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RESEARCH PAPER

Hydrogen Production from Methane by *Methylomonas* sp. DH-1 under Micro-aerobic Conditions

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Abstract Fueled by the recognition of hydrogen as a promising renewable energy source for the future, there have been many attempts to find greener and more economical ways for its production from various sources. In this study, *Methylomonas* sp. DH-1, a type I methanotroph, was found to produce hydrogen using methane as a sole carbon source, under micro-aerobic conditions; this is analogous to the partial oxidation of methane in a thermochemical process based on metal catalysts. Flask cultures of *Methylomonas* sp. DH-1 were used to investigate the effects of different culture conditions on hydrogen production, including oxygen levels, methane/oxygen ratios, and initial cell densities. *Methylomonas* sp. DH-1 could produce hydrogen at an oxygen level below 4%, regardless of the methane content in the flask, implying that the critical factor for hydrogen production is the oxygen level, rather than the methane/oxygen ratio. Moreover, *Methylomonas* sp. DH-1 shows reversibility in hydrogen production and uptake, because the strain produces hydrogen under micro-aerobic conditions, uptakes it when the oxygen levels increase, and restores the hydrogen production capability when conditions become micro-aerobic again. Under initial conditions of 30% methane, 70% air, and an OD_{600nm} of 6, hydrogen production was 26.87 μmol and its yields per methane and dry cell weight were 14.98 mmol-H_2/mol-CH_4 and 101.53 μmol-H_2/g DCW, respectively, after 24 h of cultivation.

Keywords: *Methylomonas* sp. DH-1, methanotroph, hydrogen, methane, micro-aerobic conditions

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1. Introduction

Hydrogen, which is well known as a substitute for the conventional energy sources, has been considered a potential energy carrier for the future and its production has been the focus of several studies [1,2]. To date, it has mostly been derived from fossil fuels through thermochemical methods that demand significant amount of external energy input. In this context, there have been many attempts to find greener and more economical ways, such as biological production, to produce hydrogen at room temperature and atmospheric pressure and from various sources [1].

Nevertheless, most of the biohydrogen production methods are based on obligate or facultative anaerobic microorganisms such as *Clostridium butyricum*, *Enterobacter* species, cyanobacteria, or *Thermococcus onnurineus* NA1. These methods take place under absolute absence of oxygen [1-6] since most hydrogenases known to produce hydrogen are vulnerable to oxygen. Oxygen-tolerant hydrogenases

have been reported, but they are mostly hydrogen-consuming and not suitable for hydrogen production [7-9]. Such sensitivity to oxygen has been an obstacle to the commercialization of these methods, as well as their low yield and productivity, requiring additional facilities to secure oxygen-free conditions.

Methane (CH_4), the inexpensive and most abundant component in natural gas and biogas, is a significant contributor to global warming and attracts attention as an alternative carbon source. Transforming methane into valuable products has recently become a promising option for mitigating global warming [10-12]. Methane can be used for producing hydrogen through thermal processes, such as steam methane reforming and partial oxidation reactions. Methane-oxidizing bacteria, methanotrophs, play an essential role in the global carbon cycle by consuming methane gas, which is greenhouse-active when it is emitted into the atmosphere [13]. Moreover, they have the potential to be applicable in bioremediation, wastewater treatment, and biofuel production due to their diverse metabolic versatilities, which are not yet well understood [13].

Most methanotrophs consume methane with methane monooxygenase (MMO), which is the key enzyme for the conversion of methane to methanol, in aerobic conditions. Then, formaldehyde, a metabolite of methane, is assimilated into the cell biomass through different pathways, depending on the types of methanotrophs [13-16]. In type I methanotrophs, the ribulose monophosphate (RuMP) pathway is used for its assimilation. Among the four different types (Type I, II, X, and IV) of methanotrophs, type I methanotrophs such as *Methylomicrobium buryatense* 5GB1 and *Methylomicrobium alcaliphilum* 20Z have been reported to produce a certain amount of hydrogen under oxygen-limited, but not anaerobic conditions [16-18]. Hox hydrogenase, which produces hydrogen from NAD(P)H molecules, is predicted to be responsible for hydrogen production [18], yet it has not been further studied. And the mechanisms, specific conditions, and enzymes for hydrogen production in aerobic bacteria, such as methanotrophs, have not been well observed. [17,18].

In this study, *Methylomonas* sp. DH-1, a type I methanotroph, was found to produce hydrogen using methane or methanol as a sole carbon source under micro-aerobic conditions, in an analogous manner to the hydrogen production through partial oxidation of methane using metal catalysts in a thermochemical platform. *Methylomonas* sp. DH-1, isolated from the activated sludge of a brewery plant, is known as a highly efficient biocatalyst for the bioconversion of alkane to alcohol [19,20] due to its high growth rate and tolerance to high methanol concentration. In order to explore the applicability of *Methylomonas* sp. DH-1 as a green producer of hydrogen from methane, the detailed conditions for hydrogen production were investigated through experiments using different cell densities, oxygen ratios, and carbon sources. Moreover, hydrogen production by *Methylomonas* sp. DH-1 cells at various oxygen levels was examined for sustained cell activity and, thus, prolonged hydrogen production.

2. Materials and Methods

2.1. Culture conditions

Methylomonas sp. DH-1 used in this study was cultivated in nitrate mineral salt (NMS) medium with 30% (v/v) methane with or without 1.0 g/L or 5.0 g/L methanol as a carbon source in a sealed flask. Every liter of NMS medium (pH 6.8) contains: 1 g KNO_3, 0.62 g $Na_2HPO_4 \cdot 7H_2O$, 0.26 g KH_2PO_4, 1 mL of trace element solution, 1 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, and 1 mL of vitamin stock. Magnesium, calcium, and vitamin stocks were filtered or autoclaved separately [19].

2.2. Pre-culture for cell production

Since low cell density leads to the low production of hydrogen, each experiment was performed via two-stage cultivation to secure a sufficient cell mass. For the pre-culture, each strain was inoculated with 10% (v/v) of 50 mL working volume into a 540 mL plastic flask with a rubber cap. With 30% (v/v) CH_4 and 70% (v/v) air in 490 mL headspace, the flask was then incubated in the shaker at 30ºC and 230 rpm for one or two days until the optical density reached a certain level.

2.3. Main culture for hydrogen production

In the main culture, different gas components required for each experiment were fed into the pre-cultured flasks by gas-exchange with a 300 mL/min flow rate using a mass flow controller (Brooks, Hatfield, PA, USA). A certain amount of filtered methanol was injected with syringe if needed. Sampling was performed every 12 h. Total gas in the flask was replaced every 24 h after sampling. The detailed conditions are shown in Table 1. All sets were incubated in the shaker at 30ºC and 230 rpm.

2.4. Analytical method

Cell growth was measured in terms of optical density at 600 nm using a UV/Vis spectrophotometer (Biochrom, UK).

Methanol concentrations were analyzed using a gas chromatography (GC) system (Agilent 7890B, USA) equipped with a DB-WAX column (Agilent 122-7032, USA), connected to a Flame Ionization Detector (FID). Nitrogen was used as a carrier gas along with H_2 (35 mL/min) and air (350 mL/min), at a makeup flow of 20 mL/min.

Table 1. Experimental condition and the performance of hydrogen production in each set

Section	Set	OD_{600nm} at 0 h	Gas composition [%(v/v)]			MeOH (g/L)	Time intervals (h)			H_2 production (μmol)	H_2 yield (mmol/ mol CH_4)		Specific H_2 production rate (μmol/g DCW/h)	
			CH_4	N_2	O_2		Sampling	Gas exchange	Culture time		0-24 h	24-48 h	0-24 h	24-48 h
3.1	Set 1	2	30	56	14	N.A[a]	12	24	48	7.55	0	3.65	0	1.11
	Set 2	2	30	63	7	N.A.	12	24	48	0.78	0	0.77	0	0.71
	Set 3	2	30	70	0	N.A.	12	24	48	0	0	0	0	0
	Set 4	6	30	56	14	N.A.	12	24	48	48.56	14.98	10.19	4.23	4.46
	Set 5	6	30	63	7	N.A.	12	24	48	40.2	30.17	18.44	53.74	11.58
	Set 6	6	30	70	0	N.A.	12	24	48	0	0	0	0	0
3.2	Set 7	6	30	40	30	N.A.	12	N.A.	48	0	0	0	0	0
	Set 8	6	30	10	60	N.A.	12	N.A.	48	0	0	0	0	0
3.3	Set 9[b]	6	30	56	14	N.A.	2	N.A.	6	23.68[c]/6.24[d]	64.84[c]	17.86[d]	155.88[c]	16.24[d]
3.4	Set 10	6	30	56	14	1	12	N.A.	24	12.81	3.85[e]	N.A.	1.43	N.A.
	Set 11	6	0	86	14	5	12	N.A.	24	8.88	2.37[e]	N.A.	1.22	N.A.

[a]Not applicable
[b]Set 9 was pre-cultured for 2 days with gas exchange, one more day without gas exchange and then injected with 50 mL O_2 and 10 mL H_2 for the main experiment.
[c]H_2 uptake from 0-2 h
[d]from 2-6 h
[e]The yield was calculated from total amount of carbon sources.

Hydrogen, oxygen, nitrogen, methane, and carbon dioxide were detected with a GC system (Younglin 6500, Korea) equipped with 80/100 Porapak N and 45/60 Mol sieve 13X (Supelco, 13052-U/ 13047-U, USA) to the Thermal Conductivity Detector (TCD). Argon was used as the carrier gas with a flow rate of 15 mL/min.

3. Results and Discussion

3.1. Hydrogen production under micro-aerobic conditions

Methylomonas sp. DH-1 is a methane assimilating bacterium that grows under aerobic conditions and consumes oxygen for the oxidation of methane. In batch cultures of *Methylomonas* sp. DH-1 without gas exchange, oxygen decreases to a critical level and cultivation becomes micro-aerobic or reaches a fermentative stage. After a day without gas exchange, the strain unexpectedly produced hydrogen. Therefore, it is speculated that the strain can produce hydrogen only when some substrates, such as oxygen, are limited. Since oxygen ratios and levels appear to be the most crucial factors for hydrogen production, the following three conditions were examined: in Set 1, aerobic condition was the control and gas exchange occurred with 30% methane and 70% air, so the total ratio of oxygen was 14%. Sets 2 and 3 both involved gas exchange with 30% methane, but the total ratio of oxygen was 7% (35% air and 35% nitrogen) and 0% (70% nitrogen) for the introduction micro-aerobic and anaerobic conditions, respectively (Table 1). In order to obtain sufficient cell mass to investigate the effects of cultivation conditions on hydrogen production, the pre-culture was grown to an OD_{600} of 2 for one day under aerobic conditions (30% methane and 70% air); this culture was then used for the main experiments. Under oxygen-limited conditions (Sets 2 and 3), the cells hardly grew, as expected, with no increase in cell density. Hydrogen production was observed after 36 h in Set 1 and Set 2 (Fig. 1). Set 2 did not produce hydrogen any faster than Set 1, with less amount of 0.3 μmol. In particular, hydrogen was not detected in Set 3 under anaerobic conditions, indicating that oxygen is required for hydrogen production through methane conversion. The results were interesting for the following reasons. First, the strain has a system to produce hydrogen from methane as a sole carbon source, which can be further applied for hydrogen production from methane in non-methanotrophic bacteria. Moreover, most microorganisms used for hydrogen production require absolute anaerobic conditions [2-6]. However, the hydrogen production in this strain was higher in the presence of oxygen than in anaerobic conditions. This oxygen tolerance can be studied and used for anaerobic hydrogen-producing strains.

In addition, more hydrogen production was expected with increased cell mass, as well as faster oxygen consumption. Therefore, pre-cultures with an increased cell density (OD_{600} of 6) were used for main cultures with the same conditions of headspace gas in order to investigate the effects of oxygen levels and cell mass. More hydrogen was produced with a higher cell density when oxygen concentration was lower than 4%, which is 0.7 mmol in

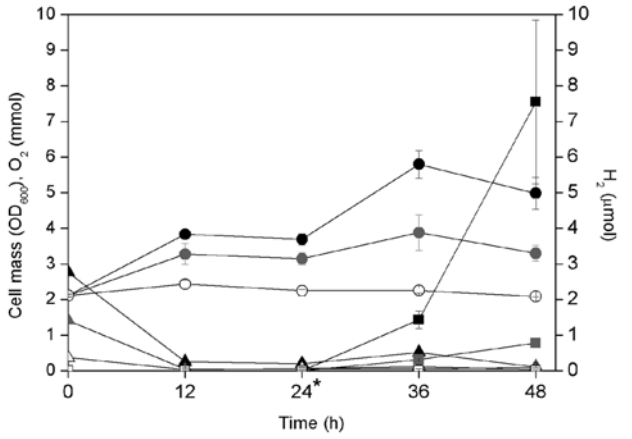

Fig. 1. Hydrogen production under micro-aerobic condition with OD_{600nm} of 2; Time profiles of hydrogen production by 3 sets of *Methylomonas* sp. DH-1 in shaking flask cultivation. Symbols and colors are: circle (●), cell growth; triangle (▲), oxygen; square (■), hydrogen; black (●), Set 1; grey (●), Set 2; white (○), Set 3. Data represent the mean of 3 replicates and the detailed growth condition and the final amount of produced hydrogen of each set are displayed in Table 1. Asterisk in x-axis is marked for the time of gas exchange after sampling.

490 mL headspace, while hydrogen production in Set 4 under low oxygen conditions began earlier than in Set 1 (Fig. 2). This indicates that the amount of hydrogen produced by *Methylomonas* sp. DH-1 using methane is more related to the cell mass than to the oxygen ratio in the feed gas. Moreover, based on the results from Sets 3 and 6 with decreasing cell density and no hydrogen production, it can be concluded that the metabolism of active cells is required for hydrogen production.

The yield of hydrogen per cell and specific production rate of hydrogen were also higher in Sets 4-5 than in Set 1-2 (Table 1). Also, the greatest amount of hydrogen production in *Methylomonas* sp. DH-1 in Set 4 was 48.56 μmol.

3.2. Hydrogen production under hyper-aerobic conditions

As stated previously, active cells are necessary for prolonged hydrogen production, thus requiring oxygen for methane conversion. Hyper-aerobic conditions, which have a higher concentration of oxygen, were applied to confirm the effects of oxygen levels. Sets 7 and 8 both had gas exchange with 30% methane, but the total ratio of oxygen was 30% (30% oxygen and 40% nitrogen) and 60% (60% oxygen and 10% nitrogen) in order to produce the hyper-aerobic conditions. After growing the pre-cultures to the OD_{600} of 6 in 2 days, each set was gas-exchanged with the respective settings, without further gas exchange (Table 1). Unlike the control, the strain still grew with the remaining oxygen in Sets 7 and 8 even after 48 h (4 days of total cultivation). The final compositions of oxygen were over 7% and 20% in Sets 7 and 8, respectively, a lot higher than the 1% present in the control, and there was no hydrogen production (Fig. 3). Based on these results, it can be confirmed that the most critical factor for hydrogen production in *Methylomonas* sp. DH-1 is the oxygen level rather than the methane/oxygen ratio. However, the growth curves of Sets 7 and 8 were not particularly different from each other even with different oxygen levels (Fig. 3). This indicates that there is a particular range for the uptake of

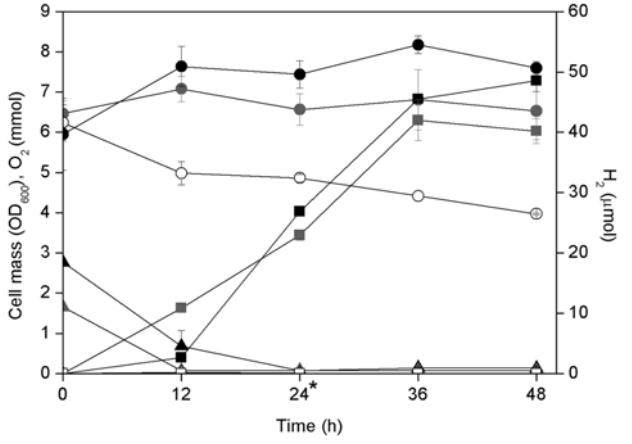

Fig. 2. Hydrogen production under micro-aerobic condition with OD_{600nm} of 6; Time profiles of hydrogen production by 3 sets of *Methylomonas* sp. DH-1 in shaking flask cultivation. Symbols and colors are: circle (●), cell growth; triangle (▲), oxygen; square (■), hydrogen; black (●), Set 4; grey (●), Set 5; white (○), Set 6. Data represent the mean of 3 replicates and the detailed growth condition and the final amount of produced hydrogen of each set are displayed in Table 1. Asterisk in x-axis is marked for the time of gas exchange after sampling.

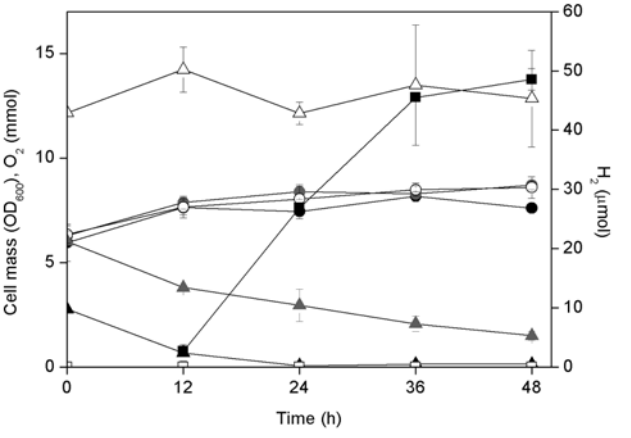

Fig. 3. Hydrogen production under hyper-aerobic condition; Time profiles of hydrogen production by 3 sets of *Methylomonas* sp. DH-1 in shaking flask cultivation. Symbols and colors are: circle (●), cell growth; triangle (▲), oxygen; square (■), hydrogen; black (●), Set 4; grey (●), Set 7; white (○), Set 8. Data represent the mean of 3 replicates and the detailed growth condition and the final amount of produced hydrogen of each set are displayed in Table 1. The data of Set 4 is the same as in Fig. 2.

oxygen in *Methylomonas* sp. DH-1, since the significantly higher oxygen concentration did not affect its growth. Even if unknown hydrogenases in *Methylomonas* sp. DH-1 are responsible for hydrogen production and tolerant to oxygen, they may still be vulnerable to high concentrations. Although there was no hydrogen production under hyper-aerobic conditions, there is a possibility of improvement on the fixed ratio of 30% methane and 14% oxygen for the basic cultivation of *Methylomonas* sp. DH-1, since the set fed with a higher oxygen ratio was able to grow for a longer time without additional gas supply.

3.3. Hydrogen uptake under aerobic conditions

Most hydrogenases in hydrogen-producing bacteria are also capable of hydrogen uptake [21]. However, there have been few studies on hydrogen-producing methanotrophs, and hydrogen uptake has not been investigated. Therefore, in this study we investigated whether the putative hydrogenase in *Methylomonas* sp. DH-1 can uptake as well as produce hydrogen, and whether its hydrogen-producing ability can be restored under micro-aerobic conditions. The strain was pre-cultured for two days and produced hydrogen for the next 24 h without additional gas exchange. An increase of hydrogen production from 7.24 μmol to 11.14 μmol was observed in *Methylomonas* sp. DH-1 when injected with 50 mL of extra air into the headspace of the flasks, but no hydrogen uptake was observed. Since the total percentage of oxygen was still low as 50 mL of air increased the oxygen level by only 2%, this may only prolong the micro-aerobic period. In addition, hydrogen production was faster than expected as the amount of hydrogen increased after only 2 h (Fig. S1). When injected with 50 mL of oxygen (10% oxygen in total), hydrogen concentration appeared to decrease for 2 h before increasing again, as the oxygen concentration decreased to 4% (Fig. S2). The results were more evident when 10 mL hydrogen (2% in total) and 50 mL oxygen were both injected (Set 9). The OD_{600} kept increasing for 6 h and the hydrogen amount slightly decreased for 2 h from 204.12 μmol to 180.44 μmol as the oxygen concentration decreased to below 4%. Hydrogen production then increased again to 186.68 μmol for the next 4 h (Fig. 4). This indicates that the produced hydrogen can be uptaken by *Methylomonas* sp. DH-1 when the oxygen concentration becomes sufficient again. This also shows its reversibility in hydrogen production and uptake since the strain produces hydrogen under micro-aerobic conditions, uptakes it with sufficient oxygen levels, and produces hydrogen again when the conditions become micro-aerobic once more. This reversibility may give some insight into understanding the mechanism of hydrogen production in *Methylomonas* sp. DH-1, compared to other hydrogen-producing/uptaking hydrogenases.

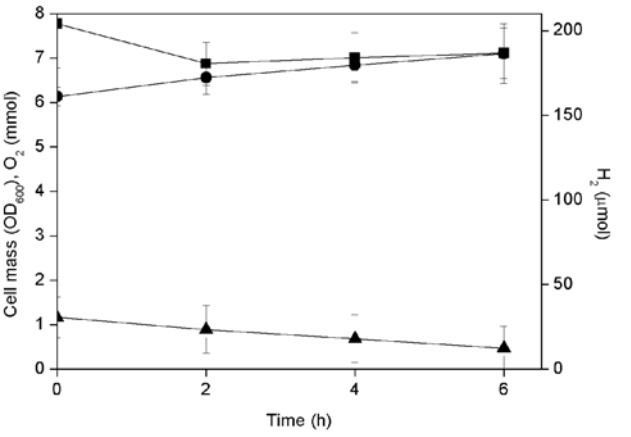

Fig. 4. Hydrogen uptake under aerobic condition with the injection of 50 mL oxygen and 10 mL hydrogen; Time profiles of hydrogen uptake and production by Set 9 of *Methylomonas* sp. DH-1 in shaking flask cultivation. Symbols and colors are: circle (●), cell growth; triangle (▲), oxygen; square (■), hydrogen; Data represent the mean of 3 replicates and the detailed growth condition and the final amount of produced hydrogen of each set are displayed in Table 1.

3.4. Hydrogen production with methanol consumption

Methylomonas sp. DH-1 is known for its high tolerance to methanol and can grow on methanol as a sole carbon source [19,22]. The effect of methanol in hydrogen production as a co-substrate or as a sole carbon source was examined since methanol can be used as a solution to overcome the mass transfer limitation from gas to liquid when using methane as the sole carbon source for methanotrophs. In this regard, experiments with different conditions were conducted as follows: Set 10 was gas-exchanged with 30% methane and 70% air and was also injected with 1.0 g/L of filtered methanol. 30% methane of 490 mL headspace equals 6 mmol of carbon source, and 1.0 g/L methanol in 50 mL working volume equals 1.5 mmol of carbon source. Therefore, Set 11 was gas-exchanged with 30% nitrogen and 70% air and injected with 5.0 g/L methanol (7.5 mmol of total carbon source).

Even when the amount of methane was sufficient (Set 10), methanol was also consumed by *Methylomonas* sp. DH-1. Besides, less hydrogen was produced in Set 10 and far less in Set 11 than in Set 4 after 24 h with methanol consumption. However, oxygen was consumed faster in Sets 10 and 11 than in Set 4, with a faster production of carbon dioxide (Fig. 5). This may be due to the oxidation of the produced formate from methanol consumption in Sets 10 and 11 (Fig. S3). The two sets fed with methanol as a co-substrate (Set 10) or as a sole carbon source (Set 11) could both produce hydrogen directly after 12 h with accelerated oxygen consumption. On the contrary, considering hydrogen production, the yield over carbon sources and the

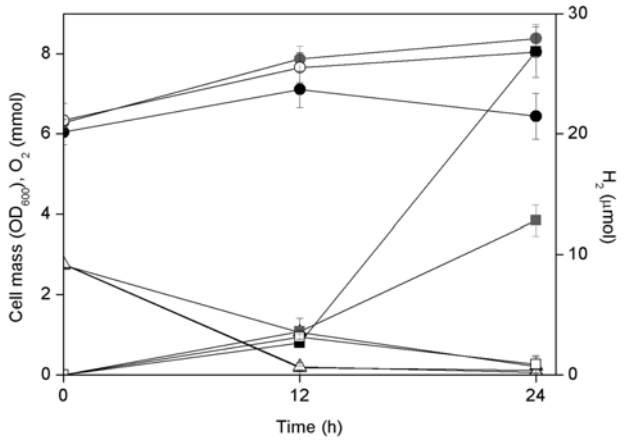

Fig. 5. Hydrogen production with methanol consumption; (A) Time profiles of hydrogen production by 3 sets of *Methylomonas* sp. DH-1 in shaking flask cultivation. Symbols and colors are: circle (●), cell growth; triangle (▲), oxygen; square (■), hydrogen; black (●), Set 4; grey (●), Set 10; white (○), Set 11. Data represent the mean of 3 replicates and the detailed growth condition and the final amount of produced hydrogen of each set are displayed in Table 1. The data of Set 4 is the same as in Fig. 2.

hydrogen production rate per cell were both the highest in Set 4 (Table 1). This can be assumed that the fast consumption of oxygen and carbon sources in Set 10 and Set 11 led to better cell growth, but not to hydrogen production. The results show that there is a significant effect of methanol consumption on the overall decrease in hydrogen production in *Methylomonas* sp. DH-1.

4. Conclusion

There have been continuous efforts to solve environmental problems such as global warming and land, air, and ocean pollution. Thus, biorefinery, centered in bio-based production processes, has extensively been developed as an eco-friendly process for sustainable production of chemicals, polymers, and renewable energy sources such as 1,3-propanediol, polyhydroxyalkanoates, ethanol, butanol, biodiesel, and hydrogen [1,2,23-27].

The amount of hydrogen produced in this study was very low, but the detailed conditions for hydrogen production by *Methylomonas* sp. DH-1 were examined using various oxygen ratios and methanol as a co-carbon source along with methane, which has not been studied in other methanotrophs. Even though the strain grows well under aerobic condition, an oxygen-limited condition bears a greater similarity to its natural condition in sludge from brewery plants. There may be a reason that the strain produces hydrogen under oxygen starvation stress for $NAD(P)^+$ regeneration, such as to cope with a decrease in pH due to the incomplete oxidation of organic acids [17]. This environmental pressure may have forced the strain to adapt to this system [21].

According to the genome research conducted in this study, *Methylomonas* sp. DH-1 may contain a form of nickel-iron (Ni-Fe) hydrogenase (AYM39_19615-19620) with an identity of over 70% with the one in *M. alcaliphilum* 20Z (MEALZ_3726), which can endure micro-aerobic conditions and produce hydrogen under these conditions. However, a significant portion of the hydrogen-producing mechanisms in these type I methanotrophs has not yet been clearly revealed. Therefore, further studies on this topic are required with particular attention to how *Methylomonas* sp. DH-1 could produce and uptake hydrogen depending on culture conditions such as oxygen levels.

Also, the ability to reverse or restore hydrogen production observed in this study is desirable since cell growth and hydrogen production could be performed simultaneously by manipulating oxygen concentration. In addition, the hydrogen production activity could be preserved even if oxygen is supplied for maintaining cell activity. Although the hydrogen production rate is not sufficient to be commercialized, improvements are to be expected through a greater understanding of hydrogen production mechanisms, as well as from further studies on reactor development such as the use of a continuous flow reactor.

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The authors declare no conflict of interest.

Neither ethical approval nor informed consent was required for this study.

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