Preliminary study of methane oxidizing bacteria isolation and selection on three rice agroecosystems

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Abstract. The agricultural sector contributes to releasing methane (CH\textsubscript{4}) as greenhouse gas emissions from lowland rice cultivation. One effort to reduce CH\textsubscript{4} emissions in paddy fields is the utilization of methane-oxidizing bacteria from paddy fields. The study aimed to obtain isolates of methane oxidizing bacteria from isolating and selecting their oxidation ability on three rice agroecosystems. The research was conducted at the Laboratory of the Indonesian Agricultural Environment Research Institute (IAERI), Pati, Central Java, Indonesia. Soil sampling was carried out in three rice agroecosystems, namely technical irrigation (SI), rainfed (TH), and organic rice cultivation (OF) rice fields in Pati Regency. Isolation and purification of bacteria used Nitrate Mineral Salts (NMS) with 1% methanol. Parameters collected were characteristics of morphology colonies, staining of gram bacteria and measuring methane oxidation ability. The results obtained 15 bacterial isolates from three different rice agroecosystems. The highest percentage of CH\textsubscript{4} concentration reduction from three rice different agroecosystems during 15 days of incubation were SI5 16, TH6 23, and OF3 23%, respectively. Methane oxidizing bacteria are expected as a technology to reduce CH\textsubscript{4} emissions from rice fields.

1. Introduction

Paddy fields are one of the sources of greenhouse gases (GHGs) such as methane (CH\textsubscript{4}) and nitrous oxide (N\textsubscript{2}O). Rice cultivation under continuously flooded water management caused the soil to become anaerobic conditions (limited oxygen). Anaerobic conditions in the rice field and low redox potential of the soil were the optimum conditions for producing CH\textsubscript{4}[1]. Nitrogen fertilizer application in the rice field could increase crop yield and influence CH\textsubscript{4} and N\textsubscript{2}O emissions of the soil [2-5]. Organic and inorganic fertilizers can increase the activities of soil microorganisms and enzymes and available nutrients in the soil [6]. The amount and way of application N-fertilizer affected to emit CH\textsubscript{4} and NO\textsubscript{2} gases [7].

The three mechanism ways of releasing CH\textsubscript{4} emissions from the soil into the atmosphere are through: 1) diffusion of CH\textsubscript{4} dissolved from the soil into the atmosphere due to differences in gradient concentration, 2) through air bubbles that out of the soil gaps (ebullition) and 3) transporting CH\textsubscript{4} through aerenchyma tissue in the root and stem of plant [8]. The release of CH\textsubscript{4} from an ecosystem is generally affected by plant types, microbial communities, soil properties and their interactions. More than 80% of the total CH\textsubscript{4} emissions from lowland rice is transported through the aerenchyma in rice plants [9,10].
Archaea methanogens as CH₄-producing bacteria are able to invite the presence of other bacteria, such as methanotrophic bacteria (methane oxidizing bacteria, MOB) that utilize CH₄ as a source of carbon in their metabolic processes [11]. The presences of these bacteria in the roots and rhizosphere of rice plants have positive impacts to reduce CH₄ emissions in rice field and released into the atmosphere. These bacteria in the rhizosphere of rice plants has a positive impact to reduce CH₄ emissions that released into the atmosphere. These bacteria have the specific enzyme namely methane monoxygenase (MMO) which is able to oxidize CH₄ into methanol, formaldehyde, formate and carbon dioxide (CO₂) [1,12], being able to grow in aerobic (oxygen) and anaerobic (without oxygen) conditions in several species [1,13], and at neutral soil pH and included at mesophilic bacteria (25°C) and low salinity [14]. The optimum conditions can increase the MMO enzymatic activity in the oxidizing methane into methanol. The two methane monoxygenase types consist of soluble methane monoxygenase (sMMO) enzyme and particulate methane monoxygenase (pMMO) enzymes [1,11]. The process of methane oxidation is predominantly catalyzed by the pMMO enzyme [15]. The study aimed to obtain isolates of methane oxidizing bacteria and selecting their methane oxidation ability on three rice agroecosystems.

2. Materials and methods

2.1. Soil sampling

Soil samples were taken from three rice fields, namely technical irrigation of rice field (SI), rainfed (TH) and organic rice cultivation (OF) on three villages in Pati Regency (table 1). In technical irrigation (SI) and rainfed (TH) locations, the soil samples were taken diagonally at the plant root area by using 50 mL syringe (± 3–10 cm depth). In organic rice cultivation (OF) location, soil samples were taken around the rhizosphere area. Liquid or mud samples were placed into a sterile bottle, then stored into a plastic sample, and labeled for each location. Isolation and selection activities were conducted at IAERI laboratory, Jaken Subdistrict, Pati Regency, Central Java Province of Indonesia. The research was started in January to June 2018.

| Table 1. | Soil sampling location description of soil sampling in three rice agroecosystems in Pati, Central Java. |
| Location description | Brati, Kayen | Sidomukti, Jaken | Tambahmulyo, Gabus |
| Type of field | Technical irrigation rice field (SI) | Rainfed (TH) | Organic rice cultivation (OF) |
| Coordinate | 6°54′8′′S, 111°1′39′′E | 6°46′56′′S, 111°12′3″E | 6°49′27′′S, 111°2′44″E |
| Area (ha) | 0.35 | 0.18 | 0.14 |
| Rice variety | Inpari 32 | Inpari 32 | Mentik Wangi |
| Days after transplanting | 42 | 60 | 56 |
| Water irrigation | Flooded | Flooded | Intermitten irrigation |
| Water sources | Irrigation channel and rainfall | Ground water and rainfall | Ground water and rainfall |
| Water level | 3.5 cm | 2.5 cm | 1.2 cm |
| Cropping pattern | Paddy-paddy-paddy | Paddy-paddy-corn | Paddy-paddy-soybean |
| Organic fertilizer | Farmyard manure | Patigan - compost | Farmer's biofertilizer |
| Anorganic fertilizer | Phonska, Urea, KCl, ZA | Urea, KCl, SP36 | - |
| Pesticides | Herbicides | Herbicides | - |
| Insecticides | Insecticides | Insecticides | - |
| Fungicides | Fungicides | Fungicides | - |
| Bactericides | Molluscicides | Molluscicides | - |
| Molluscicides | IAERI's Bioperticides | - | - |

Remarks: S = South/Latitude, E = East/Longitude.
2.2. Isolation and purification
Isolation of methane oxidizing bacteria from soil was carried out by growing them in NMS (Nitrate Mineral Salts) medium with 1% methanol. Composition of 1 L of NMS were bacto agar 20 g, MgSO\(_4\).7H\(_2\)O 1 g, CaCl\(_2\).2H\(_2\)O 0.2 g, KNO\(_3\)/NaNO\(_3\) 1 g, KH\(_2\)PO\(_4\) 0.272 g, Na\(_2\)HPO\(_4\).12H\(_2\)O 0.717 g, NH\(_4\)Cl 4 g, Methanol 1% and Trace element (TE) 0.5 mL (TE/1 L): Na\(_2\)EDTA 0.5 g, FeSO\(_4\).7H\(_2\)O 0.2 g, H\(_2\)BO\(_3\) 0.03 g, CoCl\(_2\).6H\(_2\)O 0.02 g, ZnSO\(_4\).7H\(_2\)O 0.01 g, MnCl\(_2\).4H\(_2\)O 0.03 g, NaCl 6H\(_2\)O 0.02 g, CaCl\(_2\).2H\(_2\)O 0.01 g [16]. Forty five mL liquid NMS in the incubation bottle was added 5 mL inoculum (mud rice field/TH & SI) or 5 g (soil rice field/OF). Standard CH\(_4\) 11.52 ppm was also added with the composition of headspace in the bottle near to 50% CH\(_4\) and 50% air by syringe (dilution series 1). Incubation was conducted for 5 days with 150 rpm shaker and then dilution series 2, 3, 4, 5, 6 and 7 were made for each sample with 2 replications. The dilution series 5, 6, and 7 were inoculated onto the NMS medium to make a duplo (2) growing plates with pour plate method. Time for incubation was 7 to 15 days in room temperature. Colonies of bacteria were purified in the same medium and repeated until getting pure culture. Pure culture is a culture that containing only one type of bacteria.

2.3. Characteristics of bacterial colonies morphology and gram staining
Morphological characteristics of bacteria colonies were observed such as color, optics, shape, margins and elevation. Shape, margin and elevation of bacteria colonies can be directly determined with a stereo microscope. The procedure of gram bacterial staining used Gram A B C D in smear preparations of bacterial isolates. Gram staining involves four processes: namely staining with a water-soluble dye called crystal violet (Gram A), lugol (Gram B) the binding of crystal violet to the cell wall, and alcohol 96% (Gram C) as decolorization, and counterstaining with safranin (Gram D). Binocular microscope was used to know color of staining membrane cell and shape of cell bacteria. The staining of bacterial membrane cell gave colors on the bacterial cell walls. The cell wall of gram-positive bacteria will absorb purple, whereas pink in gram-negative bacteria [17].

2.4. Methane oxidation ability
Methane oxidation activity was tested to determine the ability of methanotrophic bacteria in reducing CH\(_4\) from obtained isolates. This test was done by measuring the concentration of CH\(_4\) gas remaining in the headspace using gas chromatography techniques [18]. One mL of the inoculum of isolate 105 cfu mL\(^{-1}\) (NMS medium + 1% methanol) was inoculated into 15 mL of liquid NMS medium (without 1% methanol) in a 35 mL volume of the bottle. The gas in the headspace was made up of 50% CH\(_4\) and 50% air. The air in the bottle was taken by syringe 10 mL and added 10 mL of CH\(_4\) 11.52 ppm standard. As control was used 10 mL of NMS medium in the bottle with the same headspace composition and 1 mL NMS + 1% methanol medium without inoculum of bacteria. Incubation was carried out for 15 days on a shaker at room temperature (27-30°C). Measurement of CH\(_4\) concentration was carried out at 7, 10 and 15 days during the incubation period. Sample gas was taken by syringe 5 mL and measured using GC Shimadzu 14 A with FID (Flame Ionization Detector) for analyzing CH\(_4\) gas concentration.

2.5. Data analysis
Methane concentration from isolates bacteria (treatment) was compared to CH\(_4\) concentration of control (without isolate). Calculation of the percentage reduction in CH\(_4\) concentration in the growing medium of methane oxidizing bacteria was calculated by equation (1):

\[
\frac{\text{Control concentration (ppm)} - \text{Treatment concentration}}{\text{Control concentration (ppm)}} \times 100\% \quad (1)
\]

where:
Control = liquid NMS without methanol + 1 mL NMS with methanol + CH\(_4\) standard
Treatment = liquid NMS + inoculum of bacteria + CH\(_4\) standard
3. Results and discussion

3.1. Isolation and purification in three rice agroecosystems

The results of isolation and purification processes from three different rice agroecosystems obtained 15 bacterial colonies. The number of isolates obtained from the locations of technical irrigation (SI), rainfed (TH), and organic cultivation (OF) rice fields were each 5 (namely SI1, SI2, SI3, SI4, and SI5), 6 (namely TH1, TH2, TH3, TH4, TH5, and TH6), and 4 (namely OF1, OF2, OF3, and OF4), respectively. Fifteen isolates candidates from three rice agroecosystems were able to grow on NMS media with 1% methanol. These isolates are expected as methanotrophic bacteria that could oxidize CH₄ as a carbon source in its metabolic process. The special characteristics of these bacteria have an enzyme methane monoxygenase (MMO) which can convert methane into CO₂ and H₂O as end products of metabolic processes [1,12].

3.2. Characteristics of bacterial colonies morphology and gram staining

The parameters of colonies morphology observed from 15 isolates were color, shape, margin, and elevation colony as well as gram staining of bacteria as shown in table 2. The colonies morphology of methanotrophic bacteria very diverse in color, optical, shape, margin, and elevation colony of bacteria, as well as shape of bacterial cells. Some methanotrophic bacteria do not produce pigments, while some methane-oxidizing bacteria were able to produce pigments, such as yellow, brown, white, orange, and pink to red [11,19], yellow to purple, yellow to orange, purple to pink, and pink to purple [20]. Methanotrophic bacteria are gram negative bacteria utilizing CH₄ as a carbon source [1,11]. Gram negative (diderm) bacteria have two layers surrounding a thin layer of peptidoglycan located in the periplasmic space between the plasma membrane and the outer membrane of the bacterial cell. In the gram staining test, the bacteria absorb the pink color [17,21]. The identified methanotrophic cells are bacilli, cocci, coccobacilli, elliptoidal or pleomorphic, usually 1 to 2 μm size [19].

Table 2. Characteristics of colonies morphology and gram staining of bacteria.

| Isolates | Color    | Optic   | Shape    | Margin | Elevation | Gram bacteria | Shape of cell |
|----------|----------|---------|----------|--------|-----------|---------------|---------------|
| TH1      | White    | Transparent | Irregular | Lobate | Raised    | Negative      | Bacillus      |
| TH2      | Cream    | Transparent | Irregular | Undulate | Flat      | Negative      | Coccus        |
| TH3      | Cream    | Translucent | Irregular | Lobate | Flat      | Negative      | Coccus        |
| TH4      | Brown    | Translucent | Irregular | Undulate | Flat      | Negative      | Bacillus      |
| TH5      | Cream    | Opaque   | Irregular | Entire | Convex    | Negative      | Bacillus      |
| TH6      | Brown    | Translucent | Irregular | Undulate | Raised    | Negative      | Coccus        |
| OF1      | White    | Transparent | Irregular | Lobate | Flat      | Negative      | Bacillus      |
| OF2      | White    | Transparent | Irregular | Serrate | Flat      | Negative      | Coccus        |
| OF3      | Cream    | Opaque   | Irregular | Lobate | Convex    | Negative      | Bacillus      |
| OF4      | Dark brown | Opaque   | Irregular | Lobate | Umbonate | Negative      | Bacillus      |
| SI1      | White    | Transparent | Irregular | Lobate | Flat      | Negative      | Bacillus      |
| SI2      | White    | Transparent | Irregular | Lobate | Flat      | Negative      | Coccus        |
| SI3      | Brown    | Translucent | Circular | Undulate | Umbonate | Negative      | Coccus        |
| SI4      | Yellow   | Opaque   | Circular | Entire | Convex    | Negative      | Bacillus      |
| SI5      | Cream    | Opaque   | Irregular | Rhizoid | Raised    | Negative      | Bacillus      |

Remarks:
TH (rainfed), OF (organic rice cultivation), and SI (technical irrigation rice field)

Optical: opaque, translucent, and transparent

Shape of colonies: circular, irregular, spindly, filamentous, and rhizoid

Margin: entire, lobate, undulate, serrate, filamentous, and curled.

Elevation: flat, raised, convex, umbonate, and pulvinate

Shape of cell: bacillus, coccus, vibrio, and spiral

Staining cell: pink/red (gram negative), and blue (gram positive)
3.3. Methane oxidation ability

Percentage values of CH\textsubscript{4} concentration reduction from each of the 15 selected isolate results have differences in CH\textsubscript{4} oxidation abilities in incubation bottle with the inoculum addition of bacteria and standard CH\textsubscript{4} gas with CH\textsubscript{4} standard and air headspace ratio of 50:50%. The percentage of CH\textsubscript{4} concentration reduction from the 15 isolates is presented in Figure 1. The isolate ability to oxidize methane of the cultures is assessed by gas chromatography to monitor methane concentration and oxygen levels, and increase of carbon dioxide levels [22].

![Figure 1. Percentage of CH\textsubscript{4} concentration reduction from 15 isolates.](image)

Figure 1. Percentage of CH\textsubscript{4} concentration reduction from 15 isolates.

Methane concentration reduction of 15 isolates from three different rice agroecosystems resulted in different percentages among isolates. A value of 0, which means there was no decrease in methane concentration by inoculated isolates. Seven days incubation was the best time to reduce CH\textsubscript{4} concentration in the bottle culture with the highest of CH\textsubscript{4} concentration reduction compared to 10 and 15 days incubation for 12 isolates except for OF1, SI2 and SI4 isolates showing the highest CH\textsubscript{4} concentration reduction at 15 days incubation. This time was the log phase of bacterial growth where there is an increase in the number of bacterial cells that requiring methane as a carbon source. Decreasing of CH\textsubscript{4} concentrations at 7, 10, and 15 days incubation were 9 to 43, 3 to 23, and 1 to 16%, respectively. The highest average percentage values of CH\textsubscript{4} concentration reduction during 15 days incubation from the three rice agroecosystems were 23, 16, and 23 and resulted by TH6, SI5, and OF3 isolates under rainfed irrigation, technical irrigation, and organic rice field cultivations, respectively.

The difference in methane oxidation ability of each isolate is also influenced by the type of MMO enzyme that is owned. In addition, the oxygen availability can also affect the methane oxidation process in rice fields. The oxidation of methane to methanol by MMO requires two electrons and oxygen, and NADH is an electron donor in the process [23]. Oxygen is a very important limiting factor for the methanotrophic bacteria to convert methane into carbon dioxide, water, cell biomass, and get energy for growth [1,24]. Oxidation process of methanotrophic bacteria was begun with oxidizing methane into methanol by releasing oxygen bonds with the enzyme MMO [25].

4. Conclusions

The bacterial isolation from 3 different rice agroecosystems found 15 isolates consisting of 5 isolates (namely SI1, SI2, SI3, SI4, and SI5) from technical irrigated, 6 isolates (TH1, TH2, TH3, TH4, TH5, and TH6) from rainfed, and 4 isolates (OF1, OF2, OF3, and OF4) from organic rice field cultivations, respectively. The highest percentage of CH\textsubscript{4} concentration reduction during 15 days incubation were resulted by TH6, SI5, and OF3 isolates under rainfed irrigation, technical irrigation, and organic rice field cultivations, respectively. The utilization of methane oxidizing bacteria is expected as a technology to reduce CH\textsubscript{4} emissions and enhance productivity of rice fields.
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