Analyzing cellulolytic bacteria diversity in mangrove ecosystem soil using 16 svedberg ribosomal ribonucleic acid gene

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BACKGROUND AND OBJECTIVES: Soil is an essential abiotic component serving as a habitat for numerous organisms, including cellulolytic bacteria commonly found in mangrove ecosystems. This bacteria could produce active enzymes needed to improve environmental quality by accelerating the organic matter decomposition. The unique mangrove environment may contain new types of cellulolytic bacteria with new characteristics. Despite several mangrove areas being explored as sources of cellulolytic bacteria, there is currently unexplored data on its diversity in Aceh Province, Indonesia. Accordingly, it is necessary to analyze the molecular biological approach, namely the 16 svedberg ribosomal ribonucleic acid gene, to identify the diversity of cellulolytic bacteria and analyze the phylogenetic relationships between them.

METHODS: Bacteria isolates were collected from mangrove soil at six research locations with three replications. A purposive sampling method was applied to determine the research location. Isolates from soil samples were streaked and purified in carboxymethyl cellulose as selective media for cellulolytic bacteria. Molecular identification adopted 16 svedberg ribosomal ribonucleic acid gene sequencing, and the sequencing data were matched with GenBank data. Phylogenetic analysis and genetic distance between species were evaluated using molecular evolutionary genetics analysis.

FINDINGS: Thirteen isolates were sequenced, and nine species of cellulolytic bacteria dominated by the Bacillus genus were identified. These species exhibited an identity value of 97.77–100 percent when compared to data from GenBank, and B. velezensis was found to have a close relationship with B. amyloliquefaciens at a value of 0.002 percent. Interestingly, the non-rehabilitated mangrove areas had more bacterial species than the rehabilitated ones. Two Bacillus genus had different nucleotide bases, proving they were distinct species.

CONCLUSION: Nine cellulolytic bacteria species were identified; the two closely interspecies genetic distance related were B. velezensis and B. amyloliquefaciens, whereas the farthest were Bacillus sp1. and Bacillus sp2. Small genetic distances of interspecies indicate a close relationship between species. In comparing the two sampling sites, the non-rehabilitated mangrove contains higher bacterial cellulolytic species than the rehabilitated and Bacillus-dominated site. The findings provide valuable insights into the diversity of cellulolytic bacteria in mangrove ecosystems. The abundance of bacterial species could serve as sources of cellulase enzymes with different characteristics, essential in an environmental aquatic management.
INTRODUCTION

The mangrove ecosystem is a unique marine environment characterized by distinctive features, such as high productivity and providing a habitat for diverse aquatic organisms (Thatoi et al., 2013; Hu et al., 2022). Bacteria are the most abundant and diverse microorganisms found in the mangrove ecosystem, and its soil plays a crucial role in providing essential nutrients, such as carbon, nitrogen, and phosphorus, which contributes to high productivity (Becker et al., 2020; Palit et al., 2022; Saneha et al., 2023). However, as a heterotrophic microorganism, bacteria serve as primary decomposers within the ecosystem and are instrumental in decomposing organic matter, mineralization, and nutrient cycling for plant growth (McGuire et al., 2012; Liu et al., 2019; Pringgenies et al., 2023). In mangrove ecosystem, fallen vegetation litter contributes to the abundance of cellulose on the soil surface. These are rich in polysaccharides, such as cellulose and hemicellulose, and are major components of plant cell walls and a primary carbon source in the carbon cycle (Furusawa, 2019). Cellulolytic bacteria can digest cellulose and are widespread microorganisms (Watanabe and Tokuda, 2010). Cellulolytic bacteria is one of the essential bacteria types commonly found in mangrove soil; they contribute carbon sources to improve soil fertility. The diversity of bacteria in the ecosystem serves as an indicator of water fertility, and each species has its unique function and role, such as cellulolytic bacteria, which produces cellulase enzyme that accelerates cellulose degradation (Biswa et al., 2020; Nimnoi and Pongsilp, 2022). Microbial enzymes involved in plant cell wall degradation convert these polysaccharides into digestible components (Ejaz et al., 2021). Bacteria participate directly in the nutrient cycle and provide insight into soil environmental quality through decomposition (Hafich et al., 2012). Studying highly active cellulolytic bacteria is essential to understanding microbial cellulose degradation (Talia et al., 2012). Interest in cellulase enzymes has grown in recent years due to its application in bioenergy and biofuel production, alongside other industries, such as beverage, paper, and textile (Srivastava et al., 2015; Ejaz et al., 2021). Safika et al. (2018) stated that cellulolytic bacteria has been proposed as a cost-effective method for reducing fibrous feed and increasing its digestibility, as opposed to using commercial cellulase enzymes.

In the fisheries sector, cellulose degradation in feed ingredients can enhance fish growth by improving digestibility (Kurniawan et al., 2019). The isolation of cellulolytic bacteria from mangrove organic materials, like sediments and leaf litter, has been reported in several studies (Behera et al., 2014; Yahya et al., 2014; Chantarasiri, 2015; Kurniawan et al., 2018; Ningsih et al., 2014). Cellulolytic bacterial diversity isolated from the mangrove ecosystem using the 16 svedberg ribosomal ribonucleic acid (16s rRNA) gene has been carried out. Pramono et al. (2021) reported the species found in mangrove soil, namely Fictibacillus nanhaiensis; Kurniawan et al. (2019) recorded two species, namely Vibrio parahaemolyticus and Bacillus amyloliquefaciens from mangrove soil. There are three species of cellulolytic bacteria from mangrove soil in Malaysia, namely Anoxybacillus sp., Bacillus subtilis, and Paenibacillus dendritiformis (Naresh et al., 2019). Bacterial diversity can be assessed using two approaches: cultivation and non-cultivation, with metagenomic analysis being a useful tool to overcome the limitations of cultivation-based methods (Glogauer et al., 2011). In studying bacterial diversity in forest soil enriched with cellulolytic bacteria, the 16S rRNA gene sequencing analysis is commonly employed (Talia et al., 2012). This method is useful for analyzing the diversity of microorganisms (Izquierdo et al., 2010). Diverse studies of cellulolytic bacteria in Indonesia’s mangrove ecosystems have been carried out for morphology approach and molecular biology. However, there is no information about the diversity of cellulolytic bacteria in the soil of rehabilitated and non-rehabilitated mangrove areas in Aceh Besar and Banda Aceh, Aceh Province, Indonesia. Those areas have different bacterial populations, productivity, and soil characteristics, including organic carbon (OC) content and soil texture, making those interesting subjects to study. Non-rehabilitated mangrove areas exhibited higher OC content and cellulolytic bacterial populations than rehabilitated ones. Specifically, the OC content and bacterial populations in non-rehabilitated and rehabilitated mangrove areas were 1.21 percent (%) and 0.90%, categorized low and very low OC content (Dewiyanti et al., 2021), and 5.07 × 107 colony form unit/gram (CFU/g) and 3.47 × 107 (CFU/g), respectively. High bacterial diversity can be associated with the stability and fertility of an ecosystem. The suitable environmental management should be applied in mangrove ecosystem to increase
productivity and fertility. One way is to analyze the presence of cellulolytic bacteria and their species richness. Furthermore, the distinct environmental characteristics of mangrove soil harboring cellulolytic bacteria have encouraged several studies to discover new bacterial strains capable of producing cellulase enzymes with unique properties. Therefore, the current study aims to analyze the molecular biological approach, namely the 16S rRNA gene, to identify the diversity of cellulolytic bacteria species and to analyze the phylogenetic relationships between them in the mangrove ecosystems, Banda Aceh and Aceh Besar. This study was carried out in the northern coast of Aceh Province, Indonesia, in 2021–2022.

MATERIALS AND METHODS

Study area

Cellulolytic bacteria isolates were collected from the soil samples in rehabilitated and non-rehabilitated mangrove areas in the northern coast of Banda Aceh and Aceh Besar, Aceh Province, Indonesia. The rehabilitated mangrove is the vegetation planted after the tsunami catastrophe in 2004, while the non-rehabilitated is the ecosystem that was not destroyed by the tsunami. The rehabilitated mangroves were dominated by *Rhizophora* sp., but the three common species in the non-rehabilitated ecosystem included *Rhizophora* sp., *Avicennia marina*, and *Sonneratia alba*. Identifying these isolates was performed through molecular biological techniques, specifically the phylogenetic analysis of the 16S rRNA gene at the Research Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala (USK).

Sample handling

The study involved using bacterial isolates sourced from the soil samples. These isolates were purified and then tested for their ability to produce cellulase enzymes in the rehabilitated and non-rehabilitated mangrove ecosystems. Then, the isolates producing cellulase continued to the molecular biology step. The soil samples were collected from six locations, with three located in the non-rehabilitated area and the remaining in the rehabilitated mangrove region in Aceh Besar and Banda Aceh. Stations one, two, and three were identified in Lambadeuk, Dayah Teungoh, and Gampong Pande (rehabilitated); four, five, and six were in Ruyung, Lamreh, and Gampong Lampanah (non-rehabilitated). The study area’s geographic location and soil sampling location are depicted in Fig. 1. Table 1 explains the location and coordinates of the study area.

Production of carboxymethyl cellulose (CMC) liquid media

The preparation of selective media for cellulolytic bacteria was carried out by weighing specific amounts of various ingredients, such as 1 gram (g) of CMC, 0.02 g of magnesium sulfate heptahidrate (MgSO$_7$H$_2$O), 0.05 g of potassium dihydrogen phosphate (KH$_2$PO$_4$), 0.075 g of potassium nitrate (KNO$_3$), 0.002 g of ferrous sulfate (FeSO$_4$), 0.004 g of calcium chloride (CaCl$_2$), 0.2 g of yeast extract, and 0.1 g of glucose, which were then added to 100 milliliter (mL) of distilled water and placed in an Erlenmeyer flask. Subsequently, one bacterial isolate was introduced into the flask and then centrifuged. The Erlenmeyer flask containing the liquid media was covered with aluminum foil and plastic wrap before transporting to the research laboratory at the Faculty of Veterinary Medicine, USK, for bacterial deoxyribonucleic acid (DNA) extraction.

DNA extraction

This process involved using the commercially available gDNA Presto™ Bacteria Mini kit (Geneaid) to extract the DNA separately. The purified total DNA of 50 microliter (µL), ~200 microgram per milliliter (µg/mL) was eluted and then used as a template for Polymerase Chain Reaction (PCR) testing (Sari et al., 2017). To begin the extraction process, a pellet was resuspended by vortexing in 200 mL of extraction buffer. Subsequently, 20 mL of proteinase K was added to the mixture, which was then incubated at 37 degree Celcius (°C) for 30 minutes (min), with the sample being inverted every 10 min, during the incubation period. The present study outlines a DNA extraction and purification protocol using a column-based method. Initially, 200 g of Genomic bind (GB) buffer was added to the sample, and the mixture vortexed for 10 seconds (s) before being incubated at 70°C for 10 min. The elution buffer is preheated to 70°C for the subsequent step, where 200 mL of absolute ethanol is added to the mixture and lysed using a shaker. The resulting sample is then transferred to a column tube of 2 mL capacity and centrifuged at 14,000–16,000 revolution per min (rpm) for 2 min. The supernatant is then discarded, and the pellet is transferred to a new 2 mL tube. Next, 400 mL of wash1 buffer is added to the column and centrifuged at
Diversity of cellulolytic bacteria in mangrove ecosystems soil

14.000–16.000 rpm for 30 s. The resulting supernatant is then discarded, and the process is repeated using 600 mL of wash buffer (containing ethanol). After the final centrifugation at 14.000–16.000 rpm for 30 s, the column is transferred to a 1.5 mL tube. Following this, preheated elution buffer (30–50 mL) is carefully added to the center of the column, and the DNA tube is allowed to incubate at room temperature for 3–5 min before being centrifuged at 14.000–16.000 rpm for 1 min. Finally, the purified DNA tube is stored at −20°C to prevent degradation until it is used in the PCR for DNA amplification.

**DNA amplification**

A study conducted by Sari et al. (2017) involved obtaining approximately 50 µL of pure DNA at a concentration of 200 µg/mL, which was then utilized as a template for PCR testing. The adopted primers were 63Forward (F) 5’ (AGA GTT TGA TCM TGG CTC AG) 3’ and 1387Reverse (R) 3’ (TAC GGY TAC CTT GTT ACG ACT T) 5’, thereby amplifying the 16S rRNA gene with a length of approximately 1500 base pair (bp). Meanwhile, 30 nanogram (ng) of DNA was combined with a total of 25 mL mixture containing 10 picomole (pmol) of each primer and 12.5 mL of the main mix.

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Table 1: Location and coordinates of the study area

<table>
<thead>
<tr>
<th>No.</th>
<th>Location</th>
<th>Coordinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gampong Lambadeuk</td>
<td>05°32'35.8&quot;(North) N 95°14'30.9&quot;E (East)</td>
</tr>
<tr>
<td>2</td>
<td>Gampong Dayan Teungoh</td>
<td>05°33'50.3&quot;N 95°18'13.3&quot;E</td>
</tr>
<tr>
<td>3</td>
<td>Gampong Pande</td>
<td>05°34'15.3&quot;N 95°18'46.4&quot;E</td>
</tr>
<tr>
<td>4</td>
<td>Gampong Ruyung</td>
<td>05°36'08.2&quot;N 95°29'46.8&quot;E</td>
</tr>
<tr>
<td>5</td>
<td>Gampong Lamreah</td>
<td>05°36'30.0&quot;N 95°32'18.1&quot;E</td>
</tr>
<tr>
<td>6</td>
<td>Gampong Lampanah</td>
<td>05°35'25.1&quot;N 95°40'20.9&quot;E</td>
</tr>
</tbody>
</table>

Fig. 1: Geographic location of the study area along with soil sampling locations with red dot in the mangrove ecosystem
(Kapa Biosystems, Boston, Massachusetts, United States). The PCR amplification process involved 25 cycles and a pre-denaturation temperature of 95°C for 5 min. During each of the 25 cycles, denaturation was performed at 95°C for 1 min, followed by annealing at 50°C for 30 min, and extension at 72°C for 2 min. A final extension step was performed at 72°C for 10 min. In determining the purity and size of the amplification products, 1.2% weight/volume (w/v) agarose gel electrophoresis was used in 1× Tris-Acetate-ethylene diamine triacetic acid (EDTA) buffer (TAE buffer), potential of hydrogen (pH) = 8.3 (containing 40 milliMolar (mM) Tris-hydrochloric acid (HCl), 40 mM acetate, 1.0 mM EDTA), and the Gel Doc XR+ System (Bio-Rad) was used for analysis. TAE is commonly used as a buffer for nucleic acid electrophoresis.

DNA electrophoresis and sequencing
The Gel Doc XR+ System from Bio-Rad was used for analyzing the gel electrophoresis results. The resulting electrophoresis bands were visualized using an ultra-violet (UV) transilluminator, such as the UVITEC Fire-Reader V10-Plus machine. The presence of a single, clear band with a size of relatively 1500 bp indicates good PCR product visualization. PCR products showing satisfactory electrophoresis results were sent to First BASE Laboratories, in Malaysia for further analysis. Cycle sequencing was performed using the PCR Kapa 2G Fast ReadyMix kit with dye, and the reaction product was sequenced with Dye Terminator (3′-labeled dideoxy nucleotide triphosphate).

Identification and Phylogenetic tree construction
To identify the bacteria species, the 16S rRNA gene based on sequencing techniques commonly employed for detecting and classifying bacteria (Ntushelo, 2013). The 16S rRNA gene sequence is the most common genetic marker applied for almost all bacteria due its function has not changed over time, suitable for bacterial classification, and has sufficient variation to distinguish between taxa (Ntushelo, 2013; Manjul and Shirkot, 2018). The Clustal W program in the molecular evolutionary genetics analysis (MEGA X) version was used to align the obtained sequencing results alongside the sequencing data from GenBank (Tamura et al., 2013). The edited sequencing alignments were 1386 bp; it is used to construct a phylogenetic tree with 1000× bootstrap support (Gusakov et al., 2011; Naresh et al., 2019; Sari et al., 2017). However, to confirm the species similarity with existing databases, the Basic Local Alignment Search Tool (BLAST) was used to analyze the obtained sequencing results and determine the similarity between DNA sequencing and that of bacteria. Meanwhile, the BLAST-nucleotide, programmed at the National Center for Biotechnology Information (NCBI), was adopted to analyze the homology of 16S rRNA gene sequencing using data in the GenBank database. Using the kimura-2-parameter (K2P) model, genetic distances were estimated, and bacterial relationships were presented in a phylogenetic tree constructed using the Neighboring Joining (NJ) method with 1000× bootstrap and the K2P model. The MEGA X program was used to construct the phylogenetic tree based on the methods that Kumar et al. (2018) and Tamura et al. (2013) adopted.

RESULTS AND DISCUSSION
Composition of cellulolytic bacteria species in soil on mangrove ecosystems
The 16S rRNA gene was amplified from cellulolytic bacteria using the primer pair 63F and 1387R, yielding a fragment size of 1500 bp upon alignment of the DNA ladder (Fig. 2). After alignment, the 16S rRNA gene fragment size was determined to be 1386 bp. This primer pair is capable of amplifying genes of approximately 1300 bp, resulting in an average size of ±1350 bp for the 16S rRNA gene fragment (Faturrahman, 2005; Kusumaningrum et al., 2016). The 16S rRNA is commonly used and selected as a method of bacterial identification because of its distribution in all bacteria species, its function that never changes, and this gene is large enough to reach 1500 bp in size (Safika et al., 2018).

The sequencing results were compared to the GenBank database, revealing species similarities ranging from 97.77% to 100%. The specific similarity values for each species are presented in Table 2. This indicates that the values between 97.77% and 100% of the sequencing matched the database. For instance, the isolates BTM121, BTM622, BTM632, and BTM511 were identified as B. altitudinis with 100% homology, whereas isolate BTM113 was identified as Bacillus sp1. with 97.77% homology, and Bacillus sp2. with 99.86%. According to Petti
I. Dewiyanti et al. (2007), a genus is considered similar assuming the similarity score is 97%, and a species is deemed one supposing the similarity obtained is 99%. However, assuming Bacillus sp1. from isolate BTM113 does not reach the 99% to 100% homology threshold, it may suspect a new species, and there are nucleotide base differences compared to GenBank. The same sequencing result has not been recorded or registered in GenBank. Additionally, isolate Bakteri tanah mangrove (BTM)432 and BTM321 were identified as Brevibacillus sp., and Pseudomonas aeruginosa, with 99.57% and 98.85% similarity to the data in GenBank. The highest individual composition is B. altitudinis followed by B. subtilis.

Briefly, nine species of cellulolytic bacteria were identified among the 13 sequenced isolates obtained from the mangrove ecosystem soil (Table 2). These include B. altitudinis, B. amyloboulimiae, Bacillus sp1., Bacillus sp2., B. safensis, B. subtilis, B. velezensis, Brevibacillus sp., and P. aeruginosa. However, among these species, Bacillus was the most commonly found genus, with four isolates identified as B. altitudinis, and two identified as Bacillus sp. and B. subtilis. This finding is consistent with previous studies, such as Shome et al. (1995) who identified 38 bacterial isolates, and reported that Bacillus sp. is the most dominant species (>50%) isolated from mangrove sediments in South Andaman. Yahya et al. (2014) reported that Bacillus sp. is the most abundant bacteria involved in the decomposition process of mangrove litter in the coast of Pasuruan Palace, thereby exhibiting the ability to degrade cellulose. Vásquez and Millones (2023) reported two species of bacteria that produce cellulase enzymes: B. safensis

Fig. 2: Visualization of DNA from each cellulolytic bacterial isolate on geldoc Description: 1 kilobyte (kb) = 250 DNA Ladder (bp)

Table 2: Species of cellulolytic bacteria identified using 16S rRNA

<table>
<thead>
<tr>
<th>No. of species</th>
<th>Sample code</th>
<th>Species</th>
<th>BLAST (%)</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BTM113</td>
<td>Bacillus sp1.</td>
<td>97.77</td>
<td>1386</td>
</tr>
<tr>
<td>2</td>
<td>BTM512</td>
<td>Bacillus sp2.</td>
<td>99.86</td>
<td>1386</td>
</tr>
<tr>
<td>3</td>
<td>BTM 121</td>
<td>B. altitudinis</td>
<td>100</td>
<td>1386</td>
</tr>
<tr>
<td></td>
<td>BTM 622</td>
<td>B. altitudinis</td>
<td>100</td>
<td>1386</td>
</tr>
<tr>
<td></td>
<td>BTM 632</td>
<td>B. altitudinis</td>
<td>100</td>
<td>1386</td>
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<tr>
<td></td>
<td>BTM 511</td>
<td>B. altitudinis</td>
<td>100</td>
<td>1386</td>
</tr>
<tr>
<td>4</td>
<td>BTM211</td>
<td>B. amyloboulimiae</td>
<td>99.78</td>
<td>1386</td>
</tr>
<tr>
<td>5</td>
<td>BTM533</td>
<td>B. safensis</td>
<td>100</td>
<td>1386</td>
</tr>
<tr>
<td>6</td>
<td>BTM123</td>
<td>B. subtilis</td>
<td>99.93</td>
<td>1386</td>
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<tr>
<td></td>
<td>BTM621</td>
<td>B. subtilis</td>
<td>99.93</td>
<td>1386</td>
</tr>
<tr>
<td>7</td>
<td>BTM611</td>
<td>B. velezensis</td>
<td>99.93</td>
<td>1386</td>
</tr>
<tr>
<td>8</td>
<td>BTM431</td>
<td>Brevibacillus sp.</td>
<td>99.57</td>
<td>1386</td>
</tr>
<tr>
<td>9</td>
<td>BTM321</td>
<td>P. aeruginosa</td>
<td>98.85</td>
<td>1386</td>
</tr>
</tbody>
</table>
and *B. subtilis*. *Bacillus* is a genus of Firmicutes group; Basak *et al.* (2016) found that Bacteroidetes, Acidobacteria, Firmicutes, Actinobacteria, Nitrospirae, Cyanobacteria, Plantomycetes, and Fusobacteri are groups of microorganisms mostly found in mangrove sediments in Dhulibhashani, India. *Brevibacillus* sp. is a type of cellulytic bacteria that can produce cellulase enzymes. Liang *et al.* (2009) reported that strain JLX of *Brevibacillus* sp., can break down various unique polymers, including cellulose, and utilizes various carbohydrates, such as cellulose, cellobiose, glucose, and xylose. Several studies have reported that *P. aeruginosa* is another cellulase-producing bacteria found in soil (Gunavathy and Boominathan, 2015; Gunavathy and Boominathan, 2016) and sawdust (Agarwal et al., 2014). Kurniawan *et al.* (2018a) identified *P. aeruginosa* as one of the cellulose-degrading bacteria found in mangrove forest soil in Bangka Island, Indonesia. The diversity of cellulytic bacteria varies in each area of the mangrove ecosystem; various species can be found in different mangrove ecosystems due to differences in their environmental characteristics, such as soil and water. The higher the species diversity and abundance of bacteria, the faster process of organic matter degradation. The bacterial richness indicates ecosystem fertility because they uniquely functions as a decomposer, such as cellulytic bacteria, which produces cellulase enzyme that accelerates cellulose degradation (Biswas *et al.*., 2020). Several studies have been conducted on mangrove soil’s bacteria. For example, Kurniawan *et al.* (2019) found cellulytic bacteria using molecular biology of the 16S rRNA gene, including TBL1 isolate with 85% similarity to *Vibrio parahaemolyticus*, and strains HY3 and TBL2 with 98% similarity to *B. amyloliquefaciens*. Pramono *et al.* (2021) identified isolate LG2 as the species *Fictibacillus nanhaiensis* strain JSM 082006, which produced the highest cellulase enzyme activity. Naresh *et al.* (2019) successfully identified three species using the 16S rRNA gene: *Anoxybacillus* sp., *B. subtilis*, and *Paenibacillus dendritiformis*, and Biswas *et al.* (2020) discovered strains T2-D2 as *Bacillus* sp. and E1-PT as *Pseudomonas* sp. The composition of cellulytic bacteria found in mangrove sediments is high, reaching 87.87% (Pramono *et al.*, 2021). In the fisheries sector, several cellulyase bacteria were found to have potential as probiotics. According to Yanbo and Zirong (2006) and Soltani *et al.* (2019), *Bacillus* sp. is one of the bacteria believed to increase the digestibility of fish because these bacteria are classified as probiotics, so they are often applied to feed to increase feed digestibility. The species of *Bacillus* often applied as a probiotic is *B. subtilis* (El Dakar *et al.*, 2007; Olmos *et al.*, 2020; Truong *et al.*, 2021), and this bacterium was found at the study site. The use of *Bacillus* sp. as a probiotic bacterium in fish feed is believed to enhance their digestive capacity by converting unutilized feed into a digestible form (Putra *et al.*, 2021; Widanarni *et al.*, 2022). This approach can be an alternative to producing dual-function feed and indirectly improve feed quality.

**Genetic distance of cellulytic bacteria species**

Table 3 shows the genetic distance within-species (intraspecies) values, and the values of interspecies distances. Four isolates detected as the *B. altitudinis* species exhibited a species distance of zero, meaning that these isolates are identical. Similarly, two isolates identified as the *B. subtilis* species exhibited a within-species distance of zero. *Bacillus* sp. has a interspecies distance value of 0.084%, indicating differences between the two species (*Bacillus* sp1. and *Bacillus* sp2.). The interspecies genetic distance is 0.069% between *Bacillus* sp2. and *B. amyloliquefaciens*, 0.067% between *Bacillus* sp2. and *B. altitudinis*, and 0.067% between *B. velezensis* and *Bacillus* sp2. The closest intraspecies distances were found to be between *B. velezensis* and *B. amyloliquefaciens* at 0.002%, followed by *B. safensis* and *B. altitudinis* at 0.004%, *B. velezensis* and *B. subtilis* at 0.004%, as well as *B. subtilis* and *B. amyloliquefaciens* at 0.006%. Maduppa *et al.* (2018) stated that a smaller genetic distance implies a closer a relationship between species and vice versa.

In a study by Fan *et al.* (2017), *B. velezensis*, *B. methylotrophicus*, and *B. amyloliquefaciens* were found to have a high degree of similarity in their nucleotide base sequence, depicting that they belong to the same clade. Furthermore, Hossain *et al.* (2015) reported that a recent phylogenomic study has shown that the genomes of *B. methylotrophicus*, *B. velezensis*, *B. oryzicola*, and *B. amyloliquefaciens* are highly similar. A specific strain isolate code of *B. amyloliquefaciens* (DSM 23117T), first identified as *B. amyloliquefaciens* in 2008 (Wang *et al.*, 2008), was later revised as *B. amyloliquefaciens* sub-sp. plantarum in 2011 (Borris *et al.*, 2011), and finally
reclassified as *B. velezensis* in 2016 based on DNA hybridization, and phenotypic and phylogenetic analyses (Dunlap et al., 2016). Although some recent publications and GenBank data sets still refer to this strain as *B. amyloliquefaciens*, it was later confirmed as *B. velezensis* strain using molecular methods (Fan et al., 2017; Liu et al., 2019; Santiago et al., 2021).

Despite having similar morphology, physiology, phenotypic properties, and 16S rRNA gene sequences, *B. velezensis* and *B. amyloliquefaciens* can be distinguished based on their fatty acid (FA) cellular composition, as noted by Huynh et al. (2022). Both *B. velezensis* and *B. amyloliquefaciens* belong to the *B. subtilis* group, which suggests a close taxonomic relationship (Fan et al., 2017).

### Phylogenetic tree of cellulolytic bacteria

Using the NJ method with 1000× bootstrap repetitions, the phylogenetic analysis consistently revealed that *B. velezensis* is closely related to *B. amyloliquefaciens* and *B. subtilis* and showed that *B. subtilis* is closely related to *B. safensis* (Fig. 3). Recently, Vásquez and Millones (2023) reported the close phylogenetic relationship of cellulolytic bacteria of *B. subtilis* and *B. safensis* isolated from solid waste of palm forests, Peru. Constructing the phylogenetic tree from 13 cellulolytic bacteria isolates divided into nine species, and two main clades (groups). Clade one consists of *B. altitudinis*, *B. safensis*, *B. amyloliquefaciens*, *B. subtilis*, *Bacillus* sp1. (BTM113), *Bacillus* sp2. (BTM512), *B. velezensis*, and *Brevibacillus* sp. Meanwhile, clade two consists of *P. aeruginosa* at 1000× bootstrap. One main clade was then divided into two subclades, which separated *Brevibacillus* sp. Species of *A. hydrophila* (access code LR991675.1), an outgroup species in the phylogenetic tree (Fig. 3). The *Bacillus* and *Brevibacillus* genera gather in the same class and order, namely the Bacilli class and the order Bacillales, but in different families. *Bacillus* belongs to the Bacillaceae family while *Brevibacillus* belongs to the Paenibacillaceae family. Both families are the phylum Firmicutes. Sulistiyani et al. (2021) reported two families and three genera identified using the 16S rRNA gene, namely Bacillaceae (genus *Bacillus* and *Fictibacillus*), and Paenibacillaceae (genus *Brevibacillus*). These families can produce cellulase, xylanase, amylase, and lipase enzymes (Shanti and Roymon, 2018; Powthong and Suntornthitcharoen, 2017).

Comparatively, there are five species of cellulolytic bacteria in the rehabilitated mangrove area: *B. amyloliquefaciens*, *Bacillus* sp1., *B. subtilis*, *B. altitudinis*, and *P. aeruginosa*. The non-rehabilitated area has six species of cellulolytic bacteria, including *Bacillus* sp2., *B. subtilis*, *B. altitudinis*, *B. velezensis*, *B. safensis*, and *Brevibacillus* sp. Based on the analysis, *Bacillus* sp1., *B. amyloliquefaciens*, and *P. aeruginosa* were not found in non-rehabilitated areas, while *Bacillus* sp2., *B. safensis*, *B. velezensis* and *Brevibacillus* sp. were also not found in the rehabilitated areas. The phylogenetic trees of the rehabilitated and non-rehabilitated mangrove areas, shown in Fig. 4a and Fig. 4b, respectively, consist of two main clades, with one serving as an outgroup. The species used as an outgroup in this phylogenetic tree is *A. hydrophila* (access code LR991675.1).

### Table 3: Within-species and interspecies distance values of cellulolytic bacteria

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>12</th>
<th>13</th>
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</thead>
<tbody>
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<td>1</td>
<td><em>B. altitudinis</em></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>B. altitudinis</em></td>
<td>0.000</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>B. altitudinis</em></td>
<td>0.000</td>
<td>0.000</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>B. altitudinis</em></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>5</td>
<td><em>B. amyloliquefaciens</em></td>
<td>0.030</td>
<td>0.030</td>
<td>0.030</td>
<td>0.030</td>
<td>0.0</td>
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</tr>
<tr>
<td>6</td>
<td><em>Bacillus</em> sp1.</td>
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<td>0.045</td>
<td>0.045</td>
<td>0.045</td>
<td>0.019</td>
<td>0</td>
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<td></td>
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<tr>
<td>7</td>
<td><em>Bacillus</em> sp2.</td>
<td>0.057</td>
<td>0.067</td>
<td>0.067</td>
<td>0.067</td>
<td>0.069</td>
<td>0.084</td>
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<td></td>
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<tr>
<td>8</td>
<td><em>B. safensis</em></td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
<td>0.004</td>
<td>0.032</td>
<td>0.048</td>
<td>0.067</td>
<td>0</td>
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</tr>
<tr>
<td>9</td>
<td><em>B. subtilis</em></td>
<td>0.029</td>
<td>0.029</td>
<td>0.029</td>
<td>0.029</td>
<td>0.006</td>
<td>0.021</td>
<td>0.066</td>
<td>0.031</td>
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<td>10</td>
<td><em>B. subtilis</em></td>
<td>0.029</td>
<td>0.029</td>
<td>0.029</td>
<td>0.029</td>
<td>0.006</td>
<td>0.021</td>
<td>0.066</td>
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<tr>
<td>11</td>
<td><em>B. velezensis</em></td>
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<td>0.028</td>
<td>0.028</td>
<td>0.028</td>
<td>0.002</td>
<td>0.019</td>
<td>0.067</td>
<td>0.030</td>
<td>0.004</td>
<td>0.004</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td><em>Brevibacillus</em> sp.</td>
<td>0.969</td>
<td>0.969</td>
<td>0.969</td>
<td>0.969</td>
<td>0.960</td>
<td>1.006</td>
<td>0.914</td>
<td>0.963</td>
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<td>0.957</td>
<td>0.960</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td><em>P. aeruginosa</em></td>
<td>5.428</td>
<td>5.428</td>
<td>5.428</td>
<td>5.428</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
The present study found that the number of cellulolytic bacteria species was higher in the non-rehabilitated mangrove area than in the rehabilitated one. Preliminary studies suggested that this difference in species richness was due to lower OC in the rehabilitated mangrove soil. The OC in the non-rehabilitated and rehabilitated mangrove were 1.23% and 0.90% (Dewiyanti et al., 2021), followed by a higher percentage of silt fraction (fine fraction) in non-rehabilitated mangrove (43%) than in

Fig. 3: Phylogenetic tree of 9 cellulolytic bacterial sequences isolated from mangrove soil using NJ method
rehabilitated (31%). Soil organic C content is higher in fine soil with small porosity (silt) than in coarse soil with big porosity (sand). Carney and Matson (2005) mentioned that fine-textured soils support more microbial biomass and species than their coarse-textured. Huang et al. (2019) supported this view, stating that bacterial diversity tends to increase with higher organic matter content in the soil. A higher number of mangrove vegetation in non-rehabilitated than in rehabilitated area was assumed to contribute more litter production that can increase OC content. There were 70 individuals/100 square meter (m²) in non-rehabilitated than 39 individuals/100 m² in rehabilitated. Litter production significantly contributes OC into the soil, promoting the diversity of cellulolytic bacteria (Leff et al., 2012). Another factor that could explain the higher number of bacterial species in the non-rehabilitated areas is the more extensive root systems in these ecosystems. Batubara (2021) reported that the rhizosphere, the soil layer where roots grow, is enriched with nutrients from root exudates (materials released from living root cells, such as sugars, amino acids, organic acids, and FA) and other soil organisms. Bacteria tend to dominate in the rhizosphere due to organic matter abundance; many bacteria species form symbiotic relationships with plant roots, as these exudates serve as the primary food source for microorganisms.
and their activities (Raaijmakers et al., 2009; Sulistiyani et al., 2020). A previous study investigating cellulolytic bacteria: *B. altitudinis* and *B. safensis* showed a higher cellulolytic index (CI) than other bacteria found in mangrove non-rehabilitated areas. Dewiyanthi et al. (2022) reported that *B. safensis* had CI index of 4.82, followed by *B. altitudinis* with 2.09 CI index, categorized as a high CI index. A high CI index indicates high cellulase production (Naresh et al., 2019). Enzyme produced by microorganism could be good indicators to ecosystem response’s to environmental changes (Luo and Gu, 2015).

**Composition of nucleotide bases in cellulolytic bacteria**

The sequencing of 13 isolate samples resulted in nucleotides with a base length of 1386 bp, and Guanine (G) being the most abundant nucleotides, followed by Adenine (A), Cytosine (C), and Thymine (T). The nucleotide base composition for each cellulolytic bacterial species is presented in Table 4. The nucleotide composition had an average of 20.18% (T), 23.51% (C), 25.06% (A), and 31.26% (G). The GC content of the first, second, and third codon positions increased, with mean GC and AT contents of 27.39%, and 22.62%, respectively. The average nucleotide composition is shown in Table 5.

After conducting a nucleotide alignment, the two *Bacillus* sp. isolates were discovered to be distinct species, as revealed by Multalin (multiple sequence alignment) program (Fig. 5). The interspecies were identified in both isolates, with nucleotide base changes observed at several points, denoted by the following base sequence numbers 9, 11, 12, 16, 17, 28, 29, 74, 75, 118, 119, 120, 121, 122, etc. The nucleotide base of these two species had a variation of 106 base sequence points (7.7%) due to nucleotide base substitution. These nucleotide base variations are highlighted in blue and black (Fig. 5), and were primarily caused by nucleotide base changes, including transition and transversion substitutions. Transitions are changes between A and G bases (purines) or disparities between C and T bases (pyrimidines), while transversions are alterations.

### Table 4: Nucleotide base composition and accession number registered in GenBank of cellulolytic bacteria

<table>
<thead>
<tr>
<th>No.</th>
<th>Bacterial isolate</th>
<th>Species</th>
<th>Accession number</th>
<th>T (%)</th>
<th>C (%)</th>
<th>A (%)</th>
<th>G (%)</th>
<th>GC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BTM121</td>
<td><em>B. altitudinis</em></td>
<td>OQ152604</td>
<td>20.00</td>
<td>23.78</td>
<td>25.02</td>
<td>31.20</td>
<td>54.98</td>
</tr>
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<td>2</td>
<td>BTM632</td>
<td><em>B. altitudinis</em></td>
<td>OQ152605</td>
<td>20.00</td>
<td>23.78</td>
<td>25.02</td>
<td>31.20</td>
<td>54.98</td>
</tr>
<tr>
<td>3</td>
<td>BTM511</td>
<td><em>B. altitudinis</em></td>
<td>OQ152606</td>
<td>20.00</td>
<td>23.78</td>
<td>25.02</td>
<td>31.20</td>
<td>54.98</td>
</tr>
<tr>
<td>4</td>
<td>BTM622</td>
<td><em>B. altitudinis</em></td>
<td>OQ152607</td>
<td>20.00</td>
<td>23.78</td>
<td>25.02</td>
<td>31.20</td>
<td>54.98</td>
</tr>
<tr>
<td>5</td>
<td>BTM211</td>
<td><em>B. amyloliquefaciens</em></td>
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<td>24.84</td>
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<td>6</td>
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<td><em>Bacillus</em> sp1</td>
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<td>31.42</td>
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<td><em>Bacillus</em> sp2</td>
<td>OQ152610</td>
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<td>53.31</td>
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<tr>
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<td><em>B. subtilis</em></td>
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<td>23.64</td>
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<td>54.98</td>
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<td><em>B. subtilis</em></td>
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<td>25.02</td>
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<td>54.98</td>
</tr>
<tr>
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<td><em>B. velezensis</em></td>
<td>OQ152613</td>
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<td>23.64</td>
<td>24.80</td>
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<tr>
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<td>25.38</td>
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</table>

### Table 5: Average nucleotide composition

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<tr>
<th>Nucleotide</th>
<th>Average</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>T %</td>
<td>20.18</td>
<td>19.93</td>
<td>20.87</td>
</tr>
<tr>
<td>C %</td>
<td>23.51</td>
<td>22.62</td>
<td>23.78</td>
</tr>
<tr>
<td>A %</td>
<td>25.06</td>
<td>24.80</td>
<td>25.82</td>
</tr>
<tr>
<td>G %</td>
<td>31.26</td>
<td>30.55</td>
<td>31.64</td>
</tr>
<tr>
<td>GC %</td>
<td>54.76</td>
<td>53.31</td>
<td>55.20</td>
</tr>
</tbody>
</table>
Fig. 5: Sequence alignment of *Bacillus* sp. (BTM113 and BTM512) using Multalin

Fig. 6: Sequence alignment of *B. subtilis* (BTM123 and BTM621) using Multalin
between a purine and pyrimidine base (Elyvra et al., 2009; Nandy, 2009; Ubaidillah and Sutrisno, 2009). Specifically, the nucleotide chain polymorphism in Bacillus sp. includes 74 nucleotide transitions and 32 transversions. Among these substitutions, nucleotide transitions were more commonly found than transversions (Kochzius and Nuryanto, 2008). The nucleotide composition of Bacillus sp1 (BTM113) was 20.65% (T), 22.98% (C), 24.95% (A), and 31.42% (G), and the highest average was the G+C pair with a value of 27.2% while the lowest is the A+T pair (22.8%), the G+C nucleotide pair composition was also higher in Bacillus sp2. (BTM512). Assuming both species have a higher A+T nucleotide base value based on composition, the species will have many similarities due to independent parallel substitutions. Conversely, supposing both species have a higher G+C nucleotide base composition, the species will have fewer similarities (Lam and Morton, 2006). Transition mutations were found to occur more frequently and easily than transversion mutations, which explains why transitions were more commonly found among substitutions (Graur and Li, 2000). Notably, the average content of the G+C nucleotide base was greater than that of the A+T nucleotide base in each identified species of cellulolytic bacteria, with values of 27.39% (GC) and 22.62% (AT), respectively. This formula also occurred in each identified species of cellulolytic bacteria. This finding is consistent with previous research suggesting that G+C nucleotide pairs are more prevalent in bacterial genomes.

Meanwhile, two isolates, identified as B. subtilis, and the four isolates, identified as B. altitudinis, were confirmed through alignment to be the same species. Figs. 6 and 7 show that the nucleotide base variations in these isolates were identical, indicating that they belonged to the same species.

CONCLUSION

The study results revealed cellulolytic bacteria in mangrove soil for rehabilitated and non-rehabilitated, Banda Aceh and Aceh Besar, Aceh Province, Indonesia.
Based on the alignment using MEGA X and comparing to the BLAST database in GeneBank, nine species were identified from the 13 isolates successfully sequenced using the 16S rRNA gene: *B. altitudinis*, *B. amyloliquefaciens*, *Bacillus sp1.*, *Bacillus sp2.*, *B. safensis*, *B. subtilis*, *B. velezensis*, *Brevibacillus sp.*, and *P. aeruginosa*. The similarity of data sequencing and GenBank database ranged from 97.77% to 100%. The isolates BTM121, BTM622, BTM632, and BTM511 were identified as *B. altitudinis* with 100% homology, whereas isolate BTM113 was identified as *Bacillus sp1.* with 97.77% homology, and *Bacillus sp2* with 99.86%. Species of *B. amyloliquefaciens*, *B. safensis*, *B. subtilis*, *B. velezensis*, and *P. aeruginosa* have 99.78%, 100%, 99.93%, 99.57%, and 98.85% homology, respectively. The species obtained are considered similar due to similarity achieving 97%, and categorized as same species with database if the similarity obtained 99%. Further analysis revealed that *B. velezensis* is closely related to *B. amyloliquefaciens*, with inter-specific genetic distance of 0.002%. The farthest interspecies genetic distance was 0.084% related between *Bacillus sp1.* and *Bacillus sp2*. Small genetic distances of interspecies indicate a close relationship between species. Research studies were located at two kinds of mangrove habitats (rehabilitated and non-rehabilitated mangrove) with different characteristics, for example, substrate fraction, OC, and mangrove species. Previous research showed that non-rehabilitated mangrove contain higher OC, silt fraction, and mangrove vegetation species and abundance. This condition makes the non-rehabilitated mangrove soil acquire more bacterial cellulolytic species than the rehabilitated habitat. The characteristics of a suitable soil environment strongly support the life and diversity of cellulolytic bacteria, so an action is needed to increase the presence of cellulolytic bacteria in mangrove soils by applying cellulolytic species that produce high enzyme cellulase. The bacteria diversity is a critical aspect to the functioning of the ecosystem, managing the ecosystem while maintaining diversity may be one of the approaches to ensure the stability and productivity of the mangrove ecosystem in the study area. Notably, *Bacillus* is a genus commonly found in the study area, and two isolates from the *Bacillus* sp. were identified as different species, as proven by different nucleotide bases that have been aligned. *Bacillus sp1.* could be a novel species due to low homology percentage and the absence of nucleotide bases identical to the aligned data on GenBank. *B. subtilis* found is a cellulolytic bacterium often applied in the industrial, animal husbandry, and fishery sectors.

**AUTHOR CONTRIBUTIONS**

D. Darmawi, as a corresponding author, developed the study idea and concepts and supervised the manuscript. I. Dewiyanti, as the first author, has contributed to study conceptualization, surveys, data collection and analysis, result interpretation, and manuscript preparation. Z.A. Muchlisin contributed to the research design, result interpretation, and manuscript review. T.Z. Helmi contributed to the result interpretation and manuscript review.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

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ABBREVIATIONS

16S rRNA 16 svedberg ribosomal ribonucleic acid
% Percent
µg Microgram
µL Microliter
°C Degree Celsius
A Adenine
BTM Bakteri tanah mangrove (mangrove soil bacteria) as isolate code
BLAST Basic local alignment search tool
bp Basepair
C Cytosine
CaCl₂ Calcium chloride
CFU Colony form unit
CI Cellulolytic index
CMC Carboxymethyl cellulose
DNA Deoxyribonucleic acid
DSM 23117T Isolate code of B. amyloliquefaciens
E1-PT Isolate code of Pseudomonas.
E East
EDTA Ethylene diaminetraacetate acid
etc et cetera
F Forward
FeSO₄ Ferrous sulphate monohydrate
FA Fatty acid
G Guanine
g Gram
GB Genomic bind
HCL Hydrogen chloride
HY3 Isolate code of B. amyloliquefaciens
JLX Genus in the Brevibacillus
JSM Strain code
K2P Kimura-2-parameter
KH₂PO₄ Potassium Dihydrogen Phosphate
KNO₃ Potassium nitrate
LG2 Isolate code of Fictibacillus nanhaiensis
LR991675.1 Access code of A. hydrophila
m² Square meter
MEGA Molecular evolutionary genetics analysis
MgSO₄·7H₂O Magnesium sulfate heptahydrate
min Minute
mL Milliliter
mM miliMolar
Multalin Multiple sequence alignment
N North
NCBI National Center for Biotechnology Information
ng Nanogram
NJ Neighbouring joining
No Number
OC Organic carbon
PCR Polymerase chain reaction
pH Potential of hydrogen
pmol Picomole
R Reverse
rpm Revolution per minute
s Second
T2-D2 Isolate code of Bacillus sp.
T Thymine
TAE buffer Tris-acetate-EDTA buffer
TBL1 Isolate code of Vibrio parahaemolyticus
TBL2 Isolate code of B. amyloliquefaciens
USK Universitas Syiah Kuala
UV Ultra Violet
w/v Weight/volume
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