حذف متان به‌وسیلهٔ سویه‌های *methylocystis* جداسازی‌شده در محیط کشت طراحی‌شده به همراه ارزیابی توانایی سویه‌ها در شرایط نامطلوب

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چکیده

مقدمه: متانوتروف‌ها، میکروارگانیسم‌هایی هستند که در کاهش ۱۵ درصد از غلظت کل متان محیطی نقش دارند؛ این باکتری‌ها می‌توانند متان را به متانول اکسید کنند. متان، گاز گلخانه‌ای آلایندهٔ جو است که همراه با توسعهٔ جوامع، غلظت آن افزایش یافته و تهدیدی جدی برای محیط و بقای حیات روی کرهٔ خاکی است. نامساعدی محتمل آب و هوای آینده به همراه کاربرد گستردهٔ انواع مختلف آفت‌کش‌ها، استفاده از باکتری‌های متانوتروف مقاوم به این شرایط را ضروری می‌کند. مطالعهٔ حاضر روی جداسازی و بررسی اختصاصی باکتری‌های متانوتروف *Methylocystis* تمرکز یافته است.

مواد و روش‌ها: پس‌از جداسازی سویه‌های باکتریایی از باتلاق‌ها، رودخانه‌ها و شالیزارها، نمونه‌ها با کشت در محیط‌های غنی از گاز متان تغلیظ و با رقیق‌سازی متوالی و پاساژهای متناوب در محیط‌های مایع و جامد، تخلیص شدند؛ ضمن شناسایی مولکولی، محیط‌کشت مناسب سویه‌های *Methylocystis* به نام *Methylocystis* Culture (MOC) طراحی شد. به‌منظور ارزیابی بقا، سویه‌ها در شرایط مختلف با تغییر پارامترهای مهمی مانند دما، pH، شوری و خشکی در MOC کشت داده شدند؛ همچنین سویه‌ها از نظر مقاومت به آفت‌کش‌ها بررسی شدند. کاهش گاز متان در فضای فوقانی محیط‌کشت هر سویه، ضمن تطابق با نمونه‌های کنترل به‌عنوان اکسیداسیون متان آن سویه در نظر گرفته شد.

نتایج: سرعت اکسیداسیون گاز متان برای محیط‌کشت‌های چند سویه به‌طور *in vitro* تعیین شد. منحنی رشد سویه‌ها ترسیم شد. طبق نتایج، متانوتروف‌های *Methylocystis* قدرت بقای زیادی در شرایط مختلف داشتند، به آفت‌کش‌های رایج مقاوم بودند و بیشتر از ۷۵ درصد گاز متان را طی دورهٔ چهارده‌روزه مصرف کردند.

بحث و نتیجه‌گیری: باکتری‌های متانوتروف *Methylocystis* می‌توانند در کاهش انتشار متان به جو و تغییرات جوی آینده اهمیت داشته باشند.

واژه‌های کلیدی: توانایی، سازگاری، اکسیداسیون متان، متانوتروف، بهینه‌سازی، آفت‌کش، شالیزار، رودخانه، باتلاق

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Methane Removal of Isolated *Methylocystis* Strains in the Culture Medium Designed by Evaluating Strain Capacity under Adverse Conditions

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Abstract

Introduction: Methanotrophs are microorganisms that play an important role in reducing up to 15% of environmental CH_4 concentration. These bacteria can oxidize methane to methanol. Methane is an atmosphere-polluting greenhouse gas with increasing atmospheric concentrations and is considered a serious threat to the environment and the sustenance of life on the planet. Possible unfavorable future weather and the extensive use of different types of pesticides necessitate the use of methanotrophic bacteria that are resistant to these conditions. The present study focused on the isolation and specific study of *Methylocystis* methanotrophic bacteria.

Materials and Methods: The bacterial strains were isolated from different environments such as wetlands, rivers, and paddy fields. The isolated strains were concentrated with Methane gas enriched cultures and purified by serial dilution-extinction, alternating liquid, and solid subcultures. Then, molecular identification was performed and a suitable culture medium was designed for *Methylocystis* strains in the name of *Methylocystis* Culture (MOC). Strains were cultured in MOC to evaluate their viability in different conditions by changing important parameters such as the temperature, pH, salinity, and drought. In addition, they were affected by pesticides. The methane gas reduction in the upper space of the culture medium of each strain, in accordance with the control samples, is considered as the methane oxidation of that strain.

Results: Methane gas oxidation rate was determined *in vitro* for the cultures of several strains. The growth curve of the strains was plotted. According to the results, *Methylocystis* methanotrophs had high survivability in different conditions and were resistant to common pesticides, and consumed more than 75% of methane gas over a period of 14 days.

Discussion and Conclusion: *Methylocystis* methanotrophic bacteria can greatly reduce the release of methane into the atmosphere and alter the fate of the atmosphere.

Key words: Ability, Compatibility, Methane Oxidation, Methanotroph, Optimization, Pesticide, Rice field, River, Wetland.

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Introduction

Today, increasing methane gas has become a major concern for human societies. As a greenhouse gas pollutant, it could warm the atmosphere 34 and 72 times as that of CO_2 over the past 100 and 20 years, respectively (1). A slight increase in the earth's temperature also causes numerous problems such as climate change, biodiversity loss, and diseases such as malaria, yellow fever, and even other types of unknown diseases.

Methanotrophs are one of the most important eco-friendly bacteria, specifically for consuming 15% of methane as an atmospheric pollutant. Because of their special enzymes, they can oxidize methane to methanol and reduce the rate of available methane gas (2).

Methanotrophs are divided into two main groups, the first of which is Gammaproteobacteria including *Methylococcus*, *Methylomonas*, *Methylosphaera*, *Methylosoma*, *Methylomicrobium*, *Methylothermus*, *Methylohalobius*, *Methylosarcina*, and *Methylobacter* species (3) and uncultured filamentous *Candidatus* Crenothrix polyspora and *Candidatus* Clonothrix Fusca strains (4, 5). The second group of methanotrophs is Alphaproteobacteria including *Methylosinus*, *Methylocapsa*, *Methylocella*, and *Methylocystis* species (6). *Verrucomicrobia* is another recently-identified aerobic methanotroph of the genus *Methylacidiphilum* (7, 8). These microorganisms are gram-negative bacteria consisting of anaerobic and aerobic species, which play a central role in methane consumption since they possess a broad spectrum of methane monooxygenase enzymes in soluble and particulate forms (9).

Given the predominance of *Methylocystis* strains in nature, these bacteria are expected to be more compatible with nature among the methanotrophs (10). Whittenbury et al. (11) have reported on these bacteria. The characteristics of these bacteria were determined by Bowman et al. (12). *Methylocystis* has a greater diversity than what is known. These microorganisms are type II methanotrophic bacteria classified in the phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, and the family Methylocystaceae in Dedysh's classification (13, 14).

The main sources of methane are stagnant waters, bogs, factory wastewater, etc., on which researchers have focused to reduce methane by methanotrophs, including *Methylocystis*. In addition, the consumption of agricultural pesticides is increasingly on the rise, and their final terminations are stagnant waters such as wetlands.

Methylocystis bacteria investigated in the present study were isolated from different places, particularly contaminated and drying wetlands, and evaluated in terms of the applicability of strains under different conditions, to reduce methane contamination.

Materials and Methods

Sampling and Culture Conditions: The specimens were isolated and encoded from different geographical regions of Iran in late October and early November 2017, including samples from the Qurigol wetland (37° 91' 90" N, 46º 71' 22" E) with an altitude of 1905 m coded ABZ1, west of the Anzali wetland (37º 47' 87" N, 49º 37' 31" E) with an altitude of -29 m coded ABZ2, Astara paddy area (38º 37' 19" N, 48º 85' 74" E) with an altitude of -24 m coded ABZ3, Astarachai at the border between Iran and the Republic of Azerbaijan (38° 38' 91" N, 48° 63' 44" E) with an altitude of 560 m coded ABZ4, and river Baliqluchai in Ardabil (which flows into the Ghara Sou river and continues to the Aras River on the Iranian-Azerbaijani border) at 38º 10' 48 "N, 48º 14' 28" E with an altitude of 1489 m above sea level coded ABZ5 (Fig. 1).

Fig. 1- Map showing sampling regions

The growth in quantity and quality of the cultures were evaluated in different NMS, 10, dNMS, AMS, and M2 culture media in liquid culture bottles (11, 15, 16, 17). The growth quality of strains in culture medium 10 was better than the others. Therefore, this culture medium was suitable for the tests. The composition of culture medium-10 was (per litre of distilled water) as follows:

NH_4Cl (0.5 g); $MgSO_4.7H_2O$ (0.1 g); Na_2HPO_4 (0.53 g); KH_2PO_4 (0.3 g); $CaCl_2.6H_2O$ (0.01 g); $FeSO_4.7H_2O$ (5 mg) and 1 ml of trace elements solution containing $ZnSO_4.7H_2O$ (0.44 mg); $CuSO_4.5H_2O$ (0.20 mg); $MnSO_4.2H_2O$ (0.17 mg); $Na_2MoO_4.2H_2O$ (0.06 mg); H_3BO_3 (0.10 mg) and $CoCl_2.6H_2O$ (0.08 mg). The medium pH was adjusted to 7.0.

Liquid media were prepared in one-fifth of sterile penicillin bottles and capped using a butyl rubber stopper. Methane 99.99% (20 ml) replaced an equivalent amount of air inside the bottle with a syringe. Finally, the bottle cap was completely sealed with parafilm to prevent gas leakage. In the preparation of solid media, 1.5% agarose (Difco) was added to the culture medium-10, and after the culturing, the samples were placed in a gas-tight jar, of which one-third of the volume comprised methane gas. It should be noted that the initial sample volume was 2 g of wet soils from the sampling sites. Liquid media were incubated in a slow shaker with 60 rpm at 28 °C. Microbial growth in culture media was controlled on alternate days from the third day onwards based on turbidity.

Enrichment and purification with different methods: Prior to the purification, enrichment was carried out by the incubation of the samples for 6-8 weeks at 25 °C under methane/air mixture in 1:1 ratio. Afterward, 4-6 passages were made in liquid culture media to determine the range of growth period completion to reach the stationary phase. After these steps, purification was performed by serial dilution-extinction and alternating liquid and solid subcultures. For dilution, 8-column wells with 12 rows were used for the possibility of testing multiple samples. The total volume of each well was 300 µL containing 50 µL of the sample and 250 µl of liquid medium-10, which were incubated in equivalent 30% pure methane conditions. After incubation, dilutions from 10^{-5} to 10^{-8} were sub-cultured into solid media. The newly-formed colonies in the solid media

were examined by a stereomicroscope and transferred to new media. Liquid culture media were also employed for periodic bacterial enrichment. Upon the first observation of turbidity, an aliquot was transferred to the new culture medium. Fungal contamination is always present due to the long-term growth of methanotrophs. Therefore, anti-fungal compounds of Cycloheximide, Terbinafine Hydrochloride, Amphotericin B, Fluconazole, and Nystatin (Sigma-Aldrich, USA) were examined for the elimination of yeasts, mycelia, and various soil fungi associated with methanotrophs. Parallel to the culture of the strains, bacteria-free culture media were also prepared and incubated in similar conditions. The purity of the strains was controlled by methane gas and carbon resources-free media, where bacteria should not reasonably grow. Because in this case, if bacteria grow in the culture medium, they cannot be methanotrophs. The purity of methanotrophic cultures was confirmed by microscopic observation.

Molecular Identification: For this purpose, bacterial DNA extractions were performed for 14-day-old (early stationary phase) culture media, using Genomic DNA Extraction Kit from Gram-Negative Bacteria (*Gene Transfer Pioneers*). Polymerase chain reaction (PCR) test was performed with 16S rRNA specific primer pairs called Type.IF and Type.IR for type I methanotrophs, and primer pairs Type.IIF and Type.IIR for type II methanotrophs. These sequences are conserved in most methanotrophic bacteria. The sequence of primers is presented in Table 1 (18). PCR products were subjected to electrophoresis.

Table 1- Primers selected for PCR

Primer Name	Sequence	Number of bases	Primer Type
Type.IF	ATGCTTAACACATGCAAGAACG	22 bases	Forward
Type.IR	CCACTGGTGTTCCTTCMGAT	20 bases	Reverse
Type.IIF	GGGAMGATAATGACGGTACCWGGA	24 bases	Forward
Type.IIR	GTCAARAGCTGGTAAGGTTC	20 bases	Reverse

PCR Amplification: PCR was done with 20 µL mixtures in 200 µL micro-tubes using Eppendorf thermal cycler model 5345. These mixtures consisted of 20 ng of pure DNA templates, 1 unit of Top DNA polymerase (0.2 µL) *(Bioneer Inc., Daejeon, South Korea)*, 10 pmol of each forward and reverse primer, 250 µM of dNTPs, 30 mmol of KCl, 10 mmol of Tris-HCl (pH 9.0), and 1.5 mM of $MgCl_2$, which reached a final volume of 20 µL using deionized water. PCR reactions were carried out after the initial denaturation stage at 95 °C for 5 min. Subsequently, 35 cycles were performed at 95 °C for 20s, 54 °C for 20s, and 72°C for 30s, followed by a 5 min final extension at 72 °C. A negative control was provided to confirm that the observed PCR amplicons were not due to contamination. Final products were electrophoresed on 1.5% agarose gel using EvaGreen fluorescent DNA Stain. The PCR product was sequenced and Accession Numbers were provided for each strain from the GenBank database.

Evaluation of Culture Medium for *Methylocystis* Strains: Culture medium 10 was suitable for *Methylocystis* strains, so to test each compound at a certain concentration, base culture medium 10 was employed in which compounds at different concentrations replaced those at a certain and defined concentration under normal conditions. These compounds were tested at specific concentrations for each *Methylocystis* strain, as seen in Table 1. All chemicals were of analytical grade and purchased from Merk, Germany, and Sigma-Aldrich, USA.

According to the culture method and incubation of liquid media, different specimens were prepared in two replicates with initial inoculation of bacteria as $OD_{620\ nm}$: 0.03 in the culture medium. Therefore, the growth rate of bacteria in different cultures was determined by the turbidimetry method. The mean OD of each strain was calculated at different concentrations of the compounds tested, and the tested concentration supporting the highest OD was used as the appropriate concentration of each compound to prepare the MOC culture medium. In order to confirm the suitability of the MOC culture medium for isolated strains, it was compared with culture medium 10 by preparing each strain in both culture media under the same conditions followed and compared by the measurement of OD growth.

The culture medium was designed based on the change in the concentration of different compounds and investigating the better growth of *Methylocystis* strains.

Determining Bacterial Growth Curves: To do this, the MOC liquid media were prepared and incubated in sufficient numbers in optimal conditions of the described parameters. The initial bacterial inoculation was 0.03 at $OD_{620\ nm}$. The test for the selected strains was performed by a shaker at 60 rpm after a 20-day incubation and $OD_{620\ nm}$ was measured at regular 2-day intervals (19). The OD-measured media were discarded due to changes in the headspace gas composition caused by opening the cap which was not used in subsequent optical density measurements.

Methane Oxidation Test: The MOC media was employed for testing with an initial inoculation equal to those of the previous tests on a shaker at 60 rpm under similar incubation conditions. This test was carried out by Gas Chromatography (GC) (PerkinElmer, USA) with a Flame Ionization Detector (FID) (detector temperature 250 °C) and a glass column (1.8 m length, 2 mm diameter). The gas chromatography device was calibrated via the external standard method (i.e., by injecting samples with specific volumes of methane gas, the calibration diagram was drawn). Gas samples (600 µL) were injected into the GC by a Hamilton syringe. The amount of methane gas in bacteria-containing culture media was analyzed after reaching the stationary phase (Completion of the 14-day period). The amount of consumed gas in the bottles or the extent of methane oxidation was calculated in terms of micromoles methane per litre air per hour (µM/L/h) based on the amounts of initial and end-period gas. Control samples (bacteria-free culture media) were tested in the same conditions.

Determination of pH, Temperature, and Salt-Tolerance Ranges and Their Optima in the Growth Medium: In this test, different parameters were measured separately with the one-factor-at-a-time (OFAT) approach. To assess the resistance of bacteria to different conditions, the MOC culture media containing the relevant strains were exposed to temperatures of 5, 9, 15, 20, 25, 30, 37, 45, and 50 °C. The strains were also sub-cultured in MOC media with pH values of 2, 3, 3.5, 4.5, 5.5, 6, 6.5, 7, 7.2, 7.5, 8, 9, and 10, prepared by 0.1 M potassium phosphate buffer at 25 °C. They were then cultured in the MOC media prepared with NaCl percentages of 0.6%, 1.5%, 3%, 4.5%, and 6% (20). Optimal evaluation parameters were determined as well.

The growth ability in microbial cultures was evaluated based on the resulting turbidity and was compared with growth at normal temperature and pH levels.

Drought and Heat Tolerance Evaluations: For the evaluation of drought tolerance, the media were dried from the stationary phase with a volume of 5 ml in a sterile plate and then re-cultured. In the heat tolerance test, the strains present in the liquid culture

media from the stationary phase were incubated at 80°C for 20 min, and these strains were re-cultured (20).

Examination of Spore Presence: Four-month-old liquid media were stained with malachite green.

It should be noted that all tests were performed in two replicates, and three replicates in suspicious cases to confirm the results.

Pesticide Tolerance Test: *Methylocystis* bacteria were exposed to commonly-used pesticides including the herbicides Glyphosate, Imazethapyr, and Paraquat, the insecticide Haloxyfop-R methyl, and the fungicides Neutralized copper sulphate and Dicopper chloride trihydroxide, Thiophanate-methyl with the corresponding percentage of the active ingredient of the compounds as follows: 41%, 10%, 20%, 10.8%, 18%, 35%, and 70% (purchased from commercial sources). This procedure was performed using the polystyrene wells with 8 columns and 12 rows. Each well was filled separately with different amounts of pesticides (100, 50, 25, 10, 5, 2, 0.6, and 0.15 µL), liquid MOC medium, and various strains of *Methylocystis* with a final volume of 300 µL. The wells were incubated with pure methane equivalent to 30% of the upper space in a gas-tight jar without shaking for 14 days. After this period, the wells were sub-cultured in a solid MOC media with net methane to determine their survivability and growth. Non-pesticide control samples and non-bacterial control specimens were also prepared for this test.

Accession Numbers: The nucleotide sequences obtained from PCR based on 16S rRNA were deposited to the GenBank database. The Accession Numbers were: for strain ABZ1: MK719950, for strain ABZ2: MK719948, for strain ABZ3: MK719944, for strain ABZ4: MK719947, and for strain: ABZ5: MK719946.

Results

Five strains of *Methylocystis* methanotrophic bacteria were isolated in two wetland regions, a river, and a rice field. From a total of 45 samples and with methanotrophic selective methods, five obtained methanotrophic strains were related to *Methylocystis* species. The rest of the strains were of different genes of Gammaproteobacterial methanotrophs and one strain was of *Methylosinus* genus, which demonstrates an obvious dominance of these species among other methanotrophic bacteria. Data uniformity was verified through consecutive tests.

Molecular Identification: The molecular analysis with two types of primer pairs (type I and type II) specific for methanotrophs resulted in the production of PCR products with only type II specific primer pairs (Fig. 2). The band formation area in them was also about 525 bp. Fig. 2 shows the image of the corresponding electrophoresis gel, which represents the presence of the specific methanotrophic 16S rRNA gene in strains with sample codes ABZ1, ABZ2, ABZ3, ABZ4, and ABZ5.

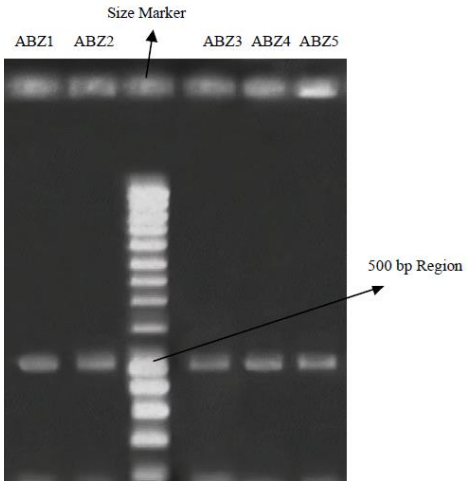

Fig. 2- PCR product electrophoresis gel of 16S rRNA gene amplification with type II primer with the pair of Type.IIF and Type.IIR primers, samples with codes ABZ1, ABZ2, ABZ3, ABZ4, and ABZ5 form the band; 100-base pair DNA Size Marker and negative control are seen in this electrophoresis gel

Sequencing and blasting with NCBI indicated that the research strains were from the genus *Methylocystis*, which did not grow in media lacking methane and any other carbon sources. Fig. 3 shows the neighbour-joining tree based on the 16S rRNA gene sequences.

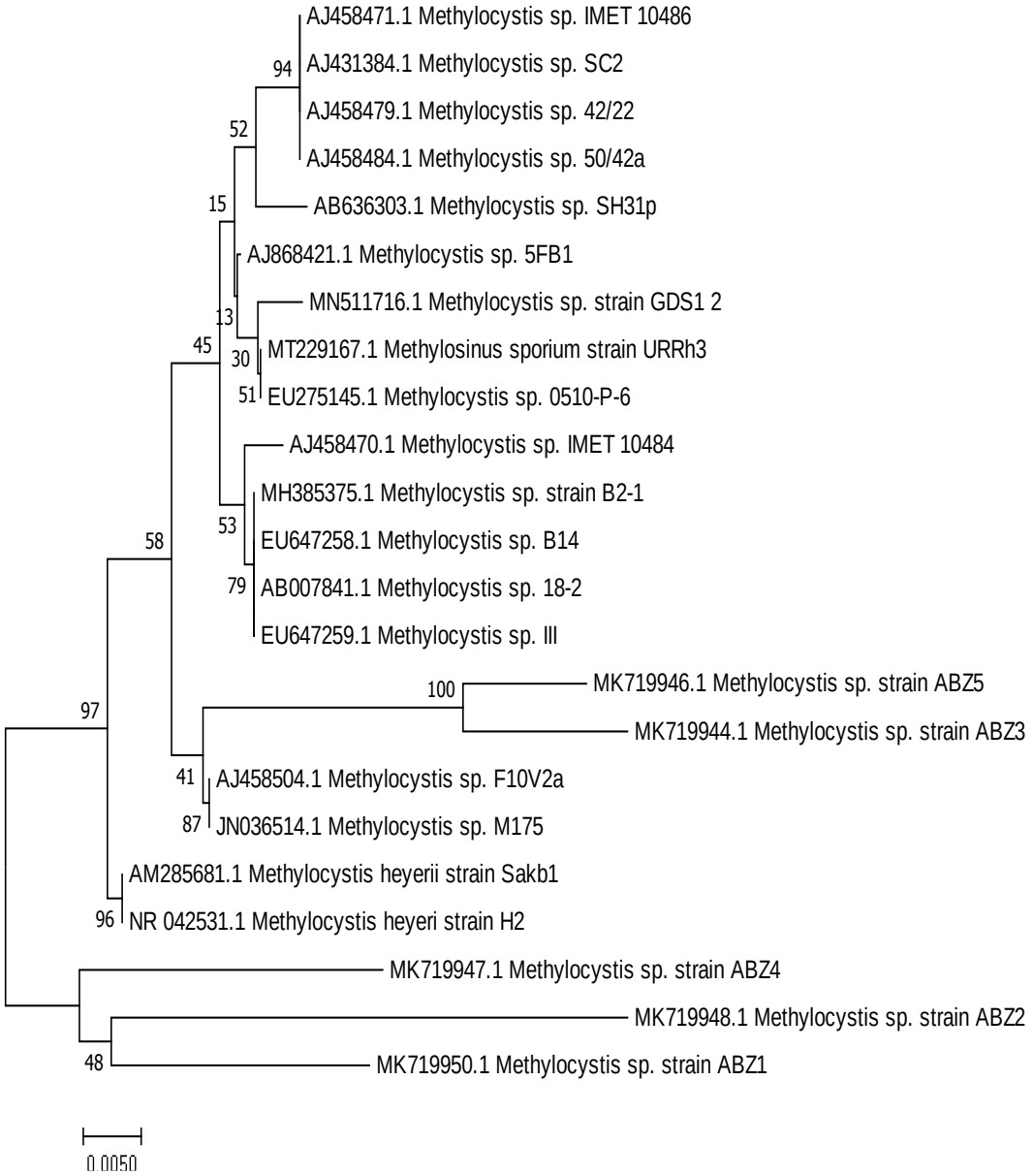

Fig. 3- Rootless phylogenetic tree produced by Mega version 7 based on 16S rRNA sequence of Isolated Strains; Types of ABZ strains are relevant to this study.

Results of Culture Medium Optimization: None of the antifungal compounds used had a growth inhibitory effect on methanotrophs by 50 mg/L, even Cycloheximide did not have a significant growth inhibitory effect on methanotrophs and could be used as a useful compound in the purification of methanotrophic strains. The mean OD results of each strain at different concentrations of the compounds are shown in Table 2.

Table 2- Average $OD_{620\ nm}$ Results of *Methylocystis* Strains with Different Compounds during the 14-day Growth Period

Basic composition	Variable composition	The amount of variable composition	Strain					Average $OD_{620\ nm}$
			ABZ1	ABZ2	ABZ3	ABZ4	ABZ5	
Culture Medium 10	NH_4Cl [a]	0.25 g [b]	0.32	0.404	0.361	0.29	0.465	0.368
Culture Medium 10		0.5 g	0.294	0.401	0.348	0.276	0.422	0.348
Culture Medium 10		1 g	0.281	0.349	0.332	0.28	0.403	0.329
Culture Medium 10	KNO_3 [c]	0.25 g	0.301	0.356	0.345	0.275	0.410	0.337
Culture Medium 10		0.5 g	0.3	0.32	0.32	0.269	0.413	0.324
Culture Medium 10		1 g	0.292	0.305	0.296	0.241	0.394	0.305
Culture Medium 10	$MgSO_4.7H_2O$	0.1 g	0.303	0.401	0.401	0.332	0.440	0.375
Culture Medium 10		0.2 g	0.324	0.39	0.4	0.335	0.44	0.377
Culture Medium 10		0.4 g [b]	0.31	0.422	0.45	0.33	0.455	0.393
Culture Medium 10	Na_2HPO_4/KH_2PO_4	0.36/0.4 g	0.249	0.288	0.303	0.301	0.419	0.312
Culture Medium 10		0.55/0.6 g	0.283	0.38	0.356	0.304	0.428	0.35
Culture Medium 10		0.82/0.9 g [b]	0.344	0.4	0.411	0.322	0.438	0.383
Culture Medium 10	$CaCl_2.6H_2O$	0.1 g	0.322	0.385	0.433	0.275	0.42	0.367
Culture Medium 10		0.2 g	0.325	0.404	0.431	0.3	0.42	0.376
Culture Medium 10		0.4 g [b]	0.32	0.406	0.446	0.325	0.444	0.388
Culture Medium 10	$FeSO_4.7H_2O$	5 mg	0.319	0.434	0.4	0.291	0.42	0.372
Culture Medium 10		20 mg [b]	0.31	0.4	0.432	0.331	0.499	0.394
Culture Medium 10		100 mg	0.275	0.39	0.381	0.257	0.457	0.352
Culture Medium 10	½ X Trace elements solution[d]	-	0.23	0.301	0.424	0.263	0.269	0.297
Culture Medium 10	2X Trace elements solution[d]	-	0.095	0.042	0.079	0.065	0.05	0.066
Culture Medium 10	Optimal culture medium (MOC) [e]	-	0.334	0.42	0.451	0.329	0.479	0.402

- The incubation temperature of all media was 28°C at 60 rpm of the shaker.
- The results shown are the average of each test with two repetitions.

[a] The culture medium composition of each row includes the medium-10 with the application of the specified compound in each row to the specified volumes.

[b] These amounts of the relevant compounds were used in the optimum culture medium.

[c] KNO_3 replaced NH_4Cl in the media of those rows.

[d] In Culture medium-10. Change in the number of trace elements reduced or inhibited the growth of methanotrophs. Therefore, the number of trace elements used in the culture medium-10 was used in culture medium MOC.

[e] The optimum medium was prepared from different compounds that produced good OD with 1 X Trace elements solution used in Culture medium-10, and tested for different strains under similar incubation conditions in the 14-day growth period.

The composition of the MOC culture Medium was determined (per litre of distilled water) as follows:

NH_4Cl (0.25 g); $MgSO_4.7H_2O$ (0.40 g); Na_2HPO_4 (0.82 g); KH_2PO_4 (0.90 g); $CaCl_2.6H_2O$ (0.40 g); $FeSO_4.7H_2O$ (20 mg) and 1 ml of trace elements solution containing $ZnSO_4.7H_2O$ (0.44 mg); $CuSO_4.5H_2O$ (0.20 mg); $MnSO_4.2H_2O$ (0.17 mg); $Na_2MoO_4.2H_2O$ (0.06 mg); H_3BO_3 (0.10 mg) and $CoCl_2.6H_2O$ (0.08 mg).

The medium pH was adjusted to 7.0. As a result of the evaluation, the composition of the trace elements solution in the MOC was prepared according to the culture medium-10 composition (Table 3).

Table 3- Comparison of Designed Culture Medium MOC with Culture Medium 10 (Control) during the 14-day Growth Period of Isolated *Methylocystis* Strains with $OD_{620\ nm}$

Culture medium	Test Strains					Average $OD_{620\ nm}$
	ABZ1	ABZ2	ABZ3	ABZ4	ABZ5	
10	0.304	0.412	0.39	0.285	0.425	0.363
MOC	0.334	0.42	0.451	0.329	0.479	0.402
Percentage difference of OD (turbidity)[a]	9.8%	1.9%	15.6%	15.4%	12.7%	10.7%

[a] Culture medium MOC compared to culture medium 10

The results of the last row of Table 3 show that the mean growth density of isolated strains in culture medium MOC was higher than culture medium 10 and the strains had better growth.

Capability Assessment of the Isolated Strains: The results of Methane oxidation of isolated *Methylocystis* strains grown in MOC culture media are shown in Table 4. In this table, the oxidation rate of methane, the volume of oxidized methane in the laboratory culture medium, and the percentage of residual methane in the laboratory culture medium for each strain can be detected

Table 4- Methane Oxidation of *Methocytosis* Strains

Strain	ABZ1	ABZ2	ABZ3	ABZ4	ABZ5
Methane oxidation rate (µm/L/h)[a]	19.4473	19.262	21.1187	20.0006	20.994
Methane oxidation amount (ml)[b]	15.93	15.78	17.3	16.39	17.2
Methane residual in culture medium (%)[b]	20.3	21.05	13.45	18	13.95

[a] micromoles methane per litre air per hour (µM/L/h) based on the amounts of initial and end-period gas
[b] After a period of 14 days with 60 rpm Shaking. These rows show the remaining methane in the bottles after subtracting the consumed methane from the total methane added to the bottles.

The growth temperature evaluation also represented optimal growth for all five strains of *Methylocystis* at 28 °C. They did not need NaCl for growth. After drying, however, they lost their growth ability in liquid media, and spores were not observed after staining. Interestingly, all the strains survived against different concentrations of herbicides and fungicides after 15 days of incubation and formed colonies subsequently in subcultures. They did not have any growth reduction compared to control tests. Other results are presented in Table 5 and Fig. 4 to compare the differences between strains.

Table 5- Results of Stability Test for *Methylocystis* Strains

Strain	ABZ1	ABZ2	ABZ3	ABZ4	ABZ5	*Methylocystis parvus*[a]	*Methylocystis echinoides*[a]
pH range[b]	3.5-7.5	3.5-7.5	3.5-7.5	3.5-9	3.5-7.5	5.0-9	5.0-9
Optimum pH[b]	6-7.2	6.2-7	6-7	6-7	6-7	6.5-7	6.5-7
Temperature range (ºC)[b]	15-30	20-30	20-30	20-30	9-40	20-37	20-28
Optimum temperature (ºC)[b]	28	28	28	28	28	28-32	25-28
Tolerance of 80(ºC)[c]	-	-	-	-	-	-	-
NaCl tolerance	Up to 1.5%	No growth	Up to 0.6%	Up to 0.6%	Up to 0.6%	Up to 2.5%	Up to 1%
NaCl requirement[b]	-	-	-	-	-	-	-

+, 100% is positive; -, 100% is negative.
[a]Data from Bowman et al.
[b]For growth
[c]Time: 20 min

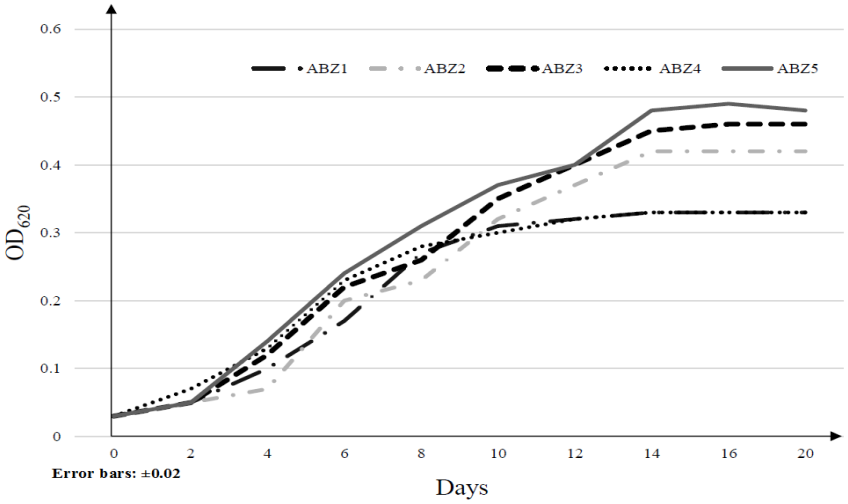

Fig. 4- Bacterial growth curves of methanotrophic strains of ABZ1, ABZ2, ABZ3, ABZ4, and ABZ5 (according to the results of OD_{620nm} at different growth times)

Fig. 5- presents a general conclusion from the data of *Methylocystis* strains in this study as a feature of these bacteria.

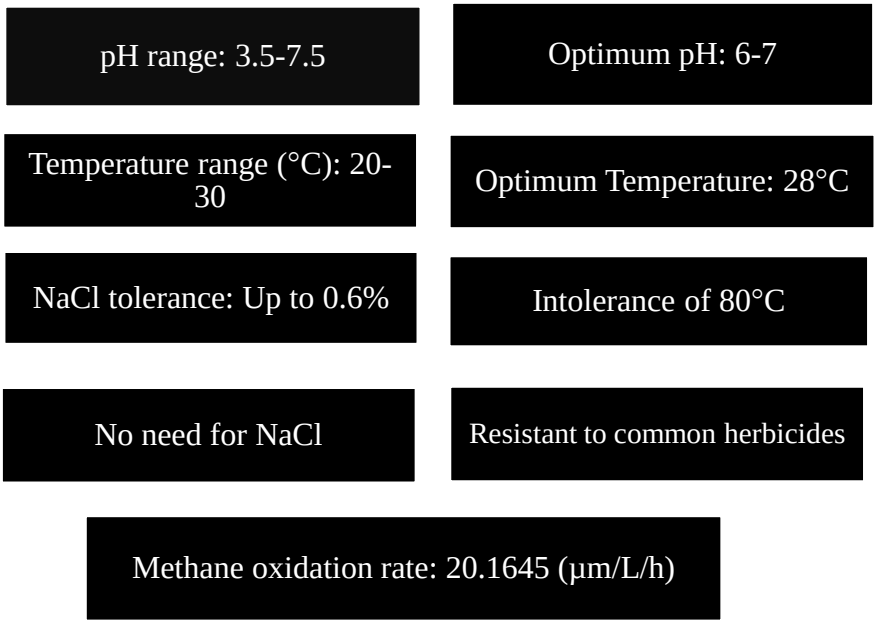

Fig. 5- Characteristics of *Methylocystis* strains from the general data of this study

Discussion and Conclusion

The data from a high amount of methane oxidation during the growth period with a low shaking was the promising point of this research for environmental cleaning of methane. The amount of methane oxidation was examined at the bacterial growth period in a low-rpm shaker incubator to imitate similar conditions in wetlands and stagnant waters. Besides, low-rpm shaking led to low aeration for the cultured strains. Low shaking reduces the growth and oxidation rate of these bacteria but compared to similar studies conducted

using high shaking up to 200 rpm, the results of the study of methane oxidation are significant (21).

In the present study, the isolation of methanotrophs in water-saturated soils, soils with water or sludge, and wetlands with decayed plant residues were associated with the abundance of *Methylocystis* strains. *Methylocystis* strains occur at a high level of methane, a condition that occurs during methane high availability in forests or upper soils due to immersion in water or increased deposition. It is predicted that in future climatic conditions, *Methylocystis* strains will be predominant and abundant (22).

These methanotrophs have high compatibility. For example, they have both pMMO and sMMO enzymes, where sMMO is expressed only at a low ratio of copper to biomass, but pMMO is expressed at a high ratio of copper to biomass (9), all representing compatibility for survival in a copper-deficient media. The other compatibility is being microaerophilic, that is, they do not require much oxygen and are in accordance with the conditions of the experiments performed in the present study (23). Another compatibility is the presence of methanobactin in this type of methanotrophs, which stores excess copper in the cell first known in the *Methylocystis* species (24).

For purification, various types of fungicides were used for methanotrophs, which eliminated various yeasts and fungal mycelia.

The band formation only in ~ 500 bp region of electrophoreses gel with the primer pairs Type.IIF and Type.IIR verified that all the isolates were type II Methanotroph (18).

The increase in growth in the designed culture medium compared to other culture media also leads to rapid screening of the medium, during which the process of separation is facilitated. Strains are maintained for a long time and biotechnological potentials can be evaluated.

Adding 1 g / l of salt compounds, for example KNO_3, increases the amount of salt in the culture medium. Although *Methylocystis* strains are salt-tolerant, halophilic strains are generally less common. Increasing the amount of salt in the culture medium can elevate the osmotic pressure not leading to the desired result (25). This study sought to maximally dilute the prepared culture medium. The high concentration of phosphate buffer in the MOC culture medium was associated with satisfactory growth of *Methylocystis* strains. In this regard, Whittenbury et al. (11) pointed out that further growth of strains is inhibited at phosphate concentrations above 2 g / l and ammonium chloride above 0.5 g / l. The values of these compounds in the MOC culture medium did not exceed those of Whittenbury's study. It was said that high phosphate concentrations selectively inhibit Malate dehydrogenase (MDH) activity in relevant cells, and high concentrations of phosphate also inhibit pMMO activity in cells. The use of ammonium in the tested culture media can explain that ammonium is a competitive chemical with methane (26). The bacteria growing in the ammonium medium should have a higher selectivity for methane than those that prefer nitrate medium, which is due to the high ability of these strains and possibly other species of *Methylocystis* (10). Besides, in the present study, the optimum growth of *Methylocystis* strains was also provided in an ammonium-containing environment. The concentration of ammonium chloride of more than 0.25 g / l reduced the growth of some strains and the concentration of more than 0.5 g / l showed a significant reduction in the growth of all strains. The strains differ in their sensitivity to ammonium ions. Adding large amounts of ammonium salts to

laboratory cultures could affect methane oxidation due to osmotic stress (25). High concentrations of iron ions inhibit the growth of bacteria, including methanotrophs, but iron ions help to use copper ions for methanotrophs (27).

The results of the present study also showed that iron concentration was central in the culture medium of *Methylocystis* strains. Raising or lowering the concentration outside a range reduced the growth of a number of strains. $CaCl_2.6H_2O$ is a compound for providing calcium available in the culture medium. Until recently, no harmful effect of calcium ions on methanotrophic growth has been observed. This ion is essential in the activity of MxaF, the largest subunit of the MDH enzyme (28). According to the results, it was observed that the need for calcium ions for *Methylocystis* strains was high. Magnesium ions are used to synthesize bacterial protoplasm and have not yet been shown to have a growth inhibitory effect on methanotrophs. In the present study, increasing the concentration of Mg^{2+} also had a positive effect on the growth of *Methylocystis* strains. The use of one-half of the usual amount of solution of rare elements in the culture medium significantly reduced the growth of all strains, where copper deficiency in the culture medium could be the most important cause. Doubling the solution of rare elements in the culture medium even inhibited growth, which could be due to the toxicity of copper available in the culture medium (29). The strains were sensitive to changing conditions of the culture medium. In this study that was conducted mostly on *Methylocystis* strains, the widest spectrum of changes occurred in the experiment of different amounts of iron and phosphate, but even though the strains are of the same genus, the types of strains showed different responses to the changing conditions. The members of this genus are physiologically different, which does not seem illogical due to the phylogenic diversity of this genus.

The chemical compounds such as bromoxynil (herbicide) and methomyl (insecticide) had a short-term inhibitory effect on methanotrophs (30). In the present study, bacteria were affected by pesticides for two weeks. In this test, the ratio used was even ten times as that of the agriculture application, but none of them had a lethal effect on *Methylocystis* strains, which is important. Future studies should investigate the long-term effects of pesticides because the long-term decomposition of pesticides has shown greater effects on methanotrophs (31).

Our observations also indicated the high viability of *Methylocystis* strains suggesting that they may also be able to reduce atmospheric methane in unfavourable conditions. The pH data showed a wider growth pattern. These strains did not show significant differences from the findings of Bowman et al. (12). The differences lie, however, in the growth pH range of isolated strains which is more acidic than their findings. Also, strain ABZ4 had a wider growth temperature range. In both studies, the ability of strains to grow in different conditions is evident.

The long growth periods, purification problems, and sensitivity of the tests required a high degree of precision and diligence in this research aiming at environmental clean-up. The inability in the spore formation and the lack of growth and survival after drying of cells are other noteworthy results of the research. In this regard, future research can focus on the applicability of such strains in natural environments. It is highly valuable to isolate and identify methanotrophic strains that can form spores and cysts, have drought and heat resistance, and consume high levels of methane in unfavourable environments (32). The application of such studies could reduce the polluting gases of the earth's atmosphere.

The results of this research with selective isolation and the design of a specific medium to *Methylocystis* strains indicate that they have high methane oxidation with aeration limitations and optimized compounds, and can grow and survive under different conditions. In other words, *Methylocystis* species are of higher applicability and more value than previously defined (33).

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