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Isolation and identification of heterotrophic bacteria with methane oxidation potential from various ecosystems as biological agents for reducing methane emissions

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Abstract. *Widiastuti E, Santosa DA, Anwar S. 2026. Isolation and identification of heterotrophic bacteria with methane oxidation potential from various ecosystems as biological agents for reducing methane emissions. Biodiversitas 27 (4): d270423. https://doi.org/10.13057/biodiv/d270423.* Methane is the second-largest greenhouse gas after carbon dioxide, contributing to global warming. Methane-oxidizing bacteria can help reduce methane emissions. However, the availability of methane-oxidizing bacteria isolated from various Indonesian ecosystems remains very limited, leaving a knowledge gap regarding their diversity and functional potential. This study aimed to isolate and identify bacteria from the swamp, river, and cattle manure ecosystems. Sediment samples were collected at 1-10 cm depth from five points in each ecosystem. Bacteria were isolated using a nitrate mineral salt medium supplemented with methane gas as a carbon source during incubation. The bacteria obtained were subjected to further analysis, namely morphological observation, pathogenicity testing through hypersensitivity and hemolysis analysis, indirect testing of Particulate Methane Monooxygenase (pMMO) and Soluble Methane Monooxygenase (sMMO) enzyme activity. Methane oxidation activity was quantified in three replicates for each isolate using gas chromatography. Molecular identification involved Polymerase Chain Reaction (PCR) amplifying the 16S rRNA gene. There are nine potential bacterial isolates with diverse morphological forms. In vitro tests show that all bacteria are non-pathogenic, with methanol production indirectly indicating pMMO enzyme activity, showing an average concentration of 1.80 mM, while sMMO activity is negative. The three best bacteria, Rw3, Rw4, and Sn1, had potential CH_4 oxidation capabilities of 66.61, 62.39, and 62.55%, respectively, under laboratory conditions. The three bacteria were identified as *Achromobacter* sp., *Exiguobacterium* sp., and *Burkholderia* sp., with similarity values of 97.41%, 99.88%, and 98.85%, respectively. Although these three genera are not classified as methanotrophs, they exhibit potential methane-oxidizing activity under laboratory conditions. Overall, this study highlights the potential role of diverse heterotrophic bacterial genera in oxidizing methane, making them relevant biological candidates in the methane cycle.

Keywords: 16s rRNA gene, climate change, enzyme activity, microorganism, phylogenetic

INTRODUCTION

A global phenomenon that has been the center of attention in recent decades is the increase in greenhouse gas emissions, which has driven global climate change (Filonchyk et al. 2024). Greenhouse gases trap heat in the atmosphere and contribute significantly to global warming (Jeffry et al. 2021). The main greenhouse gases contributing to global warming include methane (CH_4), carbon dioxide (CO_2), and nitrous oxide (N_2O) (IPCC 2023). Methane is the second most influential greenhouse gas after carbon dioxide (Li et al. 2022). Methane is a potent greenhouse gas that increases by about 1% annually (Aimen et al. 2018). In 2021, the global average atmospheric methane concentration reached 1909.3 ppb, up from just 774 ppb in 1850 (Nisbet et al. 2021). The main sources of atmospheric methane include natural and anthropogenic activities, such as agricultural practices, livestock farming, natural gas leaks, and the extraction and consumption of fossil fuels (He et al. 2024).

Methane emissions are largely produced by methanogenic archaea in the anaerobic zone of the soil (Prasitwuttisak et al. 2022). However, most methane is not directly released into the atmosphere because methanotrophic bacteria consume it (Sakai et al. 2023). In general, methanotrophic bacteria are classified into Types I-II-X. Type I is characterized by the production of particulate methanemonoxygenase and cells that are rod-shaped or coccus (Tveit et al. 2023). Type II organisms are characterized by the presence of soluble methane monooxygenase and a crescent-shaped morphology (Guerrero-Cruz et al. 2021). Type X bacteria appear as paired cocci and share characteristics with Type I and Type II bacteria (Khmelenina et al. 2019). Methanotrophic bacteria oxidize CH_4, which is influenced by various environmental factors, including pH, humidity, soil particle size, temperature, and nutrient availability (Zheng et al. 2024). The presence of methanotrophic bacteria can reduce CH_4 emissions (Li et al. 2021) through genes encoding methane monooxygenase (MMO) enzymes, in both particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO) forms, which effectively catalyze CH_4 oxidation (Sakai et al. 2023). The MMO enzyme consists of three subunits: α, β, and γ (Khider et al. 2021). The function of these three parts is as

the active site for catalyzing CH_4. Methanotrophic bacteria act as natural biofilters and are potential biological agents in mitigating global climate change (He et al. 2024).

Although the role of true methanotrophs has been extensively studied, most previous research has focused on obligate methanotrophs and growth-dependent methane oxidation mechanisms using CH_4 as the primary carbon source. This approach limits our understanding of the contribution of other microorganisms that coexist in methane-rich ecosystems, particularly non-methanotrophic or heterotrophic bacteria. As a result, the potential role of heterotrophic bacteria in methane oxidation has been understudied in methane-cycle research. Heterotrophic bacteria may oxidize methane via co-metabolic mechanisms. Some microorganisms use the main substrate for cell growth, and some enzymes are induced to transform growth substrates, which can also catalyze the conversion of non-growth substrates (Tran et al. 2013). Important major co-metabolic reactions involve microbial oxygenase enzymes, such as methane monooxygenase (MMO), toluene mono- and dioxygenase, ammonia monooxygenase, biphenyl oxygenase, and other monooxygenase enzymes (Nzila 2013). Research conducted by Li et al. (2016) showed the ability of Pseudomonas sp. to degrade Methyl tert-Butyl Ether (MTBE) through co-metabolism on n-alkane (C5-C8) growth substrates.

Meanwhile, research conducted by Rani et al. (2021) showed that several genera, such as *Hyphomicrobium*, *Burkholderia*, *Methylobacterium*, *Paenibacillus*, *Rahnella*, and *Pseudomonas* can grow on methane, utilizing 74.33% of it, indicating that certain heterotrophic bacteria can oxidize methane via a co-metabolic mechanism. This condition indicates the need for further research on the role of heterotrophic bacteria living in various methane-rich ecosystems to determine their ability to oxidize methane. This study aims to isolate methane-oxidizing bacteria from three ecosystems (swamp, river, and cattle manure), evaluate the presence of pMMO and sMMO enzyme activities and methane oxidation capacity, identify isolates, and assess their phylogenetic relationships.

MATERIALS AND METHODS

Sample collection

Sediment samples were collected from three ecosystems with different environmental conditions and organic matter inputs as methane sources. These ecosystems include a swamp area (4°00'36.23"S, 122°31'33.51"E) in Kendari City, Southeast Sulawesi Province, Indonesia; the Cisadane River (6°32'42.59"S, 106°43'2.41"E); and a cattle manure disposal site (6°33'18.01"S, 106°42'58.54"E) in Bogor, West Java, Indonesia. Samples were collected using a random sampling method from 0-10 cm at five points in each ecosystem, representing environments with predominantly reducing conditions and high organic matter availability, which promote methane production and support the presence of heterotrophic bacteria with methane oxidation potential. The samples were stored in plastic containers and kept at 4°C in a refrigerator for 24 hours before further analysis at the Soil Biology Laboratory, Department of Soil Science and Land Resource, Faculty of Agriculture, Institut Pertanian Bogor, Indonesia.

Isolation of methanotrophic bacteria

Isolation of methanotrophic bacteria was performed using the enrichment method with Nitrate Mineral Salt (NMS) medium (Stępniewska et al. 2017) containing a complete composition ($CaCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, Na_2HPO_4, NH_4Cl, KNO_3, $FeSO_4 \cdot 7H_2O$, H_3BO_4, Na_2EDTA, $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$). Each sediment sample was replicated three times for enrichment. A total of 1 g of the sample was inoculated into 50 mL of NMS medium in 125 mL serum bottles, which were then sealed with rubber stoppers. Each serum bottle was filled with 37.5 mL of methane gas, which was injected into the vial using a sterile gas-tight syringe, adjusting the gas composition in the headspace to 50% methane and 50% air. The bottles were incubated for 6-10 days at 100 rpm on a shaker at room temperature, in the dark. In the sediment sample enrichment process, NMS controls without CH_4 were not included, so the isolates obtained came from NMS medium enriched with methane. After incubation, 1 mL of the sample was placed in a test tube containing 9 mL of 0.85% NaCl, then homogenized with a vortex until a first dilution (10^{-1}) was obtained. Dilutions were carried out until a fifth dilution (10^{-5}) was obtained. Then, 0.1 mL was taken from each test tube, and the results of 10^{-3} to 10^{-5} dilution were poured into a petri dish containing NMS agar medium using the spread plate technique and then incubated at room temperature in a dark room for 5-6 days (Rusmana and Akhadiya 2009). The growing colonies were streaked again on the same medium using the quadrant method until a single isolate was obtained. All isolates were then analyzed for their morphological shape, followed by growth testing on nutrient broth liquid medium in 10 mL vials with adjusted pH (4, 5, 6, 7, 8, 9) by observing turbidity to determine the range of each bacterium's ability to grow at a specific pH.

Analysis of hemolysis and hypersensitivity

Hemolysis and hypersensitivity tests are used to assess the safety of bacterial isolates during initial evaluation, ensuring they are safe for animals (hemolysis test) and plants (hypersensitivity test). The hemolysis test was performed on blood agar medium. Isolated bacteria were inoculated onto blood agar media in the form of dots and incubated at room temperature (27-30°C) for 24 hours. Hemolysis was observed as a clear zone around the bacterial colony. If there is a clear zone, the bacteria are pathogenic/hemolytic. The hypersensitivity test was performed by growing bacteria in nutrient broth media, then injecting 1 mL of the suspension using a sterile needle directed at the underside of a tobacco leaf (*Nicotiana tabacum* L.). When injecting, it is important to ensure that there are no leaf tears or leakage of the suspension from the tissue (Triwidodo 2021). Observation for hypersensitivity reactions was performed after 48 hours. If the leaf shows

necrosis at the injection site, the bacteria are potentially pathogenic to the plant.

Analysis of indirect indications of methanol production and soluble methane monooxygenase (sMMO)

Analysis of methane monooxygenase enzymes, divided into pMMO and sMMO, was conducted to determine whether each isolate possessed the enzyme activity required for methane oxidation. The pMMO enzyme activity test was performed indirectly by measuring SNP reaction products, which indicate methanol production, a product of the initial oxidation of methane by pMMO. Bacterial isolates were cultured on NMS medium and CH4 until colonies grew (5-10 days). The pMMO analysis was performed by taking a 0.1 mL liquid sample, adding 0.6 mL of SNP reagent (10% sodium nitroprusside, potassium ferricyanide, 5% NaOH, aquades), and diluting to a final volume of 1.5 mL with distilled water. The sample was vortexed for 10-20 seconds at maximum speed and left to stand for 15 minutes. The sample was then analyzed at 481 nm using a spectrophotometer (Zhan et al. 2010), with two replicates per isolate. The standard solution used was methanol reacted with SNP reagent at varying concentrations, which were then processed to generate a calibration curve. The absorbance values of the standard solutions yielded the regression equation $y = 0.0612x + 0.0003$ ($R^2 = 0.9997$), which was used to estimate methanol concentration in the samples. Because pMMO analysis is an indirect test, it has several limitations, including the possibility that other compounds in the culture that react with the SNP reagent can affect color formation. The sMMO analysis was performed by growing bacteria on NMS medium until colonies grew (5-10 days). The Petri dish was placed upside down, then opened by lifting the part of the dish containing the colony. Several naphthalene crystals were sprinkled inside the dish lid, and it was stored upside down at 30°C for 15 minutes. After 15 minutes, the colony surface was sprayed with o-dianisidine solution (5 mg/mL) for 2 to 3 seconds, then covered with a new dish lid and incubated again for 15 minutes (the dish is not inverted) (Graham et al. 1992). If the colony produced naphthol, a purple-red color would form, indicating a positive result (Baskaran et al. 2023).

Analysis of methane oxidation

The oxidation activity of CH_4 from the isolate was assessed by quantifying the residual methane gas in the vial using gas chromatography. A total of 50 mL of bacterial suspension was placed in a 125 mL vial, which was tightly sealed with a rubber stopper. Each vial was filled with 37.5 mL of methane gas, injected using a sterile gas-tight syringe, and the gas composition in the vial headspace was adjusted to 50% methane and 50% air. The control treatment was made using sterile NMS media without bacteria. Each sample was prepared in triplicate and then incubated for 12 days on a shaker at room temperature (27-30°C) in the dark. The remaining methane concentration was measured at the end of the incubation period by gas chromatography (Liu et al. 2018). Methane measurements were performed using gas chromatography equipped with a Flame Ionization Detector (FID). This system was controlled by a computer (PC), and the data results were processed and stored on another computer. The sample gas and standard gas were fed into the sample loop, then injected into the main column using ultra-pure N_2 carrier gas (>99.9999%) at a flow rate of 50 mL/minute. The main column was a stainless-steel cylinder with an inner diameter of 3 mm and a length of 4 m, filled with 5A molecular sieve (60/80 mesh). The sample loop and column were maintained at 70°C in the oven.

The data were statistically analyzed using Analysis of Variance (ANOVA) to evaluate the effect of bacterial isolates on methane oxidation activity. This experiment used various isolates, with each treatment replicated three times. Data normality was tested using the Shapiro-Wilk test. When the data were normally distributed ($p>0.05$), ANOVA was performed, and further analysis was conducted using Tukey's test. Statistical data processing was performed using SPSS software.

Molecular identification of bacteria

DNA extraction was performed using the cetyltrimethylammonium bromide (CTAB) method. The 16S rRNA gene fragment was amplified using a GeneAmp PCR System 9700 machine with a pair of universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Santosa 2001). The PCR process consisted of a pre-denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. The PCR product was analyzed using 1% agarose gel electrophoresis at 50 volts for 50 minutes, and the DNA bands were visualized with a UV transilluminator and compared with a 100 bp DNA marker.

The PCR product was then purified prior to sequencing using Centricon®-100 columns (PN N930-2119). The Centricon-100 columns were assembled, then 2 mL of deionized water was added to prepare the ultrafiltration membrane. The entire volume of the PCR product was added to the column and centrifuged at 3000 × g for 10 minutes. After centrifugation, the waste container was removed and replaced with a collection tube. The column was then inverted and centrifuged again at 270 × g for 2 minutes to collect the purified PCR product, yielding approximately 40 µL. The PCR product was sent to First Base Asia Laboratory for nucleotide sequencing. The sequence data were edited using BioEdit software and analyzed for homology using the Basic Local Alignment Search Tool-Nucleotide (BLAST-N) tool available on the National Center for Biotechnology Information (NCBI) website to identify closely related species based on sequence similarity. Genetic distance matrix analysis was performed using the p-distance method, and phylogenetic tree reconstruction was conducted using the Molecular Evolutionary Genetics Analysis (MEGA) 11 application with the maximum likelihood method and 1,000 bootstrap replications to assess the reliability of the tree topology.

RESULTS AND DISCUSSION

Abundance of bacteria

Nine potential bacterial isolates were successfully isolated on NMS (Nitrate Mineral Salt) medium from three ecosystems (swamp, river, and cattle manure). Five isolates were obtained from the swamp (Rw1, Rw2, Rw3, Rw4, Rw5), and two isolates were obtained from each the river (Sn1, Sn2) and a cattle manure disposal site (Pt1, Pt2). The number of isolates was determined based on distinct morphological characteristics, where colonies with similar morphology were considered a single isolate.

Differences in environmental characteristics across ecosystems affect microbial community diversity, including organic content, temperature, pH, and the availability of carbon substrates necessary for microbial growth. Swamp sediment is generally rich in organic matter and, under anaerobic conditions that support microbes in the carbon cycle, including those capable of oxidizing methane, yields more isolates than other sources. Type I methanotrophic bacteria, including genera such as *Methylomonas*, *Methylococcus*, *Methylomagnum*, and *Methylocucumis*, as well as Type II methanotrophs, such as *Methylocystis* spp. and *Methylosinus* sp., were successfully isolated in wetlands (Mohite et al. 2024).

In river ecosystems, nutrient availability for microbes is relatively dynamic because it is highly dependent on nutrients carried by the water flow. Water flow can cause nutrients to be lost as they are swept away by the current, allowing only certain microbes to live and thrive. Meanwhile, the total number of isolates from cattle manure is influenced by environmental conditions that are often extreme, such as high ammonia concentrations, fluctuating pH, and the presence of toxic compounds from animal metabolism, which can inhibit the growth of most microbes, leaving only those with high tolerance to survive. The different morphological characteristics reflect the diversity of the bacterial types shown in Table 1.

The enrichment process to grow bacteria was carried out only in NMS medium enriched with methane, without NMS controls without CH_4 or NMS + CH_4 controls with dead cells. Therefore, the growth of isolates at the enrichment stage cannot be used as direct evidence of obligate dependence on methane as the sole carbon source. Hemolysis and hypersensitivity tests were performed as an initial biological safety screening to ensure that the potential bacteria did not have pathogenic properties against humans or plants in the environment. The results of hemolysis analysis on blood agar medium for 24 hours revealed that all bacteria were non-hemolytic, as indicated by the absence of a clear zone around the bacterial isolates. Hemolytic bacteria can degrade components of blood agar medium, producing clear zones that indicate α-hemolysin and β-hemolysin activity (Mogrovejo et al. 2023). Both toxins are highly dangerous because they can degrade components of human blood cells (Amaria et al. 2023).

Meanwhile, the hypersensitivity test results showed that all isolates were non-pathogenic, as indicated by the absence of necrosis on tobacco leaves after inoculation for 5-6 days. The hypersensitivity reaction is a rapid and localized cell death program. Each microbe can produce enzymes and secondary metabolites in varying amounts and with varying characteristics (Perry et al. 2022). These active compounds are what make microbes potentially pathogenic to plants, so comprehensive microbial selection is necessary to prevent the spread of disease in the environment. In addition, growth tests at various pH levels were conducted to evaluate the isolates' ability to survive and grow in environmental conditions with different pH levels, reflecting their adaptation to pH variations in their native ecosystems. The test results showed that all isolates grew well at neutral pH (pH 7), as indicated by uniform turbidity in the medium. In contrast, isolates Rw4, Rw5, and Pt2 also grew optimally under acidic conditions (pH 4). These findings indicate that some isolates have the physiological flexibility to survive in ecosystems with lower pH conditions, which could be an important factor in their environmental applications.

Indirect indications of methanol production and sMMO test results

Methanotrophic bacteria use the enzyme methane monooxygenase (MMO) to oxidize methane. MMO exists in two forms, namely particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO) (Ross and Rosenzweig 2017). In this study, methanol production was indirectly assessed using the SNP reagent, along with a qualitative plate-based assay for sMMO (Table 2).

In this study, methanol production was analyzed as an indirect indication using SNP reagents. The average methanol production measurement from nine isolates was 1.80 mM, with the highest value in isolate Rw5 at 2.14 mM. The measured methanol production value indicates the potential presence of the pMMO enzyme but does not definitively prove pMMO enzyme activity; further studies are needed to use controls without CH_4 and dead-cell controls to ensure that methanol production originates from living-cell activity. In the common methanol oxidation pathway, the pMMO enzyme catalyzes the release of dioxygen, with one oxygen atom binding to methane to form methanol and the other being reduced to H_2O. The methanol dehydrogenase enzyme oxidizes methanol to form formaldehyde, which is then oxidized to formate by the formaldehyde dehydrogenase enzyme. Next, formate is oxidized into carbon dioxide by the formate dehydrogenase enzyme (Thallaj 2015). The pMMO enzyme cannot oxidize naphthalene to naphthol, unlike sMMO. Based on the test results, none of the bacterial isolates showed a purple/red color change, indicating that the sMMO enzyme is active. This test was conducted without an sMMO positive control; negative results were based solely on the testing conditions used. Therefore, a sMMO methanotrophic strain positive control is required in further studies to validate the sMMO plate test. The sMMO enzyme is only found in certain methanotrophic strains, such as those in the genera *Methylocella* and *Methyloferula*, which grow in environments with low copper levels (Li et al. 2021).

Table 1. Morphological characteristics and pathogenity tests

Characteristics	Nine potential bacterial isolates								
	Rw1	Rw2	Rw3	Rw4	Rw5	Sn1	Sn2	Pt1	Pt2
Color	White	Milky white	Cream	White	Yellow	Yellow	Yellow	Yellow	Cream
Shape	Circular	Irregular	Irregular	Circular	Irregular	Irregular	Circular	Circular	Circular
Margin	Entire	Undulate	Undulate	Entire	Undulate	Undulate	Entire	Undulate	Entire
Elevation	Convex	Flat	Flat	Flat	Flat	Flat	Convex	Convex	Flat
Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Mucoid	Smooth	Smooth	Smooth
Size	Small	Small	Medium	Medium	Medium	Medium	Punctiform	Small	Medium
pH	5-9	5-9	5-9	4-9	4-9	5-9	5-9	5-7	4-9
Hypersensitivity	-	-	-	-	-	-	-	-	-
Hemolysis	-	-	-	-	-	-	-	-	-

Note: Nine bacterial isolates were obtained from three sampling sites, consisting of five isolates from the swamp (Rw1-Rw5), two from the river (Sn1-Sn2), and two from a cattle manure (Pt1-Pt2)

Table 2. Indirect indications of methanol production and sMMO test results in bacterial isolates

Ecosystems	Isolates	Methanol production (mM)	pMMO concentration (µM)	sMMO activity
Swamp	Rw1	1.74	1.28	-
	Rw2	1.84	1.35	-
	Rw3	1.48	1.09	-
	Rw4	1.78	1.30	-
	Rw5	2.14	1.57	-
River	Sn1	1.84	1.35	-
	Sn2	2.05	1.50	-
Cattle Manure	Pt1	1.45	1.06	-
	Pt2	1.89	1.54	-

Methane oxidation activity

The results of methane oxidation activity tests showed that all bacterial isolates oxidized methane, as measured by gas chromatography after 12 days of incubation. The data were normally distributed, as indicated by the Shapiro-Wilk test ($p>0.05$). Analysis of variance (ANOVA) showed a significant effect of bacterial isolates on residual methane concentration ($p<0.001$). Tukey's HSD post hoc test indicated that all bacterial treatments resulted in significantly lower methane concentrations compared to the control ($p<0.05$), although no significant differences were observed among some isolates. Methane oxidation is the initial step in the utilization of methane as a carbon and energy source for bacterial growth. The higher an isolate's methane oxidation activity, the lower the remaining methane concentration in the culture chamber. The lowest residual methane was observed with bacterial isolates Rw3, Rw4, and Sn1, which reduced gas levels by 66.61, 62.39, and 62.55%, respectively, compared to the control treatment. The results of methane oxidation analysis by each bacterial isolate are shown in Figure 1.

All bacterial isolates showed potential for CH_4 oxidation during the 12-day incubation period compared to the control treatment, which had a methane concentration of 10.92 mg CH L^{-1}. These results indicate that all isolates tested have potential as methane-oxidizing bacteria, albeit with varying degrees of efficiency on a laboratory scale (Figure 1). However, the molecular and biochemical basis for CH_4 utilization in each bacterial isolate could not be determined, but several bacterial genera allow CH_4 utilization through co-metabolism mechanisms. These findings indicate that CH_4 is likely converted into intermediate compounds, although CO_2 measurements as the final product of methane oxidation were not performed. Isolate Rw3 had the highest methane oxidation potential, producing the lowest residual CH_4 concentration of 3.65 mg CH_4 L^{-1}, followed by Sn1 at 4.09 mg CH_4 L^{-1} and Rw4 at 4.11 mg CH_4 L^{-1}. These values indicate that the three isolates have the potential to oxidize more than 60% of methane compared to the control. Other isolates showed moderate methane oxidation potential. Isolate Sn2 produced a residual CH_4 concentration of 4.47 mg CH_4 L^{-1}, followed by Rw1 (5.00 mg CH_4 L^{-1}), Pt1 (5.09 mg CH_4 L^{-1}), Pt2 (5.38 mg CH_4 L^{-1}), and Rw5 (5.54 mg CH_4 L^{-1}). Although the residual CH_4 concentration in this group was higher than that of isolates Rw3, Sn1, and Rw4, all isolates still showed a significant decrease in CH_4 concentration compared to the control, confirming the potential for methane oxidation activity during incubation. Meanwhile, isolate Rw2 showed the highest residual CH_4 concentration among the test isolates, namely 6.53 mg CH_4 L^{-1}, indicating the lowest methane oxidation potential compared to other isolates, but still lower than the control. Overall, the differences in residual CH_4 concentrations produced by each isolate reflect variations in methane oxidation ability during the incubation period.

Molecular identification of potential bacteria

The best isolates, Rw3, Rw4, and Sn1, based on methane oxidation activity results, underwent further analysis of 16S rRNA using universal primers 27F and 1492R. DNA sequence identification results showed similarities with available bacterial sequences in the NCBI GenBank database, as determined by BLAST (Table 3). Isolate Rw3 showed 97.41% similarity to *Achromobacter* sp. BL5, *Achromobacter xylosoxidans* JCM 9656, and *Achromobacter aegrifaciens* B1. This value indicates that isolate Rw3 may be a new species within the *Achromobacter* genus, as its similarity value is <98%. The 16S rRNA gene of isolate Rw4 shows 99.88% similarity to *Exiguobacterium* sp.

IAE53 and *Exiguobacterium indicum* X10, while *Exiguobacterium enclense* FJ31 has a similarity of 99.76%. Meanwhile, isolate Sn1 shows 98.85% similarity to *Burkholderia* sp. ZYBZ-2 and *Burkholderia cepacia* MSMB10, while *Burkholderia diffusa* B33 shows 98.69% similarity. In general, a 16S rRNA gene similarity value ≥98.7% indicates species similarity, while a value <98.7% with a 95% threshold indicates similarity at the genus level (Rossi-Tamisier et al. 2015).

The bacterial isolates Rw3, Rw4 and Sn1 are potential candidates for reducing methane (CH4) emissions from swamp and river ecosystems. The three bacteria are not included in the 13 genera of methanotrophic bacteria previously identified, namely *Methylomonas*, *Methylobacter*, *Methylosphaera*, *Methylomicrobium*, *Methylosarcina*, *Methylosoma*, *Methylocaldum*, *Methylococcus*, *Methylocystis*, *Methylohalobius*, *Methylocella*, *Methylocapsa*, and *Methylosinus*. However, these three bacteria were able to utilize methane as a carbon source after being incubated for 12 days in vials containing NMS liquid medium and methane.

Although the molecular and biochemical basis for CH_4 utilization in the genera *Achromobacter*, *Burkholderia*, and *Exiguobacterium* has not been established, indirect methane utilization by heterotrophic bacteria through co-metabolism is possible. Co-metabolism occurs through oxygenase enzymes produced for the metabolism of other substrates that can play a role in the oxidation of methane as a non-growth substrate. *Achromobacter* sp. has been reported to grow and oxidize methane by 75% after 10 days of incubation (Sanei et al. 2021). *Achromobacter* also has the potential to accelerate the conversion of methanol to simpler compounds, thereby enhancing the overall efficiency of methane oxidation in environments with methanotrophic and methylotrophic communities (Yousaf et al. 2024). The genus *Burkholderia*, which consists of five different species (*Burkholderia* sp., *B. cenocepacia*, *B. vietnamiensis*, *B. gladioli*, and B. *cepacia*), is capable of oxidizing methane (Rani et al. 2021) and can act as rhizobacteria that promote plant growth (Wallner et al. 2022). Overall, the presence of these heterotrophic bacteria has the potential to enhance methane oxidation at the microbial community level through co-metabolism and metabolic interactions, thereby contributing to the mitigation of CH_4 emissions in natural ecosystems. Table 4 presents the genetic distance matrix based on p-distance analysis of the 16S rRNA gene for isolates and reference bacteria.

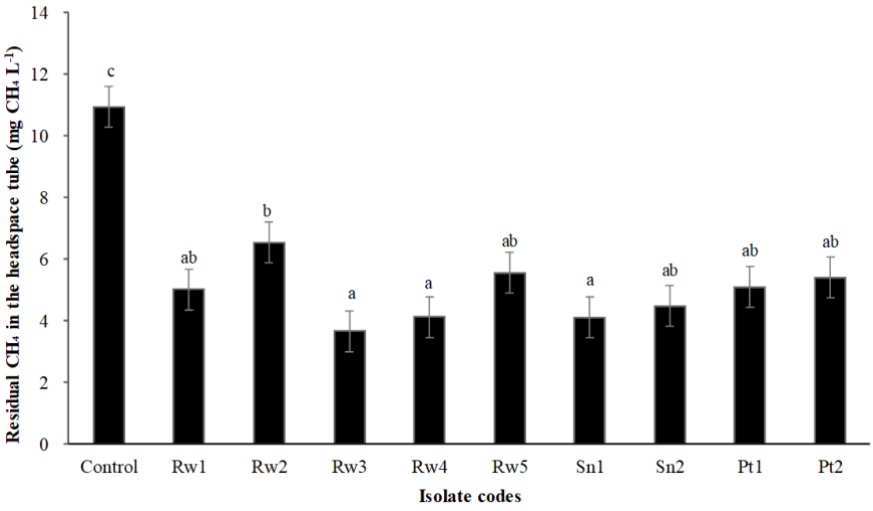

Figure 1. CH_4 consumption by potential methane-oxidizing bacteria. Values are presented as mean ± standard deviation (n = 3). Error bars represent standard deviation. Different letters above the bars indicate statistically significant differences among treatments based on Tukey's test ($p<0.05$); bars sharing the same letter are not significantly different

Table 3. Analysis of 16S rRNA gene similarity

Isolates	Reference bacteria	Access code	Similarity
	Achromobacter sp. BL5	KX179627.1	97.41%
Isolate Rw3	*Achromobacter xylosoxidans* JCM 9656	LC506135.1	97.41%
	Achromobacter aegrifaciens B1	KP241015.1	97.41%
	Exiguobacterium sp. IAE53	MK414999.1	99.88%
Isolate Rw4	*Exiguobacterium indicum* X10	OR342593.1	99.88%
	Exiguobacterium enclense FJ31	OR551428.1	99.76%
	Burkholderia sp. ZYBZ-2	PV888669.1	98.85%
Isolate Sn1	*Burkholderia cepacia* MSMB10	EF114400.1	98.85%
	Burkholderia diffusa B33	PP474289.1	98.69%

Table 4. Matrix of genetic distance values for the 16S rRNA gene sequence in samples with reference bacteria using the p-distance model

	1	2	3	4	5	6	7	8	9	10	11
Exiguobacterium enclense FJ31											
Achromobacter aegrifaciens B1	0.246										
Exiguobacterium indicum X10	0.000	0.246									
Burkholderia diffusa B33	0.164	0.098	0.164								
Burkholderia cepacia MSMB10	0.164	0.098	0.164	0.000							
Achromobacter xylosoxidans JCM 9656	0.246	0.000	0.246	0.098	0.098						
Exiguobacterium sp. IAE53	0.000	0.246	0.000	0.164	0.164	0.246					
Burkholderia sp. ZYBZ-2	0.164	0.098	0.164	0.000	0.000	0.098	0.164				
Achromobacter sp. BL5	0.246	0.000	0.246	0.098	0.098	0.000	0.246	0.098			
Isolate Rw4	0.049	0.279	0.049	0.197	0.197	0.279	0.049	0.197	0.279		
Isolate Sn1	0.164	0.098	0.164	0.000	0.000	0.098	0.164	0.000	0.098	0.197	
Isolate Rw3	0.238	0.008	0.238	0.090	0.090	0.008	0.238	0.090	0.008	0.270	0.090

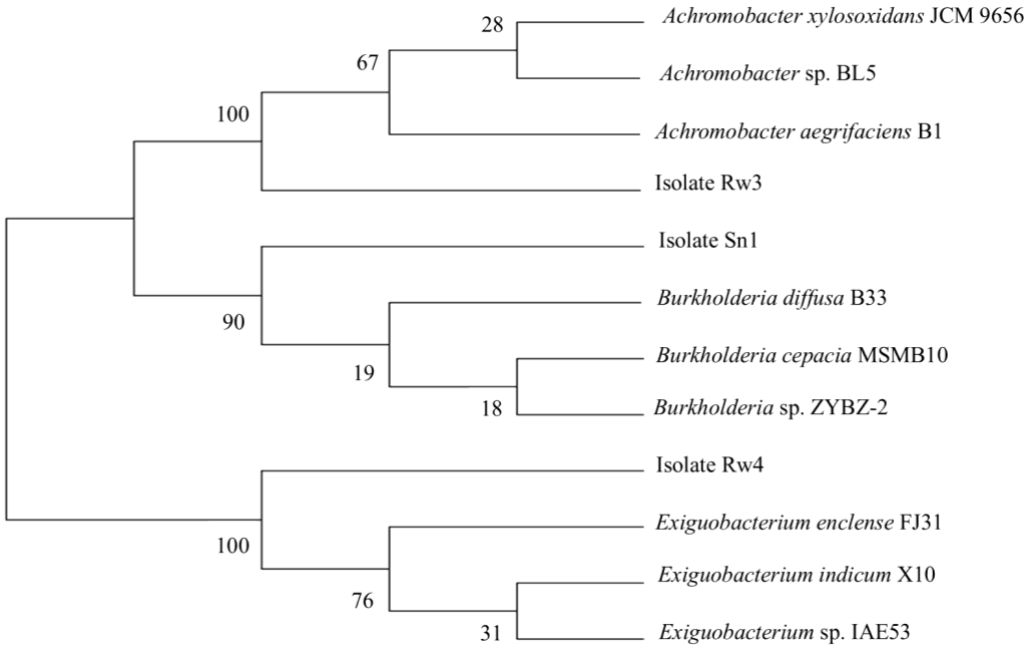

Figure 2. Phylogenetic tree reconstructed using the maximum-likelihood method with Mega 11 (1000x bootstrap)

Genetic distance analysis was performed in MEGA 11 using the p-distance model to determine the genetic relatedness between isolates and reference bacteria based on the 16S rRNA gene. The results showed that isolate Rw3 had the smallest genetic distance to all *Achromobacter* bacteria (0.008) and the largest genetic distance to *Exiguobacterium* bacteria (0.270). The low genetic distance indicates that isolate Rw3 is closely related to *Achromobacter*. The large genetic distance between *Achromobacter* and *Exiguobacterium* is due to differences in taxonomy and phylum, with *Achromobacter* belonging to the phylum Proteobacteria (Melnichuk et al. 2019), while *Exiguobacterium* belongs to the phylum Firmicutes (White III et al. 2019) with low G + C content, which includes a diverse group of facultative anaerobic Gram-positive bacteria, with varying morphologies, ranging from small rods to cocci (López et al. 2021).

Genetic distance analysis was performed in MEGA 11 using the p-distance model to determine the genetic relatedness between isolates and reference bacteria based on the 16S rRNA gene. Isolate Rw4 had the smallest genetic distance (0.049) to *Exiguobacterium* and the largest (0.279) to *Achromobacter*. This indicates that isolate Rw4 is more closely related to the genus *Exiguobacterium* than to other genera. Meanwhile, isolate Sn1 showed the smallest genetic distance (0.000) to *Burkholderia* and the largest (0.197) to isolate Rw4. A distance value of 0.000 indicates sequence identity with *Burkholderia*, signifying a very close phylogenetic relationship. Conversely, the large distance between Sn1 and Rw4 indicates significant genetic differences, as the two isolates belong to phylogenetically distinct groups of bacteria: *Burkholderia* (Proteobacteria) and *Exiguobacterium* (Firmicutes). A small genetic distance value indicates a close evolutionary relationship, while a

larger value indicates greater genetic differences (Nei and Kumar 2000; Battistuzzi et al. 2020). Figure 2 shows a reconstructed phylogenetic tree illustrating the relationship between the isolates obtained and the reference bacteria, based on maximum likelihood analysis.

The phylogenetic tree was reconstructed using several methods (Unweighted Pair Group Method with Arithmetic Mean (UPGMA), neighbor-joining, minimum evolution, maximum parsimony, and maximum likelihood) to test the consistency of the topology. The tree shown was constructed using the maximum-likelihood method in MEGA 11 and was tested with 1000 bootstrap replicates to assess branch robustness. Low bootstrap values on some internal nodes indicate uncertainty in the more detailed phylogenetic placement within the genus. However, the placement of isolates at the genus level is still supported by 16S rRNA sequence similarity. Isolate Rw3 formed a clade with *Achromobacter* species (e.g., *A. xylosoxidans* JCM 9656, *Achromobacter* sp. BL5, and *A. aegrifaciens* B1), with moderate bootstrap support at the internal node (values at the internal node of the *Achromobacter* clade: 28-67). The position of Rw3 clustered with *Achromobacter* is consistent with the results of similarity and p-distance analyses, which show a small genetic distance to *Achromobacter* (Rw3 is closest to *A. xylosoxidans*). This indicates the phylogenetic affiliation of Rw3 within the *Achromobacter* clade.

Isolate Sn1 is located in the *Burkholderia* clade, which is distinct from the *Achromobacter* clade, closely associated with *B. diffusa* B33, *B. cepacia* MSMB10, and *Burkholderia* sp. ZYBZ-2; sufficiently strong bootstrap values support the *Burkholderia* subclade at the main node (≈90), although some internal nodes show lower support (values of 19 and 18 at internal branches). The position of Sn1 at the center of the *Burkholderia* clade supports its molecular identification as a *Burkholderia* member and is consistent with the similarity/genetic distance values (Sn1 is identical or very similar to *Burkholderia* in the 16S rRNA matrix). Isolate Rw4 forms a strong, separate clade with *Exiguobacterium* (node at the main split of the *Exiguobacterium* clade, supported by bootstrap = 100; internal values 76 and 31 at its branches). The relationship between Rw4 and *Exiguobacterium* is consistent with a similarity value ≥99% and a very small genetic distance (p-distance ≈0.000-0.049), so Rw4 most likely belongs to the *Exiguobacterium* clade.

Based on molecular identification and phylogenetic analysis, isolates Rw3, Rw4, and Sn1 were identified as *Achromobacter*, *Exiguobacterium*, and *Burkholderia* bacteria, respectively. Although isolation was performed using NMS medium with methane as the carbon source, which is theoretically selective for obligate methanotrophs, the results of 16S rRNA gene identification showed that the isolates obtained belonged to the heterotrophic genus. This finding indicates that heterotrophic bacteria from sediment samples can grow by indirectly utilizing methane through a co-metabolism mechanism during the enrichment phase.

The results of genetic distance analysis and phylogenetic grouping consistently showed differences in evolutionary relatedness between isolates, reflecting the diversity of bacterial communities potentially involved in the utilization of methane as an energy source. The three isolates have been stored in the Indonesian center for Biodiversity and Biotechnology (ICBB) microbe collection as *Achromobacter* sp. ICBB 10124, *Exiguobacterium* sp. ICBB 10125, and *Burkholderia* sp. ICBB 10127. Although these three bacteria do not belong to the methanotrophic bacterial genus, the potential of heterotrophic bacteria also plays an important role in accelerating the utilization of carbon compounds in the environment. The observed reduction in methane represents functional evidence on a laboratory scale, but cannot yet explain the underlying molecular mechanisms.

The main limitation of this study is the undetectable canonical methanotrophic genes such as pmoA and mmoX, because bacterial identification was based solely on 16S rRNA gene analysis. Therefore, the observed CH_4 loss mechanism cannot yet be conclusively proven, whether through direct biological oxidation, methane carbon assimilation through co-metabolism, or the influence of abiotic factors in a closed system. Thus, the isolates obtained in this study are potential bacteria or bacteria that may contribute to methane transformation, not obligate methanotrophs. Further research is needed for further analysis in tracing carbon flow mechanisms through mass balance analysis ($CH_4 \rightarrow CO_2$ and intermediate compounds) to confirm the CH_4 transformation pathway, analysis of stable isotope markers (e.g., $^{13}CH_4$) to confirm the uptake of methane carbon into microbial biomass, genomic analysis, and functional genes related to methane oxidation, as well as trials on a microcosm or field scale and co-culture experiments with classical methanotrophs. This is important to understand how heterotrophic bacteria contribute to the methane metabolism network, both directly and indirectly, as well as their role in microbial community dynamics and their potential application in methane emission mitigation strategies in natural environments.

In conclusion, three heterotrophic bacterial isolates, namely Rw3 (*Achromobacter* sp.), Rw4 (*Exiguobacterium* sp.), and Sn1 (*Burkholderia* sp.), were identified as the most effective methane-oxidizing isolates in this study. Isolates Rw3, Rw4, and Sn1 have the potential to oxidize methane by 66.61, 62.39, and 62.55%, respectively, compared to the control treatment, making them promising laboratory candidates for methane oxidation. Further studies are needed to uncover the basic mechanisms of methane oxidation they perform and to evaluate their effectiveness under field-scale and long-term application conditions.

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