Int. j. adv. multidisc. res. stud. 2024; 4(5):275-278

Received: 29-07-2024
Accepted: 09-09-2024

International Journal of Advanced Multidisciplinary Research and Studies

ISSN: 2583-049X

Morphological Characteristics of Methanotroph Bacteria Isolated from Sediment Samples in Ponds

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DOI: https://doi.org/10.62225/2583049X.2024.4.5.3228
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Abstract

Semarang is one of the largest cities in Indonesia, and it was a coastal city with 33 km of coastline in 2016. The high emission and waste in Semarang City affect water pollution which impacts the presence of microorganisms such as bacteria. Isolation of anaerobic bacteria has been explored due to the difficulties of handling. One type of anaerobic bacteria is methanotroph bacteria. The lack of literature on methanotroph bacteria in the aquatic environment which in this study is specific to ponds, it is necessary to explore. The study aimed to isolate and determine the characteristics of methanotroph bacteria in ponds at Tirang Beach. Research methods used the exploratory and purposive sampling methods. Sampling was conducted at ponds in Tirang Beach at 3 stations consisting of 3 sampling points. Moreover, water sampling was employed to determine the variables of the aquatic environment and sediment samples for bacterial isolation. Bacterial isolation was carried out with liquid NMS media and agar, followed by Gram staining techniques to determine the characteristics of methanotroph bacteria. The abundance obtained from isolation with NMS agar media was 2.27 x 10^2 CFU/mL with 6 isolates that were successfully purified. Gram observation results produced 6 isolates with red cell walls or Gram-negative bacteria. The resulting bacterial cell forms are coccus, cocobacillus, and bacillus.

Keywords: Gram Stain, Methanotrophs, NMS Media, Tirang Beach

1. Introduction

Semarang City is the capital city of Central Java Province. Semarang City has an area of 373.78 km^2 (Semarang City Central Statistics Agency, 2023) [16]. The density of urban and industrial activities in Semarang City makes Semarang produce waste and gas emissions from the energy industry, transportation, solid waste, processing industry, and liquid waste treatment. The amount of waste and emissions produced can affect the environment both from the declining air quality and the quality of the water and coastal environment in Semarang City. Semarang, one of the largest cities in Indonesia and a coastal city, had a coastline of 33 km in 2016 (Semarang City Environment Service, 2018; Ginanjar *et al*., 2022 [6]). Ecosystems in coastal Semarang City include beaches, mangroves, and ponds. The high emissions and waste from Semarang City can affect water pollution which can also have an impact on the presence of microorganisms particularly bacteria in the waters. Many studies have discussed the isolation of aerobic bacteria on the coast of Semarang City. However, the isolation of anaerobic bacteria is still under-explored. One type of anaerobic bacteria is methanotroph bacteria. Metanotroph bacteria is one of the anaerobic bacteria that use methane gas as its carbon energy source (Nonci *et al*., 2015) [13]. Methanotroph bacteria can be found in land, freshwater, and seawater environments. Research on methanotroph bacteria in the aquatic environment is still very limited. It is important to explore methanotroph bacteria by knowing the characteristics of these bacteria. The characteristics of methanotroph bacteria are known by Gram check using a microscope. Characterization of bacteria using this microscope aims to determine the morphology of bacteria. (Gustiana *et al*., 2021) [8]. The lack of literature on methanotroph bacteria in the aquatic environment which in this study was more specifically carried out in ponds, it is necessary to explore it. The purpose of this study was to isolate and determine the characteristics of methanotroph bacteria in ponds at Tirang Beach.

2. Materials and Methods

Materials and Research Tools

Materials in the study were sediment samples for isolation and identification of bacteria, Nitrate Mineral Salt (NMS) media for bacterial isolation, methane gas, and gasp to maintain anaerobic conditions. Tools in the study were sediment cores and plastic zippers for sediment sampling, Petri dishes for bacterial isolation, Bio Safety Cabinet for bacterial isolation, autoclave for sterilization of media and bacterial isolation tools, anaerobic Jar for anaerobic bacteria incubation, anaerobic chamber for anaerobic bacteria isolation.

Research Procedure

Research method with an exploratory method with purposive sampling method. Sampling was conducted in ponds at Tirang Beach, Semarang City in January 2024. Sampling was conducted at 3 stations on different ponds. Sampling of each station is done at three sampling points—water sampling to determine the variables of the aquatic environment and sediment samples for bacterial isolation. Sample analysis was conducted in the Tropical Marine Biotechnology laboratory of FPIK UNDIP in January-May 2024. The following is a map of the sampling location (Fig 1).

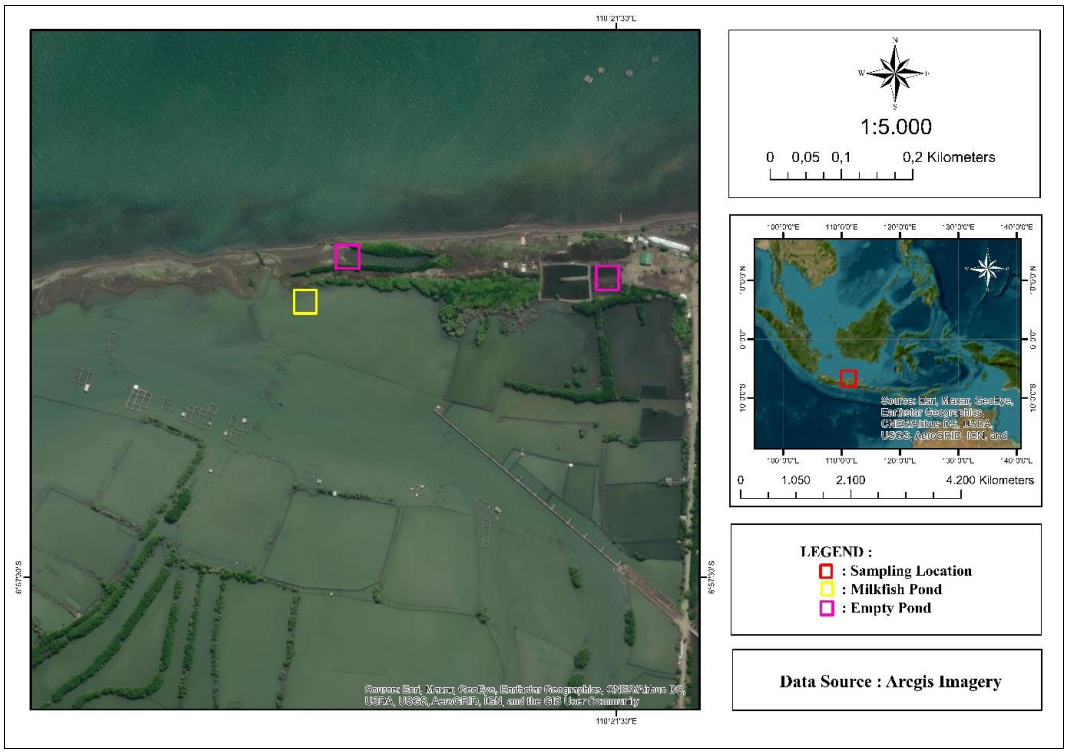


Fig 1: Map of Sampling Location

Bacterial Isolation

Isolation with liquid NMS media is carried out with samples in vial bottles that are incubated using a shaker. Incubation was carried out for 10 to 14 days (Nonci *et al*., 2015) [13]. Incubation was carried out under headspace conditions with 50% air and 50% methane gas, then grown on NMS agar media (Rusmana and Akhdiya, 2009) [14]. Incubation of NMS agar media is done anaerobically using an anaerobic jar.

Microscopic Observation of Bacteria

Microscopic bacterial observation procedures were carried out through Gram staining techniques. The Gram staining method used is coloring bacteria with a basic dye in the form of crystal violet, fixating the color to strengthen the color attachment with iodine, washing with alcohol or acetone, and re-staining with safranin (Harahap *et al*., 2021) [10].

3. Results and Discussion

Results

Measurement of Environmental Variables

Based on the research that has been done, the results of measurements of environmental variables on ponds in Tirang Beach are presented in Table 1.

Table 1: Results of Measurement of Physical Parameters of Ponds in Tirang Beach

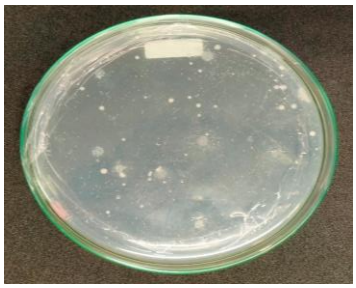
Variables	Quality Standard	Station 1	Station 2	Station 3
Sediment pH		5.8	6.3	5.8
Dissolved Oxygen (mg/L)	>5	3.8	3.5	1.9
Salinity (‰)	Natural	25	30	35
Temperature (°C)	Natural	35.5	34.5	34.8
Depth		52	29	32

Source: Research Data 2024

Description: Appendix VIII. Seawater Quality Standard for Marine Biota according to Government Regulation (Peraturan Pemerintah). Number 22 Year 2021.

Isolation of Bacteria with NMS Media

Bacteria that have been isolated and incubated with liquid NMS media produce colors from clear to cloudy. Isolation is continued with NMS agar media with the appearance of bacteria in the naked eye white and pink with a coccus shape. The following is an example of the results of bacterial isolation on NMS agar media (Fig 2).



Source: Research Documentation

Fig 2: Bacterial Isolation Results with NMS Agar Media

The abundance of bacteria with NMS agar media in ponds in Tirang Beach is presented in Table 2.

Table 2: Results of NMS Media Bacterial Isolation

Station	Sample Code	Number of Colonies	*Total Plate Count* (CFU/mL)
I	T1.S	44	$4.4x10^1$
II	T2.S	9	-
III	T3.S	183	$1.83x10^2$
Total		**227**	$2.27x10^2$

Description: T1.S= Milkfish pond
T2.S; T3.S = Empty ponds

Microscopic Observation of Bacteria

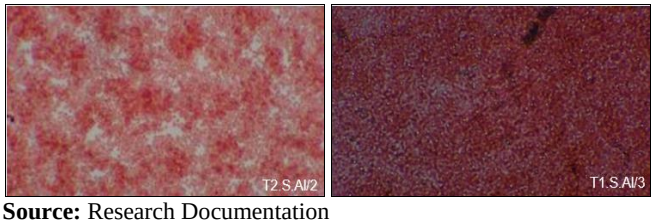
Bacteria isolated on NMS agar media produced Gram-negative bacteria totaling 6 isolates. More details are presented in Table 3.

Table 3: Result of Microscopic Observation on NMS Agar Media

Station	Isolate Code	Color	Gram	Shape
Station 1	T1. S. Al/1	Red	(-)	*Coccus*
	T1. S. Al/2	Red	(-)	*Coccus*
	T1. S. Al/3	Red	(-)	*Coccus*
Station 2	T2. S. Al/1	Red	(-)	*Coccobacillus*
	T2. S. Al/2	Red	(-)	*Coccobacillus*
Station 3	T3. S. Al	Red	(-)	*Bacillus*

Source: Research Data 2024

Examples of Gram staining results on bacteria on NMS agar media can be seen in the following figure:



Source: Research Documentation

Fig 3: Example of Bacterial Gram Staining Results on NMS Agar Media

Discussion

Environmental Variables of Tirang Beach Ponds

The results of sediment pH measurements at stations 1, 2, and 3 were 5.8; 6.3; and 5.8, respectively. Methane oxidation rate occurs at sediment pH between 4 to 5 and methane bacterial activity can still be detected in slightly alkaline sediments (Kharitonov *et al*., 2021) [12]. The sediments in Tirang Beach Pond may be capable of oxidizing methane gas. Dissolved oxygen measurements at stations 1, 2, and 3 were 3.82 mg/L; 3.53 mg/L; and 1.92 mg/, respectively. These results indicate that dissolved oxygen levels are not too high which can occur due to the activity of microorganisms and aquatic biota that require oxygen. Dissolved oxygen is needed by microorganisms for the decomposition of ammonia and nitrite (Fuadi *et al*., 2020) [5]. The results of salinity measurements at stations 1, 2, and 3 are 25‰; 30‰; and 35‰ which shows optimal results. The results of water temperature measurements at stations 1, 2, and 3 are 35.5°C; 34.5°C; and 34.8°C, respectively. While the optimal temperature for the growth of methanotroph and methanogen bacteria ranges from 25 to 30 ° C (Dunfield *et al*., 1993) [3]. Pond depth at stations 1, 2, and 3 were 52 cm; 29 cm; and 32 cm, respectively. Methane gas increases in concentration at sediment depths of up to 40 cm (Hanson and Hanson, 1996) [9] so sediment collection was carried out at a depth of 40 cm.

Isolation of Bacteria with NMS media

NMS media is a selective media used for the isolation of methanotroph and methanogen bacteria. Bacteria that have been isolated with liquid NMS media are continued with isolation on NMS agar media. Bacteria grown on NMS media will grow if there is methane gas added to the sample (Whittenbury *et al*., 1970) [20]. Bacteria that have been incubated on liquid NMS media show a change in color from clear to slightly cloudier, this can indicate the development of bacteria. The same statement was conveyed by Amanda *et al*. (2019) [1], the change in color of the sample to cloudy after the incubation period indicates that the bacteria have grown. Bacterial development on NMS agar media is visible after 3 to 5 days of incubation. Physically, the bacteria produced from isolation with NMS agar media are coccus or circle-shaped with a single colony. The growth of methanotroph bacteria has slow growth compared to other types of bacteria and most do not show colony morphology (Svenning *et al*., 2003) [18]. The color of the bacteria is white and pink. Generally, methanotroph bacteria that grow on NMS agar media have different colors, namely white, yellow, pink, and orange (Waskitho *et al*., 2023) [19]. The abundance generated from isolation with NMS agar media is $2.27x10^2$ CFU/mL with 6 isolates successfully purified.

Microscopic Observation of Bacteria

Gram staining technique can be used to identify and determine the characteristics of bacteria based on differences in cell walls and bacterial shape. Gram staining can be observed microscopically using a microscope with a certain magnification. Gram staining is done using various dyes that have their respective functions. The Gram staining technique was first developed by Hans Christian Gram from Denmark (Harahap *et al*., 2021) [10]. The Gram staining technique was first introduced in 1882. The results of Gram observation of bacteria on NMS media produced 6 bacterial

isolates with red cell walls. Bacteria that have red cell walls indicate that these bacteria are Gram-negative bacteria. Gram-negative bacteria have a thin peptidoglycan layer while Gram-positive bacteria have a thicker peptidoglycan layer. The cross-linked and layered peptidoglycan layers dehydrate with the addition of decolorization thus trapping crystal violet and iodine in the cells. After decolonization, Gram-positive bacterial cells remain purple while Gram-negative bacteria lose their primary color, namely purple, and take a positively charged dye, namely safranin, so that Gram-negative cells become red (Smith and Hussey, 2005) [17]. Gram-negative bacteria consist of two layers of cell wall, the inner layer is peptidoglycan and the outer peptidoglycan is an additional outer membrane consisting of proteins, phospholipids, and lipopolysaccharides (Schlegel, 1993) [15]. Gram-positive bacteria have a cell wall layer with thick peptidoglycan and are very protective, in Gram-positive bacteria many antibiotics work effectively against microorganisms on Gram-positive, this happens because the peptidoglycan layer has Gram-positive cell wall components (Fauziah, 2023) [4]. The physical characteristics shown in the isolate with NMS media, which has a red cell wall layer, indicate that the resulting isolate is a methanotroph bacterium. All species of methanotroph bacteria known today are Gram-negative bacteria (Karlsen *et al*., 2011) [11]. The shape of bacterial cells resulting from Gram staining of bacteria on NMS media is coccus or round, coccobacillus and bacillus or rod-shaped. The currently described form of methanotroph bacteria has the form of straight rods, curved, cocci, and sarcina such as a great and filaments (Bowman, 2006; Stoecker *et al*, 2006; Danilova *et al*., 2016 [2]).

4. Conclusion

Based on the research that has been done, bacterial isolation with NMS agar media produces an abundance of $2.27x10^2$ CFU/mL. The characteristics of methanotroph bacteria that have been isolated are Gram-negative with coccus, coccobacillus, and bacillus cell forms.

5. Acknowledgments

The researcher would like to thank 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. Then to all those who have helped the author in research, preparation, criticism, and suggestions in research.

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