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Characteristic and activity of cellulolytic bacteria isolated from mangrove soil in Northern Coast of Aceh Province, Indonesia

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Abstract. *Dewiyanti I, Darmawi D, Muchlisin ZA, Helmi TZ, Arisa II, Rahmiati R, Destri E, Fanisha S. 2022. Characteristic and activity of cellulolytic bacteria isolated from mangrove soil in Northern Coast of Aceh Province, Indonesia. Biodiversitas 23: 6587-6599.* The cellulolytic bacteria as decomposing cellulose bacteria is related to the soil fertility and productivity of the mangrove ecosystem, and the presence and activity of enzymes produced can evaluate them. The study aims to analyze the cellulolytic bacteria characterization, cellulase enzyme activity, and production and to evaluate the molecular biology of cellulase bacteria isolated from the mangrove soil on the northern coast of Aceh Province, Indonesia. This study was conducted at six locations, and soil samples were collected randomly with three replications. The results showed that the purified bacteria produced 39 isolates that grew in CMC selective media. The common bacteria discovered have an irregular colony shape, undulate colony edge, raised colony elevation, and cream pigmentation. The bacilli cell is the most common shape, while 22 bacteria out of the 39 isolates showed cellulase activity. The cellulolytic index (CI) ranged from 0.31 to 4.82, and the highest CI was BTM533 (4.82), followed by BTM622 (2.09). The quantitative analysis showed that the highest specific cellulase activity of BTM533 and BTM622 were at 36 hours and 42 hours with the value of 0.042 U mg^{-1} and 0.129 U mg^{-1}. Among the bacteria isolates, two cellulolytic bacteria with high cellulase activity were identified as *Bacillus safensis* and *B. altitudinis* using the 16S rRNA gene molecularly.

Keywords: 16S rRNA gene, Aceh Province, *Bacillus*, cellulolytic bacteria, mangrove soil

INTRODUCTION

Mangroves are a complex tropical marine ecosystem with habitat characteristics influenced by tides and salinity (Noor et al. 2015; Srikanth et al. 2015), thus forming a transition between the terrestrial and marine environments (Arifanti et al. 2022). They provide some ecological functions, i.e., protect the coastline from erosion (Harefa et al. 2022), provide habitat for aquatic and terrestrial fauna (Thomas et al. 2017), and serve as nursery and spawning ground for some commercial fauna (mud crab, fish, and prawn) (Abu El-Regal and Ibrahim 2014; Jacobs et al. 2019). Although the ecosystem has moist and muddy soil that is poor in oxygen, it is rich in organic matter (Indawan et al. 2017).

The productivity of mangrove vegetation is determined by the nutrients available in the soil, and the decomposition process carried out by microorganisms such as cellulolytic bacteria (Soares-Junior et al. 2013; Naresh et al. 2019; Biswas et al. 2020). Bacteria are directly involved in the nutrient cycle in the soil and play an important role in the decomposition of organic matter and mineralization of organic compounds (McGuire et al. 2012; Thatoi et al. 2013). Microorganisms such as bacteria are potential sources of enzyme production and proteolytic, amylolytic, and cellulolytic, which are the common bacteria that produce enzymes in the mangrove ecosystem (Behera et al. 2014; Setyati et al. 2014). Bacteria are commonly observed for enzyme production because they are easy to isolate and produce extracellular enzymes quickly (Bhatt et al. 2020).

Enzymes are divided into two, namely intracellular enzymes that are synthesized in living cells and play a vital role in the cell, and extracellular enzymes that are synthesized inside the cell and function outside the cell, such as protease, amylase, cellulase, and lipase (Sulman and Rehman 2013; Orsi et al. 2018). Cellulase is a group of enzymes consisting of three major components: endoglucanase, exoglucanase, and β-D-glucosidase (Anoop Kumar et al. 2019; Gupta et al. 2012). Cellulolytic bacteria produce cellulase that degrades the cellulose substrates and converts them into simpler products such as glucose (Biswas et al. 2020). Cellulose is a polysaccharide composed of monomers of glucose units bonded together by β-1,4-glycosidic bonds (Behera et al. 2017), and it is the most abundant carbohydrate (Anggara et al. 2021) and plant biomass. Moreover, the culture media commonly applied for bacteria cellulose production require carbon, nitrogen source, and pH (Saichana et al. 2015). Commonly, carbon sources added to depict higher bacterial cell biomass were maltose, lactose, sucrose, glucose, and fructose (Mohapatra et al. 2020).

Enzymes derived from microbes are relatively more stable and diverse than plants and animals (Gurung et al. 2013; Nguyen and Nguyen 2017). Therefore, bacterial cellulose plays an essential role, and some have important applications in food, animal feed, and research. For example, cellulolytic bacteria reduce fibrous feed and increase digestibility (Sari et al. 2017), and the degradation of cellulose in fish feed raw materials can promote and increase fish growth (Zhou et al. 2013; Kurniawan et al. 2018a). Previous studies showed cellulolytic bacteria can be isolated from mangrove ecosystems such as soil (Behera et at. 2014; Chantarasiri 2015; Naresh et al. 2019; Biswas et al. 2020) and leaf litters (Kurniawan et al. 2018b). Moreover, several research studies have been carried out on the characterization, cellulase activity, and identification of cellulolytic bacteria. For example, three species of cellulolytic bacteria, namely *Anoxybacillus* sp., *Bacillus subtilis*, and *Paenibacillus dendritiformis*, were found in the mangrove soil of northern Malaysia (Naresh et al. 2019).

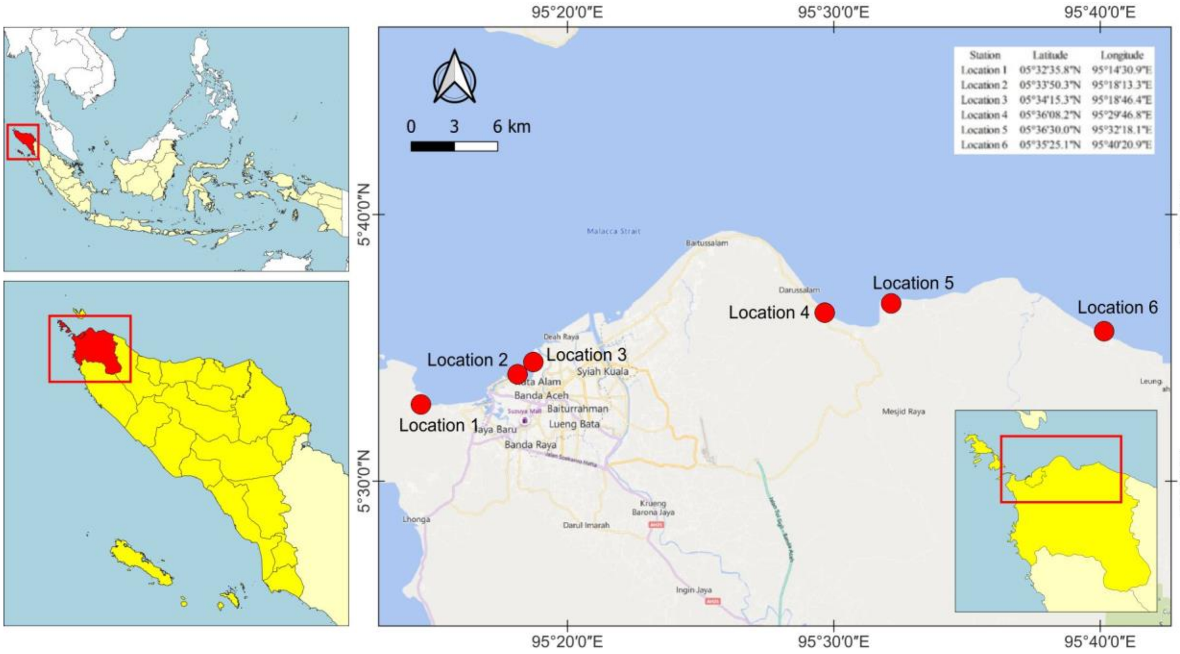
Furthermore, two targets of cellulolytic strain (*Bacillus* sp. and *Pseudomonas* sp.) were isolated from the soil mangrove Bangladesh (Biswas et al. 2020). However, studies on cellulolytic bacteria and their cellulase activity on different soil characteristics that compare rehabilitated and unrehabilitated mangroves in Banda Aceh and Aceh Besar have not been examined. Currently, there is no observation of bacteria that have the potential as cellulose-degrading bacteria and their ability to produce cellulase enzymes from mangrove soil in Aceh.

On the northern coast of Aceh, the two types of mangrove ecosystems, namely the rehabilitated and unrehabilitated areas (without human intervention), have different characteristics. Unrehabilitated mangrove ecosystems are natural vegetation not planted after damage by the tsunami in 2004. These two types of ecosystems contain different soil characteristics, i.e., the soil organic carbon (SOC) content is very low in the rehabilitated (0.9%) and low in unrehabilitated mangrove (1.23%) (Dewiyanti et al. 2021). The differences in soil characteristics of both areas can affect the presence of cellulolytic bacteria and their cellulase activity; thus, it is important to be studied. Therefore, this study aims to analyze the properties of cellulolytic bacteria isolated from the mangrove soil on the northern coast of Aceh Province, cellulase enzyme activity, and analyze the species with high cellulase activities using a molecular approach identified by the nucleotide analysis of 16S rRNA gene.

MATERIALS AND METHODS

Study area

This study was conducted at two types of habitats, namely unrehabilitated and rehabilitated mangrove areas located on the northern coast of Banda Aceh and Aceh Besar districts, Aceh Province, Indonesia. The rehabilitated mangrove area is the vegetation planted after the tsunami catastrophe in 2004, while the unrehabilitated is the ecosystem that was not destroyed by the tsunami. The mangrove rehabilitated were dominated by *Rhizophora* sp., while the most common three species in the unrehabilitated ecosystem include *Rhizophora* sp., *Avicennia marina*, and *Sonneratia alba*. The purposive sampling method was used to determine six sampling locations, where locations 4, 5, and 6 were in the unrehabilitated and locations 1, 2, and 3 were in rehabilitated mangrove areas, as shown in Figure 1.



Station	Latitude	Longitude
Location 1	05°32'35.8"N	95°14'30.9"E
Location 2	05°33'50.3"N	95°18'13.3"E
Location 3	05°34'15.3"N	95°18'46.4"E
Location 4	05°36'08.2"N	95°29'46.8"E
Location 5	05°36'30.0"N	95°32'18.1"E
Location 6	05°35'25.1"N	95°40'20.9"E

Figure 1. Map showed research location (red dot)

Mangrove soil collecting

Soil samples were taken from the mangrove ecosystems at 6 sampling locations, consisting of three soil samples from each rehabilitated and unrehabilitated location with three replications randomly. Locations 1, 2, and 3 were at Lambadeuk Village, Dayah Teungoh, and Pande Village as the mangrove was rehabilitated. Mangroves were unrehabilitated at Ruyung Village (location 4), Lamreh Village, and Lampanah Village locations 5 and 6 (Figure 1). Different soil characteristics of each mangrove area must be considered in determining the sampling location. Subsequently, the samples were mixed to form a composite sample, where a total of 100 g of soil were taken using a sterilized spatula and put into sterilized plastic sample bags. Finally, the soil samples were brought to the laboratory using a cool box to analyze cellulolytic bacteria characterization and their cellulase activities.

Screening of cellulolytic bacteria

Selective medium for cellulolytic bacteria was made by mixing 1 g of CMC, 0.02 g of $MgSO_4.7H_2O$, 0.05 g of KH_2PO_4, 0.075 of KNO_3, 0.002 of $FeSO_4$, 0.004 of $CaCl_2$, 0.2 g of yeast extract, 1.5 g of bacto agar, and 0.1 g of glucose. These materials were put into 100 mL distilled water (Khalila et al. 2020). The medium was sterilized in an autoclave at a temperature of 121°C for 15 minutes and poured into a sterilized petri dish.

A total of 1 g of mangrove soil sample was weighed and diluted from 10^{-1} to 10^{-6} dilution using a physiological solution of NaCl. Subsequently, 1 mL of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were spread on the selective medium enriched with 1% CMC (carboxymethyl cellulose) using a scattering rod in duplicate incubated at 30°C for 48 hours. Since the dilution series before 10^{-3} and after 10^{-6} will not give accurate results (Benard et al. 2015), the bacteria that grew at the 10^{-6} dilution were further purified. Subsequently, bacterial isolates were purified on CMC selective medium using the streak quadrant method and incubated at 30°C for 24 hours. Then, streaking was repeated to obtain a single and pure isolate colony. The purified isolates were characterized and included macroscopic and microscopic observations. The macroscopic observations included a colony, margin/edge, elevation, and pigmentation, while microscopic observations comprised bacteria cell shape and gram staining. The isolates were characterized at the Microbiology Laboratory, Biology Department, FMIPA, Universitas Syiah Kuala (USK).

Qualitatively enzyme assay

The pure isolates of cellulolytic bacteria obtained were tested qualitatively to determine their abilities to produce cellulase enzymes using the spot technique. The isolates were inoculated in the selective medium and incubated at 37°C (Biswas et al. 2020) for 24 hours, and the cellulolytic activity was carried out using the Congo Red method. The plates were stained with 1% (w/v) Congo Red and allowed to stand for 15 minutes to observe clear zone formation (Khalila et al. 2020). Subsequently, the plates were washed with 2M NaCl and incubated for 24-48 hours to complete the formation of the clear zone. The bacteria isolate the formation of a clear zone around the colony indicated that decomposed CMC. The diameter of the clear zone formed was measured with a digital caliper. The cellulolytic index (CI) was calculated by comparing the value of the clear zone diameter with the colony diameter (Kasana et al. 2008). The value of CI is categorized as low when ≤ 1 cm, medium at 1-2 cm, and high when ≥ 2 cm (Choi et al. 2005). The CI for each purified isolate obtained was calculated using the equation below (Bradner et al. 1999):

$$\text{Cellulolytic Index (CI)} = \frac{\text{clear zone diameter (cm)-colony diameter (cm)}}{\text{colony diameter (cm)}}$$

Enzyme activity and specific enzyme activity (Quantitatively enzyme assay)

Based on glucose levels, the crude extract was used to test the cellulase activity at Microbiology Laboratory, Biology Department, Institut Pertanian Bogor (IPB) University. The glucose levels were measured using a 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The enzyme production was started by inoculating one loop of rejuvenated bacteria colonies into 1% CMC liquid media, which were incubated for 24 hours. Subsequently, one ml of bacteria culture was inoculated into 100 mL of 1% CMC liquid media and incubated for 54 hours at room temperature in a shaking incubator. Finally, the samples were centrifuged at room temperature for 10 minutes at 10,000 rpm. The supernatant was used as a crude enzyme extract (EEK), and as many as 0.5 mL of EEK and 0.5 mL of CMC substrate were added to measure its activity using a DNS reagent. In total, 2 mL (0.5 mL of EEK, 0.5 mL of CMC, and 1 mL of DNS) sample of bacterial culture was taken each 6 hours intervals for 54 hours. The substrate used to measure cellulase activity was 1% CMC dissolved in 0.1 M phosphate buffer pH 7.0 and incubated for 30 minutes. Approximately 1 mg mL^{-1} of glucose was used as standard sugar (the control); the standard will be used as a reference to determine the amount of glucose formed from the breakdown of the cellulase enzyme. The reducing sugar produced from the reaction was calculated from the Optical Density (OD) measured by a spectrophotometer with a wavelength (λ) of 540 nm. Meanwhile, the cellulase enzyme activity was calculated using the formula below:

$$\text{Cellulase activity (U/mL)} = \frac{\text{Xsample - Xcontrol x Diluent Factor x 1000}}{\text{Molecular weight of glucose x incubation time}}$$

Where; Xsample is the glucose concentration of the sample (mg mL^{-1}), Xcontrol is the glucose concentration of the control (mg mL^{-1}), and the molecular weight of glucose is the 180.156 g mol^{-1}

The specific activity of the cellulase was calculated by comparing the enzyme activity to the total protein concentration. Meanwhile, the enzyme's total protein content was determined using the Bradford method (Kruger 2002). In this study, the standard curve of protein was prepared using various Bovine Serum Albumin (BSA) concentrations. When reacting the sample with Bradford's reagent, total protein content was calculated by observing the optical density (OD). Then, the specific activity was calculated using the equation:

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme Activity (U/mL)}}{\text{Total protein (mg/mL)}}$$

Effect of salinity, pH, and carbon source on cellulolytic bacteria

One mL of two days of cellulolytic bacteria CMC culture was inoculated into media containing different salinities, pH, and carbon sources. Treatment for salinities were 0% (without the addition of NaCl), 2.5% (2.5 g of NaCl), 5% (5 g of NaCl), 7.5 % (7.5 g of NaCl), 10% (10 g of NaCl), while for carbon sources were 0.2 g of glucose, fructose, and sucrose, respectively. Furthermore, 0.5 mL bacteria of CMC culture was inoculated into each Erlenmeyer tube containing various pH concentrations. The test media with different pH concentrations were made by adding 1% HCl or NaOH to form a liquid CMC solution with a pH of 2, 4, 6, 8, and 10. From now on, each Erlenmeyer containing different treatments was covered with sterile cotton, sealed using parafilm, and incubated for 2 days at 37ºC using an orbital shaker at 150 rpm. The growth of each isolate was observed by measuring the optical density (OD) using a spectrophotometer at a wavelength (λ) of 581 nm.

DNA sequencing and phylogenetic tree construction

Molecular identification was carried out at Riset Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala (USK). The 16S rRNA gene amplification and sequencing were investigated into two isolates with high cellulolytic index (CI). Meanwhile, the 16S rRNA gene sequence is the most common genetic marker applied for almost all bacteria due its function has not changed over time (Manjul and Shirkot 2018). The isolated DNA was extracted separately using the commercial gDNA Presto™ Bacteria Mini kit (Geneaid) with slightly modified. Approximately 50 µL ~ 200 µg mL^{-1} of total pure DNA was eluted and adopted as a template for testing polymerase chain reaction (PCR) (Sari et al. 2017). The tubes containing pure DNA were stored at -20°C to prevent degradation and were used in the amplification stage by PCR. The primers adopted for PCR were 63F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1387R 3' (TAC GGY TAC CTT GTT ACG ACT T) 5', which amplified 16S rRNA gene that has a length about 1,500 bp. A 30 ng of DNA was added to a total of 25 µL of the reaction mixture consisting of 10 pmol of each primer containing 12.5 µL of the master mixture (KAPA Biosystems, Boston, MA, USA)

The PCR amplification stage was carried out for 25 cycles, and the process was conditioned at a pre-denaturing step at a temperature of 95°C for 5 min. Further, it was denatured with 25 cycles at 95°C for 1 min, 50ºC annealing for 30 s, and 72°C extensions for 2 min with a final step at 72°C for 10 min. The amplification product was checked for purity and sized by 1.2% (w/v) agarose gel by electrophoresis in 1 x Tris-Acetate-EDTA (TAE) buffer pH 8.3 (40 mM Tris-HCL, 40 mM acetate, 1.0 mM EDTA and analyzed with Gel Doc XR+System (BIO-RAD). The amplification product was sequenced by Macrogen Inc., Korea, using Dye Terminator (3'-dye labeled dideoxy nucleotide triphosphate).

The sequencing results were used to determine the similarity of DNA sequences with bacteria DNA using the basic local alignment search program tool (BLAST). Meanwhile, the data on the GenBank database was adopted to analyze the 16S rRNA gene sequence homology using the BLAST program at the National Center Biotechnology Information/NCBI (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequence was aligned and analyzed using the CrustalW program, and a phylogenetic tree was constructed applying the MEGA X version (Kumar et al. 2018). The phylogenetic tree was created using the Maximum Likelihood (ML) method with 1000x bootstrap with the Hasegawa-Kishino-Yano model (Kumar et al. 2012). Moreover, some sequencing results of *Bacillus* sp. from NCBI were aligned and included in the phylogenetic tree for comparison

RESULTS AND DISCUSSION

Characterization of cellulolytic bacteria

Based on morphological characteristics, 39 isolates were successfully isolated from mangrove soil, consisting of 17 isolates from rehabilitated and 22 isolates from unrehabilitated mangroves. A total of 9 isolates were obtained at locations 1, followed by 4 isolates from locations 2 and 3, while 5, 10, and 7 isolates were obtained from locations 4, 5, and 6, respectively. Locations 1, 2, and 3 were in mangrove rehabilitated dominated by *Rhizophora* sp., while locations 4, 5, and 6 were in the unrehabilitated, with *Rhizophora* sp., *Avicennia marina*, and *Sonneratia alba.* as the common species. The soil of the rehabilitated mangrove contained more sand fraction than the other location. The macroscopic and microscopic characteristics were used to differentiate the isolates. The macroscopic characterization showed that the colonies were irregular, circular, and filamentous shapes, and the edges were undulate, lobate, entire, and filamentous forms (Figure 2). Based on macroscopic observations, the predominant characteristics of the 39 purified isolates were an irregular shape, undulate edge, raised elevations, and cream pigmentation. These results were similar to the report reported by Khalila et al. (2020), where more colonies were obtained with an irregular shape, raised elevation, and cream color. Chantarasiri (2015) discovered that the colony pigmentations vary from white to cream, yellow, and pale brown.

The microscopic characterization was carried out by gram staining and bacterial cell shape. The result showed that the isolates were categorized as gram-positive and negative, where BTM512, BTM513, BTM521, BTM531, BTM612, BTM613, BTM621, BTM631 were gram-negative, and others were positive. Gram staining is the most widely used technique for classifying bacterium (Becerra et al. 2016). In this study, it was shown that the gram-positive were the most commonly cellulolytic bacteria. That is in line with Behera et al. (2014), who reported that almost all isolates are gram-positive bacteria. A previous study discovered that the bacterium could retain the purple color through gram staining of crystal violet during decolorization. In contrast, gram-negative bacteria are red due to the absorption of red color from safranin and lose the purple color of crystal violet (Oje et al. 2012). Based on the cell shape, the isolates of BTM312 and BTM512 were cocci cells form, while BTM511, BTM513, BTM521, BTM531, BTM612, and BTM631 were spiral cells form, and others were bacilli (Table 1), and the most common type found in the study location was bacilli cell form. All cellulolytic bacteria isolated from Peatland, Kubu Raya District, Indonesia, had a rod (bacilli) cell shape (Kotimah et al. 2020). Meanwhile, Haldar and Nazareth (2018) discovered that the cocci (28.3 %) cell forms were generally fewer than the bacilli/rods (71.6 %).

Qualitative enzyme assay

Bacteria isolates from mangrove soil produced cellulase enzymes by showing the clear zone (Figure 3). In this study, the 22 isolates that showed a clear zone (halo zone) from 39 isolates had been successfully purified as cellulolytic bacteria. The highest number of isolates producing cellulase enzymes were discovered at location 1, with 6 isolates, followed by location 5, with 4 isolates, and locations 6, 2, 3, and 4, with 4, 3, 2, and 2 isolates, respectively.

Table 1. Macroscopic and microscopic characterization of cellulolytic bacteria

Isolate code	Macroscopic characteristics			Microscopic characteristics		
	Colony shape	**Colony edge**	**Colony elevation**	**Colony pigment**	**Gram**	**Cell shape**
BTM111	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM112	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM113	Irregular	Undulate	Flat	Cream	Positive	Bacilli
BTM121	Irregular	Lobate	Flat	Cream	Positive	Bacilli
BTM122	Irregular	Lobate	Flat	Cream	Positive	Bacilli
BTM123	Circular	Entire	Convex	Cream	Positive	Bacilli
BTM131	Filamentous	Filamentous	Raised	Turquoise	Positive	Bacilli
BTM132	Irregular	Undulate	Flat	Cream	Positive	Bacilli
BTM133	Irregular	Undulate	Flat	Cream	Positive	Bacilli
BTM211	Circular	Entire	Convex	Cream	Positive	Bacilli
BTM212	Irregular	Lobate	Flat	Cream	Positive	Bacilli
BTM221	Irregular	Lobate	Raised	Cream	Positive	Bacilli
BTM231	Filamentous	Filamentous	Raised	Turquoise	Positive	Bacilli
BTM311	Circular	Entire	Convex	Cream	Positive	Bacilli
BTM312	Filamentous	Filamentous	Raised	Turquoise	Positive	Cocci
BTM321	Filamentous	Filamentous	Raised	Turquoise	Positive	Bacilli
BTM331	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM411	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM412	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM421	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM431	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM432	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM511	Irregular	Undulate	Flat	Cream	Positive	Bacilli
BTM512	Irregular	Lobate	Flat	Cream	Negative	Cocci
BTM513	Circular	Entire	Convex	White	Negative	Spiral
BTM514	Circular	Entire	Convex	Cream	Positive	Bacilli
BTM521	Circular	Entire	Convex	White	Negative	Spiral
BTM522	Circular	Entire	Convex	Cream	Positive	Bacilli
BTM523	Irregular	Lobate	Raised	Cream	Positive	Bacilli
BTM531	Circular	Entire	Convex	White	Negative	Spiral
BTM532	Circular	Entire	Convex	Cream	Positive	Bacilli
BTM533	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM611	Filamentous	Filamentous	Raised	Cream	Positive	Bacilli
BTM612	Circular	Entire	Convex	White	Negative	Spiral
BTM613	Irregular	Undulate	Flat	Cream	Negative	Bacilli
BTM621	Circular	Entire	Convex	Cream	Negative	Bacilli
BTM622	Irregular	Undulate	Raised	Cream	Positive	Bacilli
BTM631	Circular	Entire	Convex	White	Negative	Spiral
BTM632	Irregular	Undulate	Raised	Cream	Positive	Bacilli

Note: BTM512: Bacterial soil mangrove; 5: sampling location; 1: 1st depth; 2: repetition. BTM621: Bacterial soil mangrove; 6: sampling location; 2: 2nd depth; 1: repetition.

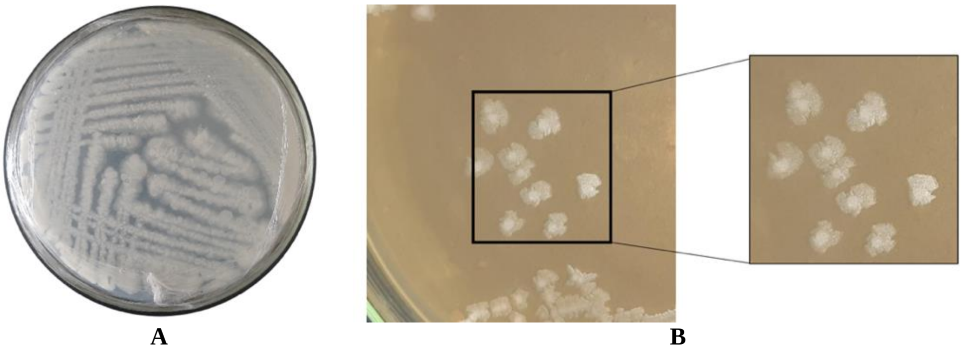


Figure 2. Screening of cellulolytic bacteria. A. Isolate of BTM622, B. Colony morphological characterization of BTM622 isolate

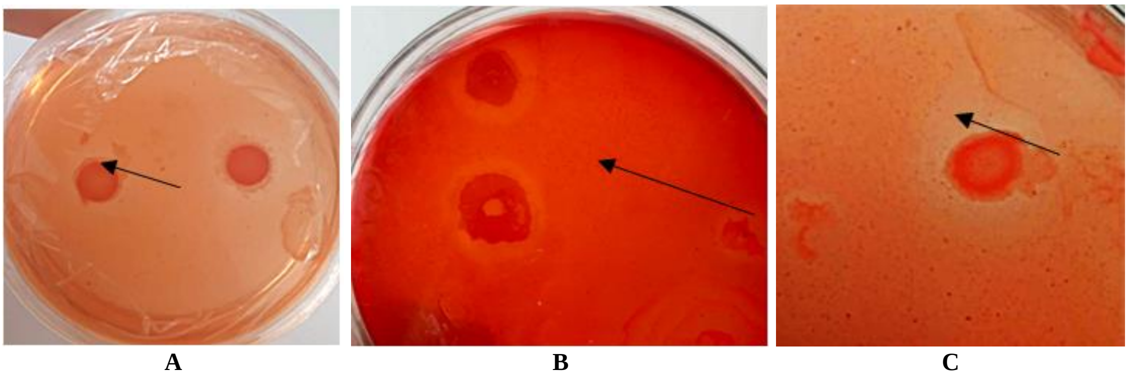


Figure. 3. Selection results of cellulolytic bacteria producing cellulase enzymes. A. Bacteria colony, B. CMC media was flooded with congo red, C. Clear zone

The number of isolates obtained was more than in previous studies, where 15 cellulolytic bacteria were isolated from the mangrove soil of Mahanadi River (Behera et al. 2014), and 7 isolates were from mangrove soil in Tropical mangrove, North Malaysian (Naresh et al. 2019). However, the number of isolates obtained was similar to Kurniawan et al. (2018a), who reported 22 isolates of cellulolytic bacteria from the mangrove of South Bangka, Indonesia. The cellulolytic bacteria in the studied area were less than the number of cellulose-degrading bacteria from water and sediment (35 isolates) of Uppanar estuary, India (Kalaiselvi et al. 2013).

The cellulolytic activity is indicated by the ability of bacteria to hydrolyze CMC substrates. Bacterial colonies that hydrolyze CMC will form a clear zone (halo zone) around the colony after being soaked in 1% (w/v) Congo red, which does not color the bacteria but the culture media. Congo red staining is commonly used in many studies to screen cellulase microorganisms (Liang et al. 2014). The clear zone was produced by 1% Congo red solution because it cannot bind to the media without β-1,4glycosidic bonds contained in cellulose polymers (Jo et al. 2011). Each isolate has different clear zone (halo zone) sizes, which indicates that the activity of cellulolytic bacteria with the largest clear zone diameter also has high cellulase enzyme activity.

Table 2 shows the cellulolytic index (CI) obtained from each isolate that produces cellulase enzymes, where the CI ranged from 0.31 to 4.82. The highest CI was BTM533 isolate (4.82), followed by BTM622 isolate (2.09), and the highest average CI was in location 5 (1.38), located in mangrove unrehabilitated. Behera et al. (2014) recorded CI values of 1.18 - 2.5 from the bacteria isolates from the mangrove soil of the Mahanadi River Delta. Naresh et al. (2019) stated that the CI of bacteria isolates KFY-40 from mangrove soil was 3.40 ± 0.01, which has the highest cellulolytic index. Moreover, Karthika et al. (2020) reported *Bacillus toyonensis* showed cellulose activity of 10 mm, followed by *Bacillus anthracis* as many as 0.8 cm. According to Choi et al. (2005), the CI is low when the value of CI ≤ 1, medium when 1-2, and high when ≥ 2. The difference in CI values could be caused by different types of isolates with the ability to produce cellulase enzymes. The greater the hydrolysis capacity, the higher the cellulase enzymes production by isolate culture (Naresh et al. 2019). The ability of bacteria to degrade cellulose varies based on the type of bacterium strain. Sometimes, cellulase activity was not detected in various liquid media containing CMC and other cellulose materials, thus showing the enzyme concentration produced by this strain is very low, or the ability of the strain to secrete cellulase is weak (Liang et al. 2014).

The diameter of clear zones is generally larger than that of the colony because the cellulase enzyme is secreted into the surrounding environment by cellulose-degrading bacteria. Moreover, the clear zone's size indicated the cellulolytic bacteria's potential in the cellulose decomposition process (Meryandini et al. 2009). Both isolates BTM533 and BTM622 had high CI, which showed that the isolates could degrade the carboxymethyl cellulose, indicating a high ability of extracellular cellulase production. Therefore, they were selected as bacteria candidates that potentially produce cellulase.

The BTM533 and BTM622 with a higher CI as qualitative assay than other isolates were isolates from unrehabilitated mangrove soil. The highest average of CI at location 5 was assumed due to the soil moisture and soil organic carbon (SOC) content, followed by location 6. Both locations had medium moisture and higher organic carbon than other locations. Based on previous research was done by Dewiyanti et al. (2021), the average soil organic carbon and soil moisture in locations 5 and 6 were 1.35%; 6.31%, and 1.17 %; 6.65%, respectively.

Extracellular enzyme production and activity interacted with the quantity and quality of substrates such as soil temperature, moisture, organic carbon, nitrogen, and pH soil. Soil moisture and temperatures will affect plant growth and soil organic matter input, affecting soil microbe communities and activities (Yang et al. 2018). These showed that enzymatic processes by microorganisms are associated with their environment. Furthermore, organic matter in the marine ecosystem is affected by extracellular enzymes (Orsi et al. 2018).

Enzyme activity and specific enzyme activity (Quantitatively enzyme assay)

Measurement of cellulase enzyme activity was quantitatively tested on BTM533 and BTM622. The incubation time for the cellulase activity was 2.5 days (54 hours) to obtain the optimum time for producing the cellulase enzymes. The measurements were carried out 9 times at the 6th, 12nd, 18th, 24th, 30th, 36th, 42nd, 48th, and 54th hours (h). The quantitative assay showed that the cellulase and specific cellulase activities of BTM533 isolate were highest at the 36th incubation time with the value 0.003 U mL^{-1} and 0.042 U mg^{-1}. The isolate BTM622 had cellulase and specific cellulase activity higher than BTM533 with the value 0.013 U mL^{-1} and 0.129 U mg^{-1} at 42 hours. Commonly, the enzyme activities decreased until the end of the observation time.

BTM533 isolate started producing the cellulase and specific cellulase at 6 hours with the value of 0.001 U mL^{-1} and 0.011 U mg^{-1} (Figure 4); however, the BTM622 isolate started to produce enzyme at 12 hours with the value of specific cellulase activity was 0.002 U mg^{-1} (Figure 5). The cellulase activity and specific cellulase activity of BTM533 fluctuated due to the physiological characteristic of the bacteria. Between 24 hours and 30 hours, the glucose availability in the medium was sufficient to support the bacteria growth, proved by decreasing the cellulase activity. Following time, the bacteria secrete more enzymes to break down cellulose into glucose and provide food for bacteria. There are several peaks in the exponential phase due to the repeated secretion of enzymes produced by bacteria into the substrate (Warly et al. 2019). Nutrition composition in the medium is related to cellulase production (Goyal et al. 2014), and nutrition optimization can increase cellulase activity produced by bacteria (Sadhu et al. 2014).

The specific cellulase of BTM622 was highest with the value 0.129 U mg^{-1} at 42 hours, and it decreased sharply to 0.072 U mg^{-1} at the end of incubation time (54 hours). Enzyme activity fluctuated due to different cell growth phases and incubation time. When the enzyme activity increases, cell growth will experience an exponential phase; otherwise, when the enzyme activity decreases, the cell has reached a stationary phase and is followed by a death phase. Furthermore, the presence of catabolic repression decreases enzyme activity, and repression catabolic is a condition where cells use glucose as a carbon source, suppressing enzyme production (Abalos et al. 1997).

Each isolate has a different ability to produce cellulase enzyme hourly, decreasing cellulase production due to the limitation of nutrition and the effect of other enzyme products (Dos Santos et al. 2012). For example, Iqbalsyah et al. (2019) reported that the bacteria cellulase activity from *Geobacillus* isolated from a geothermal Jaboi, Weh Island was low at 14 hours (0.29 U g^{-1}) but increased significantly between 38 hours and 48 hours (0.57 U g^{-1} to 1.62 U g^{-1}). That showed that the cellulase enzyme activity in this study was lower than the previous reports, such as Sonia and Kusnadi (2015), who obtained the highest cellulase activity of 0.08 U mL^{-1} at 24 h from isolate OS-16 isolated from Bromo-Tengger desert. The highest cellulase activity was 1.74 U g^{-1} at 72 hours, and specific cellulase activity was 0.44 U mg^{-1} at 48 hours from *Geobacillus* bacteria isolated from Geothermal, Weh Island (Iqbalsyah et al. 2019). Khotimah et al. (2020) also reported that the cellulase activity of SB1.1.1 isolate had the highest enzyme activity (11.17 U mL^{-1}) isolated from Teluk Bakung Peatland, Kubu Raya District, Indonesia.

Meanwhile, the four phases of bacteria growth, namely the lag, the log (exponential), stationary, and the death phase, affected the enzymes produced. The result showed that the lag phase occurred from 6 to 12 hours when the bacteria were still adapting to the new environment. The phase where the bacteria growth begins to change at 30 hours and 36 hours significantly was called the log phase. At this phase, the bacteria begin to replicate DNA, and cells begin to grow, which takes place rapidly up to twice the original number (Rolfe et al. 2012). The BTM533 and BTM622 enter the death phase after 36 hours and 42 hours of incubation, where the number of dead cells is more than that of living cells. An increase in enzyme production was associated with increased cell growth, which indicated that cellulolytic bacteria actively used cellulose during the growth phase (Seo et al. 2013).

Specific cellulase activity included protein concentration (mg mL^{-1}). The protein concentration of isolate BTM533 and BTM622 ranged from 0.000 to 0.075 mg mL^{-1} and 0.078 and 0.197 mg mL^{-1} (Table 3). The highest specific cellulase was 0.129 U mg^{-1} produced by BTM622 isolate at 42 hours, and it also had the highest protein concentration (0.197 mg mL^{-1}). The specific cellulase activity and protein concentration fluctuated assumed due to the enzyme sample containing cellulase proteins and other proteins, which are also measured because the sample is still a crude extract enzyme that has not been purified (Murtiyaningsih and Hazmi 2017). Therefore, the enzyme still contains other components or proteins that are inhibitors that can interfere with the function of the enzyme.

Effect of salinity, pH, and carbon source on cellulolytic bacteria

BTM533 isolate had a good tolerance to salinity concentration (2.5%) with an optical density (OD) value of 0.688, followed by 5% salinity with an OD value was 0.612. BTM622 had the highest OD (0.354) at the salinity was 5%, followed by 10% with an OD was 0.297. BTM533 and BTM622 isolates grew well at 2.5% and 5% salinity concentrations added into the media culture (Figure 6.A). Different salt concentrations will affect the growth of cellulolytic bacteria, and each species has adapted to salt levels in their environment. A previous study reported that the highest cellulase activity was found at 10% salt concentration (0.246±0.031 U mL^{-1}) (Rachamontree 2017).

Furthermore, Fitri et al. (2021) found that actinobacteria had the highest optical density (OD) at 10% and 5% salinity concentrations. Measurement using OD value can provide the value of bacterial population growth and enzyme activity, and it has significantly dealt with bacterial samples in high cell abundances. Estimating microbial growth parameters based on absorbance measurements as optical density has advantages, i.e., fast and relatively easy to obtain compared to the counting technique (Dalgaard and Koutsoumanis 2001). The higher the absorbance value, the more bacteria growth. BTM533 and BTM622 are cellulolytic bacteria isolated from mangrove soil with the DHL (salinity) contained was 10 and 7.7 µscm^{-1} (5.627 ppt and 4.25 ppt).

Table 2. Colony diameter, clear zone diameter, and cellulolytic index of cellulolytic bacteria isolated from mangrove soil

Isolate codes	Clear zone diameter (cm)	Colony diameter (cm)	Cellulolytic index (CI)
BTM111	0.81 ± 0.06	0.28 ± 0.21	1.88
BTM113	0.90 ± 0.43	0.33 ± 0.06	1.75
BTM121	1.01 ± 0.05	0.64 ± 0.04	0.58
BTM122	1.20 ± 0.07	0.84 ± 0.07	0.43
BTM123	0.53 ± 0.09	0.26 ± 0.06	1.06
BTM133	1.27 ± 0.11	0.96 ± 0.11	0.33
BTM211	0.46 ± 0.65	0.16 ± 0.22	1.97
BTM212	0.99 ± 0.25	0.39 ± 0.08	1.56
BTM221	1.03 ± 0.47	0.79 ± 0.43	0.31
BTM321	0.75 ± 0.05	0.30 ± 0.02	1.53
BTM331	0.71 ± 0.25	0.46 ± 0.06	0.55
BTM431	1.04 ± 0.01	0.56 ± 0.06	0.86
BTM432	0.39 ± 0.54	0.21 ± 0.08	0.83
BTM511	1.05 ± 0.16	0.63 ± 0.06	0.63
BTM512	0.98 ± 0.08	0.45 ± 0.21	1.20
BTM514	1.28 ± 0.03	0.92 ± 0.08	0.40
BTM533	0.99 ± 0.03	0.17 ± 0.04	4.82
BTM611	0.67 ± 0.09	0.32 ± 0.01	1.08
BTM613	0.82 ± 0.10	0.46 ± 0.09	0.81
BTM621	1.18 ± 0.18	0.69 ± 0.07	0.71
BTM622	0.84 ± 0.32	0.27 ± 0.17	2.09
BTM632	1.05 ± 0.28	0.61 ± 0.26	0.74

Table 3. Protein concentration of BTM533 and BTM622

Incubation Time (h)	Specific cellulase (U mg^{-1}) BTM533	Protein concentration (mg mL^{-1}) BTM533	Specific cellulase (U mg^{-1}) BTM622	Protein concentration (mg mL^{-1}) BTM622
0	0.000	0.000	0.000	0.080
6	0.011	0.068	0.000	0.078
12	0.005	0.069	0.002	0.100
18	0.010	0.075	0.019	0.086
24	0.026	0.054	0.032	0.089
30	0.003	0.065	0.042	0.088
36	0.042	0.066	0.048	0.100
42	0.000	0.080	0.129	0.197
48	0.000	0.066	0.106	0.093
54	0.000	0.051	0.072	0.091

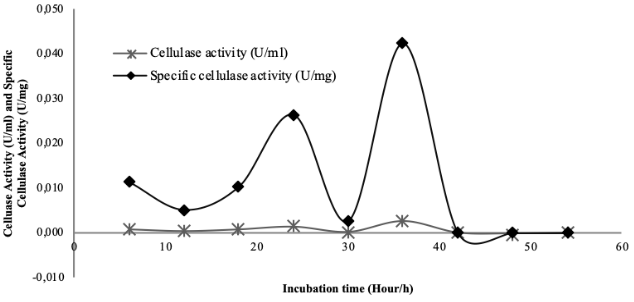


Figure 4. Cellulase activity and specific cellulase activity of BTM533 isolate

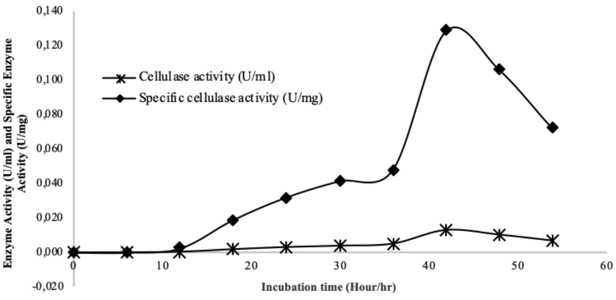


Figure 5. Cellulase activity and specific cellulase activity of BTM622 isolate

The OD value decreased with increasing NaCl concentrations. In this study, both isolates showed salt tolerance of up to 5%, probably because the bacteria are grown on selective and nutrient-rich media, where these media hinder the effect of NaCl. It is possible that NaCl will bind with other organic materials in the culture media, thus reducing the effect of NaCl on bacterial growth. In addition, the rate of bacterial growth in nutrient-rich media depends on the concentration of nutrients in it and on the environment that limits these nutrients (Agwu et al. 2017).

Furthermore, the highest optical density (OD) of BTM533 and BTM622 were at pH 6, and the value were 1.055 and 0.916, respectively. pH is assumed to be an important parameter influencing enzyme activities and bacteria growth. pH in the present study reported that cellulolytic bacteria grew luxuriantly at pH 6 and followed at pH 8. The pH ranged from 3 to 10 was the maximum cellulase activity (Liang et al. 2014), and the optimal growth of actinobacteria pH ranged from 6 to 8 (Akond et al. 2016). Furthermore, Dobrzy'nski et al. (2022) recorded the highest cellulase activity of *Bacillus* sp. 8E1A strain was at pH 7.0 (0.520 U mL^{-1}). Dissimilar to Dar et al. (2021), *B. altitudinis* RSP75 showed good cellulolytic activities at pH 5.0, which was the suitable pH for cellulose degradation.

Three types of carbon sources were treated for this study: glucose, fructose, and sucrose. The highest optical density (OD) was bacterial media added sucrose for both isolates, and the value were BTM533 (0.717) and BTM622 (0.738) and followed by dextrose as much as 0.712 and 0.735. The type of sugars such as glucose, fructose, and sucrose influences bacterial cellulolytic production. A recent study explained that sucrose is a good carbon source for growing cellulolytic bacteria, as shown by higher optical density than glucose and fructose. Castro et al. (2012) have explained that bacterial cellulose production is affected by carbon source type and concentration, and each bacterium has a different reaction to the carbon source in their culture media. Furthermore, the treatment carried out was not only media with sucrose added, but glucose and fructose were also determined to measure the utilization of carbon sources. Sucrose is a disaccharide consisting of glucose, fructose, and bacteria break down sucrose to metabolize it (Ramírez et al. 2017). Glucose, fructose, sucrose, mannitol, and maltose were reported to be suitable carbon sources for cellulase production (Premalatha et al. 2015). Each cellulolytic bacterium produces a cellulase enzyme complex that varies based on the genes possessed and the carbon source used (Meryandini et al. 2009).

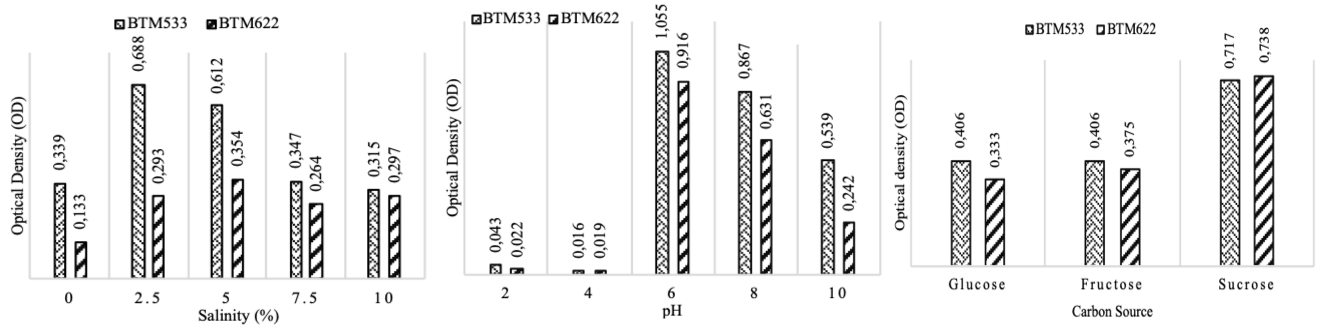
DNA sequencing and phylogenetic tree of BTM533 and BTM622

DNA sequencing and a phylogenetic tree were analyzed for 2 isolates with higher cellulolytic index than other isolates. In this study, the phylogenetic tree was inferred using the Maximum Likelihood (ML) method showing two clusters. The percentage of replicate trees, where the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method, and the rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

This analysis involved 13 nucleotide sequences, including 11 sequences from the NCBI GenBank. The phylogenetic tree showed that the isolate of BTM533 related to *Bacillus safensis* and BTM622 was *Bacillus altitudinis*. Comparison of the 16S rRNA gene showed that strain BTM533 was closely related to 95% similar identity to *B. safensis* strain P-NA1-2, *B. safensis* strain IMJ7, *B. safensis* strain SH10, *B. safensis* strain MK-12.1, and *B. safensis* strain LSRBMoFPIKRGCFTRI40, while strain BTM633 a revealed similarity of 99% to *B. altitudinis* strain MY.d, *B. altitudinis* strain B1-2a, *B. altitudinis* strain GOES10, *B. altitudinis* strain L1, and *B. altitudinis* strain NPB34b. *Pseudomonas aeruginosa* was clustered as an outgroup in the different branches. The phylogenetic tree is depicted in Figure 7. The 2 isolates were deposited in the GenBank NCBI database under the accession numbers OP363153 and OP363154 (Table 4).

Table 4. Genetic identification of 2 bacteria cellulolytic and their accession number

Bacterial isolate	Identified species	GenBank acc. number
BTM622	*B. altitudinis* strain BTM622	OP363153
BTM533	*B. safensis* strain BTM533	OP363154



A B C

Figure. 6. Growth pattern of BTM533 and BTM622 isolates in liquid media with different concentrations (A) salinity (%), (B) pH, and (C) carbon sources

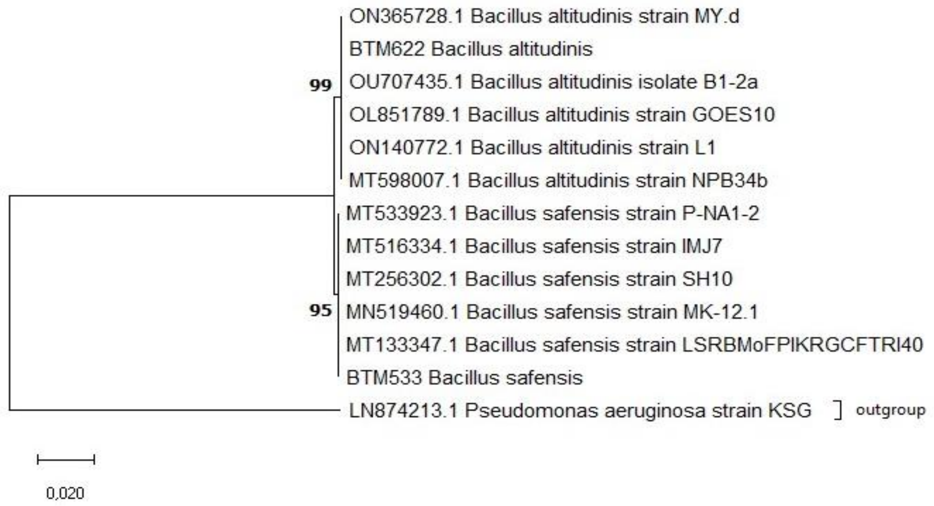


Figure 7. Phylogenetic tree of BTM533 and BTM622 isolates relationship to sequences from other bacteria in the same species

Both BTM533 and BTM622 isolates were closely related to *B. safensis* and *B. altitudinis*. Dobrzy'nski et al. (2022) reported the potential lignocellulose identified by 16S rRNA gene sequencing was *Bacillus* (strain 8E1A) which had the highest size of the clear zone (25 mm). Bacteria produced from mangrove leaf litter are diverse, but the most dominant species was *Bacillus* sp. (Yulma et al. 2017). Furthermore, *Bacillus* sp. plays a vital role in this area's decomposition process of mangrove leaf, litter, and dominant genus (Yahya et al. 2014). Approximately 20.83% of the total soil bacteria, i.e., *Bacillus anthracis* strain ALA1, *Bacillus cereus* strain ALA3, and *Bacillus thuringiensis* strain ALA5 isolates from three regions produced amylase, cellulase, and inulinase and they have maximum enzyme activity (Aarti et al. 2020). Meanwhile, *Bacillus* sp. can produce cellulase enzymes in small and large quantities to degrade cellulose (Meryandini et al. 2009). In the fisheries aspect, *B. safensis* strain NPUST1 increases the digestive ability of fish, such as growth, weight gain, and feed efficiency (Wu et al. 2021). Furthermore, the role of the *Bacillus* sp. as probiotic bacteria can enhance the growth and immune function of aquatic biota and help maintain the density of beneficial bacteria (Soltani et al. 2019).

B. safensis was categorized into *B. pumilus* group and closely related to *B. altitudinis*, *B. xiamenensis*, and *B. invictae*, the living habitats of those bacteria include terrestrial and marine environments (Lateef et al. 2015). *B. safensis* is a bacterium that has good physiological adaptations, which allows it to survive even in diverse and extreme environments and may have a severe impact on other bacteria; it can live and thrive in a wide variety of habitats, i.e., salt deserts, industrial waste, oil-contaminated sites, plants, and animal waste, as well as soil (Liu and Avendaño 2013; Kothari et al. 2013). Meanwhile, *B. safensis* can produce a source of industrial enzymes such as amylase (Kothari et al. 2013), cellulase (Khianngam et al. 2014), protease (Berrada et al. 2012), and lipase (Kumar et al. 2014). A present study reported that *B. safensis* (BTM533) has high growth in 2.5% NaCl. However, this bacterium could still grow at a salinity of 10% NaCl, and pH 6, as Kothari et al. (2013) conveyed that *B. safensis* strain VK is a salt-tolerant and can survive and grow in 14% NaCl and the pH range from 4 to 8. Moreover, Khianngam et al. (2014) stated that PH27 isolate related to *B. altitudinis* has a cellulolytic index (CI), which was 2.80, showing the cellulase activity of 0.170±0.004 U mL^{-1}. These values are almost similar to this study, where the CI was 2.09, and the cellulase activity was 0.129 U mL^{-1} in the same species identified molecularly. Also, *B. altitudinis* strain RSP75 showed the highest cellulolytic activities (2.8 cm) identified by 16S rRNA gene sequencing (Dar et al. 2021).

In conclusion, out of the 22 isolates that showed clear zone and cellulase activity, 39 were purified from mangrove soil. The morphology of bacteria colonies was dominated by irregular colony shape, undulate colony edge, raised colony elevation, and cream pigmentation. The bacilli cell form and gram-positive were common microscopic characteristics obtained in both rehabilitated and unrehabilitated mangroves. Furthermore, cellulolytic bacteria that were isolates from mangrove soil had low, middle, and high cellulolytic indexes. Cellulase produced by cellulolytic bacteria was higher in unrehabilitated areas than in rehabilitated areas because carbon organic was highest in unrehabilitated mangroves. The assay observation proved that the best salinity, pH, and carbon sources for cellulase production were recorded at 2.5%-5% salinity, 6 pH, and sucrose as carbon sources, respectively. The two isolates with high cellulase activity are closely related to *Bacillus safensis*, which has a similarity of 95% and a similarity of 99% belonging to *Bacillus altitudinis* based on 16S rRNA gene sequencing. Both isolates from mangrove unrehabilitated. However, they had low cellulase activity and specific cellulase activity in the quantitative assay.

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