WWW.IJRPR.COM

International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Isolation of Methanotrophic Bacteria from Tirang Beach Mangrove Ecosystem, Semarang, Indonesia

Zallva Arsyta Hartanto[a]*, Aninditia Sabdaningsih[b], Haeruddin[b]

[a] *Master Program of Aquatic Resources Management, Universitas Diponegoro*
[b]*Department of Aquatic Resources Management Study Program, Universitas Diponegoro*
**Email: haeruddindaengmile@lecturer.undip.ac.id*

A B S T R A C T

Mangrove is one of the productive coastal ecosystems and rich in biodiversity. Mangroves have a function as a significant carbon sink. Methane produced from the decomposition of organic matter under anaerobic conditions is one of the more harmful greenhouse gases when compared to carbon dioxide.However, when effectively captured and utilized as a renewable energy source, methane can significantly reduce reliance on fossil fuels, thereby mitigating its negative environmental impact. This study aims to determine the activity and identification of methanotroph bacteria and their role in the mangrove ecosystem of Tirang Beach Semarang. This study aims to isolate and characterize bacteria from certain environments that potentially have methane monooxygenase (MMO) enzyme activity. Isolation was done using culture method with Nitrate Mineral Salt (NMS) media and identification was carried out using morphology characterization and Gram staining. MMO enzyme activity was analyzed through absorbance measurement with a spectrophotometer UV-VIS. The results showed the presence of a number of isolates with variations in morphology and Gram characteristics, which included Gram-positive and Gram-negative bacteria. A total of 4 Gram-positive and 3 Gram-negative bacterial isolates were identified. Total Plate Count (TPC) was also calculated to provide a quantitative cultureable bacteria in the sample environment, with the highest TPC recorded at 2.275 x 10-2 CFU/mL and the lowest at 9.5 x 10-1 CFU/mL.. From the enzyme activity analysis, it was found that some isolates showed significant MMO activity, indicating their potential in methane oxidation. The highest enzyme activity was observed in isolate 3.1.B with an absorbance value of 0.2608 and a K*Abs value of 133.27. This study provides initial insight into the potential of indigenous microorganisms for biotechnology applications related to methane sequestration.

Keywords: *Bacteria, Mangrove, Methane, Methanotroph*

1. Introduction

Mangrove ecosystems are ecosystems that are influenced by tides and are located in the estuary. The existence of mangrove ecosystems has a very complex role both from the biological, physical, chemical, and economic aspects (Zega *et al*.,2024). The capacity of mangrove forests to store carbon is very high. One of them is the mangrove area of Tirang Beach, Semarang. The role of the mangrove ecosystem is as a CO_2 absorber and reservoir. Substrates in mangrove ecosystems have enormous carbon sequestration potential. Part of the amount of carbon in the form of CO_2 is utilized by mangroves for the photosynthesis process, the other part is in the form of gas in the atmosphere which if the amount is too much can cause adverse effects on the global climate. The photosynthesis process that occurs in mangrove ecosystems converts inorganic carbon (CO_2) into organic carbon in the form of vegetation material. According to Farista and Virgota (2021), mangrove forests have an average carbon content of 1.023 MgC per hectare. Organic soil in mangrove forest areas at a depth of about 3 m can store 49%-98% of carbon. The increase of carbon elements in the form of gases such as charcoal acid gases, exhaust gases (CO), methane (CH_4), and greenhouse gases can trigger the global climate. Methane is a gas formed from anaerobic decomposition of organic matter. Methane is a 15% contributor to greenhouse gases and global warming. Sources and absorption of global methane gas can come from wetlands, oceans, termites, rice field agriculture, livestock, landfill, biomass burning, fossil fuel use and coal and gas oil mining (Milich, 1999). Methanogens are divided into three groups: hydrogenotrophs, acetrophs, and methylotrophs, where hydrogenotrophs use carbon dioxide for energy and H_2 as an electron donor. Acetrophs require acetic acid and are also used by sulfate-reducing bacteria, while methylotroph methanogens produce methane gas using non-competitive substrates or methyl-group compounds (Oremland and Polcin, 1982). Methane (CH_4) can be used as alternative energy and is a renewable gas. Sources of methane gas can come from biological processes (biogenic), and physico-chemical processes (thermogenic and pyrogenic). Biogenic methane is usually produced by wetlands, agriculture, and livestock. Methanogenesis or methane gas production is the last stage of organic carbon mineralization (Zhuang, 2014). In sedimentary layers that have high oxygen levels, aerobic bacteria will break down carbohydrates using oxygen. In this layer, proteins are decomposed into ammonia and then oxidized into nitrate (NO3) by nitrifying bacteria. Between the oxygen-rich and oxygen-poor layers, NO3 and MnO2 release oxygen to decompose carbohydrates. After oxygen is reduced, the mineralization process occurs by reducing iron. When electron acceptors are depleted, sulfate-reducing bacteria and methanogenic bacteria will compete for energy to decompose organic carbon.

In anaerobic environments, sulfate-reducing bacteria (SRB) generally outcompete methanogenic archaea due to their higher affinity for common electron donors, such as hydrogen and acetate, and the greater thermodynamic favorability of sulfate reduction compared to methanogenesis. However, in environments where aquatic plants are present, the diffusion of oxygen through plant roots (a process known as radial oxygen loss) can alter the redox conditions in the sediment. This localized oxygenation can create micro-oxic zones that suppress the activity of SRB, reducing sulfate availability or altering competitive dynamics. Under such conditions, methanogenic archaea may thrive in anaerobic niches where sulfate competition is minimized, allowing them to effectively produce methane (Ulumudin, 2019). Increased methane gas in wetland environments, especially in the Tirang Beach Mangrove Area, Semarang, is formed under anaerobic conditions and is influenced by the activity of rhizosphere bacteria, namely methanogenic bacteria that act as methane producers and methanotrophic bacteria that utilize methane gas as a carbon source. Based on this description, further research is needed to determine the importance of methanotrophic bacteria in the absorption of organic carbon in mangrove ecosystem wetlands. Therefore, this study aims to isolate methanotrophic bacteria from mangrove sediments in the Tirang Beach area.

2. Materials and Methods

Study Area : This study was conducted in the Tirang Beach Mangrove Area, Semarang, Indonesia.

Sample Collection : Sampling was collected by composite sampling method which is a method of taking three samples from three points per station with the aim to be more efficient and the sample is considered to have represented each station. Sediment samples were taken in the mangrove ecosystem with a depth of 10-30 cm using a sediment core tool. The sediment samples were then put into a plastic zipper and then stored in a coolbox. Sediment samples were taken at six different stations (Fig.1).

Fig 1- Map of sampling locations in the mangrove ecosystem of Tirang Beach (red: station 1, yellow: station 2, green: station 3, purple: station 4, pink: station 6).

2.1 Bacterial Isolation

Bacterial isolation was carried out using the pour plate method. The sediment sample was previously diluted 10^{-1} by weighing 1 gram of sediment sample and putting it into a test tube containing 9 mL of distilled water and then homogenizing it. The dilution results were taken 1000 µL then poured into a petri dish and isolated with NMS media. Bacterial isolation was carried out using Nitrate Mineral Salt (NMS) media. NMS media is specifically designed to support the growth of methanotroph bacteria by providing the nutrients needed and creating optimal environmental conditions for these bacteria. The composition of NMS media includes 1.3 g/l NaO_3; 0.13 g/l $MgSO_4$; 0.65 g/l $Na_2HPO_4.2H_2O$; 0.286 g/l KH_2PO_4; 0.0507 g/l $CaCl_2.6H_2O$; 2.6 mg/l $FeSO_4.7H_2O$; 1.3 ml/l Trace Metal Solution; and 20 g Pure Agar while the composition of Trace Metal Solution includes 500 mg/l EDTA; 200 mg/l $FeSO_4$. $7H_2O$; 10 mg/l $ZnSO_4.7H_2O$; 20 mg/l $MnCl_2.4H_2O$; 30 mg/l H_3BO_3; 20 mg/l $CoCl_2.6H_2O$; 1 mg/l $CaCl_2.2H_2O$; 2 mg/l $NiCl_2.6H_2O$ and 3 g/l $Na_2MoO_4.2H_20$ (Whittenbury & Wilkinson, 1970). Bacterial isolation samples will then be purified, Gram stain test, and molecular identification as well as determining the phylogenetic tree of the species that have been found.

2.2 Gram Staining

Gram staining test is done to determine the characteristics of bacteria based on their cell walls (Gram negative and Gram positive) by dropping one drop of distilled water on a glass object which is added with one loop of sample culture then fixed over a fire. Gram staining is done by adding one drop of Gram a then rinsed with distilled water and waiting for one minute, then dripped with Gram b then rinsed with distilled water and waiting for one minute. Then dripped with Gram c rinsed with distilled water, then observed under a microscope with a magnification of 1000x. Red bacteria indicate that the bacteria are Gram negative, and purple indicates that the bacteria are Gram positive. Alcohol will increase the porosity of the cell wall by dissolving the outer layer lipids in Gram Negative cells. Thus, the Crystal Violet (CV-I) complex will be more easily removed from the peptidoglycan layer that is not strongly cross-linked. Therefore, the effect of alcohol washing facilitates the release of unbound CV-I complexes, which causes the cells to lose color or become colorless. Because only Gram-negative cells experience color loss so that their cells absorb the counter dye. While Gram-positive retains the purple color of the primary dye (Post et al., 2005).

2.3 Isolate Selection Based on Methane Monooxygenase (MMO) Activity

Selection of MMO activity was carried out using the pMMO (Particulate Methane Monooxygenase) stage. pMMO is an enzyme that plays a role in the oxidation process of methane to methanol by methanotrophic bacteria (Ross & Rosenzwig,2017) . This selection was carried out by growing bacteria in NMS liquid media in an airtight bottle. The airtight bottle was added with 1 mL of a 10-1 dilution sample, 50 mL of liquid NMS media, and 12 mL of methane gas. The sample was incubated for 7 days. Measurement of pMMO activity was carried out by taking a sample of 0.1 mL, adding 0.6 mL of SNP reagent and adding distilled water to a total volume of 1.5 mL. The sample was vortexed at maximum speed for 10-20 seconds and left for 15 minutes. Furthermore, the sample was measured with a spectrophotometer with a wavelength of 481 nm (Anthony, 1982).

3. Result and Discussion

Bacterial isolation was carried out using NMS agar media and liquid NMS media then calculated using the TPC (Total Plate Count) method. The results of bacterial isolation obtained through NMS media can be seen in Table 1.

Table 1. Bacterial Isolation of 6 mangrove sediment samples taken at 6 stations in Tirang Beach

Sampling site	**Sample Code**	**Inoculation Volumes (mL)**	**Number of colonies**	**Dilution**	***Total Plate Count* (CFU/mL)**
1	1.1	1	4	10^{-1}	40
	1.2	1	15	10^{-1}	150
***Total Plate Count* (CFU/mL)**					0.95×10^{2}
2	2.1	1	8	10^{-1}	80
	2.2	1	17	10^{-1}	170
***Total Plate Count* (CFU/mL)**					1.25×10^{2}
3	3.1	1	14	10^{-1}	140
	3.2	1	10	10^{-1}	100
***Total Plate Count* (CFU/mL)**					1.2×10^{2}
4	4.1	1	27,5	10^{-1}	275
	4.2	1	18	10^{-1}	180
***Total Plate Count* (CFU/mL)**					2.275×10^{2}
5	5.1	1	15	10^{-1}	150
	5.2	1	8	10^{-1}	80
***Total Plate Count* (CFU/mL)**					1.15×10^{2}
6	6.1	1	32,5	10^{-1}	325
	6.2	1	2	10^{-1}	20
***Total Plate Count* (CFU/mL)**					1.725×10^{2}

Code 1.1 - 6.1 in the sample is the result of isolation using NMS agar media by growing bacteria from dilution (10^{-1}) directly into NMS agar media. Code 1.2 - 6.2 in the sample is the result of isolation of liquid NMS media which is then grown again on NMS agar media. Isolates that have been obtained from isolation on NMS media are then carried out bacterial purification or purification on NMS agar media.

Each petri was taken colonies that showed different morphology. The results obtained from purification, namely the morphological characteristics of diverse colonies, can be seen in Table 2.

Table 2. Morphological Characteristics of Bacterial Colonies on NMS Media

No	Sample code	Isolate color	Shape	Margin	Elevation
1	1.2.a	Ivory white	*Circular*	*Entire*	*Raised*
2	2.1.e	Ivory white	*Circular*	*Entire*	*Raised*
3	2.2.a	Ivory white	*Circular*	*Entire*	*Raised*
4	3.1.b	Ivory white	*Circular*	*Entire*	*Raised*
5	3.1.c	Ivory white	*Circular*	*Entire*	*Flat*
6	4.2.a	Ivory white	*Circular*	*Curled*	*Convex*
7	4.2.b	Ivory white	*Circular*	*Entire*	*Raised*
8	5.1.a	Pink	*Filamentous*	*Filamentous*	*Flat*
9	5.2.a	Ivory white	*Circular*	*Entire*	*Flat*
10	6.1.d	Ivory white	*Irregular*	*Undulate*	*Convex*
11	6.1.e	Ivory white	*Circular*	*Entire*	*Flat*

The bacterial isolate sample code has a meaning, namely the first number indicates the station, the second number indicates the planting of bacteria through direct dilution using agar media (1) and planting isolated bacteria using liquid media (2). Then the last letter shows the isolate that is purified in one petri. The results of the bacterial isolation stage show the diversity of bacterial colony morphology which can be seen from the shape, margins, and elevations that are not only one type. The results of bacterial isolation from sediment samples produced several 6 pale white isolates derived from NMS agar media and 6 clear-colored isolates grown on liquid NMS media and then grown again on NMS agar media. The next process is to find pure bacteria by purifying bacteria on NMS agar media and then purifying again with nutrient agar media. There are 7 bacterial isolates that can be transferred, these isolates come from bacteria that grow in the initial isolation process on NMS agar media which are then selected based on their different morphological characteristics. Morphological observations shown in Table 2 show that most bacterial isolates have a round colony shape with flat edges and some have a shape like a scrub root, the color produced by bacterial colonies is dominated by ivory white. This can be caused by nutritional factors from bacterial growth media. According to Rinihapsari et al. (2023), bacterial colonies show remarkable morphology, which is influenced by various factors, including growth media and temperature. The composition of the growth medium plays an important role in determining the availability of nutrients for bacteria, which directly affects the growth pattern and morphology of the colonies. For example, the concentration of nutrients and agar in the media can cause variations in colony size, shape, and texture.

The pure bacterial isolates obtained were then Gram stained with the aim of knowing the cell structure of the bacteria that had been isolated by knowing the type of Gram Bacteria obtained. The results of Gram staining of 7 pure bacterial isolates can be seen in Table 3.

Table 3. Gram staining results of 6 sediment samples taken at 6 stations in the Tirang Beach Mangrove Area

No	Sample Code	Coloring results	Gram	Cell shape
1	2.1.e	Purple	Positive	Bacil
2	3.1.b	Red	Negative	Cocci
3	3.1.c	Purple	Positive	Bacil
4	4.2.a	Red	Negative	Cocci
5	5.1.a	Purple	Positive	Cocci
6	6.1.d	Purple	Positive	Cocci
7	6.1.e	Red	Negative	Cocci

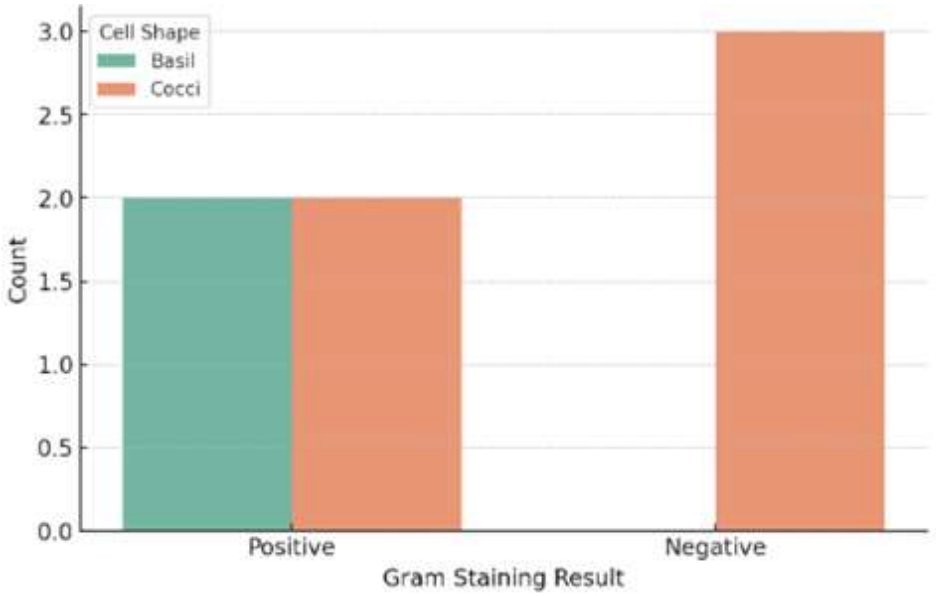

Fig-1 distribution of bacteria based on Gram stain and bacterial form

The Gram staining results in the diagram above show that there are 4 isolates of Gram-positive bacteria and 3 isolates of Gram-negative bacteria, with 5 isolates of bacteria showing a coccus-shaped cell structure and 2 isolates of bacteria in the form of bacilli. These isolates are the results of purified isolates that have been selected. Gram-positive bacteria will appear purple under a microscope because these bacteria have thick peptidoglycan and are able to bind purple, while Gram-negative bacteria will appear red under a microscope because these bacteria have a very thin peptidoglycan layer. According to Hamidah et al., (2019), Gram-positive bacteria have 2 structures, namely peptidoglycan and a fairly thick membrane. The peptidoglycan layer binds the crystal violet color. The thickness of the peptidoglycan layer owned by Gram-positive bacteria is 20-80 nm, while the thickness of the peptidoglycan layer owned by Gram-negative bacteria is 5-10 nm with lipoprotein, outer membrane and polysaccharides as the main composition.The results of the MMO activities that have been carried out using a spectrophotometer can be seen in the table 4.

Tabel 4. MMO activity results on spectrophotometer

No Sample	Abs	K*Abs
2.1.E	0.2319	118.50
6.1.E	0.2602	132.96
5.1.A	0.2423	123.82
3.1.B	0.2608	133.27
4.2.A	0.2436	124.48
6.1.D	0.2368	121.00
3.1.C	0.2368	97.754

The spectrophotometer results show the MMO (methane monooxygenase) enzyme activity in methanotroph bacteria through the measurement of absorbance values (Abs) and K*Abs The absorbance value reflects the intensity of light absorbed by the sample, where higher values indicate the possibility of greater enzyme activity. Based on the data, the absorbance values range from 0.2319 in sample 2.1.E to 0.2608 in sample 3.1.B, with sample 3.1.B having the highest absorbance value, indicating the highest MMO enzyme activity. In addition, the K*Abs value, which is calculated based on a certain constant, shows that the highest activity is found in sample 3.1.B with a value of 133.27, while the lowest activity is in sample 3.1.C with a value of 97.754. This confirms that sample 3.1.B has the most optimal enzyme activity.

Variations in enzyme activity between samples can be caused by several factors, such as methane substrate concentration, pH conditions, temperature, or the presence of inductors that affect MMO enzyme expression and efficiency. The optimal enzyme activity in sample 3.1.B was likely produced by favorable environmental conditions or the appropriate substrate concentration. The MMO enzyme plays an important role in the oxidation of methane to methanol, so these results indicate the potential utilization of methanotroph bacteria for further applications in methane bioconversion. Overall, these data provide a preliminary picture of the differences in MMO enzyme activity in various samples and the importance of optimizing conditions to increase enzyme activity. If required, further analysis can be conducted to understand the specific factors that influence these results.

4. Conclusion

The results of bacterial isolation from the sediment of the mangrove ecosystem of Tirang Beach Mangrove Area, Semarang obtained 7 bacterial isolates consisting of 3 Gram negative bacteria and 4 Gram positive bacterial isolates. The results of the calculation of the largest TPC found at station 4 amounted to 2.275 x 10^2 CFU/mL and the lowest at station 1 which amounted to 9.5 x 10^1 CFU/mL. Based on the data obtained, the sample with the highest absorbance value is 3.1.B, with an absorbance value of 0.2608 and a K*Abs value of 133.27. This indicates that sample 3.1.B has the highest methane monooxygenase (MMO) enzyme activity among all the samples tested.

Acknowledgment

The authors would like to thank the Master Program in Water Resources Management, Diponegoro University for the support and facilities provided during this research. Special thanks are given to the laboratory staff and teaching staff for their assistance and guidance during the research. Special thanks are given to the Ministry of Education, Culture, Research and Technology of the Republic of Indonesia for providing a grant under the Postgraduate Research – Master Thesis Research (PPS-PTM) scheme with contract number 449A-64/UN7.D2/PP/IV/2023.

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