Diversity of cellulolytic bacteria from mangroves in Blanakan, West Java, Indonesia

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Abstract. Indonesia has the highest percentage of mangrove ecosystems in the world (23%). Cellulolytic bacteria are cellulose-degrading bacteria that have great potential and have a fast growth rate compared to other microbes. Information on bacterial diversity in mangrove areas, especially in Indonesia, needs to be improved. This research aims to identify the diversity of cellulolytic bacteria originating from the Blanakan mangrove area in Central Java. Water samples were collected at six observation stations. Isolation and screening of cellulolytic bacteria were carried out using the spread plate method on carboxymethyl cellulose (CMC) media. The cellulase index was obtained through an antagonist test using Congo red. Isolates that have cellulolytic power were characterized based on morphological and physiological characteristics and then grouped into genera based on Bergey's Manual of Determinative Bacteriology. The results of isolation and purification showed that 24 isolates grew on CMC media, most of which were yellow and cream, with smooth edges, a convex surface and a round shape. Nine isolates showed a clear zone and cellulase index. The two isolates that had the highest cellulolytic power were isolate BVIw1a and isolate BVIw1b (station point 1250 m from the mangrove area), with cellulase indices of 4 and 4.37, respectively. The results of the physiological and morphological characterization of the nine isolates showed that five isolates were Gram-positive and four isolates were Gram-negative, as well as five isolates in the form of coccus and four isolates in the form of bacillus. Based on Bergey's Manual of Determinative Bacteriology, the two isolates with the highest cellulolytic power belong to the genus *Bacillus* and the genus *Aeromonas*. Both genera have good potential in bioremediation strategies to reduce environmental pollution.

Key Words: *Aeromonas*, *Bacillus*, cellulase, Indonesia, mangrove.

Introduction. The total area of mangroves in the world is estimated to reach 137760 km^2 and is distributed in 118 countries. Indonesia has extensive mangrove forests, accounting for 23% of the world's total mangroves and has the highest percentage of the global mangrove ecosystem (Spalding et al 2010; Ministry of Environment and Forestry 2017; Arifanti 2020; Ministry of Maritime Affairs and Fisheries of the Republic of Indonesia 2021). The mangrove area located in Blanakan District, West Java, Indonesia, covers 303.25 ha and directly borders the Java Sea, with 20.03% consisting of a mangrove ecosystem (Indrayanti et al 2015). Blanakan District has an altitude of 0-2 m above sea level, with an average temperature of 27°C and rainfall ranging from 1600-2300 mm (Indrayanti et al 2015; Muhammad et al 2012).

Mangrove forests are a natural resource with important roles from a social, economic and ecological perspective. One of the functions of mangrove forests in ecological terms is being a boundary between land and sea ecosystems, as well as a breeding ground for various groups of microorganisms (Behera et al 2016a). Mangrove leaves and litter fall throughout the year and, over time, turn into humus with the help of bacteria. The existence and diversity of bacteria in mangrove ecosystems are influenced by various factors, including salinity, pH, climate, vegetation, nutrition, and location factors, among others (Hrenović et al 2003; Prakash et al 2015; Palit et al 2022). The existence of bacteria in the mangrove ecosystem plays an important role, especially in decomposing mangrove leaf litter. Bacteria decompose litter enzymatically through the active role of cellulolytic enzymes. Some bacteria genera that play a role in the cellulose decomposition process include *Cytophaga* and *Sporocytophaga* (Das et al 2006; Ratrinia et al 2020). The decomposition process by these bacteria is able to convert indigestible mangrove leaf fibers into fibers that are more easily digested, providing food for other organisms in the mangrove forest (Yahya et al 2014; Yulma et al 2017).

Cellulolytic bacteria are bacteria that can hydrolyze cellulose complexes into smaller oligosaccharides and ultimately into glucose. Some cellulose-degrading bacteria are aerobic mesophilic and thermophilic bacteria (*Cellumonas* sp., *Celvibrio* sp., *Microbispora bispora*, and *Thermomonospora* sp.), anaerobic mesophilic and thermophilic bacteria (*Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Bacteroides succinogenes, Ruminococcus albus, Ruminococcus flavefaciens,* and *Clostridium thermocellum*). Cellulolytic bacteria are often isolated from soil containing leaf litter. The soil is rich in organic matter, and the leaf litter has a fairly complex polysaccharide content. These conditions cause soil with leaf litter to become a good habitat for various microorganisms (Rosado & Govind 2003). According to Sutiknowati (2010), in the mangrove ecosystem, bacteria act as primary decomposers, functioning in the release and binding of nutrients from sediment to become nutrients needed by mangrove plants.

Information on bacterial diversity in mangrove areas, especially in Indonesia, needs to be improved. Several studies have succeeded in identifying the characteristics of bacteria in the Tarakan City Mangrove area (Yulma et al 2017), the Peniti Mangrove area, Segedong Mempawah District (Rudiansyah et al 2017), and the Pasuruan Palace Coastal Mangrove area (Yahya et al 2014). More information is needed about the diversity of bacteria in the Blanakan mangrove area. This area is classified as having a high level of mangrove degradation (Heriyanto 2012) and is experiencing conversion into ponds with an average increase in aquaculture activities of around 33.02% per year. The bacteria in the mangrove area may have special characteristics that are not found in other mangrove areas. Based on these conditions, it is necessary to carry out research to determine the diversity of bacteria in the Blanakan mangrove area, focusing on cellulolytic bacteria. Cellulolytic bacteria are one of the potential cellulose-degrading microbes and have a fast growth rate compared to other microbes. This characteristic is due to the cellulase enzyme produced by these bacteria. These cellulolytic enzymes can be used to degrade waste substrates to reduce pollution in water (Chasanah et al 2013). Cellulase is one of the main industrial enzymes with various uses, such as in food, paper, textiles, pharmaceuticals, detergents, and biofuels (Imran et al 2019; Rodrigues & Odaneth 2021). This research aims to identify the diversity of cellulolytic bacteria originating from the Blanakan mangrove area in Central Java.

Material and Method

Material and equipment. Sampling was carried out in the mangrove area located in Blanakan sub-district, Subang, Central Java in 2021. Bacterial cellulolytic isolation and testing were carried out at the Microbiology Laboratory, Department of Biology, IPB, Bogor, Indonesia. The equipment used in this research included a cool box, sample bottles, pH indicator paper, air/water temperature meter, and a centrifuge. The materials used in this research include water samples from the Blanakan mangrove area, carboxymethyl cellulose (CMC) media, agar, Congo red, Gram stain, indole, citrate, urea, glucose, H_2O_2, NaCl, HCl, alcohol and distilled water.

Sampling. Samples were taken from the Blanakan mangrove area at 6 observation stations. Station 1 is the initial section closest to the mangrove area (0 m). Station 2 is part of the beach (250 m). Stations 3 to 6 are, respectively, 500, 750, 1000, and 1250 m from the mangrove area. In each station, a 200 mL sample of water was collected. The sample was taken from 1-2 m under the water surface. Sampling was carried out two times at each station, summing 12 water samples. All samples were put into sample containers, stored in a coolbox, and transported to the laboratory for isolation of growing bacteria. When collecting samples, the physical and chemical properties of water were measured, including water temperature, air temperature and pH.

Isolation and purification. Isolation and screening of cellulolytic bacteria were carried out using the spread plate method on CMC media. CMC media was obtained by dissolving 15 g agar in 5 g carboxymethyl cellulose, 1g KH_2PO_4, 5 g $MgSO_4.7H_2O$, and 2 g yeast extract in 1000 mL. The mixed ingredients were then sterilized and 5 mL were poured into each test tube, and 15 mL into each Petri dish. All samples obtained were subjected to dilutions of 10^{-1}, 10^{-2}, and 10^{-3}, then each sample dilution was inoculated on the media and incubated at 22°C for 24 h. After the bacterial colony grew, it was separated by regrowing the bacteria. The way to produce these bacteria is by re-inoculating one loop into new CMC media and then incubating it. The bacterial colonies that grow are purified using the quadrant streaking method until a single and uniform bacterial colony or pure culture is obtained. The results of isolation and purification obtained several colony isolates that were morphologically different, for example, color, edges, surface, and colony shape. The bacteria that have been isolated may be cellulolytic because the CMC medium used for isolation and purification is a selective medium for the growth of cellulose-degrading bacteria.

Cellulase index measurement. The cellulase index was obtained through an antagonist test using Congo red. Isolates that grew on CMC media and were morphologically different were inoculated into Petri dishes containing CMC agar media and then incubated for 2x24 h. After the bacteria were produced, they were dripped with 0.1% Congo red solution, left for 30-60 min, and washed using 1 M NaCl to remove any remaining color on the media. The clear zone around the bacterial colony was observed and its diameter measured. If there was a clear zone, the bacteria had cellulolytic power with a certain cellulolytic index (CI). CI was measured by calculating the ratio between the clear zone's diameter and the bacterial colony's diameter. The cellulolytic index value is classified as low if ≤1, medium if between 1-2, and high if ≥2 (Choi et al 2005). According to Choi et al (2005), the formula for calculating CI is the following:

$$CI = \frac{\text{Clear Zone's Diameter} - \text{Colony's Diameter}}{\text{Colony's Diameter}}$$

Morphological and physiological tests. All isolates with cellulolytic power were then characterized to determine the cells' morphology, biochemistry and physiology. Several characterizations were carried out to determine morphological characteristics, including the colony's shape, color, surface, and edges. Meanwhile, characterization to determine physiological properties includes indole, MR-VP, citrate, H_2S, urea, glucose broth and oxidase tests. Biochemical characteristics include catalase, Gram and cell shape tests. This characterization intends to group isolates with cellulolytic power into certain groups, referring to Holt et al (1994).

Observed parameters. Several parameters observed included the morphological appearance of the colony (color, edges, surface, colony shape, and cell shape) and physiological appearance, including the results of the indole, MR-VP, citrate, H_2S, urea, glucose broth, and oxidase tests. In addition, clear zone measurements and cellulolytic index calculations were also carried out. Data analysis was carried out on the isolate's ability to have cellulolytic properties, as indicated by forming a clear zone. Analysis was also carried out on the physiological and morphological characterization of each isolate with high cellulolytic power.

Results and Discussion. The existence of bacteria in the aquatic environment is largely determined by the physical and chemical factors of the water. The values of physical and chemical water factors at 6 observation stations according to the sampling points are presented in Table 1.

Table 1

Physical and chemical factors of water in the Blanakan mangrove area

No	*Location*	*Air temperature (°C)*	*Water temperature (°C)*	*pH*
1	1	33	29	6
2	2	30	28	6
3	3	33	28	6
4	4	33	28	6
5	5	31	28	6
6	6	33	29	6
	Average	32.2	28.3	6

Note: 1-6 - observation station (0 m to 1250 m from the mangrove area).

Table 1 shows that the average water temperature in the Blanakan mangrove area is 28.3°C and the air temperature is 32.2°C. The air temperature is not much different from that in the North Coast of the Pemalang mangrove area, which ranges between 27.91-28.42°C (Poedjirahajoe et al 2017). This temperature range is categorized as good for mangrove growth. Temperature is a factor that greatly influences cellulase activity because it is related to enzyme activity (Behera et al 2016b, 2017). Rudiansyah et al (2017) added that cellulolytic bacteria groups can generally be found in a temperature range of 27-36°C and are capable of carrying out degradation activities with relatively high yields. Thus, the water temperature of the Blanakan mangrove area is very suitable for the growth of mangroves and several cellulase-forming bacteria.

The acidity level (pH) is one of the factors that greatly influences the presence of nutrients in the soil. The average pH obtained at the observation stations is 6. Water conditions with a pH of 5-7 indicate that the waters are in a good range, within the normal limits for mangrove vegetation and aquatic biota and is the optimum pH for cellulolytic bacteria (Wahyuni et al 2015; Poedjirahajoe et al 2017; Fauziah & Ibrahim 2021; Batubara et al 2022). The activity of cellulose-degrading bacteria is influenced by pH, and soil acidity can inhibit enzyme activity in bacteria (Khairiah et al 2013). Thus, the pH in the Blanakan mangrove area is considered optimum and very productive for the growth of decomposing bacteria.

Isolation and purification. The results of the isolation and purification of water samples obtained from the Blanakan mangrove area showed that 24 selected isolates could grow on CMC media. These isolates had cellulolytic power because of their ability to grow on CMC media (Figure 1).

CMC media is a selective medium for cellulose-degrading bacteria. The isolates that can grow on CMC media can use cellulose as a carbon source and are cellulolytic bacteria (Murtiyaningsih & Hazmi 2017; Wahyuningsih & Zulaika 2019). CMC is the best substrate for cellulase production because it can induce bacteria to produce cellulase enzymes (Idiawati et al 2015). Colony morphology was observed for the 24 isolates with cellulolytic power. Observations on the morphological characteristics of the colony were carried out to facilitate the process of identifying bacterial types (Wardhani et al 2020). The results of observing the morphological characteristics of 24 isolates aged 24 hours are presented in Table 2. Table 2 shows that the colonies are mostly yellow and cream, with slippery edges, a convex surface, and a round shape. Based on the morphology, there are four large groups of colonies: cream-colored colonies, with slippery edges, convex surfaces, and round shapes; yellow colonies, with slippery/wavy edges, flat/convex surfaces, and round/irregular shapes; orange colonies, with slippery edges, convex surfaces, and round; and white colonies, with wavy edges, flat surface, and irregular shape. The morphological characteristics of the colonies in the Blanakan mangrove area are similar to cellulolytic bacteria isolated from banana peel waste samples (round shaped, flat edges and flat surface) and from the Sungailiat mangrove ecosystem (cream colored, round shaped, jagged edges and slightly elevation) (Sari et al 2018; Khulud et al 2021).

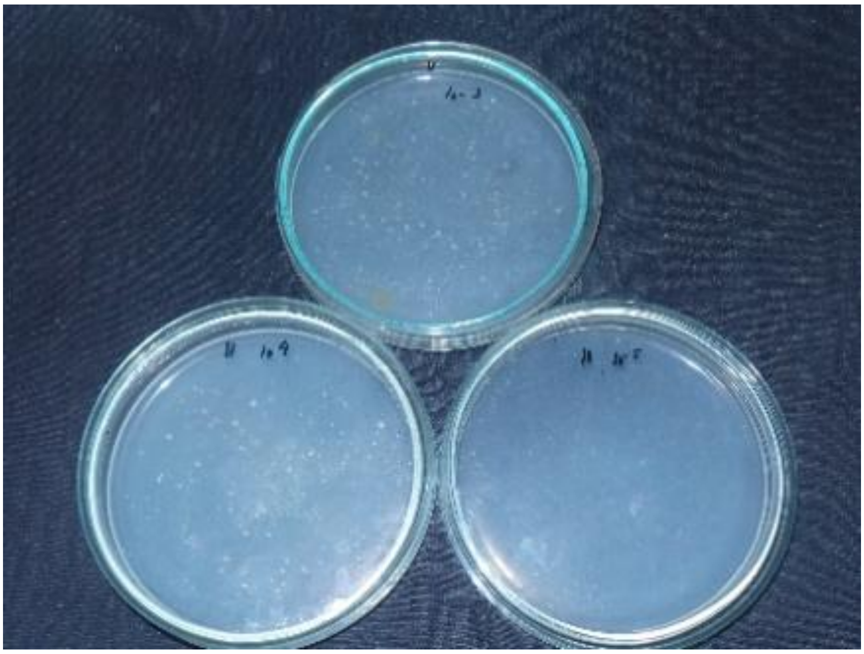

Figure 1. Several isolates on CMC media.

Table 2

Colony morphological characteristics of 24 isolates

Location	*Isolate code*	*Colony characteristics*			
		Color	*Edge*	*Surface*	*Shape*
1	BIw1a	Cream	Slippery	Convex	Round
	BIw1b	Cream	Slippery	Convex	Round
	BIw2a	Cream	Slippery	Convex	Round
	BIw2b	Cream	Slippery	Convex	Round
2	BIIw1a	Yellow	Slippery	Convex	Round
	BIIw1b	White	Wavy	Flat	Irregular
	BIIw2a	Yellow	Slippery	Flat	Irregular
	BIIw2b	Yellow	Slippery	Flat	Irregular
3	BIIIw1a	Yellow	Slippery	Convex	Round
	BIIIw1b	Yellow	Slippery	Convex	Round
	BIIIw2a	Yellow	Wavy	Flat	Irregular
	BIIIw2b	Yellow	Wavy	Flat	Irregular
4	BIVw1a	Cream	Slippery	Convex	Round
	BIVw1b	Yellow	Slippery	Convex	Round
	BIVw2a	Orange	Slippery	Convex	Round
	BIVw2b	Orange	Slippery	Convex	Round
5	BVw1a	Cream	Slippery	Convex	Round
	BVw1b	Cream	Slippery	Convex	Round
	BVw2a	Orange	Slippery	Convex	Round
	BVw2b	Cream	Slippery	Convex	Round
6	BVIw1a	Yellow	Slippery	Convex	Round
	BVIw1b	Orange	Slippery	Convex	Round
	BVIw2a	Yellow	Wavy	Flat	Irregular
	BVIw2b	White	Wavy	Flat	Irregular

Note: B - Blanakan; I-VI - sampling location (from 0 to 1250 m from the mangrove area); w - water sample; 1 – 1st intake; 2 - 2nd intake; a, b - 2 best isolates from each location.

Cellulase index measurement. 24 isolates that successfully grew on CMC media were tested for cellulolytic power using the Congo red staining method. In addition to using Congo red, we also used iodine to determine the degree of cellulolytic power presented (Andriani et al 2012). The formation of a clear zone around the colony indicates cellulolytic properties. This cellulolytic index shows the isolate's ability to degrade cellulose. A clear zone forms around the isolate because the isolate has cellulolytic power by utilizing cellulose in CMC media. Cellulolytic potential can be determined in secreting cellulase enzymes by testing the cellulolytic index based on the clear zone visible around bacterial colonies growing on the CMC medium (Shovitri & Zahidah 2013). Figure 2 shows the clear zone formed by one of the isolates from the Blanakan Mangrove area. Based on the 24 isolates tested for cellulolytic power, 9 isolates showed a clear zone and cellulase index (Table 3). The cellulolytic or hemicellulolytic index is the ratio between the diameter of the clear zone and the diameter of the colony (Meryandini et al 2010). The clear zone produced by the isolate is caused by the reaction of sodium benzidindiazo-bis-1-naphthylamine-4-sulfonate (Congo red), which interacts strongly with the β-1,4-glycosidic bond in CMC (Arifin et al 2019).

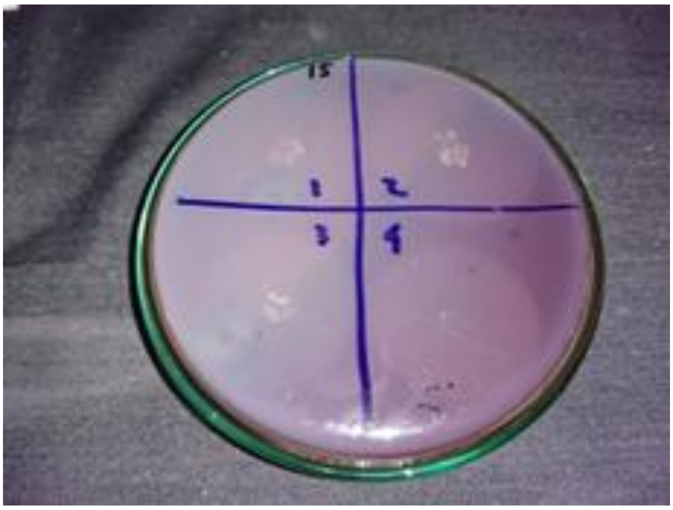

Figure 2. Formation of a clear zone around the isolate

Table 3

Clear zone diameter and cellulase index of 9 isolates

No	*Isolate code*	*Diameter (mm)*		*Cellulase index*
		Colony	*Clear zone*	
1	BIw1a	8	20	1.5
2	BIIw1b	6	8	2.3
3	BIIw2a	5	10	3
4	BIIw2b	3	7	3.3
5	BIIIw2a	8	27	2.3
6	BIIIw2b	8	28	2.5
7	BVIw1a	9	45	4
8	BVIw1b	8	43	4.37
9	BVIw2a	8	35	3.37

The formation of a clear zone is related to the solubility of the cellulase enzyme, with a higher solubility producing a larger clear zone. The diameter of the clear zone is generally larger than the diameter of the colony because the cellulase enzyme is secreted into the surrounding environment by cellulose-degrading bacteria. The 9 isolates that showed a clear zone had a diameter or cellulase index that varied widely among isolates, from 1.5 to 4.37. The lowest clear zone with a cellulase index of 1.5 was shown by isolate BIw1a, while isolate BVIw1b showed the highest cellulase index of 4.37. A greater cellulolytic index means a more potent enzyme produced by the bacterial isolate (Mulyasari et al 2015). The high or low cellulase index or clear zone diameter is caused by each bacterial species' ability to produce different cellulases by hydrolyzing the CMC substrate. It also depends on the genes, as cellulase production is influenced by the properties of cellulase, the cloning and expression of cellulase genes, and the carbon source used (Meryandini et al 2010; Sadhu & Maiti 2013; Sari et al 2018).

The final results obtained in the degradation process are influenced by several factors, namely pH, access to carbon (compatibility of enzyme conformation with substrate), redox reactions that occur, and product concentration. Based on the size of the cellulolytic index, research by Dar et al (2015) grouped the level of degradation into three groups, namely: high degradation, with a cellulolytic index of more than 4 cm; moderate degradation, with a cellulolytic index of 2-3.9 cm; and low degradation, with a cellulolytic index of less than 2 cm. Table 3 shows two isolates with a relatively high cellulolytic index, namely isolates BVIw1a and BVIw1b. The finding of 9 isolates (37.5%) that had a cellulase index from the Blanakan mangrove area shows that there is greater potential for the presence of cellulolytic bacteria when compared with research from the North Coast mangrove area of Semarang, where there were only two isolates that showed cellulase activity out of 18 isolates (11.11%) (Djarod et al 2017). The potential of mangrove areas as a source of bacteria that produce cellulase enzymes is also supported by the results of research by Sinatryani et al (2014), which found that cellulolytic bacteria were abundant in the Bancaran and Gunung Anyar mangrove areas (Sinatryani et al 2014).

The abundance of cellulolytic bacteria in mangrove areas is related to organic particles or litter, a living place for bacteria, fungi and other microorganisms. Mangrove litter buried in mud experiences decomposition by various organisms to produce detritus and minerals for soil fertility and a source of phytoplankton life (Mahmudi et al 2008). Dead plants are mainly comprised of cellulose, which tends to be hydrolyzed into glucose using the cellulase enzyme produced by cellulolytic bacteria. Hydrolysis of cellulose in plant materials using cellulase enzymes from cellulolytic bacteria can overcome pollution problems and produce economically valuable products (Masfufatun 2009).

Physiological and morphological characterization. Physiological and morphological characterization was performed on 9 isolates with clear zones or cellulolytic power (Table 4). Characterization is intended to group the isolate into one of the bacterial classification groups according to Holt et al (1994).

Table 4

Physiological and morphological characterization of cellulase-producing isolates

Isolate code	*Indole*	*MR*	*VP*	*Citrate*	*H_2S*	*Urea*	*Glucose broth*	*Oxidase*	*Catalase*	*Gram*	*Cell form*
BIw1a	-	-	+	-	-	+	without gas (+)	-	+	Positive	Coccus
BIIw1b	-	-	-	-	-	-	without gas (+)	+	+	Positive	Bacillus
BIIw2a	-	-	-	-	-	-	without gas (+)	-	+	Negative	Coccus
BIIw2b	-	-	-	-	-	-	-	-	+	Positive	Coccus
BIIIw2a	-	-	+	-	-	-	-	+	+	Positive	Bacillus
BIIIw2b	-	-	-	-	-	-	-	-	+	Negative	Bacillus
BVIw1a	-	-	-	-	-	-	-	-	+	Positive	Bacillus
BVIw1b	-	+	+	+	-	-	without gas (+)	-	+	Negative	Coccus
BVIw2a	-	-	-	-	-	-	-	-	+	Negative	Coccus

Note: - : negative; + : positive.

Of the 9 isolates, 5 were Gram-positive and 4 were Gram-negative. Based on cell shape, there were 5 isolates in the form of coccus and 5 isolates in the form of bacillus or stems (Figure 3).

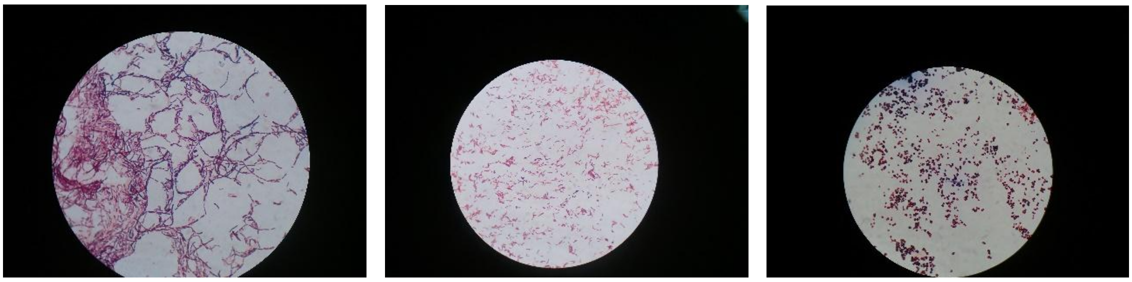

Figure 3. Coccus and stem cell forms of some isolates.

The two isolates with the highest cellulolytic power were BVIw1a and BVIw1b, with cellulolytic power of 4 and 4.37, respectively. The characteristics of isolate BVIw1a belong to the genus *Bacillus*. This is also supported by similar characteristics of isolates in other studies which were grouped into the genus Bacillus, namely that they were Gram-positive, bacillus or rod-shaped, produced catalase enzymes, were unable to produce oxidase, were unable to produce indole and H_2S, were unable to ferment VP, and were unable to hydrolyze citrate (Batubara et al 2022; Ananda et al 2023). *Bacillus*, which have cellulolytic power, are also often found in the waters of various mangroves in Kraton, Pasuruan. Some species are *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus subtilis* (Kurniawan et al 2019). The *Bacillus* genus studies have shown its potential in bioremediation strategies, being one of the best solutions for reducing heavy metals in various environments (Goyal et al 2019; Wróbel et al 2023).

The BVIw1b isolate has the characteristics of a Gram-negative bacterium, is round, produces catalase, and has physiological properties, including a positive MR result, negative VP, and negative H_2S. This characterization places the isolate in the genus *Aeromonas*. In addition, the characters shown by isolate BVIw1b are also the same as those of cellulolytic bacteria originating from Peniti mangrove forest soil, Mempawah Regency, bagasse waste, and sedimentary waters in the Tonle Sap Lake littoral zone, Cambodia, which were identified as belonging to the genus *Aeromonas* (Chasanah et al 2013; Rudiansyah et al 2017; Chantarasiri 2021; Chusniasih et al 2023). The *Aeromonas* genus comprises Gram-negative-bacteria that live in water and in terrestrial environments. This genus is closely related to humans and animals, and, based on research results, shows potential in bioremediation strategies, industrial biocatalysts, and polyester production (Canellas & Laport 2023).

Conclusions. Water samples were obtained from 6 observation stations in the Blanakan Mangrove area. Examining physical and chemical factors showed that the average water temperature was 28.3°C, the air temperature was 32.2°C, and the pH was 6. The results of isolation and purification showed that 24 isolates grew on CMC media, most of which were yellow and cream, with slippery edges, convex surfaces, and round shapes. Among the 24 isolates tested for cellulolytic power, 9 showed a clear zone and cellulase index. The 2 isolates with the highest cellulolytic power were BVIw1a and BVIw1b (station point 1250 m from the mangrove area), with cellulase indices of 4 and 4.37, respectively. The finding of isolates with a cellulase index from the Blanakan mangrove area shows that there is great potential for cellulolytic bacteria. The physiological and morphological characterization results of the 9 isolates showed that 5 isolates were Gram-positive, 4 isolates were Gram-negative, 5 isolates were coccus, and four isolates were in the form of bacillus. The two isolates with the highest cellulolytic power (BVIw1a and BVIw1b) belong to the genera *Bacillus* and *Aeromonas*. Both genera have good potential in bioremediation strategies to reduce environmental pollution.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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