Isolation and Characterization of Cellulase-Producing Bacteria from Lake Chitu Soil Sediment for their Potential Application in Agriculture and Other Industries Oromia Regional State, Ethiopia

Nebeyu Tefera Kebede

A Thesis Paper Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Masters in Applied Biology (Applied Microbiology)

Office of Graduate Studies

Adama Science and Technology University

Ethiopia, Adama

May,2024

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DECLARATION

I declare that this Master Thesis paper named **Isolation and Characterization of Cellulase-Producing Bacteria from Lake Chitu Soil Sediment for their Potential Application in Agriculture and Other Industries Oromia Regional State, Ethiopia** is my original work and has not been submitted to any university for the same purpose. The references utilized in this proposal are fully recognized via suitable citations.

Nebeyu Tefera

Name of student	Signature	Date

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The major advisor /supervisor of this research thesis, here by certify that I closely advised/supervised the student while developing and analyzing this research thesis and read the thesis which is entitled "Isolation and Characterization of Cellulase-Producing Bacteria from Lake Chitu Soil Sediment for their Potential Application in Agriculture and Other Industries Oromia Regional State, Ethiopia" prepared by under my guidance. There for I recommend of the thesis to the department for further review and evaluation.

Major Advisor: Seid Mohammed (PHD)

Signature: _____

APPROVAL PAGE

We the advisors of the thesis entitled: **Isolation and Characterization of Cellulase-Producing Bacteria from Lake Chitu Soil Sediment for their Potential Application in Agriculture and Other Industries Oromia Regional State, Ethiopia** and developed by Nebeyu Tefera here by certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

_____	_____	_____
Major Advisor	Signature	Date
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Co-advisor	Signature	Date

APPROVAL OF BOARD OF REVIEWERS

We the undersigned members of the board of examiners of the thesis by Nebeyu Tefera have read and evaluated the thesis entitled ― Isolation and Characterization of Cellulase-Producing Bacteria from Lake Chitu Soil Sediment for their Potential Application in Agriculture and Other Industries Oromia Regional State, Ethiopia examined the candidate during open defense. This is therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Masters of Science in Microbiology.

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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Post Graduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

Department Head	Signature	Date
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Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

MALDITOF-MS	Matrix-Assisted Laser Desorption-Ionization-Time of Flight Mass Spectrometry
(SIM) medium	Sulfur, Indole, Motility
16SRNA	16S Ribonucleic acid
AGU	Anhydro-d-glucopyranose unit
ASTU	Adama Science and Technology University
CMC	Carboxymethyl cellulose
CPBILCS	Cellulose producing bacterial isolate from lake Chitu sediment
MEGA 11	Molecular Evolutionary Genetics
MR	Methyl Red
MR-VP	Methyl Red -Voges Proskauer
pH	Potential of Hydrogen
RNA	Ribonucleic acid
TSI	Triple sugar iron
VP	Voges-Proskauer

ABSTRACT

Cellulases are enzymes naturally produced by many organisms and used for cellulose hydrolysis. They are well-known for their widely employed industrial application. The recent study was aimed at isolating and screening cellulase producing bacteria from soil samples. Soil sediments were collected from Lake Chitu and transported into Applied Microbiology laboratory.Astu Cellulase producing bacteria were isolated and screened from soil sediments. Various biochemical tests, Protein profile analysis, and molecular characterization were conducted for the identification of these cellulase producing bacteria. Data analyses were conducted using Micro Soft excel, and Origin 2019b software. The sequence analyses were conducted using MEGA 11 version. In this study, a total of 10 cellulase producing bacteria isolates out of 45 isolates were obtained from soil sediments of Lake Chitu. From these isolates, better cellulase producers were identified as Bacillus cereus CPBILCS-9, Bacillus licheniformis CPBILCS-14, Proteus vulgaris CPBILCS-4, and Pseudomonas sp. CPBILCS-13 using matrix assistance lesser desorption time of flight mass spectrometer (MALDI TOF MS). One of the identified cellulase producing bacterial isolates was distinguished as Bacillus tequilensis CPBILCS-15 using 16Sr RNA gene sequence. In this study, it was also noticed that carbon source like glucose, lactose, and sucrose were employed for the growth of Bacillus tequilensis CPBILCS-15 at suitable temperature (37^{o}C) and pH7.5. In conclusion, potential cellulose producing bacterial isolates can be screened from their natural ecosystems and employed for the production of cellulase which used for various industrial applications.

Key words: Bacteria, Cellulase, Isolates, & Producing

1. INTRODUCTION

1.1. Background of the study

Enzymes are highly efficient biological catalysts studied for their industrial scale catalysis because of their numerous advantages (Bhat & Bhat, 1997). They can be found in practically all naturally existing organisms, from microorganisms to plants and animals. Due to their presence in very small quantity in plants and animals, they cannot be exploited for industrial applications (Singh et al., 2019). In contrast, the production of enzymes from microorganisms has several advantages, such as easy handling, rapid replication under controlled conditions, easy genetic manipulation, high production yield, and so forth (Bharathi & Rajalakshmi, 2019).

Cellulose is the most prevalent biological compound in both terrestrial and aquatic ecosystems (Demirbas, 2008). Since cellulose is the major common waste product from the agriculture sector, there has been a lot of interest in using it as feed and an energy source. It is made up of D-glucose units connected by ß-1, 4-glycosidic linkages to form a linear chain. Moreover, the most prevalent organic substance in the world is cellulose. It is commonly known that the most prevalent source of renewable carbon and energy on Earth. In essence, cellulose makes up the structural element of green plants, algae's primary cell walls (Aktar et al., 2016).It makes up the majority of plant biomass. Each year, 4×10^9 tons of cellulose are produced by plants. It is also regarded as one of the planet's most significant sources of carbon, with both marine and terrestrial plants biosynthesizing it annually at a rate of 1.5 trillion tons (Ohkuma, 2003)

Cellulases are produced by many microorganisms include bacteria, fungi, yeasts and ctinomyces (Savalia & Dungrechiya, 2022). Among these, microbial cellulases, particularly those produced by bacteria, have significant benefits over those produced by plants and mammals due to several important properties. Microbial cellulase are high in demand due to their specificity of reaction, low energy consumption, hydrolytic and synthetic activities, high yield, and ease of genetic manipulation.

Cellulases from various sources have distinctive features as they exhibit optimum pH and temperature, solubility depending on the amino acid composition (Aktar et al., 2016). In recent years, researchers have been paying attention to various bacteria that produce cellulases because of their high growth rate and resistance to an extreme environment when compared to fungi and

has a good potential in cellulase production. Bacteria belonging to the genera *Clostridium*, *Cellulomonas*, *Bacillus*, *Ruminococcus*, *Bacteroides*, *Acetovibrio*, *Streptomyces*, and *Paenibacillus* have been found to produce different types of cellulase when incubated under aerobic or anaerobic conditions (Liang et al., 2014).

Numerous microorganisms that can degrade cellulose have been isolated, screened, and identified. However, many studies have put more emphasis on cellulose degrading bacteria from agricultural, industrial, and municipal wastes because the cellulases that they produced are easy to extract, and some of the bacterial cellulases have been used as commercial cellulase (Ajeje et al., 2021).

The major industrial applications of cellulases are in textile industry for bio-polishing of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness. Besides, they are used in animal feeds for improving nutritional quality and digestibility, in processing of fruit juices, in baking etc. Utilisation in deinking of paper is yet another emerging application (Tolan and Foody, 1999). The cellulases that are used so far for the above-mentioned industrial applications are those from fungal sources (Tolan & Foody, 1999).

1.2. Statements of the problem

Enzymes are now being used in a growing number of industrial operations. Although enzymes are found in all living organisms, most industrial enzymes currently in use are obtained from microorganisms. Ethiopia has a lot of potential when it comes to discovering new enzymes that might be very useful in many industrial operations. The East African region has a distinct microbial biodiversity that could provide new enzymes for industrial applications. Furthermore, because of the availability of extremely unique habitats, such as alkaline environments, hot springs, etc. Ethiopia is working to boost sectors such as textiles, leather, detergents, pulp, and paper. Enzymes like amylase, lipase, and protease are required as an input in these industries. Various sectors in the region currently import large amounts of enzymes for usage in the leather tanning, textile, and brewing industries. Other industries hesitate to import enzymes due to high cost. For example, in Ethiopia more than 150,000 kg of bating enzyme is imported annually with a cost of US$ 900,000 -1,000,000 (Scarlat et al., 2015). This is a severe economic burden that is having a negative impact in the country's economy. Environmental effect is another impact which is posed by importing those synthetic enzymes.

1.3. Objectives of the study

1.3.1. General objective

- To isolate and characterize cellulase producing bacteria from soil sediment of lake chitu.

1.3.2. Specific objectives

- To isolate cellulase producing bacteria from soil sediment.
- To screen potential cellulase producing bacterial isolates from isolated bacterial strains.
- To characterize potential cellulase producing isolates using microscopic and biochemical tests.
- To determine the culture condition (temperature, pH, carbon source and nitrogen sources) for cellulase producing bacteria.
- To identify the isolates using MALDI-TOF-MS and 16sRNA.

2. LITERATURE REVIEW

2.1. Cellulase Enzyme

The breakdown of cellulose into accessible sugars is catalyzed by cellulases. The term "cellulases" refers to a class of enzymes called "glycosyl hydrolases," which includes endoglucanase, exoglycanase, and glucosidase. Despite having different ways of breaking down cellulose, these enzymes must work together to fully hydrolyze cellulosic biomass enzymatically (Yang et al., 2014)

The hydrolysis of cellulose is catalyzed by a class of enzymes called "cellulase" (endo-1,4-β-glucanase), which is mainly produced by bacteria, protozoa, and fungi. Numerous industries, such as pulp and paper, textile, bioethanol, wine and brewing, food processing, animal feed, and agriculture, use cellulases extensively (Kuhad et al., 2011). Cellulase completely breaks down cellulose into glucose units. These units can then be fermented further to produce bioethanol or smaller polysaccharides. Considering the commercial significance of cellulase, it is imperative to discover a feasible way to increase its activity.

2.2. Sources of Cellulose

Thousands of monosaccharide units of glucose monomers make up the polysaccharide known as cellulose. The primary structural element of plants is cellulose. The vital substance that provides carbon to living organisms is cellulose. In the natural world, it is a biopolymer (Dixit et al., 2011). cellulose is a component of the cellular structure of many natural fibers, including cotton and higher plants. Except for the terminal end, every cellulose molecule has three hydroxyl groups per anhydro-d-glucopyranose unit (AGU) in its lengthy chains. The cellulose is the excipient. Plant polysaccharides are being thoroughly researched for use in pharmaceutical applications because they meet many of the standards expected of pharmaceutical excipients, including non-toxicity, stability, availability, and renewability (Lavanya et al., 2011)

2.3. Types of cellulase enzymes and their function

Cellulase enzymes can be of three primary types:1. Endoglucanases: these enzymes decompart cellulose molecules into smaller fragments by breaking down their internal bonds.2. Exoglucanases: at the ends of the cellulose chains, these enzymes cleave off the glucose units. 3.

Cellobiohydrolases: these enzymes function similarly to exoglucanases, but they eliminate pairs of glucose units as opposed to single units(Sammond et al., 2012).

2.3.1. Endoglucanase

Also known as endo-1,4-beta-glucanase or cellulase, is an enzyme that catalyzes the hydrolysis of the internal cites of a cellulose chain and subsequently produce oligosaccharides of various lengths. Cellulose is a polysaccharide that is found in plant cell walls, and endoglucanase plays a critical role in the breakdown of cellulose into smaller sugar molecules. This enzyme is produced by various microorganisms, such as bacteria and fungi, and is also used in various industrial applications, including biofuel production, textile processing, and food and feed industries (Mandel's & Reese, 1957). Endoglucanases can randomly hydrolyze the glycosidic bonds of cellulose, whereas exoglucanases are known to break the cellulose chain from their reducing or non-reducing ends.(Rahman et al., 2018).

2.3.2. Exoglucanases

Enzymes called exo-β-1,4-glucanases or cellobiohydrolases act processively on either the reducing or the nonreducing ends of a cellulose chain to liberate cellobiose as the major product are responsible for breaking down cellulose, a significant constituent of plant cell walls. These enzymes specifically cleave the β-1,4-glycosidic bonds in cellulose chains, resulting in the release of cellobiose, a disaccharide made up of two glucose units. Exoglucanases are widely employed in commercial processes like bioremediation and biofuel synthesis because they are essential to the breakdown of cellulose (Fraga Vidal et al., 2021).

2.3.3.*β*-glucosidase

Act on oligosaccharide to form glucose.

Table 1: Application of Cellulases in Various Industries

2.4.1. The Paper & Pulp Sector

Due to their use in biomechanical pulping processes, cellulases have become increasingly popular in the paper and pulp industry. This has led to better paper quality with less energy usage. The paper and pulp sector might profit from cellulases in a variety of ways that would make their

operations more sustainable and efficient. Biodegradable paper products, enzymatic deinking, and biomodification of fiber properties are all part of this category. The importance of ongoing research and development in cellulase technology for the success of the industry (Imran et al., 2019).

2.4.2. Bioethanol Industry

The use of cellulases to convert lignocellulosic materials like sugarcane bagasse, corncob, rice straw, *Prosopis juliflora*, *Lantana camara*, switchgrass, sawdust, and forest residues into biofuel has garnered significant attention in current research. This process involves several steps, including mechanical, chemical, or biological pretreatment, hydrolysis of polymers to produce easily metabolized sugars, and the subsequent separation and purification of the desired products. Enzymatic hydrolysis is favored over acid or alkaline hydrolysis due to its operation under mild conditions (pH 4-6 and 45–50°C) and its lack of corrosion issues (Fraga et al., 2021)

2.4.3. Wine and Brewery Industry

Cellulases and related enzymes have significant applications in the wine and brewery industry, improving fermentation processes and enhancing the quality and yields of alcoholic beverages. Glucanases are added to hydrolyze glucan, reduce viscosity, and improve filterability, while pectinases, glucanases, and hemicellulases improve color extraction, clarification, and filtration. Commercial enzyme preparations have resulted in better maceration, improved color extraction, easy clarification, filtration, enhanced wine quality, and improved stability (Sukumaran et al., 2005)

2.4.4. Food Processing Industry

Cellulases have a wide range of potential applications in the food processing industry, particularly in the extraction, clarification, and stabilization of fruit and vegetable juices. They are part of a complex of macerating enzymes used to improve juice yield and process performance without additional investment. These enzymes are beneficial for reducing the viscosity of nectars and purees from tropical fruits and increasing the stability and texture of the resulting products. Additionally, the addition of enzymes like pectinases and β-glucosidases to

citrus fruits can enhance their texture, flavor, and aroma (L. M. J. de Carvalho et al., 2008) membrane technology. The use of macerating enzymes, including cellulases, xylanases, and pectinases, has been shown to improve the extraction and clarification of fruit and vegetable juices (Baker & Wicker, 1995)

2.4.5. Animal Feed Industry

Cellulases have been found to have potential applications in the animal feed industry. (Dhiman, et al., 2002). They can improve the nutritional value of grain feed and agricultural silage by breaking down specific feed ingredients, removing antinutritional elements, and producing additional di gestive enzymes like proteases, amylases, and glucanases. For instance, dietary fiber, which can act as an anti-nutritional factor for animals, is composed of non-starch polysaccharides like cellulose, arabinoxylans, and other plant components like waxes, chitins, pectins, lignin, resistant dextrins, and oligosaccharides. These enzymes can break down these components and improve the nutritional value of the feed (Ali et al., 1995)

Table 1:Bacteria from Lake Chitu soil sediment for their Potential Application in Agriculture and Other industries.

Industry	Applications
Agriculture	Plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence on mineral fertilizers
Bioconversion	Conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein, and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high-quality fodder
Detergents	Cellulase-based detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; ant redeposition of ink particles

Fermentation	Improved malting and mashing; improved pressing and color extraction of grapes; improved aroma of wines; improved primary fermentation and quality of beer; improved viscosity and filterability of wort; improved must clarification in wine production; improved filtration rate and wine stability
Food	Release of the antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruit juices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture, flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits
Pulp and Paper	Co additive in pulp bleaching; biomechanical pulping; improved draining; enzymatic deinking; reduced energy requirement; reduced chlorine requirement; improved fiber brightness, strength properties, and pulp freeness and cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, and sanitary paper
Textile	Biostoning of jeans; biopolishing of textile fibers; improved fabrics quality; improved absorbance property of fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics; restoration of colour brightness
Others	Improved carotenoids extraction; improved oxidation and colour stability of carotenoids; improved olive oil extraction; improved malaxation of olive paste; improved quality of olive oil; reduced risk of biomass waste; production of hybrid molecules; production of designer cellulosomes

3. MATERIALS AND METHOD

3.1. Description of the study area

Soil sediments were taken from Lake Chitu (the Great Lake), which is located in a tropical soda lake located some 287 km south of Addis Ababa at a geographical position of $7^{o}24'$ N $38^{o}25'$E and altitude of 1600 m above sea level. It was situated at about 1 km southwest of Lake Shala in the Abijata-Shala Lakes National Park of the Ethiopian Rift Vally. The research was carried out at Adama Science and Technology University, which is situated in Adama City.

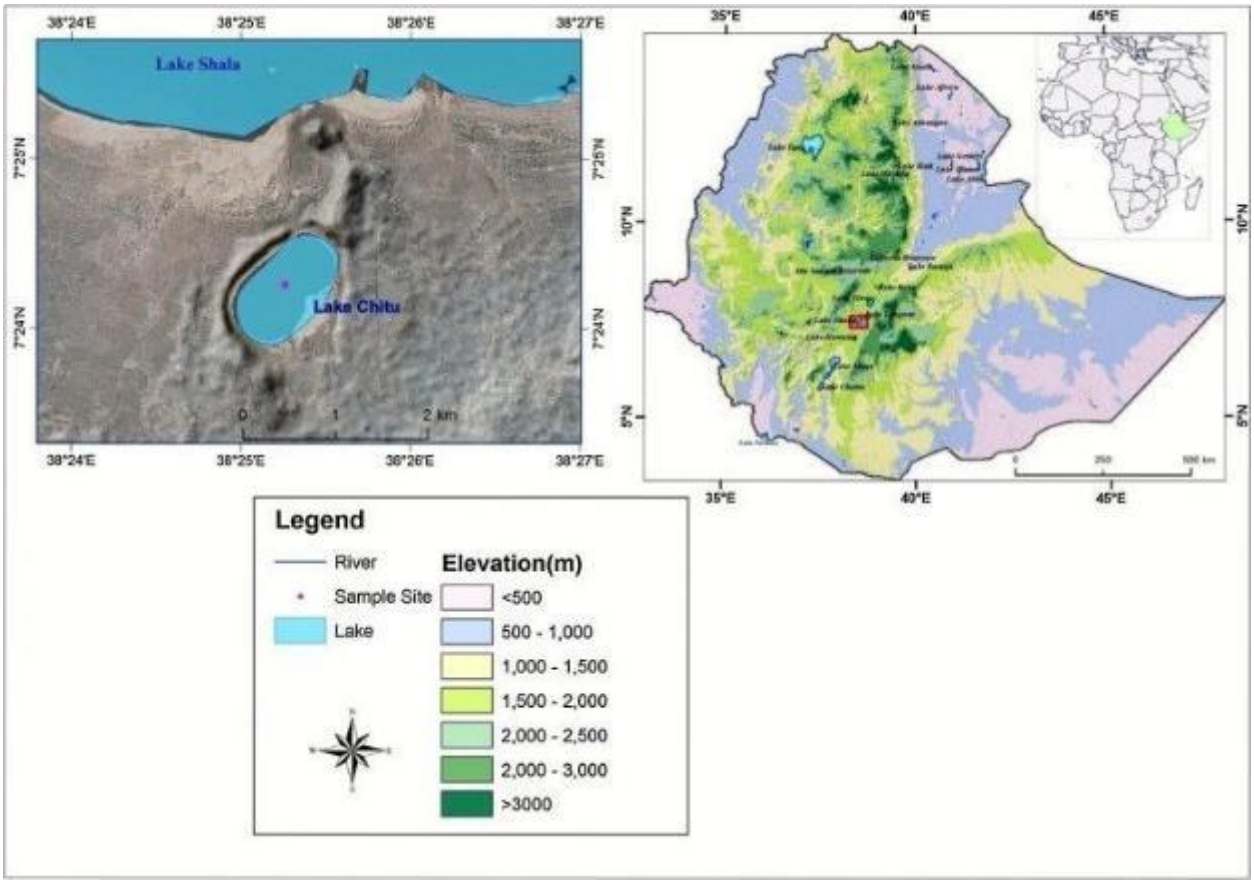

Figure 1: Location Map (Nebeyu,2024)

3.2. Sample collection

A total of 10 soil sediment samples were collected from various places (from 5 points, each of them was 50 g) using a plastic bag. These soil sediment samples were collected from Lake Chitu. The collected samples were labelled with the collection date and sample number and transported

to the ASTU Applied Biology laboratory for further analysis. The samples were preserved until used for further analysis.

3.3. Isolation of cellulase producing bacterial isolates

A 1g of each of the three (large, medium, small) soil sediments from Lake Chitu were collected. A 10 ml of sterile saline water (0.85% NaCl) was used to suspend 1ml of water and the mixture was vortexed. A serial dilution was performed from 10^{-1} to 10^{6}. From the dilutions, a 0.1 ml sample was applied onto nutrient agar medium (Peptone, 5g/l; Beef extract, 3g/l; NaCl, 8 g/L; Agar, 15g/l and 6.8 ± 0.2 pH) and incubated at 37°C for 24 h (Malik & Naz, 2019). During inoculation, spread plate technique was employed. Pure cultures were prepared by taking a single colony and transfer into another fresh nutrient agar medium. These pure cultures were maintained on slant agar at 4^{o}C until used for further analysis.

3.4. Screening of cellulase producing potential isolates from collected three zones of soil sediment

Pure isolated bacterial cultures were screened for cellulase activity using CMS agar. The CMS agar medium pH 7 was prepared with Carboxymethyl cellulose (CMC) powder 1.2, Dipotassium phosphate (K_2HPO_4) 1.2, Magnesium sulfate.$7H_20$ ($MgSO_4$) 0.036, Ammonium sulfate $(NH_4)_2SO_4$ 0.3, Gelatin 0.24, and Agar 15g in distilled water. Then, the agar plates were incubated at 37°C for 24 h and stored at 4°C. After incubation, the plates were submerged with 0.1% (w/v) Congo-red solution for 15 min and discolored with 1 M NaCl solution. The degradation zone around the bacteria indicated that the strains could hydrolyze CMC. Enzyme activity of the isolated strains was confirmed by several types of assays like iodine solution, Congo-red, and filter-paper degradation assay. Those bacteria that formed clear zones on CMC plates were subject to filter-paper degrading activity assay.

Characterization of lipase producing isolates

3.5. Cauterization of lipase producing isolates

Identification and characterization of bacteria were carried out using microscopic characterization, biochemical tests, and proteomic identification (MALDI–TOF MS).

3.5.1. Microscopic identification

Gram staining methods were used to distinguish the isolated strains as Gram-positive or Gramnegative bacteria. The cells were heated to adhere to the slide and stained with crystal violet (1%, w/v) for 1 min before being mordant with an iodine solution for 1 min. The stained cells were then washed with ethanol. Gram-positive cells retain the stain, whereas Gram-negative cells do not. The preparation was counterstained with a contrasting red dye, safranin (1%, w/v), for 45 sec to contrast the two results. Gram-negative cells turned red and were easily seen. Finally, the slide was examined using a microscope with an oil emulsion objective of 100X magnification (Gram, 1884).

3.5.2. Biochemical characterization

Different biochemical tests were performed to identify the isolated bacteria strains and described as follows.

3.5.2.1. Indole test

This test was used to identify microbes that can break down tryptophan into indole. Sterilized tubes containing tryptophan broth (4 ml) were inoculated with a loopful of the isolates and incubated at 37^{o}C for 24-28 h. At the end of the incubation period, 0.5 ml of Kovac's reagent (w/v) was added. The presence/absence of a ring was used to judge as a positive/negative test for indole production ability (Dawodu & Akanbi, 2021).

3.5.2.2. Methyl Red Voges Proskauer test: The MR-VP test was performed as Bhagya et al. (2019) prescribed. Briefly, methyl Red and Voges Proskauer test (MR-VP) broth prepared in two sets was inoculated with a loopful of the bacterial isolates and incubated for 48 h at 30°C. Two or three drops of methyl red alcoholic solution were added to the first tube. A distinct red colour indicated a positive reaction to the MR test. In the second set of tubes, α-naphthol solution (5 percent solution in 70% ethyl alcohol) (v/v) was added and shaken gently for 15 min. The positive reaction of acetyl methyl carbinol production was indicated by the development of red colour. This showed a positive result for the VP test

3.5.2.3. Citrate utilization test: Bacterial isolates were inoculated on Simmons citrate agar medium, and slants were incubated at 37°C for 48 h. Discoloration of the medium from green to blue due to a pH change indicates a positive reaction. Citrate present in the Simmons medium was used as its carbon and energy source (Bhagya et al., 2019).

3.5.2.4. Catalase test

A drop of 3% hydrogen peroxide solution was added to the sterile slide containing a loopful of the organism. Foaming or bubble indicates a positive result (Dawodu & Akanbi, 2021).

3.5.2.5. Urease test: The bacterial isolates were inoculated to the urea agar plates and incubated for 24 to 48 h. The change of color of the medium to pink indicate positive test for Urease production (Bhagya et al., 2019).

3.5.2.6. Triple sugar iron (TSI) test: Slants of triple sugar iron medium were prepared in the test tubes by autoclaving. Using sterile technique; small amount of the experimental bacterium from 24 h old pure culture was inoculated into the tubes by means of a stab and streak inoculation method with an inoculating needle. The screw caps were not fully tightened, and the tubes were incubated for 24 h at 37°C. Fermentation is indicated by yellowing of the butt and the slant of Triple Sugar Iron (TSI) agar medium. If gas was formed during the fermentation, it is shown in the butt either by the formation of bubbles or cracking of the agar (Aditi et al., 2017)

3.5.2.7. Motility test: Semisolid nutrient agar medium tubes were prepared, and bacterial culture was stabbed into the center of tubes containing semi solid medium and motility was observed by its growth in medium (Bhagya et al., 2019).

3.5.2.8. Hydrogen sulfide producing test

Sulfide indole motility (SIM) medium was used to study hydrogen sulfide production. SIM agar stab was prepared; the stab was inoculated with bacterial isolates. After 21 inoculation of Desulfovibrio genus of bacteria, stabs were incubated at 30°C for 48 h. After 48 h of incubation, black coloration along the line of stab inoculation indicated H_2S production (Bhagya et al., 2019). Hence, it indicates the potential of the bacteria to reduce sulfur containing compounds.

3.6. MALDI–TOF MS

Identification of cellulase producing bacterial isolate using MALDI–TOF MS to assess all isolates. The samples were first inoculated into nutrient agar for 24 h at 37°C. Each colony was transferred to a microplate (96 MSP, Bruker®– Billerica, USA). The bacterial sediment was covered with a lysis solution (70% formic acid; Sigma–Aldrich®). Then a 1-L aliquot of matrix solution (alpha-ciano-4-hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma–Aldrich®) was added. The spectra of each sample were generated in a mass spectrometer (MALDI–TOF LT Microflex, Bruker®) equipped with a 337 nm nitrogen laser in a linear path, controlled by the Flex Control 3.3 (Bruker®) program. The spectra were collected in a mass range between 2000 and 20,000 m/s, and then were analyzed by the MALDI Biotyper 2.0 (Bruker®) program, using the standard configuration for bacteria identification, which compared the spectrum of the samples with the references in the database.

3.7. Determination of Growth requirment

3.7.1. Determination of suitable carbon source for bacterial isolate

Different carbon sources bagasse, glucose, mollasse, and sucurose were used. A 1% each these carbon sources were added into CMC containing fresh bacteria. The media was incubated at 37°C for 48 hours and optical density (OD) was measured (A et al., 2017)

3.7.2. Determination of suitable nitrogen source for bacterial isolate

In this study, various 1% (w/v) nitrogen sources like yeast extract, tryptone, ammonium chloride and ammonium sulphate were added to the mineral salt medium (Dipotassium hydrogen phosphate 1.73 g/l, Potassium dihydrogen phosphate 0.68 g/l, Magnesium sulfate heptahydrate 0.1 g/l, Sodium chloride 4 g/l, Ferrous sulfate heptahydrate 0.03 g/l, Ammonium nitrate 1 g/l, Calcium chloride 0.02 g/l and Glucose 5 g/l) to evaluate the effect of nitrogen source on enzyme activity. Remaining parameters were unaltered (Sagar et al., 2013)

3.7.3. PH

The optimal substrate and carbon source concentration are taken, and the broth pH is balanced at 6.0, 7.0, 8.0, 9.0, and 10.0 in the various conical flask using 1 N HCl and 1 N NaOH as well as autoclaved. Cultures are inoculated and cultivated at specific temperatures. The cell mass was separated by centrifugation after 24 hours of fermentation at 37°C with 150 rpm shaking, and cell-free culture filtrate was obtained as an enzyme source (Arju Hossain et al., 2021)

3.8. Genomic DNA Extraction `and PCR Sequence preparation

Genomic DNA was extracted from bacterial pure cultures using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions.The primer pairrD1 and fD1 (Weisburg et al. 1991, J. Bacteriol. 173, 697-703), were used to generate a 1500bpPCR product. The IGS region between 16S and 23S rRNA was amplified using FGPS1490 and FGPS132 primers (La Guerre et al. 1996.Applied and Environmental Microbiology 62:2029–2036). The PCR condition was a 95 degree initial denaturation for 5 minutes followed by35 cycles of denaturation at 95 degrees for 30 seconds, annealing at 55 degrees for 1 minute and extension at 72 degrees for 30 seconds with a final extension at 72 degrees for 5 minutes. The same primers were then used to sequence the PCR product on the ABI 3730XL genetic analyzer.

3.9. Data analysis

Experiments were performed in triplicate and the optimization data was analyzed and interpreted using Microsoft Excel 2010 and SPSS version 26 Software. Identification of bacterial species was performed ABIS version 12 online and MALDI-TOF MS.

4. RESULTS AND DISCUSSION

4.1. Isolation of cellulase producing bacteria

In this study, 45 isolates were obtained from the soil sediments of Lake Chitu. Of these isolates, 35 out of 45 isolates were ignored because they are non-cellulolytic. The ten isolates were cellulase-positive when grown on CMC agar using iodine staining. Some of their cultures were depicted in Figure 1 (Figure a, b, c, d, e and f). In the previous study, (Sethi et al., 2013) certain cellulase producing bacterial isolates identified from soil samples. Other study also showed that cellulase producing certain strains (C_1, C_2 and C_3) were screened from molasses samples, which obtained from a sugar industry area located in Katakhali region of Rajshahi city area in Bangladesh (Islam & Roy, 2018).

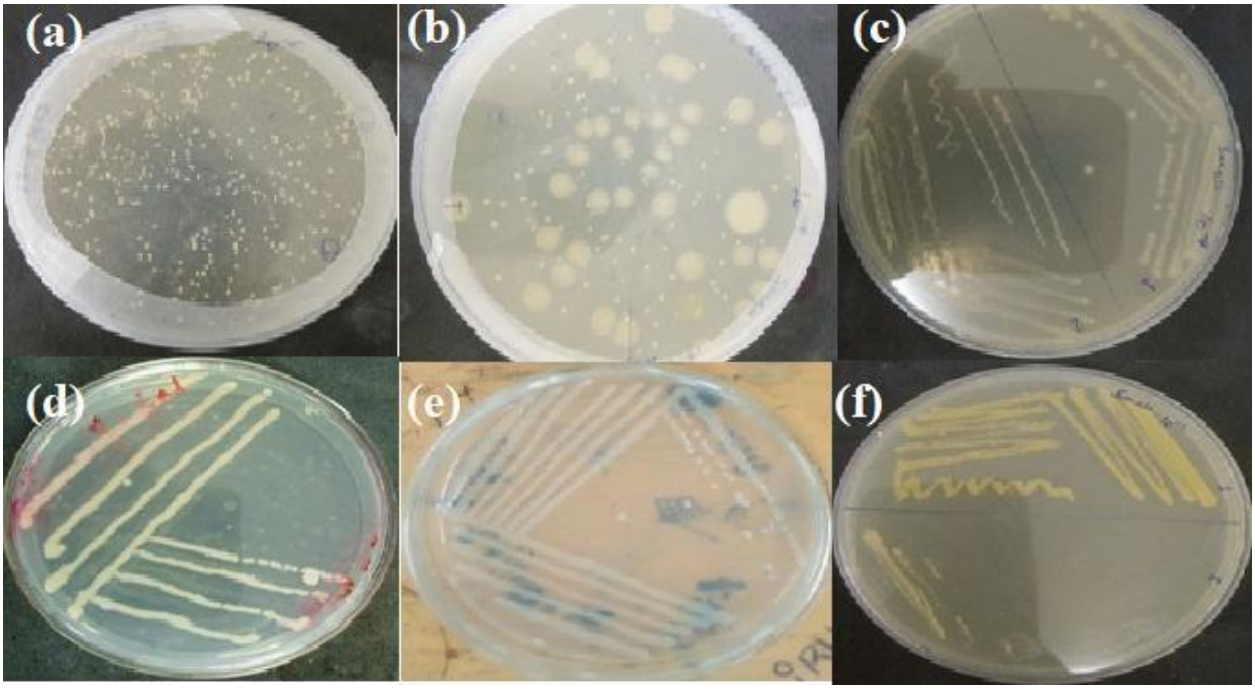

Figure 2: Pure culture isolated from Lake Chitu soil sediments after grown on CMC media. a and b indicate the colony from serial dilution. c, d, e, and f indicate the pure culture after grown on CMC culture media.

4.2. Morphological characterization

The cellulase-producing bacterial isolates were identified based on morphological characterization as Gram-positive rod shaped purple in color (Figure 3a & b)and the gram negative rod shaped pink in color (Figure c& d) were observed after gram staining as illustrated in Figure 3a & b bacillus rod shape appeared .It indicates that bacillus sp CPBILCS-4, CPBILCS-15 respectivily.Figure 1c & d indicate pink color from gram staining and indicates that gram negative bacteria isolate CPBILCS-13, and CPBILCS-9, respectively.

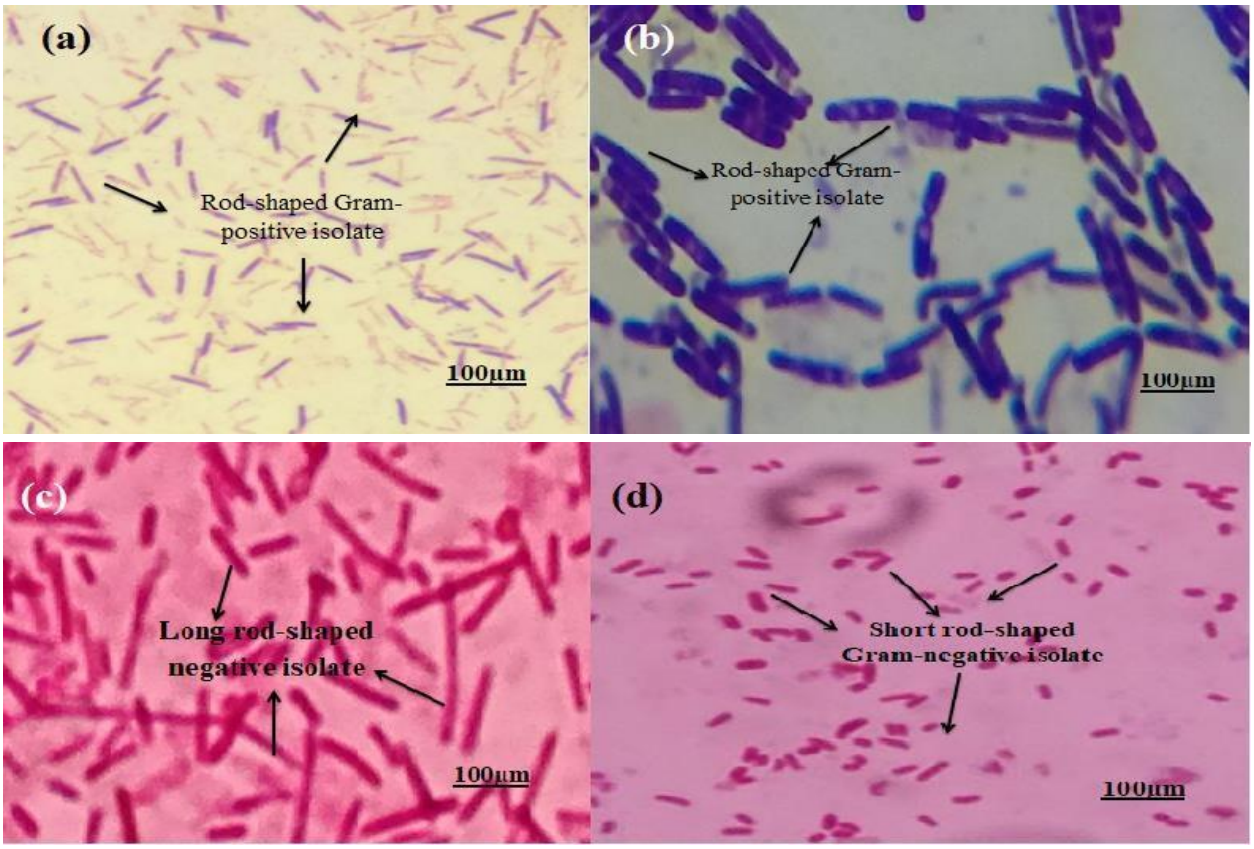

Figure 3: Morphological characterization of cellulase-producing isolates. (a) Long rod-shaped Gram-positive (CPBILCS-4), which was obtained from soil sediments, (b) long rod-shaped Gram-positive (CPBILCS-15), (c) long rod-shaped Gram-negative (CPBILCS-13), and (d) short rod-shaped Gram-negative (CPBILCS-9).

4.3. Screening of cellulase producing isolates

Ten bacterial isolates were screened for cellulolytic activity. From of these isolates showed cellulose utilization zones on agar plates after iodine solution staining. In agreement with this study (Arju Hossain et al., 2021) Out of 43 isolates, four isolates were efficient cellulase

producers and analyzed for secondary screening. In Gram staining technique, the three showed zone of clearance and were found to be gram-positive and one isolate showed the maximum zone of clearance and was found to be gram-negative bacteria after grown on CMC media. According to Gohel et al. studies, the best results are obtained using grams of iodine, accompanied by congo red staining (Gohel et al., 2014). Out of these strains, 4 isolates showed hydrolyzing zones on agar plates containing CMC-Na after iodine solution staining (Figure 4). The hydrolyzing zone diameter and colony diameter are indicated in figure 4.

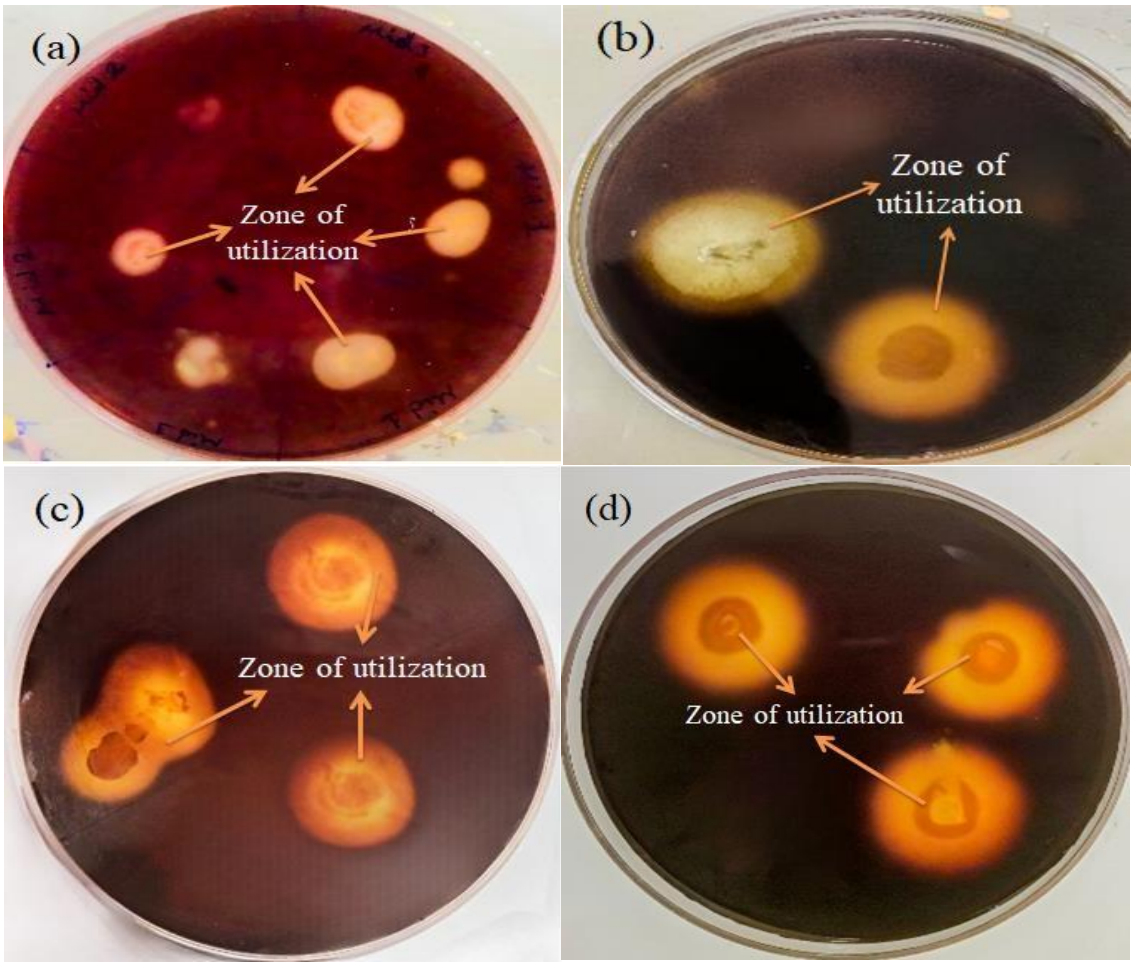

Figure 4: Cellulase producing isolates after growing on CMC. (a) CPBILCS-4 (b) CPBILCS-9, (c) CPBILCS-13 (d) *Bacillus* sp. CPBILCS-15 isolate which produce better zone cellulose utilization.

4.4. Biochemical characterization of the four selected isolates

According to morphological and biochemical tests, the selected strains were identified to be two *bacillus* sp. CPLCi-4, *pseudomonas* spp. CPLCI-13, and *proteus vulgaris sp. 11*, respectively. In

filter paper degradation test, these strains were observed to complete degrade the filter-paper which indicates the cellulolytic properties of the isolated bacterial strains. Biochemical characterization of the four isolates were performed and the result of the four selected isolates was described in Table-4. The isolates showed positive result for most biochemical tests such as citrate utilization, urease, and catalase but showed negative for indole production, sucrose, and lactose fermentation.

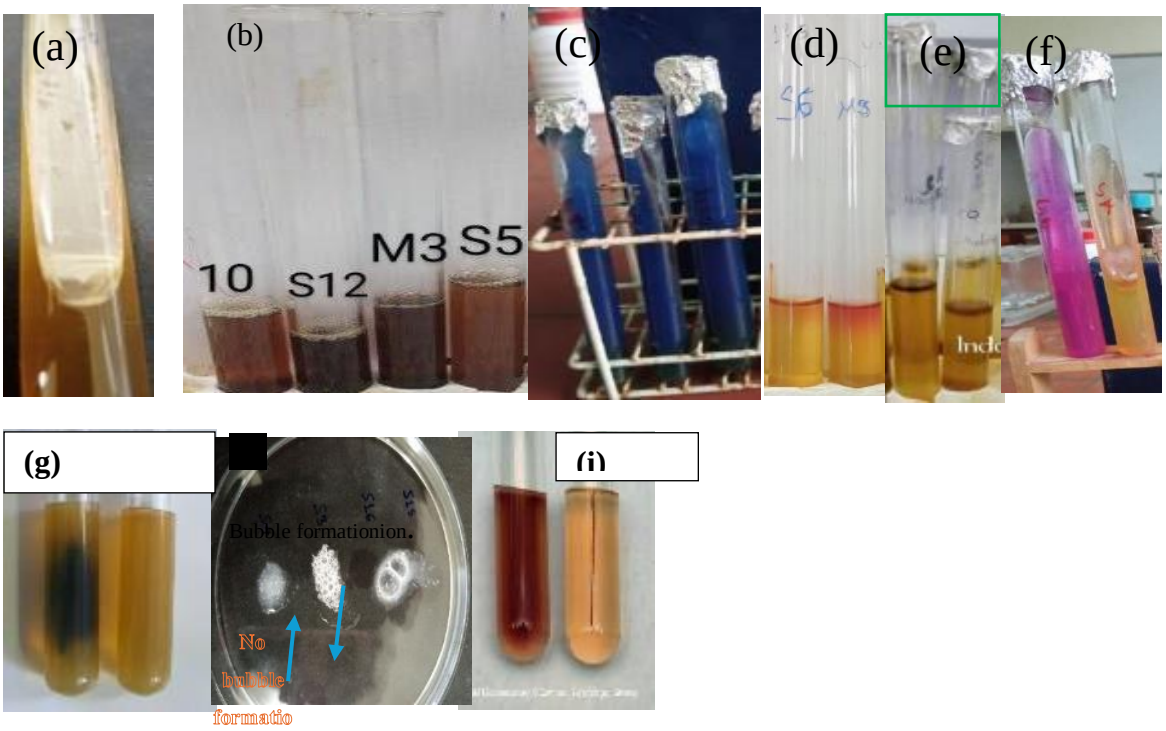

Figure 5: Biochemical tests (a) indicate triple sugar fermenter (b) indicates Voges-Proskauer test (c) indicates citrate test (d) indicates methyl red test (e) the indole tests (f) indicate urease tests (g) indicates motility test indicates (h) catalase test (i) motility test.

4.5. Cellulase producing bacterial isolate identification based on protein profile approach (MALDI–TOF MS)

In this study, certain cellulase producing bacterial isolates (CPBIs) were identified based on protein profiles (Table 2). These isolates were identified as *Bacillus, Pseudomonas,* and *Proteus* spp. These isolates were frequently obtained from Lake Chitu soil Sediments. They are designated as *Bacillus cereus* CPBILCS-9, *Bacillus licheniformis* CPBILCS-14, *Proteus vulgaris* CPBILCS-4, and *Pseudomonas sp*. CPBILCS-12 (Table 2). Since CPBILCS-12 contains less than two mass-to-charge ratio (m/z), the isolate could be new species as indicated in the Table 1.

Similarly, the previous study showed that cellulase producing strain were identified to be *Paenibacillus* sp. (C_1), *Bacillus* sp. (C_2) and *Aeromonas* sp. (C_3), respectively, where C_1 and C_2 was Gram-positive and C_3 was Gram-negative bacteria based on biochemical and morphological characteristics (Islam & Roy, 2018). In line with this study, 43 of the isolates of cellulase producing bacterial strains were screened using the same methods (MALDI TOF MS) and they were identified as *Bacillus licheniformis* (30, 69.8%), *Paenibacillus thiaminolyticus* (11, 25.6%), *Bacillus cereus* (1, 2.3%) and *Brevibacillus borstelensis* (1, 2.3%) (Guta et al., 2024). Other study identified certain cellulase producing bacterial strain as *Bacillus subtilus*, *E. coli*, *Pseudomonas fluorescens*, and *Serratia marscens* based on the morphological and biochemical characterization (Sethi et al., 2013). However, the methods may not be convenient and reliable for identification bacterial isolates to genus and species levels (Kshikhundo & Itumhelo, 2016).

Table 2: Biochemical test and protein profile based approach for cellulase producing bacterial isolate identification

S.N.	Endophyte isolates	Various biochemical tests							Protein profile based identification			
		Gram staining	Indole test	MR test	Citrate test	Urease test	VP test	Catalase test	Genus	Closest species	Score	Most likely isolates types
1	CPBILCS-4	-ve	+ve	-ve	+ve	+ve	-ve	+ve	*Proteus*	*vulgaris*	2.09	*P. vulgaris* CPBILCS-4
2	CPBILCS-9	+ve	-ve	+ve	**+ve**	-ve	+ve	+ve	*Bacillus*	*cereus*	2.14	*B. cereus* CPBILCS-9
3	CPBILCS-13	-ve	-ve	-ve	+ve	-ve	-ve	+ve	*Pseudomonas*	*aeuroginosa*	1.83	*Pseudomonas* sp. CPBILCS-13
4	CPBILCS-14	+ve	-ve	-ve	+ve	-ve	-ve	+ve	*Bacillus*	*licheniformis*	2.07	*B. licheniformis* CPBILCS-14
5	CPBILCS-15	+ve	-ve	-ve	+ve	**-ve**	+ve	+ve	*Bacillus*	*subtilis*	1.67	*B.* sp CPBILCS-15

Key note: CPBILCS indicated that Cellulase producing bacterial isolates which obtained from Lake chitu soil sediments.

4.6. Phylogenetic Tree construction for newly isolated cellulase producing bacterial isolate

The recent isolates exhibited sequence similarity with bacillus species, as indicated in Table 3 and **Figure 6:** The highest pairwise sequence similarity (100%) was predicted between *B. subtilis* NCIB 3610^{T} (ABQL01000001) and *B. tequilensis* CPBILCS-15, the recent isolate (Figure 6, Table 2). T

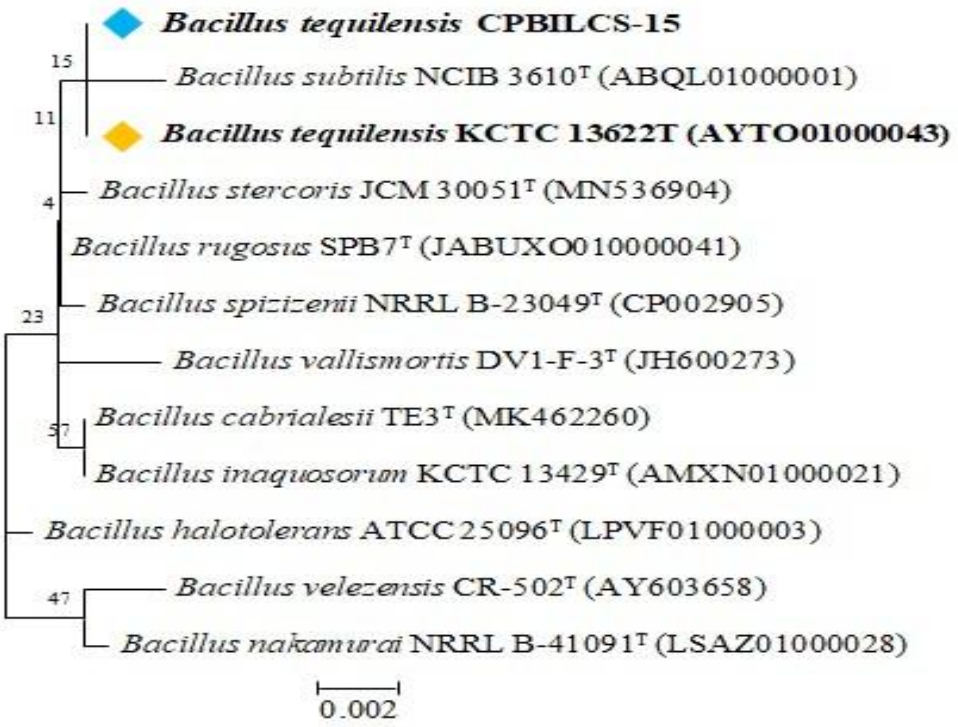

Figure 6 Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-2405.78) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This

analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1659 positions in the final dataset.

Evolutionary analyses were conducted in MEGA11 (Felsenstein, 1985; Tamura & Nei, 1993)

Table 3: Sequence similarities between the recent cellulase producing bacterial isolates and the other strains

S.N.	Name	Strain	Accession	Pairwise Similarity (%)
1	*B. tequilensis*	KCTC 13622^{T}	AYTO01000043	The recent
2	*B. subtilis*	NCIB 3610 ^{T}	ABQL01000001	99.93
3	*B. cabrialesii*	TE3^{T}	MK462260	99.93
4	*Ba. inaquosorum*	KCT C 13429 ^{T}	AMXN01000021	99.93
5	*B. stercoris*	JCM 3 0051^{T}	MN536904	99.93
6	*B. spizizenii*	NRRL B 23049^{T}	CP002905	99.85
7	*B. halotolerans*	ATCC 25096^{T}	LPVF01000003	99.71
8	*B. velezensis*	CR-502^{T}	AY603658	99.69
9	*B. vallismortis*	DV1-F-3^{T}	JH600273	99.64
10	*B. nakamurai*	NRRL B 41091^{T}	LSAZ01000028	99.63
11	*B. mojavensis*	RO-H-1^{T}	JH600280	99.56
12	*B. siamensis*	KCTC 13613^{T}	AJVF01000043	99.49
13	*B.amyloliquefaciens*	DSM 7^{T}	FN597644	99.4
14	*B. atrophaeus*	JCM 9070^{T}	AB021181	99.19
15	*B. licheniformis*	ATCC 14580 ^{T}	AE017333	98.75
16	*Bacillus glycinifermentans*	GO-13^{T}	LECW01000063	98.68
17	*B. paralicheniformis*	KJ-16^{T}	KY694465	98.60
18	*B. haynesii*	NRRL B-41327^{T}	MRBL01000076	98.60
19	*B. swezeyi*	NRRL B-41294^{T}	MRBK01000096	98.53
20	*B. sonorensis*	NBRC 101234^{T}	AYTN01000016	98.46
21	*B. aerius*	24K ^{T}	AJ831843	98.09
22	*B. altitudinis*	41KF2b	ASJC01000029	97.14
23	*B. xiamenensis*	HYC-10 ^{T}	AMSH01000114	97.07
24	*B. safensis subsp. safensis*	FO-36b	ASJD01000027	96.99
25	*B. safensis subsp. osmophilus*	BC09^{T}	KY990920	96.99

26	*B. pumilus*	ATCC 7061^T	ABRX01000007	96.92
27	*B. zhangzhouensis*	DW5-4^T	JOTP01000061	96.92
28	*B. australimaris*	NH7I_1^T	JX680098	96.85

The next highest pairwise sequence similarity of the recent isolate predicted against *B.* subtilis NCIB 3610^T (ABQL01000001) (99.93%) (Figure 6, Table 3). Considerable amount pairwise sequence similarity were predicted among *B. tequilensis* CPBI & *B. cabrialesii* TE3^T (MK462260) (99.92%), *B. spizizenii* NRRL B-23049^T (CP002905) (99.85%), *B. halotolerans* ATCC 25096^T (LPVF01000003) (99.71%), *B. velezensis* CR-502T (AY603658) (99.69%), *B. nakamurai* NRRL B-41091^T (LSAZ01000028) (99.63%) and *B. vallismortis* DV1F-3^T (JH600273) (99.63%) (Figure 6, Table 3). A previous study showed that sequence similarities were detected among PHA-producing bacterial strains, *such as Bacillus* sp. BPPI-14 & *Bacillus* sp. BPPI-19 and other *Bacillus* strains like *B. aryabhattai* B8W22^T (EF114313) (100%) & *B. megaterium* NBRC 15308^T (JJMH01000057) (99.86%), respectively (Mohammed et al., 2019).

4.7. Effect of growth condition against *B. tequilensis* CPBILCS-15

Certain growth condition such as carbon sources, pH values, temperatures, and others can affect the growth of bacterial species. Some the agro wastes are also utilized as a main carbon souces for diversities of bacterial growth. These growth conditions are employed for various bacterial cells to be effectively carried out their cell division and cellular formation (Ebu et al., 2023; Mohammed et al., 2019, 2020; Mohammed & Ray, 2022; Mor & Sivan, 2008; Nedi et al., 2024a).

The recent study showed that various CDW (g/L) and OD_{600nm} were recorded against *B. tequilensis* CPBILCS-15 isolate. The minimum and maximum CDW (g/L) were 0.4313± 0.04400 and 0.6550± 0.02326, respectively (Figure 6a; Additional file- Table A_1). As indicated in **Figure 7a:** Additional file- Table A_1, considerable amount of CDW (g/L) was documented for the same isolates at pH8 (0.6450±0.00500). Based on the optimization of growth conditions, the recent capacity of our isolate to produce cellulase enzymes, which could be employed for various industrial applications, will be determined. Optimization of mycolytic enzymes such as

Chitinase, $\beta_{1,3}$-glucanase and cellulase against *Bacillus subtilis* can give maximum biocontrol agent efficiency (Narasimhan et al., 2013).

As shown in the Figure 7a, great significant variations ($p<0.05$) were predicted among various pH values (e.g. pH 6 & pH 6.5, pH 6 & pH 7, pH 6 & pH 8, pH 6.5 & pH 8, pH 7 & pH 7.5, etc) in terms of CDW (g/L) against *B. tequilensis* CPBILCS-15 isolate. However, no variations for CDW (g/L) were detected against *B. tequilensis* CPBILCS-15 between pH6.5 & pH7 (*p=0.638*), and pH7.5 & pH8 (*p=0.662*) in terms of CDW (g/L) (**Figure 7a, additional file: Table A_2**).

Similar study exhibited that pH range from 6 to 11 affect the growth of bacterial strains such as *Bacillus* sp. BPPI-14 and *Bacillus* sp. BPPI-19 (Mohammed et al., 2019) which obtained from plastic waste landfill. Various CDW (g/L) were also reported for these Polyethylene degrading bacterial isolates such as *Bacillus* sp. PDI-21, *P. aeruginosa* PDI-1, and *P. balearica* PDI-17 (Nedi et al., 2024). Previous study demonstrated that change in pH values in the media found to affect the growth of *E. coli* ATCC 25922^T, *P. putida* KT2440^T and *P. pseudoalcaligenes* CECT 5344^T (Sánchez-Clemente et al., 2018). The pH change in the rumen animal also reported to affect the growth rate various microbioms and determine their competition the gut (Russell et al., 1979).

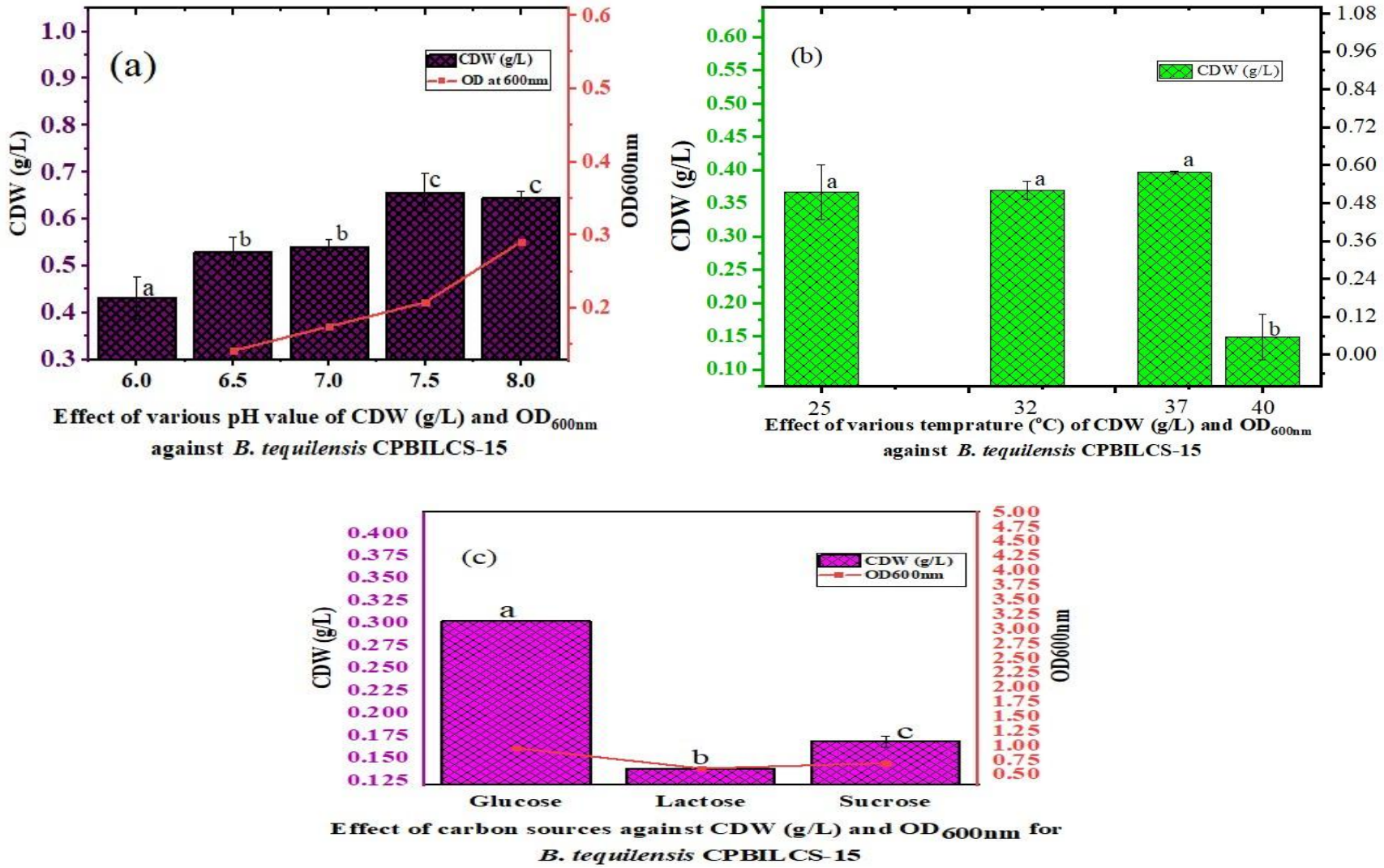

Figure 7: Effect of various growth conditions against *B.tequilensis* CPBILCS-15 isolate, a cellulase producer. (a) Effect of various pH values against *B. tequilensis* CPBILCS-15 for CDW (g/L) and OD 600nm. (b) Effect of various temperature against *B.tequilensis* CPBILCS-15 isolate for CDW (g/L). (c) Effect of various carbon sources against CDW (g/L) and OD_{600nm} for *B.tequilensis* CPBILCS-15. Data represented mean ± SD of three replicate (n = 3) of CDW (g/L), and $OD_{600\ nm}$. Mean of CDW (g/L) within a column sharing the same superscript letter(s) are not significantly different at 95% confidence interval by using LSD test (P N 0.05).

Importance of this experiment especially in figure (c) was to show that B.tequilensis grow best in monosaccharides sugars like glucose because glucose is the optimum carbon source for bacteria.as it supplies both the energy and material resources necessary for strain growth. But when the graph came to lactose it showed a decline in growth and a little better on sucrose but the way it declined on both Discarride sugars compared to glucose is that disaccharides are complex and take time to break down when compared with glucose(monosaccharide).

4.7.1. DNS Method:

The DNS (Dinitrosalicylic Acid) method is a colorimetric assay used to measure the activity of cellulase, an enzyme that breaks down cellulose into simpler sugars. The method was based on the reduction of 3,5-dinitrosalicylic acid (DNS) by the reducing sugars produced during cellulase activity.

4.7.2. Reagents for Cellulase Activity

Here are the common reagents used in the DNS method for measuring cellulase activity:

1. Cellulose substrate: Carboxymethyl cellulose (CMC) or other cellulose derivatives.
2. DNS reagent: 3,5-Dinitrosalicylic acid (DNS) dissolved in sodium potassium tartrate solution.
3. Buffer solution: A suitable buffer, such as citrate or phosphate buffer, to maintain the optimal pH for cellulase activity.
4. Cellulase enzyme: The enzyme preparation containing cellulase activity.
5. Distilled water: Used to prepare the reaction mixture and for dilutions.

Temperatures are the other growth parameter which affects the growth of certain microbial cells. For instance, some mesophilic and thermophilic microbial cells may produce cellulase at a suitable temperature range. Similar study indicated that thermostable cellulases producing *Bacillus* and *Geobacillus* strains can produce cellulase at elevated temperatures (60^{o}C) (Rastogi et al., 2010). In this study, the minimum 0.149467± 0.034314 g/L) and maximum (0.396±0.0015 g/L) biomass were obtained against *B. tequilensis* CPBILCS-15. The biomass (g/L) obtained at 40^{o}C was less which indicated that *B. tequilensis* CPBILCS-15 could be a mesophilic isolate and produce lower cellulase enzymes at this particular temperature point. Previous study exhibited that various temperature range can be employed for the growth various *Bacillus* and other species (Mohammed et al., 2019). Variation observed ($p<0.005$) among 25 & 40^{o}C, 32 & 40^{o}C, and 37 & 40^{o}C for *B. tequilensis* CPBILCS-15 isolate. However, no variations ($p>0.05$) were detected among 25 & 32^{o}C, 25 & 37^{o}C, 32 & 37^{o}C of incubated temperature.

Certain carbon sources were utilized by certain microbial cells for their cellular formation. Previous study shown that glucose, sucrose, molasses, lactose and other agro-wastes were used for diversities of bacterial growth with various CDW (g/L) (Mohammed et al., 2019). The specific rate of substrate consumption occurred on fructose, glucose, and galactose at 30 °C (Fonseca et al., 2013) against *Kluyveromyces marxianus* CBS 6556. The recent study exhibited that glucose which is simple carbon source gave maximum CDW (g/L) (0.302±0.00058) and OD_{600nm} (0.956±0.01326) (Figure 6c, Additional file: Table A_5). As indicated in Figure 6c and Additional File: Table A_5, less CDW (g/L) and OD_{600nm} were obtained against lactose (0.138±0.001 g/L, 0.6077±0.0136) and sucrose (0.168±0.0062450) for *B. tequilensis* CPBILCS-15, a cellulase producing isolate at pH7.5 and 37^{o}C. This might be due to their double sugars which is difficult for microbial diversities for breaking down. Similarly it was predicted that *K.s marxianus* CBS 6556 shown smaller growth on disaccharides sucrose and lactose (Fonseca et al., 2013) since cells need to perform a hydrolysis step before disaccharides can enter the glycolytic pathway, which might decrease the rate with which the substrate is metabolized.

From this study, it was realized that the recent isolate can produce cellulase using glucose as the main carbon source at pH7.5 and 37^{o}C. In line with this study, it was reported that certain carbon sources such as cellobiose, xylan, and *Leptochloa fusca* (kallar grass) can produce cellulase, and other enzymes against *Cellulomonas biazotea* (Rajoka & Malik, 1997). Other carbon souces like steam pretreated spruce, willow, corn stover and delignified lignocellulose (Solka

Floc) employed for the biosyhnthesis cellulase and hemicellulase enzymes against *Trichoderma reesei* RUT C30 (Juhász et al., 2005). Study conducted by indicated that carbon sources like fructose, galactose, glucose, glycerol, mannitol, and sucrose were found to be better for producyion cellulase using *Gluconacetobacter xylinus* ATCC 53524^{T} (Mikkelsen et al., 2009). As indicated in Figure 6C, the CDW (g/L) against glucose, lactose, and sucrose exhibited very significant variation (*p=0.000*) for *B. tequilensis* CPBILCS-15 isolate.

5. Conclusion

Screening the diversity of bacterial species employed for cellulase production is essential for various industrial applications. The same enzymes are also important for the removal of certain cellulose agro-wastes. Generally, the following points are driven regarding the current study about isolating and screening cellulase-producing bacterial species: Cellulases are the essential natural product obtained from various bacterial species. These bacterial isolates were identified as *P. vulgaris* CPBILCS-4, *B. cereus* CPBILCS-9, *Pseudomonas sp*. CPBILCS13, *B. licheniformis* CPBILCS-14, and *B.* sp CPBILCS-15 based on protein profiles (MALDI TOF MS tools). Other various cellulase-producing isolates were also identified based on biochemical tests. Most of the recent cellulase-producing isolates were found to be Grampositives. Some of them were Gram-negative. pH can be used to affect the growth of cellulaseproducing isolates. It was found that over 0.431333± 0.044004 to 0.655± 0.023259 CDW (g/L) were recorded for *B. tequilensis* CPBILCS-15 isolate. Over 0.149467± 0.034314 to 0.396333± 0.001528 CDW (g/L) were recorded for the same isolate. These isolates can utilize carbon sources such as glucose, lactose, and sucrose. The study showed that over 0.138± 0.001 to 0.302333± 0.000577 CDW (g/L) were obtained from glucose against *B. tequilensis* CPBILCS-15 isolate. However, over 0.608± 0.013614 to 0.956067 OD600nm was detected against *B. tequilensis* CPBILCS-15 isolate. This study concluded that cellulase production could be enhanced due to the involvement of potential cellulase-producing bacterial isolates when certain growth conditions such as carbon source, suitable pH values, and temperature.

6. Recommendation

Based on the findings of the research the following recommendations are made:

- The optimization of the cellulase producing bacteria's growth on salt solution of these bacteria's was not studied to its full right leaving a gap in the knowledge of cellulase producing bacteria from lake Chitu, so further investigation of the ability of this study is necessary.
- The enzymatic activities of the cellulase producing bacterial isolates should be quantitatively assessed.
- Enzyme assay for the cellulase producing bacterial isolates should be made to fill the gap.

7.References

Aditi, F. Y., Rahman, S. S., & Hossain, Md. M. (2017). A Study on the Microbiological Status of

Mineral Drinking Water. *The Open Microbiology Journal*, *11*(1). https://doi.org/10.2174/1874285801711010031

Ajeje, S. B., Hu, Y., Song, G., Peter, S. B., Afful, R. G., Sun, F., Asadollahi, M. A., Amiri, H.,

Abdulkhani, A., & Sun, H. (2021). Thermostable Cellulases / Xylanases From Thermophilic and Hyperthermophilic Microorganisms: Current Perspective. In *Frontiers in Bioengineering and Biotechnology* (Vol. 9). https://doi.org/10.3389/fbioe.2021.794304

Aktar, L., Khan, F. I., Islam, T., Mitra, S., & Saha, M. L. (2016). Isolation and characterization of indigenous lipase producing bacteria from lipid-rich environment. *Plant Tissue Culture and Biotechnology*, *26*(2). https://doi.org/10.3329/ptcb.v26i2.30574

Ali, S., Hall, J., Soole, K. L., Fontes, C. M. G. A., Hazlewood, G. P., Hirst, B. H., & Gilbert, H. J.

(1995). Targeted expression of microbial cellulases in transgenic animals. In *Progress in Biotechnology* (Vol. 10, Issue C). https://doi.org/10.1016/S0921-0423(06)80111-5

Arju Hossain, Md., Akash Ahammed, Md., Islam Sobuj, S., Kubra Shifat, S., & Dipta Somadder, P. (2021). Cellulase Producing Bacteria Isolation, Screening and Media Optimization from Local Soil Sample. *American Journal of Microbiological Research*, *9*(3). https://doi.org/10.12691/ajmr-9-3-1

Bharathi, D., & Rajalakshmi, G. (2019). Microbial lipases: An overview of screening, production and purification. In *Biocatalysis and Agricultural Biotechnology* (Vol. 22). https://doi.org/10.1016/j.bcab.2019.101368

Bhat, M. K., & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances*, *15*(3–4), 583–620.

Demirbas, A. (2008). Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. *Energy Conversion and Management*, *49*(8). https://doi.org/10.1016/j.enconman.2008.02.020

Fraga Vidal, R., Arísticas Ribalta, R. C., Martínez Valdés, L. T., Lafargue Gámez, M., Montes Alvarez, A., Rubio Sánchez, A., Dubreucq, E., & Moreau, B. (2021). Construction of a Novel Chimeric Dextransucrase Fused to the Carbohydrate-Binding Module CBM2a. *Catalysts*, *11*(10). https://doi.org/10.3390/catal11101179

Gohel, H. R., Contractor, C. N., Ghosh, S. K., & Braganza, V. J. (2014). A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. *International Journal of Current Microbiology and Applied Sciences*, *3*(5).

Imran, M., Bano, S., Nazir, S., Javid, A., Asad, M. J., & Yaseen, A. (2019). Cellulases Production and Application of Cellulases and Accessory Enzymes in Pulp and Paper Industry: A Review. *Biological Research*, *4*(1).

Kuhad, R. C., Gupta, R., & Singh, A. (2011). Microbial cellulases and their industrial applications. In *Enzyme Research* (Vol. 2011, Issue 1). https://doi.org/10.4061/2011/280696

Lavanya, D., Kulkarni, P. K., Dixit, M., Raavi, P. K., & Krishna, L. N. V. (2011). Sources of cellulose and their applications—A review. *International Journal of Drug Formulation and Research*, *2*(6), 19–38.

Liang, Y. L., Zhang, Z., Wu, M., Wu, Y., & Feng, J. X. (2014). Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by Paenibacillus terrae ME27-1. *BioMed Research International*, *2014*. https://doi.org/10.1155/2014/512497

Malik, T., & Naz, A. (2019). Isolation and Characterization of Thermophilic Alkaline Lipase Produced by Different Bacterial Species from Oil-contaminated Soil Suitable for Detergent Industries. In *Focus on Medical Sciences Journal* (Vol. 5, Issue 2).

MANDELS, M., & REESE, E. T. (1957). Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals. *Journal of Bacteriology*, *73*(2). https://doi.org/10.1128/jb.73.2.269-278.1957

Ohkuma, M. (2003). Termite symbiotic systems: Efficient bio-recycling of lignocellulose. In *Applied Microbiology and Biotechnology* (Vol. 61, Issue 1). https://doi.org/10.1007/s00253-002-1189-z

Rahman, M. S., Fernando, S., Ross, B., Wu, J., & Qin, W. (2018). Endoglucanase (eg) activity assays. *Cellulases: Methods and Protocols*, 169–183.

Sammond, D. W., Payne, C. M., Brunecky, R., Himmel, M. E., Crowley, M. F., & Beckham, G. T. (2012). Cellulase linkers are optimized based on domain type and function: insights from sequence analysis, biophysical measurements, and molecular simulation. *PloS One*,

7(11). https://doi.org/10.1371/journal.pone.0048615

Savalia, H. J., & Dungrechiya, A. (2022). identification and Optimization Study of lipase Producing Bacteria isolated from Municipal Waste and Bio-deteriorated Waste. *Journal of Pure and Applied Microbiology*, *16*(4). https://doi.org/10.22207/JPAM.16.4.27

Scarlat, N., Dallemand, J. F., Monforti-Ferrario, F., & Nita, V. (2015). The role of biomass and bioenergy in a future bioeconomy: Policies and facts. *Environmental Development*, *15*. https://doi.org/10.1016/j.envdev.2015.03.006

Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2016). Review article microbial cellulases in industrial applications. *Annals of Applied Bio-Science*, *3*(4).

Singh, R. S., Singh, T., & Pandey, A. (2019). Microbial Enzymes—An Overview. In *Advances in Enzyme Technology, First Edition*. https://doi.org/10.1016/B978-0-444-64114-4.00001-7

Sukumaran, R. K., Singhania, R. R., & Pandey, A. (2005). Microbial cellulases - Production, applications and challenges. In *Journal of Scientific and Industrial Research* (Vol. 64, Issue 11).

Tolan, J. S., & Foody, B. (1999). *Cellulase from Submerged Fermentation*. https://doi.org/10.1007/3-540-49194-5_3

Yang, W., Meng, F., Peng, J., Han, P., Fang, F., Ma, L., & Cao, B. (2014). Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production. *Electronic Journal of Biotechnology*, *17*(6), 262–267.

7. Annex section

Table A_1: Mean value and SD of various pH values against CDW (g/L) and OD_{600nm} for *B. tequilensis* CPBILCS-15

CDW (g/L) against various pH values								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) against B. tequilensis CPBILCS-15 at pH6	3	.4313	.04400	.02541	.3220	.5406	.39	.48
CDW (g/L) against B. tequilensis CPBILCS-15 at pH6.5	3	.5293	.03057	.01765	.4534	.6053	.50	.56
CDW (g/L) against B. tequilensis CPBILCS-15 at pH7	3	.5401	.01585	.00915	.5007	.5795	.52	.55
CDW (g/L) against B. tequilensis CPBILCS-15 at pH7.5	3	.6550	.02326	.01343	.5972	.7128	.63	.68
CDW (g/L) against B. tequilensis CPBILCS-15 at pH8	3	.6450	.00500	.00289	.6326	.6574	.64	.65
Total	15	.5602	.08856	.02287	.5111	.6092	.39	.68

Table A_2 Multiple Comparisons and Post Hoc Test at 0.05 LCD confidence interval for various pH values against CDW (g/L) and OD600nm for *B. tequilensis* CPBILCS-15

Multiple Comparisons						
Dependent Variable: CDW (g/L) against various pH values LSD						
(I) Coding	(J) Coding	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) against B. tequilensis CPBILCS-15 at pH6	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6.5	-.09800*	.02217	.001	-.1474	-.0486
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7	-.10877*	.02217	.001	-.1582	-.0594
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7.5	-.22367*	.02217	.000	-.2731	-.1743
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH8	-.21367*	.02217	.000	-.2631	-.1643
CDW (g/L) against B. tequilensis CPBILCS-15 at pH6.5	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6	.09800*	.02217	.001	.0486	.1474
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7	-.01077	.02217	.638	-.0602	.0386
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7.5	-.12567*	.02217	.000	-.1751	-.0763
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH8	-.11567*	.02217	.000	-.1651	-.0663
CDW (g/L) against B. tequilensis CPBILCS-15 at pH7	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6	.10877*	.02217	.001	.0594	.1582
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6.5	.01077	.02217	.638	-.0386	.0602
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7.5	-.11490*	.02217	.000	-.1643	-.0655
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH8	-.10490*	.02217	.001	-.1543	-.0555
CDW (g/L) against B. tequilensis CPBILCS-15 at pH7.5	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6	.22367*	.02217	.000	.1743	.2731
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6.5	.12567*	.02217	.000	.0763	.1751
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7	.11490*	.02217	.000	.0655	.1643

	CDW (g/L) against B. tequilensis CPBILCS-15 at pH8	.01000	.02217	.662	-.0394	.0594
CDW (g/L) against B. tequilensis CPBILCS-15 at pH8	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6	.21367*	.02217	.000	.1643	.2631
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH6.5	.11567*	.02217	.000	.0663	.1651
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7	.10490*	.02217	.001	.0555	.1543
	CDW (g/L) against B. tequilensis CPBILCS-15 at pH7.5	-.01000	.02217	.662	-.0594	.0394

*. The mean difference is significant at the 0.05 level.

Table A3: Mean value and SD of various temperature against CDW (g/L) for *B. tequilensis* CPBILCS-15

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
cdw (g/L) at 25oC against B. tequilensis CPBILCS-15	3	.366767	.0404888	.0233762	.266187	.467346	.3342	.4121
cdw (g/L) at 32oC against B. tequilensis CPBILCS-15	3	.369333	.0133167	.0076884	.336253	.402414	.3540	.3780
cdw (g/L) at 37oC against B. tequilensis CPBILCS-15	3	.396333	.0015275	.0008819	.392539	.400128	.3950	.3980
cdw (g/L) at 40oC against B. tequilensis CPBILCS-15	3	.149467	.0343140	.0198112	.064226	.234707	.1274	.1890
Total	12	.320475	.1064203	.0307209	.252859	.388091	.1274	.4121

Table A_4 Multiple Comparisons and Post Hoc Test at 0.05 LCD confidence interval for various temprature against CDW (g/L) for *B. tequilensis* CPBILCS-15

Multiple Comparisons						
Dependent Variable: Temperature LSD						
(I) coding	(J) coding	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) at 25oC against B. tequilensis CPBILCS-15	cdw (g/L) at 32oC against B. tequilensis CPBILCS-15	-.0025667	.0223475	.911	-.054100	.048967
	cdw (g/L) at 37oC against B. tequilensis CPBILCS-15	-.0295667	.0223475	.222	-.081100	.021967
	cdw (g/L) at 40oC against B. tequilensis CPBILCS-15	.2173000*	.0223475	.000	.165767	.268833
cdw (g/L) at 32oC against B. tequilensis CPBILCS-15	cdw (g/L) at 25oC against B. tequilensis CPBILCS-15	.0025667	.0223475	.911	-.048967	.054100
	cdw (g/L) at 37oC against B. tequilensis CPBILCS-15	-.0270000	.0223475	.261	-.078533	.024533
	cdw (g/L) at 40oC against B. tequilensis CPBILCS-15	.2198667*	.0223475	.000	.168333	.271400
cdw (g/L) at 37oC against B. tequilensis CPBILCS-15	cdw (g/L) at 25oC against B. tequilensis CPBILCS-15	.0295667	.0223475	.222	-.021967	.081100
	cdw (g/L) at 32oC against B. tequilensis CPBILCS-15	.0270000	.0223475	.261	-.024533	.078533
	cdw (g/L) at 40oC against B. tequilensis CPBILCS-15	.2468667*	.0223475	.000	.195333	.298400
cdw (g/L) at 40oC against B. tequilensis CPBILCS-15	cdw (g/L) at 25oC against B. tequilensis CPBILCS-15	-.2173000*	.0223475	.000	-.268833	-.165767
	cdw (g/L) at 32oC against B. tequilensis CPBILCS-15	-.2198667*	.0223475	.000	-.271400	-.168333
	cdw (g/L) at 37oC against B. tequilensis CPBILCS-15	-.2468667*	.0223475	.000	-.298400	-.195333
*. The mean difference is significant at the 0.05 level.						

Table A5: Mean value and SD of various carbon sources against CDW (g/L) and OD600nm for *B. tequilensis* CPBILCS-15

CDW (g/L) and OD at 600nm	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) against *B. tequilensis* CPBILCS-15 for Glucose	3	0.302333	0.0005774	.0003333	.300899	.303768	.3020	.3030
CDW (g/L) against *B. tequilensis* CPBILCS-15 for Lactose	3	0.138000	0.0010000	.0005774	.135516	.140484	.1370	.1390
CDW (g/L) against *B. tequilensis* CPBILCS-15 for sucrose	3	0.168000	0.0062450	.0036056	.152487	.183513	.1610	.1730
OD600nm against *B. tequilensis* CPBILCS-15 for Glucose	3	0.956067	0.0132549	.0076527	.923140	.988994	.9418	.9680
OD 600nm against *B. tequilensis* CPBILCS-15 for Lactose	3	0.607667	0.0136137	.0078599	.573848	.641485	.5970	.6230
OD 600nm against *B. tequilensis* CPBILCS-15 for sucrose	3	0.697000	0.0265141	.0153080	.631135	.762865	.6700	.7230
Total	18	0.478178	0.3077290	.0725324	.325148	.631208	.1370	.9680

Table A_6 Multiple Comparisons and Post Hoc Test at 0.05 LCD confidence interval for various carbon sources against CDW (g/L) and OD600nm for *B. tequilensis* CPBILCS-15

Multiple Comparisons						
Dependent Variable: CDW (g/L) and OD at 600nm LSD						
(I) coding and analysis	(J) coding and analysis	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CDW (g/L) against B. tequilensis CPBILCS-15 for Glucose	CDW (g/L) against B. tequilensis CPBILCS-15 for Lactose	.1643333*	.0110773	.000	.140198	.188469
	CDW (g/L) against *B. tequilensis* CPBILCS-15 for sucrose	.1343333*	.0110773	.000	.110198	.158469
	OD600nm against *B. tequilensis* CPBILCS-15 for Glucose	-.6537333*	.0110773	.000	-.677869	-.629598
	OD 600nm against B. tequilensis CPBILCS-15 for Lactose	-.3053333*	.0110773	.000	-.329469	-.281198
	OD 600nm against B. tequilensis CPBILCS-15 for sucrose	-.3946667*	.0110773	.000	-.418802	-.370531
CDW (g/L) against B. tequilensis CPBILCS-15 for Lactose	CDW (g/L) against B. tequilensis CPBILCS-15 for Glucose	-.1643333*	.0110773	.000	-.188469	-.140198
	CDW (g/L) against B. tequilensis CPBILCS-15 for sucrose	-.0300000*	.0110773	.019	-.054135	-.005865
	OD600nm against B. tequilensis CPBILCS-15 for Glucose	-.8180667*	.0110773	.000	-.842202	-.793931
	OD 600nm against B. tequilensis CPBILCS-15 for Lactose	-.4696667*	.0110773	.000	-.493802	-.445531

	OD 600nm against B. tequilensis CPBILCS-15 for sucrose	-.5590000*	.0110773	.000	-.583135	-.534865
CDW (g/L) against B. tequilensis CPBILCS-15 for sucrose	CDW (g/L) against B. tequilensis CPBILCS-15 for Glucose	-.1343333*	.0110773	.000	-.158469	-.110198
	CDW (g/L) against B. tequilensis CPBILCS-15 for Lactose	.0300000*	.0110773	.019	.005865	.054135
	OD600nm against B. tequilensis CPBILCS-15 for Glucose	-.7880667*	.0110773	.000	-.812202	-.763931
	OD 600nm against B. tequilensis CPBILCS-15 for Lactose	-.4396667*	.0110773	.000	-.463802	-.415531
	OD 600nm against B. tequilensis CPBILCS-15 for sucrose	-.5290000*	.0110773	.000	-.553135	-.504865
OD600nm against B. tequilensis CPBILCS-15 for Glucose	CDW (g/L) against B. tequilensis CPBILCS-15 for Glucose	.6537333*	.0110773	.000	.629598	.677869
	CDW (g/L) against B. tequilensis CPBILCS-15 for Lactose	.8180667*	.0110773	.000	.793931	.842202
	CDW (g/L) against B. tequilensis CPBILCS-15 for sucrose	.7880667*	.0110773	.000	.763931	.812202
	OD 600nm against B. tequilensis CPBILCS-15 for Lactose	.3484000*	.0110773	.000	.324265	.372535
	OD 600nm against B. tequilensis CPBILCS-15 for sucrose	.2590667*	.0110773	.000	.234931	.283202
OD 600nm against B. tequilensis	CDW (g/L) against B. tequilensis CPBILCS-15 for Glucose	.3053333*	.0110773	.000	.281198	.329469

CPBILCS-15 for Lactose	CDW (g/L) against B. tequilensis CPBILCS-15 for Lactose	.4696667*	.0110773	.000	.445531	.493802
	CDW (g/L) against B. tequilensis CPBILCS-15 for sucrose	.4396667*	.0110773	.000	.415531	.463802
	OD600nm against B. tequilensis CPBILCS-15 for Glucose	-.3484000*	.0110773	.000	-.372535	-.324265
	OD 600nm against B. tequilensis CPBILCS-15 for sucrose	-.0893333*	.0110773	.000	-.113469	-.065198
OD 600nm against B. tequilensis CPBILCS-15 for sucrose	CDW (g/L) against B. tequilensis CPBILCS-15 for Glucose	.3946667*	.0110773	.000	.370531	.418802
	CDW (g/L) against B. tequilensis CPBILCS-15 for Lactose	.5590000*	.0110773	.000	.534865	.583135
	CDW (g/L) against B. tequilensis CPBILCS-15 for sucrose	.5290000*	.0110773	.000	.504865	.553135
	OD600nm against B. tequilensis CPBILCS-15 for Glucose	-.2590667*	.0110773	.000	-.283202	-.234931
	OD 600nm against B. tequilensis CPBILCS-15 for Lactose	.0893333*	.0110773	.000	.065198	.113469
*. The mean difference is significant at the 0.05 level.						