amenyogbe *et al.* (2021)	Kuebutornye *et al.* (2020)	Kavitha *et al.* (2018)	Rasool *et al.* (2017)	Croos *et al.* (2019)	Islam and Roy (2018)	Islam and Roy (2019)
Morphology	Rod		Circular/	Rod			Rod	Rod
Colony colour	White		Irregular				White	
Gram's	+	+	White	+	+	+	+	
Oxidase	+		+	+	-			
Catalase	+	+		+	+	+	+	+
MR test	-		+	+			-	-
VP test	+	+	-	-	+		+	+
Gelatinase	-	-	-					
Starch hydrolysis	+				+			
Citrate agar	+	+	+	+	+		+	+
Sucrose fermentation	+		+	+			+	+
Dextrose fermentation	-			+				
Starch fermentation	+	+						
Glucose fermentation	+		+	+			+	+
H2S test	-		+	-			-	-
Motility test	+			+	+	+	+	+
Urase test	+	-		-		+		
Indole test	-		+	-				

(Amenyogbe *et al.* 2021). Since, to reach the small intestine they have to pass through from the stressful conditions of stomach where pH can be as low as 1.0 (Chou and Weimer 1999; Çakir *et al.* 2003). Our analysis found that the *B. cereus* exhibited a moderate level of resistance to acidic conditions up to a pH of 3. In a separate study, researchers found that isolates of *B. velezensis, B. subtilis*, and *B. amyloliquefaciens* shown the potential to undergo sporulation and resist pH levels as low as 1 (Kuebutornye *et al.* 2020). After 3 hrs of incubation at pH 2, *B. cereus* was determined to be the most acid-tolerant strain (Dabiré *et al.* 2022a).

Auto aggregation is a criterion used to assess the ability of probiotic strains to bind to epithelial cells and mucosal surfaces. Self-aggregation can also influence the ability of a bacteria to colonise the gastrointestinal tract ecosystem, giving it a competitive edge (Kos *et al.* 2003; Nikoskelainen *et al.* 2003). Previous research findings stated that *B. cereus* strain exhibited the highest auto aggregation ability of 34.49% (Kuang *et al.* 2024). Additional research has shown that the coaggregation capabilities of *Bacillus* strains can range from 9 to 48% (Samson *et al.* 2020). The results we obtained indicate that FB-2 (*B. cereus*) exhibits a high potential as a probiotic bacterium, displaying the most effective aggregation property of 30.03±2.89% among the isolates. Patel *et al.* (2009) also reported that *B. subtilis* showed best auto aggregation followed by *Bacillus megaterium* and *B. thuringiensis* exhibiting less auto aggregation and adhesion. *B. cereus* strains can survive longer in GI tract than *B. clausii* and *B. subtilis* because *B. cereus* spores can efficiently adhere to human epithelial cells owing to their hydrophobicity (Lee *et al.* 2019).

The γ-hemolytic isolates (FB-1, FB-2, and FB-3) examined in the present research did not exhibit any activity that caused the lysis of red blood cells on the blood agar. This safety measure is vital during the characterisation process, since haemolytic bacteria have the potential to degrade the epithelial layer of host cells and disrupt the functioning of the defence system (A. Nurhidayu 2012). Neither γ-haemolysis nor α-haemolysis are believed to provide any risk, while β-haemolysis is thought to be hazardous (Kuebutornye *et al.* 2020). A study found that the bacterial strains *B. subtilis, B. cereus*, and *B. amyloliquefaciens* did not exhibit any haemolytic activity (γ-haemolysis) when exposed to human blood on a blood agar medium (Kavitha *et al.* 2018). Lee *et al.* (2017) has made similar observations for *Bacillus* strains. In an experiment conducted by Dabiré *et al.* (2022a), six *Bacillus* strains were shown to exhibit no haemolysis when cultured on sheep blood agar plates. Another study also reported similar outcomes for two strains of probiotic *B. cereus* (Nwagu *et al.* 2020).

An antibiotic susceptibility experiment was conducted in the study to assess the appropriateness of these bacterial isolates for future probiotic production. Result demonstrated that the most effective isolate in the present investigation FB-2 was highly susceptible to all except for amoxicillin which indicated that the isolate was susceptible to a sufficient number (75%) of antibiotics tested. In a study, *B. cereus* was susceptible to 11, intermediate susceptible to 5 and resistant to 6 antibiotics among 22 (Amenyogbe *et al.* 2021). Another study revealed that *B. velezensis, B. subtilis* and *B. amyloliquefaciens* were highly susceptible to 15 out of 17 antibiotics tested (Kuebutornye *et al.* 2020).

The assay for cellulase enzyme was performed by the ability of individual isolates to hydrolyse cellulose into reducing sugars (Karthika *et al.* 2020). The data from a research indicated that the isolate *B.cereus* is a more efficient cellulase producer when compared to *B. subtilis*. The 4.0% inoculums volume resulted in maximum cellulase activity of 4.12 units/ml (Vimal *et al.* 2016). Microbial productions of cellulase enzymes - exoglucanase, endoglucanase and bglucosidase of *B. cereus* were 3.52±0.002, 0.34±0.002, and 1.99±0.002 unit/ml respectively (Karthika *et al.* 2020). In our findings, cellulase activity of native isolate *B. cereus* was 1.56 units/ml which align with the above results. The optimization of media and conditions is needed to enhance enzyme production capacity. The microbial enzyme from FB-2 (*B. cereus*) can be a potential substitute for commercial enzymes in multiple industries.

In conclusion, the cellulase degrading bacteria isolated from fermented bamboo, a traditional food in the hill tracts of Bangladesh displays promising biochemical and probiotic properties. According to the data, *Bacillus cereus* is the most suitable isolate and has the potential to be used as probiotic-starter cultures for the development and promotion of products that can have a positive effect on human and animal health. Nevertheless, the optimal utilisation of these methods will necessitate additional in vivo investigations. Furthermore, its capacity to generate cellulase enzyme has the potential to broaden its scope of applications.

Conflicts of Interest

The authors declare that there is no conflict of interest in this research.

Acknowledgements

This study was funded and supported by Program Based Research Grant (PBRG), National Agricultural Technology Program-Phase II Project, Bangladesh Agricultural Research Council (Sub-Project ID- 155).

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