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Original Article

Screening and characterization of novel methane oxidizing bacterial strains from oil contaminated soils in Khuzestan, Iran

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Abstract

Background & Objectives: Based on information about the types and numbers of microorganisms in the surface soil samples, the distribution range of underlying oil and gas reservoirs can be judged. In the present study, methane oxidizing bacteria were isolated and characterized from oil contaminated soils in Khuzestan, Iran and their growth was optimized in the presence of methane as sole carbon and energy sources.

Materials & Methods: Bacteria were isolated in nitrate mineral salt (NMS) medium in the presence of 50% air and 50% methane. The cultures were incubated on a shaker for 10 days at 30 °C in dark place, and every two days, the gas mixture was replaced. The isolated bacteria were characterized based on biochemical and molecular identification tests. Then the optimum growth conditions was detected in different pH values and incubation temperatures in NMS medium containing methane.

Results: Three Gram-negative rods were isolated from soil samples that were able to grow in isolation condition. The isolates were characterized as *Achromobacter* and *Sphingomonas* spp. The strains could also grow in the NMS medium with a high methanol concentration (3%). The optimal pH and temperature for the isolates were 7.4 and 30 °C respectively.

Conclusion: Methanotrophic strains that were isolated in the present study were able to grow and oxidize methane in high ranges of temperature and pH and can be proposed for the removal of mono-carbon compounds such as methane and as biological detectors for prospecting for oil and gas reservoirs.

Keywords: Methane oxidizing bacteria, Oil contaminated soils, *Achromobacter*, *Sphingomonas*.
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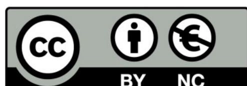

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Original Article

غربالگری و شناسایی سویه های جدید باکتریایی اکسید کننده متان از خاک های آلوده به نفت خوزستان

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چکیده

سابقه و هدف: با استفاده از اطلاعات به دست آمده از نوع و پراکندگی میکروارگانیسم ها در نمونه های خاک های سطحی می توان به میزان توزیع مخازن نفت و گاز پی برد. هدف از این پژوهش، جداسازی و شناسایی باکتری های اکسید کننده متان از خاک های آلوده به نفت در خوزستان و بهینه سازی دما و اسیدیته در حضور متان بود.

مواد و روش ها: باکتری ها در محیط نمک های معدنی و نیترات (NMS) در حضور ۵۰ درصد هوا و ۵۰ درصد متان جداسازی شدند. محیط های کشت به مدت ۱۰ روز بر روی شیکر در دمای ۳۰ $^{\circ}C$ در تاریکی گرمخانه گذاری شدند و هر ۲ روز یک بار مخلوط گازها تجدید کشت شد. جدایه ها با استفاده از ویژگی های بیوشیمیایی و مولکولی شناسایی شدند. همچنین بهینه سازی رشد باکتری ها در دما و اسیدیته های گوناگون در محیط کشت NMS در حضور متان به عنوان منبع کربن انجام شد.

یافته ها: در شرایط مورد بررسی، سه باسیل گرم منفی از نمونه های خاک جداسازی گردید. جدایه ها متعلق به گونه هایی از *اکروموباکتر* و *اسفنگوموناس* بودند. تمامی سویه ها قادر به رشد در محیط NMS دارای غلظت بالای متانول (۳٪) بودند. بهترین شرایط pH و دمای بهینه رشد باکتری های جداسازی شده به ترتیب ۷/۴ و ۳۰ $^{\circ}C$ به دست آمد.

نتیجه گیری: سویه های متانوتروف جداسازی شده در بررسی حاضر قادر به رشد و اکسیداسیون متان در محدوده وسیع اسیدیته و دما بودند و می توانند برای حذف ترکیبات تک کربنه مانند متان و به عنوان شناساگر بیولوژیک مخازن نفت و گاز پیشنهاد شوند.

واژگان کلیدی: باکتری های اکسید کننده متان، خاک های آلوده به نفت، *کروموباکتر*، *اسفنگوموناس*.

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Introduction

Renewable energy fuels that are produced by microbial biomasses are among the best sources of bioenergy (1). Petroleum reservoirs are sub-surfaces of short-chain and long-chain (C1-C30), linear, cyclic, or branched alkanes. The diverse activities and abundance of microbial populations in soil needs to be identified to predict the presence of oil degrading microorganisms in the area where supposed to be the oil and gas reservoirs (1, 2). These microorganisms can use short-chain alkanes (C1-C4) as their only carbon and energy source (3). Methanotrophs are a group of methylotrophs which metabolize methane (CH_4) as the only source of energy and carbon. On the other hand, these bacteria are able to use formaldehyde as an important cellular carbon source in anaerobic condition (4-6). With respect to different characteristics, such as Guanine and Cytosine DNA content, phylogeny, arrangement of intracellular membranes, pathways of carbon assimilation, and phospholipid fatty acid (PLFA) composition, methanotrophs can be categorized into two major types: Gammaproteobacteria (type I) and Alphaproteobacteria (type II). There are different genera for type I methanotrophs: *Methylomicrobium, Methylococcus, Methylobacter, Methylomonas, Methylosoma, Methylocaldum, Methylosarcina, Methylothermus, Methylosphaera, Clonothrix,* and *Crenothrix*. On the other hand, *Methylocapsa, Methylocella, Methylosinus,* and *Methylocysti*s are the genera of type II methanotrophs (7, 8). In the methane oxidation pathway, methane monooxygenase has been recognized as the primary enzyme. Particulate methane monooxygenase (pMMO), which is a membrane-associated enzyme produced by all methanotrophs. In the event of copper limitation, a soluble methane monooxygenase (sMMO) is produced by some methanotrophic strains (9). Methane monooxygenase (MMO) facilitates methane-to-methanol oxidation as the first step of the process. Methanol is converted into either acetyl coenzyme A to produce energy or converted into formaldehyde that assimilated by the cell (10, 11). A surface exploration technology is Microbial Prospecting for Oil and Gas (MPOG), which depends on the detection of diversities of microbial communications in soil. For hydrocarbons, geomicrobial prospecting is an explorative method, which involves the seepage of light gaseous hydrodrocarbons from oil or gas reservoirs to the surface and use of these compounds by hydrocarbon-oxidizing bacteria (HCO) (12). This research was carried out to enrichment and identification of methanotrophic bacteria from the oil contaminated soils in Iran and optimization of their growth pH and temperature in the presence of methane as the sole carbon source.

Material and Methods

*Samples collection***:** Oil contaminated samples were included 60 soil samples taken from Masjed Soleyman, 55 soil samples from Ahvaz, and 34 soil samples from Omidieh which were oil-rich areas in Khuzestan, South of Iran. Samples were taken from 0-100 cm depths of the soils and were kept at 4 °C in sterilized tubes before experimental stages (13). *Culture medium:* Nitrate mineral salt (NMS) medium (ATCC® MD-1306), solidified by 1.5% (w/v) agar, was used for isolation and purification of bacteria (13, 14). *Isolation and enrichment of methane-oxidizing bacteria:* Isolation of methanotrophic bacteria was conducted using the enrichment technique in NMS medium. For this purpose, 1 g of each soil sample was dissolved in distilled water

(50 ml). After incubation for 2 hours on a shaker at 30 °C and 1 hour resting time, 10% of the upper soil suspension volume was added to 30 ml NMS medium. To prevent fungal contamination, 0.1 mg/ml of cyclohexamide was added and the medium atmosphere was filled with 50% methane (99.99% purity) and 50% air in a gas-tight jar as shown in Fig. 1 to stimulate the growth of methane oxidization by bacteria (9, 15). The NMS medium containing soil suspension without a carbon source, as well as the NMS medium filled with the composition of air and methane without soil suspension were considered as negative controls (13, 14). The jar containing the culture media was incubated on a shaker at 30 °C for 10 days in dark place, and every two days, the gas mixture was replaced. The bacterial isolates were then purified on NMS agar plates and incubated in the gas-tight jar with gas composition described above for two weeks at 30 °C and methane-utilizing colonies were taken (16, 17). The microscopic morphology of the bacterial isolates was detected following Gram staining (Hucker method), and capsule staining (Nigrosine method). Then standard biochemical tests were done for initial identification of the bacterial Genera (18). Also methanol utilizing ability test was performed by inoculation of the bacteria into NMS medium containing 3% methanol and evaluation of colony formation after 10 days incubation at 30 °C (9, 15). The isolates were finally inoculated in broth media containing 50% (v/v) glycerol for long-term storage at the temperature of -70 °C (18). *Optimum temperature and pH:* In order to select the suitable temperature and pH according to the growth rate, bacterial suspensions equivalent to 0.5 McFarland at a rate of 1% were inoculated in flasks containing NMS broth medium. Then, the media were incubated at the temperatures of 22 °C, 30 °C, and 40 °C respectively, in the presence of methane as the sole carbon source. Every two hours, the optical densities of the media were read at the wavelength of 600 nm with a visible spectrophotometer (Shanghai Lengguang Technology) and the bacterial growth curves were prepared within 72 hours. The experiments were repeated in the optimal temperature while the pH of media were set at 4.7, 7.5, and 9 respectively, by using NaOH or HCl, and the growth curves were prepared again as described above. *Phylogenetic analysis:* For DNA extraction, an extraction kit (GeneAll, South Korea) was employed. The PCR assay was conducted to amplify 16S rRNA gene in the extracted DNA template (20 ng) by using the following primers: 27f: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492r: 5′- TACGGYTACCTTGTTACGACTT -3′ with the following protocol: incubation for 5 minutes at 94 °C; Taq addition; 30 cycles of incubation for 1 minute at 94 °C, 1 minute at 45 °C, and 2 minute at 72 °C; and a final cycle of 1 minute at 94 °C; 1 minute at 45 °C, and 10 minutes at 72 °C. The products were then detected by electrophoresis on 1% agarose gel.

Fig 1. The apparatus that was used for obtaining 50% methane and 50% air for bacterial growth (9, 15).

The NCBI BLAST tool was used to compare 16S rRNA sequences with GenBank sequences. Then, CLC Main Workbench 6 program (Qiagen) was used to align DNA sequences to the sequences of closely related organisms. Finally, using MEGA 6 program, phylogenetic trees were plotted (19-21).

Results

Isolation of methane-oxidizing bacteria: Three methane-oxidizing bacteria were obtained from the samples during 3 mounts (March to May 2019) and named Msa (isolated from Masjed Soleyman at soil depth of 34 cm), RA (isolated Ahvaz at soil depths of 20 cm) and RY (isolated Ahvaz at soil depths of 10 cm). Although the growth rate and colony development on NMS agar was partially different among different isolates, the methanotrophic bacteria were slow growing, and it took an average of 12 days to form colonies on NMS media. *Characterization of isolates:* Standard procedures were applied to identify the strains. According to morphological and biochemical analyses, all three stains were Gram-negative rods and belonged to genera *Sphingomonas* and *Achromobacter*. The characteristics of the isolates are shown in Table 1. *Phylogenetic analysis:* Analysis of 16S rRNA gene sequences shows that that the organisms belonged to *Sphingomonas* and *Achromobacter* Genera. The MSa isolate exhibited the highest similarity to *Achromobacter xylosoxidans* (99.8% similarity), RA isolate was related to

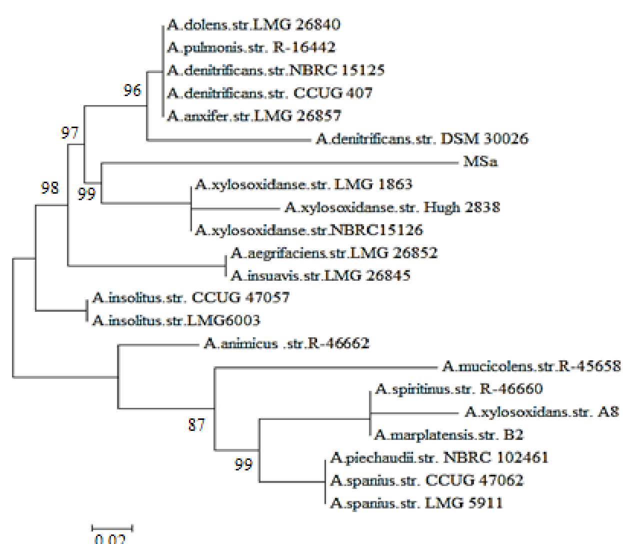

Fig. 2. The phylogenetic tree of the MSa isolate that shows a strain of *Achromobacter xylosoxidans*.

Table 1. Biochemical and morphological characteristics of the strains.

Characteristic	MSa	RA	RY
Colony morphology	Opaque-circular	Opaque-fusiform	Opaque-fusiform
Cellular morphology	Rod	Rod	Rod
Color of colonies	Opalescent Grey	Yellow	Yellow
Gram staining	–	–	–
KOH test	+	+	+
Catalase activity	+	+	+
Oxidase activity	+	–	–
Motility	+	+	–
Urease test	–	–	–
Citrate utilization	+	–	+
Indol production	–	–	–
Methyl red test	–	–	–
Voges–Proskauer test	–	+	+
Starch hydrolysis	+	+	+
Esculin hydrolysis	–	+	+
Tween 80 hydrolysis	+	+	+
Gelatin hydrolysis	+	+	–
Nitrite from nitrate	+	–	+
Zn test on negative NO_2 test	–	+	–
Gas from nitrate	–	–	–
H_2S production	–	–	–
Growth in 3% NaCl	+	+	+
Phenylalanine deaminase	–	–	–
DNase	–	–	–
Growth at 42 ºC	+	+	+
Growth at 4 ºC	–	–	–
Carbon source for growth: Methanol 3%	+	+	+
Ethanol 3%	D	+	+
Utilization of: Glucose	+	+	+
Fructose	D	–	+
Galactose	+	+	+
Xylose	–	+	+
Sucrose	–	–	+
Maltose	–	–	+
α-ketoglutarate	+	–	+
L-serine	+	+	–
L-malate	+	–	+
D-malate	+	–	–
Glycerol	D	+	–
Trehalose	–	–	–
Tartrate	–	–	–
D-lyxose	–	–	–
L-arabinose	D	–	+
Melibiose	D	+	–
Aconitate	+	–	+

+ positive; - negative; D differentiate

Sphingomonas paucimobilis (99.7% similarity), and RY strains was belonged to *Sphingomonas sanguinis* (99.8% similarity). The phylogenetic trees that was prepared using MEGA 6 program, indicates the situation of the bacteria among related strains are shown in Figs. 2-4.

Optimum growth temperature and pH: Analysis of pH and temperature showed that although the strains could grow at 22-40 ºC, the optimal temperature was 30 ºC (Fig. 5). Also, based on the findings, although the strains could grow at pH of 4.7-9, the optimal pH was 7.5 (Fig. 6).

Discussion

Microbial processes highly influence different industries, environmental protection, and many aspects of the economy. In recent years, interest to these processes and their practical application in exploration of hydrocarbon deposits has rapidly increased (3). The study on the distribution of hydrocarbon degrading bacteria may lead to prospection for hydrocarbon deposits as well as identification of hydrocarbon generation and migration (1).

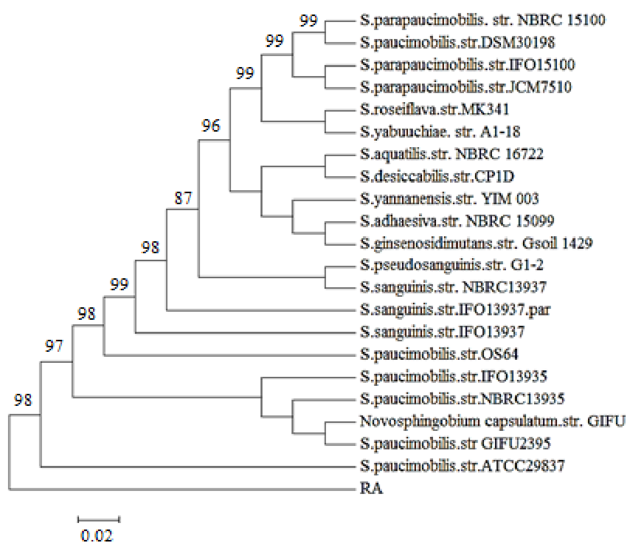

Fig. 3. The phylogenetic tree of the RA isolate that shows a strain of *Sphingomonas paucimobilis.*

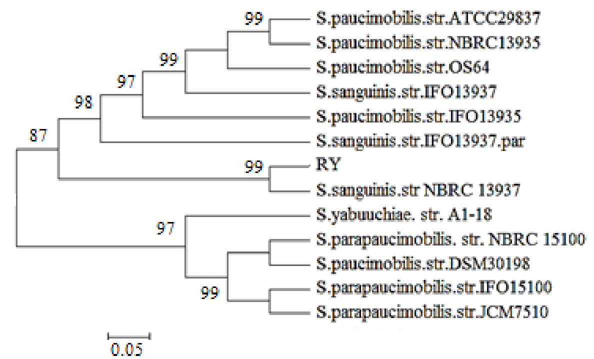

Fig. 4. The phylogenetic tree of the RY isolate that shows a strain of *Sphingomonas sanguinis.*

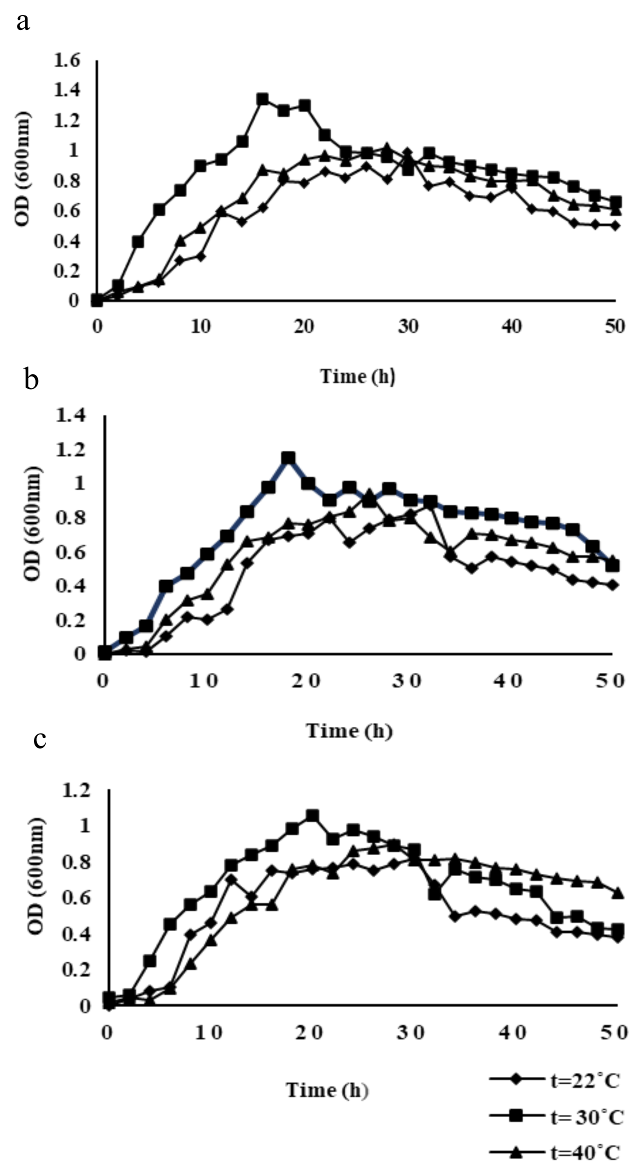

Fig. 5. Growth curves of *Achromobacter xylosoxidans* (a), *Sphingomonas paucimobilis* (b), and *Sphingomonas sanguinis* (c) in different temperatures.

Due to the presence of major oilfield areas in Iran and the need to explore new techniques to help the exploration of oil and gas resources, this study aimed to isolate the Methane oxidizing bacteria from some areas of Khuzestan province, Iran which can be used in future as detector to identify presence of oil and gas resources. Methanotrophic bacteria can use compounds without any carbon-carbon bonds (C1 compounds) as the only source of carbon and energy; therefore, the abundance of these bacteria in soil would be a prediction for the presence of oil and gas reservoirs (4, 5, 22).

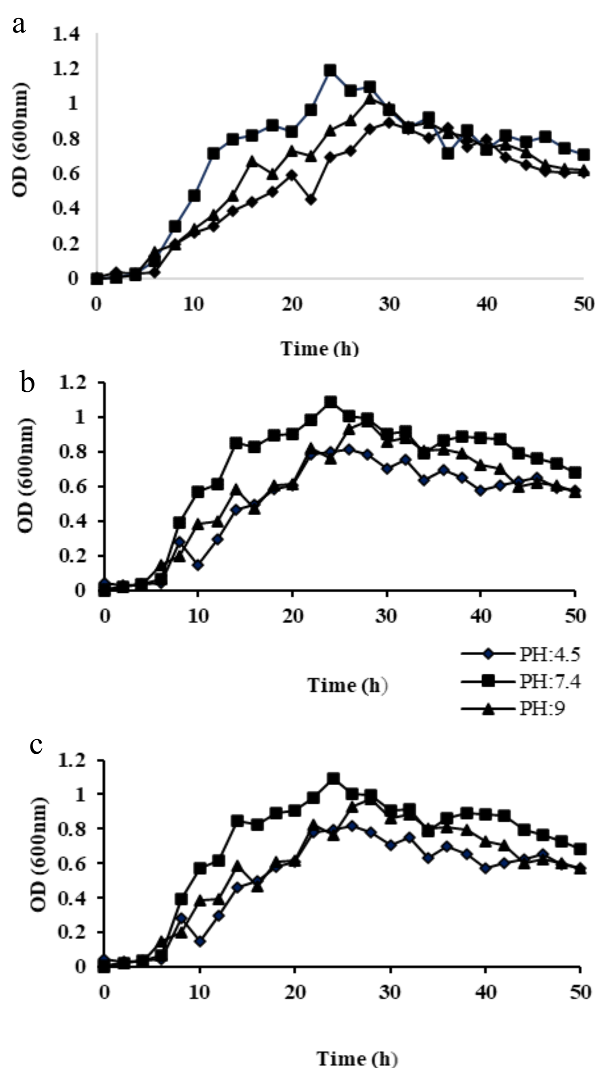

Fig. 6. Growth curves of *Achromobacter xylosoxidans* (a), *Sphingomonas paucimobilis* (b), and *Sphingomonas sanguinis* (c) in different pH values.

Detection of hydrocarbons is done in both soils and sea surfaces. (23). Using bacteria such as methanotrophs for tracing of oil and gas reservoirs has some imitations including frequently contamination of cultures with fungi; difficulties in achievement of pure cultures; no discrimination in phenotypic features; and lack of alignment to the International Code of Nomenclature of Bacteria. A high yield rate is obtained in the case of hydrocarbon anomalies. To delineate hydrocarbon prospects, isolation of various methane-oxidizing bacteria in subsoil strata is part of the microbial prospecting method. The great amount of these hydrocarbon-oxidizing bacteria in the soil is indicative of the presence of hydrocarbons on the surface; accordingly, they are regarded as indicative microbes (24). On the other hand, methane is one of the major sources of the powerful greenhouse gases. An increased concentration of methane in the atmosphere results in global warming and climate change. Methanothrophic bacterial communities are effective on controlling the methane cycle that would lead to control the climatic changes and warming (25). In the present study, *Achromobacter xylosoxidans* was isolated from Masjed Soleyman and *Sphingomonas* spp. were isolated from Ahvaz petroliferous regions. Heyer et al. (2002) isolated type II methane-oxidizing bacteria (MOB) from different environments, including rice paddies, pristine and polluted freshwaters and sediments, mangrove roots, upland soils, brackish water ecosystems, moors, oil wells, water purification systems and livestock manure. 16S rRNA gene sequences indicated that the isolates were belonged to *Methylocystis* spp., *Methylosinus sporium* and *Methylosinus trichosporium* (26). Cho Suk et al. (2014) isolated *Sphingomonas* spp. from landfills with the largest source of methane as a biofilter for

decomposing methane or odor-producing compounds. They reported MD2 strain in *Sphingomonas* spp. as a novel methane-oxidizing strain (27). Tambekar et al. (2011) also isolated *Achromobactor xylosoxidans* from Lonar Lake for methanol remediation and control of global warming. They used selective enrichment media as in the present study to increase the microbial population capable of using methane gas (28). The isolates obtained in this study were able to grow on medium containing 3% methanol as well as the ability to grow on nutrient agar. Rusmana and Akhdiya (2009) and Kim et al. (2008) isolated methanotrophic strains from agricultural fertilizer and rice fields respectively. The isolates were only able to use methane and methanol as carbon sources and were unable to grow on nutrient agar medium (15, 16). Also, Jhala et al. (2014) isolated five methanotrophic strains including *Bacillus aerius* AAU M 8, *Rhizobium* sp. AAU M 10, *Bacillus subtilis* AAU M 14, *Paenibacillus illinoisensis* AAU M 17 and *Bacillus megaterium* AAU M 29 from wet rice fields. These isolates were well grown in the presence of methane as the sole carbon source on media containing methanol, methyl acetate and trichloroethylene and all isolates were capable of growing on nutrient agar medium. They reported the isolates as facultative methylotrophs (7) which similar to the present isolated bacteria grew on complex carbon sources as well as mono-carbon compounds. It has been shown that physicochemical factors including chemical composition and the concentration of the hydrocarbon, temperature, pH, water activity, oxygen, nutrient availability and salinity in addition to biological factors such as adaptation due to prior exposure, seeding, biosurfactant production, and immobilization of the cells influence the degradation of hydrocarbons by microorganisms (3). The best temperature for growth of all three methanotrophic isolates in the presence of methane was determined in this study at 30 ºC although the strains were also able to grow at temperatures of 40 ˚C as well as 22 ˚C. The warm climate of Khuzestan seems to be influence the high temperature adaptation of these bacteria. Rodrigues et al. (2009) selected three temperatures of 20 °C, 30 °C and 40 °C to evaluate the best growth and methane oxidation temperatures by a methanotrophic strain, *Methylosinus trichosporium* OB3b which is different from the strains that was isolated in the present study. Their results showed that the strain was able to grow and oxidize methane at all three temperatures but required more time at 20 °C to adapt to the conditions and reached its highest growth rate after 30 hrs (29). Similarly, in the present study, a longer time was required for all 3 isolates to reach the highest growth point at 22 °C than 30 °C and 40 °C, which is consistent with the results of Rodrigues et al. (2009). Also Abushammala et al. (2014) reported the best temperature for the growth and oxidation of methane at 30 ˚C and stated that the growth rates decreased at 40 ˚C (6). The best pH of growth of all three methanotrophic isolates in the present study was 5.7 although the present methanotrophic strains were able to grow in all three acidic, alkaline and neutral pH values. Other studies have also reported wide ranges of pH for the growth and methane oxidization by methanotrophs such as *Methylosinus trichosporium* (29) and methanotrophic consortia presented in soils of upland forests and forested peatlands (30). Abazari et al. (2020) isolated and identified a *Methylococcus* sp. from Hoz-e Soltan Salt Lake in Iran. The isolated strain was able to grow in 3.3% salinity

and acidic pH of 3.5, and reduced more than 75% of methane from culture medium within 10 days (31). The advantage of methanotrophs that has been isolated from the environmental samples in Iran in the present study and by Abazari et al. (2020) is the ability of them to grow and remove methane in different environmental conditions such as wide ranges of temperature and pH.

Conclusion

Methanotrophs play an important role in controlling global warming by means of methane removal. The methanotrophic isolates that obtained from different areas in Khuzestan, Iran were able to grow and oxidize methane in high ranges of temperature and pH and are proposed to use for mono-carbon compounds removal and as biological detectors for MPOG.

Etical Consideration

Authors of all ethics including non-plagiarism, dual publishing has complied with data distortions and data making in this article.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

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