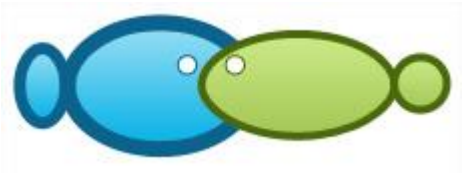

Selection of proteolytic *Vibrio* from mangrove sediments

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Abstract. The mangrove environment is very bacterially rich. One of these microorganisms is *Vibrio*. *Vibrio* is detrimental to humans and animals due to its ability to produce large amounts of proteases. The *Vibrio* isolated and identified in this study were proteolytic and lack of thermostable direct haemolysin Mangrove sediments were collected from Probolinggo City, Indonesia. Three of the locations were close to the estuary. Bacteria were isolated from sediment and grown on TCBSA media before being inoculated into TSA media for colony selection. BAP media was used to test pathogenicity, while SMA media was used to test proteolytic activity. Microbact Biochemical Identification kits were used for biochemical analysis, and 16S rRNA universal primers 5'TACGGYTACCTTGTTACGACTT 3 1492R and 27F 5' AGAGTTTGATCMTGGCTCAG 3' were used for genetic identification. In the BAP medium, 10 isolates did not display any identifiable zones. In addition, the 10 isolates were tested for proteolytic activity on SMA medium, with four of them, SdA34, SdB31, SdB33, and SdC14, achieving the greatest clean zones. Biochemical, morphological, and 16S rRNA investigations indicated that 3 bacterial isolates (SdA34, SdB31, and SdB33) belonged to the genus *Bacillus*, and were homologous to the species *Bacillus cereus*. One isolate (SdC14) is related to *Vibrio proteolitycus* and *Vibrio parahaemolitycus*.
Key Words: bacteria, estuary, identification, isolation, proteases.

Introduction. Different varieties of microbial communities interact with the mangrove ecosystem, resulting in a more diversified mangrove habitat. Microbes play an important part in the preservation of mangrove ecosystems (Gomes et al 2011). Based on various reports, *Vibrio* may be detected in its natural environment in mangrove sediments (Venugopal & Saramma 2006; Grisi & Gorlach-Lira 2010; Subhashini et al 2012; Manjusha et al 2013; Yahya et al 2014; Rocha et al 2016; de Menezes et al 2017; Ambeng et al 2019; de Fretes et al 2019). Three bacterial orders were detected in mangrove sediments: Actinomycetales, Bacillales, and Vibrionales (Dias et al 2009). Furthermore, the enzyme production test on these three sets of bacterial orders resulted in the synthesis of four distinct types of enzymes, including amylase, protease, esterase, and lipase. The most abundant producers of these enzymes, notably amylase and protease, are Vibrionales. This bacteria produces enzymes such as proteases, agarases, L-glutaminase, alginate lyase, and amylase (Manjusha et al 2013). Vibrionales are a type of bacteria capable of producing high-efficiency extracellular protease enzymes for industrial use (Venugopal & Saramma 2006).

Protease enzymes catalyze the hydrolysis process, which breaks down protein molecules into peptides and amino acids. Enzymatic hydrolysis can lower the size of a peptide, increasing its characteristics and protein quality. Microbe-derived protease is preferable than plant-derived protease, because it can be produced in large numbers quickly, is relatively inexpensive, and is sustainable (Sharma et al 2017). The goal of this research is to locate and isolate *Vibrio* bacteria capable of producing protease enzymes in mangrove sediments.

Material and Method

Description of the study sites. The study was carried out between April and July 2021. Mangrove sediments were collected from the Probolinggo mangrove forest, East Java province, Indonesia (N - 7°44'39.8616; E - 113°13'56.2188) (Figure 1). Purposive random sample was used to identify the sampling location, which consisted of three sites with robust mangrove vegetation and *Avicennia* vestiges. In addition, pH, temperature, and sediment conductivity were measured. In the mangrove region, three points of a 5x5 m transect were sampled. The distance between transects was between 50 and 100 m. Each transect was sampled three times. Using a sediment scoop, 100 g of sediment samples were collected at depths ranging from 10 to 20 cm. The distance between the sediment and the nearest river flow has been used to calculate how many samples were collected at each sediment sampling station. Sediment samples were saved in sterile plastic sample trays and stored at 4°C, until they arrived at the Biological Science Central Laboratory of the University of Brawijaya Malang in East Java, Indonesia.

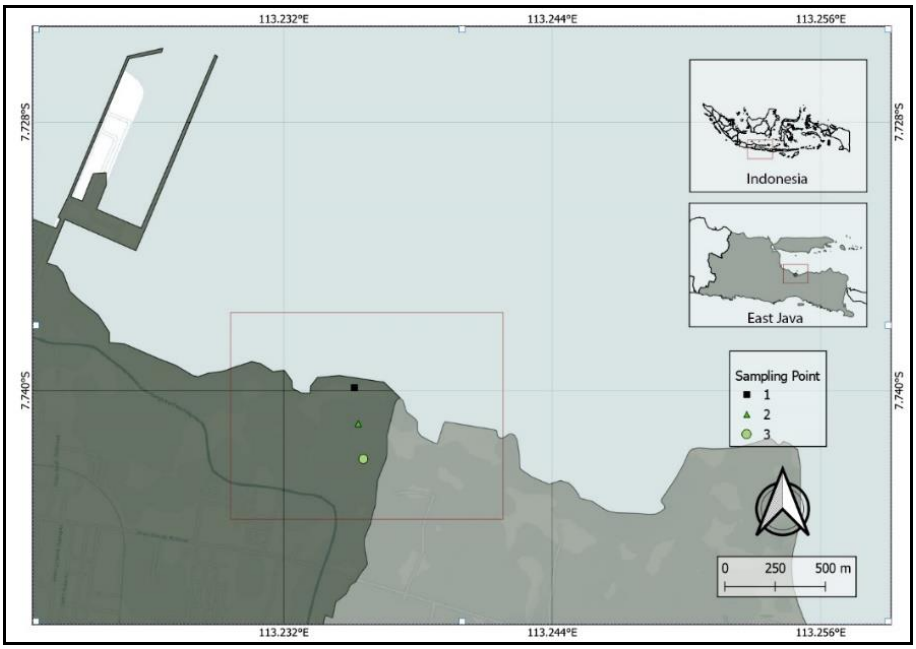

Figure 1. Location of sediment sampling at Probolinggo Mangrove Forest.

Sediment sampling. Sediment samples were collected from several locations and blended into a single sample. After homogenizing 25 g of the composite, it was diluted in 25 mL of alkaline peptone water (APW). This sediment solution was the first dilution (10^{-1}). The same dilution process was used for serial dilutions to 10^{-5}. The sediment samples were inoculated on petri dish selective media, preferably TCBSA (Thiosulphate Citrate Bile Salt Agar, Himedia M189), and incubated overnight at 37°C. 5 selected colonies from the TCBSA plate were reinoculated on a petri dish plate using TSA (tryptone soya agar, Oxoid CM0131B) medium.

Isolation and selection of bacteria. The streak plate method was employed to assess pathogenicity using BAP (Blood Agar Plate, acquired from the Agarindo Biology Laboratory in Indonesia) and incubated overnight at 37°C. Clear zones do not form in *Vibrio* colonies cultivated on BAP medium if bacteria lack tdh (thermostable direct haemolysin) (Buxton 2005). Furthermore, the *Vibrio* isolate without a distinct region was chosen for the following test. The proteolytic activity test determines which *Vibrio* isolates generate the most proteases, as measured by the diameter of the clear zone created on SMA (Skim Milk Agar, Himedia M763) media using the modified method from Prabakaran et al (2015) and Baehaki et al (2011).

Characterization and identification of bacteria. Bacteria were identified using microscopic morphological criteria as well as physiological qualities revealed by biochemical tests. Gram staining was used to examine cell shape, size, and color under a microscope (Ambeng et al 2019). Bacteria and other microorganisms can be classified depending on their enzymatic and metabolic functions. Bacteria may live on a wide range of metabolites, which are recognized by microbial interactions with reagent test agents, producing reagent colors. Certain tests will elicit cellular reactions. To identify bacterial isolates, a variety of biochemical reaction tests can be utilized (Venkateswaran 1999).

Microbact Biochemical Identification kits were used to characterize the chosen bacteria isolates (Oxoid). The 16S rRNA technique was used to identify *Vibrio* bacteria at the molecular level (Dorsch et al 1992). The 16S rRNA sequence was amplified using the Polymerase Chain Reaction (PCR) technique using universal primers 1492R 5'TACGGYTACCTTGTTACGACTT 3' and 27F 5' AGAGTTTGATCMTGGCTCAG 3', followed by sequencing at 1st BASE Laboratories Sdn Bhd, Malaysia. The DNA sequencing findings from this technique were then compared to NCBI Genbank data for bacterium kinds using the Nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, using the ClustalW tool in MEGA 11 software, we were able to rebuild the phylogenetic tree of isolated bacteria by aligning the nitrogen base sequences and comparing them using the neighbor joining method.

Statistical analysis. Microsoft Office Excel 2019 was used to tabulate the data. Normality and homogeneity have been verified. One-way ANOVA was used to compare variables between data, followed by a Duncan post-hoc test, with significance set at $p<0.05$. IBM PASW Statistic 18 software was used for statistical analyses.

Results and Discussion. The research location is a naturally occurring mangrove forest close to extensive whiteleg shrimp (*Litopenaeus vannamei*) ponds. Two big ponds were operational at the time of sampling, as evidenced by the operation of the pond's waterwheel, whereas the other three were inert. According to observations, Rhizophora and *Avicennia* species dominated the mangrove vegetation at sampling site 1 (Figure 2). A puddle was present at the site. The water quality parameters at the time of testing were DO 2.2 mg L^{-1}, water pH 6.4, soil pH 5, temperature 28^{o}C, and salt 29 ppt.

Figure 2. Mangrove condition in the sampling location.

The mangrove vegetation at sampling point 2 was nearly identical to that of sampling point 1, where *Rhizophora* and *Avicennia* prevailed. There is a small stream that serves as drainage for the water in the pond. In sampling point 2, water quality measurements reveal a DO content of 2.4 mg L^{-1}, a pH of 6.3 in the water, a pH of 4.7 in the soil, a

water temperature of 28°C, and a salinity of 32 ppt. A minor stream with a higher flow of water than the stream at sampling point 2 was present at sampling point 3. This river is thought to be a water intake channel for ponds. In this sampling point, the DO was 2.6 mg L^{-1}, pH 6.4, soil pH 5, temperature 28°C, and salinity 30 ppt. The flora at sample point 3 was dominated by *Sonneratia* and *Rhizophora*.

The soil texture of mangrove sediments from each of the study's sample locations shows no significant variation. The soil texture of the mangrove sediment at position I (A) was composed of 0.77% sand, 43.51% dust, and 55.72% clay. Location II (B) was composed of 0.91% sand, 46.02% dust, and 53.07% clay. Position III (C) presented 1.32% sand, 44.51% dust, and 54.17% clay.

Isolation of bacteria from mangrove sediment. The results of bacterial culture from mangrove sediments on TCBSA medium are presented in Figure 3 and Table 1. Each TCBSA-inoculated sand sample generated a different number and color of colonies: SdA1=20, SdA2=30, SdA3>50, SdB1=18, SdB2=40, SdB3>50, SdC1=6, SdC2=7, SdC3=12. Colors include yellow (Y), green (G), greenish yellow (GY), and black (B). Convex forms are classified into three types: irregular convex (IrC), smooth convex (SC), and smooth umbonate (SU).

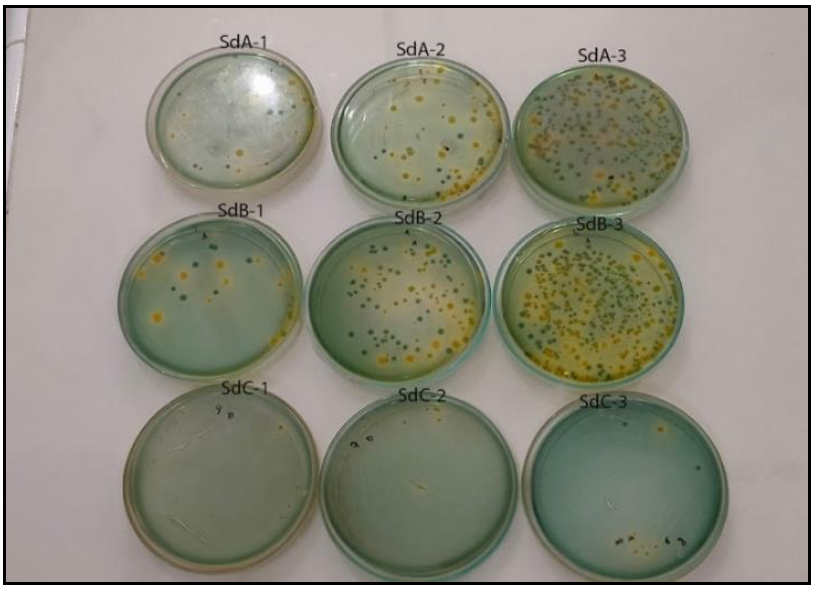

Figure 3. *Vibrio* bacterial colonies from mangrove sediments.

Table 1

Mangrove sediment bacterial colony data on TCBSA media

Point sampling	*Repetition*	*Code*	*Number of colonies*	*Color*	*Shape*
I	1	SdA1	20	Y, G, GY, B	IrC, SC, SU
	2	SdA2	30		
	3	SdA3	>50		
II	1	SdB1	18	Y, G, GY	IrC, SC, SU
	2	SdB2	40		
	3	SdB3	>50	Y, G, GY, B	
III	1	SdC1	6	Y, G, GY	IrC, SC
	2	SdC2	7		IrC, SU
	3	SdC3	12		SC, SU

Note: Y - yellow; G - green; GY - greenish yellow; B - black; IrC - irregular convex; SC - smooth convex; SU - smooth umbonate.

Purification or reinoculation of isolates on TCBS medium was done depending on the colonies found on each isolate from the prior study location. Pure bacteria isolate cultures were obtained from each mangrove sediment sample, with the following results based on the similarity of isolate characteristics, particularly color and colony morphology: SdA1=5, SdA2=10, SdA3=10, SdB1=8, SdB2=10, SdB3=10, SdC1=3, SdC2=3, SdC3=5, resulting in a total of 64 isolates from reinoculation. Inoculants were then cultivated on BAP media.

Blood agar plate (BAP) media selection. Bacteria colonies were seen to proliferate, with varied clear zones generated by each isolate depending on the findings of BAP media selection (Figure 4). This growth was classified into four groups: group 1 (no clear zone created), group 2 (a small clear zone diameter formed), group 3 (a medium clear zone diameter formed), and group 4 (a big clear zone diameter formed) (produced a large clear zone diameter). Group 1 had 8 isolates, group 2 had 9 isolates, group 3 had 23 isolates, and group 4 had 24 isolates. In addition, 8 isolates from group 1 were chosen from the BAP media screening to continue the screening using skim milk agar (SMA) media to examine the proteolytic activity.

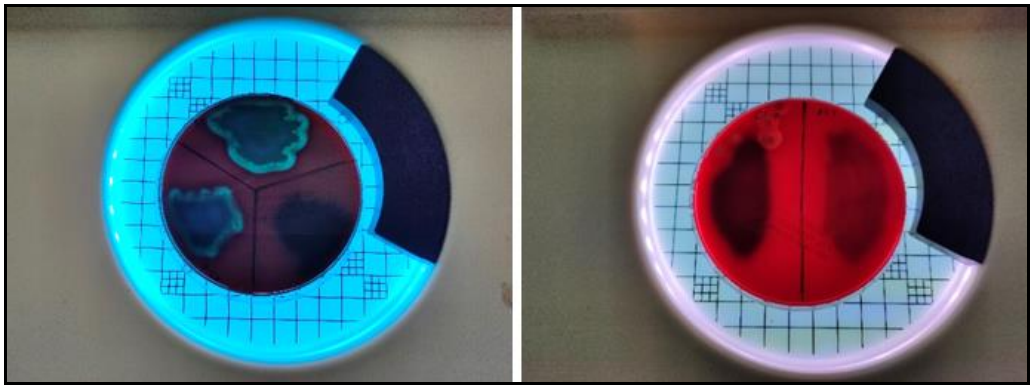

Figure 4. Bacterial isolates inoculated on blood agar plate.

Skim milk agar (SMA) media selection. Based on the findings of the proteolytic assay on 8 isolates, 4 isolates (SdA34, SdB31, SdB33, and SdC14) had the largest clear zone (Figure 5).

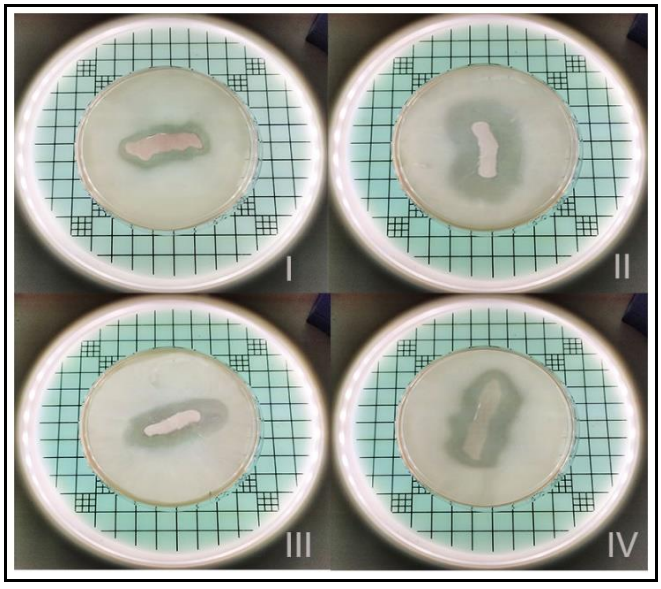

Figure 5. Isolates of bacteria that produced the largest clear zone on SMA media after 24 h (I=SdA34, II=SdB31, III=SdB33, IV=SdC14).

The 4 isolates were chosen using SMA media, and the morphological and biochemical parameters of the bacteria were determined.

Identification of bacteria. Table 2 displays the test results. Bacterial identification was conducted by molecular genetics using the 16S rRNA technique (Dorsch et al 1992). Furthermore, based on the identification of 16S rRNA, the sequence was amplified using the Polymerase Chain Reaction (PCR) technique using universal primers 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 1114R (5'CCCGGAACCCAAAAACTTTG-3') and 765R internal primers (5'-CCCGGAACCCAAAAACTTTG-3'; '-CTGTTTGCTCCCCACGCTTTC-3'). The DNA was amplified well at a length of roughly +1400p (Figure 6). Following that, the PCR products were sequenced in 1st BASE.

Table 2

Morphological and biochemical test results of bacterial isolates from mangrove sediments

No	*Characteristics*	*Isolates*			
		SdA34	*SdB31*	*SdB33*	*SdC14*
	Morphology				
1	Smooth convex	-	+	-	-
2	Irregular convex	+	-	-	-
3	Smooth umbonate	-	-	+	+
4	Yellow	-	-	+	+
5	Green	+	-	-	-
6	Green yellowish	+	-	-	-
7	Gram	-	-	-	-
8	Straight cells	+	-	+	+
9	Bent cells	-	-	+	+
10	Motilities	+	+	+	+
	Biochemical properties				
11	Reduction of nitrate	+	+	+	+
12	Oxidase	+	+	+	+
13	Indole production	+	+	+	+
14	Voges-Proskauer	-	-	-	-
15	Methyl red	+	+	+	+
16	Spore	-	-	-	-
17	Lysin	+	-	-	-
18	Glucose	-	-	-	-
19	Manitol	+	+	+	+
20	Xylosa	-	-	+	-
21	H_2S	-	-	-	-

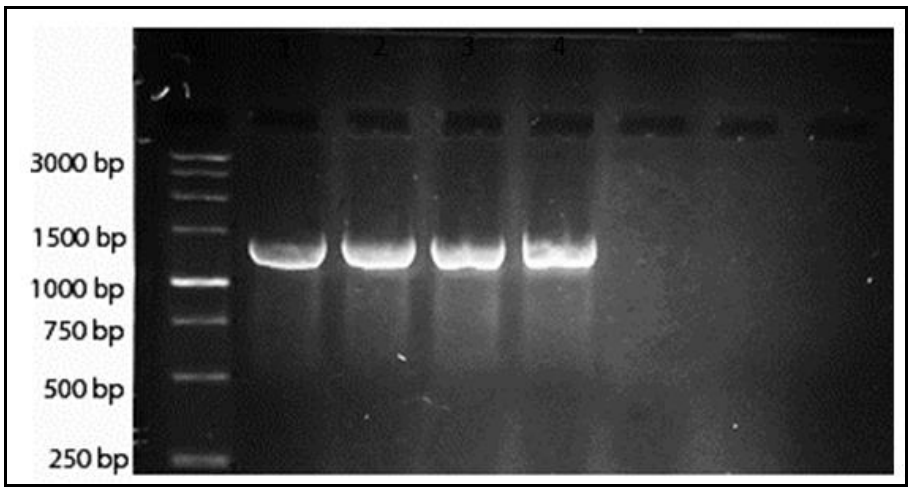

Figure 6. Results of DNA amplification electrophoresis of markers (M), SdA34 (1), SdB31 (2), SdB33 (3), SdC14 (4) are shown from left to right.

BLAST was used to compare the sequences of isolated proteolytic bacteria strains from sand to those of bacterial strains housed in the NCBI GenBank. According to the BLAST findings, isolates SdA34, SdB31, and SdB33 appear to be members of the phylum Firmicutes, genus *Bacillus*, but isolate SdC14 appears to be a member of the phylum Proteobacter, genus *Vibrio*. The phylogenetic tree was constructed using the 16S rRNA gene sequence (Figure 7).

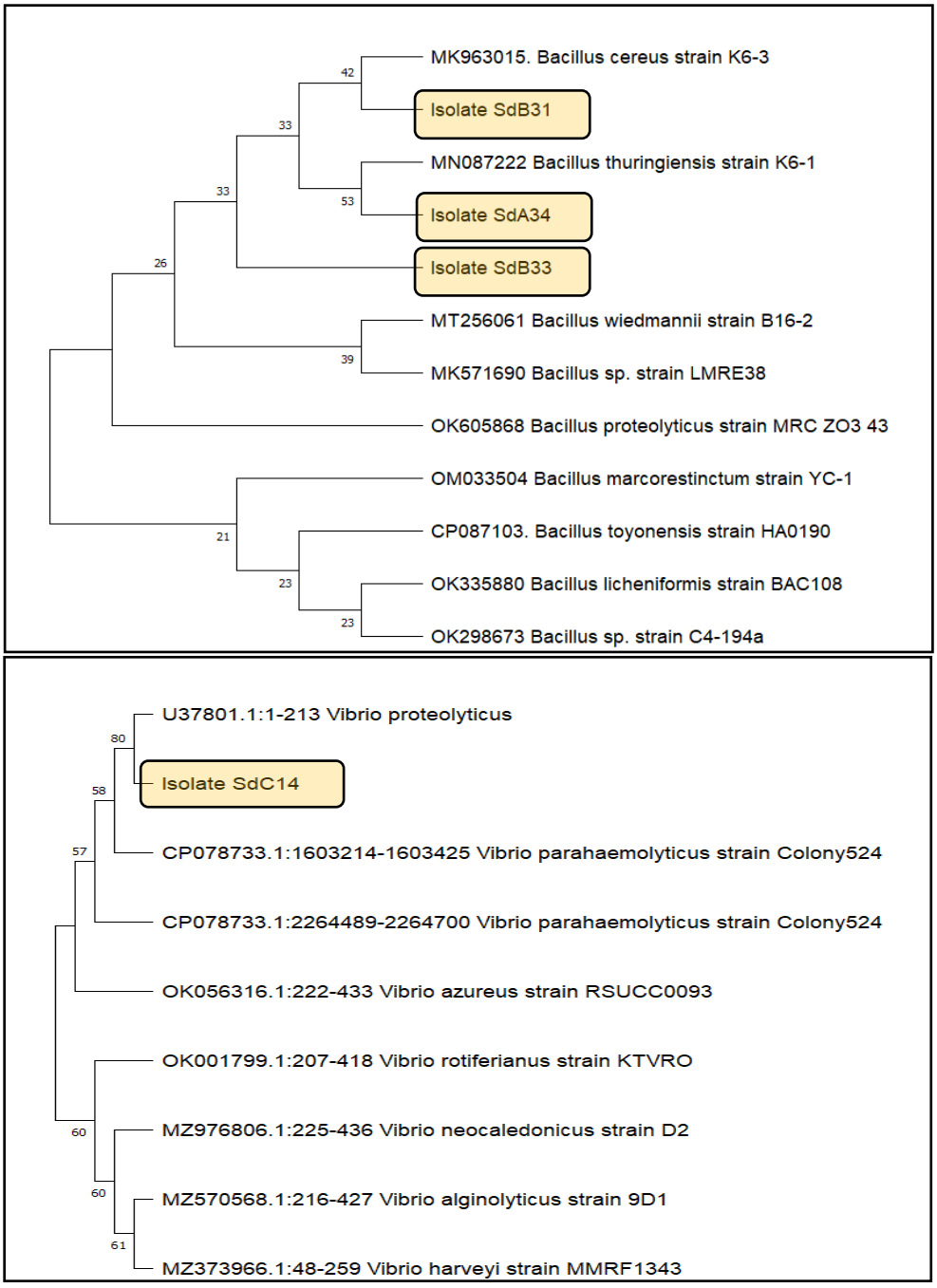

Figure 7. The phylogenetic tree of sediment proteolityc bacteria was generated using the neighbor-joining method and compared to those in other homologous species of bacteria.

In this study, the water and soil quality parameters in the mangrove ecosystem were excellent for the formation of *Vibrio* bacteria. Islamiah et al (2017) noted that *Vibrio* bacteria were found in soils with a pH of 6-7, a salinity of 30 ppt, and temperatures ranging from 28 to 37°C. According to Behera et al (2016), mangrove soil bacteria thrive at pH levels ranging from 5 to 7 and a maximum temperature of 40°C. According to

Yulma et al (2017), at 28-30 ppt salinity, 28-30°C temperature, pH between 7.6-7.8, and DO between 5.53-6.15 mg L^{-1}, there was a great variety and amount of bacteria in mangrove debris. The three principal mangrove genera are *Rhizophora*, *Sonneratia*, and *Avicennia*. Yahya et al (2014) observed *Avicennia marina, Rhizophora apiculata, Avicennia alba,* and *Sonneratia alba* are among the plant species isolated from mangrove waters and that *Vibrio* bacteria are more common in *Avicennia alba* vegetation.

In this research, the bacterial colony data showed that the closer the sample station was near running water, such as streams, the more bacteria colonies developed on TCBSA culture media. Point I had the highest colony abundance, followed by points II and III. De Menezes et al (2017) observed a large number of colonies in estuarine waters and sediments. This is due to the estuary's favorable natural conditions for *Vibrio* growth.

SdA34, SdB31, SdB33, and SdC14 are 4 of 8 isolates selected from group 1 due to morphological similarities. According to Buxton (2005), pathogenic strains exhibit haemolysis, which results in the formation of a clear zone surrounding the bacteria colony propagating on BAP or wagatsuma media, a phenomenon known as the Kanagawa phenomenon. In bacteria colonies cultivated on wagatsuma media, microbes lacking tdh do not exhibit specific zones (Sakazaki et al 2006).

Morphological and biochemical data show that the colony properties discovered in this study were identical to those of the genus of *Vibrio*. Several of the microbiological analytical parameters investigated in this study are connected to the nature of bacteria in the genus *Vibrio*. This includes being gram-negative, having a curved rod shape (comma shape), being facultative anaerobes, being positive for oxidase, not generating spores, being motile, and possessing polar flagella (De Souza Valente & Wan 2021).

Isolates SdA34, SdB31, and SdB33 belong to the *Bacillus* genus and are homologues of *Bacillus cereus* and *Bacillus thuringiensis*. Although the TCBS media employed in this work is a selective medium designed to cultivate bacteria from the *Vibrio* genus, it does not rule out the growth potential of bacteria from other genera, particularly those from salty substrates (marine). Chen et al (2012) identified 7 genera among the 158 strains isolated from the Jiulong River estuary sediments: *Pseudomonas* (28%), *Aeromonas* (24%), *Pseudoalteromonas* (19%), *Shewanella* (13%), *Bacillus* (11%), *Vibrio* (4%), and *Psychrobacter* (4%), among others (1%). *Bacillus* species produce the majority of proteases, primarily neutral and alkaline proteases (Rao et al 1998). Zebua et al (2020) discovered that 3 of the 10 isolates from mangrove sediment belong to the *Bacillus* genus.

Vibrio bacteria, in particular, are known to produce protease enzymes. *V. cholerae, V. fluvialis, V. mimicus, V. parahaemolyticus, V. alginolyticus, V. metschnikovii,* and *V. vulnificus* are several species of proteolytic *Vibrio* that are pathogenic to humans and fish. Others proteolytic vibrio species, including *V. aestuarianus, V. anguillarum, V. carchariae, V. harveyii, V. pelagius, V. penaecida, V. proteolyticus,* and *V. tubiashii,* are nonpathogenic to humans and fish (Shinoda & Miyoshi 2011). Although serine proteases and cysteine proteases are other types of proteolytic enzymes, vibrio-produced proteolytic enzymes are generally classified as zinc metalloproteases (Miyoshi et al 2002; Venugopal & Saramma 2006; Shinoda & Miyoshi 2011).

Conclusions. Microorganisms are abundant in mangrove sediments. They could be used as a source of protease enzymes for biotechnology applications. Based on the results of bacterial selection using TCBSA, blood agar plate (BAP), and skim milk agar (SMA) media, 4 proteolytic bacteria candidates were identified. Furthermore, biochemical, morphological, and 16S rRNA analyses revealed that 3 bacterial isolates (SdA34, SdB31, and SdB33) were *Bacillus* members, homologous to the species *Bacillus cereus*, while one isolate (SdC14) is related to *Vibrio proteolitycus* and *Vibrio parahaemolitycus*.

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

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