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Potential of Methanotroph bacteria as non-symbiotic nitrogen fixation in rice paddy fields in Morowali regency

Potential of Methanotroph bacteria as non-symbiotic nitrogen fixation in rice paddy fields in Morowali regency

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Abstract. Nitrogen makes up about 78% of all the gases in the atmosphere. Although it is very abundant in the atmosphere, nitrogen cannot be used directly by plants. The aims of this study were to isolate, characterize and identify the morphology and physiology of methanotrophic bacteria, calculate the total population of methanotrophic bacteria and analyze the chemical properties of paddy rice soil in Morowali Regency. Results Based on the gram reaction test, 11 isolates were gram negative, 11 isolates were catalase positive, 11 isolates were capable of nitrogen fixation, five isolates were yellowish white, five isolates were white, one isolate was yellow, six isolates had a convex elevation, and five isolates had a flat elevation. Based on the identification results, the Witaponda sub-district produced five isolates, three isolates from Bumiraya, two isolates from Bungku Barat and one isolate from Bungku Tengah District. Based on the results of the calculation of the number of bacteria found in Witaponda subdistrict as much as $54.05x10^6$ Cfu/mL Bumiraya as much as $40.21x10^6$ Cfu/mL, Bungku Barat as much as $12.06x10^6$ Cfu/mL, Bungku Tengah as much as $11.65x10^6$ Cfu/mL. The results of the analysis of soil chemical properties showed that pH, CEC, organic C, P205, K were classified as low in both irrigated and rainfed rice fields.

1. Intruduction

The natural fixation of nitrogen from the atmosphere can be carried out by soil bacteria so that the availability of plant nitrogen elements remains sufficient. Chemical treatment or natural fixation of nitrogen is necessary to convert nitrogen gas into a form that plants can use. The natural fixation of nitrogen from the atmosphere can be carried out by soil bacteria so that the availability of plant nitrogen is sufficient even though it grows in marginal areas with very low nutrient content such as coastal areas. One of the bacteria that can do this is rhizobacteria [1].

Biological nitrogen fixation as part of nitrogen input to support plant growth has decreased due to the use of inorganic fertilization. In the soil, especially in the rhizosphere, there are various kinds of microorganisms that live and are beneficial for the growth of a plant, one of which is methanotrophic bacteria. The rhizosphere is a root area that provides various organic materials that can stimulate microbial growth. The rhizosphere is an excellent habitat for microbial growth. Organic materials released by the roots can be in the form of root exudates, sugars, amino acids and organic acids [2].

Application of methanotrophic bacteria as a nitrogen fixing agent helps in the provision of N nutrients in the soil, so as to provide optimal growth. This is in accordance with the opinion [3].

Methanotrophic bacteria have the ability to fix nitrogen gas as nitrogen source. Initially, only methanotrophic bacteria type II and X were known to be able to fix N2 [4], but research by [5] proved that the ability to fix N2 was widespread in methanotrophic bacteria, including type I and type II. Methanotrophic bacteria types I and II have nifH genes and nitrogenase activity ranging between 0.4 and 3.3 nmol/min/mg protein.

Acidophilic methanotrophic bacteria are known to have nifH and nifD genes which have nitrogenase enzymes, including Methylocella and Methylocapsa which are members of Alphaproteobacteria. Methylococcus capsulatus (Gammaproteobacteria) and the Methylosinus/Methylocystis (Alphaproteobacteria) group, including type I and type II methanotrophic bacteria, have been shown to have the nifH and nifD genes [6]. Evaluation results over the last ten years in several places around the world show that nitrogen fixing bacteria that live freely on roots and in plant tissues in rice, such as Pseudomonas sp., Enterobacteriaceae, Bacillus, Azotobacter, Azospirillum, and Herbaspirillum have been proven capable of fixing nitrogen [7].

The presence of bacteria in the rhizosphere area which is a group of Rhizotobacteria bacteria which functions to increase plant growth. Rhizobakteri are known as Plant Growth Promoting Rhizotobacteria (PGPR) because they have a significant effect on plant growth, produce IAA (indole Acetid Acid) hormones, dissolve soil phosphate, produce ACC deaminase (Amino Cyclepropane Carbolic Deaminase) [8]. This hormone plays an important role in the mechanism of cell expansion, namely in root initiation, division, elongation, and cell differentiation as an agent or signal carrier in plant response so that it can stimulate plant height growth.

The bacterial population in the rhizosphere has more than one type of bacteria, each of which has a different character. The character of a bacterium is a characteristic that is possessed by a certain type of bacteria to distinguish it from other types of bacteria. The process of characterizing bacteria can be carried out by making observations based on colony morphology, gram type and biochemical tests [9].

Based on these assumptions, it is necessary to conduct research on the potential of nitrogen-fixing methanotrophic bacteria in lowland rice which is expected to increase rice production and also support the realization of environmentally friendly agriculture. The research objectives include (1) To obtain isolates of methanotrophic bacteria capable of fixing nitrogen (2) To calculate and test population number of matenotrophic bacteria in irrigated and rainfed lowland rice (3) Analyzing the chemical properties of the soil in irrigated and rainfed lowland rice

2. Methodology

This research was conducted from April to October 2021 at the Bioscience and Plant Reproduction Biotechnology Laboratory, Department of Agricultural Cultivation, Faculty of Agriculture, Hasanuddin University. Soil analysis was carried out at the Laboratory of Soil Chemistry and Fertility, Department of Soil Science, Faculty of Agriculture, Hasanuddin University.

2.1. Preparation section

The soil sample used is a soil sample from Witaponda District, (121º37'41.877"E - 213'32.459"S) Bumiraya, (2º13'04.3"S – 121 41'57.1"E West Bung, (2º26'32.4"S - 121 54'17.0"E and Middle Bung. 2º26'27.5"S - 121"5417.0."E - 2º13'32.459"S.

2.2. Isolation and observation of isolate morphological characteristics

Soil sampling was carried out compositely in the rice root zone (about 5-10 cm deep). After that, the paddy field mud is put into plastic and tightly closed to prevent oxygen from getting into the plastic, then immediately taken to the laboratory for analysis (Figure 1).

Figure 1. Sampling of soil from irrigated rice fields (a) soil samples from rainfed rice fields (b).

2.3. Isolation and purification

Isolation of methanotrophic bacteria was initiated by making a series of 10^1 to 10^8 dilutions for each sample with two replications. Furthermore, each sample was grown on selective NMS media (Hanson and Hanson.19[10] which had been prepared and sterilized previously, with the composition: 1.0 g MgSO4.7[23]O; 1.0 g KNO_3 ; 0.717 g Na_2HPO_4.12 H_2O; 0.272 g KH_2PO4; 0.2 $CaCL_2$.$6H_2O$; 4.0 g NH_4CL; 0.5[8]ml trace Element Solution with composition (per 1 liter of distilled water[15]0.5 gr Na2EDTA; 0.2 g $FeSO_4$.$7H_2O$; 0.03 g H_3BO_3 ; 0.02 g $CoCL_2$ $6H_2O$; 0.01 $ZnSO_4$.$7H_2O$; 3.0 mg Mn CL2 .$4H_2O$; 3.0 mg Na2 MoO_4 ; 2.0 mg $NiCl_2$.$6H_2O$; 1.0mg $CaCl_2$ $2H_2O$. Then incubated for 10-14 days. Each growing colony was scraped again on the same medium, and this was done repeatedly until a single colony was obtained [10].

2.4. Isolation and morphological characteristics of isolates

The identification of isolates based on morphological criteria and the resulting pigment refer to Miyadoh's atlas and Bargey's manual. Elevation, seen with convex indicators, flat or concave morphology on the surface of the media.

2.5. Isolates based on gram's reaction

Incubation that has obtained a single colony is then analyzed by testing the gram reaction with the aim of which bacteria are gram-positive and gram-negative by scratching using a needle and then smearing it on the object-glass. After that, it was given a 3% KHO solution and stirred until smooth for about 5 seconds, and the slimy colonies included a positive reaction (gram-negative bacteria). On the other hand, not slimy bacteria had a negative reaction (gram-positive bacteria).

2.6. Catalase test

Observations were made in Laminar Air Flow. Open the wrapping on the culture media resulting from the isolation of bacteria. Ose needles to be used are dipped in 96% alcohol, drained and then sterilized by flaming over the Bunsen. Last aerated. Gently pick up bacteria using a needle loop. Put the bacteria on the slide, add a drop of H_2O_2, and observe the reaction; if bubbles form, the response is positive, but if no bubbles occur, the response is negative.

The results of the catalase test from 4 locations showed that 10 isolates were able to degrade hydrogen peroxide and 1 isolate was unable to degrade hydrogen peroxide. Hydrogen peroxide is a toxic substance that is harmful to plants because it can destroy cells quickly. Some bacteria have catalase or peroxidase enzymes that are able to convert hydrogen peroxide into water and oxygen [11].

2.7. Nitrogen fixation test

Making Bur'ks Media Considering Burk's media materials namely; 0.20 g MgSO4, 0.05 g K_2HPO_4, 0.15 g KH_2PO_4, 1.3 g CaSO4 are mixed to make it homogeneous. Preparation of FeMo solution materials, namely; 0.145 g FeCl3, 2 g Na2MoO4 diluted with 100 mL Aquades. Put 1000 mL of distilled water into the erlenmeyer, mix 1.3 g of Burks's media, 1 mL of FeMo solution, 2 g of sucrose, 17 g of agar into the erlenmeyer and heat on a hot plate. Burks's media is sterilized using an autoclave at 121 ℃ for 15 minutes. Allow the Burk's media to cool to ±45-50 ℃. Then pour the media into the petri dish, then incubate it until it solidifies for 10-14 days at room temperature. Take 1 ose of methanotrophic bacteria isolates of the bacteria to be tested, streak them on burk media and incubate for 48 hours at room temperature. Isolates that grow are marked + (positive), while those that do not grow are coded – (negative).

2.8. Calculation number of colonies

Colony counting is usually done manually by marking and counting the bacterial colonies in a petri dish. [12] Before carrying out the calculation of the number of colonies, dilution was carried out first, then they were grown on NMS medium in a petri dish, then incubated. Dilution is usually done in decimal, namely 10^1, 10^2, 10^3, and so on until the dilution level is 10^8

3. Results and discusion

Results Based on the gram reaction test, 11 isolates were gram negative, 11 isolates were catalase positive, 11 isolates were capable of nitrogen fixation, five isolates were yellowish white, five isolates were white, one isolate was yellow, six isolates had a convex elevation, and five isolates had a flat elevation. Based on the identification results, the Witaponda sub-district produced five isolates, three isolates from Bumiraya, two isolates from Bungku Barat and one isolate from Bungku Tengah District (Table 1).

Table 1. Colony character/morphology, gram reaction, catalase test and *nitrogen fixation test*.

No	Districts	Type of Rice Field	Gram Reaction	Catalase Test	Nitrogen Fixation Test	Isolate Code	Colony Shape	Colony Colour	Elevation
1		Irrigated	-	+	+	WPM 1	Circular	Yellowish White	Convex
2		Irrigated	-	+	+	WPM 2	Circular	Yellowish White	Convex
3	Witaponda	Irrigated	-	+	+	WPM 3	Irregular	Yellow	Convex
4		Irrigated	-	+	+	WPM 4	Irregular	Yellowish White	Convex
5		Irrigated	-	+	+	WPM 5	Circular	Yellowish White	Convex
6		Irrigated	-	+	+	BRM 1	Irregular	White	Flat
7	Bumiraya	Irrigated	-	+	+	BRM2	Irregular	White	Flat
8		Irrigated	-	+	+	BRM3	Circular	Yellowish White	Convex
9		Rainfed	-	+	+	BBM1	Circular	White	Flat
10	Bungku Barat	Rainfed	-	+	+	BBM2	Irregular	White	Flat
11	Bungku Tengah	Rainfed	-	+	+	BTM1	Irregular	White	Flat

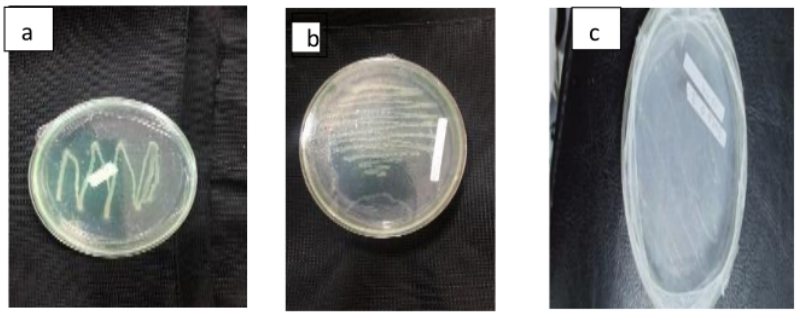

Figure 2. The results of observing the color of the isolates, the results of isolates are yellow (a) the results of isolates are yellowish white (b) the results of isolates are white (c).

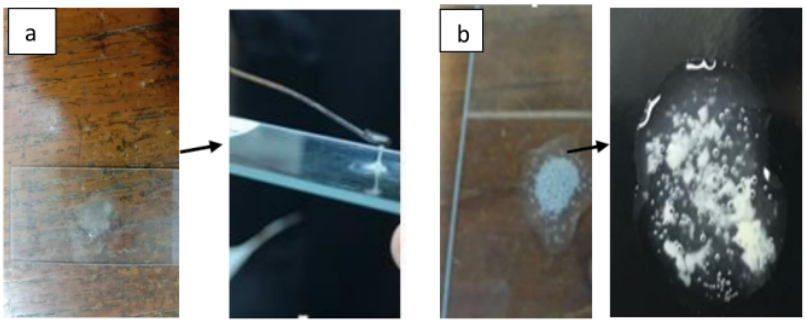

Figure 3. The results of the isolate test, the results of the gram reaction test, produce mucus (a) the results of the catalase test produce air bubbles (b).

Table 2. Calculation results of the number of methanotrophic bacteria.

Subsdistrict	Dilution rate (Cfu/mL)			Number of Bacteria
	10^4	10^5	10^6	Cfu/mL
Witaponda	$2.95x10^6$	$1.20x10^7$	$4.3x10^7$	$54.05x10^6$
Bumiraya	$2.57x10^6$	$1.15x10^7$	$3.2x10^7$	$40.21x10^6$
Bungku Barat	$2.50x10^6$	$1.01x10^7$	0	$12.6x10^6$
Bungku Tengah	$2.45x10^6$	$9.2x10^7$	0	$11.65x10^6$

Table 2 shows the results of elemental analysis of soil C-organic content in irrigated rice fields having values ranging from 1.53-2.00% (low category) in rainfed rice fields having values ranging from 1.76-1.81% with (low category) low). It can be concluded that the C-organic value in irrigated and rainfed rice fields is categorized as low because the value obtained is less than 2%.

Table 3. Content of C-Organic, N, C/N, P2O5 and K2O in irrigation locations (Witaponda, Bumiraya) and rain-fed locations (West Bungku, Central Bungku.

No.	Types of Rice Fields	Subsdistrict	Soil Analysis				
			C-Organik (%)	N (%)	C/N	P_2O_5 mg/100g	K_2O cmol / kg
1	Irrigation	Witaponda	1.53	0.18	8.37	0.25	18.25
		Bumiraya	2	0.24	8.28	0.23	10.24
2	Rainfed	Bungku Barat	1.76	0.18	9.88	0.22	14.75
		Bungku Tengah	1.81	0.21	8.75	0.32	32.55

Elemental analysis results of soil N content in irrigated rice fields have values ranging from 0.18-0.24% (high category) in rain-fed rice fields have values ranging from 0.18-0.21% (high category). It can be concluded that the N value in irrigated and rainfed rice fields is categorized as high because the values obtained range from 0.15 to 0.75 2%.

Observations The results of the analysis of phosphorus (P) content in irrigated rice fields have values ranging from 0.23–0.25 mg/100g (very low category) in rainfed rice fields having values ranging from 0.22-0.32 mg/100g with an average - (very low category). It can be concluded that P2O5 in irrigated in irrigated and rainfed rice fields is categorized as very low because the value obtained is less than 15 mg/100g.

Table 3 shows that the K2O content in irrigated rice fields has values ranging from 10.24–18.25 mg/100g (low category) because the values obtained range from 10-20mg/100g. In rain-fed rice fields, West Bungku sub-district has a value of 14.75 mg/100g (low category) Middle Bungku sub-district has a value of 32.55 mg/100g (medium category) because the values obtained range from 20-40 mg/100.

Table 4. Content of Ca, MgNa. And pH at irrigation locations (Witaponda, Bumiraya) rainfed locations (West Bungku, Middle Bungku).

No.	Field Type	Subdistrict	Soil Chemical Analysis				
			pH H_20	KTK cmol / kg	Ca cmol /kg	Mg cmol / kg	Na cmol / kg
1	Irrigation	Witaponda	6.35	16.51	18.35	10.22	28.25
		Bumiraya	6.57	19.27	24.36	18.32	25.34
2	Rainfed	Bungku Barat	6.48	15.29	28.62	14.21	21.85
		Bungku Tengah	6.52	17.41	16.21	32.25	35

Table 4. The pH (H_2O) value of the soil at the study site shows that the pH of the soil in irrigated rice fields has a value ranging from 6.35-6.57% (in the slightly acidic category) in rain-fed rice fields it has a value ranging from 6.48-6.52 % with (very sour category). It can be concluded that the pH values in irrigated and rainfed rice fields are categorized as slightly acidic because the values obtained are in the range of 5.6-6.5%.

One of the indicators in determining the status of soil fertility is Cation Exchange Capacity, because soil colloid is a determinant of the number of cations available and exchanged with the amount available for plants [13]. Table 4 shows that the soil CEC in irrigated rice fields in the Witaponda sub-district has a value of 16.51 cmol/kg in the low category because the values obtained range from 5-16 cmol/kg. Bumi Raya sub-district has a value of 19.27 cmol/kg in the medium category because the values obtained range from 17-24 cmol/kg in rain-fed rice fields in Bungku Barat sub-district which has a value of 15.29 cmol/kg in low category because the values obtained range from 5-16 cmol /kg. Bungku Tengah District has a value of 17.41 cmol/kg in the moderate category because the values obtained range from 17 – 24 cmol/kg.

It was observed from the analysis that soil Ca in irrigated rice fields in the Witaponda sub-district had a value of 18.35 cmol/kg in the high category because the values obtained ranged from 11-20 cmol/kg. Bumi Raya sub-district has a value of 24.36 cmol/kg in the very high category because the value obtained is above 20 cmol/kg in rainfed rice fields in Bungku Barat sub-district has a value of 28.62 cmol/kg in the very high category because the value obtained is above 20 cmol /kg. Subdistrict.

Bungku Tengah has a value of 16.21 cmol/kg in the high category because the values obtained range from 11-20 cmol/kg The results of elemental analysis of Mg content in irrigated rice fields had values ranging from 10.22-18.32 cmol/kg (very high category in rainfed rice fields having values ranging from 14.21-32.25 cmol/kg with (very high category) It can be concluded that the Mg values in irrigated and rainfed rice fields are categorized as very high because the values obtained are above 8 cmol/kg.

Table 4 shows that elemental analysis of Na content in irrigated rice fields has values ranging from 25.34-28.25 cmol/kg (very high category) in rain-fed rice fields having values ranging from 21.85-35.00 cmol/kg (very high category). tall). It can be concluded that the value of Na in irrigated and rainfed rice fields is categorized as very high because the value obtained is above 1 cmol/kg.

Burgy's Manual of Determinative Bacteriology classify methanotrophic bacteria into gram-negative bacteria. Gram-negative bacteria have a thin peptidoglycan so that it is easily extracted by ethanol (alkali) and increases the permeability of the bacterial cell wall. [4]The gram test using 3% KOH solution will dissolve the cell wall of gram-negative bacteria and cause mucus to secrete which is genetic material (DNA). Furthermore, [10] reported that methanotrophic bacteria are gram-negative bacteria, are aerobic, anaerobic and uses methane as a carbon source for energy.

Differences in gram properties are influenced by the content of the cell wall, namely For gram-positive bacteria, the peptidoglycan content is thicker when compared to gram-negative bacteria [14] at the structure of the cell wall of gram-positive bacteria consists of thick peptidoglycan, while the structure of the cell wall of gram-negative bacteria contains lots of lipids.

Table 1 shows that all isolates are able to bind nitrogen from the air. Testing for nitrogen fixing bacteria was carried out using selective Burk's media which did not contain nitrogen elements so that the bacteria had to bind nitrogen in the air. This is in accordance with the opinion [3] which states that Methanotrophic bacterial isolates BGM3 and BGM9 play a role in nitrogen fixation because they have the nifH and nifD genes that provide dinitrogenase reductase enzymes (Fe protein) and the α submit of dinitrogenase (Fe-Mo protein). Administration of methanotrophic bacteria as a nitrogen fixing agent assist in the provision of N nutrients in the soil, so as to provide optimal growth.

Nitrogen is an essential nutrient for plants, which has the property of being lost if it is in the soil, this is caused by several factors such as evaporation (volatilization), nitrification, denitrification or washing by water and erosion. Non-symbiotic nitrogen fixation can be carried out by free-living bacteria such as Herbaspirillum Enterobacteriaceae, Azospirillum, Azotobacter, and Bacillus which have been shown to be able to carry out nitrogen fixation [15]. Azotobacter can produce growth-promoting substances, namely gibberellins and cytokinins so that they can stimulate plant growth, especially in the roots of plants [16].

Table 1 shows that the Witaponda sub-district produced five isolates with isolate codes WPM1, WPM2, WPM3, WPM4, WPM5. Bumi Raya District produced 3 isolates with isolate codes BRM1, BRM2, BRM3. Bungku Barat District produced two isolates with isolate codes BBM1 and BBM2 and Bungku Tengah District produced 1 isolate with code BTM1. Table 2 shows that the total bacterial population of the rice rhizosphere in Witaponda District was higher than in Bumiraya, West Bungku and Middle Bungku Districts. Soil conditions that are frequently flooded produce more methanotrophic bacteria populations (Table 2.2) when compared to locations that are rarely flooded (rainfed). Locations that are stagnant cause the overhaul of organic matter around the roots, for example straw, roots which decays anaerobically in flooded paddy fields

The low C-organic is thought to be due to intensive paddy field management (continuous rice cultivation) with intensive fertilization without returning organic matter, causing soil organic matter to be depleted. Low levels of organic C in paddy soil are also caused by monoculture cropping patterns, excessive use of inorganic fertilizers and no return or addition of organic matter to the soil [17]

According to [18], the level of carbon content in the soil is influenced by the activity of microorganisms in breaking down soil organic matter, evapotranspiration or being involved in harvesting. This condition of the land needs to increase its C-organic content by adding organic matter in several ways, for example returning crop residues, applying manure, applying biological fertilizers and biochar.

Flooding and irrigation also affect the amount of K-total in the soil, excessive irrigation results in percolation which dissolves K in the soil resulting in leaching. The K_2O value in irrigated rice fields is in the moderate criteria, it is suspected that the potassium nutrient from the fertilizer applied in the soil is easily leached by surface runoff and the level of its content is strongly influenced by the flow of irrigation water which carries sufficient alkaline elements including potassium elements into the paddy soil. But if there is an intensive cycle of water supply, the irrigated paddy soil can also lose [19]. This is supported by the opinion of [20], that irrigation water contains a lot of alkaline elements such as sodium, calcium, potassium and magnesium, but if there is a regulation of giving often the paddy soil will quickly lose alkaline elements because alkaline elements are elements that are easily washed

4. Conclusion

Based on the results of the characteristics of the Witaponda sub-district, five isolates with isolate codes WPM1, WPM2, WPM3, WPM4, WPM5 were produced. Bumiraya sub-district as many as three isolates with isolate codes BRM1, BRM2, BRM3. West Bungku District produced two isolates with the isolate code BBM1, BBM2 and Bungku Tengah District as much as one isolate with the code BTM1.

Based on the results of the calculation of the number of soil bacteria found in the Witaponda subdistrict as much as $54.05x10^6$ Cfu/mL in Bumiraya subdistrict as much as $0.21x10^6$, Cfu/mL in West Bungku subdistrict as much as $12.06x10^6$ Cfu/mL in Bungku Tengah subdistrict as much as $11.65x10^6$ Cfu/mL. The results of the analysis of chemical properties show that pH, CEC, organic C, P205, K, are classified as low in both irrigated and rainfed rice fields.

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