Short Communication

Lipid accumulation capability of typical non-acclimated activated sludge microbial consortia using methane gas as secondary carbon source

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Abstract
This work experimentally demonstrates that wastewater activated sludge microbial consortia can utilize methane resulting in lipid content enhancement. Activated sludge was cultivated using a synthetic wastewater as culture media. After initial purging with air, analytical grade methane gas was added in the culture headspace. The cultures were cultivated in batch-mode for 120 hours at 25°C in 500-mL bioreactors with 125-mL working liquid volume. Results indicate that methane gas was utilized by activated sludge microbial consortia under a similar pattern of microbial respiration as the control cultivated only with air. The activated sludge in the methane-purged bioreactors showed lipid enhancement (0.123 ± 0.037 mg lipid per mg biomass) and biomass growth (0.626 ± 0.163 mg biomass per mg glucose plus methane), which were higher than those in the control runs (0.009 ± 0.034 mg lipid per mg biomass and 0.225 ± 0.133 mg biomass per mg glucose).

Keywords
biofuels, biogas, bioprocess, lipids, methanotrophs, natural gas

1 | Introduction

Methane is the primary constituent of natural gas (>85%)1 and biogas (~65%).2,3 Reduced natural gas prices have adversely impacted the value of using methane within natural gas and biogas (via anaerobic digestion) for
commercial purposes (combustion or manufacturing). Thus, industries often flare natural gas and biogas instead of pursuing marketable benefit. For example, in 2016, around $2.1 \times 10^{11}$ ft$^3$ of natural gas was flared and vented in the United States. In the past, biogas was considered a good option to offset electricity (biogas to power generator) by displacing natural gas; however, this option may not be economically viable in some regions (such as the United States) due to low natural gas prices. For this reason, this work proposes to evaluate the capability of a typical wastewater treatment plant (WWTP) activated sludge (AS) microbial consortia to accumulate lipids via the cofeeding of methane gas with wastewater influents. The global commercial lipid market (currently at $1B/y and expanding) can offer as much as a 4-fold up-valuing of both natural gas and biogas. In addition to market considerations, developing other conversion pathways for chemicals of renewable origins aligns well with the principles of green and sustainable engineering.

An interesting class of aerobic microorganisms is methanotrophs, which can utilize gaseous methane as a carbon source for sustaining cell growth. Methanotrophs have been of research and commercial interest for several years; mainly, as a means of remediating (via biotreatment) chlorinated solvent—contaminated media. For bioremediation systems, methane is feed to methanotrophic consortium to initiate the formation of methane monooxygenase, which is able to breakdown chlorinated solvents, such as trichloroethylene. Several large-scale field studies were successfully performed during the 1980s and 1990s. More recently, with the growing commercial interest in lipids for both production of biofuels (biodiesel and green diesel) and green, sustainable products, such as paints and other polymers, a new wave of interest in methanotrophs has emerged. These works are focusing on the use of cheap methane sources (biogas and natural gas) to serve as a carbon source for the methanotrophs toward the eventual production of moderate levels of lipids over the typical lipids levels within heterotrophic bacterial cultures. Lipids have various functions in microbial cells depending on the type of lipid. Neutral lipids (ie, triacylglycerides-TAGs, waxes) are the energy storage materials of the cells, while polar lipids (eg, phospholipids) serve as structural components of the cell wall. Lipid accumulations in methanotrophs in excess of 20% (w/w) have been widely reported, making methanotrophs an appealing option to up-value biogas and/or natural gas into biofuel and chemical precursors. Moreover, the large-scale culturing of methanotrophs is a proven concept and they have been commercialized to produce methanol; use their oxygenases cultured in large fermenters to treat full-scale polluted aquifer systems; biofuels; and single cell proteins. In all cases of these commercial applications of methanotrophs-to-product systems, methane was used to grow the methanotrophs for later harvesting of the targeted cell constituents of value.

Several works on methane-utilizing bacteria have been published in the recent years: production of polyhydroxyalkanoates (PHAs) from anaerobic digester sludge; treatment of high-nitrogen wastewater; electricity production from simulated biogas; electrochemical production of microbial protein; photoautotroph-methanotroph culturing for CO$_2$-CH$_4$ utilization; immobilized methane-utilizing bacteria; and bubble-column reactor design for poly-3-hydroxybutyrate (PHB) production from CH$_4$; and CH$_4$ to methanol via immobilized methane-utilizing bacteria.

The envisioned concept of this present work is that the methane within biogas generated at a municipal WWTP could be directly used or supplemented with natural gas for the production of lipids via wasted AS microbial cells. The concept hypothesizes that a high-molar carbon-to-nitrogen ratio (C/N) via the addition of methane can induce additional lipid production within AS over a similar system simply being fed a standard strength municipal wastewater influent. The use of high C/N ratios was previously shown to be feasible with sugars (such as glucose and xylose) and organic acids (such as acetic, propionic, and butyric acid) as carbon sources. That is, this study explores the utility of methane gas as carbon source for lipid enhancement in an AS microbial consortium without any attempt to optimize via prolonged microbial acclimation to assess the presence and capacity of methanotrophs in a typical AS. The scope of this work was limited to using analytical grade methane gas and a two-level experimental design (control vs cultivation with methane gas). Nonetheless, this work demonstrates the potential of AS lipid enhancement as an alternative conversion pathway for methane—particularly using biogas that is often produced within the same wastewater treatment facilities. A key interest area is that the past remediation-oriented work almost always required the seeding of methanotrophs into the targeted systems. This work aimed to explore how significant the methane oxidizer populations were within a typical AS consortium and the lipid yield improvement achievable without acclimation attempts to “optimize” the consortium toward methane oxidation.
2 | METHODOLOGY

2.1 | Activated sludge inoculum preparation

The AS samples were collected from the sampling well of the sludge return line from the aeration basin of a local municipal WWTP (East WWTP, Lafayette, Louisiana 70501, USA). The samples were collected and stored in plastic bottles while being transported to the laboratory, wherein the AS samples were immediately processed for the experiments. Prior to cultivation runs, the AS sample was centrifuged to separate the solids. The liquid phase was discarded. Around 500 mL of recovered wet solids from the grab sludge sample after centrifugation was added to 2 L of a synthetic wastewater (SWW) and thus the simulated influent and methane provided the envisioned cofed system. This resulted in AS initial solids concentration of \( \sim 4.5 \text{ g/L} \) in the SWW in all the culture experiments.

2.2 | SWW preparation

The SWW was prepared based on the formulation used in previous works on AS\textsuperscript{38-41} (in 1-L deionized water): 0.5-g glucose, 37-mg ammonium sulfate (to make C/N of 30 with glucose), 0.15-g gelatin, 0.21-g starch, 0.07-g yeast extract, 0.01-g casamino acids, 1.5-g NaH\textsubscript{2}PO\textsubscript{4}, 1-g K\textsubscript{2}HPO\textsubscript{4}, and 5-mL trace minerals solution. A C/N of 30 is typical level in biological processes of WWTPs.\textsuperscript{42} Very high C/N (such as 70\textsuperscript{36}) was avoided to eliminate the dominance of glucose-induced lipid accumulation. The glucose loading of 0.5-g/L has an equivalent COD of 530 mg/L, which is within typical levels in influents of biological wastewater processes.\textsuperscript{42} The trace minerals solution contained the following: (in 1 L deionized water): 0.5-g ethylenediaminetetraacetic acid (EDTA), 3-g MgSO\textsubscript{4}•7H\textsubscript{2}O, 0.5-g MnSO\textsubscript{4}•H\textsubscript{2}O, 1-g NaCl, 0.1-g FeSO\textsubscript{4}•7H\textsubscript{2}O, 0.1-g CaCl\textsubscript{2} anhydrous, 0.1-g ZnSO\textsubscript{4}•7H\textsubscript{2}O, and 0.01-g CuSO\textsubscript{4}•5H\textsubscript{2}O (all chemicals used from Fisher Scientific, USA).

2.3 | Cultivation experiments

The AS microbial consortia in SWW purged with their corresponding headspace gas were cultivated in three-neck round-bottom 500-mL glass flasks as shown in Figure 1. The liquid volume was 125-mL leaving most of the flask volume for headspace gas. Compressed air from a laboratory air compressor was used to purge the sample headspace. For the samples intended for methane cultivation, the methane gas was added after the initial compressed air purge. To make sure that air (specifically oxygen) was not completely purged out, the timing of purging methane gas was determined prior to executing preparation steps. Methane gas supplied from an analytical grade (99%) compressed gas cylinder (Matheson Gas, USA). At the start of cultivation, the headspace gas compositions were measured to make sure that the initial headspace in the methane-purged setups contained air (oxygen). Three replicates were made for both the control and the methane headspace treatment. The setups were incubated in a water bath at 25°C on top of a magnetic stirring platform. To ensure contact of the headspace gas with the AS microbial consortia in the liquid, the stirring was set at level sufficiently high to induce vigorous mixing at liquid-gas interface and to have gas bubbles visually observable diffusing into the liquid phase. The cultivation runs were set for 120 hours, which is an AS cultivation period used in previous works of similar bioprocess.\textsuperscript{36,37}

2.4 | Chemical analyses

The composition of the headspace gas in each cultivation reactor setup was monitored for the consumption of oxygen and methane gas and for the production of culture off-gas such as carbon dioxide and hydrogen. This was done using gas chromatography instrument (GC 6890N by Agilent USA and fitted with a Fix Gas GC column by Restek USA) with thermal conductivity detector and calibrated with a standard mix of CH\textsubscript{4}, CO\textsubscript{2}, CO, H\textsubscript{2}, O\textsubscript{2}, and N\textsubscript{2} gases. Gas samples of 1.5 mL total volume per sample were taken from each flask setup using gas-tight syringe (Gastight #1005, Hamilton Co., USA). The volume measurements for methane, oxygen, and carbon dioxide were normalized by the volume of nitrogen, which was essentially an inert headspace component. Hence, any change in the amounts of the component gases would still be comparable with each other.

To verify glucose loading and to account for its consumption, the concentrations of glucose and volatile fatty acids (VFAs) were measured using liquid chromatography instrument (Agilent 1100 Series LS using a UV detector and fitted
with Rezex ROA-Organic Acid H+ 300 x 7.8 mm column by Phenomenex, USA). The VFAs in the calibration were acetic acid, propionic acid, and butyric acid.

The following procedures were performed for the analysis of the control and methane-cultivated setup samples collected at the start ($t = 0$ hours) and at the end of experiments ($t = 120$ hours). Total solid biomass concentration was determined gravimetrically through centrifugation followed by freeze-drying (FreeZone; Labconco). The total solid biomass was reported as gram per liter. The dried biomass solids were then pulverized and prepared for lipid content determination through an accelerated solvent extractor (ASE 300; Dionex) with a solvent system consisted of chloroform-methanol. The lipid extracts were recovered by evaporating the chloroform and methanol through purging with nitrogen (TurboVap; Biotage). The lipid content was reported as weight percent (% weight lipid extract/weight total solids). The fatty acids profiles of the extracted lipids were determined by gas chromatography-mass spectroscopy (GC/MS) analysis of the esterified lipids. The procedure for the esterification of the lipids into fatty acid methyl esters (FAMEs) was based on previous works. A FAMEs standard covering fatty acid carbon length of eight to 24 (F.A.M.E. Mix C8-C24; Sigma-Aldrich) was used in the calibration of the GC/MS quantitation step. The fatty acid profiles were expressed as weight percent of each fatty acid relative to the total of the esterified lipid (% weight/weight esterifiable lipid).

### 3 Results and Discussion

Gas component profiles during the AS cultivation are shown in Figure 2. From Figure 2A, it can be seen that methane and oxygen were consumed accompanied by the production of carbon dioxide as an end product. The patterns of oxygen consumption and carbon dioxide production within the methane-charged system were similar to the patterns exhibited in the control samples (see Figure 2B). The carbon dioxide yield for the methane-charged systems was approximately the same (actually slightly less) than the control, but it had more input carbon than the control. This provides evidence of carbon conversion into microbial component (lipids and other cell products). Over 60% of the methane was utilized over the first 70 hours of incubation; however, at the 70-hour incubation, the headspace oxygen levels reached practically zero levels, which appear to have ceased methanotroph conversion of the methane. Potentially, more oxygen could have yielded an even higher extent of methane utilization. These results indicated that methane gas can be utilized by the nonacclimated standard AS microbial consortium. Methane was essentially consumed after 3 days. Glucose as the common and primary carbon source in all runs (control and methane-charged) was completely consumed in all setups at the end of
FIGURE 2  Headspace gas time-profile during cultivation runs showing consumption of methane and oxygen and production of carbon dioxide in the methane-charged samples, A, compared with the control samples, B. Component gas volumes are normalized by the volume of nitrogen gas which was the internal reference inert component. Note that all error bars are included but some are too small overlaid with the data average markers. The following are the corresponding average initial headspace volumetric compositions (%v/v) of the flask setups: methane-charged contained $16 \pm 0.8\%$ O$_2$, $64 \pm 2.3\%$ N$_2$, $20 \pm 2.8\%$ CH$_4$, and control contained $21 \pm 0.1\%$ O$_2$, $79 \pm 0.1\%$ N$_2$.

TABLE 1  Biomass and lipid yield estimates relative to substrates for the control and the methane-charged runs

| Run                  | Biomass yield (mg/mg) | Lipid yield (mg/mg) |
|----------------------|-----------------------|---------------------|
| Control\(^a\)        | 0.225 ± 0.133         | 0.009 ± 0.034       |
| CH$_4$-charged\(^b\) | 0.626 ± 0.163         | 0.123 ± 0.037       |

\(^a\)Yield estimates relative to the consumption of glucose.  
\(^b\)Yield estimates relative to the consumption of both glucose and methane.

cultivation (~0.5-0 g/L for 120 hours). The measured pH levels of the initial cultures ($t = 0$ hours) were around 6.8 and of the final cultures ($t = 120$ hours) were around 6.5 for both control and methane-charged bioreactors. During the 48- to 72-hour period, significant consumption of methane occurred while oxygen was not significantly consumed (Figure 2A). This may indicate the occurrence of anaerobic oxidation of methane. Studies have shown that some bacterial species common in aquatic and wastewater streams may use sulfate and metals, which were present in the SWW used in the culture media, as terminal electron acceptors at low levels of oxygen when consuming methane.$^{43,44}$

As a result of the utilization of methane gas, the AS in the methane-charged bioreactors contained appreciably higher lipid content and biomass levels than the control samples (see Figure 3A,B). Using initial cultures ($t = 0$ hours) as reference, the following changes occurred by the end of cultivation: for the control runs, an average biomass increase of 6.40% (w/w initial) and an average lipid increase of 3.57% (w/w initial) and for methane-charged runs, an average biomass increase of 19.23% (w/w initial) and an average lipid increase of 48.88% (w/w initial). Even though the average lipid content of the control at 120 hours was essentially equal to the lipid content at the start (Figure 3A), the biomass concentration increased, resulting to a slight increase in the actual amount (not the weight %) of lipid produced. The corresponding biomass and lipid yields are summarized in Table 1. These yield estimates agree with literature on the ranges of biomass and lipid yields for sugar$^{36}$ and methane$^{8}$ substrates. Genetically modified obligate pure culture methanotrophs can even achieve biomass yields (biomass/CH$_4$) greater than one.$^{45}$ To account for the fate of glucose as carbon source, the concentrations of VFAs in the liquid phase were also measured (Figure 3D). Notably, the methane-charged cultures produced more VFAs than the control. This seems contradictory to the observed higher yield of lipid and biomass in the methane-charged samples compared with the control, that is, VFAs are common intermediate carbon sources in microbial cultures. The observation of lesser VFAs concentration in the control should have been manifested as higher yield in the biomass. A plausible explanation may be the possible production of exopolysaccharides (EPS)$^{46}$ instead of VFAs by the microbial consortia in AS. EPS was not measured in this work and probably have been washed out when the AS biomass was being prepared for dry-biomass analysis, but previous works on wastewater AS cultivated at high C/N detected and measured significant levels of EPS on AS microbial consortia.$^{47}$

The lipid fatty acid profiles measured as FAMEs are shown in Figure 3C. The fatty acid profile of the methane-charged runs is essentially similar to the fatty acid profile of the control runs, indicating that the main effect of methane feeding is lipid enhancement without significant alteration of the profile of fatty acids in the lipid produced. Moreover, the fatty acid profile of both the methane-charged and the control at 120 hours were similar in pattern as the fatty acid profile of
the lipid extracted from the culture samples at the beginning of the runs \((t = 0\) hours). These patterns indicate that the changes in lipid content and biomass concentrations in both the control and methane-charged runs (Figure 3A,B) did not appreciably affect fatty acid profiles. However, it must be noted that this is not a general pattern, that is, previous works on AS lipid-enhancement showed that fatty acid profiles in the extracted lipids tend to approach certain profiles at the end of cultivation amid varying starting fatty acid profiles depending on cultivation conditions such as carbon source, C/N level, and pH buffering.\(^{37,40}\) Fatty acid profiles may be key considerations for applications of the lipid extract or the lipid-enhanced AS. This is particularly true if the lipid produced will be used for biofuel production. For example, biodiesel qualities (i.e., cetane number, lubricity, cold flow properties, and oxidative stability) are dictated by the fatty acid profile of the lipid feedstock.\(^{48,49}\) On the other hand, green diesel characteristics produced through catalytic cracking is more independent of the fatty acid profile of the feedstock. However, unsaturated fatty acids tend to more reactive (i.e., faster reaction rates).\(^{50}\) Another area of application where lubricity of AS lipid has been found beneficial is the formulation of drilling fluids for oil and gas exploration\(^{51}\)—this application does not require extraction of the lipid. Too much unsaturation in the AS lipid fatty acids, however, may result to oxidative instability especially when the number of double bonds in a chain are two or more.\(^{48}\) The higher proportion of unsaturated fatty acids in the lipids produced could also find application in the manufacture of paint\(^{52}\) as well as polymers\(^{53}\) and adhesives.\(^{54}\)

### 4 CONCLUSIONS AND FUTURE PERSPECTIVES

The initial findings in this work prove that a typical AS microbial consortium can utilize methane gas to accumulate lipids without special acclimation or methanotroph seeding. These findings are consistent with previous works. Lamb and Garver\(^{55,56}\) three decades ago showed that methane-utilizing mixed cultures can be selectively isolated from AS and be used in batch and continuous cultivations. A more recent study on methanotrophs showed that genetic engineering of a pure culture obligate methanotrophs results to significant increase in biomass and lipid yields\(^{45}\) supporting the finding in this work that lipid yields may be improved. With WWTP constantly wasting and inputting varied carbon and microbial sources within the influents resulting in a fairly dynamic chemical and microbial matrix within an AS plant, these results are encouraging in that the envisioned concept may be easily applied to perhaps produced commercial lipids at a typical WWTP. However, several aspects of the bioprocess concept are still unknown. First, further studies should identify the microbial groups acting as biological agents for the observed performance of AS consortia, that is, identify the methanotrophs within the AS capable of accumulating lipids. Second, evaluate various process conditions...
such as actual natural gas and biogas as substrates instead of analytical-grade methane. This will consequentially evaluate any adverse effects of impurities in these gas streams when fed directly to AS for lipid enhancement. The culture media may also be an important consideration since microorganisms need nutrients other than carbon sources to grow. Another aspect is the liquid-gas interfacial dynamics to enhance the transfer of methane into the liquid phase where the microorganisms thrive. A consequence of this line of inquiry is the possibility of using attached growth design that intermittently exposes the AS microbial consortia to the liquid nutrients and to the gaseous headspace for methane supply.

The results of this work may also have process design implications worth considering. Take a typical biogas produced from anaerobic digestion containing around 65% v/v CH₄² (natural gas may have at least 85% v/v CH₄)³ as feed to the microbial culture of AS in this work. As methane is being consumed for biomass and lipids production in AS, CO₂ is being produced and the headspace fraction of CO₂ increases, which may have negative effect on the AS microbial consortia. A possible solution to this issue is the integration downstream of algae cultivation stage that consumes the CO₂. Alternatively, this integration may be implemented in reverse—feed biogas to algae culture to convert CO₂ to O₂ hence creating a gas rich in CH₄ with sufficient O₂ to be used as final electron acceptor by AS during lipid accumulation. This latter integration is similar to the proposed process by van der Ha et al⁵⁷ that demonstrated the use of a pure methane-oxidizing Methylocystis parvus culture to use algae effluent gas consisting of CH₄ and O₂.

The use of microbial platform for the conversion of CH₄ to bioproducts has been spurred by the low prices of CH₄ as natural gas or biogas, and reimagining the conversion pathways with CH₄ as primary feedstock has been considered a key path to future bioeconomy.⁵⁸ A preliminary technoeconomic analysis done by Fei et al¹¹ shows that low prices of natural gas can make methane-utilizing lipid-producing microbial processes economically competitive with petroleum-derived diesel. This was the result of a best-case scenario of $100/ton CH₄ with an estimated raw material cost per gal of diesel at $0.7/gal. Around 60% of petroleum-derived diesel may be replaced by methane-derived diesel if all 1.1 × 10¹⁴ ft³ global annual natural gas is converted to diesel.¹¹ Another economic aspect on the use AS microbial consortia for methane-to-lipid process is the cost offset on the treatment of AS from wastewater treatment facilities. According to the literature compilation study conducted by Mateo-Sagasta et al,⁵⁹ around 25,188 metric tons of dry sewage sludges are produced globally per year from wastewater treatment, and their use has been majorly in agricultural land application and thermal conversion. In the United States, however, around 30% of national sewage sludge production goes to landfills.⁵⁹ The use of AS microbial consortia for lipid production from methane may potentially up-value sewage sludge.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Dhan Lord B. Fortela contributed to the conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing the original draft—review and editing. Wayne Sharp contributed to conceptualization, investigation, methodology, writing—review and editing. Emmanuel Revellame contributed to the conceptualization, funding acquisition, methodology, visualization, writing—review and editing. Andrei Chistoserdov contributed to the conceptualization, funding acquisition, writing—review and editing. William Holmes contributed to the investigation, methodology, writing—review and editing. Daniel Gang contributed to the conceptualization, methodology, writing—review and editing. Rafael Hernandez contributed to the conceptualization, formal analysis, writing—review and editing. Mark Zappi contributed to the conceptualization, formal analysis, funding acquisition, methodology, writing—review and editing.

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