Lignolytic Activity of Bacteria Isolated from Mangrove Sediment in Tirang Beach, Semarang as a Consortium Agent for Bioremediation of Methylene Blue Dyes

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Abstract. Lignin is an abundant phenolic compound that has –OH groups and is found in many sources such a tree. Lignolytic enzyme plays an important role in dye bioremediation. Methylene blue is a dye generally applied in the industry to produce a blue color. However, it also causes pollution and waste. To prevent environmental pollution, it is necessary to process this waste. Bioremediation is an environmentally friendly method for dealing with dye waste. This research aims to isolate bacteria from mangrove sediment that can break down lignin and remove methylene blue color. The research methods include isolating and purifying the bacteria, testing for lignin-degrading enzymes, decolorization test were performed with a spectrophotometer at 650 nm wavelength, and analyzing bacteria's genetic information. The results of the isolation process showed that 17 potential bacteria were isolated from mangrove sediments. Among them, 6 bacteria were tested positive for lignin-degrading enzymes, with degrading zone sizes ranging from 6.5 to 14 mm. The decolorization test, conducted at a wavelength of 650 nm, revealed that 3 selected bacteria had decolorization rates of 88% for isolate 4.1.1, 74% for isolate 3.2.1, and 82% for isolate 2.2.1. The consortium of bacteria achieved a decolorization rate of 90%. Molecular identification using 16s RNA identified the species as *Exiguobacterium profundum*, *Vibrio alginolyticus*, and *Pseudomonas aeruginosa*.

1 Introduction

Mangrove ecosystem had an important role for maintaining the stability of the surrounding environment by mitigating physical dynamics. Mangroves important role due to their physico-chemical dynamics, which contribute to the high diversity of microorganisms. This, in turn, leads to an abundance of nutrients for microorganisms, aiding in pollution control [1].

Mangrove bacteria produce extracellular enzymes, including the laccase enzyme plays an important role in the bioremediation of pollutants. Laccase is a type of oxydase enzyme that

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is commonly found in microorganisms and plants. It has various potential applications, one of which is the degradation of methylene blue. Another study reported that laccase is effective in breaking down dyes due to its oxidative properties [2]. Laccase has been extensively studied and has shown positive results in treating dye waste, such as methylene blue, safranin, and crystal violet. Mangrove sediment bacteria also produced lignolytic enzyme and had a lot of potential [4]. Lignin in mangroves originates from roots, root substrates, and leaf litter, as mangroves were one of the plants that contain high amount lignocellulose.

This research aims to investigate bacteria that have the ability to break down lignin and remove dyes from water. The study is particularly interested in the problem of dye waste in the textile industry, which is currently receiving a lot of attention due to its urgent nature and potential environmental pollution risks. One specific dye that will be examined is Methylene blue, as it is readily available and commonly used in the textile industry. The presence of dye materials in waste can lead to pollution and pose a threat to human health. In addition to that, its impact on living organisms can also result in death. Currently, efforts are being made to develop dye waste treatment methods to neutralize it and prevent it from posing a threat to the environment.

Based on the description above, the research aims to determine the following the activity of lignin-degrading enzymes, the ability of bacteria to remove color from dyes, and the effectiveness of both consortia and individual isolates in breaking down dyes.

2 Materials and methods

2.1 Samples collection

Researchers conducted sediment sampling in the mangrove ecosystem area at Tirang Beach, Tugurejo, Tugu, Semarang City, Indonesia. The sample was collected with shovel in a depth of 5 cm [5]. The collected samples were then stored in ziplock bags in room temperature (27°C) and labeled with a sample name. Sediment sample preserved in cool box transported to laboratory for bacterial isolation. Preparation of sample sediment sample must directly homogenize to sterile seawater for isolation and purification of bacteria.

2.2 Screening and bacteria isolation

The process of sample purification involved performing serial dilution up to the 10^{-5} dilution. Each dilution consisted of 9 mL of sterile seawater. Diluted sample of 10^{-2}, 10^{-3}, and 10^{-4} was the selected dilution for bacterial isolation. Spread plate method was used for isolation in this research. This involved pipetting 100 µL of the diluted sample onto the prepared medium. Samples that were already in the agar medium were also subjected to the spread plate technique. The plates were then incubated in an incubator in temperature 34°C for 24 h. The purification process is conducted using Zobell marine 2216 agar media, which consisted of 15 g of bacteriological agar, 1 g of yeast extract, and 4 g of peptone per liter. Additionally, other salt mineral components such as 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0,5 g of $MgSO_4.7H_2O$, 0,5 of Na_2SO_3, and 0,5 of NaCl were also added for 1.000 mL composition to the media. The method of purification involved dividing the agar plate into four quadrants [3].

2.3 Lignolytic assay

The ligninolytic activity test was conducted with Kirby Buaer method. Each paper disk was soaked in 30 µL of the test sample. The test was prepared on Zobell agar media that were enriched with 1% tannic acid. Positive results of the lignolytic test indicated by the presence of a brown to reddish brown zone because activation of phenol oxidase from bacteria degrading lignin [6].

2.4 Bacterial growth

The growth of bacteria was studied by cultured isolates into Zobell broth. The growth rate was conducted using a spectrophotometer at a wavelength of 600 nm with pure zobell broth as a blanko. The spectrophotometer analysis indirectly measured bacteria biomass. Iincreased turbidity in a culture serves as an additional indicator of bacterial growth and cell numbers (biomass). Utilizing a spectrophotometer reveals a decrease in transmitted light corresponding to an increase in cell population. Measurements were taken at intervals of 2, 4, 6, 8, 12, 18, 24, 36, 48, and 72 h [7].

2.5 Decolorization assay

Decolorization method based on Zobell broth 2216E media were used along with 1% methylene blue (100 mg·L-1) [11]. The mixture then shakes at a speed of 100 rpm. The absorbance value of the samples after centrifugation were measured with a spectrophotometer at a wavelength of 650 nm. The absorbance measurement at various time intervals, including the 0, 2, 4, 6, and 24 hours. The blank of decolorization assay was Zobell Broth added 1% methylene blue (100 $mg \cdot L^{-1}$) and measured with spectrophotometer for initial point. Decolorization test was measured with equation:

$$\%Decolorization = \frac{Initial\ absorbance - final\ absorbance}{Initial\ absorbance}\ X\ 100 \quad (1)$$

2.6 Antagonist Assay

The antagonist assay streaked the isolate into three streak sections zone in petri dish. Antagonist test was used Zobell agar 2216E and the isolate were stored in incubator at 34℃.

2.7 Molecular Identification

Molecular identification is conducted on bacterial isolates that exhibit positive results in enzymatic tests for their activity. The MEGA 11 software is used for sequence analysis and the creation of phylogenetic trees. The neighbor-joining method is employed to construct the phylogenetic tree. The bacterial DNA extraction procedure is performed and utilizes the Geneaid Kit [9]. After the extraction, then the 16s RNA sample were amplified with polymerase chain reaction [10]. The PCR formula with mixed 1.5 µL of DNA template, 15 µL of Gotaq green Master Mix (Promega), 3 µL of forward primer, 3 µL of reverse primer, and 7.5 µL of ddH2O, resulting in a total volume of 30 µL of PCR sample. Denaturation stage was set up to 95℃, annealing set up to 55℃, and elongation set up to 72℃ and repeated until 30 cycles. After PCR, electrophoresis with 1% gel agarose was conducted to determine the length of DNA bands and the purity of DNA. The PCR result was then sequenced by PT. Genetika Science company. The sequencing results were processed with MEGA 11 software

and aligned using alignment tools. The aligned sequences were saved in Fasta format. The alignment results were examined using BLAST on the National Center for Biotechnology Information (NCBI) website. The BLAST was accessed through the NCBI website at https://blast.ncbi.nlm.nih.gov/Blast.cgi [11].

3 Result

3.1 Purified Bacteria

The results of mangrove sediment bacteria showed there were 17 isolates. These isolates were obtained by isolating the endogenous bacteria using Zobell Agar 2216 E media with the addition of mineral salt. The characterization results revealed that the dominant isolate had a circular shape, and it was white and creamy white in color. It had a convex elevation, size from punctiform to large, and it had entire margins (Figure 1).

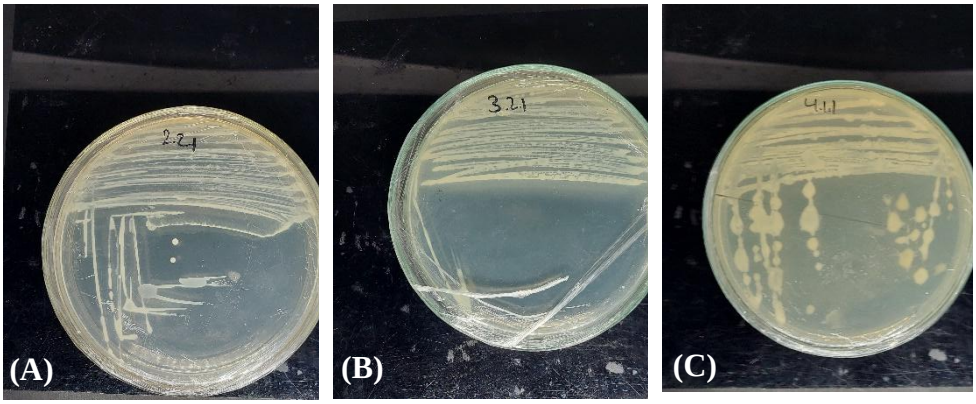


Fig. 1. Purified bacteria from mangrove sediment (A) isolate 2.2.1, (B) isolate 3.2.1, and (C) isolate 4.1.1.

3.2 Lignolytic Assay

The results of the lignolytic test using the disk diffusion method are shown in Table 1. Positive results are indicated by the formation of a brown or reddish-brown zone.

Table 1. Lignolytic activity of bacterial isolates from mangrove sediment.

No	Isolate code	Time (hour)		
		24 h	48 h	72 h
1	SDm 2.2.1	8.75 ± 0.35 mm	9.6 ± 1.1 mm	9 ± 0.6 mm
2	SDm 3.1.3	7 ± 0.35 mm	7.2 ± 0.4 mm	7 ± 0.92 mm
3	SDm 3.1.6	8 ± 0.33 mm	8.7 ± 0.30 mm	14 ± 1.4 mm
4	SDm 3.1.7	9.23 ± 0.32 mm	11.5 ± 1 mm	9 ± 1.2 mm
5	SDm 3.2.1	8.17 ± 0.24 mm	11.6 ± 0.78 mm	13.4 ± 0.7 mm
6	SDm 4.1.1	0 ± 0 mm	4 ± 0.15 mm	6.5 ± 0.7 mm

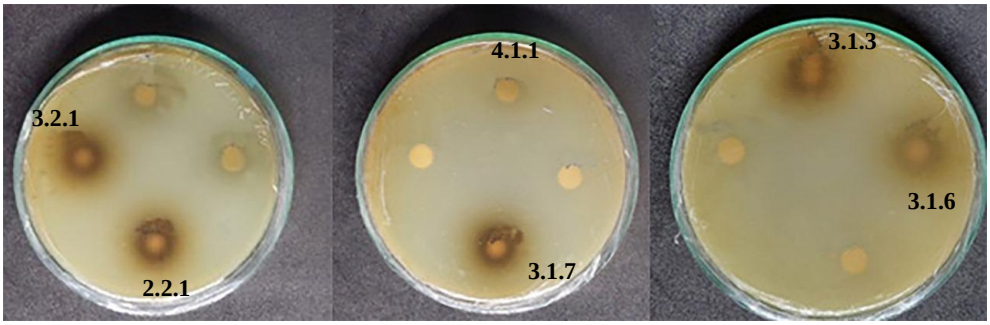


Fig. 2. Lignolytic test results for isolates 3.2.1, 2.2.1 (a), isolates 4.1.1, 3.1.7 (b), isolates 3.1.6, 3.1.3 (c).

Lignolytic activity test showed that 6 out of the 17 isolates positive for lignolytic enzymes. The isolate with the code 4.1.1 had the lowest value of 6.5, while the highest value of 14 mm was observed in isolate 3.1.6. The formation of a brown color indicates bacterial activity in degrading lignin, as observed in isolates 3.2.1 and 2.2.1 in figure 2a), isolate 3.1.7 in figure 2b), and isolates 3.1.6 and 3.1.3 in figure 2c). Isolate 4.1.1 had a late lignolytic activity than other isolate because it has appeared more than 24 h.

3.3 Antagonist assay

The antagonist assay revealed that the three isolates tested did not exhibit antagonistic activity, suggesting that the three isolates were working together. This is indicated by the lack of an inhibition zone from each streak on the media used (Figure 3).

Fig. 3. Antagonist assay of isolate 4.1.1 (A), isolate 2.2.1 (C), and isolate 3.2.1 (D).

3.4 Bacterial Growth

Table 2. Bacterial growth kinetics of methylene blue dye degrading bacteria

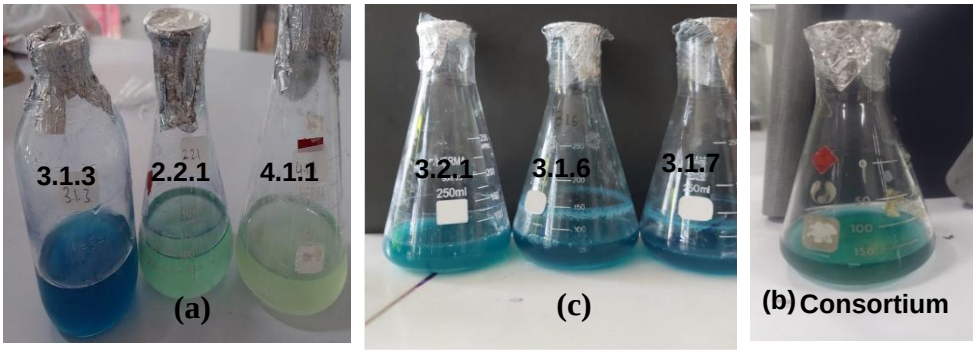
Hour	Isolate Code								
	SDm 3.2.1			SDm 4.1.1			SDm 2.2.1		
	Λ	μ	Tg	Λ	μ	Tg	λ	μ	Tg
0	0.011	0.282	2.46	0.011	0.253	2.68	0.012	0.265	2.62
2	0.259			0.1796			0.318		
4	0.294			0.2395			0.366		
6	0.570			0.5815			0.453		
12	1.166			0.7675			0.976		
18	1.753			1.004			1.433		
24	1.814			1.235			1.816		
36	1.46			1.3436			1.677		
48	1.129			1.228			1.075		
72	0.827			0.9805			0.889		

Note: The μ symbol indicates growth rate (hour^{-1}), λ indicate absorbance, and Tg indicate generation time (hour)

The bacterial growth curve for both isolates indicated that the logarithmic or exponential phase occurred at 18 h. The generation time for isolate 3.2.1 was 2.46, 4.1.1 is 2.68 while for isolate 2.2.1 it was 2.62. The bacterial growth compared from lignolytic activity from Table 2 and pre-screening decolorization activity from Figure 3 with the highest and lowest lignolytic activity. The isolate with code 4.1.1 was a slow metabolism to degrading lignin because the activity had appeared after more than 24 h incubation.

3.5 Decolorization Assay

The decolorization activity in this study was evaluated qualitatively by observing color changes in Zobell broth 2216E media. The media was supplemented with 0.5% Methylene blue. Quantitative analysis was also performed using a spectrophotometer at a wavelength of 650 nm. The results of the lignin decolorization test are presented in Figure 4 and percentage of decolorization presented in Table 3.



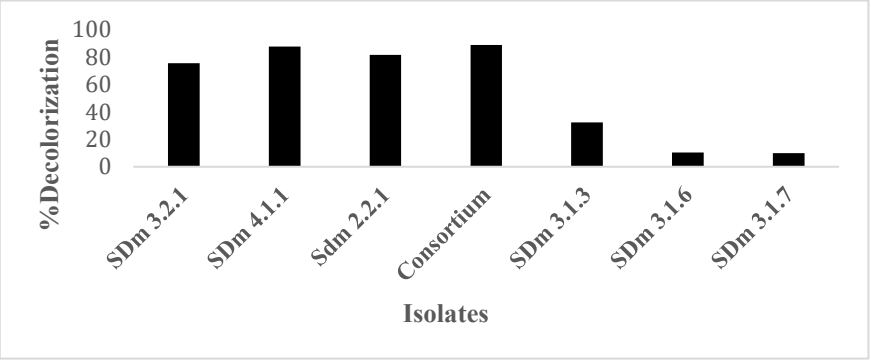


Fig. 4. Decolorization assay of methylene blue by bacterial isolates (a) consortium (b) and %decolorization by bacterial isolates and consortium (c).

Table 3. Biodecolorization activity from spectrophotometer absorbance result.

Hour	Isolate Code			
	SDm 3.2.1	**SDm 4.1.1**	**SDm 2.2.1**	**Consortium**
0	1.82	1.82	1.82	1.82
2	1.567	1.433	1.669	1.547
4	1.375	0.908	0.896	0.923
6	1.156	0.684	0.647	0.326
8	0.726	0.433	0.581	0.224
24	0.443	0.224	0.332	0.199

Isolates 2.2.1, 3.2.1, and 4.1.1 were produced the highest decolorization activity. These isolates were combined as consortium produced the highest decolorization activity. The decolorization results can be observed by the clarity produced on the media by the isolate in Figure 4a and consortium in Figure 4b. Decolorization percentage of methylene blue from isolates shows that there were 3 isolates with highest activity of decolorization. Isolate 4.1.1 had 88%, 3.2.1 had 76%, and 2.2.1 had 82% decolorization. Higher decolorization represents that these isolates could be candidates as bioremediation agent for dye waste.

3.6 Molecular Identification

The sequencing results produce similar species based on the nucleotide bases that are obtained (Table 4). These results can be seen in Table 4. The results of the BLAST indicated that isolate with code 4.1.1 was identified *as Exiguobacterium profundum,* 3.2.1 as *Vibrio alginolyticus,* and 2.2.1 *as Pseudomonas aeruginosa.*

Table 4. Result of Sequencing analysis.

Isolate Code	**Nucleotide Length**	**Species**	**Percent Identification**	***Query Cover***	***Accesion Number* (BLAST NCBI)**
SDM 4.1.1	1440 bp	*Exiguobacterium profundum*	99.77%	99%	OR062352.1
SDM 3.2.1	1371 bp	*Vibrio alginolyticus*	98.46%	99%	KU052619.1
SDM 2.2.1	1453 bp	*Pseudomonas aeruginosa*	97.53%	100%	MT131240.1

Based from sequencing analysis it shows that percent identification had more than 97% of each isolate. These percent result can be affected by base pair length, alignment method, and alignment editing in software. Percent identification indicate that the sample had mostly same structure sequence of base pair compared with database from NCBI.

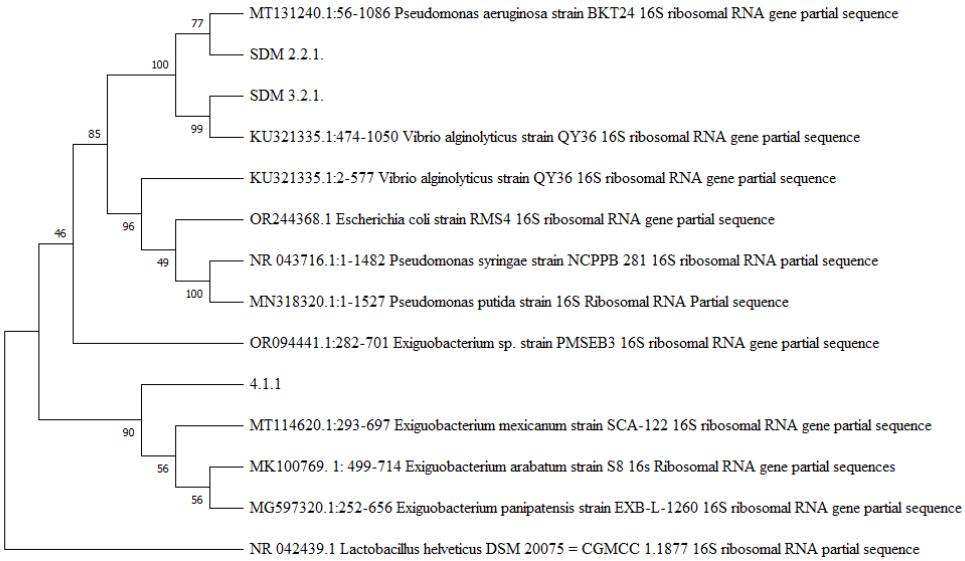


Fig. 5. Phylogenetic tree of isolate 4.1.1, 3.2.1, and 2.2.1 with clustal W alignment result.

Phylogenetic results showed that isolate had similarity and confirmed as *Exiguobacterium profundum*, *Vibrio alginolyticus,* and *Pseudomonas aeruginosa* (Figure 5). Tablent identification on Table 4 also confirmed that selected isolates with high decolorization activity had high similarity more than 97%.

4 Discussion

Characterization was conducted in order to gather isolates that were deemed to be pure. The criterion for purity is based on obtaining one type of characterization from each petri dish. Purified isolate purposed to get a single type of bacteria and not contaminated by others bacteria. Contamination was significant factor to get pure isolates and highly avoided as it can affect the results of characterization, effectivity of enzymatic assays, and molecular identification. The different morphology shows different isolates. The purified isolate determined by same morphology in each petri dish. Bacteria isolation resulted 17 potential bacteria that already purified.

The results of this lignolytic activity show varying abilities to degrade lignin. The ability to degrade lignin is not only measured by the size of the reddish-brown zone and the intensity of the color produced. Reddish-brown zone was a positive enzymatic reaction of lignolytic and an indicator of oxidative reaction from lignin and indicate the phenolic compound from lignin. The degradation of lignin is influenced by the presence of three main enzyme : laccase, lignin peroxidase, and manganese peroxidase. Lignin peroxidase and manganese peroxidase are extracellular peroxidase enzymes that require H_2O_2 for lignin degradation, while laccase enzymes assist in degrading the lignin structure alongside lignin peroxidase and manganese peroxidase [10].

The six isolates that have confirmed lignolytic activity are hypothesized to possess three enzymes involved in the degradation of lignin. The enzyme responsible for degrading

methylene blue in the form of lignin peroxidase is capable of catalyzing oxidative degradation. This degradation is correlated with hydrogen peroxide, which then activates the N-demethylation stage and leads to the decolorization of the dye to breakdown the double bond of methylene blue. Manganese peroxidase and laccase also play a role in enhancing and assisting the degradation process carried out by lignin peroxidase. The resulting degradation product from the enzymatic reaction is smaller and less toxic than the original dye, and its composition depends on the specific dye and its structure.

Enzyme production from bacteria affected by temperature, pH, and growth phase of bacteria. Temperature affected to enzyme activation because enzyme would be denaturated if exceeds optimal temperature and enzyme natural character also thermolabil. The optimum temperature for bacteria culture around 30-35℃ and this research incubated in incubator at 35℃. Low temperature would decrease the enzyme activation and did not accelerate chemical reaction on it. The pH of media would affect to enzyme chemical structure and bacteria survivability, and bacterial growth phase had a significant effect to production of metabolites.

During the stationary phase, bacteria stop growing and enter the metabolic synthesis stage. In this stage, bacteria synthesize compounds for self-defense and produce enzymes. The phases of microbial growth can differ depending on the type of microbe, and the stationary phase is the ideal stage for testing bacteria. Therefore, the reason for testing the isolates at 18–24 hours was because they were in the stationary phase, which is when bacteria start to synthesize their metabolism optimally on that phase [15].

The presence of a brighter color compare d to the control indicates high decolorization activity. Bacteria had an enzymes and secondary metabolites related to lignin, which enable them to degrade dyes [14]. The degradation between lignolytic activity and dyes were lignin enzyme such lignin peroxidase, mangan peroxidase, and laccase had oxidative mechanism to breakdown the aromatic rings and other chemical bond in dyes. The decolorization test was conducted using a consortium of three isolates (4.1.1, 3.2.1, 2.2.1), which was able to degrade methylene blue. The isolates in the consortium showed better results in decolorization and had shorter degradation times. The consortium capable to producing more effective biodegradation due to the synergy of the three isolates, which can degrade the substance through the enzymes they produce. The other three isolates had low values in terms of decolorizing methylene blue, likely because they were unable to degrade it. Decolorization activity correlated with bacterial growth, indicate had a correlation. Based on Figure 4c and Table 2, the highest decolorization activity occurred at 24 h and it is related to result of bacterial growth in 24 h that on a stationary phase. Bacterial kinetic growth and decolorization activity showed that going synchronized. In the beginning of kinetic growth and decolorization at 2-4 h indicate that the decolorization activity shows small degradation from the decrease of absorbance but in the next 24 h, it showed the most significant biodegradation activity. Bacterial growth and enzyme activity have a negative correlation which is the decolorization activity does not affected to bacterial growth in hour. Enzyme activity affected by nutrient abundant on media, pH, and temperature. Nutrient abundant, pH, and temperature are the most affected to enzyme activation. Nutrient will support the bacterial growth to multiply meanwhile pH bacteria will affect to enzyme activation. Synthesize metabolism including enzyme between exponential and stationary phase related to it is density on broth culture. Synthesize of bacteria metabolism related to nutrition, age, genus, and environment factor [8].

The electrophoresis results obtained from the three candidate isolates of the consortium were found to be 1500 bp. According to the BLAST analysis, the isolate with code 4.1.1 was identified as *Exiguobacterium profundum*, 3.2.1 as *Vibrio alginolyticus*, and 2.2.1 as *Pseudomonas aeruginosa*. If the percent identification value is ≥ 97. Similarly, if the BLAST analysis shows an identification percentage of 98-99% and a query cover value of 99%.

The study confirmed that *Exiguobacterium profundum* has lignolytic enzyme activity. Another study also investigated and found that *Exiguobacterium* sp. had the ability to degrade lignin. This discovery could be a starting point for determining the biodegradability of dye waste. Another interesting finding from this research is that this species can also degrade high levels of methylene blue. A consortium has been tested, which suggests that this bacterium has potential as a bioremediation agent [14].

Vibrio alginolyticus is a gram-negative bacterium that is a pathogen for shrimp. It is halophilic and is commonly found in marine environments. *Vibrio* sp. had extensively studied and found to be widely used as a heavy metal degrader. It also possesses enzymatic activity similar to lignin, which has only recently been investigated. This research confirmed that *Vibrio alginolyticus* has lignolytic activity, which has been utilized as a degrader of methylene blue dye.

Pseudomonas aeruginosa plays important roles in the environment as a degrader. Through genomic analysis, *Pseudomonas sp*. possesses laccase activity, an enzyme involved in lignin degradation. Previous researchers have confirmed the lignin degradation activity of *Pseudomonas aeruginosa* through lignolytic enzyme tests, decolorization experiments, and genomic analysis. Based on this information, *Pseudomonas aeruginosa* has the potential to be used as a bioremediation agent.

5 Conclusion

Bacteria found in the sediment of Tirang Beach have the ability to break down lignin. Out of the 17 isolates, it was discovered that 6 isolates or 35% from collected isolates tested positive for lignolytic enzymes, with diameters ranging from 6-14 mm. The six isolates were capable of degrading methylene blue dye, as indicated by their ability to degrade the colour. The isolates 4.1.1, 3.2.1, and 2.2.1 demonstrated the highest decolorization ability, with percentages of 88%, 76%, and 82% respectively. The consortium isolates had a decolorization percentage until 90%, with faster degradation activity and efficiency compared to individual isolates. The species isolated with code 4.1.1. were *Exiguobacterium profundum*, the species isolated with code 3.2.1. were *Vibrio alginolyticus*, and the species is isolated with code 2.2.1. were species of *Pseudomonas aeruginosa*.

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