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Ph.D. Dissertation

Optimization of methanol synthesis from methane by *Methylomonas* sp. LM6, a newly isolated and characterized strain from rice paddy soil

Graduate School of Chosun University

Department of Environmental Engineering

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논에서 새로 분리한 ***Methylomonas*** **sp. LM6**를 이용한 메탄으로부터 메탄올 생합성의 최적화

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Supervisor : Prof. Si Wouk Kim

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Abstract

Optimization of methanol synthesis from methane by *Methylomonas* sp. LM6, a newly isolated and characterized strain from rice paddy soil

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Methanotrophs, also called methane-oxidizing bacteria (MOB), are a group of bacteria that use methane as their sole source of carbon and energy under aerobic or microaerophilic conditions. They share a general pathway for the metabolism of methane to carbon dioxide and the first step in methane metabolism is the oxidation of methane to methanol via methane monooxygenase (MMO). Therefore, MOB has great potential in the biotechnological utilization of methane, a readily renewable carbon source, for the production of chemicals and sustainable renewable energy. Recently, this unique capability has attracted great attention for applied microbiologists and biochemical engineers.

A methane-oxidizing bacterium was isolated from the rice paddy field and characterized. It is a Gram-negative, motile and pink-orange pigmented. The morphology of the cells was short rods (approximately 1.3 X 0.73 μm) and they occurred as single or sometimes in pairs. It could grow up to 40℃ with an optimum at 27 - 30℃ and also grow in a pH range of 5.0-8.0 with an optimum of 5.5-7.0. The specific growth rate and generation time were found to be 0.07 h^{-1} and 9.48 h. Based on 16S rDNA sequencing analysis demonstrated that the isolate (NCBI accession number KX774627) was belonged to family *Methylococcaceae* of the class

Gammaproteobacteria and was most closely related to the *Methylomonas koyamae* with 97% gene sequence homology. In addition, the major cellular fatty acids were $C_{14:0}$, $C_{16:1\ w5c}$ and $C_{16:0.}$ Therefore, based on morphological, physiological, molecular and phylogenetic properties, the strain is considered to represent a new strain within the genus *Methylomonas* and named as *Methylomonas* sp. LM6.

Methylomonas sp. LM6 is an obligate methanotrophic bacterium that grows on methane and methanol as sole carbon and energy source. Here we have presented the high-quality draft genome sequence of *Methylomonas* sp. LM6, consisting of a chromosome (4,894,002 bp) with 56.43% G+C content and a plasmid (186,658 bp). A total of 4,490 genes were predicted in the genome of this strain, 4,337 of which are protein coding genes and the total length of protein coding regions is 4,432,392bp. 2,779 of protein coding genes were assigned to a putative function, the remaining were annotated as hypothetical proteins. Furthermore, the genome distance between *Methylomonas koyamae* type strain JCM 16701 (NZ_BBCK01000001) and LM6 is estimated to 97.0% based on a two-way average nucleotide identity. This value showed that the strain LM6 belongs to the species *Methylomonas koyamae*. The strain LM6 possessed two particulate methane monooxygenase operons (*pmoABC*, XXXX_3758-XXXX_3760; *pmoCAB*, XXXX_3996-XXXX_3998) encoding a particulate methane monooxygenase (pMMO) which oxidizes methane to methanol, whereas the soluble methane monooxygenase operon was not found in this genome.

This research also investigated an approach to methanol biosynthesis from methane. Methanol production was carried out utilizing whole cells of *Methylomonas* sp. LM6 as the biocatalyst. Methane to methanol conversion using bacterial cells has been carried out to replace the high cost chemical processes. In addition, biological conversion has advantages as it requires

less energy since it is conducted at ambient temperatures and high conversion efficiency with environmentally friendly processes. MMO is the key enzyme that catalyze the oxidation of methane to methanol. Methanotrophic bacteria can accumulate methanol by inhibiting the activity of methanol dehydrogenase (MDH).

In this work, bioconversion of methanol from methane using newly isolated cells of *Methylomonas* sp. LM6 as the biocatalyst was investigated. The cells were cultured on nitrate mineral salt (NMS) medium with a supply of methane/air (6:4, v/v) mixture in a tightly sealed Erlenmeyer flask. The optimal pH and temperature for growth were pH 7.0 and 30℃, respectively, and the copper concentration was 2 μM. Cells were harvested by centrifugation and washed three times using 20 mM phosphate buffer (pH 7.0). To produce methanol, MDH activity should be inhibited. Therefore, 60 mM of potassium phosphate, 60 mM of $MgCl_2$, 90 mM of NH_4Cl, and 3 mM of EDTA were used as MDH inhibitors and added to 70 mL serum bottle containing 10 mL NMS medium (pH 7.0), supplied with methane/air (6:4, v/v) mixture for the maximum synthesis of methanol. The reaction temperature was maintained at 30℃, and the added cell concentration and Na-formate in the reaction mixture were 0.68 mg of dry cell wt/mL and 80 mM, respectively. During 24 h reaction, 11.3 mM of methanol has been accumulated in the reaction mixture. Also, further optimization of the parameters and synergistic effects of several MDH inhibitors such as potassium phosphate, $MgCl_2$, NH_4Cl and EDTA significantly improved methanol accumulation up to 22.8 mM.

Furthermore, one of the major challenges in this study is the enhancement of methane gas solubilization in reaction mixture. Therefore, several organic solvents were tested to improve methane solubility but not to inhibit cell growth. 1-octanol was the most effective

solvent enhancing methanol synthesis by increasing methane gas transfer between gas and aqueous phases, without cell growth inhibition.

In a previous study, maximum methanol accumulation in a batch reaction by *Methylomonas* sp. LM6 was 22.8 mM. In this study, we found that methanol production significantly increased when 1-octanol was added to the reaction medium. 500 mL Erlenmeyer flask containing 100 mL NMS reaction mixture (pH 7.0) and 2 mL 1-octanol was supplied with methane/air (6:4, v/v) and agitated at 180 rpm for 8 h at 30°C. After removal of 1-octanol, high methane concentrated reaction mixture was treated with not only 90 mM NH_4Cl, 80 mM Na-formate and 0.68 mg dry cells/mL, but also 60 mM potassium phosphate, 60 mM $MgCl_2$, and 3 mM EDTA as MDH inhibitor. The reaction mixture was incubated at 30°C for 24 h without additional methane. Then, the produced methanol concentration increased from 22.8 to 50.1 mM. 1-octanol did not inhibit the cell activity and was recovered from the reaction mixture and reused for further experiments. These results show that 1-octanol enhances solubility of methane from 80.03 mM to 130.80, about 1.6 times higher than that in control and also increases methanol production by approximately 2 times higher when compared to the control (without 1-octanol). It was concluded that methane bioconversion to methanol using 1-octanol as a methane solubilizer is an effective way and have a potential for use in industrial application.

초록

메탄 산화 박테리아(MOB)라고도 불리는 Methanotroph 는 호기성 또는 미호기성 조건에서 메탄을 탄소와 에너지의 유일한 원천으로 사용하는 박테리아 그룹이다. 그들은 메탄과 이산화탄소의 물질 대사를 위한 일반적인 경로를 공유하며 메탄 대사의 첫 번째 단계는 메탄산화효소(MMO)를 통한 메탄을 메탄올로의 산화이다. 따라서 MOB 는 화학 물질 및 지속 가능한 재생 에너지 생산을 위해 쉽게 재생 가능한 탄소원인 메탄의 생물공학적 이용에 큰 가능성을 가지고 있다. 최근에, 이 특별한 능력은 응용되는 미생물 학자들과 생화학 엔지니어들에게 많은 관심을 받고 있다.

논에서 메탄 산화세균이 분리되어 특성이 규명되었다. 그람 음성, 운동성 및 pink-orange 색소가 있는 종이다. 세포의 형태는 짧은 로드(약 1.3X0.73μm)였고 단일 또는 때때로 쌍으로 나타났다. 최적온도는 27-30℃이고 40 ℃까지 자랄 수 있으며, pH 범위는 5.0-8.0 이고 최적 범위는 5.5-7.0 이다. 비 성장 속도 및 생성 시간은 0.07h-1 및 9.48h 인 것으로 나타났다. 16Rdna 염기서열 분석 결과에 의하면 분리균주 (NCBI accession number KX774627)는 *Gammaproteobacteria* 계열의 *Methylococcaceae* 에 속하였고 *Methylomonas koyamae* 와 가장 밀접하게 관련되어 97%의 유전자 서열 상 동성을 보였다. 또한 주요 세포

지방산은 C14:0, C16:1 w5c and C16:0 이었다. 따라서 형태학적, 생리학적, 분자적 및 계통학적 특성에 기초하여, 이 균주는 *Methylomonas* 속의 새로운 균주를 나타내는 것으로 간주되며 *Methylomonas* sp. LM6 으로 명칭이 붙여졌다.

Methylomonas sp. LM 은 메탄과 메탄올을 유일한 탄소와 에너지원으로 자라는 절대적인 메탄영양세균이다. 여기서 우리는 *Methylomonas* sp. LM6 의 양질의 유전체 서열을 보여 주었다. 56.43 %의 G + C 함량을 가진 염색체 (4,894,002 bp)와 플라스미드 (186,658 bp)로 구성되어있다. 총 4,490 개의 유전자가 이 균주의 게놈에서 예측되었으며, 이 중 4,337 개는 단백질 코딩 유전자이고 단백질 코딩 영역의 총 길이는 4,432,392bp 이다. 2,779 개의 단백질 코딩 유전자가 추정 기능에 할당되었고, 나머지는 가상의 단백질로 주석 처리되었다.또한 *Methylomonas koyamae* 형 균주 JCM 16701 (NZ_BBCK01000001)과 LM6 사이의 게놈 거리는 양방향 평균 뉴클레오티드 동일성을 기준으로 97.0%로 추정되었다. 이 값은 LM6 균주가 *Methylomonas koyamae* 종에 속한다는 것을 보여준다. LM6 균주는 메탄을 메탄올로 산화시키는 미립자 메탄산화효소(pMMO)를 암호화하는 2 개의 미립자 메탄산화세균 오페론 (*pmoABC*, XXXX_3758-XXXX_3760; *pmoCAB*,

XXX_3996-XXXX_3998)을 보유하고 있지만 가용성 메탄산화세균 오페론은 이 게놈에서 발견되지 않았다.

이 연구는 또한 메탄으로부터 메탄올 생합성에 대한 접근법을 연구하였다. 메탄올 생산은 생체촉매제인 *Methylomonas* sp. LM6 의 전체 세포를 이용하여 수행되었다. 박테리아 세포를 이용한 메탄-메탄올 전환은 고비용의 화학 공정을 대체하기 위해 수행되었다. 또한 생물학적 전환은 상온에서 수행되고 환경 친화적인 공정으로 높은 전환 효율로 수행되므로 에너지를 덜 필요하므로 장점이 있다. MMO 는 메탄의 메탄올로의 산화를 촉매 하는 주요 효소이다. *Methanotrophic* 박테리아는 메탄올 탈수소효소(MDH)의 활성을 억제함으로써 메탄올을 축적할 수 있다.

이 연구에서 새로 분리된 *Methylomonas* sp. LM6 의 세포를 생체 촉매로 사용하여 메탄으로부터 메탄올을 생체 전환시키는 방법을 연구하였다. 세포를 단단히 밀봉 된 삼각 플라스크에 methane/air (6:4, v/v) 혼합물을 공급하여 질산 미네랄 염(NMS)배지에서 배양하였다. 성장을 위한 최적의 pH 와 온도는 각각 pH 7.0 과 30℃이고, 구리 농도는 2 μM 이다. 세포를 원심분리에 의해 수확하고 20 mM 인산 완충액 (pH 7.0)을 사용하여 3 회 세척하였다. 메탄올을 생산하기 위해서는 MDH 활성이 저해 되어야 한다. 따라서 MDH

억제제로 60 mM of potassium phosphate, 60 mM of MgCl2, 90 mM of NH4Cl, and 3 mM of EDTA 를 사용하고 최대 메탄올 합성을 위해 methane/air (6:4, v/v) 혼합물이 공급된 10 mL NMS 배지 (pH 7.0)를 함유한 70mL serum bottle 에 넣었다. 반응 온도는 30℃를 유지하였으며, 반응 혼합물 중의 첨가된 세포 농도 및 Na-formate 는 0.68 mg dry cells wt / mL 및 80mM 이었다. 24 시간 반응 동안, 11.3mM 의 메탄올이 반응 혼합물에 축적되었다. 또한, potassium phosphate, MgCl2, NH4Cl and EDTA 와 같은 여러 가지 MDH 억제제의 매개 변수 및 상승 효과를 더욱 최적화하여 최대 22.8mM 의 메탄올 축적을 크게 개산하였다. 또한 이 연구에서 주요 과제 중 하나는 반응 혼합물에서의 메탄 가스 가용화의 증진이다. 따라서 메탄 용해도를 향상 시키지만 세포 성장을 억제하지 않기 위해 여러 유기 용매를 시험 하였다. 1-octanol 은 세포 성장 억제 없이 가스와 수성 단계 사이의 메탄 가스 전달을 증가시킴으로써 메탄올 합성을 향상시키는 가장 효과적인 용매였다.

이전 연구에서, *Methylomonas* sp. LM6 에 의한 회분식 반응에서의 최대 메탄올 축적은 22.8mM 이었다. 이 연구에서 1-octanol 이 반응 매질에 첨가 될 때 메탄올 생성이 유의하게 증가한다는 것을 발견했다. 100 mL 의 NMS 반응 혼합물 (pH 7.0)과 2 mL 의 1-octanol 이 들어있는 500 mL 삼각 플라스크에 메탄 / 공기 (6 : 4, v / v)를 공급하고 180

rpm 으로 30 ° C 에서 8 시간 동안 교반 하였다. 1- octanol 을 제거한 후, 고 메탄 농축 반응 혼합물을 MDH 억제제로서 90mM NH4Cl, 80mM Na- formate 및 0.68mg dry cells / mL 뿐만 아니라 60 mM potassium phosphate, 60 mM MgCl2, and 3 mM EDTA 로 처리하였다. 반응 혼합물을 추가의 메탄 없이 24 시간 동안 30 ℃에서 배양 하였다. 그 다음, 생성 된 메탄올 농도는 22.8mM 에서 50.1 mM 로 증가했다. 1- octanol 은 세포 활성을 억제하지 않았고 반응 혼합물로부터 회수되어 추가 실험을 위해 재사용되었다. 이러한 결과는 1- octanol 이 80.03 mM 에서 130.80mM 의 메탄 용해도를 대조군에 비해 약 1.6 배 증가 시켰으며 대조군 (1- octanol 제외)에 비해 메탄올 생산량을 약 2 배 증가 시킨다는 것을 보여준다. 메탄 가용화 제로서 1- octanol 을 사용하는 메탄올로의 메탄 생체 전환은 효과적인 방법이며 산업적 응용에 사용될 가능성이 있다고 결론 지었다.

I. General Introduction

1. Obligate methylotrophs

Obligate methylotrophic bacteria are defined as microorganisms that have the ability to utilize, compounds that are more reduced than carbon dioxide and contain no carbon-carbon bonds, as sole carbon and energy source (Colby et al., 1979). This includes molecules like one-carbon compounds such as methane, methanol and monomethylamine. Due to the lack of much information about obligate methylotrophs physiology and biochemical taxonomic criteria, has made it challenging to apply, conventional and traditional approaches to their classification. In addition, obligate methylotrophic bacteria are found as motile and non-motile forms. Motile obligate methylotrophic bacteria are widespread and their metabolism has been studied thoroughly. They are related to genus *Methylophilus*. Alternatively, non-motile obligate methylotrophic bacteria are less diverse and little information available on their metabolic pathways. Additionally, obligate methylotrophs are gram-negative, polarly flagellated rod capable of rapid growth with methane or methanol. Some organisms can also utilize formaldehyde or methylamines. The ribulose mono phosphate pathway utilized for carbon assimilation. The obligate methylotrophic bacteria are subdivided into two major groupings: obligate methane utilizers (methanotrophs) and obligate methanol/ methylated amine utilizers as shown in Fig. 1.

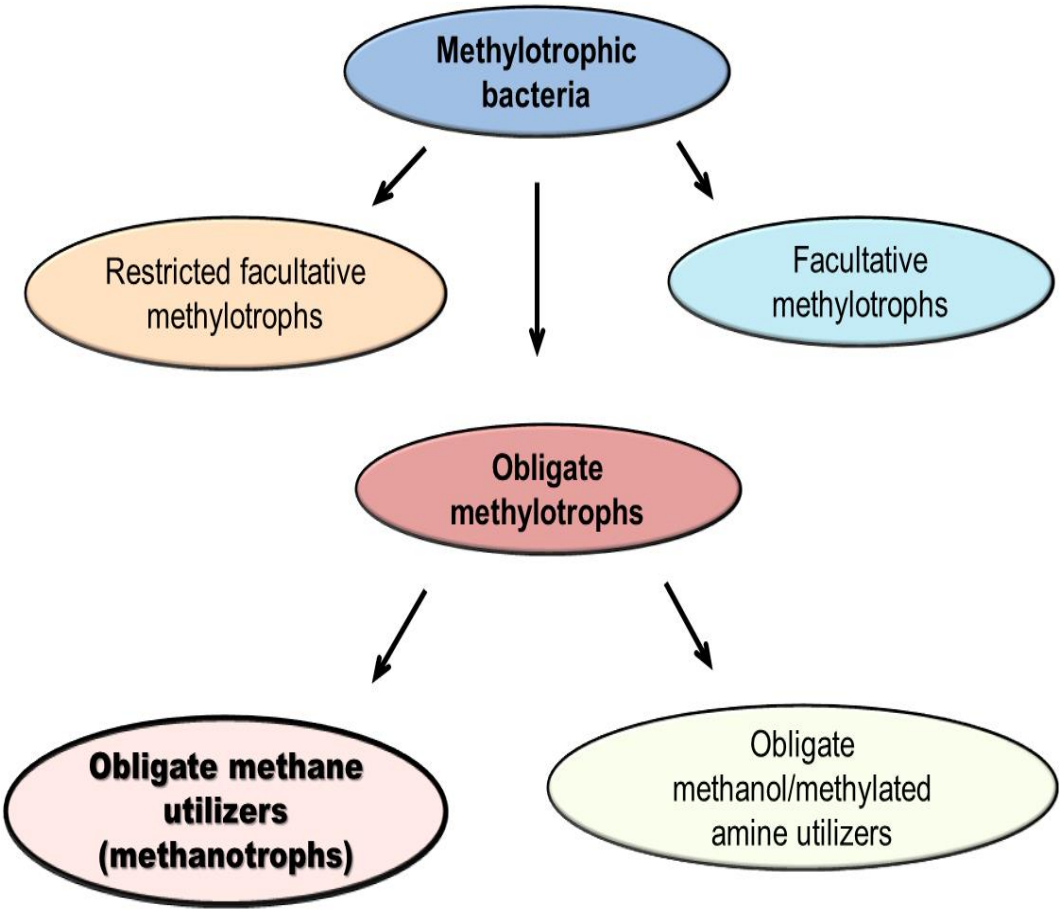

Fig.1-1. The following diagram illustrates the major grouping to subdivide the methylotrophic bacteria (Murrell et al., 1992).

1.1. Methane – Utilizing Bacteria

Methane – Utilizing Bacteria or Methanotrophic bacteria are a diverse group of organisms comprising various genera. All methanotrophs utilizes methane, via methane monooxygenase, as sole carbon and energy source under aerobic or sometimes microaerophilic conditions.

Methanotrophs had been isolated, characterized and described in the literature by Whittenbury and his colleagues at Edinburgh. Five important groups or "genera" were described: *Methylomonas, Methylobacter, Methylococcus, Methylosinus,* and *Methylocystis*. However, now methane utilizing bacteria have been broadly split into two groups: (type I and type II), on the basis of the major carbon assimilation pathway for oxidation of methane and their intracytoplasmic membrane arrangements. Recently, one of the groups, type I, is further subdivided into type X (also called type Ia and type Ib), that consist of methanotrophs capable of autotrophic CO_2 fixation. In the type I group, 14 genera of the family *Methylococcaceae* (*Methylomonas, Methylobacter, Methylosphaera, Methylomicrobium, Methylosarcina, Methylosoma, Methylovulum, Methylomarinum, Methyloglobulus, Methyloprofundus, Methylococcus, Methylocaldum, Methylogaea, Methyloparacoccus*) (Deutzmann et al., 2014; Hoefman et al., 2014b; Tavormina et al., 2015) and 3 genera of the family *Methylothermaceae* (*Methylothermus, Methylohalobius, Methylomarinovum*) are described. In the class of type II group, 5 genera such as *Methylocystis, Methylosinus, Methylocella, Methylocapsa* and *Methyloferula* are described. Type I MOB are abundantly found in the environments with high O_2 and limited CH_4 concentrations, whereas type II MOB prefer environments with high CH_4 but limited O_2 concentrations (Henckel et al., 2000; Shrestha et al., 2008). It indicates that the availability of CH_4 and O_2 seems to be the important factor for the community dynamics of

methanotrophs. The genus *Methylomonas*, which belongs to the type I MOB group, are strictly aerobic, obligate methane utilizers with type I intracytoplasmic membranes (ICMs) and use ribulose monophosphate (RuMP) pathway for carbon assimilation. Table 1-1 explains in detail about the primary categorization of type I, type II, and type X.

Table 1-1. Characteristics of type I, type II, and type X methanotrophs

Characteristic	type I	type II	type X
Family	*Methylococcaceae*	*Methylocystaceae*	
Member genera	*Methylosphaera* *Methylobacter* *Methylomicrobium* *Methylomonas*	*Methylocystis* *Methylosinus*	*Methylococcus* *Methylocaldum*
Cell morphology	Short rods, usually occur singly; some cocci or ellipsoids	Crescent-shaped rods, rods, pear-shaped cells, sometimes occur in rosettes	Cocci, often found as pairs
Membrane arrangement			
Bundles of vesicular disks	+	–	+
Paired peripheral membranes	–	+	–
Motility	±	–	±
Major carbon assimilation pathway	RuMP pathway	Serine pathway	RuMP pathway
Growth at 45 ℃	–	–	–
Resting stages formed			
Exospores	–	Some strains	–
Cysts	Some strains	Some strains	Some strains
Nitrogen fixation	–	+	+
Major PLFAs	14.0, 16:1w7c, 16:1w5t	18:1w8c	16:0, 16:1w7c
Proteobacterial subdivision	Gamma	Alpha	Gamma
G+C content of DNA (mol%)	49–60	62–67	59–65

+ = positive; – = negative.

Data from (Hanson and Hanson, 1996).

(1) Morphological and physiological criteria

Obligate methanotrophic bacteria have extensively diverse cellular shapes. These comprise slender rods of various dimensions, e.g.: curved or “vibroid” rods, pear-shaped cells, coccobacilli, cocci, and also cells that form spores. Some methanotrophic bacteria also displayed polar growth or budding (Hirsch, 1974).

① Motility and flagellar arrangement

Many, but not all, the methane utilizing bacteria are found motile at some stage during their growth cycle. However, few coccal methanotrophs are also discovered to be nonmotile. Most of the type I organisms, such as *Methylomonas* and *Methylobacter* spp. are motile with a single polar flagellum. In addition, type II organisms belonging to the *Methylosinus* group are motile, but with a polar tuft of flagella, though *Methylocystis* strains are discovered so far are nonmotile.

② Colony pigmentation

Colony colour changes with physiological or sometimes with age of the cells. For example, spore-forming organisms often found with darker colony colour, and generally by the formation of yellowish to brown pigmentation. Although all the cells are not affected in this way. Methanotrophic bacteria also produce a wide range of colony pigments, such as, soluble and insoluble. Insoluble pigments consist of white, off-white, and cream (e.g., *Methylomonas albus*); yellow (e.g., *Methylococcus luteus*) to light brown (e.g., *Methylobacter vinelandii*). In contrast, water-soluble pigmented methanotrophic bacteria are found very rare in the environment, but *Methylosinus sporium* and *Methylomonas methanica* identified with green to sapphire or brown to black pigments, respectively especially on iron-deficient media (Whitenbury et al., 1970).

③ Colony morphology

Morphological features, colony colour and size are useful in identifying the methanotrophic bacteria. For example, organisms that are selectively isolated at 45℃, of the two, one will be observed with cinnamon brown (*M. thermophillus*) with small (1-1.5 mm diameter), emerge as smooth, shiny, convex, whereas the other larger in size (≥ 3 mm diameter) and found as light brown (*M. vinelandii*).

④ Growth on various carbon sources

Carbon source such as malate, acetate, yeast extract, or succinate was used for enhancing the methane-grown cells by Whittenbury and his colleagues (1970). However, many methanotrophic bacteria cannot utilize these carbon sources and have proved a difficult test to interpret. Although several pink pigmented methane utilizers bacteria such as, *M. methanica*, *M. rubra*, and *M. rosaceous* on glucose as sole carbon and energy source were detected.

⑤ Growth temperature

Several types I methanotrophs belonging to the *Methylomonas*/*Methylobacter* groups grow at 37℃whereas type II methanotrophs appear to have different growth temperature. The brown pigmented *Methylomonas vinelandii*, *Methylococcus thermophillus* and the nonpigmented *Methylococcus capsulatus* are the groups of bacteria that can grow at 45℃ or above. In addition, *Methylococcus thermophillus* can grow in excess of 55℃. Furthermore, at low temperature, pink pigmented taxa, such as, *Methylomonas methanica* can grow at 15℃ or lower.

(2) Taxonomic structure of the Methanotrophs

The descriptions of individual organisms or groups of methanotrophs were difficult to explain due to inadequate information regarding their molecular studies. Later, Whittenburry and Krieg (1984) predicted that obligate methane-utilizing bacteria belong to a family, the *Methylococcaceae*. A brief description of the family, the *Methylococcaceae* and genera *Methylomonas* is given below.

① The *Methylococcaceae*

Genus *Methylococcus* and Genus *Methylocaldum* represent the type X methanotrophs, a subset of type I methanotrophs with a tendency of growing in high temperatures. The type species of *Methylococcus* is *Methylococcus capsulatus*, isolated from sewage sludge by Foster and Davis (1966) (reference). However, later investigations using immunological analysis and fatty acid analysis and genomic characteristics (Andreev and Galchenko, 1978; Galchenko and Nesterov, 1981;Bezrukova et al., 1983; Meyer et al., 1986, Bowman et al., 1991a; Bowman et al., 1991b) explained that the genus was made up of two groups. Although, this nomenclatural problem was resolved again when only *Methylococcus capsulatus* and *Methylococcus thermophilus* were saved in *Methylococcus* (Bowman et al., 1993b). In addition, the other species were moved to the genus *Methylobacter*, the original name coined for them by Whittenbury et al. (1970b).

These ***Methylococcaceae*** are the diverse groups of the rod and coccal shaped bacteria sharing the ability to utilize methane (via methane monooxygenase) as a sole carbon and energy source under aerobic or microaerophilic conditions. They are gram-negative, catalase and oxidase positive. These strains occur and generally found in an aerobic environment where

methane is available abundantly, i.e., rice field, forest, wastelands, water, and in muddy areas. Strains of this family consist of intracytoplasmic membranes when grown on methane. In addition, the membrane arrangements are consisting of bundles of vesicular disks or paired membranes.

Furthermore, growth temperature ranges vary among strains of this family. Few are observed to be thermophilic or thermotolerant, which cannot grow at 30°C, but can grow at 45-55°C. Some strains are very sensitive to the oxygen of the air and also require decreased oxygen level (microaerophilic conditions) for strain growth.

② The Genus *Methylomonas*

They are motile or nonmotile and motile cells have a single polar flagellum. Strains are gram-negative, aerobic, oxidase and catalase positive. The principal source of carbon and energy sources are methane, methanol and formaldehyde. Growth generally occurs at ≤ 20-37°C, although not more than 40°C. Strains are coccobacilli or straight rods, 0.5-0.7×0.7-2.0 μm. The mole% G + C of the DNA is 50-54.

The genus *Methylomonas* are found abundantly in various semi-neutral or slightly alkaline environments, such as rice paddies, water-saturated soils, volcanic areas, fresh and marine waters, lakes and sediments (Auman et al., 2000; Auman & Lidstrom, 2002; Hutchens et al., 2004; Lin et al., 2004; Lu¨ke et al., 2010; Dianou et al., 2012). However, the presence of *Methylomonas* in acidic boreal forest soils and Sphagnum-dominated wetlands were observed in several cultivation-independent studies (Morris et al., 2002; Jaatinen et al., 2005; Chen et al., 2008; Kip et al., 2011a).

1.2. Physiology and biochemistry of methane utilizing bacteria

The factors that control methane metabolism and the ecology of methane utilizing bacteria require more knowledge of the physiology of different groups of methanotrophs. As discussed above, there are three types of aerobic methanotrophs are recognized in environment, i.e., (i) type I methanotrophs are gamma proteobacteria that utilize methane monooxygenase (MMO), the enzyme for primary methane oxidation, and that utilize the ribulose monophosphate (RuMP) cycle, that converts formaldehyde into other compounds, (ii) type II methanotrophs that belong to the alpha proteobacteria, observed to have rings of pMMO-harboring membranes at the periphery of the cells, and utilize the serine cycle, an alternative pathway for converting formaldehyde into products, and (iii) type X methanotrophs, belonging to the genus *Methylococcus* (gamma-proteobacteria) and features characteristic of the other two types: they have stacked membranes and the RuMP cycle, but they also have elements of the serine cycle and sMMO. Here the physiology and biochemistry of methanol utilization by type I bacteria, with emphasis on the regulation of the formaldehyde fixation discussed further.

First observations on the ribulose monophosphate cycle of formaldehyde fixation were made by Quale and his colleagues (Johnson and Quayle, 1965).

RuMP cycle can be divided into three steps: Step 1 (fixation), in this step, the aldol condensation of three molecules of ribulose-5-phosphate by HPS to give three molecules of hexulose-6-phosphate , and finally isomerized by HPI to fructose-6-phosphate; Step 2(cleavage), one of the molecules of fructose-6-phosphate produces two C_3 compounds; Step 3 (rearrangement) the regeneration of three molecules of ribulose-5-phosphate is observed here from the two remaining molecules of fructose-6-phosphate, from stage 1 and the one molecule of glyceraldehyde-3-phosphate produced in stage 2.

2. Methane oxidation by methanotrophs

2-1. Methane monooxygenase

Methane monooxygenase (MMO), is a metalloenzyme capable of oxidizing the C-H bond in methane and belongs to the class oxidoreductase enzymes (EC 1.14.13.25). There are two forms of MMO known, the soluble, or sMMO, and the particulate type better known as pMMO, that are responsible for the oxidation of methane to methanol. Also, they mainly exist in depending on the level of copper ions present in the environment.

(1) Particulate Methane Monooxygenase (pMMO)

Ribbons and Michalover (1970) represented the first report on cell-free methane-oxidizing activity associated with the particulate fractions, extracted from *M. capsulatus*. This enzyme is a fascinating enzyme that is capable of oxidizing straight chain hydrocarbons from C1 to C5 (Chan and Yu, 2008; Lieberman and Rosenzweig, 2004). They have large protein complex along *PmoA*, *PmoB* and *PmoC*, and copper ions. Nonetheless, precious structure considers from various species have showed up demonstrating the trimeric design of the pMMO edifices, each comprising of one duplicate of the *PmoA*, *PmoC*, and *PmoC* subunits, however with only 2–3 coppers and one zinc particle for every monomer There are many models proposed for the active site and also it's working mechanism. Importantly, on the basis of crystal structures (Fig. 1-2 (a)), it has been suggested that the active site of the enzyme is present at the water-exposed dicopper site of *PmoB* (Balasubramanian et al., 2010). Nevertheless, biophysical and biochemical studies implicated hydrocarbon hydroxylation site at site D within the transmembrane domain. In addition, recently in site D, efficient methane hydroxylation and propene epoxidation were shown to mediate by preparing a peptide–tri copper complex

depending on the domain of PmoA lining at the interface between the PmoA and PmoC subunits (Chan et al., 2013).

(2) Soluble Methane Monooxygenase (sMMO)

Soluble MMO was first reported by (Colby and Dalton) in 1976. The sMMO complex was comprised of three protein segments: hydroxylase (MMOH, 251 kDa), the β unit (MMOB, 15.9 kDa), and the reductase□(MMOR, 38.6 kDa) (Fig. 1-2 (b)). Each of which is fundamental for successful substrate hydroxylation and NADH oxidation. MMOH is a homodimeric protein comprising of two arrangements of α, β, and γ subunits containing a di-iron site, which is essential for catalyzing the conversion of methane with oxygen to methanol. MMOR contains a bound flavin adenine dinucleotide (FAD) and a [2Fe-2S]-ferredoxin (Fd) cofactor, which helps in activation of MMOH by transferring reducing power to MMOH utilizing NADH. MMOB is responsible for regulating the structure and reactivity of the non-heme di-iron site of MMOH (Friedle et al., 2010). X-ray crystallography of the sMMO demonstrates that the three subunits, α2β2γ2 form a dimer shape. Likewise, a wide gorge running along the dimer interface with an opening in the focal point of the atom. A large portion of the promoters includes helices from the

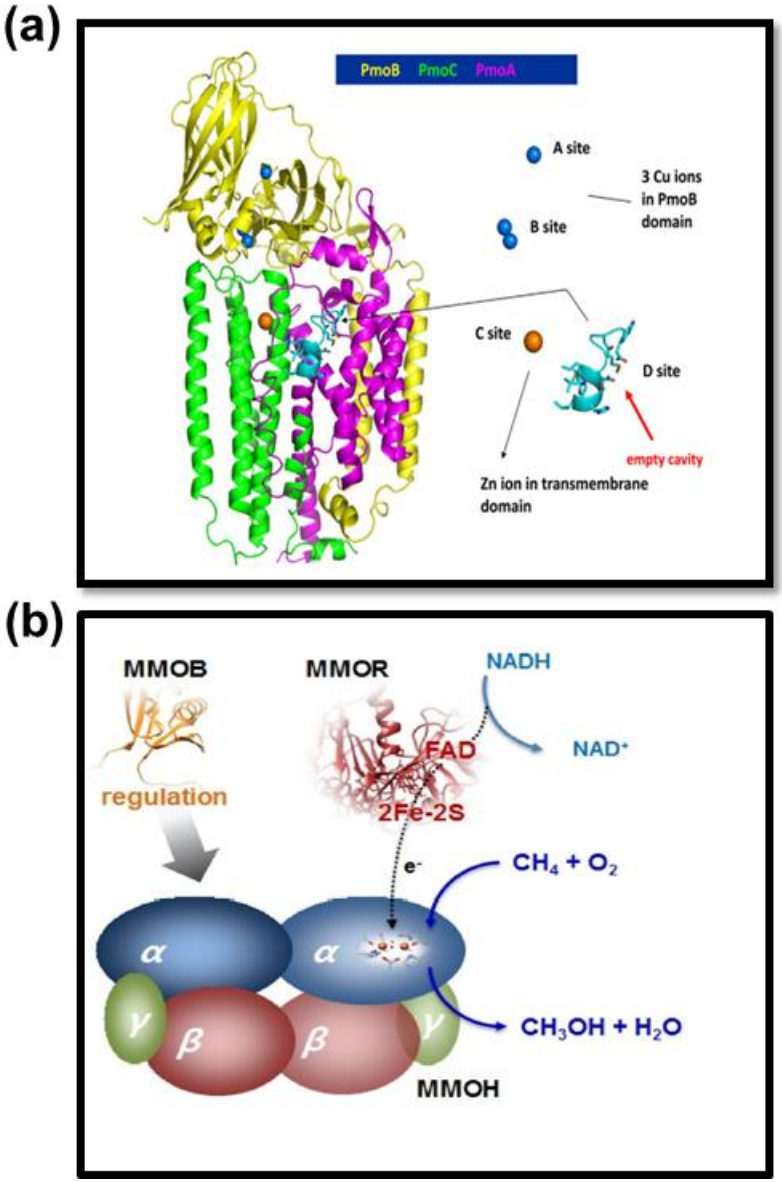

Fig.1-2. The overview of the particulate methane monooxygenase (pMMO) reported by Lieberman and Rosenzweig with tri-copper cluster and the di-iron cluster in the intermembrane pmoA and pmoC subunits of the enzyme (Lieberman and Rosenzweig, 2005; Pham et al., 2015) (a). Soluble methane monooxygenase consisting of three subunits (b) (Friedle et al. 2010) α and β subunits with no support from the γ subunit. Additionally, the collaborations with the promoters take after ribonucleotide reductase R2 protein dimer, looking like a heart.

3. Biological conversion of methane to methanol

All aerobic methanotrophic bacteria share general pathway, CH_4 is oxidized to CO_2 in final step with methanol, formaldehyde and formate as intermediates. The enzyme reactions in this pathway are catalyzed by methane monooxygenase MMO, methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FalDH), and formate dehydrogenase (FDH), respectively.

Methane (CH_4) is as of now accepting incredible consideration owing to its extreme effect on the earth as a greenhouse gas (GHG). CH_4 is a primary component of biogas (produced as a result of anaerobic digestion), including flammable gas, shale gas, and landfill gasses. Along these lines, there is a critical need to diminish the unsafe natural impacts because of these CH_4. In addition, methane is costly to store, transport, and distribute due to their gaseous form nature at ambient temperatures with a boiling point of −164 °C. Although they can be compressed into liquefied natural gas (LNG), and natural gas (CNG), but these processes need high energy and extensive capital uses. However, once again, these compressed CNG and LNG are hazardous due to their high pressure (21–25 MPa) and low temperature (−160 °C) for the environment. Therefore, in order to address these issues, it is necessary to convert CH_4 into important and easily handled liquid fuels such as methanol, ethanol, and butanol etc.

Recently, the most common method utilized for CH_4-to-liquid fuel technology is the two-step thermochemical conversion process (Chuang, 2012; Park and Lee, 2013). Although, thermochemical conversion usually have many disadvantages and encounters many barriers, such as required high pressure and/or temperature, and many expensive catalysts (Park and Lee, 2013). Also, sometimes chemical catalysts can undergo sudden deactivation, and expensive purification processes are required for better thermochemical conversion (Kohn, 2012; Navarro et al., 2013).

For that reason, thermochemical processes can be replaced by a biological conversion that is recently highly attractive due to their efficient conversion reactions and cost-effective operating conditions.

In addition, conversion of CH_4 to methanol and also other useful liquid fuels may be fulfilled by different bioprocess (CH4 to methanol and then methanol to other liquid fuels) using methanotrophic bacteria (MOB), or can also be carried out by a genetically engineered organism (using either methanotrophs, AOB or acetogens) that contains the two pathways these pathways explained in Fig. 1-3.

Interestingly, groups of bacteria have been found in nature that can perform bioconversion by activating the stable C-H bond of CH_4 under ambient conditions. They are aerobic methanotrophic bacteria, which utilizes CH_4 as their source of carbon and energy and use methane monooxygenase (MMO) to perform methanol synthesis (Hanson and Hanson, 1996). The advantage of bioconversion CH_4 into methanol can be easy to store and transportation can be done more easily and safely. Also, biological conversion of methanol from CH_4 has many advantages over the chemical methods, which includes less energy consumption, higher conversion efficiency, higher selectivity, and lower equipment costs.

These methanotrophic bacteria share a general pathway through which CH_4 is finally oxidized to CO_2 with methanol, formaldehyde and formate as intermediates (Hanson and Hanson, 1996).

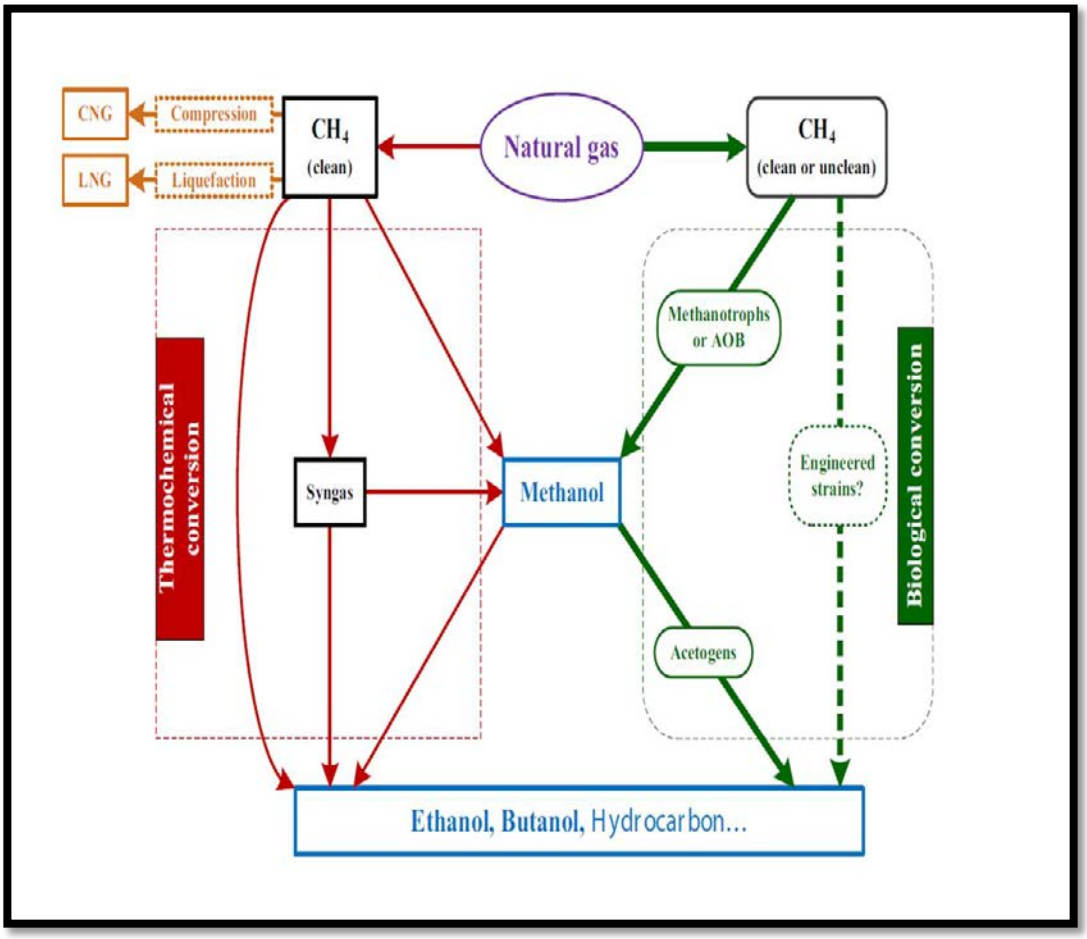

Fig.1-3. Schematic diagram representing pathways of methanol and other liquid fuel production from methane (Ge et al., 2014).

The pathway reactions are catalyzed by methane monooxygenase (MMO), methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FalDH), and formate dehydrogenase (FDH), respectively (Hanson and Hanson, 1996). In addition, few formaldehyde intermediates are assimilated via two pathways ribulose monophosphate (RuMP) or a serine pathway as explained above in introduction.

In this study, optimization of the process parameters, screening of effective MDH inhibitors, and effects of Na-formate resulted in significant enhancement in methanol production using *Methylomonas* sp. LM6. Our results suggest that *Methylomonas* sp. LM6, a Type I methanotrophic bacteria, may have potential to be utilized for industrial methanol production.

4. Research objectives

Methane has a great deal of attention from environment scientist's reports due to their abundant greenhouse gas after carbon dioxide. On the other hand, recently, isolation of methanotrophic bacteria that can be used as bio catalyst will improve the feasibility of biological conversion of CH_4 to methanol is□becoming significant. Methanotrophic bacteria have been isolated from a variety of sources such as rice field soil, natural gas fields, and methane-rich waste treatment facilities and a few reports also have isolated methanotrophs to produce methanol. In light of these findings, many researchers are focusing on developing new methods for converting and enhancing methane to methanol. In this study, a new methanotrophic bacterial strain for producing bio-methanol have been successfully isolated and characterized. This methanotrophic bacterium was utilized as catalyst and methanol productions were carried out. Additionally, the optimum MDH inhibitor conditions and optimum process parameters were determined in order to accumulate high rate of methanol. In addition, steps were taken to enhance the methanol accumulation in the media. A schematic diagram has been represented in Fig. 1-4. This thesis is divided into 4 chapters. Chapter II describes the materials and methods for isolation, characterization and methanol production. Chapter III describe the results and discussion explaining isolation of methanotrophic strain, optimization of MDH inhibitors, Na-formate, process parameters and enhancement for methanol production using organic solvents. The objective of this study has been described in the introduction section.

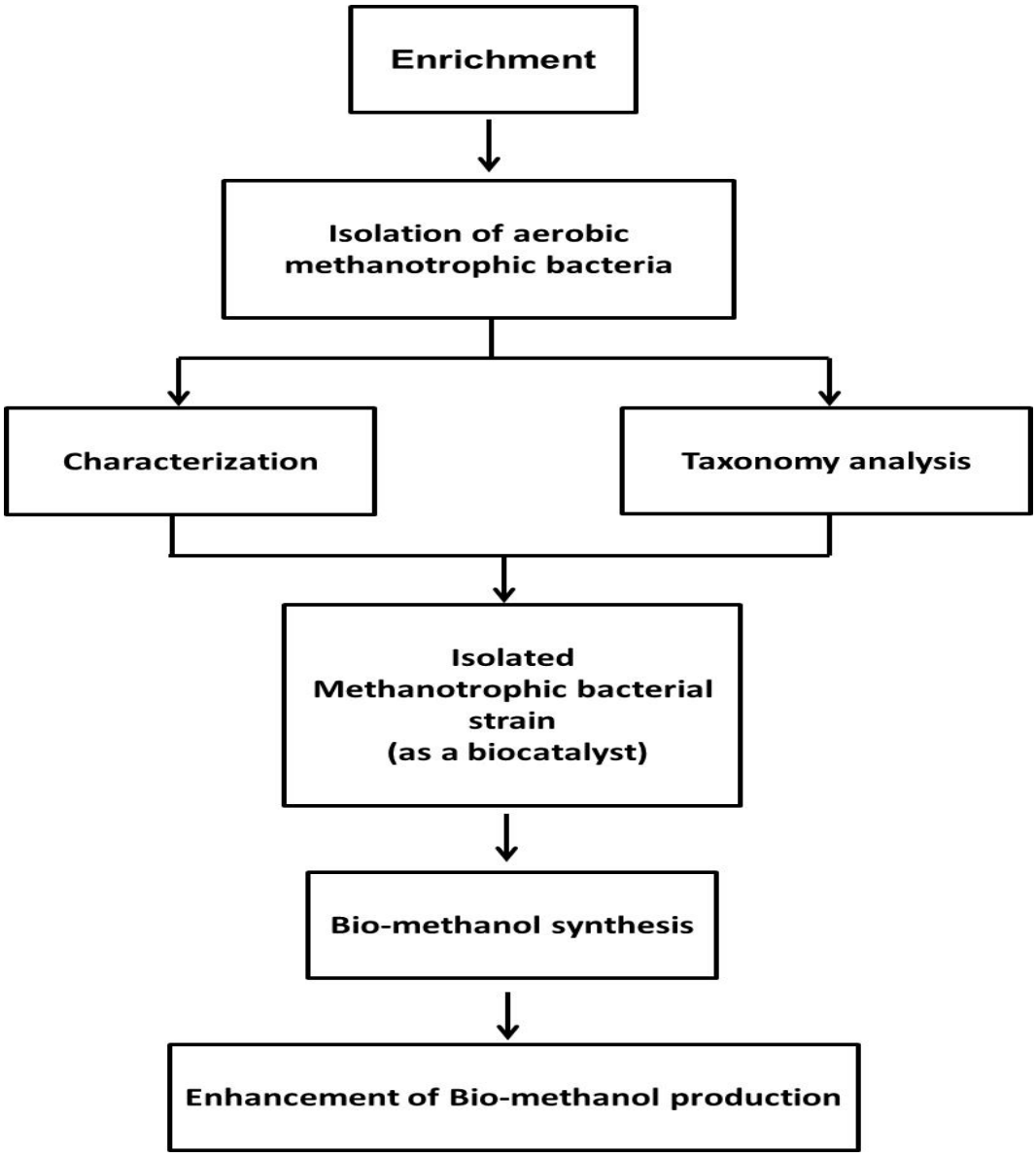

Fig.1-4. Schematic diagram of the research objective. In this study, a new methanotrophic strain isolated and methanol production were optimized.

II. Materials and methods

1. Isolation and characterization of methanotrophic bacteria

1-1. Soil samples

23 soil samples were collected from selected sites of rice paddy field (34° 53″N, 127° 8.186″ E and 35° 13.948″ N, 127° 13.23″E) around Jeollanam-do province (Fig. 2-1), the Republic of Korea.

1-2. Media and chemicals

Nitrate mineral salt (NMS) medium was used for the isolation and enrichment cultivation of methanotrophic bacteria (Deutzmann et al., 2014; Whittenbury et al., 1970). NMS medium contained (gl^{-1}): $NaNO_3$, 0.85; K_2SO_4, 0.17; $MgSO_4 \cdot 7H_2O$, 0.037; $CaCl_2 \cdot 2H_2O$, 0.007; KH_2PO_4, 0.53; Na_2HPO_4, 0.86; $FeSO_4 \cdot 7H_2O$, 0.224; and 2 mL of trace element solution. Trace element solution contained (gl^{-1}): $ZnSO_4 \cdot 7H_2O$, 0.000574; $CuSO_4 \cdot 5H_2O$, 0.0005; $MnSO_4 \cdot 7H_2O$, 0.000446; H_3BO_3, 0.000124; $Na_2MOO_4 \cdot 2H_2O$, 0.000096; and $COCl_2 \cdot 6H_2O$, 0.000096.

1-3. Enrichment and screening of methanotrophic bacteria

Each 2 gram soil sample was inoculated into 500 ml of NMS medium in a 1L Erlenmeyer flask and shown in (Fig. 2-2). The enrichment and isolation procedures were based on the method of Whittenbury (Whittenbury et al., 1970). The flask was sealed with a rubber stopper and headspace was filled with a gas mixture containing 60% (v/v) CH_4 (Korea Noble Gas CO., Korea) and 40% (v/v) air. Cells were enriched at 30 ℃ with agitation at 180 rpm for 15 days. An aliquot of the turbid suspension was then transferred to 100 ml of fresh NMS medium

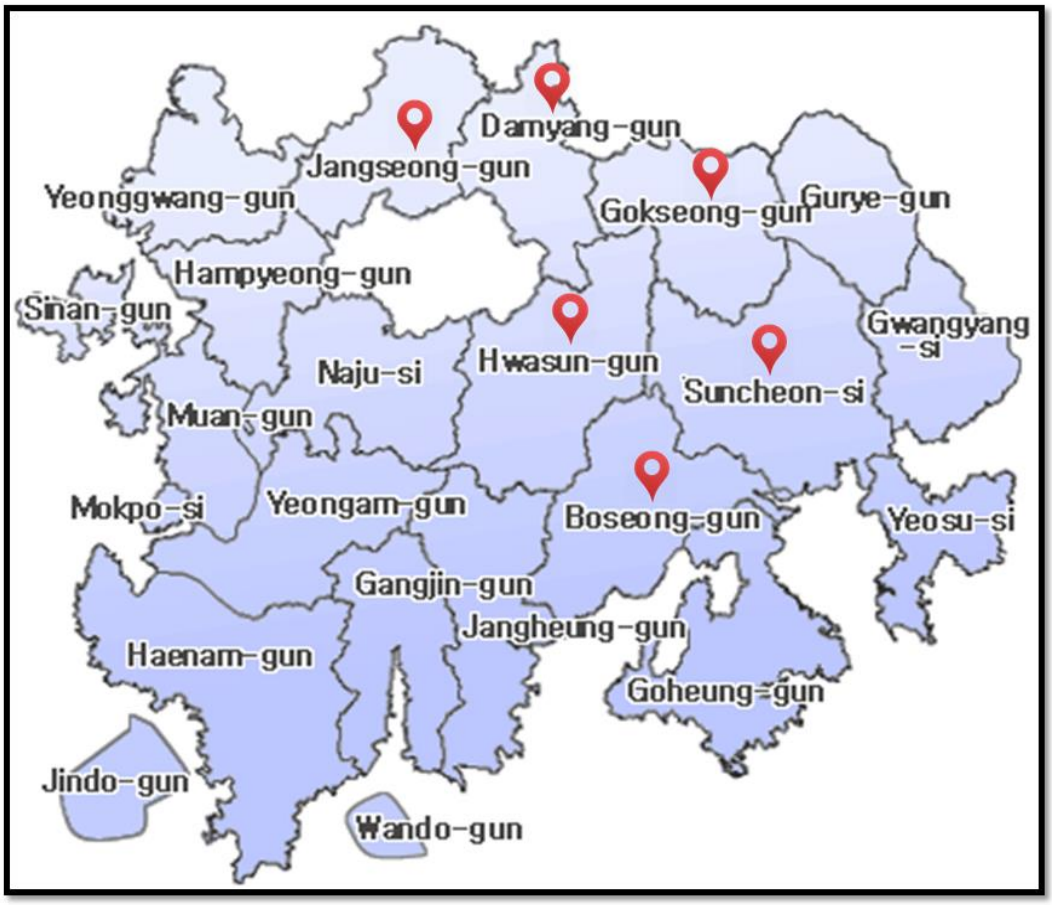

Fig. 2-1. Soil sampling sites around Jeollanam-do province.

in a 300 ml flask which was then filled with the CH_4/air mixture and incubated under the same conditions.

After two times transfers, 1 mL of suspension was serially diluted and plated on NMS agar plates containing 1.5% (w/v) noble agar (Sigma) and incubated at 30℃ in an airtight plastic box containing a CH_4/air mixture. Colonies observed within 6 days were picked, streaked on different fresh NMS agar plates and incubated under the same conditions. Finally, a bacterial strain that grew quickly was isolated and stored in 25% (v/v) glycerol at -80℃ for further study.

1-4. Morphological properties

Colony pigmentation and morphology were determined from the colonies grown on NMS agar plate. Cell morphology and size was observed by using a field emission scanning electron microscope and transmission electron microscopy.

(1) Sample preparation for SEM and TEM analysis of isolate

① **SEM procedure:**

The samples were fixed in 2.5% paraformaldehyde-glutaraldehyde mixture buffered with 0.1M phosphate (pH 7.2) for 2 hours, post fixed in 1% osmium tetroxide in the same buffer for 1 hour, dehydrated in graded ethanol, and substituted by isoamyl acetate. Then they were dried at the critical point in CO_2. Finally, the samples were sputtered with gold in a sputter coater (SC502, Polaron) and observed using the scanning electron microscope (JSM-7500F+EDS, Oxford, UK

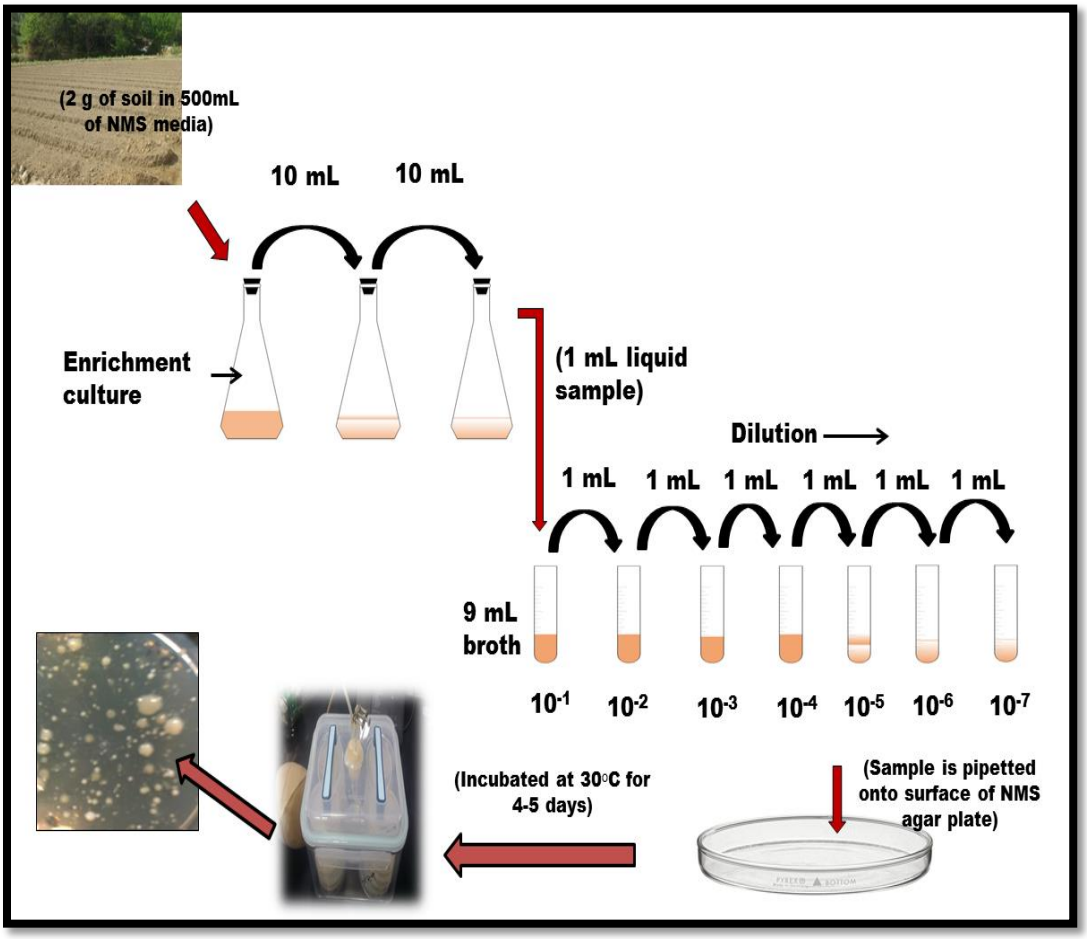

Fig.2-2. Enrichment culture and pure isolation of methane-oxidizing bacteria.

② **TEM procedure:**

For transmission electron microscopy, the tissues were fixed in 2.5% paraformaldehyde-glutaraldehyde mixture buffered with 0.1M phosphate (pH 7.2) for 2 hours, postfixed in 1% osmium tetroxide in the same buffer for 1 hour, dehydrated in graded ethanol and propylene oxide, and embedded in Epon-812. Ultra-thin sections, made by ULTRACUTE (Leica, Austria) ultramicrotome, were stained with uranyl acetate and lead citrate and examined under CM 20 (Philips, Netherlands) electron microscope.

1-5. Growth analysis

The cells generation time (g) and specific growth rate (μ) was calculated by measuring the Optical density (OD_{600nm}) of the cell suspension at regular time intervals with a spectrophotometer (DU 800, Beckman Coulter, USA).

$$\text{Specific growth rate}, \mu = \frac{(\ln A - \ln A_0)}{t - t_0}$$

$$\text{Generation time}, G = \frac{0.693}{\mu}$$

1-6. Biochemical properties

(1) Catalase activity analysis:

Catalase generally breaks down the hydrogen peroxide H_2O_2 into oxygen and water. To find out if our isolate is able to produce catalase enzyme, small inoculums of our isolate is mixed with hydrogen peroxide solution (3%) and estimated for the rapid elaboration of oxygen bubbles.

If no oxygen bubbles are formed then no catalase enzyme is present to hydrolyze the hydrogen peroxide.

(2) Oxidase activity analysis

This test was carried out by 1% (w/v) tetramethyl-*p*-phenylenediamine dihydrochloride. The development of dark purple color (indophenols) within 10 seconds shows that the test is positive and absence of color will reveal that the test is negative.

(3) Urease activity analysis

This test was performed by Stuart's urea broth method. Pure culture was inoculated into urea broth (stuarts) and incubated at 30℃ for 5 days.

1-7. Effect of pH, temperature and NaCl on bacterial growth

To find out optimal pH, NMS medium pH was adjusted from 4 to 8 with 0.1 M citric acid (acidic pH) and 0.2 M Na_2HPO_4 (basic pH) solution. To estimate the effect of agitation (140, 160, 180 and 200 rpm), temperature and salinity on cell growth, various temperature (20, 25, 30, 35, 40, and 55℃), and NaCl concentration (0.5, 1, 2, 3, 4 and 5%, w/w) conditions were optimized with supplied for 7 days.

1-8. Utilization of carbon and nitrogen sources

The entire test was done in triplicates. The isolate was examined using different carbon sources in the presence of sodium nitrate as a nitrogen source. Each of 0.2% (v/v or w/v) of methanol, ethanol, formaldehyde, monomethylamine, succinate, malate, $_{D-}$glucose, and $_{D-}$xylose

was used as a carbon source. All tests were carried out in 50 mL serum bottles containing 10mL of NMS medium. Carbon sources including volatile liquids were filter sterilized and added after autoclaving to the liquid medium. The utilization of nitrogen source was also determined in the presence of methane as a carbon source using NMS medium where potassium nitrate was replaced with the following compounds: 0.1% (w/v) of ammonium molybdate, ammonium amidosulfate, ammonium vanadate, ammonium dihydrogen phosphate, potassium nitrate, formamide, glycine, urea, yeast extract, ammonium oxalate, $_{L}$-alanine, $_{L}$-glutamine, $_{L}$-glutamic acid, $_{L}$-tryptophan, $_{DL}$-asparagine and casitone.

1-9. Molecular identification

(1) 16S rRNA, *pmoA* and *mmoXA* gene sequencing analysis

For molecular characterization of the isolate, each of 16S rRNA gene or marker gene *pmoA* and *mmoXA* was amplified using the PCR primer shown in Table 2-1. 16S rRNA and *pmoA* gene amplification were performed with pre-grown overnight cells, 1 μl each primers, 4 μl of dNTP mix (2.5 mM each), 5 μl of 10 × PCR buffer (TaKaRa, Japan), 38.75 μl of dd H_2O and 0.25 μl TaKaRA Ex Taq DNA polymerase (5 U μl^{-1}). PCR conditions for *pmoA* gene were as follows: initial denaturation for 5 min at 96℃; followed by 30 cycles of denaturation (1 min at 94℃), annealing (1 min at 56℃), and extension (1 min at 72℃); and a final extension at 72℃ for 5 min. For amplification of the *mm0X* and *mmoY* genes, PCR conditions were initial denaturation for 30 s at 94℃; followed by 30 cycles of denaturation (1 min at 92℃), annealing (1 min at 60℃), and extension (1 min at 72℃); and a final extension at 72℃ for 5 min. Whereas that for 16S rRNA gene were as follows; initial denaturation for 5 min at 98℃; 30 cycles of denaturation (30 s at 95℃), annealing (30 s at 55℃), and extension (90 s at 72℃); and a final extension at 72℃ for 10 min. The reactions were performed in 50 μl mixtures in 0.3 ml microcentrifuge

tubes. The PCR products were purified using a commercial DNA purification kit (QIAGEN, Germany), ligated to the pCR2.1 vector (Invitrogen, USA), and transformed into *E. coli* DH5α cells by chemical transformation. The recombinant plasmid was prepared from a 10 ml overnight culture in LB medium using silica spin columns (PureLink Quick Plasmid Miniprep kit; Invitrogen, USA) and sent for sequencing (Macrogen, Korea). After sequencing analysis, the 16S rRNA gene and the deduced *pmoA* sequences were compared with available sequences in the GenBank database using the NCBI tool, BLAST. Next, a phylogenetic tree was constructed using the neighbor-joining method with MUSCLE (Dereeper et al., 2008) based on bootstrap analysis of 1000 replications.

Table 2-1. PCR Primers used in this study

Target gene	**Primer**	**Sequence**	**Reference**
16S rRNA	27F	5'-AGAGTTTGATCMTGGCTCAG-3'	(Deutzmann et al., 2014)
	1492R	5'-TACGGYTACCTTGTTACGACTT-3'	
pmoA	A189f	5'-GGNGACTGGGACTTCTGG-3'	(Sasser, 1990)
	mb661r	5' -CCGGMGCAACGTCYTTACC-3'	
mmoX	*mmoXA*	5'-ACCAAGGARCARTTCAAG-3'	(Auman and Lidstrom, 2002)
	mmoXB	5'-TGGCACTCRTARCGCTC-3'	(Deutzmann et al., 2014)

(2) Whole genome sequence of the isolate

① General DNA manipulation

Total genomic DNA from strain LM6 was extracted using a Genomic BYF mini kit (iNtRON Biotechnology, Republic of Korea) following manufacturer's protocols. The plasmid was obtained using a plasmid purification mini kit (NucleoGen).

② Genome sequencing and assembly

Genome sequencing of the strain LM6 was performed using PacBio RS II single-molecule real-time (SMRT) sequencing technology (Pacific Biosciences, Menlo Park, CA). A standard PacBio library with an average of 20 kb inserts was prepared and was sequenced, yielding >187.46x average genome coverage. *De novo* assembly of the 101,766 subreads with 10,977 nucleotides on the average (1,117,183,184 bp in total) was conducted using the hierarchical genome-assembly process (HGAP) pipeline of the SMRT Analysis v2.3.0 (Chin et al. 2013). The overlapping regions at both ends of a contig were manually identified and trimmed to generate a unique stretch on both ends. Then, a new version for the contig was generated by cutting the contig into two halves and switching the first half with the second. The newly generated contig served as a reference to which raw PacBio reads were mapped using the resequencing module of the SMRT Analysis.

③ Gene prediction and annotation

Protein-coding genes were predicted by Prodigal v.2.6.3 (Hyatt et al., 2010). Signal peptides and transmembrane regions of predicted genes were predicted using SignalP v4.1

(Petersen et al., 2011) and TMHMM v2.0 (Krogh et al., 2001), respectively. BLAST-searches were performed against UniProt (Wu et al., 2006), Pfam (Finn et al., 2014) and COG (Tatusov et al., 2003) databases to functionally annotate predicted genes. Ribosomal RNA, transfer RNA and miscellaneous features were predicted using Rfam v12.0 (Griffiths-Jones et al., 2005). CRISPR's loci were predicted using CRISPR recognition tool (Bland et al., 2007). The antiSMASH web-the based tool was used for the prediction of secondary metabolite biosynthesis gene clusters (Medema et al., 2011). The graphical circular map of the complete genome was constructed and visualized using Circos v0.67 (Krzywinski et al., 2009a). The average nucleotide identity value was calculated with web-based ANI calculator (Goris et al., 2007).

1-10. Cellular fatty acid analysis

For cellular fatty acid analysis, cells were harvested at late exponential phase from NMS broth after incubation at 30 ℃. Fatty acids were saponified, methylated and extracted using the standard protocol of the Sherlock Microbial Identification System. Cellular fatty acids were analyzed by gas chromatography (model 6890; Hewlett Packard) and identified using the TSBA6 database of the MIDI (Sasser, 1990).

2. Biological conversion of CH_4 to methanol utilizing *Methylomonas* sp. LM6

2-1. Organism and growth conditions

Methylomonas sp. LM6 was cultivated in nitrate mineral salt medium (NMS) at 30^{o}C with agitation at 180 rpm till they reach their exponential/early stationary phase in a flask with a

stopper and headspace was filled with a gas mixture containing CH_4/air (6:4%, v/v). Cells were harvested by centrifugation at 15,000×g for 15 min at room temperature.

2-2. Methanol formation

Batch culture experiments were carried out in 70 mL serum bottles containing 10 mL of fresh NMS medium with 2 μM $CuCl_2$ and whole cells as an inoculum (Fig. 2-3). The reaction was initiated by replacing headspace of the vial with the same volume of CH_4/air mixture (6:4, v/v) and incubated at 30°C in a shaking incubator at 180 rpm for 24 h. After 24 h, 1 mL sample was acquired, centrifuged immediately at 4°C at 13,200×g for 10 min, filtered (0.2 μm) and the filtrates were analyzed.

2-3. Effect of MDH inhibitors

Methanol is an intermediate product in the metabolic pathway of methanotrophic bacteria and it is finally converted into CO_2 under normal conditions. Thus, accumulation and production of methanol necessitate inhibition of the MDH enzyme. A well-studied strategy for methanol accumulation is performed by MDH inhibition utilizing MDH inhibitors (Datta and Ogeltree, 1983; Dedysh and Dunfield, 2011).

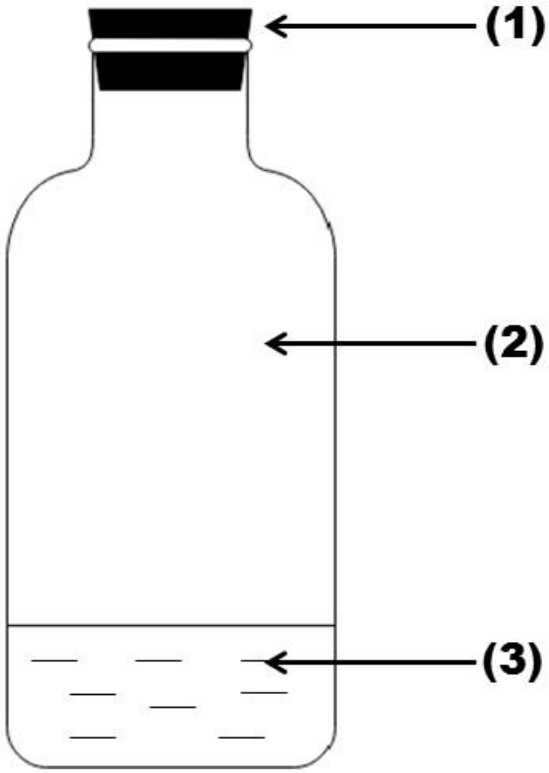

Fig. 2-3. Methanol synthesis in small scale glass serum vial. (1), butyl-rubber stopper; (2), gas phase (methane/air mixture (6:4, v/v)); (3), reaction mixture (10 mL) treated with cell biomass, MDH inhibitors and reducing agent (Na-formate).

To test for effective MDH inhibitors, inhibitors such as $MgCl_2$ (5-100 mM), potassium phosphate (10-150 mM), NH_4Cl (0-120 mM) and NaCl (10-100 mM) were optimized. These inhibitors were resuspended in NMS media containing 2 µM $CuCl_2$, whole cells (0.6 mg DCM mL^{-1}) with CH_4/air mixture (6:4, v/v)) and incubated at 30°C, 180 rpm for 24 h. All the experiments were performed in duplicates.

2-4. Effect of Na-formate on methanol production

When MDH is inhibited, an additional electron donor, usually formate, is needed to maintain cell vitality and as an (NADH) regeneration (Xumeng et al., 2014). Therefore, to improve methanol production, the effect of Na-formate (10–150 mM) as a cofactor was also evaluated. These Na-formate concentrations were resuspended in NMS media containing the various optimum MDH inhibitors discussed above (in section-2.3) and 2 µM $CuCl_2$, whole cells (0.6 mg DCM mL^{-1}) with CH_4/air mixture (6:4, v/v) and incubated at 30°C, 180 rpm for 24 h.

2-5. Effect of methane concentration and the inoculum load on methanol production

The methanol production profile was evaluated at different ratios of CH_4/air mixture (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1) for up to 36 h of incubation under optimum conditions (pH 6.8, 30ºC, and 180 rpm). The effect of altering the inoculum load was investigated by adding DCM in the ranges of 0.4–1.2 mg/ml to the reaction mixture (containing 80 mM of Na-formate and 90 mM NH_4Cl as an MDH inhibitor) in 10 mL NMS medium under optimum conditions, of CH_4 as a feed.

2-6. Optimization of process parameters

Methanol production was evaluated at pH 5.5-8.5 in Na-acetate (pH 5.5) and phosphate (pH 6.0-8.0) in 10 mL of fresh NMS medium (containing 80 mM of Na-formate and 90 mM NH_4Cl as a MDH inhibitor) and 0.6 mg DCM mL^{-1} in a 70 mL serum vial which was then filled with the CH_4/air mixture (6:4, v/v) and incubated at 30°C for 24 h. To evaluate the effect of incubation temperature on methanol production, cultures were incubated at different temperatures (20-40°C) at optimum pH with same conditions above. The effect of different agitation rates (120-200 rpm) on methanol production was evaluated at optimum pH and temperature after 24 h of incubation under the same conditions described above.

2-7. Effect of the copper concentration on methanol production

To assess the effect of copper concentrations on methanol production, whole cells were added in the 10 mL reaction mixture containing $CuCl_2$ concentration in the range of (0-10 μM) and 80 mM of Na-formate and 90 mM NH_4Cl as an MDH inhibitor in 70 mL serum vial, which was each sealed with a rubber stopper. All the vials were then incubated at 30℃ with continuous agitation at 180 rpm under an atmosphere of the CH_4/air mixture (6:4, v/v).

2-8. Synergistic effects of MDH inhibitors on methanol production

Further characterization was conducted to investigate the synergistic effect of MDH inhibitors on methanol production. The reaction mixture (100 mL), treated with 90 mM NH4Cl, 60 mM potassium phosphate, 60 mM MgCl2, 80 mM Na-formate and EDTA (0-4 mM), and 0.6 mg dry cells/mL was introduced into a 500 mL Erlenmeyer flask with a tight rubber screw cap.

The reaction was initiated by replacing 250 mL of air in the headspace of the flask with the same volume of CH_4/ air mixture (6:4, v/v) and incubated at 30°C for 24 h.

3. Enhancement of bio-methanol synthesis from CH_4 by *Methylomonas* sp. LM6

3-1. Screening and selection of organic solvent 1-octanol as methane soluble agent

CH_4 solubility by the organic solvents such as n-Hexane, paraffin oil, 1-octanol, benzene and tetraethylene glycol dimethyl ether that has been reported for the potential liquid phase CH_4 solubility were investigated (Bottger et al., 2016a, b; Han et al., 2009; Henni et al., 2006). First, the CH_4 solubility in distilled water and in organic solvents was investigated by a gas chromatography (GC-2010; Shimadzu, Japan). For this experiment, tests were conducted in a 70 mL serum vial containing 10 mL distilled water with organic solvents (1%, v/v) and were supplied with CH_4/air mixture (6:4, v/v) and incubated at 180 rpm, 30°C for 24 h. No cells were inoculated during this experiment. After incubation, 1 mL of the liquid sample was acquired at room temperature for GC analysis. Each test was performed in duplicates.

3-2. Effect of organic solvents on bacterial growth

To estimate the inhibition potential of organic solvents on the strain LM6 growth was performed. Sealed 70-mL glass vials containing 10 mL NMS medium with addition of organic solvents at 1% (v/v) and strain LM6 (1%, w/v). CH_4/air mixture (6:4, v/v) in the headspace was added as described above. The vials were incubated at 30°C with agitation at 180 rpm until the growth of the strain. Each test was performed in duplicates. Growth in each of the glass vials was indicated and compared to that of control (no organic solvents).

3-3. Effect of 1-octanol on bacterial growth

Tests to determine the inhibition potential of 1-octanol on the bacterial growth were estimated. Strain LM6 (1%, w/v) was inoculated into 10 mL of NMS medium in 70 mL sealed glass vials with a headspace containing a CH_4/air mixture (6:4, v/v) and 1-octanol (1%, v/v). The vials were incubated at 30°C with agitation at 180 rpm until the growth of the strain. Each test was performed in duplicates. Growth in each of the glass vials was indicated and compared to that of control (no organic solvents).

3-4. Methanol production using methane soluble agent, 1-octanol

The schematic diagram explaining lab-scale experimental design steps involved for the solubility of CH_4 in NMS medium (10 mL) utilizing 1-octanol (0-4%, v/v) is shown in Fig. 2-4. The CH_4 content was measured periodically. Subsequentially, for optimizing the optimal concentration of 1-octanol, methanol production was performed according to the method described in Fig.1. In the following reaction, the harvested cells (0.6 mg DCM mL^{-1}) were suspended in 10 mL acquired NMS reaction mixture (containing 2 µM $CuCl_2$, 90 mM NH_4Cl, 60 mM potassium phosphate, 3 mM EDTA, and 80 mM Na-formate) into 70 mL sealed glass flasks, without any additional CH_4/air mixture in the headspace. The flasks were incubated at 30°C for 24 h in a shaking incubator at 180 rpm. After the incubation, as described in section 2.3, 1 mL of samples were acquired, filtered (0.2 µm) and the filtrates were optimized for methanol analysis through GC. Each test was performed in duplicates.

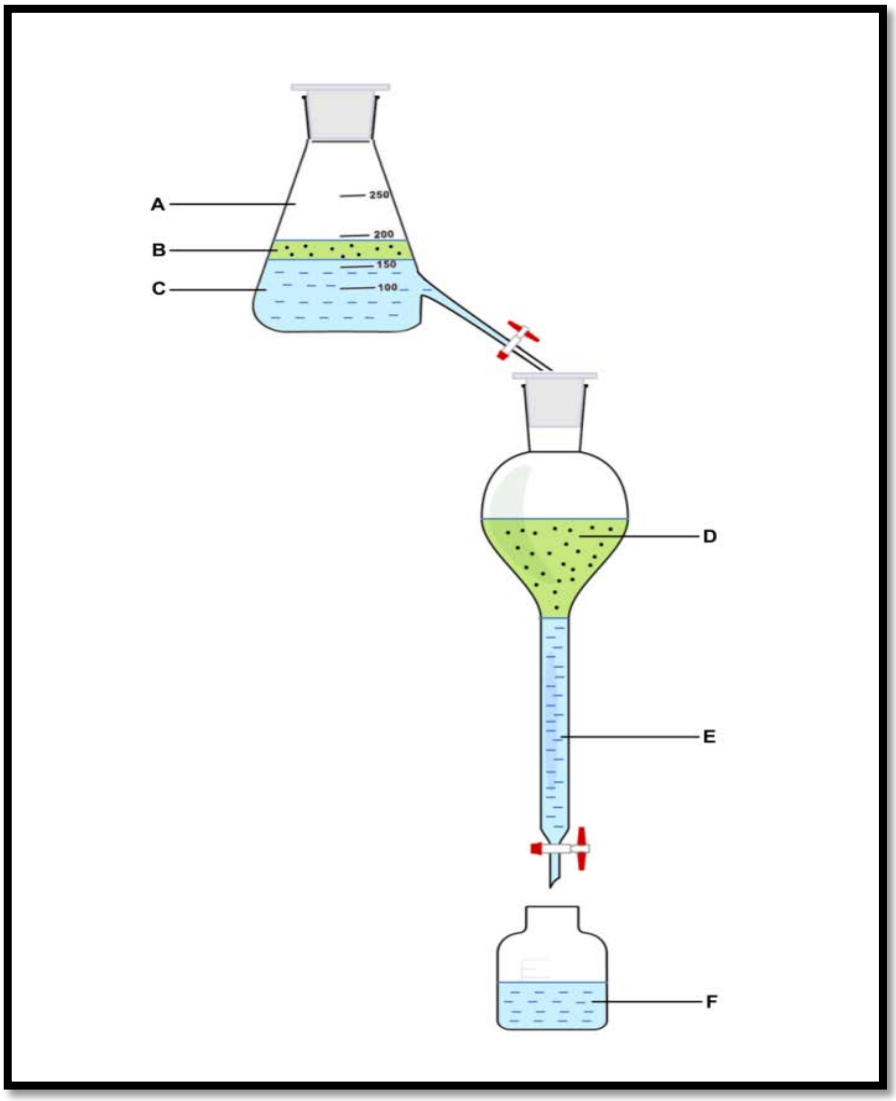

Fig. 2-4. Improvement of methane solubilization in a reaction mixture with organic solvent, 1-octanol, and removal of the solvent. A, CH_4/air mixture (6:4, v/v); B, 1-octanol; C, reaction mixture (NMS media); D, 1-octanol; E & F, reaction mixture containing solubilized CH_4. This system undergoes two steps: **Step 1**, the 1-octanol (0-4%, v/v) was added to the reaction mixture, where CH_4/air mixture (6:4, v/v) was supplied, and agitated for 24 h at 30°C, 200 rpm. After incubation, the mixture was applied into a separatory funnel and allowed to stand for 1 h at room temperature for separation; **Step 2**, the separated reaction mixture containing high concentrated CH_4 was utilized for methanol synthesis. The separated 1-octanol was stored and reused.

4. Enzyme assay

pMMO enzyme activity was determined by assaying propylene oxide (PO) concentration produced from the epoxidation of propylene by gas chromatography (Burrows et al., 1984). MDH enzyme activity was measured spectrophotometrically as described by Anthony & Zatman using a two dye linked assay system (2,6- dichlorophenol indophenol (DCPIP) as terminal acceptor and phenazine methosulfate (PMS) as an artificial electron acceptor) (Anthony and Zatman, 1965). The reaction mixture (working volume = 3mL) for spectrophotometric assay system contained: 100 mM Tris-HCl buffer (pH 9.0), 13.4 mM methanol, 3.3 µM PMS, 0.04 mM DCPIP, 1 mM KCN and 60 mM potassium phosphate. The reaction was initiated by addition of MDH, and the DCPIP reduction was measured by the decrease in A_{600} (Cox et al., 1992).

5. Analytical Methods

The CH_4 content was analyzed by gas chromatography equipped with an Agilent J&W HP-Plot Q capillary column (30 m × 0.53 mm × 40 µm) and a thermal conductivity detector. Helium was used as the carrier gas at 2 mL min rate. The temperatures of the injector, column and detector were 200, 180, and 200°C, respectively. Methanol and propylene oxide concentration was analyzed by Agilent J&W PoraBOND U capillary column (25 m × 0.32 mm × 7 µm) and a flame ionization detector was used. Helium was used as the carrier gas at a linear velocity of 42.6 cm/s, and a split ratio of 10.0. For methanol and propylene oxide analysis, the injector, column and detector temperatures were 280, 150 and 250°C, and 100, 80, and 100°C, respectively. For propylene oxide analysis, helium as carrier gas at total flow rate 30 mL min^{-1}.

III. Results and Discussion

1. Isolation and characterization of methanotrophic bacteria

1-1. Cell morphology and physiology

After several iterations of enrichment cultures, one bacterial strain that grew quickly under an atmosphere of methane/air (60:40) was isolated and used for further study. The colony is pink-orange pigmented and round with opaque form (Fig. 3-1). The isolate was motile, gram-negative, short rod (approximately 1.3 X 0.73 μm) (Fig. 3-2 (a)) and occurred as single. Temperature range for growth was 20-40 ℃ with an optimum 27-30 ℃ and pH range was 5.5-8.0 with an optimum 7.0. It could grow on 0-1.0% (v/v) methanol but was sensitive to NaCl concentration. The cell was oxidase- and urease-positive but catalase-negative. Table 3-1 shows the comparative analysis of morphological, biochemical and growth characteristics of our isolate with related genera species of methanotrophic bacteria. The isolate shared many properties with the species of genus *Methylomonas*, a type I MOB. When grown on NMS medium (pH 7.0) with methane/air mixture at 30 ℃, a specific growth rate and generation time were 0.037 h^{-1} and 18.64 h, respectively.

Table 3-1. Growth characteristics of isolate compared with other *Methylomonas* species.

Characteristics	**Isolate**	***M. koyamae***	***M. fodinarum***	***M. methanica***	***M. paludis***
Isolation source	Rice paddy field	Rice paddy field	Coal mine drainage	TCE contaminated aquifer	Acidic Sphagnum peat bog
pMMO	+	+	+	+	+
sMMO	–	–	NR	V	–
Cell size (μm)	1.3×0.73	0.9×1.7	0.7–1.0×0.8–1.2	0.6×1.0	1.0–1.5×1.0–4.0
Cell morphology	rod	rod	rod	rod	rod
Pigmentation	Pink-orange	Pink-orange	Orange	Pink	Pale pink
Motility	+	+	+	+	–
Temperature range (°C)	20-40	10-40	10-35	10-37	8-30
pH range	5.5-8.0	5.5-7.0	5.5-9.0	5.5-9.0	3.8-7.3
Growth with 1.0% methanol	+	+	+	+	+
NaCl tolerance 1.0 (%)	+	–	+	+	–
Reference	In this study	(Ogiso et al., 2012)	(Bowman et al., 1990)	(Koh et al., 1993)	(Danilova et al., 2013)

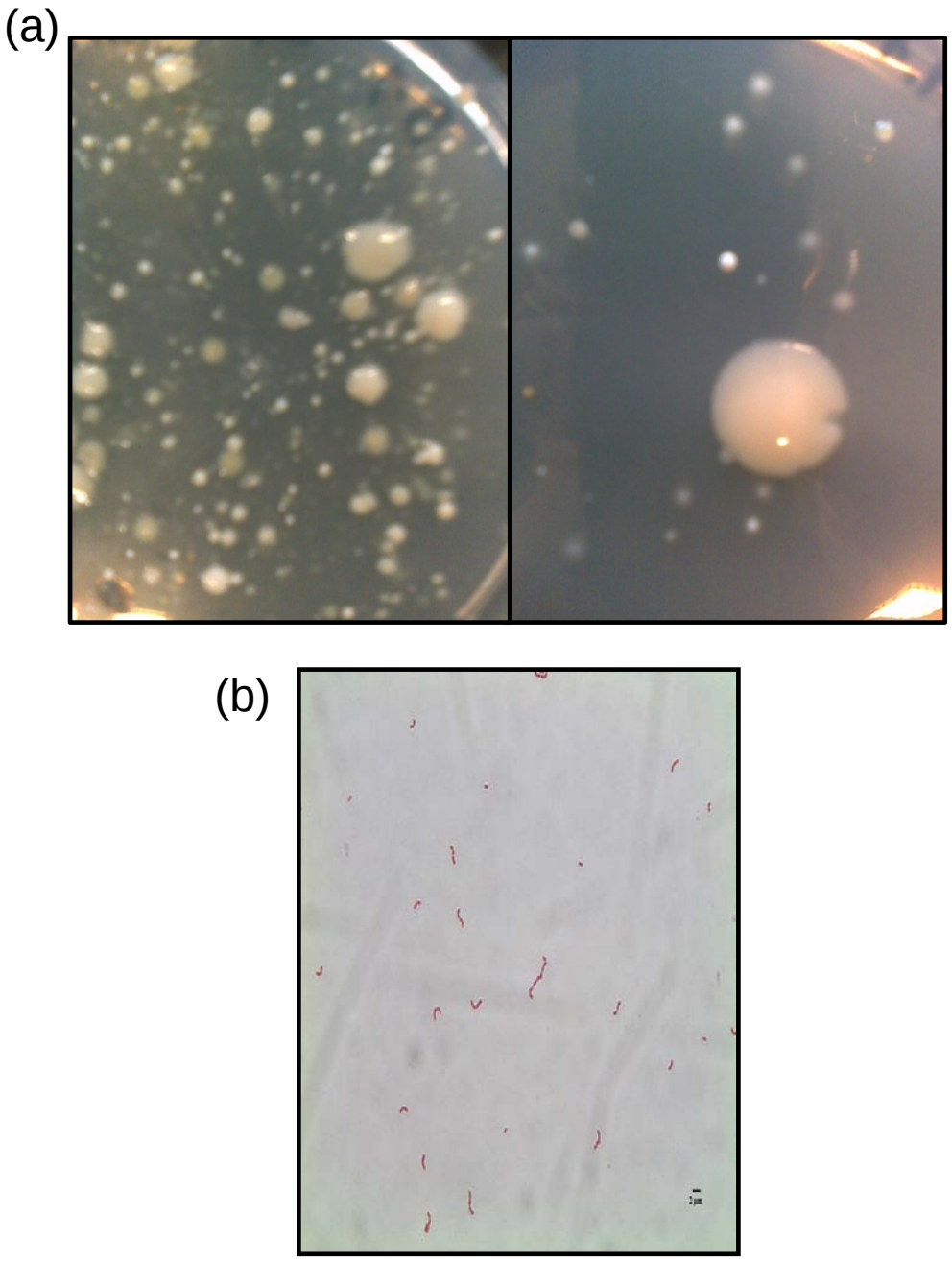

Fig. 3-1. Colony on NMS medium (a) and gram staining of the isolate (scale bar = 2 μm) (b).

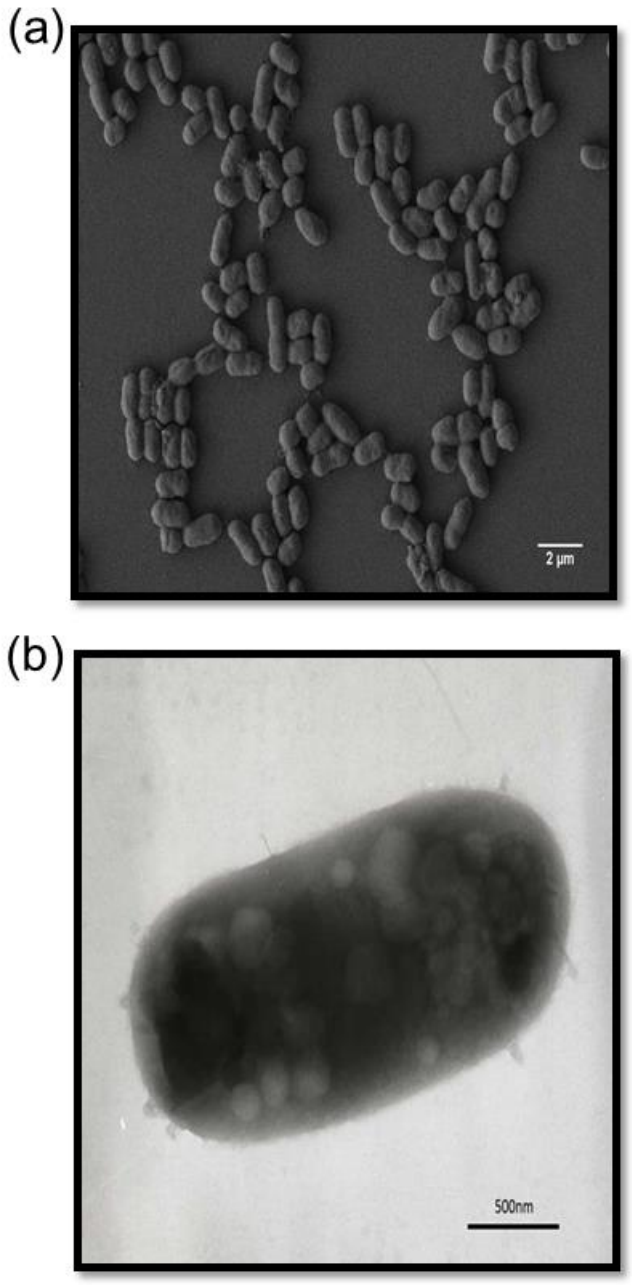

Fig. 3-2. Scanning electron micrographic image of the aggregated isolate (scale bar = 2 μm) (a). Negative staining electron micrographic image of strain LM6 (scale bar = 0.5 μm) (b).

1-2. Growth analysis

The specific growth rate and a generation time of isolate during exponential growth in NMS medium (days 2 to 5) were determined in two independent experiments and found to be 0.07 h^{-1} and 9.48 h. The doubling time of the strain was found significantly shorter when compared with the well–known methanotrophic bacteria *Methylosinus trichosporium* OB3b (0.05 h^{-1} and 13.94 h) (Fig. 3-3). The result of cell growth revealed that our strain has higher growth, and *M. trichosporium* OB3b showed slower growth rate at the same incubation conditions.

1-3. Utilization of carbon and nitrogen sources

The utilization of carbon and nitrogen source by strain LM6 is shown in Table 3-2. The results show that isolate could utilize only methane and methanol as sole carbon and energy sources but no growth was found on ethanol, formaldehyde, monomethylamine, acetate, succinate, malate, $_{D-}$glucose, and $_{D-}$xylose. In addition, ammonium amidosulfate, ammonium dihydrogen phosphate, potassium nitrate, $_{L-}$alanine, $_{L-}$glutamic acid and casitone could be used as nitrogen source for our strain.

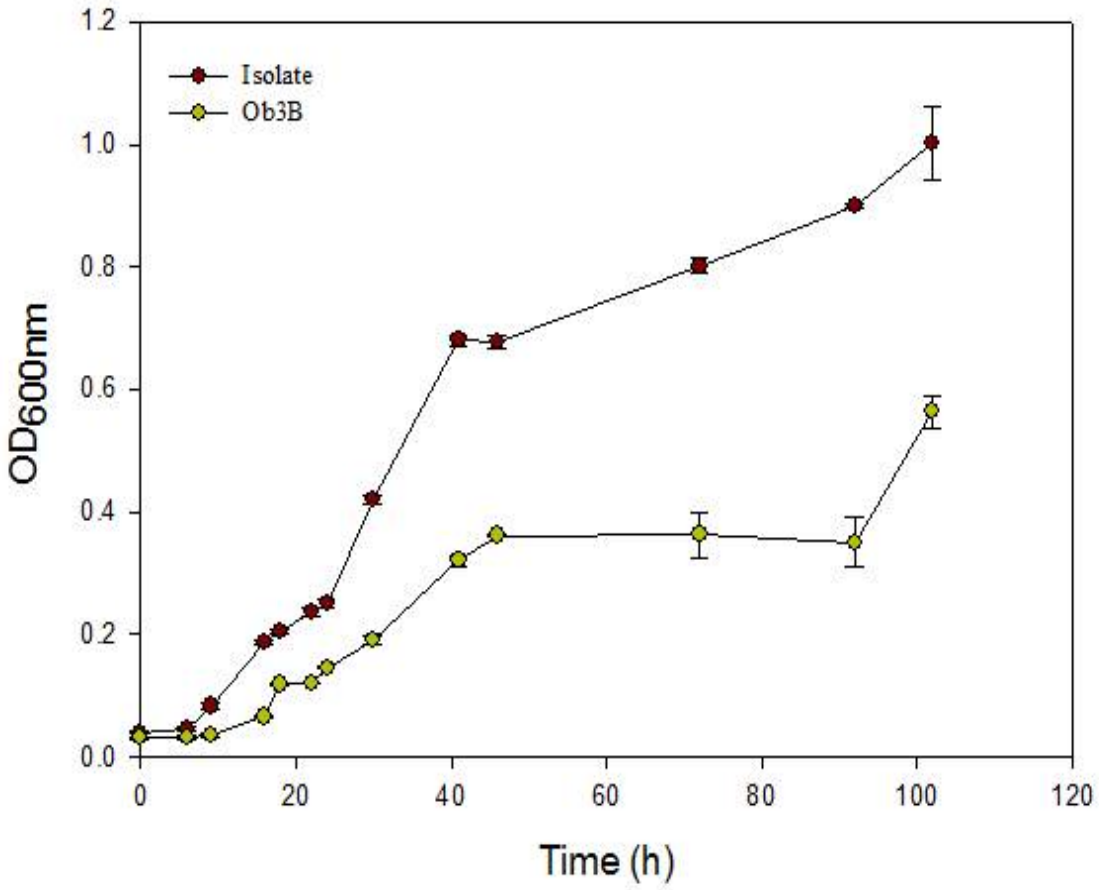

Fig. 3-3. Comparison of growth rate between the isolate and *Methylosinus trichosporium* OB3b in an Erlenmeyer flask containing NMS medium supplied with methane/air (6:4, v/v) mixture.

Table 3-2. Utilization of various carbon and nitrogen sources

Sources	Growth
Carbon sources	
methane	+
methanol	+
ethanol	-
formaldehyde	-
monomethylamine	-
acetate	-
succinate	-
D-glucose	-
D-xylose	-
malate	-
Nitrogen sources	
ammonium amidosulfate	-
ammonium dihydrogen phosphate	+
ammonium molybdate	-
ammonium oxalate	-
ammonium vanadate	+
potassium nitrate	+
formamide	-
glycine	-
urea	-
yeast extract	-
casitone	-
L-alanine	+
L-glutamine	-
L-glutamic acid	+
L-tryptophan	-
DL-asparagine	-

1-4. Molecular and phylogenetic analysis

The functional genes and phylogenetic diversity of the bacterial isolate were analyzed. The *pmoA* gene was successfully amplified by PCR, whereas *mmoX* gene was not detected. Phylogenetic analysis of 16S rRNA gene sequences revealed that the isolate was dominated by the family *Methylococcaceae* of the class *Gammaproteobacteria* (Fig.3-4). The highest level of 16S rRNA gene sequence similarity was observed between the isolate and the members of the genus *Methylomonas* and the closest relatives of the isolate is *Methylomonas koyamae* (100%). In addition, phylogenetic analysis of the *pmoA* gene indicated that all *pmoA* sequences determined were closely related to the type I methanotrophs in the *Gammaproteobacteria* and belonged to the genus *Methylomonas* (Fig.3-5). The isolate displays 96% nucleotide sequence identity with the *pmoA* gene fragment of *Methylomonas koyamae*. Based on these morphological, biochemical and molecular data, the isolate was named as *Methylomonas* sp. LM6.

1-5. Genome sequencing analysis

The genome distance between *Methylomonas koyamae* type strain JCM 16701 (NZ_BBCK01000001) and *Methylomonas* sp. LM6 is estimated to 97.0% based on a two-way average nucleotide identity. This value showed that the strain LM6 belongs to the species *Methylomonas koyamae*.

Two particulate methane monooxygenase operons (pmoABC, XXXX_3758-XXXX_3760; pmoCAB, XXXX_3996-XXXX_3998) were identified, whereas the soluble methane monooxygenase operon was not found in this genome.

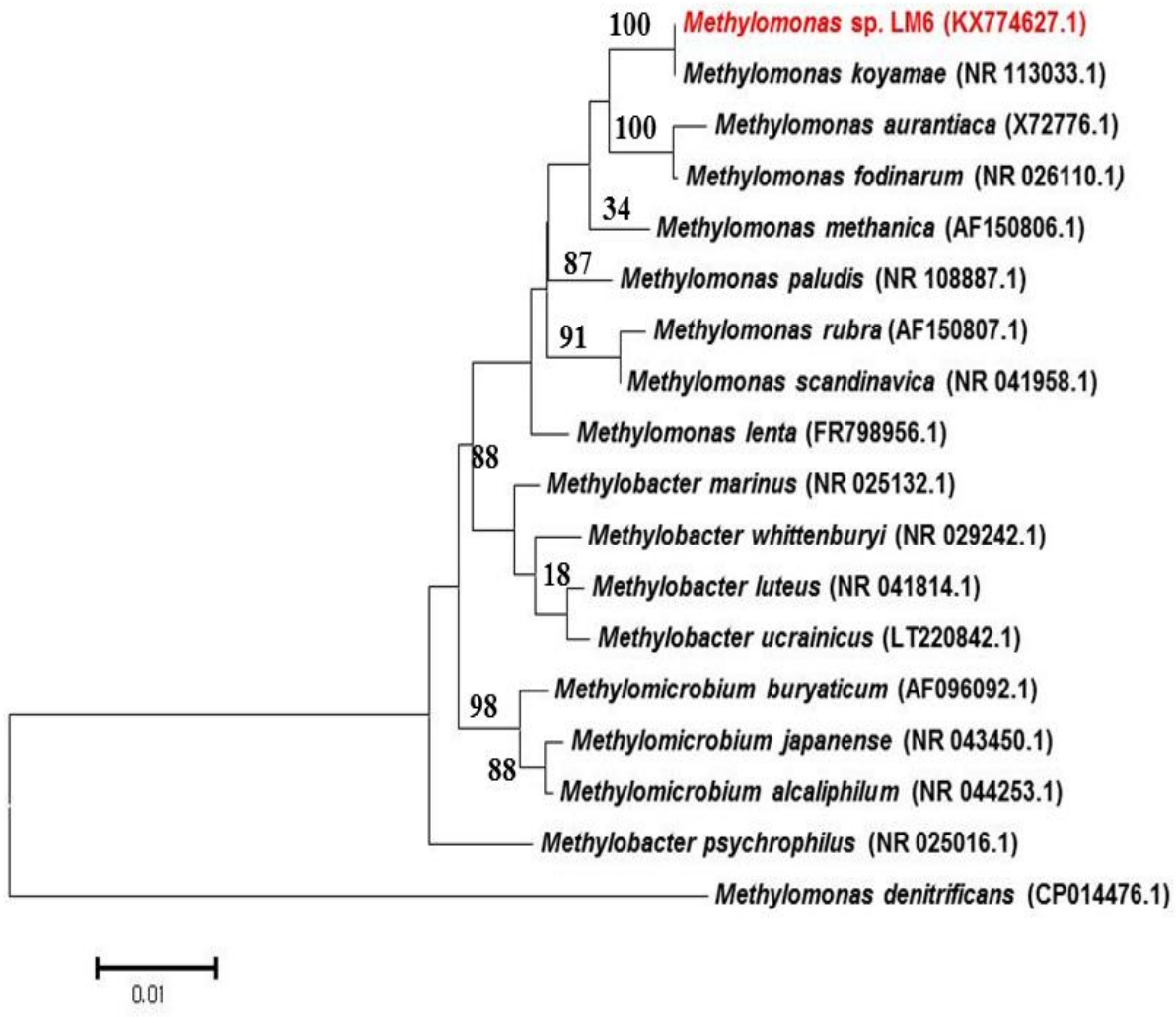

Fig. 3-4. The evolutionary history was inferred using the Neighbor-Joining method. Phylogenetic tree based on 16S rRNA gene sequences from methanotrophic bacteria belonging to *γ-Proteobacteria* displaying the relation of the isolate and another type I MOB related species. Genbank accession numbers of the strain are given in parenthesis. 0.01 substitutions per nucleotide sequence position.

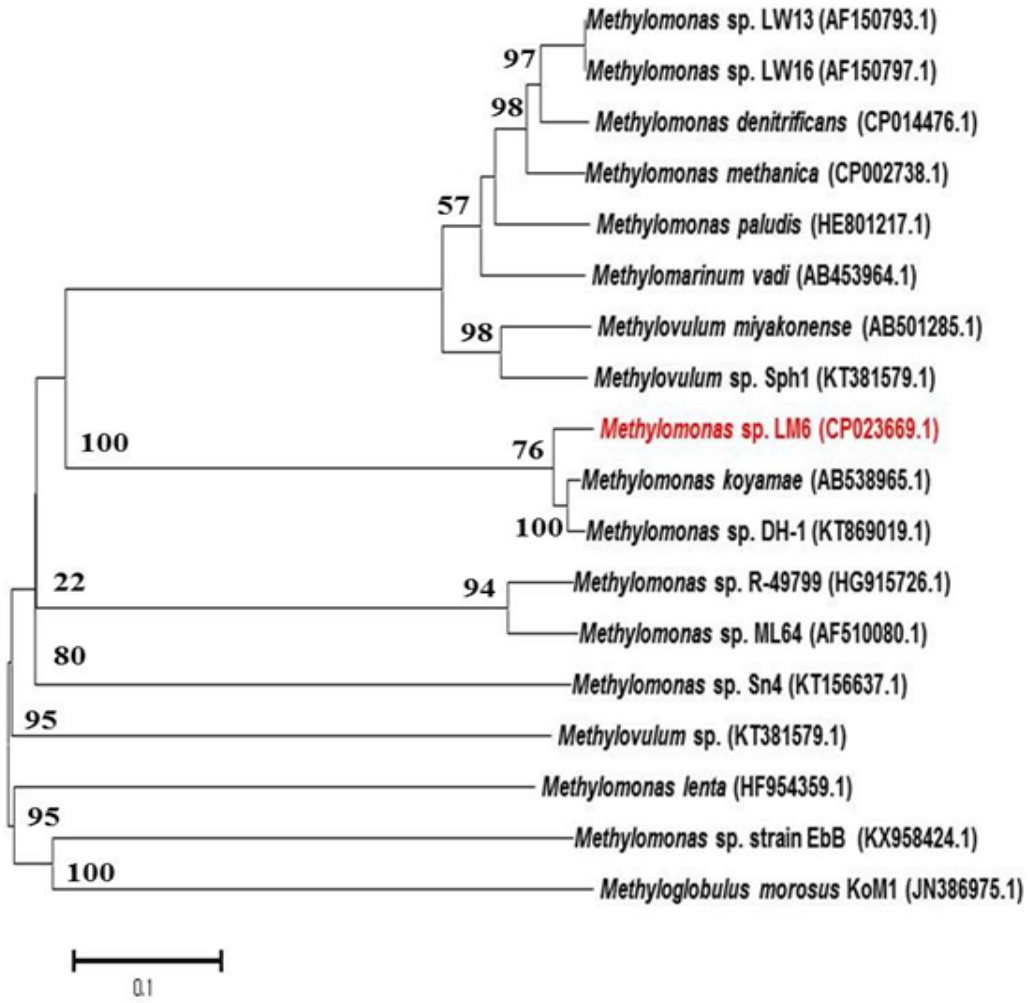

Fig. 3-5. Phylogenetic trees based on *pmoA* gene sequences presenting the relationships between the isolate and different type I MOB or methylotrophic bacteria. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.39389969 is shown. Genbank accession numbers of the strain are given in parenthesis. 0.1 substitutions per nucleotide sequence position.

The pmoABC operon is located between the genes related to electron transfer processes such as cytochrome d ubiquinol oxidase (XXXX_3744, XXXX_3746) and the [Ni,Fe] hydrogenase assembly proteins (e.g. XXXX_3772, XXXX_3786). The pmoCAB operon is located at the downstream of a type VI secretion system gene cluster (XXXX_3976-XXXX_3994). Also, the genome possesses the gene clusters for proteins involved in methanol oxidation (MxaFJGIRPSACKL, XXXX_3200-XXXX_3210) as well as them for biosynthesis of pyrroloquinoline quinone cofactor (XXXX_0572-XXXX_0576), tetrahydromethanopterin-linked pathway (e.g. XXXX_1778, XXXX_1781), and the formate oxidation to carbon dioxide (NAD-dependent formate dehydrogenase, XXXX_3478- XXXX_3482). However, unlike genomes of Methylocystis spp., the malyl-CoA/(S)-citramalyl-CoA lyase and the glycerate dehydrogenase involved in serine cycle was not detected (Stein et al., 2011, Del Cerro et. al., 2012). The genome encodes proteins involved in the complete ribulose monophosphate pathway and TCA cycle. The presence of these genes suggests that the strain incorporates the methane into the central metabolic pathway via these pathways.

Three CRISPR-associated gene clusters are encoded in this genome. One gene cluster (XXXX_1294- XXXX_1309) located at upstream of ureABCEFG encodes the type I-E CRISPR-associated proteins including the Cse1 and Cse2. Other cluster (XXXX_1943-XXXX_1948) encodes the type I-F CRISPR- associated proteins including the Csy1, Csy2, and Csy3 (XXXX_1945- XXXX_1947). The other cluster encodes the type I-U CRISPR-associated proteins including the Cas7 and Cas5/Cas6 (XXXX_2394, XXXX_2395).

It has been reported that some of *Methylomonas* spp. can fix the nitrogen (Hoefman et al., 2014). The genome also has the nitrogenase-associated genes containing nifV (XXXX_0904), nifSU, iscA-nif, nifZ (XXXX_3507-XXXX_3510), nifO (XXXX_3359), nifQ (XXXX_3355),

and nifB (XXXX_3361) as well as the nifHDKTY operon (XXXX_3424-XXXX_3429) which encodes the nitrogenase complex (Spatzal et al., 2011), the nifENX operon (XXXX_0974-XXXX_0976) which encodes protein for iron-molybdenum cofactor biosynthesis (Shah et al.,1999), and nifWZM operon (XXXX_0909-XXXX_0911) which encodes proteins for activation of iron-molybdenum proteins (Paul & Merrick, 1989). The nitrate reductase (XXXX_2172) and the nitrite reductase (XXXX_2083-XXXX_2084) were also detected, which might contribute that the strain produces the ammonium molecule or amino acids from diverse nitrogen sources.

The graphical circular map of the complete genome was constructed and visualized using Circos v0.67 (Krzywinski et al., 2009b). The genome of *Methylomonas* sp. LM6 consists of one circular chromosome of 4,894,002 bp and one plasmid of 186,658 bp with 4,337 protein coding sequences (CDSs), 48 tRNA genes, 9 rRNA genes, and an average G+C content of 56.23% (Table 3-4, Fig. 3-6 and Fig. 3-7).

The COG functional categories of the four complete genome sequences are shown in Table 3-3. Among the 4,337 CDSs of *Methylomonas* sp. LM6, 1,338 CDSs were classified into COG categories (Tatusov et al., 2003). The major categories of *Methylomonas* sp. LM6 were transcription (K), replication, recombination and repair (L), signal transduction mechanisms (T), Cell wall/membrane biogenesis (M), amino acid transport and metabolism (E), carbohydrate transport and metabolism (G) and inorganic ion transport and metabolism (P).

The *Methylomonas* sp. LM6 whole genome sequence information provided in the present study should facilitate future studies of the metabolic diversity of the genus *Methylomonas*.

1-6. Nucleotide sequence and strain accession numbers

The genome sequence of *Methylomonas* sp. LM6 was deposited into the GenBank under the accession number CP023669 (chromosome) and CP023670 (plasmid). The strain is available from the Korean Collection for Type Culture (KCTC) under the accession number KCTC 62176.

Table 3-3. Genome sizes of chromosome and plasmid in the isolate.

Label	***Size (bp)***	***Topology***
Chromosome	4,894,002	Circular
pLM6	186,658	Circular

Table 3-4. Genomic statistics of the isolate

Attribute	Chromosome		Plasmid	
	Value	% of Total	Value	% of Total
Genome size (bp)	4,894,002	100.00	186,658	100.00
Protein coding (bp)	4,281,999	87.49	150,393	80.57
DNA G+C (bp)	2,761,736	56.43	94,973	50.88
DNA scaffolds	1		1	
Total genes	4,337	100	153	100.00
Protein coding genes	4,192	96.66	145	94.77
RNA genes [a, b]	59	1.36	0	0.00
Pseudo genes	86	1.98	8	5.23
Genes with function prediction	2731	62.97	48	31.37
Genes assigned to COGs	2950	68.02	49	32.03
Genes with Pfam domains	3235	74.59	84	54.90
Genes with signal peptides	484	11.16	14	9.15
Genes with transmembrane helices	1013	23.36	27	17.65
CRISPR repeats	3	0.35	1	0.17

[a]No. of rRNA operon : 3 copies

[b]No. of tRNA genes : 48 genes

Table 3-5. COG functional categories of the isolate.

Code	Functional annotation	Count	Proportion
J	Translation, ribosomal structure and biogenesis	158	3.64
A	RNA processing and modification	1	0.02
K	Transcription	193	4.45
L	Replication, recombination and repair	269	6.2
B	Chromatin structure and dynamics	1	0.02
D	Cell cycle control, cell division, chromosome partitioning	39	0.9
V	Defense mechanisms	67	1.54
T	Signal transduction mechanisms	336	7.75
M	Cell wall/membrane biogenesis	236	5.44
N	Cell motility	112	2.58
U	Intracellular trafficking and secretion	116	2.67
O	Posttranslational modification, protein turnover, chaperones	158	3.64
C	Energy production and conversion	192	4.43
G	Carbohydrate transport and metabolism	117	2.7
E	Amino acid transport and metabolism	200	4.61
F	Nucleotide transport and metabolism	58	1.34
H	Coenzyme transport and metabolism	131	3.02
I	Lipid transport and metabolism	70	1.61
P	Inorganic ion transport and metabolism	217	5
Q	Secondary metabolite biosynthesis, transport and catabolism	82	1.89
R	General function prediction only	368	8.49
Z	Cytoskeleton	0	0
S	Function unknown	307	7.08

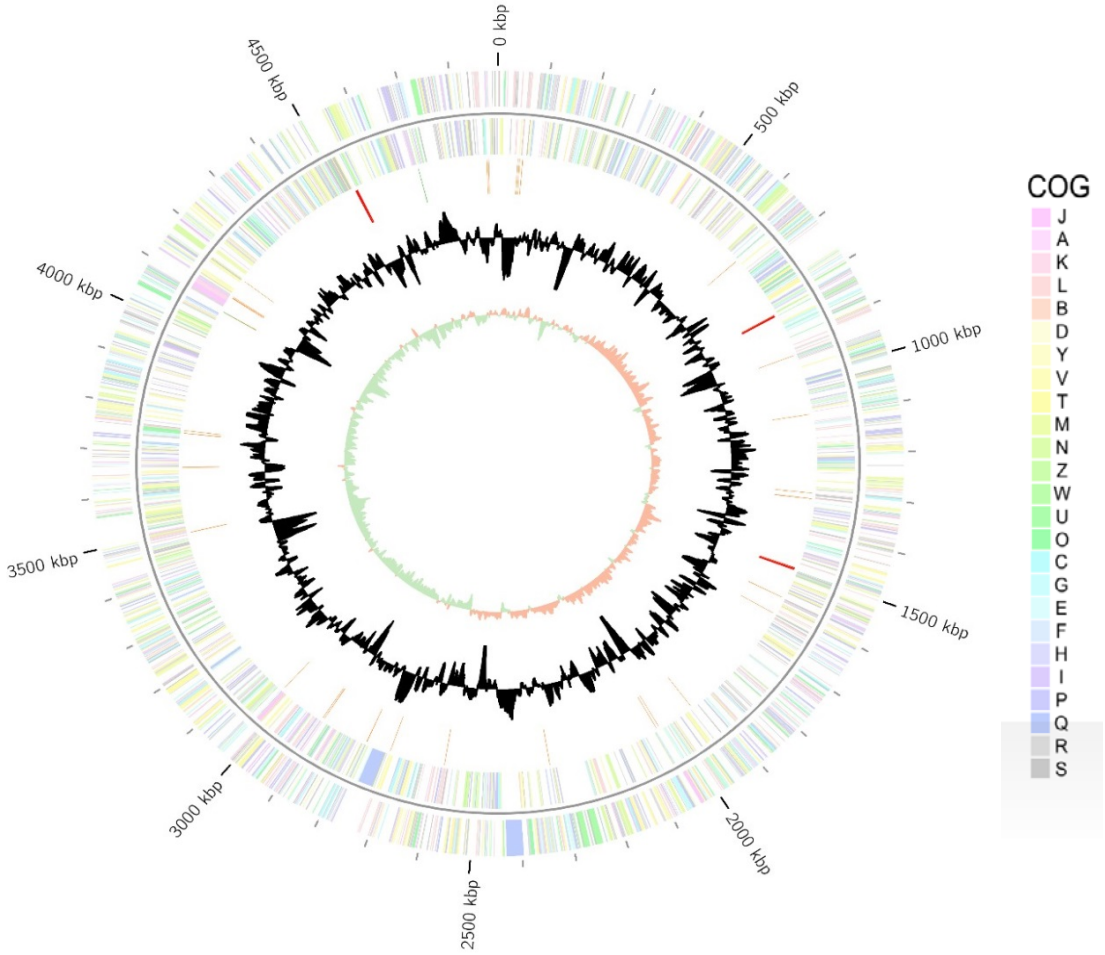

Fig. 3-6. Graphical circular map of the chromosome of the isolate. From outside to the center: Genes on forward strand (color by COG categories), Genes on reverse strand (color by COG categories), RNA genes (tRNAs orange, rRNAs red, other RNAs green), GC content (black), and GC skew (light green/orange)

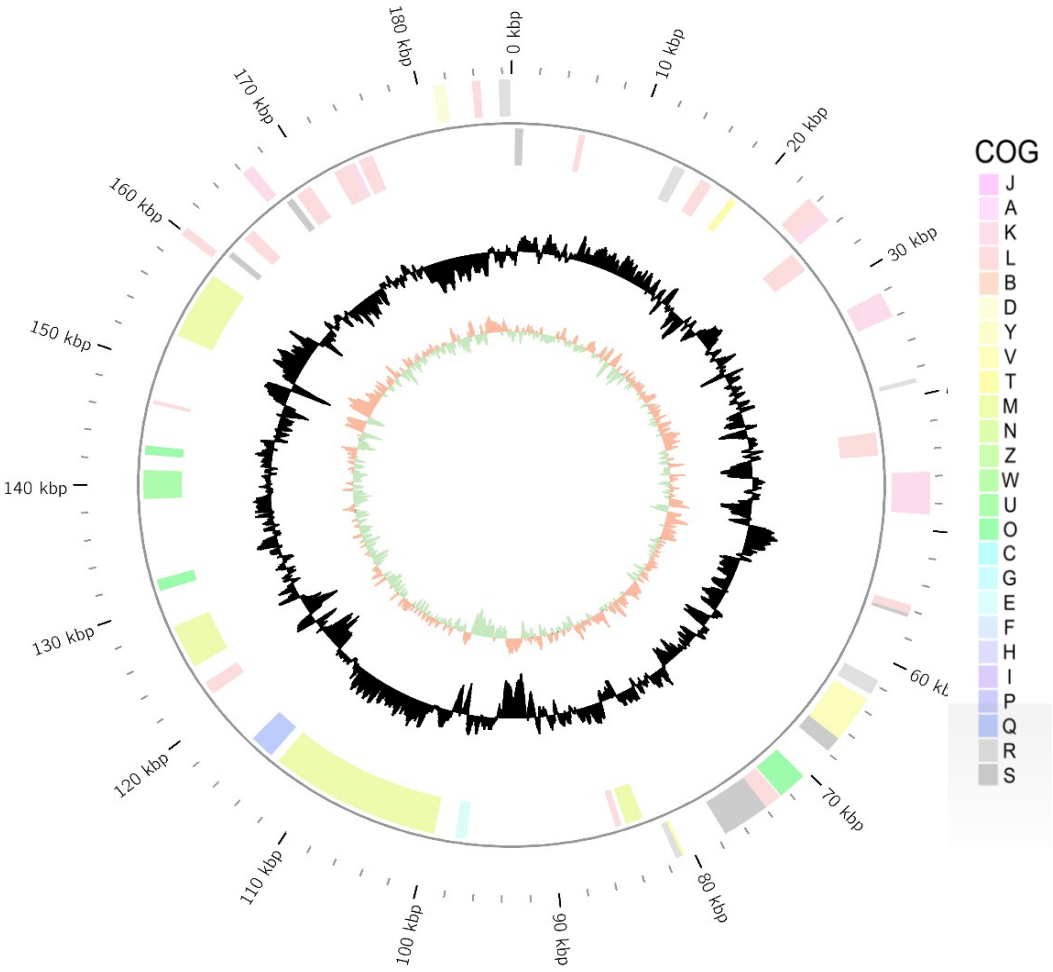

Fig. 3-7. Graphical circular map of the pLM6. From outside to the centre: Genes on forward strand (Colour by COG categories), Genes on reverse strand (Colour by COG categories), GC content (black), and GC skew (light green/orange).

1-7. Cellular fatty acid analysis

$C_{14:0}$ (21.7%) and $C_{16:0}$ (7.4%) were observed as major cellular fatty acids in isolate (Table 3-6), and interestingly much difference were noted when compared with the reference species and was quite similar to closely related *M. koyamae*. The analysis also revealed the presence of monounsaturated fatty acids of the $C_{16:1}$isomers were $C_{16:1}$ ω8c, $C_{16:1}$ ω7c, $C_{16:1}$ ω5c and $C_{16:1}$ 3-OH (48.7, 4.2, 14.4 and 1.3%), respectively.

Table 3-6. Comparison of cellular fatty acid compositions of our isolate with related *Methylomonas* species

Fatty acid	Isolate	*M. koyamae*	*M. paludis*	*M.lenta*
$C_{14:0}$	21.7	22.9	11.8	6.38–9.78
$C_{15:0}$	–	1.16	0.50	5.34–5.83
$C_{16:1}$ ω8c	48.7	39.4	22.1	40.85–42.42
$C_{16:1}$ω7c	4.2	4.35	13.9	9.10–10.47
$C_{16:1}$ ω6c	–	–	5.0	–
$C_{16:1}$ ω5c	14.4	16.7	1.8	11.67–18.26
$C_{16:1}$ ω5t	–	–	34.8	–
$C_{16:0}$	7.4	7.70	5.60	5.00
$C_{16:0}$ 3-OH	1.3	3.79	–	4.11–4.16
$C_{19:0}$ ω8c	2.3	NR	NR	NR
Reference	In this study	(Ogiso et al., 2012)	(Danilova et al., 2013)	(Hoefman et al., 2014a)

2. Methanol synthesis from methane-utilizing *Methylomonas* sp. LM6

2-1. Effect of MDH inhibitors

In order to accumulate methanol, MDH should be inhibited. Thus, the expression of highly active MMO and the accumulation of methanol by inhibition of MDH are important features of methanotrophic bioconversion of CH_4 into methanol. First, the effect of NaCl on methanol production by *Methylomonas* sp. LM6 cells was investigated at various concentrations (0-100 mM; Fig. 3-8 (a)). Methanol production increased with an increase in NaCl up to 60 mM and then continued to decrease, indicating that higher concentration might inhibit pMMO activity. The maximum amount of methanol was 3.25 ± 0.12 mM when 60 mM NaCl was added at 24 h.

Then, the effect of $MgCl_2$ on methanol production by *Methylomonas* sp. LM6 cells were investigated at various concentrations (0-100 mM; Fig. 3-8 (b)). The activity of MDH was inhibited by $MgCl_2$ and maximum methanol production was observed at 60 mM $MgCl_2$ and amount of methanol synthesis was about 4.19 ± 0.11 mM at 24 h.

Subsequently, EDTA can inhibit MDH by preventing the electron transfer from methanol to cytochrome c_L via binding to the lysyl residues located at or close to the cytochrome binding domain on a subunit of MDH (Chan and Anthony, 1992). In the case of EDTA, the methane methanol production was somewhat similar with NaCl (Fig. 3-8 (c)). Approximately 3.60 ± 0.05 mM methanol was produced when 2mM EDTA at 24 h.

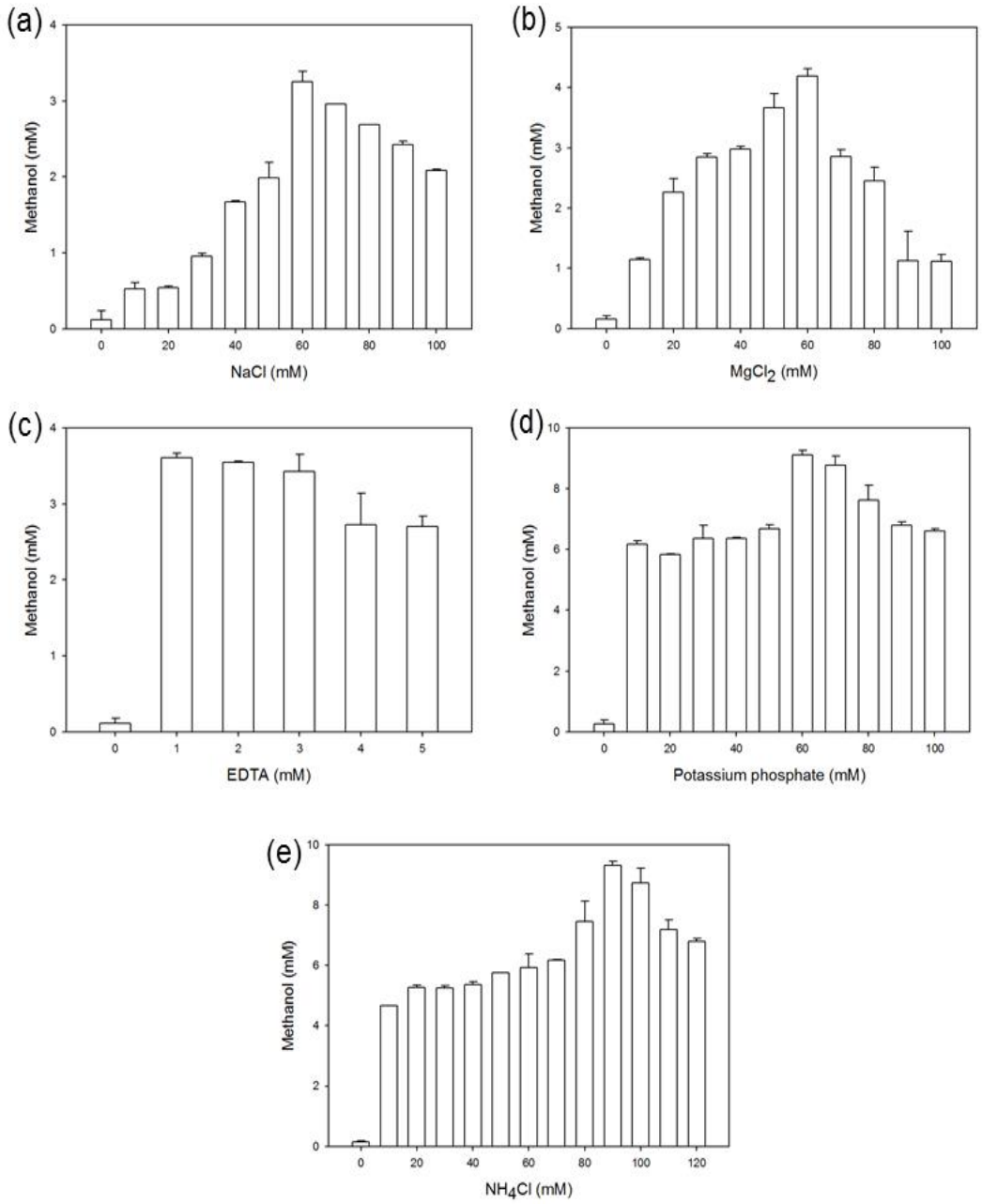

Fig. 3-8. Effects of NaCl (a), $MgCl_2$ (b), EDTA (c), potassium phosphate (d) and NH_4Cl (e) as a MDH inhibitor on methanol production by *Methylomonas* sp. LM6. n = 3 duplicate/trial per experiment.

Next, the effect of potassium phosphate addition on methanol production was investigated at various potassium phosphate concentrations (0-100 mM; Fig. 3-8 (d)). As the concentration of potassium phosphate increased, up to 60 mM, a significant increase in methanol synthesis was observed. The maximum methanol production obtained was 9.11 ± 0.14 mM at 24. The results revealed that potassium phosphate was the most effective MDH inhibitor.

Finally, the effect of ammonium chloride on methanol production was investigated at various concentrations (0-120 mM; Fig. 3-8(e)). Methanol production increased with increase in NH_4Cl concentration up to 90 mM and then started decreasing at 100mM with further increase in NH_4Cl concentration. The highest amount of methanol production was observed in this study when compared with the above MDH inhibitors. Approximately 9.31 ± 0.13 mM methanol was produced at 24 h.

2-2. Effect of Na-formate on methanol production

Subsequently, the effect of Na-formate with 90 mM NH_4Cl and 60 mM potassium phosphate in methanol production was also tested (Fig. 3-9). Addition of Na-formate generally increases the methanol production by NADH regeneration (Ge et al., 2014). Each MDH inhibitors have their own Na–formate optimum concentration in which they produced the maximum amount of methanol and above the saturation point further increase was not observed. The maximum methanol concentration (11.64 mM) was produced when 80 mM Na-formate was introduced with 60 mM potassium phosphate after 10 h. These results suggest that Na-formate as an electron donor stably enhances the activity of pMMO during CH_4 oxidation.

2-3. Effect of copper concentration on methanol production

Copper ($CuCl_2$ or $CuSO_4$), which can strongly influence methanotrophic activities, and the methanol production as the enzyme needs Cu^{2+} for active output, thus investigations were performed for determining optimum concentration for obtaining maximum methanol production. $CuCl_2$ in range of (0-10 μM) in the reaction media were tested (Fig.3-10). These results showed that the methanol synthesis was always in high amount and stable in the presence of Cu^{2+}. Maximum methanol productions at 2 μM of $CuCl_2$ (9.33 mM) were observed at 24 h.

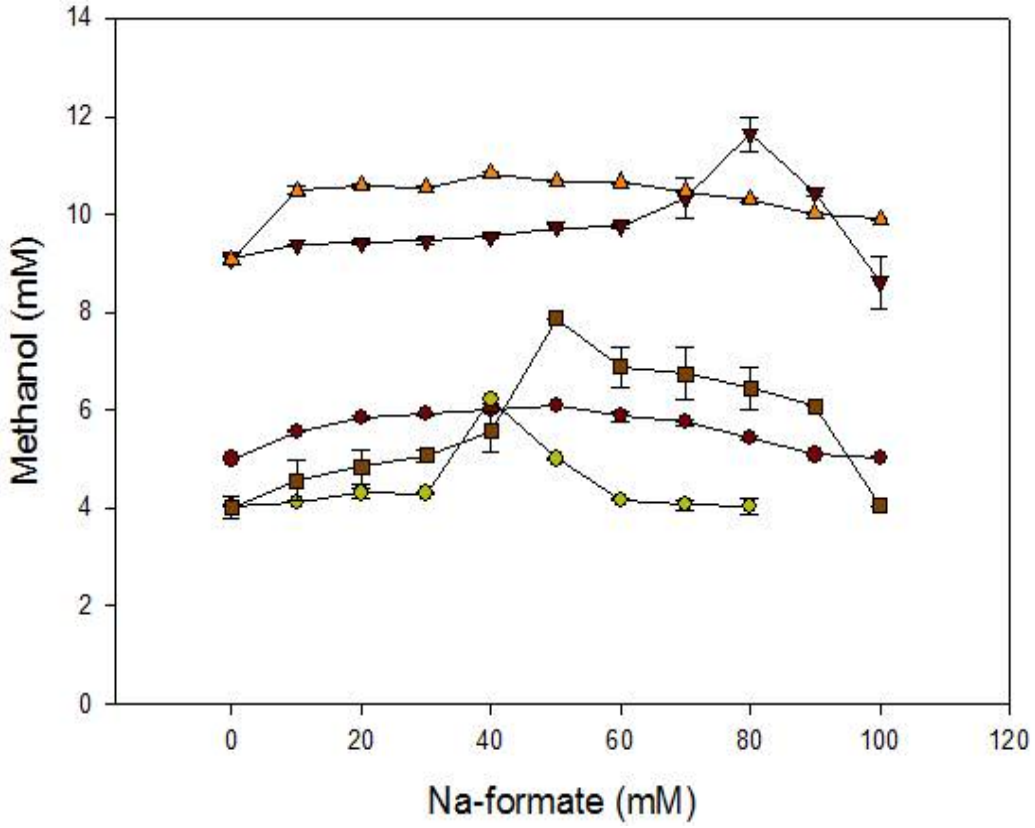

Fig. 3-9. Effects of Na-formate concentration with different MDH inhibitors on methanol production. n = 3 duplicate/trial per experiment.

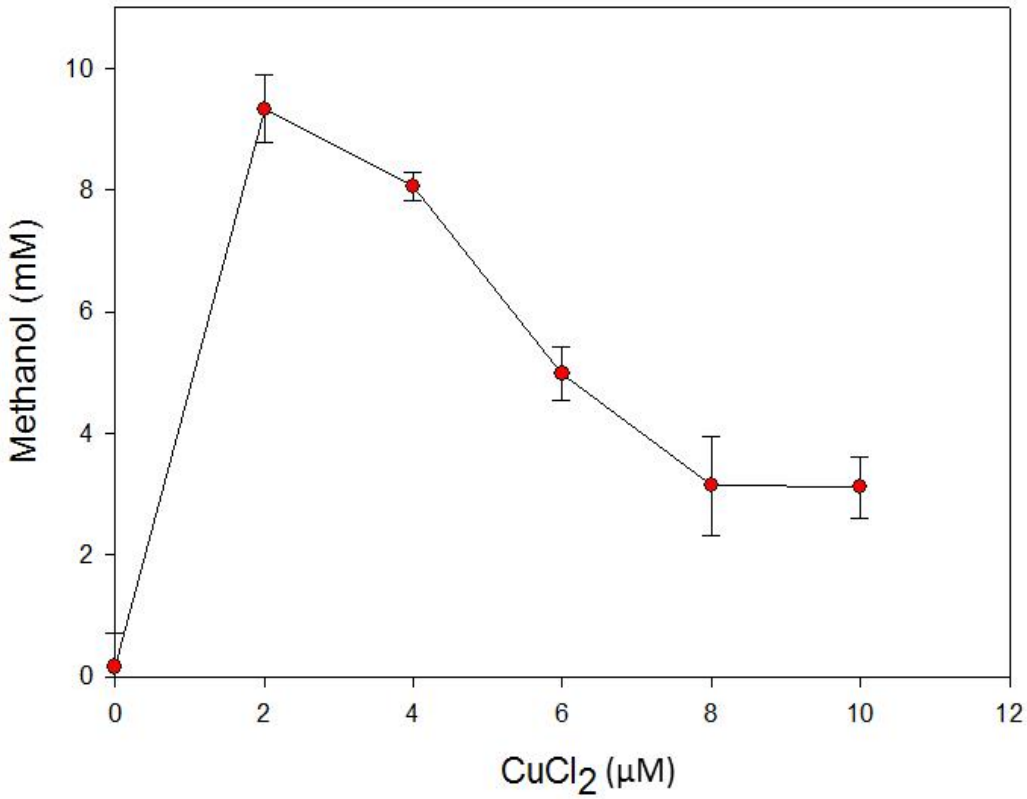

Fig. 3-10. Effect of different $CuCl_2$ concentration on methanol production. n = 3 duplicate/trial per experiment.

2-4. Effect of process parameters on methanol production

Methanol production by methanotrophic bacteria, optimization of (pH, incubation temperature, and agitation rate) could increase higher methanol production, which is inhibited at low and high pH values [8, 17, 18, 29]. To test this hypothesis, the effect of pH and temperature on methanol production by *Methylomonas* sp. LM6 with 80 mM of Na-formate and 90 mM NH_4Cl as a MDH inhibitor and in 10 mL NMS medium, pH 7.0 was observed and shown (Fig. 3-11). All the investigations were done with 80 mM Na-formate and 90 mM NH_4Cl as MDH inhibitors.

At pH values, the methanol production was observed maximum at pH 7 at 10.84 mM (Fig. 3-11(a)). In the pH range between 6.5 and 7.5, methanol production was steadily in the range of 9.65 mM, whereas acidic pH values of 5.0, 5.5, and 6.0 results showed lower methanol production. The optimum temperature for maximum methanol production was 30°C, producing a concentration of 10.49 mM (Fig. 3-11(b)).

Variation in agitation helps in influencing the nutrient availability to the cells. Thus the effect of different agitation rate on methanol production was investigated (Fig. 3-11(c)). As agitation rate increased from 120 rpm to 200 rpm, an increase in methanol production was observed (6.63 mM to 10.35 mM). At higher agitation rates up to 250 rpm, methanol production was quite stable, with a value of 10.65 mM. Overall, the maximum methanol production (10.84 mM) was observed at pH 7 after 24 h incubation at 30°C and 180 rpm.

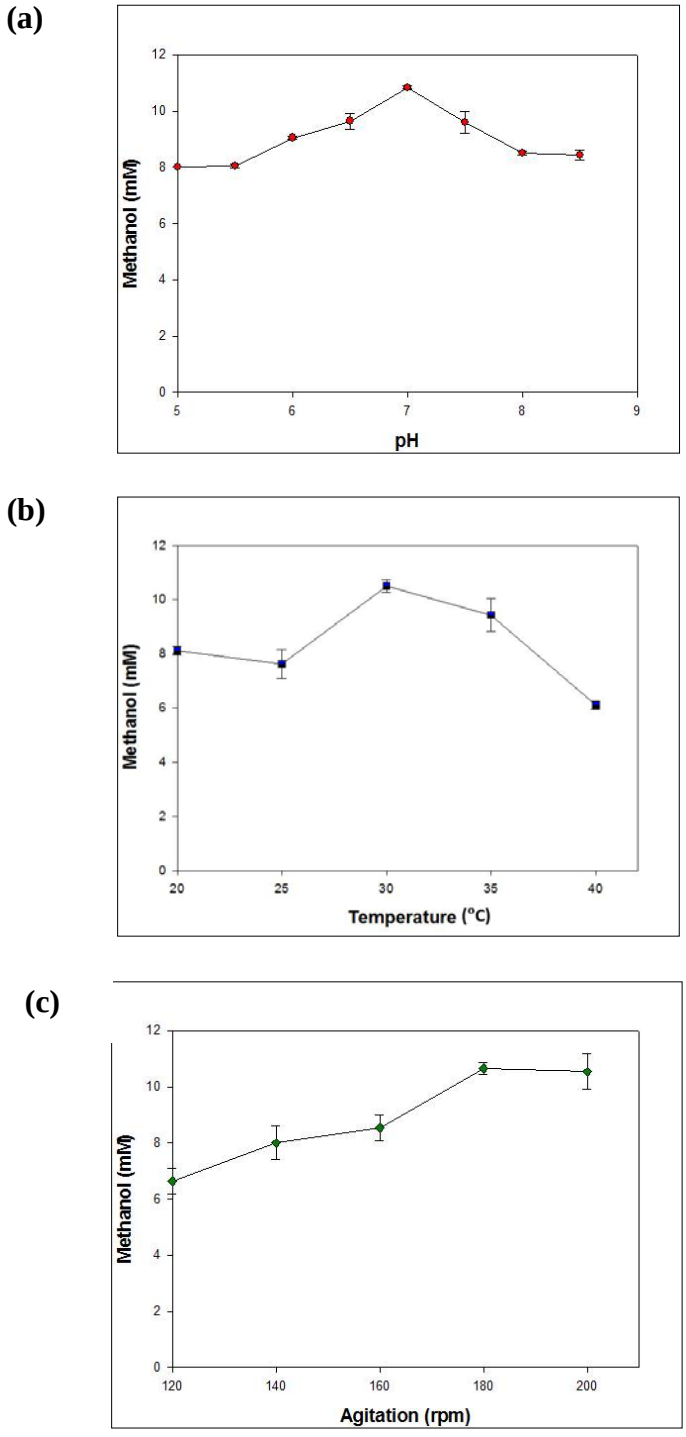

Fig. 3.11. Effect of the process parameters (a) pH, (b) temperature, and (c) agitation speed on methanol production by *Methylomonas* sp. LM6. n = 3 duplicate/trial per experiment.

2-5. Effect of methane concentration

The significant improvements in methanol production by changing the CH_4/air mixture concentration in the headspace of the serum bottle could be possible. Thus the effects of different CH_4/air mixture concentration were investigated with 80 mM of Na-formate and 90 mM NH_4Cl as a MDH inhibitor and in 10 mL NMS medium, pH 7.0 and shown in Fig (3-12).

The results showed that the methanol production was increased with increasing feed concentrations of CH_4, from 10% to 60%, and then started decreasing. The greatest enhancement in methanol synthesis occurred when CH_4 was increased from 40% to 60% under optimum conditions; the production increased from 4.22 mM to 10.49 mM.

2-6. Effect of inoculum load

The cell concentration influences the methanol synthesis by methanotrophic bacteria. The methanol synthesis by *Methylomonas* sp. LM6 was investigated using 80 mM of Na-formate and 90 mM NH_4Cl as an MDH inhibitor and in 10 mL NMS medium, pH 7.0. Methanol synthesis increased with an increase in the inoculum size till 0.6 mg DCM/mL, followed by a decrease (Fig. 3-13). An increase in methanol synthesis from 4.003 to 9.66 mM was observed with an increase in the concentration of cell inoculum from 0.2 to 0.6 mg of DCM/ml, using 60% CH_4 as a feed.

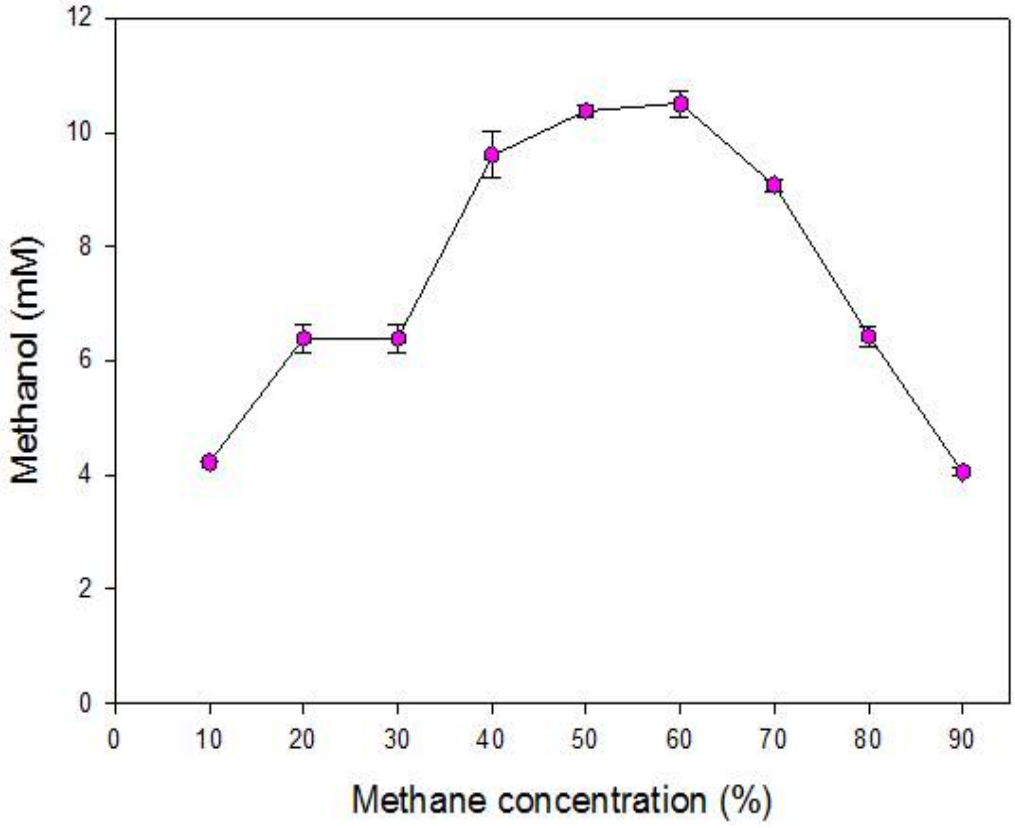

Fig. 3-12. Effect of different methane/air mixing ratios on methanol production. n = 3 duplicate/trial per experiment.

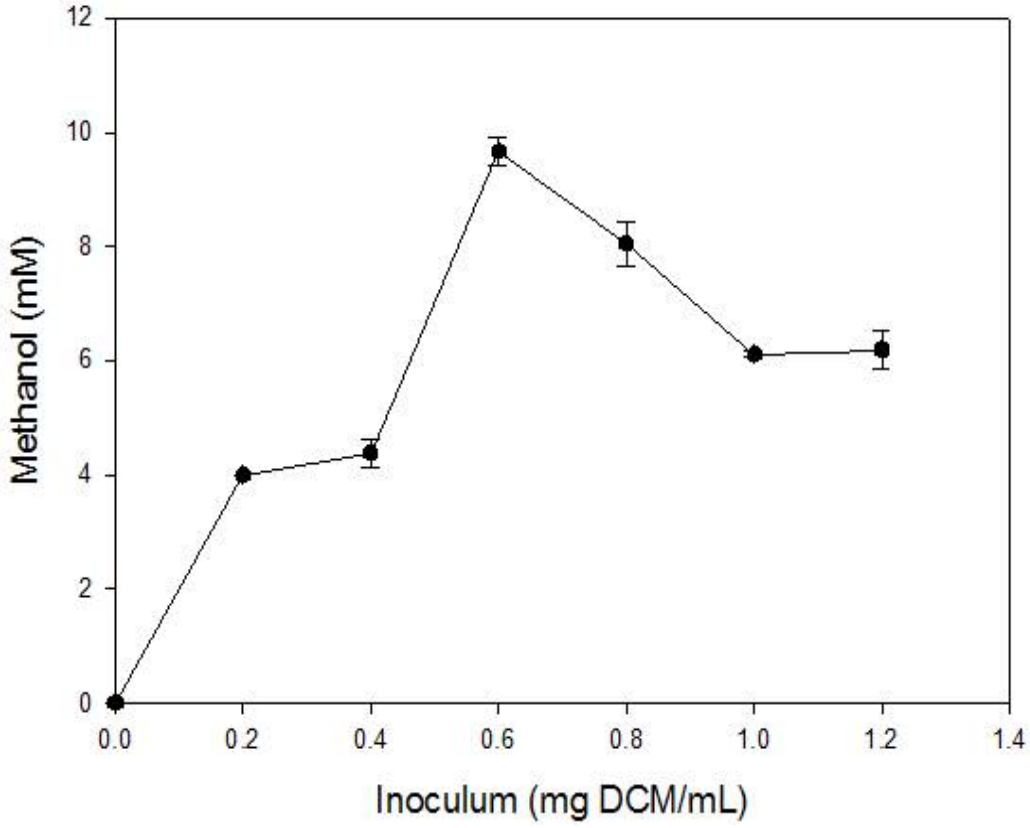

Fig. 3-13. Effect of inoculum size on methanol production. n = 3 duplicate/trial per experiment.

2-7. Synergistic effects of MDH inhibitors on methanol production

In order to increase the methanol production, the synergistic effects of MDH inhibitors on the methanol production in a 70 mL batch reactor and the CH_4 to methanol conversion rate were tested. Interestingly, our results showed that the higher production of methanol can be achieved with a simple approach of mixing inhibitors together with co-inhibitor EDTA (Fig. 3-14 (a)). Furthermore, the results showed that within 8 h, methanol started accumulated in the media and after 24 h the maximum methanol production was achieved. Moreover, it has been reported that the presence of high concentration of methanol can inhibit MMO activity (Kim et al., 2010). Remarkably, we found that in our present study high methanol concentration did not affect or inhibit pMMO activity. We observed significantly high level of methanol production (22.80 mM) at 24 h by 80 mM Na-formate, 60mM $MgCl_2$, 60 mM potassium phosphate, 90 mM NH_4Cl and 3 mM EDTA. Furthermore, the inhibitory synergistic effect of MDH inhibitors by using the optimized assay was investigated (Fig. 3-14 (b)). Interestingly, we found that the MDH activity inhibition was rapid and higher in the presence of 60 mM $MgCl_2$, 60 mM potassium phosphate, 90 mM NH_4Cl and 3 mM EDTA, respectively.

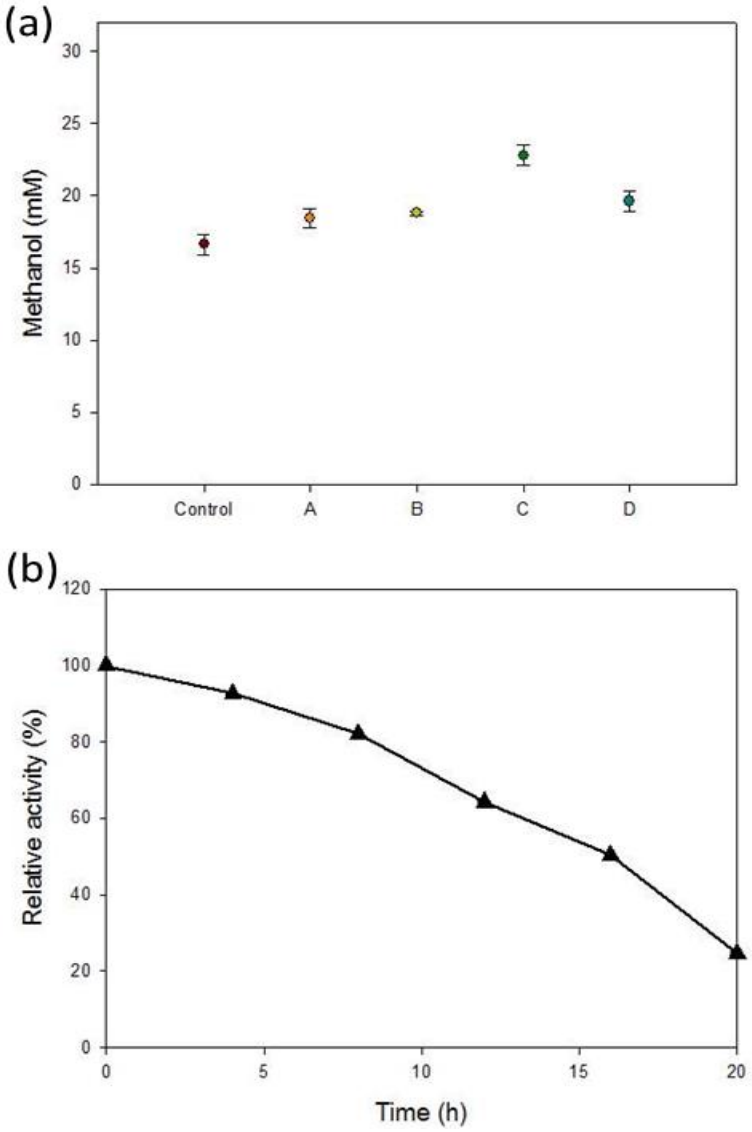

Fig. 3-14. Synergistic effects of MDH inhibitors on methanol production. (a), the reaction mixture contained 0.6 mg DCM mL^{-1}, 90 mM Na–formate and mixture of inhibitors with different EDTA in the following concentration. Control, 60 mM potassium phosphate + 90 mM NH_4Cl; A, 1 mM EDTA + 60 mM potassium phosphate + 90 mM NH_4Cl; B, 2 mM EDTA + 60 mM potassium phosphate + 90 mM NH_4Cl; C, 3 mM EDTA + 60 mM potassium phosphate + 90 mM NH_4Cl; D, 4 mM EDTA + 60 mM potassium phosphate + 90 mM NH_4Cl. (b), Inhibition of MDH activity by MDH inhibitors on methanol production. n = 3 duplicate/trial per experiment.

3. Enhancement of bio-methanol production utilizing organic solvent

3.1. Effect of organic solvents and selection of methane soluble agent

This study reports on the solubility of CH_4 in the water by various organic solvents. Fig. 3-15 shows the experimental results for the solubility of methane in pure organic solvents (1% v/v), n-Hexane, paraffin oil, 1-octanol, benzene and, tetraethylene glycol dimethyl ether, control (no solvent) in water. The solubility measurements were done in lab scale using the simple procedure and thus allowed a comparison based on the same conditions.

The solubility of methane in the water was measured at temperature 30°C. The figure shows that 1-octanol was, by far, the solvent that solubilized CH_4 the most, followed by paraffin. For example, 19.89 ± 0.118% and 18.57± 0.526% of CH_4 were dissolved in water in the presence of 1-octanol and paraffin, whereas only about 12.82 ± 0.162% of CH_4 were dissolved in only water (control) at 24 h. All the other solvents had approximately the same capacity for CH_4 solubilizing in water. However, tetraethylene glycol dimethyl ether (TGDE) had the lowest capacity for CH_4 solubilize when compared with control. In terms of higher concentration of organic solvents, once again, 1-octanol had a highest solubilizing capacity for CH_4 in water than paraffin and control. All the remaining solvents had approximately the same lower capacity of CH_4 solubilizing in water. Therefore, the study confirmed 1-octanol has the higher capacity for CH_4 solubilizing nature and can be utilized for our further study.

1-octanol is a colourless long chain alcohol used as a solvent for protective coatings, waxes and also as a raw material for plasticizers (Shingwekar, 2015). Additionally, organic solvent like paraffin was used for solubility of CH_4 into the media for cultivation of methanotrophic bacteria, *Methylosinus trichosporium* OB3b (Han et al., 2009), although, there

are no reports that cover paraffin for solubility of CH_4 on methanol production. In addition, to the best of our knowledge, this is the first report for 1-octanol as methane soluble agent for methanol synthesis.

3.2. Effect of organic solvents on bacterial growth

The screening of various organic solvents as methane soluble agent has been performed which could potentially increase CH_4 transfer between the two phases. The effects of these organic compounds, added at 1% (v/v) into the NMS medium, on the cell growth of strain LM6 are shown in Table 3-7. The results (Table 3-7) revealed that after 5-day cultivation except for 1-octanol and paraffin, the other solvents showed inhibitory effects on the cell growth of strain LM6. The inhibition of strain LM6 might have been due to either toxicity of organic solvents or strains preferred utilization of source.

3.3. Effect of 1-octanol on growth of *Methylomonas* sp. LM6

Methylomonas sp. LM6 are obligate methanotrophs and can only grow well on methane and methanol due to their ribulose monophosphate pathway of formaldehyde assimilation and incomplete Krebs cycle results in lack of α-ketoglutarate dehydrogenase and cannot grow on poly carbon compounds (Shishkina and Trotsenko, 1981). Thus, due to the obligate nature of strain LM6, 1-octanol could not be utilized as the carbon source. However, to rule out the possibility that 1-octanol can affect or inhibit the growth of strain LM6, growth curve analysis was investigated with and without 1-octanol under an atmosphere of CH_4/air mixture (6:4, v/v). The results of growth analysis were shown in Fig. 3-16. Interestingly, Strain LM6 showed growth in the presence of 1-octanol, although its specific growth rate was

decreased to about 0.03 h^{-1}, indicating that strain LM6 can tolerate and able to grow with a 1-octanol concentration on the NMS medium supplied with CH_4/air mixture. Therefore, the results revealed that 1-octanol is non-toxic and do not affect the growth of strain LM6.

Table 3-7. Effects of different organic solvents on the cell growth of *Methylomonas* sp. LM6 during 5-day cultivation.

Organic solvents	Dry weight (mg/ml)
Control	1.21
1-octanol	1.40
Benzene	0.36
n-Hexane	0.31
Paraffin	1.34
Tetraethylene glycol dimethyl ether	0.22

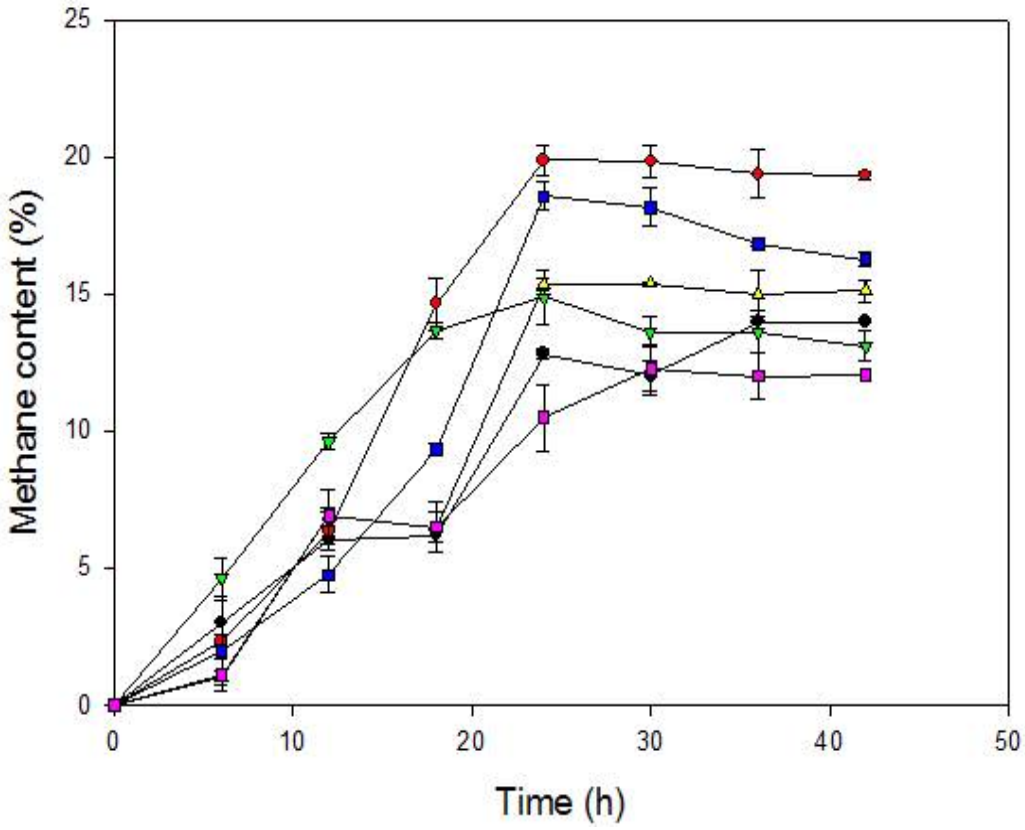

Fig. 3-15. Improvement of methane solubility by several organic solvents such as 1-octanol, benzene, hexane, paraffin oil and tetraethylene glycol dimethyl ether. The experiments were conducted in reaction mixture containing CH_4/air mixture (6:4, v/v) in the headspace of flask. Symbols: (—●—), Control; (—●—), 1-octanol; (—▼—), benzene; (—▲—), n-hexane; (—■—), paraffin; (—□—), TGDE. n = 3 duplicate/trial per experiment.

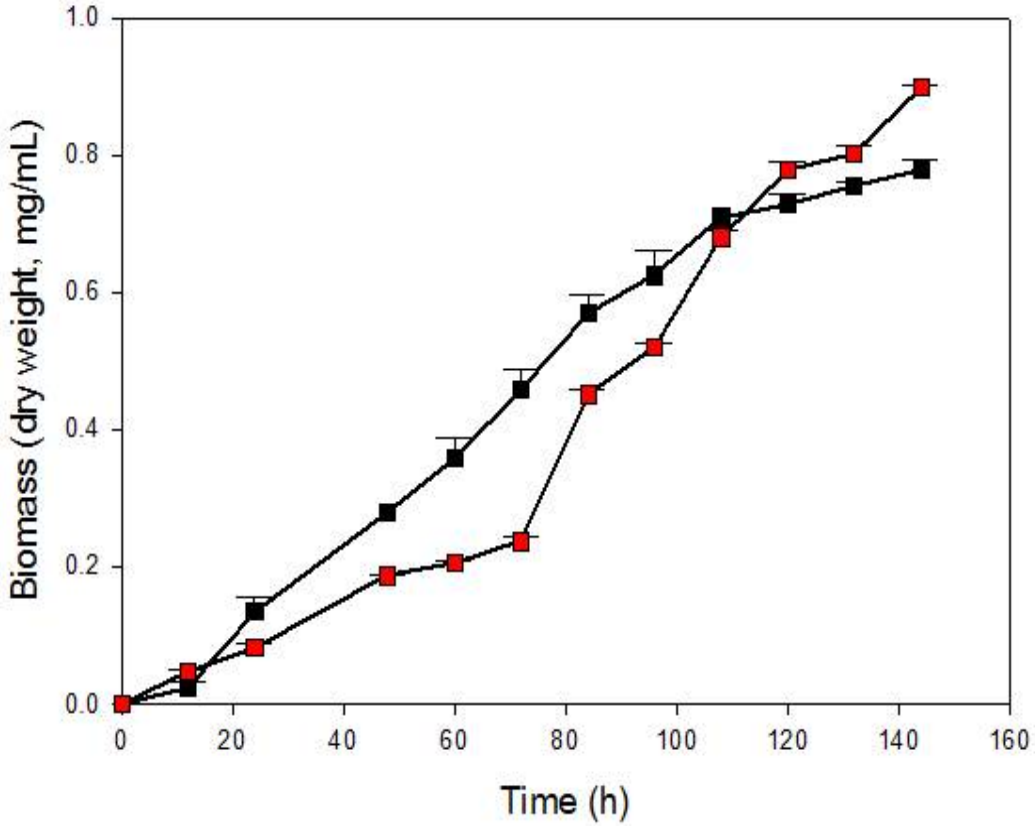

Fig. 3-16. Growth of strain LM6 with and without 1% (v/v) 1-octanol in NMS medium. Symbols: ( ), Control; ( ), 1-octanol. n = 3 duplicate/trial per experiment.

3.4 Effect of methane soluble agent, 1-octanol on methanol production

CH_4 to methanol conversion on the lab scale utilizing methane soluble agent, 1-octanol has been investigated primarily by strain LM6. To achieve effective and enhanced amount of methanol production, adequate CH_4 is required in the reaction mixture which can be achieved by utilizing methane soluble agent, 1-octanol. Therefore, in the present study, the solubility of methane was enhanced by utilizing 1-octanol concentrations (0-4%, v/v) at 30°C. Fig. 3-17 shows the effects of 1-octanol concentrations (0-4%, v/v) on methane solubility in a 70 mL batch reactor containing 10 mL reaction mixture. Also, the results show that the highest methane solubility was achieved using 3 and 4% 1-octanol and was of the same order of magnitudes, such as 27.70± 0.85 and 27.80 ± 0.06% at 24 h, but 1 and 2% 1-octanol was much less methane solubility of 19.45 ± 0.17 and 26.80 ± 0.03% at 24h, respectively. Control (0% 1-octanol) had somewhat lower capacity for methane solubility (18.003 ± 0.02%) than compared to 1-octanol. In addition, the study confirmed that higher concentrations of 1-octanol have 1.5 times higher methane solubility capacity than the control, and can be undertaken for methanol production.

Following, methanol synthesis was carried out using 10 mL acquired NMS reaction mixture (containing 2 µM $CuCl_2$, 90 mM NH_4Cl, 60 mM potassium phosphate, 3 mM EDTA, 0.6 mg DCM mL^{-1}, 80 mM Na-formate, and 1-octanol concentration (0-4%, v/v)) in tightly sealed 70 mL glass serum vial. Fig. 3-18 shows the methanol synthesis at different concentrations of 1-octanol at 24 h. This study revealed that the highest methanol synthesis was achieved using 2% 1-octanol at 24 h, however, the concentration-related decrease was observed when 3 and 4% 1-octanol were utilized. In this experiment, the methanol production increased rapidly within 24 h and a maximum methanol concentration of 50.38 ± 0.02 mM was achieved at 24 h, when 2% 1-

octanol was added. In addition, when 1-octanol was not added, methanol production was found reduced to 22.83 ± 0.03 g/L at 24 h.

Lastly, time course on dissolved methane consumption and methanol production by 1-octanol (0 and 2%, v/v) with 2 µM $CuCl_2$, 90 mM NH_4Cl, 60 mM potassium phosphate, 3 mM EDTA, 0.6 mg DCM mL^{-1}, and 80 mM Na-formate (working volume of 100 mL in a 500 mL flask) were investigated and shown in Fig. 3-19 (a). In this experiment, the methanol production increased rapidly within 24 h and a maximum methanol concentration of 50.22 ± 0.02 g/L was achieved at 24 h when 2% 1-octanol added and started decreasing at 30 h. In addition, when 1-octanol was not added, methanol production was found reduced to 22.7 ± 0.03 g/L at 24 h. Compared to control, the addition of 2% 1-octanol rapidly decreased the CH_4 consumption by increasing the methanol production. These results show that 1-octanol which enhances the methane solubility by approximately 1.6 times, also increases methanol production by approximately 2 times higher when compared to initial methanol production.

Also, to investigate the effect of 1-octanol together with MDH inhibitors on pMMO and MDH enzyme activity were examined and shown in Fig. 3-19(b). Inhibition of MDH is necessary; at the same time preserving the activity of pMMO is the foremost objective during methanol synthesis. Remarkably, results showed that 2% 1-octanol did not cause any significant loss of pMMO activity in the tested reaction mixture; however, MDH inhibitors inhibited well MDH enzyme activity by 76% at 30 h. Thus, higher and stable activities of pMMO and low activity of MDH have caused higher methanol accumulation. These results provide straightforward means for utilizing 1-octanol as methane soluble agent for the production of biological methanol and also indicating its non-toxicity to the strain LM6.

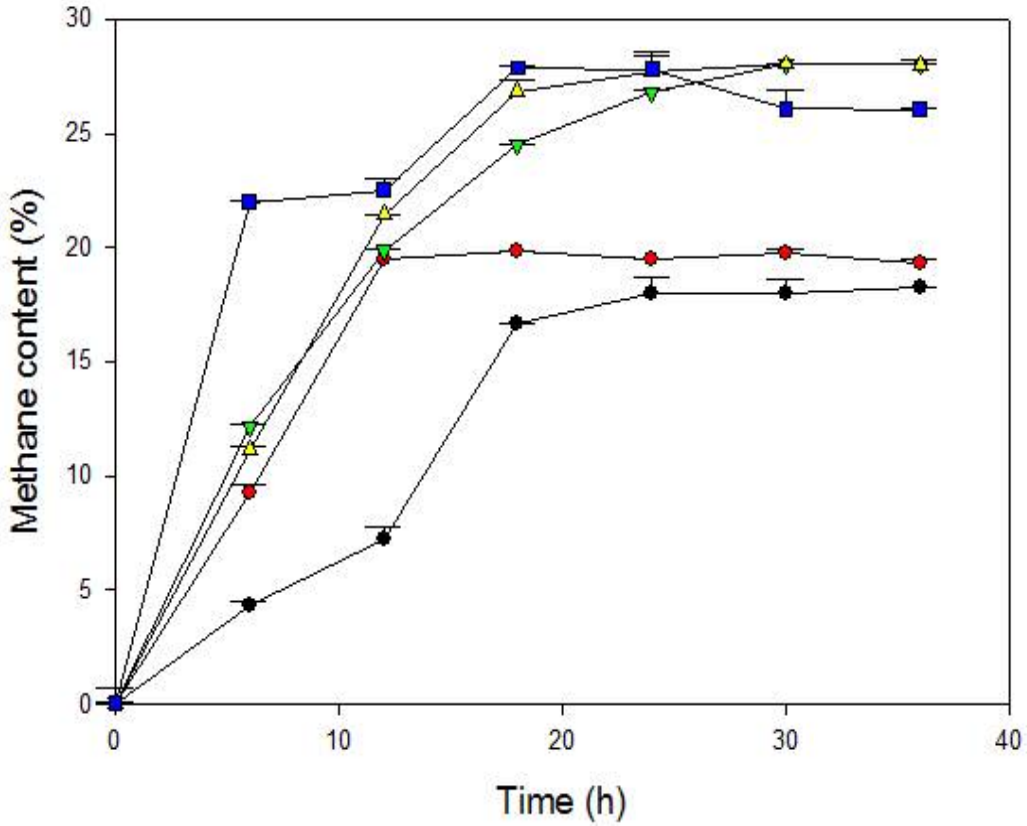

Fig. 3-17. Effect of 1-octanol concentration on dissolved CH_4 content in NMS medium. Symbols: (—●—), control (w/o 1-octanol); (—●—), 1% (v/v), 1-octanol; (—▼—), 2%; (—▲—), 3%; (—■—), 4%. n = 3 duplicate/trial per experiment.

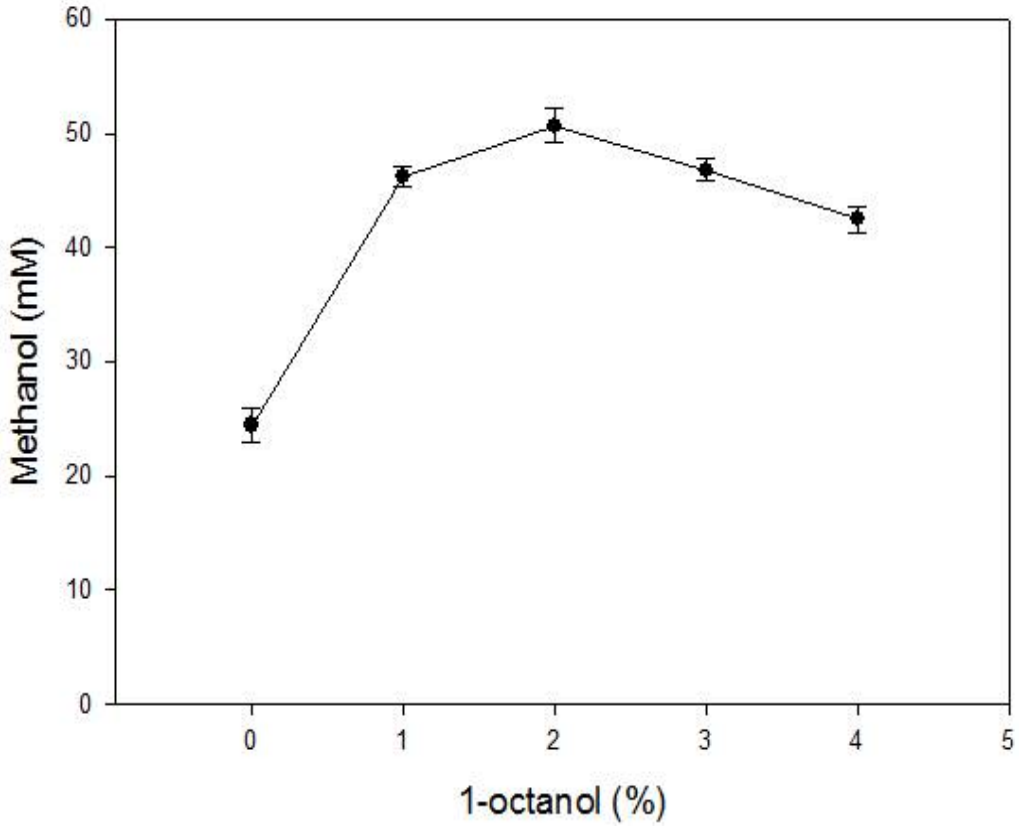

Fig. 3-18. Methanol production by various concentration of 1-octanol in a 70 mL serum bottle containing 10 mL reaction mixture with 0.6 mg dry cells/mL, 80 mM Na- formate, and 90 mM NH_4Cl, 60 mM potassium phosphate, and 3 mM EDTA as MDH inhibitors. n = 3 duplicate/trial per experiment.

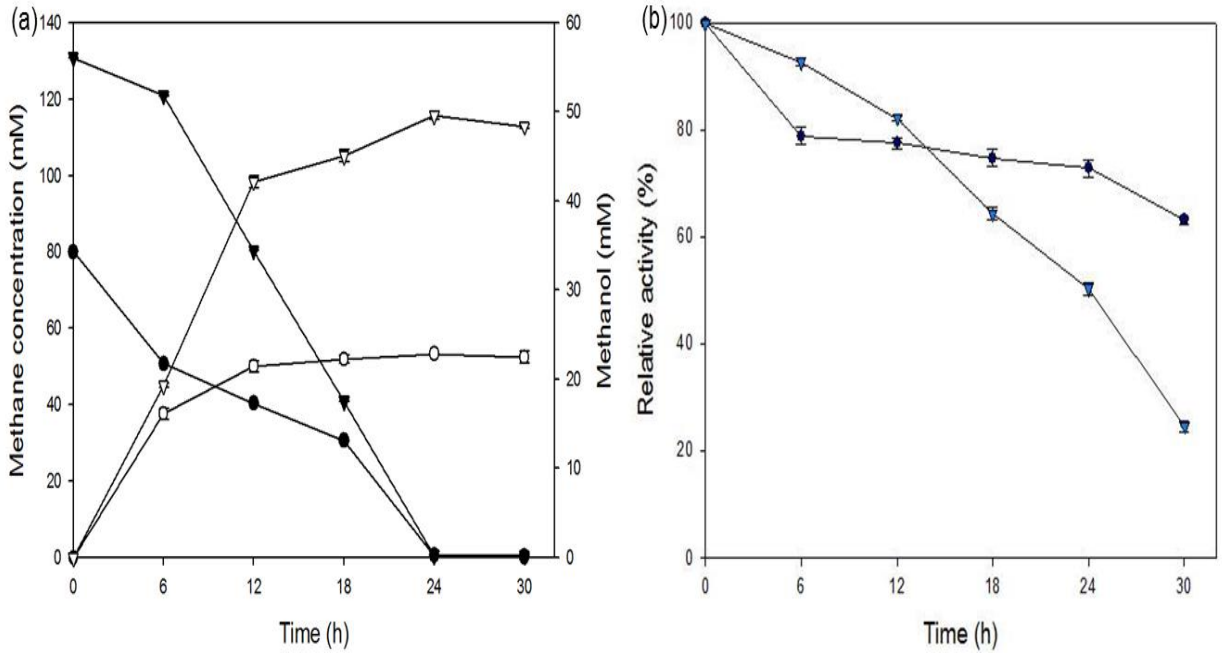

Fig. 3-19. Time dependent methanol production and methane consumption by *Methylomonas* sp. LM6 with and without addition of 1-octanol concentrations. Symbols: (—●—), Methane content (control); (—▼—), Methane content (with 2%, 1-octanol); (—○—), Methanol concentration (control); (—▽—), Methanol concentration (with 2%, 1-octanol) (a). Effect of MDH inhibitors along with 2% 1-octanol on pMMO and MDH enzyme. The initial enzyme activity was defined as 100% activity. pMMO enzyme relative activity was found to be stable and decreasing after 24 h, whereas, a decrease in MDH activity was optimized when MDH inhibitors 60 mM Potassium phosphate, 90 mM NH_4Cl, 80 mM Na-formate and 2 mM EDTA in the reaction mixture. Symbols (—●—), pMMO activity; (—▼—), MDH activity (b). n = 3 duplicate/trial per experiment.

IV.Conclusion

One strain, which could utilize methane as sole carbon source was isolated from the rice paddy field soils around Jeollanam-do province, Republic of Korea. Rice fields that are worldwide annually harvested are considered an important source of atmospheric methane. Methanotrophic bacteria play a vital role in the oxidation of methane in the rice paddy field soil surface layer. This indicates that methanotrophic bacteria can be isolated in such methane abundant soil region. Thus, this is the strong reason why we tried to isolate novel methanotrophic bacteria from rice paddy field soil region.

On the basis of morphological, biochemical and genotypic characteristics of the isolate, it was classified as a species of genus *Methylomonas*, a type I MOB. The cell was Gram-negative, motile, aerobic, short-rod, and possessed pMMO but not sMMO. Strain LM6 can utilize only methane and methanol as sole carbon and energy sources and no growth was found on other carbon sources. These results proved that the isolate is an obligate methanotroph which can utilize only methane and methanol as sole carbon and energy sources. Doubling time in NMS medium with CH_4 60% (v/v) and air 40% (v/v) was 9.48 h. The cells grew at 20–40℃ with optimum growth at 30℃ and pH 5.5–8.0, with optimum at pH 7. The strain also has salinity tolerance as it can grow well with NaCl (0–1.0%, w/w).

The composition of the cellular fatty acids of our isolate determined by using the MIDI system. The predominant cellular fatty acids of the strain LM6 were C14:0 and C16:0 with higher amounts of C14:0 (21.7), and the composition of these components was very much identical when compared to closely related *M.* koyamae sp. and different from other reference species. The highest amount of fatty acids C16:1ω8c (48.7%) were detected in strain LM6. The other fatty acid observed were summed feature 3 (C16:1ω7c/C16:1ω6c); interestingly, it was not found in other reference species. The cellular fatty acids profiles for strain LM6 was slightly

different from that of the reference species strains; however, the pattern was quite similar to those of the closely related type strain suggest that the strain is most similar to the genus *Methylomonas*. The only cellular fatty acids profile feature that distinguishes strain LM6 from other *Methylomonas* species is the high content of C16:1 ω8c shows that the strain LM6 was unique.

PCR amplification was performed using the specific primers for the *pmoA*, encoding the larger subunits of the pMMO and the key indicator gene for the MOB, and *mmoX*, encoding subunit of sMMO. The amplification of *pmoA* gene resulted in a product of about 500 bp which revealed strong evidence that the strain LM6 possesses pMMO. No *mmoX* gene encoding a subunit of sMMO was detected in strain LM6. The naphthalene oxidation test for analyzing sMMO activity in strain LM6 grown on copper free NMS medium was also negative. Hence these results suggest that sMMO is not present in cells of strain LM6. The genome of *Methylomonas* sp. LM6 consists of one circular chromosome of 4,894,002 bp and one plasmid of 186,658 bp with 4,337 protein coding sequences (CDSs), 48 tRNA genes, 9 rRNA genes, and an average G+C content of 56.23%.

Additionally, to achieve high rates of methane-to-methanol bio conversion, optimization for methanol synthesis and effective methods have been developed for enhancing methanol accumulation in batch culture utilizing *Methylomonas* sp. LM6. All the methanol dehydrogenase inhibitors (MDH) used in this study produced methanol. Methanol production was also improved by adding co–inhibitor EDTA with potassium phosphate, $MgCl_2$, and NH_4Cl_2 and accumulation was improved when Na-formate was included in the reaction mixture.

In near future, the development of a cost-effective biocatalytic method of methane-to-methanol conversion is necessary. The cost of methanol synthesis from methane will be

competitive owing to pure methane abundance, especially with the recent need for shale gas production. Therefore, in this study, we have developed a new strategy for methanol synthesis involving organic solvent, 1-octanol that can transfer methane from gas phase to liquid phase by increasing the solubility and can avoid the addition of methane again in the reaction mixture. This process can reduce the usage of methane and hence cost-effective. Additionally, results suggested that 1-octanol are non-toxic to strain LM6 and does not affect the cell growth or metabolism. The present work revealed the effects of methane solubilizing agent, 1-octanol on effective methanol synthesis using the isolated methanotrophic bacteria, *Methylomonas* sp. LM6. It was proved that 2% 1-octanol concentration increased methane solubility by 1.5 times higher and resulting in enhancing 2 times more methanol production in a small-scale batch reactor. The recovery and reuse of the solvent 1-octanol in batch reactor demonstrated a cost-effective methodology. This study shows that it is possible to produce maximum methanol synthesis utilizing newly isolated *Methylomonas* sp. LM6 by simple strategy involving organic solvent, 1-octanol. Further research in large-scale low-cost industrial methanol production is warranted.

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