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Enhancement of the cultivation process conditions of mixed culture methanotrophic Proteobacteria phylum enriched from waste activated sludge as the first step for value added recovery process

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Methanotrophs are of great interest due to their distinguish ability of recovering value-added commodities such as methanol and lipids while mitigating methane. The enhancement of methanotrophs cultivation process conditions is a pivotal step to develop a feasible methanotrophic bioreactor. In this study, multiple batch tests have been performed to evaluate the aqueous growth medium elements including nitrogen, copper, and biomass density and the gaseous headspace composition influence on methanotrophs activity and the associated microbial community. It was found that increasing copper concentration to 20 μM and nitrate concentration to 40 mM result in higher growth rate and yield. In contrast, increasing the biomass density resulted in a declination in the growth rate and yield. The 16S rRNA gene sequencing shows that the used culture was dominated by type I *Methylomonas* genus at relative abundance of 60.9% compare to 1% of the enriched population for type II and type III methanotrophs. Thereafter, the specific nitrate uptake rate has been determined to be ranging from 0.05 to 0.62 mgN-NO_3/mgTSS/day based on the cultures OD_{600}. The attained results would facilitate the continuous cultivation of type I methanotrophs dominated culture as a first step for any methanotrophic based biotechnology.

[**Key words:** Methanotrophs; Value-added products; Nitrogen source; Methane to oxygen ratio]

In a typical municipal wastewater treatment plant (WWTP), biogas is generated within the anaerobic biodegradation of the organic materials in the anaerobic digesters (1). The produced biogas mainly consists of methane (CH_4) and carbon dioxide (CO_2) with concentrations of 50–70% and 30–50%, respectively (2). Recently, the biogas emissions from WWTPs contribute by 4% to the yearly global methane budget (3). However, having a global warming potential of 25 for a 100-year time horizon makes the mitigation of CH_4 emissions pivotal for the global warming phenomena control (4). On the other hand, WWTPs are considered extensively energy demanding facilities. For instance, WWTPs consumed 4% of the total electrical demand of United States in 2014 which is expected to be increased due to the ongoing population increase (5). Therefore, it is crucial to develop sustainable technologies not only for biogas mitigation (as consortium of greenhouse gases) but also for value-added resources recovery.

Methanotrophs are the major biological methane (CH_4) sink due to their unique ability to utilize it as cellular carbon and energy source (4). Methanotrophs can integrate methane mitigation with various biotechnological applications including methanol, biopolymers, ectoine production, and biological nitrogen removal (6,7). Aerobic methanotrophs can be phylogenetically clustered into three types; type I (gamma subdivision of Proteobacteria phylum), type II (alpha subdivision of Proteobacteria phylum), and type III (verrucomicrobia phylum) (8). Type I has higher growth rates, and is more energy efficient in comparison with type II (9). Furthermore, type I methanotrophs can be easily enriched in mixed cultures under nutrients sufficient conditions (10,11). On the other hand, type III prefers to grow in extreme conditions such as acidic (optimal pH ranges from 1.5 to 3.5) and hot (optimal temperature ranges from 40°C to 55°C) environments (12,13), which limits its applications in WWTPs. Therefore, type I is more advantageous to be manipulated in methane mitigation coupled with any other biotechnological application.

As shown in Fig. 1, all aerobic methanotrophs have the same methane oxidation pathway in which methane is terminally oxidized to carbon dioxide throughout consecutive reactions catalyzed by different enzymes (14). The first step is methane hydroxylation resulting in methanol (CH_3OH). The previous reaction is energy requiring in which two electrons are needed for simultaneous methane uptake. Catalyzed by the quinoprotein methanol dehydrogenase, the resulted methanol is subsequently oxidized to formaldehyde (CH_2O) in which two electrons is generated. Later, formaldehyde is oxidized into carbon dioxide with formate (CH_2O_2) as an intermediate in which two electrons are generated from each reaction. Two electrons are consumed as reducing power for the simultaneous methane hydroxylation. Whereas, the remaining four electrons reduce the oxygen as the terminal electron acceptor generating ATP via the electron transfer chain (8). For methane assimilation pathways, type I utilize part of the produced formaldehyde into the RuMP pathway (Fig. 1).

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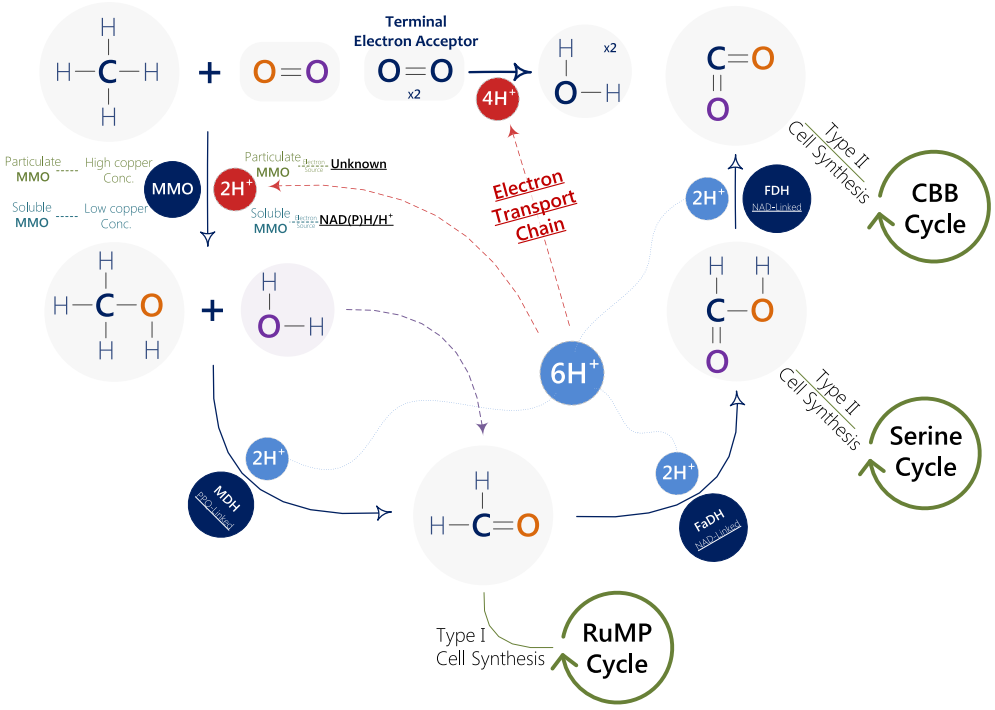

FIG. 1. Different methane oxidation pathways in methanotrophs. MMO, methane monooxygenase; MDH, methanol dehydrogenase; FaDH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; PQQ, pyrroloquinoline quinone; NAD(P)H, nicotinamide adenine dinucleotide; RuMP, ribulose monophosphate pathway; CBB, Calvin–Benson–Bassaham.

As previously mentioned, type I methanotrophs have the potential to be employed in multiple biotechnological application due to its higher growth yields and energy efficiency. Numerous factors are affecting methanotrophs performance such as nitrogen source and concentration, copper concentration, substrates concentrations, and biomass density. Therefore, the enhancement of those conditions is an important milestone on the way of developing an efficient continuous cultivation bioreactor as the first phase of any methanotrophic based biotechnology. Unfortunately, insufficient studies focused on the optimization of such conditions for methanotrophs type I enriched from mixed culture of waste activated sludge. In this study, the influence of inorganic nitrogen source, copper concentration, methane to oxygen ratio, biomass density, and methane concentrations for Proteobacteria phylum (type I methanotrophs) enriched from waste activated sludge were determined as a first step for the development of a feasible continuous methanotrophic bioreactor.

MATERIALS AND METHODS

Chemicals and operational conditions Batch experiments were performed using copper and nitrogen free nitrate mineral salts medium as previously described (15). Copper sulfate ($CuSO_4 \cdot 5H_2O$) was used to prepare copper stock solution to control copper concentration throughout the experiments. Nitrate (in the form of $NaNO_3$) and ammonium (in the form of NH_4Cl) concentrations were varied based on the targeted concentration for each experiment.

Unless otherwise specified, cells were suspended in 50 mL of MSM with specific copper and nitrogen concentrations in 250-mL sealed serum bottles capped with rubber stoppers. Methane and oxygen with purity above 99% (Praxair Technology, Inc., Danbury, CT, USA) were added to the headspace with 1:1 volumetric ratio. The serum bottles were incubated at room temperature (ranges from 25°C to 28°C) and continuously shaking at 165 rpm. Gas samples were withdrawn periodically throughout the experiments. While, liquid measurements were carried out at the start and end of each incubation.

Fresh waste activated sludge from Humber wastewater treatment plant (Toronto, Canada) were used as a seed for type I methanotrophs enrichment. The main applied pressure to select methanotrophs was by eliminating any other carbon sources but the methane which promote the dominance of methane utilizing bacteria. The obtained sludge was filtered on 100 μL filter in order to eliminate the existence of any large inert particles that may affect culture enrichment. Thereafter, the sludge was inoculated with initial biomass density of 0.25 OD_{600}. Further details about the process of culture enrichment has been previously detailed (16). After two weeks, cultures shifted completely to the pinkish and showed stable growth and methane consumption indicating type I methanotrophs dominance. Different type I methanotrophs colonies pigmentation were reported to be pinkish including *Methylomonas* genus (9,17,18). Furthermore, samples were taken and analyzed using PCR to ensure type I methanotrophs dominance. The process for the RNA extraction and PCR identification are described by AlSayed et al. (19). The used primers set in PCR identification are mentioned in Table S1. As shown in Fig. S1, lanes 1 and 2 show the presence of type I methanotrophs and the intensity of the band can relate to the genomic density of the target gene.

Growth medium enhancement Three different concatenations of sodium nitrate (10, 20, and 40 mM) and ammonium chloride (5, 10, 20 mM) were added to 50 mL MSM with 5 μM of copper sulfate. The previously enriched biomass obtained from enrichment phase was inoculated in the reaction medium with initial OD_{600} of 0.575 ± 0.075.

Copper concentration regulates the MMO enzyme expression, which is directly affect the competition between methane and ammonium (4). Thus, the effect of copper addition to the optimum nitrate and ammonium concentrations was investigated. Cultures with 40 mM nitrate and 5 mM ammonium with no copper were compared to the same cultures with copper of 10 μM. Biomass harvested from the previous phase was inoculated to the reaction medium with initial OD_{600} of 0.433 ± 0.2 after being centrifuged.

Thereafter, higher nitrate and copper concentrations were employed to identify the inhibitory and optimum concentrations. The biomass obtained from previous phases were collected and centrifuged. The nitrate enriched biomass was inoculated with initial cell density (OD_{600}) of 0.170 ± 0.039 for nitrate optimization. The used nitrate concentrations were as ranging from 10 to 160 mM added $NaNO_3$. Using the optimum nitrate concentration as the nitrogen source, the copper experiments were performed using copper concentration of 0, 20, 40, and 80 mM with initial cell density (OD_{600}) of 0.185 ± 0.038.

The influence of biomass density Initial biomass densities of OD_{600} ranged from 0.148 ± 0.01 to 2.743 ± 0.14 were used to investigate biomass density effect on type I methanotrophs. The same methane and oxygen concentrations were used in this phase. The reaction medium consisted of 40 mM of sodium nitrate and 20 μM of copper added to the MSM.

Methane to oxygen ratio effect Oxygen to methane with various molar ratios (1–4) were added to the bottles headspace using biomass harvested from previous phases. Volume of methane added was fixed in all bottles to maintain equal food to microorganisms ratio effect. Moreover, helium was added to the

headspace to fix the partial pressure in all bottles. Experiments kept running until most of the methane was consumed from one of the bottles. The initial biomass densities (OD_{600}) were equal to 1.330 ± 0.12.

Analytical methods and molecular biology analysis Regarding the Molecular biology analysis, following the growth conditions enhancement, three samples were withdrawn from the obtained cultures to quantify the dominance of type I in the mixed culture and study the associated microbial community. The detailed processes have been previously described (6).

Gas samples withdrawn from the headspace were analyzed using SRI 8610C gas chromatography (SRI Instrumentation, Torrance, CA, USA) equipped with thermal conductivity detector, methanizer and 6' molecular sieve column (Restek, Bellefonte, PA, USA) to measure methane and oxygen concentrations. The temperature program was previously described (20). Optical density (OD_{600}) at 600 nm were obtained using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, CO, USA) to determine cell densities. To analyze the liquid samples, cultures were centrifuged at 4200 ×*g* for 20 min and the supernatant was collected. Thereafter, HACH methods and testing kits were used to measure inorganic nitrogen (NH_3–N, NO_2–N, and NO_3–N).

The bacterial behavior throughout the experiments were evaluated using main three parameters. First is specific growth rate (μ) which was determined using Eq. 1.

$$\mu = \frac{\left(DCW_{initial} - DCW_{final}\right)/t}{\left(DCW_{initial} - DCW_{final}\right)/2} \quad (1)$$

where $DCW_{initial}$ is the initial dry cell weight (mg), DCW_{final} is the final dry cell weight (mg), *t* is the experiment duration (h), and μ is the specific growth rate (g $DCW_{increase}$/g $DCW_{average}$/h). Secondly, the methane uptake rate (mg-CH_4 consumed/h) was calculated by dividing the consumed methane by the incubation time. In some cases, it was normalized by the initial biomass density in order to eliminate its effect, especially in the case of comparing the results from two different experiments. The last parameter is cells observed growth yield (Y_{CH4}) which used mainly to demonstrate how efficient was the cells in converting the methane uptake into cell increase. Biomass increase was divided by methane consumed to obtain the growth yield.

RESULTS AND DISCUSSION

Type I methanotrophs behavior under nitrate and ammonium as nitrogen source Nitrogen is one of the macronutrients needed for any living organism to build their nucleic acids and proteins (21). However, some studies reported that nitrate increase has inhibitory effect on methanotrophs growth, even though, its metabolism and inhibition mechanism in methanotrophs are still unclear (22). As shown in Table 1, the increase in nitrate concentration from 10 to 20 mM resulted in a minor enhancement in bacterial activity, whereas the growth rate and methane uptake increased by 14% and 25%, respectively, by increasing nitrate concentration to 40 mM. The highest growth rate and methane uptake were equal to 0.574 ± 0.022 day^{-1} and 1.054 ± 0.084 mgCH4/h. In agreement, the tolerance to nitrate of some type I strains in pure cultures were previously investigated and all the tested strains were tolerant to 40 mM of nitrate (23). Hence, it can be deduced that the presence of nitrate with concentrations up to 40 mM as a macronutrient has a positive effect on the performance of type I methanotrophs.

It was reported that the increase in methane uptake may result in the accumulation of excessive concentration of formaldehyde known for its toxicity (22,24). This can elucidate the notable decline in the yield at higher nitrate concentration (Table 1). Another hypothesis is that the cells pushed its metabolism towards the energy production pathway at the expense of the cell synthesis to produce more electrons to maintain the higher methane uptake rate. Supportively, Oxygen uptake observed at 40 mM nitrate was 20% higher than the uptake at 10 mM (Table 1). Interestingly, the observed decline took place only throughout this phase and relativity higher yields were observed in the next phases. Therefore, it can be deduced that the cells successfully adapted with the new conditions by either developing formaldehyde detoxification mechanism and/or increasing formaldehyde oxidation rate or the cells regulating both the methane assimilation and dissimilation pathways.

As illustrated in Table 2, the amount of nitrogen utilized per average biomass (nitrogen utilization ratio) increased from 0.13 ± 0.08 for 10 mM nitrate to 0.40 ± 0.07 for 40 mM which supposed to be around 0.13 representing the cellular nitrogen (21). Moreover, the consumed C/N ratio (g carbon as CH_4 consumed/g nitrogen consumed) dropped by 50% when nitrate concentration increased from 10 to 40 mM. This drop indicates that less carbon is needed to remove the same amount of nitrogen in form of nitrate. In addition, nitrite with relatively high concentrations were observed at the end of the experiments, for instance, 18.76 ± 0.18 mgN-NO_2/L was noticed at 40 mM of nitrate (Table 2). Combined together, those findings raise the potential of the integration of methane mitigation and denitrification processes. Further investigations and enhancements are needed to confirm such potential.

As shown in Table 1, type I has a relatively higher growth rate, growth yield, and methane uptake rate at 5 mM of ammonium. It was equal to 0.536 ± 0.092 day^{-1}, 0.65 ± 0.05 $gDCW_{increase}/gCH_4$

TABLE 1. The influence of nitrogen source and concentration on type I methanotrophs growth rate and yield, and methane and oxygen uptake rates.

	Specific growth rate (day^{-1})	Observed growth yield ($gDCW_{increase}/gCH_{4\ uptake}$)	Methane uptake rate (mg_{CH_4}/h)	Oxygen uptake rate (mg_{O_2}/h)
NIT-10[a]	0.510 ± 0.003	0.68 ± 0.07	0.839 ± 0.084	2.659 ± 0.048
NIT-20	0.526 ± 0.085	0.70 ± 0.06	0.840 ± 0.059	2.676 ± 0.077
NIT-40	0.574 ± 0.022	0.52 ± 0.03	1.054 ± 0.084	3.194 ± 0.093
AMM-5[b]	0.536 ± 0.092	0.65 ± 0.05	0.857 ± 0.015	2.771 ± 0.191
AMM-10	0.460 ± 0.049	0.62 ± 0.03	0.773 ± 0.022	2.679 ± 0.063
AMM-20	0.443 ± 0.065	0.57 ± 0.09	0.721 ± 0.021	2.475 ± 0.069

[a] NIT-10, 10 mM of nitrate.
[b] AMM-5, 5 mM of ammonium.

TABLE 2. Type I methanotrophs nitrogen consumption under different nitrogen sources and concentrations.

	Final nitrogen concentration (mg-N/L)	Nitrate concn. (mg-N/L)	Nitrite concn. (mg-N/L)	N-utilization ratio ($g\text{-}N_{uptake}/gDCW_{increase}$)	Consumed C/N ratio (g C–CH_4/g-N)
NIT-10[a]	100 ± 6.8	–	2.80 ± 0.09	0.13 ± 0.08	10.05 ± 0.88
NIT-20	214 ± 5.5	–	6.82 ± 0.24	0.22 ± 0.03	5.85 ± 0.09
NIT-40	466 ± 4.9	–	18.76 ± 0.18	0.40 ± 0.07	4.71 ± 0.01
AMM-5[b]	18 ± 1.54	16.00 ± 0.93	0.18 ± 0.08	0.14 ± 0.02	9.87 ± 0.9
AMM-10	84 ± 4.8	12.00 ± 0.87	0.32 ± 0.02	0.16 ± 0.01	8.27 ± 0.28
AMM-20	220 ± 6.5	10.00 ± 0.27	0.34 ± 0.09	0.20 ± 0.05	7.19 ± 0.7

[a] NIT-10, 10 mM of nitrate.
[b] AMM-5, 5 mM of ammonium.

uptake, and 0.857 ± 0.015 mg_{CH4}/h, respectively. Moreover, increasing ammonium concentrations above 5 mM caused a decline of 17% and 11% in the growth rate and methane uptake rate. This decline was accompanied with an increase in ammonium uptake (Table 2). A lot of similarities have been reported between MMO and ammonia monooxygenase enzymes (14). Therefore, it can be concluded that the competition between ammonium and methane on the MMO enzyme is the main reason for the lower methane uptake and by consequence the lower growth rate and growth yield. The nitrogen consumption ratio was about 75 ± 3% at ammonium concentration of 5 mM. However, the nitrogen utilization ratio was equal 0.14 ± 0.02 which indicates that the amount consumed was mainly for the cellular nitrogen (Table 2). In addition, the consumed C/N ratio at the optimum ammonium concentration was equal to 9.87 ± 0.9 g C–CH_4/g-N which relatively high in comparison with nitrate.

In terms of comparing ammonium and nitrate as a nitrogen source for type I methanotrophs, the growth rate at 40 mM nitrate was 0.574 ± 0.022 day^{-1}, while at 5 mM ammonium was 0.536 ± 0.092 day^{-1}. The methane uptake was equal to 42.14 ± 3.37 $mgCH_4$ and 34.28 ± 0.62 $mgCH_4$ for 40 mM nitrate and 5 mM ammonium, respectively. In addition, the amount of methane consumed to remove 1 mg of nitrogen as ammonium was 2 times higher than nitrate (Table 2). These results show that the type I bacterial activity is better while utilizing nitrate which can be referred to the ammonium inhibitory effect discussed before.

Copper effect on type I methanotrophs behavior under different nitrogen sources Genetically, it was reported particulate methane monooxygenase (pMMO) and ammonia monooxygenase are more identical than the soluble methane monooxygenase (sMMO) (25). Therefore, ammonium inhibitory effect would be higher in pMMO expressing cultures. Therefore, the effect of copper was evaluated for the optimum concentrations for both ammonium and nitrate cultures to make the ammonium and nitrate comparison more reliable. Moreover, 10 μM of copper was tested instead of 5 μM used in the previous phase as an indication of the influence of increasing copper concentration.

In the presence of ammonium, the methane uptake rate was higher by 5 % at the copper free cultures which confirms the effect of sMMO expression on the competition between methane and ammonium (Fig. 2). sMMO is known for its lower methane uptake ability which can elucidate this minor increase (14). Furthermore, Ammonium consumption ratio increased from 80 ± 0.5% to 86 ± 1.7% by copper elimination. This observation is an evidence on the substrate competition release. Moreover, the growth rate and yield were increased from 0.576 ± 0.085 to 0.606 ± 0.022 day^{-1} and 0.79 ± 0.07 to 0.86 ± 0.08 $gCH_{4consumed}/gDCW_{increase}$, respectively, by removing the copper from the ammonium growing cultures (Fig. 2). The increased growth rate and yield confirms the positive effect of the removing the copper from the medium.

As demonstrated in Fig. 2, the methane uptake rate of the nitrate cultures with 0 and 10 μM copper was equal to 0.832 ± 0.020 and 0.950 ± 0.035 $mgCH_4$/h, respectively. Moreover, the growth rate and growth yield were increased by 8% and 5%, respectively (Fig. 2). These results can be explained by the higher methane uptake of the pMMO. Hence, copper addition has a positive effect on the bacterial activity of the nitrate growing cultures.

As shown in Fig. 2, type I methanotrophs showed better performance in cultures using nitrate as their nitrogen source regardless the expression of sMMO or pMMO. In this phase, the difference was even more obvious than the previous one due to the adaptation effect discussed before. The growth rate was almost 2 times higher at nitrate growing cultures. Moreover, the growth yield and uptake were higher by 10% and 40%, respectively. All these observation lead to that it is more advantageous to use nitrate as nitrogen source for type I methanotrophs. Furthermore, increasing copper concentration from 5 to 10 μM had a positive effect on the bacterial activity in both cultures. Specific growth rate increased by 30% due to copper concentration to 10 μM. The methane uptake rate in this phase was lower than the previous phase which is due to the lower initial biomass density. In agreement, the methane uptake/initial biomass density was equal to 3.533 ± 0.197 and 2.499 ± 0.106 $gCH_{4\ consumed}/gDCW_{initial}$ at 40 mM nitrate and 5 mM ammonium, respectively, in both phases. Therefore, 10 μM of copper sulfate was added to the upcoming phases.

Finally, it is noteworthy that the bacterial activity (uptake and growth) in the previous two phases was lower than the upcoming batch tests due to the cultures storage at 4°C in limited methane conditions. It was reported that bacterial activity especially the MMO activity is negatively affected by the storage in limited methane conditions (26,27).

Nitrate and copper concentration optimization Consistently, the addition of 40 mM nitrate resulted in the best bacterial activity. As shown in Fig. 3, it led to specific growth rate of 0.613 ± 0.035 day^{-1} and growth yield of 0.92 ± 0.06 $gCH_{4\ consumed}/gDCW_{increase}$. In comparison with cultures growing using 10 mM of nitrate, the consumed C/N ratio was 50% less which was equal to 5.05 ± 0.03. Moreover, the

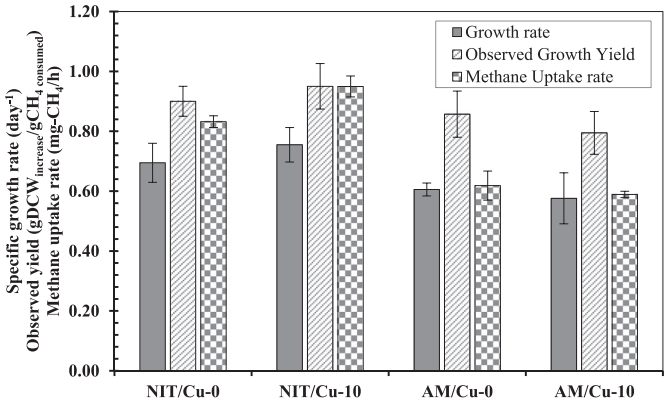

FIG. 2. The effect of copper with ammonium and nitrate as nitrogen source on type I methanotrophs, specific growth rate (day^{-1}), observed growth yield ($gDCW_{increase}/gCH_{4\ consumed}$), and methane uptake rate (mg-CH_4/h). NIT/Cu-0, nitrate 40 mM and copper 0 μM; NIT/Cu-10, nitrate 40 mM and copper 10 μM; AM/Cu-0, ammonium 5 mM and copper 0 μM; AM/Cu-10, ammonium 5 mM and copper 10 μM.

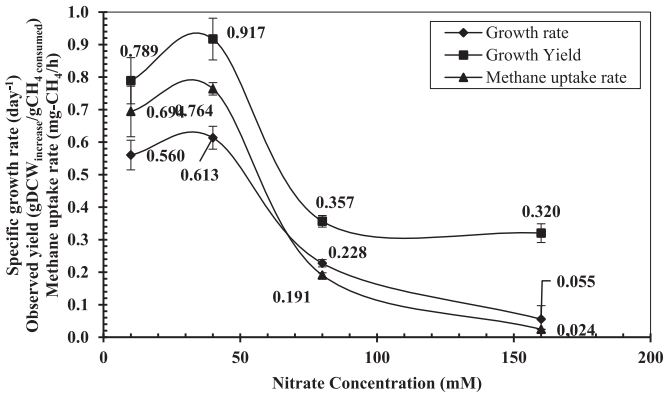

FIG. 3. Type I methanotrophs behavior under different nitrate concentrations, specific growth rate (day^{-1}), observed growth yield ($gDCW_{increase}/gCH_{4\ consumed}$), and methane uptake rate (mg-CH_4/h).

methane uptake/initial biomass was significantly equal to 13.707 ± 1.09 $gCH_{4\ consumed}/gDCW_{initial}$ which is 45% higher than uptake observed at 10 mM. It was concluded from the previous results that increasing nitrate concentration enhance the overall bacterial performance. However, a severe decline in the bacterial activity occurred when the nitrate concentration increased above 40 mM. As shown in Fig. 3, increasing nitrate concentration from 40 to 80 mM results in a decrease in growth rate and methane uptake by 90% and 75%, respectively. Moreover, almost no activity was observed while adding 160 mM of nitrate. As previously mentioned, nitrate inhibitory mechanism is still not clear, however, some studies reported that the inhibition might be due to the increased salinity (28). Also, it can be referred to the low initial carbon to nitrogen (C/N) ratio which was less than 1 for 80 and 160 mM. Collectively, it was deduced that the optimum sodium nitrate us equal to 40 mM which was used in the further experiments.

Increasing copper from 0 to 20 μM resulted in slight enhancement in the growth rate and methane uptake (Table 3). At copper concentration of 20 μM, growth rate reached 0.622 ± 0.050 day^{-1} while maintain high growth yield of 0.94 ± 0.03. The further increase in the copper concentration to 40 μM resulted in minor inhibitory effect, while, a notable inhibition in the bacterial activity was observed at copper concentration of 80 μM (Table 3). Most of the studies reported an inhibitory effect at high copper concentration, but, the inhibitory concentration varied from study to another (3,29,30). However, the cultures in this phase showed higher tolerance to copper than the previous studies. This can be referred to the effect of the associated bacterial community as most of the previous studies were hold on pure culture. Hence, it can be deduced that type I enriched from waste activated sludge are optimally grow at copper concentration of 20 μM and tolerate copper concentration up to 40 μM. Thereafter, copper sulfate was added with concertation of 20 μM in the further experiments based on these findings.

Biomass density influence Throughout the previous phases, it was noticed that decreasing the initial biomass density has resulted in a notable enhancement in the performance of type I methanotrophs. For instance, the highest growth rate in the study was obtained at the copper optimization experiment (0.622 ± 0.050 h^{-1}). This growth rate was achieved at the lowest initial biomass density (OD_{600}) equal to 0.148 ± 0.01. Thus, those observations drove us to run an experiment to assess the effect of biomass density on the bacterial activity. The results showed that the culture with the lowest optical density ratio which was equal to 0.595 ± 0.04 had the best performance. Growth rate was equal to 0.617 ± 0.077 h^{-1} while the growth yield was 0.66 ± 0.03 $gCH_{4\ consumed}/gDCW_{increase}$. Those values still lower than the obtained values at lower biomass density in copper optimization phase as demonstrated in Fig. 4. It can be concluded that type I methanotrophs growth rate and yield was positively affected by the decrease in the biomass density. On the other hand, it was observed that the methane removal ratio was the same in all bottles and equal to 83 ± 3% by the end of the incubation time. Correspondingly, the total methane uptake was almost the same

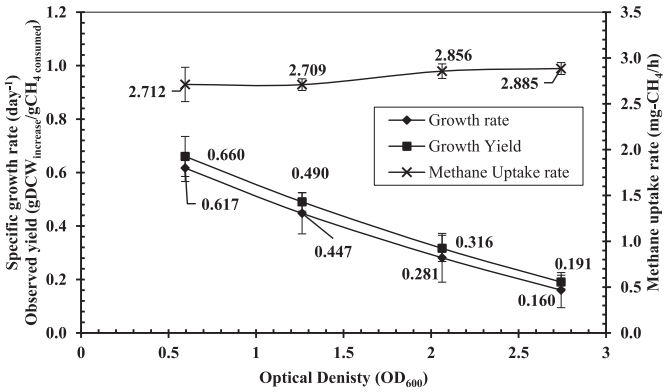

FIG. 4. Biomass density influence on type I methanotrophs, specific growth rate (day^{-1}), observed growth yield ($gDCW_{increase}/gCH_{4\ consumed}$), and methane uptake rate (mg-CH_4/h).

with only 6% higher at optical density of 2.743 ± 0.14 in comparison with 0.595 ± 0.04. However, the methane uptake rate was not the same over the incubation time. It was 1.6 times higher at biomass densities of 2.743 ± 0.14 and 2.063 ± 0.09 measured as OD_{600} than the lower biomass densities after 8 h. Whereas, the uptake rate for both cultures were declined by 2 ± 0.1 folds when measured after 21 h and remained the same at the lower biomass densities. Thus, it can be concluded that higher biomass densities (above 2 OD_{600}) are more effective in methane removal only at higher methane loading rate.

Methane to oxygen ratio effect Running the bottles at different oxygen to methane molar ratios resulted almost in the same growth rate which were equal to 0.522 ± 0.013 day^{-1}. As illustrated in Fig. 5, methane and oxygen uptake were increased by 20% and 81%, respectively. In contrast, the growth yield declined by 24% because of the increase in oxygen to methane molar ratio from 1 to 4. The increase in gases uptake can be referred the abundance

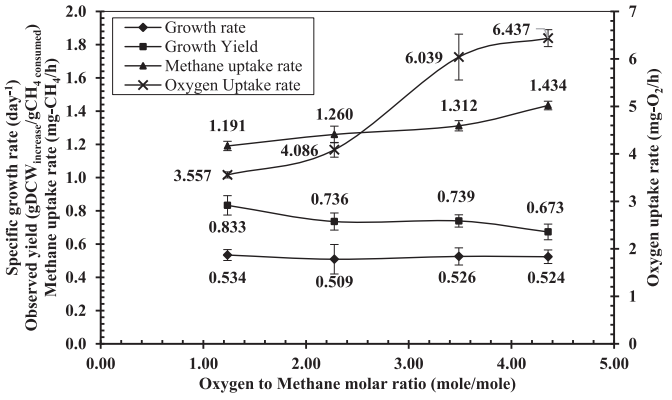

FIG. 5. Type I methanotrophs behavior under different methane to oxygen ratios, specific growth rate (day^{-1}), observed growth yield ($gDCW_{increase}/gCH_{4\ consumed}$), methane uptake rate (mg-CH_4/h), and oxygen uptake rate (mg-O_2/h).

TABLE 3. Type I methanotrophs behavior under different copper concentrations.

Initial copper concentration (μM)	Specific growth rate (day^{-1})	Growth yield ($gDCW_{increase}/gCH_{4\ uptake}$)	Methane uptake ($gCH_4/gDCW_{initial}$)
0	0.612 ± 0.019	0.94 ± 0.05	13.111 ± 0.156
10	0.613 ± 0.026	0.92 ± 0.09	13.707 ± 0.371
20	0.622 ± 0.050	0.94 ± 0.03	14.829 ± 0.186
40	0.611 ± 0.033	0.87 ± 0.08	14.062 ± 0.266
80	0.326 ± 0.060	0.54 ± 0.05	3.140 ± 0.183

of the terminal electron acceptor which stimulate the cells to uptake more methane. However, the decline growth yield indicate that the increased uptake has to some extent negative effect. As an elucidation, the increased existence of the electron acceptor stimulates the bacteria to push its metabolism more towards the energy generation stream on the expenses of the cell syntheses resulting in lower yields and the same growth rate. Thus, it is not favorable to apply oxygen to methane molar ratio higher than 1. Even though, increasing oxygen concentration result in higher methane uptake by 20%. This is because of the lower observed yields, and the observation that it was achieved with 80% higher aeration requirements and 4 times less methane loading (assuming fixed bioreactor volume) what makes it not economically feasible.

Culture behavior under the enhanced growth conditions Following the previously discussed enhancements, the enhanced conditions were applied to three bottles for 25 h. The resulted specific growth rate was equal 1.56 ± 0.13 day^{-1} which is relatively high in comparison with the previously attained growth rates using type I mixed culture (16). Furthermore, growth yield as high as 1.02 ± 0.03 $gCH_{4\ consumed}/gDCW_{increase}$ was observed.

On the other hand, 16S rRNA gene sequencing was performed to confirm type I dominance and investigate the associated microbial community. As shown in Table 4, the used culture was dominated by type I *Methylomonas* genus showing relative abundance of 60.9%. Whereas, type II and type III methanotrophs were almost washed out representing less than 1% of the enriched population. Interestingly, methylotrophs, known to preferably grow on methanol, have obvious presence in the mixed culture with relative abundance of 25.8%. As described previously, methanol is the first intermediate in the methane assimilation pathway which elucidates the methylotrophic relatively high abundance in the growing culture. Finally, the genera of *Flavobacterium* and *Pseudomonas* were detected with relative abundance of 2.4% and 4.1%. Both genera contain facultative denitrifying species which explain the high nitrate uptake observed, as discussed below. Furthermore, it promotes the possibility to integrate aerobic methane mitigation with denitrification processes. Detailed microbial analysis has been provided in Figs. S2 and S3.

In order to estimate the nitrogen (in form of sodium nitrate) uptake rate, the enhanced conditions were applied on five different biomass densities and liquid samples were withdrawn every 3–5 h and filtered for nitrogen measurements. At all the biomass densities, nitrogen uptake rate was higher during the first 6 h and under sufficient nitrogen, methane and oxygen conditions (Fig. 6). The maximum nitrogen uptake rate ranged from 5.83 ± 0.83 to 15.67 ± 0.33 mgN-NO_3/L/h for the cultures with optical densities of 0.20 ± 0.02 to 2.98 ± 0.03, respectively. Thereafter, nitrogen uptake rate declined which can be referred to the effect of biomass increase on gas diffusion, methane and oxygen limitation. The overall specific nitrate uptake rate was from 0.62 ± 0.03 to 0.05 ± 0.01 mgN-NO_3/mgTSS/day for cultures from 0.20 to 3, respectively. Those results are comparable to the specific denitrification rates in the literature. However, biomass densities in this study is lower than in the mixed liquor volatile suspended solids (MLVSS) of activated sludge processes by 10 times at least which would significantly affect the results (1). Further investigations are needed to enhance nitrogen removal and methane mitigation integration.

Representing 13% of the cells weight, nitrogen as a macronutrient is the most important macronutrient after carbon (21). In addition, nitrogen source and concentration have a notable effect on methanotrophs community structure (31). For instance, all type II and few strains of type I methanotrophs -with slower growth rates-can fix the atmospheric nitrogen (18,32). Therefore, the deterioration of the nitrate from the growing cultures may lead to a notable shift in type I cultures shifting to type II and, by consequence, a totally different behavior. Hence, nitrogen uptake rate is a decisive parameter in methanotrophic bioreactor design.

Having gaseous electron donor and acceptor makes hydraulic retention time (HRT) determined based on the nitrogen uptake rate. Typically, lower HRTs are more desirable to maintain higher flow rates with lower bioreactor volumes. In agreement, lower HRT would be beneficial for methanotrophs in which higher nitrogen (nitrate) concentrations. The maximum HRT can be a decisive factor in bioreactors operated hydraulically in fed-batch mode such as fluidized bed reactor. Such hydraulic mode can be beneficial for methanotrophic bioreactor because of the gaseous substrates, while, minimizing the water and pumping requirements. On the other hand, the minimum HRT can be determined based on the growth rate in order to prevent the bacterial washout. As discussed previously, methanotrophs growth rate is function of biomass density in addition to the substrate concentration. Further

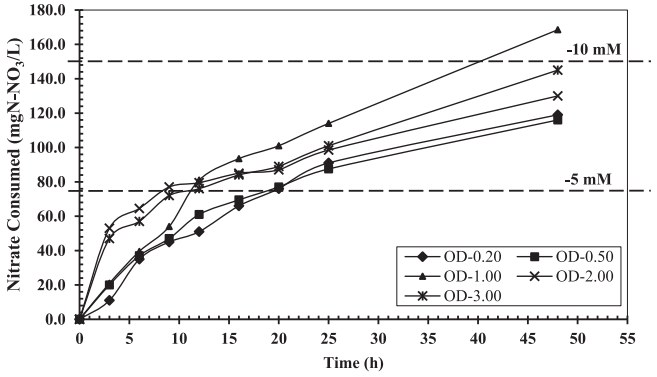

FIG. 6. Nitrogen uptake at different initial biomass densities.

TABLE 4. Taxonomic classification and relative abundance of the key bacterial community resulting from 16S rRNA gene sequences for the samples collected under the enhanced growth conditions.

Phylum	Class	Order	Family	Genus	Relative abundance (%)
Bacteroidetes	*Bacteroidia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	2.4
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	4.1
				Facultative denitrifiers	6.5
Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*	*Methylophilaceae*	*Methylobacillus*	12.8
Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*	*Methylophilaceae*	*Methylophilus*	13.0
				Methylotrophic bacteria	25.8
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Verrucomicrobium*	0.1
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Beijerinckiaceae*	*Methylocystis*	0.4
Proteobacteria	*Gammaproteobacteria*	*Methylococcales*	*Methylomonaceae*	*Methylomonas*	60.9
				Methanotrophic bacteria	61.4
				Other	6.3

investigation to identify the biomass density effect and the bacterial optimum growth rates is currently under development.

Numerous studies in the literature have been held to investigate the methanotrophs behavior under multiple operational conditions. However, most of the studies were performed using pure cultures of type II methanotrophs. In this study, enhanced values were identified for growth medium composition, biomass density, and headspace gas composition. Throughout the study, it was found that higher methane uptake and growth rate was observed while using nitrate as nitrogen source. It was concluded that the optimum nitrate concentration for type I methanotrophs is 40 mM of sodium nitrate. In addition, using 20 μM of copper sulfate resulted in the highest growth rate, growth yield and methane uptake. Moreover, the addition volumetric ratio between methane and oxygen was found to be more feasible at 1:1 due to the higher methane loading and lower aeration requirements. Regarding the biomass density, it was found that initial biomass density of 0.148 measured as OD_{600} have the higher growth rate and growth yield. Those findings identify the optimum composition for the aqueous growth culture and the gaseous headspace for any methanotrophic cultivation phase in any biotechnological application. Furthermore, higher methane uptake was observed in the higher biomass densities up to 2.743 ± 0.14. Furthermore, the maximum overall nitrate uptake rate was obtained at biomass density 0.91 ± 0.01 OD_{600} and was equal to 3.51 ± 0.07 mgN-NO_3/L/h. These attained results can be directly employed to determine the bioreactor HRT and gases loading rates to appropriately design methanotrophs cultivation bioreactor.

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