

A METHOD FOR RESOURCE RECOVERY BY MICROALGAE-METHANOTROPH COCULTURE

Field of Invention

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The present disclosure generally relates to methods of resource recovery, and more particularly relates to methods of resource recovery by microalgae-methanotroph cocultures.

Background

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The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

15 With the world's rapid population growth, the food requirement in human society is also rising. The critical challenge is the reduction in cultivation land area due to urbanization development. Thus, a land-independent technology for food production is essential for the next generation of food production. Microbial protein or single-cell protein (SCP) has been becoming an alternative solution for protein supply. It is a safe protein for animal feed or human
20 consumption.

Intensive studies have been conducted on the production of SCP by valorizing greenhouse gases (GHGs) such as CH₄ and CO₂. This approach not only reduces GHGs emissions but also adds value to those gaseous substances. CH₄ has been used as a carbon and energy
25 source for SCP production by utilizing methanotroph, which are also referred to as methane-oxidizing bacteria (MOB). Nowadays, CH₄-derived SCP production heavily relies on fossil-based feedstock, such as natural gas, and requires external O₂ supply. Substituting natural gas with renewable biogas offers a sustainable way for SCP production, reducing dependence on fossil resources. Biogas, the main product of anaerobic digestion, mainly contains CH₄ and
30 CO₂. Transforming biogas into SCP instead of its traditional use, such as burning for the heat and power industry, can create an economic driving force for the adoption of biogas in the food industry. However, the monoculture of MOB cannot take full advantage of CO₂ (the other key component of biogas) for SCP production, largely compromising carbon utilization efficiency. On the other hand, CO₂ is widely used for microalgae (MG) protein production.
35 However, the O₂ produced by MG accumulated in the reactor can inhibit MG growth. Furthermore, a low CO₂/O₂ ratio in microalgal systems should be avoided since such a condition may inhibit photosynthetic activity and CO₂ fixation by MG. In addition, soluble algae

products that are released into the liquid phase can be toxic to MG and negatively impact their growth.

To date, most studies on the co-cultivation of MOB and MG have focused on pure strain
5 cultivation in batch mode to minimize the risk of contamination. However, the potential of using
mixed cultures in larger-scale, long-term bioreactors to fully exploit the benefits of biogas for
producing SCP and other value-added products has not been fully assessed.

Therefore, there exists a need for new methods for the utilization of carbon and nitrogen
10 sources while ensuring stable and efficient bioreactor performance.

Summary of Invention

Aspects and embodiments of the invention are provided in the following numbered clauses.
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1. A method of generating a biomass mixture, comprising:
 - (a) providing a co-culture comprising microalgae and methanotrophs obtained from a waste activated sludge in a reaction vessel; and
 - (b) operating the reaction vessel under conditions that comprise feeding a gas comprising
20 CO₂ and CH₄ that is depleted of oxygen to the co-culture and subjecting the co-culture to light to generate the biomass mixture, wherein
the biomass mixture comprises proteins, lipids and glycogen in addition to the microalgae and methanotrophs.
- 25 2. The method according to Clause 1, wherein one or more of the following apply:
 - a protein content of the biomass mixture is from 40 to 60 wt%, such as from 44 to 56 wt%, such as about 50 wt%, of a cellular dry weight of the biomass mixture;
 - an omega-6 fatty acid content of the biomass mixture is from 2 to 5 wt%, such as about 2.7 wt%, of a cellular dry weight of the biomass mixture; and
 - 30 a glycogen content of the biomass mixture is from 22 to 35 wt%, such as about 28 wt%, of a cellular dry weight of the biomass mixture.
3. The method according to Clause 1 or Clause 2, wherein the co-culture further comprises heterotrophs, optionally wherein the heterotrophs comprise one or more of
35 *Ferrovibrio*, *Terrimonas*, and *Chthonobacter*.

4. The method according to any one of the preceding clauses, wherein the methanotrophs comprise one or more prokaryotes selected from the group consisting of *Methylosinus*, *Methylocystis*, *Leptolyngbya*, *Lentimicrobium*, *Melioribacter*, *Rhodoplanes*, *Methylococcus*, and *Sulfurisoma*, optionally wherein the methanotrophs comprise one or more
- 5 prokaryotes selected from the group consisting of *Methylosinus* and *Methylocystis*.
5. The method according to any one of the preceding clauses, wherein the microalgae comprise one or more eukaryotes selected from the group consisting of *Chlorella*, *Ochromonas*, and *Trichoderma*, optionally wherein the microalgae comprise one or more
- 10 eukaryotes selected from the group consisting of *Chlorella sorokiniana*.
6. The method according to any one of the preceding clauses, wherein one or more of the following operating conditions are applied:
- (ai) a pH within a range of from 6.5 to 7, such as from 6.7 to 6.8;
- 15 (aii) a temperature of from 25 to 30 °C, such as from 26 to 30 °C;
- (aiii) a hydraulic retention time (HRT) and/or a sludge retention time (SRT) of from 5 to 19 days, such as from 7 to 14 days in a continuously running process;
- (aiv) an ammonium mineral salt medium comprising ammonium chloride, magnesium sulfate (e.g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), disodium hydrogen phosphate (e.g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), potassium
- 20 dihydrogen phosphate, calcium chloride (e.g. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$), and ethylenediaminetetraacetic acid (e.g. as the free acid and/or as the ferric-sodium salt thereof, optionally wherein the free acid is used initially and the ferric-sodium salt is used after continual operation of 100 days or more).
- 25 7. The method according to any one of the preceding clauses, wherein the gas further comprises H_2S at a concentration of greater than or equal to 500 ppm.
8. The method according to Clause 7, wherein the H_2S concentration is from 750 to 10,000 ppm, such as from 1,000 to 5,000 ppm.
- 30 9. The method according to any one of the preceding clauses, wherein the volume to volume ratio of CH_4 to CO_2 is from 1.2:1 to 1.7:1, such as from 1.26:1 to 1.67:1, such as about 1.5:1.
- 35 10. The method according to any one of the preceding clauses, wherein the gas further comprises one or both of nitrogen gas and volatile organic compounds.

11. The method according to any one of the preceding clauses, wherein the co-culture comprising microalgae and methanotrophs obtained from a waste activated sludge is obtained by a method comprising the steps of:
- (bi) providing a waste activated sludge in a precursor reaction vessel;
 - 5 (bii) operating the precursor reaction vessel under conditions that comprise feeding in a gas comprising CO₂ and CH₄ that is depleted of oxygen to the waste activated sludge for a first period of time;
 - (biii) removing a biomass suspension after the first period of time and subjecting the biomass suspension to conditions that comprise feeding in a gas comprising CO₂ and CH₄
 - 10 that is depleted of oxygen to the biomass suspension for a second period of time; and
 - (biv) repeating step (biii) from 2 to 10 times until a co-culture comprising microalgae and methanotrophs is obtained.

Drawings

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Fig. 1 depicts microalgae and methanotrophs enrichment by supplying LED source and synthetic biogas into wasted active sludge.

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Fig. 2 depicts a continuous stirred tank reactor with inoculum from a 2L batch reactor.

Fig. 3 depicts a 10 L anaerobic digester (AD) for biogas production.

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Fig. 4 depicts bioreactor performance: ammonium concentration in the influent (circle); ammonium concentration in the effluent (circle with line); and total suspended solid (TSS) concentrations in the bioreactor (square with line).

Fig. 5 depicts the macromolecule compound composition of the mixed culture in the fermenter.

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Fig. 6 depicts the gas chromatograph of methanolic extract fatty acids methyl ester from the biomass.

Fig. 7 depicts total organic carbon (TOC) in the fermenter liquid phase.

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Fig. 8 depicts the fraction of soluble organic matter in the fermenter liquid phase.

Fig. 9 depicts a histogram showing the relative abundance above 0.01 based on 16S rRNA gene amplicon sequences (A) before Fe addition, and (B) after Fe addition.

Fig. 10 depicts a histogram showing the relative abundance above 0.01 based on 18S rRNA gene amplicon sequences (A) before Fe addition, and (B) after Fe addition.

- 5 **Fig. 11** depicts SB reactor biomass composition – protein, glycogen, and total lipid content change before and after Fe addition. (Triangle, circle and square are used to distinguish protein, glycogen and total lipid content, respectively. The number of patterns shows the difference in protein, glycogen, and total lipid content.)
- 10 **Fig. 12** depicts a histogram showing the relative abundance above 0.01 based on 16S rRNA gene amplicon sequences: (A) SB reactor; and (B) SB-Fe (7-day) reactor.

Fig. 13 depicts carbon fate in the (a) Control-7d and (b) Fe-7 cycle study.

- 15 **Fig. 14** depicts the microbial community at the end of the Control-7d cycle study. (a) Relative abundance (%) of prokaryotes in genus. (b) Relative abundance (%) of eukaryotes in genus. The microbial community at the end of the Fe-7d cycle study. (c) Relative abundance (%) of prokaryotes in genus. (d) Relative abundance (%) of eukaryotes in genus.
- 20 **Fig. 15** depicts the ammonium concentration of the cycle studies Control-7d, Fe-7d and Fe-3d.

- Fig. 16** depicts the biomass intracellular compound composition. (a) Protein, glycogen, and total lipid content (b) The predominant FAMES of cycle studies Control-7d, Fe-3d and Fe-7d
- 25 (Notes: one asterisk (*) stands for the *p* value between these two groups is < 0.05, while two asterisks (**) stands for the *p* value is < 0.01, n.s means there is no significant difference between these two groups).

- Fig. 17** depicts the microbial community at the end of the Control-7d cycle study: (a) relative
- 30 abundance (%) of prokaryotes in phylum; (b) relative abundance (%) of prokaryotes in class; and microbial community at the end of the Fe-7d cycle study: (c) relative abundance (%) of prokaryotes in phylum; and (d) relative abundance (%) of prokaryotes in class.

- Fig. 18** depicts the dissolved organic compounds (DOC) classification and concentrations in
- 35 the Fe-7d cycle study.

Fig. 19 depicts SB reactor biomass composition – protein, glycogen, polyhydroxybutyrate (PHB) and total lipid content in a typical 6-day cycle.

Fig. 20 depicts nitrogen fate in the batch reactor under synthetic biogas.

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Fig. 21 depicts carbon fate in the batch reactor under synthetic biogas.

Fig. 22 depicts dissolved organic carbon classification and accumulation in a cycle.

10 **Fig. 23** depicts (a) nitrogen fate in the N-7d cycle study, and (b) the biomass intracellular compound composition in the N-7d cycle study.

Fig. 24 depicts the amino acid profiles in the N-7d cycle study.

15 **Fig. 25** depicts procaryotic organism community in the three batch reactors. (A) RB. (B) SB. (C) SBS.

Fig. 26 depicts the microbial community relative abundance (%) of prokaryotes in genus.

20 **Fig. 27** depicts the microbial community relative abundance (%) of eukaryotes in genus.

Fig. 28 depicts the kinetics performance of the bioreactor under varying H₂S stress conditions over time.

25 **Description**

It has been surprisingly found that, compared to pure strain cultivation, the non-axenic operation of methanotroph (MOB) and microalgae (MG) co-culture systems may lead to the presence of heterotrophic microorganisms. These heterotrophs can play a beneficial role in promoting the stability of the culture by eliminating certain metabolites that could otherwise inhibit MOB or MG growth, such as acetate or formate. Some strains of heterotrophs have the ability to degrade short-chain alkanes, which can naturally occur in gas sources. Furthermore, heterotrophs can contribute to the quality of protein in the culture. This has important implications for the development of sustainable and efficient mixed-culture systems that can optimize the utilization of carbon and nitrogen sources for the production of high-value bioproducts. Thus, in a first aspect of the invention, there is provided a method of generating a biomass mixture, comprising:

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- (a) providing a co-culture comprising microalgae and methanotrophs obtained from a waste activated sludge in a reaction vessel; and
- (b) operating the reaction vessel under conditions that comprise feeding a gas comprising CO₂ and CH₄ that is depleted of oxygen to the co-culture and subjecting the co-culture to light
- 5 to generate the biomass mixture, wherein
- the biomass mixture comprises proteins, lipids and glycogen in addition to the microalgae and methanotrophs.

In embodiments herein, the word "comprising" may be interpreted as requiring the features

10 mentioned, but not limiting the presence of other features. Alternatively, the word "comprising" may also relate to the situation where only the components/features listed are intended to be present (e.g. the word "comprising" may be replaced by the phrases "consists of" or "consists essentially of"). It is explicitly contemplated that both the broader and narrower interpretations can be applied to all aspects and embodiments of the present invention. In other words, the

15 word "comprising" and synonyms thereof may be replaced by the phrase "consisting of" or the phrase "consists essentially of" or synonyms thereof and *vice versa*.

As noted above, the current invention is not formed from pure strains of MOB and MG, but rather derived from a source material (waste activated sludge; WAS) that contains other

20 microorganisms too. As such, the co-culture may also contain further microorganism strains that are not MOB or MG, but which may contribute to the overall effectiveness of the current method. For example, the co-culture may also include aerobic heterotrophic microorganisms that can use the organic compounds released from methanotrophs and microalgae as a substrate. Such heterotrophs may include, but are not limited to microorganisms of the genus

25 *Ferrovibrio*, *Chthonobacter*, *Terrimonas*. The genus *Ferrovibrio* are Gram-stain negative, aerobic or facultative anaerobic belonging to the family *Rhodospirillaceae*. The genus *Chthonobacter* was first proposed for describing a bacterium isolated from grass-field soil and it belongs to the family *Methylocystaceae*, which is same family with the *Methylocystis*. The genus *Terrimonas* are strictly aerobic, which is a member of the family Chitinophagaceae.

30 Without wishing to be bound by theory, it is believed that the presence of heterotrophs can play a beneficial role in promoting the stability of the culture by eliminating certain metabolites that could otherwise inhibit MOB or MG growth, such as acetate or formate. Some strains of heterotrophs have the ability to degrade short-chain alkanes, which can naturally occur in gas

35 sources. Furthermore, heterotrophs can contribute to the quality of protein in the culture. This has important implications for the development of sustainable and efficient mixed-culture

systems that can optimize the utilization of carbon and nitrogen sources for the production of high-value bioproducts.

5 In other words, the co-culture is non-axenic in nature. As such, it comprises not only methanotrophs and microalgae but also some heterotrophs that assist in consuming the metabolites produced by both methanotrophs and microalgae. As discussed herein, but without wishing to be bound by theory, it is believed that inclusion of heterotrophs can enhance the robustness of the method disclosed herein.

10 The phrase, "consists essentially of" and its pseudonyms may be interpreted herein to refer to a material where minor impurities may be present. For example, the material may be greater than or equal to 90% pure, such as greater than 95% pure, such as greater than 97% pure, such as greater than 99% pure, such as greater than 99.9% pure, such as greater than 99.99% pure, such as greater than 99.999% pure, such as 100% pure.

15 As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a compound" includes mixtures of two or more such compounds, reference to "a composition" includes mixtures of two or more such compositions, and the like.

20 The protein content of the co-culture biomass is stable at an average of 50% of cell dry weight, which is higher than that typically observed in pure strain coculture systems. In addition, the co-culture biomass contains high levels of omega-6 fatty acids, which accounts for 2.7% of the cell dry weight. Furthermore, the biomass is rich in other fatty acids that have antioxidant and antimicrobial properties.

25 The term "depleted of oxygen" is intended to refer to the gas fed into the reaction vessel, which may have less than 5 vol% O₂, such as less than 1 vol%, such as less than 0.1 vol%, such as less than 0.01 vol%, such as no O₂. It will be appreciated that the term "depleted of oxygen" further only applies to the gas that is supplied to the reaction vessel in step (b) of the method. That is, the actual environment within the reaction vessel may comprise oxygen, which may be generated *in situ* by the microorganisms within the co-culture. This oxygen may be generated, for example by MG, while the generated oxygen may be consumed by, for example, the MOB. While MG may generate oxygen, too high a concentration of oxygen may inhibit the growth of MG, so the presence of MOB to consume the released oxygen helps avoid this. In other words, oxygen is produced by the microalgae in the system, and subsequently utilized by methanotrophs. Overall, while oxygen is both produced and consumed within the system,

the resulting oxygen levels remain low but not zero, but it is noted that the oxygen may be entirely generated *in situ* within the reaction vessel.

5 The light applied to the co-culture in step (b) of the method may be from any suitable light source. This may be from an LED, from sunlight, or any other light source that can be used to maintain and grow cultures of the co-culture used herein. For example, the light source may be a LED light strip emitting warm white light.

10 In embodiments of the method, a protein content of the biomass mixture may be from 40 to 60 wt%, such as from 44 to 56 wt%, such as about 50 wt%, of a cellular dry weight of the biomass mixture. In additional or alternative embodiments of the method, an omega-6 fatty acid content of the biomass mixture may be from 2 to 5 wt%, such as about 2.7 wt%, of a cellular dry weight of the biomass mixture. In additional or alternative embodiments of the method, a glycogen content of the biomass mixture is from 22 to 35 wt%, such as about 28
15 wt%, of a cellular dry weight of the biomass mixture.

As noted above, the co-culture may further comprise heterotrophs. Examples of heterotrophs that may be present include, but are not limited to, one or more of *Ferrovibrio*, *Terrimonas*, and *Chthonobacter*.

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In embodiments of the invention, the methanotrophs may comprise one or more prokaryotes selected from the group consisting of *Methylosinus*, *Methylocystis*, *Leptolyngbya*, *Lentimicrobium*, *Melioribacter*, *Rhodoplanes*, *Methylococcus*, and *Sulfurisoma*, optionally wherein the methanotrophs comprise one or more prokaryotes selected from the group
25 consisting of *Methylosinus* and *Methylocystis*.

In embodiments of the invention, the microalgae may comprise one or more eukaryotes selected from the group consisting of *Chlorella*, *Ochromonas*, and *Trichoderma*, optionally wherein the microalgae comprise one or more eukaryotes selected from the group consisting
30 of *Chlorella sorokiniana*. As noted hereinbelow, the actual composition of the dominant eukaryotic strain may depend on the conditions in which the co-culture is kept. For example, the addition of H₂S to the gas fed into the reaction vessel of step (b), especially at higher concentrations, may have significant effects on this. Nonetheless, all of the above strains may be present in the co-culture no matter the conditions. See the examples section below for
35 further information.

The method may be run using any suitable conditions available to one with knowledge in the art. For example, one or more of the following operating conditions may be applied:

- (ai) a pH within a range of from 6.5 to 7, such as from 6.7 to 6.8;
- (aii) a temperature of from 25 to 30 °C, such as from 26 to 30 °C;
- 5 (aiii) a hydraulic retention time (HRT) and/or a sludge retention time (SRT) of from 5 to 19 days, such as from 7 to 14 days in a continuously running process;
- (aiv) an ammonium mineral salt medium comprising ammonium chloride, magnesium sulfate (e.g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), disodium hydrogen phosphate (e.g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), potassium dihydrogen phosphate, calcium chloride (e.g. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$), and ethylenediaminetetraacetic
- 10 acid (e.g. as the free acid and/or as the ferric-sodium salt thereof, optionally wherein the free acid is used initially and the ferric-sodium salt is used after continual operation of 100 days or more).

In certain embodiments of the invention, the gas (i.e. the gas fed to the reaction vessel in step

15 (b) of the method) may further comprise H_2S at a concentration of greater than or equal to 500 ppm. For example, in certain embodiments of the invention, the H_2S concentration may be from 750 to 10,000 ppm, such as from 1,000 to 5,000 ppm. It has been surprisingly found that the co-cultures disclosed herein may be able to tolerate high concentrations of H_2S gas, while still providing acceptable yields of the desired products.

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In embodiments of the invention that may be mentioned herein, the volume to volume ratio of CH_4 to CO_2 may be from 1.2:1 to 1.7:1, such as from 1.26:1 to 1.67:1, such as about 1.5:1.

In certain embodiments of the invention that may be mentioned herein, the gas (i.e. the gas

25 fed to the reaction vessel in step (b) of the method) may further comprise one or both of nitrogen gas and volatile organic compounds.

As will be appreciated, the co-culture comprising microalgae and methanotrophs obtained from a waste activated sludge may be obtained by a method comprising the steps of:

- 30 (bi) providing a waste activated sludge in a precursor reaction vessel;
- (bii) operating the precursor reaction vessel under conditions that comprise feeding in a gas comprising CO_2 and CH_4 that is depleted of oxygen to the waste activated sludge for a first period of time;
- (biii) removing a biomass suspension after the first period of time and subjecting the
- 35 biomass suspension to conditions that comprise feeding in a gas comprising CO_2 and CH_4 that is depleted of oxygen to the biomass suspension for a second period of time; and

(biv) repeating step (biii) from 2 to 10 times until a co-culture comprising microalgae and methanotrophs is obtained.

Further details of a suitable method following the above approach is set out in the examples section below.

Further aspects and embodiments of the invention will now be discussed by reference to the following non-limiting examples.

10 Examples

Materials

Synthetic biogas (40% CO₂ and 60% CH₄) was purchased from Air Liquide, Singapore. All of chemicals were purchased from Sigma-Aldrich. All one time disposable centrifuge tube, filter, weighing dishes, syringe were purchased from VWR. Biological buffers and reagents were purchased from Thermo Fisher. The column used for amino acid, glycogen, PHA testing were purchased from Agilent.

Analytical techniques

Determination of the concentrations of ammonium, OD₆₀₀, total suspended solids (TSS), and volatile suspended solid (VSS)

The concentrations of NH₄-N and total iron, OD₆₀₀, TSS, and VSS were determined by following Eaton, A. D. *et al.*, **2005**. Standard Methods for the Examination of Water and Wastewater. American Public Health Association.

Inductively coupled plasma mass spectrometry

The total iron concentration was analyzed using inductively coupled plasma mass spectrometry (iCAP Qc, Thermo Scientific, US).

Gas chromatography (GC)

A 5 ml gas sample was injected into GC with two thermal conductivity detectors (TCD_TCD) (7890A GC system, Agilent, US) to analyze the CO₂, O₂, N₂, and CH₄. The temperature of the oven, injector, and two detectors were 115, 120, 150, and 115 °C, respectively. Five columns were used to analyze CH₄, CO₂, H₂, N₂, and O₂ using argon and helium as the carrier gases (Xiao, K. *et al.*, *Appl. Sci.* **2022**, 12, 956). The hydrogen sulfide in the real biogas was detected by GC equipped with nitrogen-phosphorus detectors (NPD) and Flame Photometric Detector (FPD) connected to a gas sampler with the flow rate as 100ml/min.

Determination of the concentrations of total organic carbon (TOC) and total inorganic carbon (TIC)

TOC and TIC were measured using a TOC analyzer (TOC-5000A, Shimadzu, Japan) with the
5 combustion–infrared technique.

Liquid chromatography

Liquid chromatography with online carbon detection (LC-OCD, DOC-LABOR Dr. Huber, Germany) was utilized for the analysis of dissolved organic compounds (DOCs) and dissolved
10 organic nitrogen (DON). DOCs were classified into several main categories based on their molecular weights and chemical characteristics: (1) biopolymers (molecular weight > 20 kDa), mainly including high molecular weight (HMW) protein (PN) and polysaccharide (PS), and the proportion of PN in biopolymer can be determined); (2) humic substances (HS) with a molecular weight of 1 kDa; (3) building blocks (BB, 300–500 Da); (4) low molecular weight
15 (LMW) acids (350 Da), and LMW neutrals (350 Da), including aldehydes, ketones, and alcohols. The DOC concentration was measured in milligrams equivalent to carbon per liter (mg C/L) (Geng, Y.-K. & Zhou, Y., *J. Hazard. Mater.* **2022**, *430*, 128400).

Elemental analysis

20 The elements of C, H, N, and S of co-culture biomass (3–5 mg biomass powder) were measured by an elemental analyzer (Vario EL Cube, Elementar, Germany). The protein content can use the experimental conversion factor 6.25 of N to protein.

Statistical analysis

25 The statistical analysis was performed using IBM SPSS 28.0. The results of experiments from three cycles were presented as the mean $\pm$ standard deviation. The Student's *t*-test was utilized to identify significant differences between two groups. The statistical analysis for iron tests was carried out using ANOVA. Differences were considered statistically significant at $p < 0.05$ (at a 95 % confidence level).

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Example 1. Culture enrichment and bioreactors set-up

2 L batch reactor (Fig. 1)

A 2 L batch reactor with a 1 L working volume was built to selectively enrich microalgae and
35 methanotrophs in a modified ammonium mineral salts medium (Whittenbury, R. *et al.*, *Microbiology* **1970**, *61*, 205–218) (Fig. 1). The inoculum was waste activated sludge collected from Jurong Water Reclamation Plant, Singapore. The reactor headspace was filled up with

synthetic biogas (40% CO₂ and 60% CH₄) was used as the sole carbon source for the system. The enrichment was undertaken for a series of cycles (7-day a cycle). In each cycle, biomass was mixed by a magnetic stirrer (200 rpm) and illuminated with 6000 k LED lamps. The composition of the culture medium and trace elements are displayed in Table 1. The light source was LED light, and the pH and temperature were controlled at 6.5–7.5 and 27 ± 1 °C, respectively. The pH was controlled at 6.5–7.5 by adding 1 M HCl or 1 M NaOH. The reactor was put in a constant temperature room at 27 ± 1 °C. At the end of each cycle, 100 mL of well-mixed biomass was collected and centrifuged, and the resulting pellet was resuspended in 1 L of fresh culture medium for a new cycle.

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Table 1. The composition of mineral salt media with trace element stock solution.
(per liter, 0.55 g of Na₂EDTA·H₂O, 0.2 g of FeSO₄·7H₂O, 0.3 g of H₃BO₃, 0.01 g of ZnSO₄·7H₂O, 0.03 g of MnCl₂·4H₂O, 0.02 g of CoCl₂·6H₂O, 0.03 g of CuSO₄·5H₂O, 0.002 g of NiCl₂·6H₂O, 0.003 g of Na₂MoO₄·2H₂O).

Ammonium mineral salts (AMS) medium	g/Liter
NH ₄ Cl	0.5
MgSO ₄ ·7H ₂ O	1
Na ₂ HPO ₄ ·12H ₂ O	0.717
KH ₂ PO ₄	0.272
CaCl ₂ ·6H ₂ O	0.2
Ethylenediaminetetraacetic acid (EDTA) (Change to ethylenediaminetetraacetic acid ferric sodium salt (FeNaEDTA) at day 107)	0.005
Trace element	1 ml

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Before each cycle, pure nitrogen was used to purge the biomass suspension to eliminate oxygen in headspace and dissolved oxygen in liquid phase. The biogas was then flushed into the bottle for 15 minutes before sealing to fill up the headspace with synthetic biogas. The reactor was connected to an external gas bag filled with synthetic biogas. TSS, VSS, ammonium concentration, and gas composition were measured three times a week.

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After 7 days, a 100-200 ml biomass suspension was withdrawn and centrifuged. The collected biomass was resuspended with 1-liter fresh AMS media. Each cultivation cycle was initiated with a biomass optical density (OD) at 600 nm of around 0.1. After a couple of cycles, the gas volume in headspace and gasbag showed a noticeable reduction, the gas composition results

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also showed a decreased percentage of CO₂ and CH₄, both indicating the successful enrichment of methanotrophs and microalgae. Upon the confirmation by the microbial community results, the biomass inside the batch reactor was transferred into a PLC controlled fermenter for long-term cultivation (Fig. 2).

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5 L fermenter (Fig. 2)

The fermenter was a 5 L borosilicate glass vessel assembled with a stainless-steel impeller and connected to a PLC system with 4 peristaltic pumps (see Fig. 2). The light was provided using a standard LED light strip emitting warm white light. The dissolved oxygen concentration in the reactor was monitored. pH was controlled within 6.7-6.8 by using 1 N HCl or 2 M NaOH. The reactor was located in a room with a constant temperature and the temperature inside the vessel remained at 26-30 °C.

Synthetic biogas (40/60 CO₂/CH₄ v/v) was used as sole carbon source for the culture. The liquid phase media was supplied by a peristaltic pump with a consistent speed, and the composition can be found in Table 1 and Fe³⁺ in the form of FeNaEDTA was used to replace EDTA at the same concentration on day 100 to optimize the growth condition for methanotrophs and enhance the photosynthetic ability. The effluent pump flow rate followed the same flow rate as that of the feeding pump. The working volume of the reactor was 3 liters. The hydraulic retention time and sludge retention time were 14 days. The feed medium was purged with pure nitrogen for approximately 15 min to remove O₂ and CO₂ before it was fed into the reactor.

Example 2. Biogas production

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Biogas is the carbon source for the growth of MOB and microalgae. A lab-scale anaerobic digester was set up and received glucose and WAS to produce biogas for co-culture (Fig. 3). The key operating parameters, including pH, temperature, loading rate, and retention time, were optimized for stable and maximum biogas production. The composition of biogas, including H₂S, CH₄ and CO₂, were regularly analyzed.

The real biogas produced from the 10 L AD reactor contained 1-3% v/v volatile organic compounds, the dominant chemicals are listed in Table 2.

Table 2. The dominant volatile organic compounds (VOCs) in real biogas from 10 L AD.

Compound Name	Molecular Formula
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2-Methyl-1-butene	C ₅ H ₁₀
Benzene	C ₆ H ₆
Toluene	C ₇ H ₈
Styrene	C ₈ H ₈
1-Hexanol, 2-ethyl	C ₈ H ₁₈ O

Example 3. Parent fermenter (prepared in Example 1, see Fig. 2) performance

During the start-up period, the reactor operation was alternated between batch and continuous mode for the first 43 days, and various setup issues were resolved. Therefore, the results during the start-up period are not presented and discussed.

From day 44, the system was operated in a continuous mode. The initial ammonium concentration was 50 NH₄-N ppm in the influent to have an acclimation period. The effluent ammonium concentration gradually reduced to 0. To avoid nitrogen limitation, the ammonium concentration in the influent was increased to 70 ppm and 100 ppm on day 70 and day 130, respectively. During the stable stage, the TSS remained at 1.3 ± 0.28 g/L, and the effluent ammonium concentration remained below the detection limit (Fig. 4). The total suspended solid showed a slight drop on day 129 as biofilm formed on the glass vessel surface and blocked light source. The biomass concentration was resumed once the reactor was cleaned.

The iron addition from day 100 did not change the biomass concentration significantly and the ammonium concentration in effluent still remained 0.

Example 4. Macromolecule compound composition in biomass in fermenter

The macromolecule compound composition in the biomass in the fermenter (described in Example 1, see Fig. 2) was determined.

Determination of amino acid, glycogen, total lipid, polyhydroxyalkanoates, and fatty acid methyl esters (FAMES)

Lyophilized biomass was ground into fine powder to determine amino acid, glycogen, total lipid, polyhydroxyalkanoates, FAMES, PHB, and elemental composition. 10 mg biomass powder was hydrolyzed in HCl (5 ml, 6 M) and heated at 110 °C for 24 hours. The digested liquid samples after filtration (0.22 μm) were used to analyze amino acid profiles by a liquid chromatograph with a triple Quadruple mass spectrometer equipped with an electrospray

ionization source (1290 Infinity II, 6460 Triple Quad LC/MS, Agilent, US). Protein content was calculated as the total amino acid content.

5 Glycogen in the biomass was determined by acid hydrolysis. In brief, HCl (5 ml, 0.6 M) was added to 5 mg freeze-dried fine biomass powder and heated for 6 hours at 100 °C, followed by centrifugation at 5000 rpm for 10 minutes. The supernatant that passed through a 0.45 µm filter was injected into high-performance liquid chromatography (Agilent 1200 series) with a BC-Hi-Plex H column of 300 mm × 7.7 mm at a speed of 0.4 ml/min under 65 °C using 0.5 µM H₂SO₄ as the mobile phase. Glucose was used as the standard to build a calibration curve.

10

Total lipid was extracted from dry ground biomass following a modified gravimetrically method (Feng, Y. *et al.*, *Bioresour. Technol.* **2011**, 102, 101–105). The biomass was mixed with 3 ml chloroform:methanol (2:1) and submitted to ultrasonication for 30 minutes at 40 °C, followed by centrifugation at 4000 rpm for 8 minutes at 4 °C. The liquid phase was transferred into a glass vial with 1 ml 0.88% aqueous potassium chloride inside. Two phases were separated, and the upper layer was disposed. The lower phase was washed by a 0.5 ml solvent mixture of chloroform:methanol:water (3:48:47) thrice. Finally, the lower organic phase was transferred to a pre-weighed glass vial and dried at 40 °C using a nitrogen evaporator.

20 FAMES and PHB were extracted by acid transesterification for lipid composition analysis. A biomass powder (around 20 mg) was added to a glass vial and 2 ml 3% sulfuric acid in methanol with sodium benzoate as internal standard and 2 ml chloroform were added before heating at 100 °C for 20 hours. After that, 1 ml deionized water (DI) was added when the vial was cooled down to room temperature. There were two layers after DI was added, and the lower phase (which is the organic phase) was collected in a new vial with granular sodium sulfate inside to remove the remaining water. The final sample was filtered through a 0.22 µm filter before analysis by GC (7890B GC system, Agilent, US) with mass spectrophotometer (5977A MSD, Agilent, US) equipped column FAMEWAX with 30 m x 0.32 mm x 0.25 µm (Restek, Bellefonte, USA). The peaks that appeared before the initial 4.5 minutes were removed to avoid overlap with solvent peaks. The identification of FAMES and PHB was accomplished by comparing the fragmentation patterns of the corresponding peaks with the NIST 17. L library ([https:// chemdata.nist.gov/](https://chemdata.nist.gov/)).

Protein content calculation

35 Protein content was calculated based on the total amino acid content.

Results and discussion

Protein, glycogen, and lipid are important macro compounds to build up a microorganism cell. Fig. 5 plots a major biopolymers composition.

Protein content

5 The protein content occupied 50 ± 6 % cell dry weight on average (Fig. 5), which is higher than a pure strain co-culture of *Chlorella sorokiniana*–*Methylococcus capsulatus* that was fed on batch mode (Rasouli, Z. *et al.*, *Biochem. Eng. J.* **2018**, 134, 129-135), which achieved a protein content of 27.62%. The protein content of a reactor dominated by *Methylocystis sp.* reached 51% of dry cell weight (Valverde-Pérez, B. *et al.*, *Bioresour. Technol.* **2020**, 310, 123388). The protein extracted from *Chlorella sorokiniana* was reported to be 65% w/w initial biomass dry basis (Tejano, L. A. *et al.*, *Int. J. Mol. Sci.* **2019**, 20, 1786). Since the present disclosure used a mixed culture, it is reasonable that the protein content is lower than the average protein content of methane-oxidizing bacteria and *Chlorella sp.* The amino acid profile is presented in Table 3, and the content of essential amino acids indicates that the biomass may be suitable as animal feeds.

Table 3. Amino acid profile of the biomass in fermenter.

Classification	Amino Acid	Content (% of CDW)	
Nonessential Amino Acid	Alanine	1.07 ± 0.09	39.8 ± 5.39
	Arginine	1.6 ± 0.21	
	Asparagine	1.32 ± 0.18	
	Aspartate	13.69 ± 1.26	
	Cystine	0.09 ± 0.01	
	Glutamate	15.41 ± 3.16	
	Glutamine	1.33 ± 0.1	
	Glycine	2.03 ± 0.54	
	Proline	0.8 ± 0.14	
	Serine	2.45 ± 0.2	
Essential Amino Acid	Histidine	1.12 ± 0.08	10.03 ± 0.78
	Isoleucine	2.17 ± 0.18	
	Leucine	2.7 ± 0.19	
	Lysine	2 ± 0.08	
	Phenylalanine	0.99 ± 0.17	
	Threonine	0.34 ± 0.02	
	Valine	0.7 ± 0.2	

Glycogen content

Glycogen is the main carbon storage compound in most microorganisms. It was measured to be 0.28 ± 0.05 g/g of cell dry weight in the present disclosure. The glycogen content is comparable with a pure co-culture of *Chlorella sorokiniana*–*Methylococcus capsulatus* (32% glycogen) (Rasouli, Z. *et al.*, *Biochem. Eng. J.* **2018**, 134, 129-135). It is known that bacteria with glycogen do not break down their RNA and protein components in the absence of a carbon source. Glycogen-deficient bacteria, on the other hand, release NH_3 from their nitrogen-containing components. Therefore, although glycogen is not essential for microorganism growth, it may be likely used to protect cell integrity when external carbon source is insufficient.

Total lipid content

The total lipid content was 0.13 ± 0.01 g/g of cell dry weight. A 38% lipid and ash content was reported by using a pure strain co-culture of *Chlorella sorokiniana*–*Methylococcus capsulatus* that was fed on batch mode (Wang, J. *et al.*, *Front. Energy Res.* **2020**, 8, 563352). Wastewater was used as carbon source in Wang, J. *et al.*, *Front. Energy Res.* **2020**, 8, 563352, which led to higher carbon loading for carbon-based compounds accumulation. Notwithstanding, the lipid content in current system is similar to traditional protein sources for fish meal (9%). Supplementation of specific lipids can enhance the digestibility of single cell protein, and improve the overall sustainability of animal growth and meat quality.

FAME content

The chromatography of extracted fatty acid is shown in Fig. 6. There are 31 relatively high peak integrated and searched in library NIST 17. L (Table 4). The dominant top three FAMES can be quantified by comparing with a series of standards.

Table 4. Fatty acids profile of biomass in fermenter.

Compound Name	Molecular Formula	Retention Time	Area%	Area
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$\text{C}_{19}\text{H}_{34}\text{O}_2$	24.816	29.8%	29289423
Hexadecanoic acid, methyl ester	$\text{C}_{17}\text{H}_{34}\text{O}_2$	20.264	20.2%	19858213
9-Octadecenoic acid (Z)-, methyl ester	$\text{C}_{19}\text{H}_{36}\text{O}_2$	24.056	13.7%	13479357
7,10-Hexadecadienoic acid, methyl ester	$\text{C}_{17}\text{H}_{30}\text{O}_2$	21.382	10.9%	10760761
Methyl hexadec-9-enoate	$\text{C}_{17}\text{H}_{32}\text{O}_2$	20.606	5.2%	5113708
Methyl stearate	$\text{C}_{19}\text{H}_{38}\text{O}_2$	23.719	3.0%	2979095

9-Hexadecenoic acid, methyl ester, (Z)-	C ₁₇ H ₃₂ O ₂	20.719	3.0%	2965129
cis-13-Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	24.169	2.8%	2738359
4-n-Dodecylresorcinol	C ₁₈ H ₃₀ O ₂	15.953	1.7%	1631546
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	C ₁₉ H ₃₂ O ₂	25.886	1.1%	1128521
Eicosen-1-ol, cis-9-	C ₂₀ H ₄₀ O	16.119	1.1%	1118975
4-n-Dodecylresorcinol	C ₁₈ H ₃₀ O ₂	15.343	0.8%	826035
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O	16.734	0.8%	766949
7,10,13-Hexadecatrienoic acid, methyl ester	C ₁₇ H ₂₈ O ₂	22.473	0.7%	653135
Hexadecanoic acid, 2-hydroxy-, methyl ester	C ₁₇ H ₃₄ O ₃	27.137	0.6%	544571
Pentanoic acid, 4-oxo-, methyl ester	C ₆ H ₁₀ O ₃	9.31	0.5%	497878
Hexadecanoic acid, 14-methyl-, methyl ester	C ₁₈ H ₃₆ O ₂	21.179	0.5%	445444
9,11-Octadecadienoic acid, methyl ester, (E,E)-	C ₁₉ H ₃₄ O ₂	27.426	0.4%	407969
Butanoic acid, 3-hydroxy-, methyl ester	C ₅ H ₁₀ O ₃	8.08	0.4%	400150
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, acetate, [R-[R*,R*-(E)]]-	C ₂₂ H ₄₂ O ₂	19.574	0.4%	357297
2-Pentadecylfuran	C ₁₉ H ₃₄ O	14.76	0.3%	343900
Methyl tetradecanoate	C ₁₅ H ₃₀ O ₂	16.648	0.3%	324869
Tetracosanoic acid, methyl ester	C ₂₅ H ₅₀ O ₂	39.589	0.3%	302102
Heptadecanoic acid	C ₁₈ H ₃₆ O ₂	22.008	0.3%	288046
Octadecanoic acid, 3-hydroxy-, methyl ester	C ₁₉ H ₃₈ O ₃	28.63	0.3%	261605
Heptadecane	C ₁₇ H ₃₆	11.391	0.2%	176523
cis-10-Heptadecenoic acid, methyl ester	C ₁₈ H ₃₄ O ₂	22.398	0.2%	171351
3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate	C ₂₂ H ₄₂ O ₂	14.664	0.2%	160182
Methyl pyruvate dimethyl acetal	C ₈ H ₁₂ O ₄	6.903	0.2%	153494
Propanedioic acid, dimethyl ester	C ₅ H ₈ O ₄	8.417	0.1%	137380
Pentadecanoic acid, methyl ester	C ₁₆ H ₃₂ O ₂	18.462	0.1%	58317

The 9,12-octadecadienoic acid (Z,Z)-methyl ester was the most abundant FAME in the biomass, which occupied 2.7% w/w of the cell dry weight. It is a methyl ester form of 9,12-octadecadienoic acid, an omega 6 fatty acid and essential fatty acid for mammalian nutrition.

5 Hexadecanoic acid methyl ester, and 9-octadecenoic acid (Z)-methyl ester were accounted

for 1.6% and 0.7% of cell dry weight, respectively. These three FAMES are commonly founded in *Chlorella*.

- 9,12-Octadecadienoic acid (Z,Z)-methyl ester (C₁₉H₃₄O₂), hexadecanoic acid methyl ester (C₁₇H₃₄O₂), 9-octadecenoic acid (Z)-methyl ester (C₁₉H₃₆O₂), 7,10-hexadecadienoic acid methyl ester (C₁₇H₃₀O₂), methyl stearate (C₁₉H₃₈O₂), 9-hexadecenoic acid methyl ester, (Z)- (C₁₇H₃₂O₂), 9,12,15-octadecatrienoic acid methyl ester, (Z,Z,Z)-(C₁₉H₃₂O₂), hexadecanoic acid, 14-methyl-methyl ester (C₁₈H₃₆O₂), 2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, acetate, [R-[R*,R*-(E)]-(C₂₂H₄₂O₂)] were reported to have antioxidant and antimicrobial activity (Rachidi, F. *et al.*, *Sci. Rep.* **2021**, *11*, 930). Poly (3-hydroxybutyrate) (PHB) was also identified with a very low content (0.21% of CDW).

Example 5. Fermenter liquid phase

- The liquid phase in the fermenter (described in Example 1, see Fig. 2) was determined.

Analysis of liquid phase

- The liquid phase after 0.22 µm filtration was analyzed by a triple Quadruple mass spectrometry equipped with an electrospray ionization source (LCQQQ 6460, Agilent) and an Agilent Infinity Lab Poroshell 120 HILIC-Z column with a size of 2.1 mm × 100 mm, 2.7 µm.

Results and discussion

- The TOC of the liquid phase was measured when there was biofilm attached on the reactor surface (Fig. 7). The TOC concentration had a close relationship with the biofilm formation. The biofilm was observed on day 144, and quickly covered the reactor wall within one week, corresponding with TOC rise from 6.97 ppm to 12.02 ppm. The biofilm removal from reactor surface and equipment apparatus resulted in TOC reduction inside the fermenter.

- The composition of organic compounds in the liquid phase of fermentor is shown in Fig. 8. A majority of the dissolved organic carbon compound is biopolymer which accounted for 40.8%. The proportion of humic substances and building blocks was 33.4% of total dissolved organic carbon. The low molecular weight neutrals from C1 to C6 accounted for 16.1%. The compounds released by *Chlorella* are normally dominated by chemicals with molecular weight bigger than 10 kDa. Methanotrophs are known to produce soluble metabolic intermediates such as methanol, formaldehyde, and organic acids so the biopolymer and humic substance in the fermenter were contributed mostly by microalgae while methanotrophs were the main

contributor to the LMW neutrals. However, LMW acid was not detected in the liquid phase. The acid was likely consumed by microalgae or other aerobic microorganisms.

Example 6. Microbial community in fermenter

5

16S rRNA and 18sRNA sequencing were carried out to examine the microbial community in the fermenter (described in Example 1, see Fig. 2) at the genus level.

Sequencing

10 Before sequencing, the samples' genomic DNA was extracted using a fast DNA extraction kit for soil (MP Biomedicals, Singapore) and kept at -80°C . The sequencing and analysis of the community composition were performed in accordance with the guidelines supplied by Majorbio (Shanghai, China). For the Next-generation sequencing analysis, the 16sRNA was amplified by the primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R: (5'-
15 GGACTACHVGGGTWTCTAAT-3'), which target the V3-V4 region. The 18s was amplified by 528F (5'-GCGGTAATTCCAGCTCCAA-3') and 706R (5'-AATCCRAGAATTTACCTCT-3'). The data analysis was conducted on the Majorbio free cloud platform (www.majorbio.com).

Results and discussion

20 Methanotrophs dominated the prokaryote microbial population. *Methylosinus* was the most abundant methanotroph before day 100 (Fig. 9). After Fe addition, the microbial community measured on day 140 showed that *Methylocystis* was the dominant methanotroph (Fig. 9). These two are both from type II methanotrophs under the Family *Methylocystaceae* but different genus. Motility is one of the primary distinctions. In addition, several species of the
25 genus *Methylocystis* exhibited metabolic flexibility, such as growth on acetate and ethanol as carbon and energy sources, and it can also grow at low methane concentrations. Furthermore, several species of *Methylocystis* possess genes involved in photosynthesis. This may explain why iron addition shifted the dominant methanotrophs from *Methylosinus* to *Methylocystis* since Fe can enhance the photosynthetic activity. Furthermore, iron can enhance sMMO
30 enzyme activity and cell density of MOB.

Although CO_2 and CH_4 were used as the inorganic carbon source to support target microorganisms to grow, the 16s amplification result shows that a variety of other aerobic heterotrophic microorganisms were also present. They might use the organic compounds
35 released from methanotrophs and microalgae as substrate. A similar conclusion was published by several researchers that the MOB dominated bioreactor contained a variety of

non-methanotrophic heterotrophs (Karwautz, C. *et al.*, *The ISME Journal* **2018**, 12, 87-100; and Valverde-Pérez, B. *et al.*, *Bioresour. Technol.* **2020**, 310, 123388). In the present disclosure, *Ferrovibrio*, *Terrimonas* and *Chthonobacter* were dominant genus in heterotrophs. *Ferrovibrio* was reported as a facultative anaerobic microorganism, and low molecular weight acid can be used as a carbon and energy source for its aerobic organotrophic growth (Sorokina, A. Y. *et al.*, *FEMS Microbiol. Lett.* **2012**, 335, 19-25). *Terrimonas* is strictly aerobic and can assimilate organic carbon (e.g. glucose and sucrose). The secondary metabolism of MOB, methanol, can be consumed by the genus *Chthonobacter*. The 18s amplification result shows that more than 99% eukaryotes were *Chlorella sorokiniana* during the whole operation period (Fig. 10).

Example 7. VOCs and H₂S effect on the biomass growth and activities

It is difficult to quantify gas consumption in the continuous fermenter since the gasbag volume reduction is hard to measure accurately. The biomass growth and nutrient loading remains at a stable condition in the parent fermenter to produce a consistent culture. Three batch reactors (described in Example 1, see Fig. 1) were thus set up for a batch experiment to better understand the factors on co-culture growth and biomass composition.

Synthetic biogas was used as the baseline because it has a constant composition. The real biogas produced from the 10 L AD reactor in Example 2 contained 1-3% v/v VOCs, and the dominated chemicals are listed in Table 2. Therefore, real biogas was also used to compare the effect of VOCs on the co-culture system. As there were no sulfur compounds in the AD feed, hydrogen sulfide was not detected in the biogas. In order to prepare H₂S containing biogas, pure H₂S was injected into synthetic biogas to investigate the effect of H₂S on the system.

In the present disclosure, 3 batch reactors (described in Example 1, see Fig. 1) were prepared with 1-L working volume and 1-L headspace. The first reactor acted as a control reactor (named SB), where the biogas was synthetic biogas containing 40% CO₂ and 60% CH₄. The second reactor (named RB) was fed with real biogas (prepared in Example 2) containing 30-42 % CO₂, 44-53% CH₄, 8-12 % nitrogen, and small amount of VOCs mentioned above. The last reactor (named SBS) received synthetic biogas containing 40% CO₂ and 60% CH₄ + 500 ppm H₂S. All the three reactors were fed with the same liquid medium and operated for 7 days per cycle.

- The liquid AMS medium composition is shown in Table 1. All three reactors were operated and sampled identically. The initial biogas composition was measured 2 hours after pH adjustment when the gas-liquid equilibration was achieved. Three reactors were stirred at 260 rpm at 27 ± 1 °C. After 7 days, 100 ml culture from each reactor was withdrawn and mixed with 900 ml of fresh AMS medium. The initial OD₆₀₀ was controlled in the range of 0.1 - 0.15. The whole duration of this treatment consisted of three cycles. The performance of three reactors were closely monitored to evaluate the VOC and H₂S impact on the culture activities and microbial communities.
- At the beginning and the end of a cycle, 5 ml gas sample was collected by a gas tight syringe to analyze the headspace gas composition. 15 ml liquid phase (0.45 µm membrane filtered) was collected to measure NH₄-N concentration and pH. 10 ml culture was collected to measure TSS, VSS and OD₆₀₀. At the end of each cycle, 100 ml biomass was collected and fixed by formalin for 1 hour, followed by centrifugation at 4000 g for 15 minutes. The supernatant was disposed, and the fixed biomass was stored at -80 °C for further analysis.

Results and discussion

- The kinetics data of three reactors is summarized in Table 5. The biomass concentration in reactors RB and SBS were 13% and 25%, respectively, lower than that of reactor SB, corresponding with slightly lower specific growth rates as well. Reactor SB showed the highest biomass yield of 2.05 mg TSS/mg C, which is 24% and 15% higher than reactors SBS and RB, respectively. The specific substrate utilization in the reactors were comparable. It has been reported that the inhibition effect of H₂S on MOB was shown at 165 ppm (Xu, M. *et al.*, *Water Res.* **2020**, *184*, 116138), while the inhibition threshold for chlorella was 200 ppmv (González-Sánchez, A. & Posten, C., *J. Environ. Manage.* **2017**, *191*, 252-257.).

Table 5. Kinetics data and liquid parameters of the three reactors.

Reactor	Biomass Yield (mg TSS/mg C)	Specific Substrate Utilization (mg C/mg TSS per day)	Specific Growth Rate (day ⁻¹)	TSS (g/ L)
SB	2.05±0.31	0.07±0.01	0.14±0.03	0.63±0.07
RB	1.79±0.33	0.07±0.02	0.13±0.02	0.55±0.09
SBS	1.65±0.13	0.07±0.02	0.12±0.03	0.47±0.03

- The protein content (Table 6) in the biomass of RB and SBS was significantly higher than that of SB (both $p < 0.05$), while reactor SB had the highest glycogen content. However, it was reported that the amino acid profile decreased in MOB dominated batch reactor when the H₂S

concentration was above 1000 ppm (Xu, M. *et al.*, *Water Res.* **2020**, *184*, 116138). Although the role of H₂S in protein synthesis is unclear, it can act as a signaling molecule and play an active role in the transcriptional pathway.

5 **Table 6.** Macromolecular compound composition in three reactors (g/g of cell dry weight (CDW)).

Reactor	Protein	Glycogen	Lipid	Sum
SB	0.47±0.01	0.27±0.03	0.09±0.01	0.84±0.02
RB	0.55±0.02	0.20±0.01	0.11±0.01	0.86±0.04
SBS	0.51±0.01	0.24±0.01	0.11±0.01	0.84±0.02

The effect of VOC and H₂S was further studied and confirmed in Example 10 below.

10 **Example 8. Fe effect on the biomass growth and activities**

After the successful enrichment of the MOB-MG coculture, a series of batch experiments were conducted to quantify the kinetics of the coculture system, and to analyze the biomass and microbial community composition, as described in the following examples. The adaptation of
15 MOB-MG coculture to iron-elevated condition in the reactors described in Example 1 was studied by following the protocols in Example 4.

Reactor SB was used in this example and was operated in the same way as mentioned in Example 7. The reactor was operated for 3 cycles to collect more samples for analysis. At the
20 beginning and the end of the cycle, 5 ml gas sample, 15 ml liquid phase, and 10 ml culture were collected for measurements by following the protocol in Example 7. At the end of each cycle, 100 ml biomass was collected, fixed, and stored by following the protocol in Example 7.

After three cycles of operation, EDTA in the medium was replaced with the same concentration
25 of FeNaEDTA. Compared to the original medium, Fe concentration increased to 10 µM/L from 0.7 µM/L. The gas, liquid, biomass samples were collected following the same methods in Example 7.

To avoid ammonium depletion, the cycle duration was subsequently reduced to 2 days. At the
30 end of day 2 of each following cycle, 50 ml of the culture from the reactor was withdrawn and mixed with 950 ml fresh AMS medium respectively to meet the requirement of initial OD₆₀₀

being in the range of 0.1-0.15. The 2-day cycle was repeated for three times. The gas, liquid, and biomass samples were collected by following the protocol in Example 7.

Experiment 1 (Control-7d)

5 A batch experiment was carried out by using a similar approach to that used for coculture enrichment (see Example 1). The inoculum was obtained from enriched MOB-MG coculture, with the initial optical density (OD_{600}) controlled within the range of 0.1–0.15. The initial synthetic biogas composition was measured 2 h after pH adjustment to neutral when the gas-liquid equilibration was achieved. Every 48 h within each cycle, a 5 mL gas sample was
10 collected using a gas-tight syringe to analyze the headspace gas composition. A 15 mL liquid sample was collected and filtered through a 0.45 μ m membrane filter to measure ammonium concentration, TOC and TIC. Additionally, a 10 mL culture was taken to measure TSS, VSS, and OD_{600} . At the end of each cycle, a 100 mL culture sample was fixed with formaldehyde for 1 h, followed by centrifugation at 4000 g for 15 min. The supernatant was discarded, and the
15 fixed biomass was frozen for subsequent analysis of glycogen, total lipid, amino acid, and fatty acid methyl esters (FAMES). The batch experiment continued for four cycles, and data from the last three cycles were collected for analysis.

Experiment 2 (Fe-7d)

20 To investigate the effect of iron, the EDTA in the medium was replaced with FeNaEDTA at an equal concentration, resulting in an increase in iron concentration from 0.7 to 13.6 μ M. The batch experiment was conducted by following the same procedures as in Control-7d. Additionally, the composition of DOC in the liquid samples was analyzed. Synthetic biogas
25 was used.

Experiment 3 (Fe-3d)

Based on the observations from the preliminary experiment, which revealed a noticeable ammonium depletion beyond the third day, a batch experiment with a cycle duration of 3 days
30 was conducted using the same procedures and sampling methods as Fe-7d above. Synthetic biogas was used.

DNA extraction, sequencing and sequence analysis

Genomic DNA from biomass (15 mL) from Control-7d or Fe-7d was extracted using a FastDNA
35 Spin Kit for Soil (MP Biomedicals, Singapore) following the manufacturer's instructions. The DNA extract was checked on 1 % agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, US). The

hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R: (5' - GGACTACHVGGGTWTCTAAT-3'), which targets the V3-V4 region. The 18S rRNA genes were amplified using 528F (5'- GCGGTAATTCCAGCTCCAA-3') and 706R (5'- AATCCRAGAATTTACCTCT-3') (He, Q. *et al.*, *J. Hazard. Mater.* **2018**, 359, 222–230). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, US) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The data analysis was conducted on the Majorbio free cloud platform (www.majorbio.com).

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Results and discussion

The kinetic data of each cycle, TOC concentration and biomass concentration in SB reactor are summarized in Table 7. The biomass yield increased from 2.05 to 2.47 mg TSS/mg Carbon consumed once Fe concentration increased to 10 μ M/L. The biomass concentration doubled from 0.62 to 1.41 g/L, corresponding with a higher specific growth rate (0.14 vs 0.25 per day, respectively). Fe is an important factor in the active site of many enzymes such as the methane soluble methane monooxygenase. Meanwhile, Fe addition can increase the expression of various genes, including carbonic anhydrase, which is essential for microalgae growth. When the cycle duration was shortened to 2 days, the specific substrate utilization and specific growth rate showed a significant increase ($p < 0.05$).

In addition to the effects on kinetic performance, the intracellular compound compositions were also different (Fig. 11). The Fe addition enhanced the total lipid content in the mixture biomass. It is known that optimal iron concentrations would concurrently boost both specific growth rate and lipid content of microalgae.

With Fe addition, the 7-day operation resulted in a much lower intracellular protein content of 31% of CDW. At the same time, the glycogen showed an obvious increase from 17% to 24% of CDW while the total lipid content was comparable with the 2-day operation mode. It appears that the intracellular compounds were transformed at a certain time point and this transformation may be related to the availability of external substrates. Without wishing to be bound by theory, given the carbon/biogas supply was sufficient during the 7 days operation, it was hypothesized that nitrogen may be limited during the operation, which is further discussed in Example 9.

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Table 7. Kinetics data of SB reactor in Fe and nitrogen experiments.

Treatment	Biomass Yield (mg TSS/mg C)	Specific Utilization rate (mg C/mg TSS per day)	Substrate Rate (day ⁻¹)	Specific Rate (day ⁻¹)	Growth	TSS (g/ L)	NH ₄ consumption rate (mg N/ g TSS per day)	Total carbon (TOC, mg C/ L)	organic
SB	2.05±0.31	0.07±0.01		0.14±0.03		0.62±0.09	3.91±0	9.78±3.09	
SB-Fe (7-day)	2.47±0.21	0.1±0.01		0.25±0.02		1.41±0.17	5.16±0.35	17.72±5.35	
SB-Fe (2-day)	2.42±0.52	0.27±0.05		0.64±0.02		0.67±0.07	27.29±0.85	6.54±2.35	

When Fe was added to the SB reactor, the dominated MOB species shifted from type X methanotrophs *Methylococcus* to type II methanotrophs *Methylocystis* (Fig. 12). Meanwhile, Type I and Type X methanotrophs were below the detection limits. This finding is in agreement with previous studies that type I MOB rather than type II MOB is dominant in nitrogen-rich environment while nitrogen deficiency contributes to an abundance increase in *Methylocystis*/*Methylosinus* (Yang, Y. *et al.*, *Front. Bioeng. Biotechnol.* **2020**, *8*, 250; and Zhang, X. *et al.*, *Syst. Appl. Microbiol.* **2014**, *37*, 296-304). According to these findings, nitrogen limitation can be used to select type II methanotrophs. Besides methanotrophs, *Ferrovibrio*, *Chthonobacter*, and *Terrimonas* remained as the three dominant heterotrophs. The genus *Ferrovibrio*, which belongs to the family Rhodospirillaceae, consists of bacteria that are Gram-stain-negative and can be either aerobic or facultative anaerobic. The genus *Chthonobacter* was first proposed for describing a bacterium isolated from grass-field soil and it belongs to the family Methylocystaceae, which is the same family as the *Methylocystis*. They grow well in liquid medium with methanol, but not formate. The genus *Terrimonas* are strictly aerobic, and is a member of the family Chitinophagaceae.

Carbon transformation in a cycle study

A coculture was established after an incubation with synthetic biogas for 5 months. Then, the transformation of carbon by the coculture was monitored in typical 7-day cycles (Control-7d, Fig. 13a). Initially, synthetic biogas (634 mg-C in total) was supplied to the reactor, with 79 % in the headspace and 21 % in the liquid phase. During the cultivation cycle, the biogas in both gaseous and liquid phases was assimilated by the coculture. As a result, 35 % of the biogas was transformed into biomass at the end of the cycle. In addition, a small amount of TOC (1 % of the assimilated carbon) was detected in the liquid phase, which was likely released by the coculture.

Microbial community structure

High-throughput 16S rRNA and 18S rRNA gene sequencing was applied to the coculture samples to uncover the composition of the microbial community. The results showed that MG belonging to the genus *Chlorella* dominated the eukaryotes (97 % relative abundance to total eukaryotes, Fig. 14b). *Chlorella* is protein-rich and has potential to be used as animal feed. Furthermore, it is an advantageous organism for cultivation due to its mixotrophic metabolic capabilities and robust tolerance to environmental stressors. Meanwhile, MOB belonging to *Methylococcus* (type I, 19 % relative abundance to total prokaryotes) and *Methylosinus* (type II, 5 %) were simultaneously enriched (Fig. 14a). These two types have different preferences for nitrogen source and O₂ concentration. Predominance of *Methylococcus* in MOB populations was not surprising since ammonium concentration remained sufficient during the

enrichment period (Fig. 15). Compared with *Methylococcus*, *Methylosinus* is highly adaptable to low O₂ level (He, R. *et al.*, *Environ. Pollut.* **2020**, 258, 113676), which remained below 5 % in the headspace of the current system. Together, these results show that a coculture comprising MG and MOB was established.

5 Although biogas was used as the sole carbon source, diverse non-methanotrophic heterotrophs, including *Chthonobacter*, *Ferrovibrio*, and *Terrimonas* (25 %, 21 %, 9 % relative abundance to total prokaryotes, respectively), were present in the bioreactor. These heterotrophs were reported to use organic substrates generated by MOB during methane
10 oxidation such as methanol (*Chthonobacter albigriseus*, Kim, D. *et al.*, *Int. J. Syst. Evol. Microbiol.* **2017**, 67, 883–888), acetate, volatile fatty acids (genus *Ferrovibrio*, Sorokina, A. Y. *et al.*, *FEMS Microbiol. Lett.* **2012**, 335, 19–25), and polysaccharide (genus *Terrimonas*, Jin, D. *et al.*, *Int. J. Syst. Evol. Microbiol.* **2013**, 63, 1658–1664; and Lu, T. *et al.*, *Soil Ecol. Lett.* **2023**, 5, 230172), which are usually produced by either *Chlorella* or MOB as metabolic
15 intermediates (Sheets, J. P. *et al.*, *Bioresour. Technol.* **2016**, 201, 50–57; Strong, P. J. *et al.*, *Environ. Sci. Technol.* **2015**, 49, 4001–4018; Zhuang, L.-L. *et al.*, *Renew. Sust. Energ. Rev.* **2016**, 59, 141–148). The interactions between *Chlorella*/MOB and heterotrophs can not only ease the potential inhibition of intermediates but also improve the overall biogas utilization efficiency. This relationship can contribute to nutrient recycling and maintaining of the stability
20 of the system.

Changes in activity parameters

The MOB-MG coculture was incubated in an iron-elevated condition for 4 cycles (Fe-7d). During a typical cycle, the increased iron concentration (13.6 µM in Fe-7d compared to 0.72
25 µM in Control-7d) significantly enhanced biogas conversion efficiency (from 36 to 88 %). As a result, 85 % of the supplied biogas was assimilated into biomass by the end of the cultivation cycle (Fig. 13b), compared to only 35 % without additional iron supply. These findings highlight the importance of iron in maintaining the high activity of the coculture and, consequently, achieving high biogas conversion efficiency. Compared with cocultivation of pure strains of
30 MOB and MG (Rasouli, Z. *et al.*, *Biochem. Eng. J.* **2018**, 134, 129–135), the heterotrophs co-existing in the current system may help facilitate carbon assimilation as they could consume the released organic metabolites in the liquid phase (discussed below).

To further evaluate the impact of iron, multiple activity parameters of the coculture were
35 determined and compared between Fe-7d and Control-7d (Table 8). The Fe-7d coculture exhibited a higher specific growth rate (0.27 day⁻¹), resulting in a 140 % increase in final biomass concentration. Additionally, the biogas conversion rate almost tripled. These results

collectively demonstrate the improved overall performance of the coculture under elevated iron conditions.

Table 8. Kinetic data of the cycle studies Control-7d, Fe-3d and Fe-7d.

Treatment	Biomass yield	Specific utilization rate	substrate	Biogas conversion rate	Specific growth rate	VSS
	mg-VSS/ mg-C	mg-CO ₂ - C/mg-VSS day ⁻¹	mg-CH ₄ - C/mg-VSS day ⁻¹	mg-C day ⁻¹	L ⁻¹ day ⁻¹	g L ⁻¹
Control-7d	2.03 ± 0.25	0.06 ± 0.01	0.07 ± 0.01	26.81 ± 1.78	± 0.18 ± 0.04	0.54 ± 0.08
Fe-3d	1.44 ± 0.1	0.16 ± 0.03	0.18 ± 0.01	106.57 ± 16.84	± 0.62 ± 0.12	0.54 ± 0.06
Fe-7d	2 ± 0.1	0.06 ± 0	0.08 ± 0.01	82.39 ± 3.77	± 0.27 ± 0.03	1.31 ± 0.09

5 Notes: Control-7d: a 7-day cultivation without external Fe addition; Fe-3d: a 3-day cultivation with external Fe addition; and Fe-7d: a 7-day cultivation with external Fe addition.

Changes in biomass composition

10 In addition to the kinetic parameters, the presence of elevated iron conditions had a significant impact on the intracellular compound composition of the coculture. Surprisingly, while the Fe-7d coculture exhibited faster growth compared to Control-7d, it had a lower protein content (decreased from 0.47 to 0.31 g/g biomass). Conversely, the total lipid content increased from 9 to 14 % of the biomass (Fig. 16a). The results of Fe-3d are discussed in Example 9.

15 Furthermore, the profiles of biomass fatty acids were further analyzed in the form of FAMES. As depicted in Fig. 16b, 9,12-Octadecadienoic acid (Z,Z)- methyl ester became the most abundant FAME of in the Fe-7d biomass, with its percentage increasing from 1.5 to 4.4 % of the biomass. This particular fatty acid, known as an omega-6 fatty acid, is essential for mammalian nutrition. Additionally, the most commonly observed saturated fatty acid in
20 microorganisms, hexadecenoic acid, showed a twofold increase in content after the addition of iron. Notably, the methyl ester of linolenic acid, 9,12,15-Octadecatrienoic acid (Z,Z,Z), a plant-based essential omega-3 fatty acid, was observed to accumulate in the biomass following the addition of external iron to the system.

25 *Changes in microbial community composition*

Following the amendment of iron for a period of one month, the eukaryote community remained largely unchanged, with *Chlorella* still dominating (99 %, Fig. 14d). However, the addition of external iron resulted in a reduction in the diversity of the microorganism community at the phylum, class, and genus levels (Fig. 17), accompanied by an expansion of the MOB population (Fig. 14c). This shift was supported by the Shannon index, which is a metric used to measure the diversity of species within a community (Shannon, C. E., *Bell Syst. Tech. J.* **1948**, 27, 379–423), which showed a decrease from 2.4 to 2.1 when iron was added. Iron is crucial for the growth of both MOB and MG. In MOB, iron is an essential component of key enzymes involved in methane oxidation, such as methane monooxygenase. In MG, iron plays multiple roles in several key metabolic processes, including photosynthesis and biosynthesis of chlorophyll and other pigments. Interestingly, within the MOB population, a shift from *Methylococcus/Methylosinus* to *Methylocystis* (type II, 34 % relative abundance to total prokaryotes) was observed (Fig. 14c). In Fe-7d, ammonium was usually limited after day 3 in each cycle (Fig. 15). Previous findings indicate that type II MOB such as *Methylocystis*/*Methylosinus* are more competitive in nitrogen-limited condition (Yang, Y. *et al.*, *Front. Bioeng. Biotechnol.* **2020**, 8; and Zhang, X. *et al.*, *Syst. Appl. Microbiol.* **2014**, 37, 296–304). Therefore, nitrogen deficiency may serve as a key stimulus for the proliferation of *Methylocystis* in the iron-elevated conditions observed in the present disclosure.

In the Fe-7d, organic carbons were released, and their concentration increased along over time (Fig. 18). Further composition analysis revealed a substantial increase in biopolymers, which became the predominant fraction at the end of the cycle (52 %), followed by humic substances and LMW neutrals. It was reported that *Chlorella* typically releases organic compounds with high molecular weight (>10 kDa) (Henderson, R. K. *et al.*, *Water Res.* **2010**, 44, 3617–3624), suggesting that biopolymers are likely produced and secreted by MG in the coculture. In addition, MOB is known to produce soluble metabolic intermediates such as methanol and formaldehyde, which may be responsible for the secretion of LMW neutrals. *Methylocystis* is also capable of producing humic substances, such as like UV humic acid and visible humic acid. Overall, without wishing to be bound by theory, MG and MOB may probably be responsible for the release of these organic carbons. Interestingly, humic substances not only work as substrate for heterotrophs but also could promote *Chlorella* growth under a reasonable concentration range (below 30 ppm). Thus, *Chlorella* may benefit from the MOB-MG coculture system through the taking up of the intermediates produced by MOB.

As discussed above, the presence of heterotrophs may be strongly associated with the release of organic carbons. For instance, the high proportion of biopolymers in the released organic carbons may account for the predominance of *Terrimonas* (35 % relative abundance to total

prokaryotes), which usually utilizes polysaccharides as its substrate. The other two dominant species, *Chthonobacter* (9 % relative abundance to total prokaryotes) and *Ferrovibrio* (7 % relative abundance to total prokaryotes) are also capable of consuming LMW neutrals. Apart from enhancing biogas conversion efficiency, these considerable heterotrophs may contribute to the removal of substances that could be toxic to MG or MOB. The presence of heterotrophs in the current system enhances the stability of the symbiotic system by consuming the organic carbon released by both MG and MOB. In an intriguing comparison, when incubated in a similar iron concentration, the monoculture growth rates of *Methylococcus capsulatus* and *Chlorella sorokiniana* reached only 0.12 day⁻¹ and 0.14 day⁻¹, respectively (Kim, I. T. *et al.*, *Water* **2018**, 10, 1414; and Wan, M. *et al.*, *Appl. Microbiol. Biotechnol.* **2014**, 98, 9473–9481), notably lower than the 0.62 day⁻¹ achieved in a Fe-3d coculture system. Compared to a pure strain MOB-MG cultivation system, the non-axenic system exhibits an improved specific substrate utilization rate and carbon conversion rate, while also preserving the protein content of the biomass.

Example 9. Nitrogen availability impact on the culture growth and activities

To understand how the culture growth, intracellular and extracellular compounds changed in the system from a nitrogen sufficient condition to a nitrogen limiting condition, reactor SB was continued for three cycles by following the protocol in Example 7 except with a modified sampling strategy. The sampling was conducted on day 0, 2, 4, and 6 with the first day of each cycle as day 0. 15 ml liquid phase (0.45 µm membrane filtered) was taken to analyze pH, ammonium, TIC, TOC, organic compounds composition. Gas sample was collected in a 5 ml gas tight syringe. The biomass was also harvested on days 0, 2, 4 and 6, respectively, to acquire intracellular composition analysis. The fresh AMS medium was replenished after sampling to maintain the working volume as 1 liter.

In Fe-7d, iron addition indirectly led to the depletion of ammonium after day 3. Thus, additional two series of batch tests (Fe-3d and N-7d) were conducted to rigorously evaluate the impacts of iron and nitrogen, respectively, on the coculture.

Nitrogen effect on MOB-MG coculture

To understand how the MOB-MG coculture responded to the change of nitrogen availability, the reactor (N-7d) was conducted with the same operating conditions as Fe-7d (see Example 8) except the biomass culture sampling frequency was increased to figure out the transformation of intracellular compounds under different nutrient availabilities. A 200 mL of culture was harvested and fixed on day 1, 3, 5, and 7 for analysis of glycogen, amino acid,

total lipid, PHB, and elemental composition. The working volume was replenished with medium after sampling to maintain a volume of 1 L. On days 1, 3, 5, and 7, a 15 mL of liquid sample was taken and filtered to analyze ammonium, iron, and DON concentration. A 10 mL culture was taken to measure TSS, VSS, and OD₆₀₀. Additionally, a gas sample was collected using a 5 mL gas-tight syringe for gas composition analysis.

Results and discussion

Though the biomass concentration was higher in Fe-aided 7-day experiment, the total intracellular content in terms of protein, glycogen and total lipid was 13% lower than that of the 2-day experiment. A detailed cycle study was carried out to understand how the intracellular macromolecular compounds were converted in a 6-day cycle.

Within a cycle, the protein inside the cell accumulated from 0.25 ± 0.02 g/g of CDW on day 0 to 0.57 ± 0.05 g/g of CDW on day 2, and dipped afterwards to 0.31 ± 0.03 g/g of CDW on day 6 (Fig. 19). It was noted that ammonium in the liquid phase was reduced to a very low level at the end of day 2. The accumulation tendency of glycogen was opposite to that of protein. Nitrogen limitation is one of the most effective methods for shifting protein production to carbohydrates production in microalgae and is a necessary condition for carbohydrate and starch formation in *Chlorella* sp. PHA tends to be accumulated in methanotrophs when nitrogen source is limited. However, PHB content in the current culture was at a very low level though slight PHB increase was also observed. Glycogen was thus likely accumulated as the energy source after ammonia was being depleted. The trend of total lipid did not show much fluctuation. It is crucial to obtain the desired by-products through an optimal bioprocessing manipulation.

The kinetics data are summarized in Table 9. When ammonium was limited after day 2, the biomass concentration continuously increased from 0.62 g/L to 1.35 g/L with a significant drop in specific growth rate from 0.59 to 0.17 per day. There was a decreased trend in specific substrate utilization rate from 0.26 to 0.07 mg C/mg TSS per day. The carbon oxidation rate showed a slight increase on day 4 when the nitrogen source was limited but the carbon source was relatively abundant. Overall, the nitrogen limited condition did not stop the growth of the biomass and the consumption of gaseous carbon, which led to a relatively carbon abundant condition for the culture to synthesize carbon-based intracellular compounds (e.g. glycogen).

The nitrogen fate was calculated and shown in Fig. 20. Ammonium in the feed medium was the sole nitrogen source for biomass growth. The nitrogen was transferred from ammonium in liquid phase into cell mass mainly as protein and some as other nitrogen-based cell structure.

With the growth, some metabolites released into liquid phase showed in the form of dissolved organic nitrogen. Protein is what microorganisms use to store nitrogen sources. Hence, the protein content of CDW (triangle, Fig. 20) showed an increase trend when ammonium is sufficient in the liquid phase, but decreased after ammonium was depleted. However, the ammonium limitation did not stop the biomass growth with a consciously TSS rise. Without wishing to be bound by theory, it appears that the protein has been diverted into new biomass to support division and growth, leading to low protein content. The nitrogen source to support biomass growth was likely from intracellular protein. Overall, under a certain nitrogen source loading, the increase biomass concentration led to a decrease in protein content in cell while the total protein mass in the reactor remained stable.

Table 9. Kinetics data during a whole cycle in SB reactor.

Date	Biomass Yield (mg TSS/mg C)	Specific Substrate Utilization (mg C/mg TSS per day)	Specific Growth Rate (day ⁻¹)	TSS (g/ L)	NH ₄ -N (ppm)
Day0	NA	NA	NA	0.16±0.01	47.04±1.34
Day0-2	2.12±0.23	0.26±0.05	0.59±0.12	0.62±0.09	14.25±3.52
Day2-4	1.87±0.41	0.14±0.02	0.2±0.06	0.94±0.09	0.19±0.33
Day4-6	1.89±0.49	0.07±0.02	0.17±0.06	1.35±0.19	0.15±0.26

The carbon balance in the SB reactor within one cycle was estimated and presented in Fig. 21. Initially, carbon was mostly in the synthetic biogas with a small amount within the biomass. Carbon dioxide and methane existed in the system in two forms: gaseous in the headspace and dissolved in the medium due to gas and liquid equilibrium. Dissolved CO₂ can be known as total inorganic carbon (TIC) and dissolved CH₄ can be considered as TOC. Both forms can be used as the substance for microalgae and methanotrophs to grow. The carbon in biomass was determined by CHNS elemental analysis with a result that there was 46% carbon in the biomass. During a cultivation cycle, almost all the inorganic carbon, regardless of the forms, was assimilated by biomass, followed by organics released in both liquid phase and solid biomass.

Although TOC in lipid phase occupied a small part of the carbon pool, it can work as the substrate for other heterotrophs. Fig. 22 shows that the biopolymer was the dominant dissolved organic carbon from day 2 to day 6 with the absolute concentration and percentage of TOC showing an increment from 2.74 to 9.73 ppm C/ L and from 38% to 52%, respectively. Simultaneously, the organic substances humic substance, LMW neutrals tended to accumulate in the liquid phase. Compared with the fermenter, batch mode led to higher organics accumulation in the liquid phase as the metabolites in the fermenter can be discharged continuously. It is important to understand soluble microbial products, which are key factors in building a constant mutualistic system.

Effect of iron on the coculture

In Fe-3d, biomass concentration (0.54 g L⁻¹) reached the same level as that in Control-7d within only 3 days, without nitrogen limitation (Table 8). As previously discussed, iron is known to be an important component of some key enzymes of MOB. Furthermore, iron addition can enhance the expression of genes, such as carbonic anhydrase, which is essential for MG growth. Indeed, Fe-3d exhibited a specific growth rate 2.4 times higher than that in Control-7d (Table 8), demonstrating that the elevated iron condition (13.6 μM) significantly stimulated the growth of the coculture. This stimulation is also evident in the significantly increased specific substrate utilization rates for CO₂ and CH₄ (from 0.06 to 0.16 and from 0.07 to 0.18 mg-C/mg-VSS day⁻¹, respectively). Complementing this finding, several studies have pinpointed the optimal iron concentrations for MOB and MG growth at 18 μM and 10 μM, respectively (Karthikeyan, O. P. *et al.*, *Environ. Sci. Pollut. Res.* **2016**, 23, 4346–4357), which intriguingly align with the concentration used in the Fe-3d experiment. This alignment provides a suggestive correlation, hinting that a judicious balance of iron concentration, optimal for both MG and MOB growth, could prove to be a pivotal element for realizing high-rate biogas conversion in a coculture system.

Comparing the protein content values between Fe-3d and Control-7d (Fig. 16a), no significant difference was observed, indicating that the decrease in protein content in Fe-7d was primarily due to nitrogen limitation. Furthermore, a comparison between the Fe-7d and Fe-3d treatments revealed a trend towards increased glycogen accumulation in the presence of nitrogen depletion, as evidenced by the values of 23 % and 17 %, respectively (Fig. 16a). In Fe-3d, a higher lipid content (0.14 g/ g biomass) was obtained compared to Control-7d (0.09 g/g biomass), suggesting a potential role of iron in facilitating lipid accumulation. This finding is consistent with previous studies which found that an optimal iron concentration can enhance the lipid content of MG, as well as the specific growth rate (Liu, J.-Z. *et al.*, *Renew. Energy* **2021**, *170*, 927–935).

Effect of nitrogen on the coculture

The kinetic analysis of the N-7d test is summarized in Table 10. During the incubation, nitrogen became limited from day 3 onwards. As a result, there was a sharp decline in the specific substrate utilization rate for both CO₂ and CH₄ (0.2 to 0.07 and 0.22 to 0.1 mg-C/mg-VSS day⁻¹, respectively). In contrast, the biomass concentration continuously increased from 0.47 to 1.21 g-VSS L⁻¹. However, this increase was accompanied by a notable drop in the specific growth rate from 0.64 to 0.28 day⁻¹. These results indicate that the activity of the coculture may be partially restricted by nitrogen depletion. Previous studies showed that the concentration of chlorophyll, a key component in supporting the growth of MG, underwent a significant decrease after nitrogen depletion and remained at a low until the end of the cultivation period (Ikaran, Z. *et al.*, *Algal Res.* **2015**, *10*, 134–144; and Shrestha, N. *et al.*, *J. Appl. Phycol.* **2020**, *32*, 2795–2805). Overall, the nitrogen source is a crucial contributor to manipulate this coculture system. The fate of nitrogen in N-7d was monitored and is presented in Fig. 23a. Ammonium in the feed medium served as the sole nitrogen source for biomass growth, primarily assimilated for protein synthesis (87.7 %) and other nitrogen-based cell components (7.4 %) on the last day of N-7d. As the biomass grew, some metabolites were released into the liquid phase in the form of DON, contributing to 2 % of the total nitrogen in the system.

Table 10. Kinetic data during the N-7d cycle study.

Time	Biomass yield	Specific substrate utilization rate	Specific growth rate	VSS	NH ₄ -N	Total iron

	mg-VSS/ mg-C	mg-CO ₂ - C/mg- VSS day ⁻¹	mg-CH ₄ - C/mg- VSS day ⁻¹	day ⁻¹	g L ⁻¹	mg L ⁻¹	μM
Day1	NA	NA	NA	NA	0.11 ± 0.03	47.04 ± 1.34	7.35 ± 1.43
Day3	1.5 ± 0.22	0.2 ± 0.03	0.22 ± 0.04	0.64 ± 0.10	0.47 ± 0.04	14.25 ± 3.52	5.73 ±1.08
Day5	1.52 ± 0.25	0.11 ± 0.01	0.15 ± 0.02	0.39 ± 0.04	0.87 ± 0.06	0.19 ± 0.33	4.84 ± 0.90
Day7	1.54 ± 0.25	0.07 ± 0.01	0.1 ± 0.01	0.28 ± 0.02	1.21 ± 0.17	0.15 ± 0.26	3.23 ± 0.72

The biomass compositions were further analyzed throughout the entire incubation period. Protein content increased from 0.25 ± 0.02 to 0.57 ± 0.05 g/g biomass when nitrogen was available but decreased to 0.31 ± 0.03 g/g biomass after nitrogen depletion (Fig. 23b). These results support the notion that protein content in the coculture is regulated by the availability of nitrogen rather than iron. It was in line with previous studies, which showed that protein synthesis in cells could be ceased by an inhibitor of the ammonium transport system (Cardoso, H. & Leao, C., *FEMS Microbiol. Lett.* **1992**, *94*, 155–160). These results emphasize the vital role of ammonium in building up protein content in a MOB-MG coculture. With sufficient ammonium, the protein content of the MOB-MG coculture is comparable with traditional protein sources such as soybean, egg, and fishmeal.

The detailed amino acid profiles are shown in Fig. 24. All amino acids showed a similar trend to protein content, increasing when ammonium was sufficient and decreased when ammonium was consumed up. The proportion of essential amino acids, including Phenylalanine, Valine, Threonine, Tryptophan, Isoleucine, Leucine, Methionine, Lysine, and Histidine, was approximately 19 % of the total amino acids, which is comparable to plant-based proteins (20–22 %) (Gorissen, S. H. M. *et al.*, *Amino Acids* **2018**, *50*, 1685–1695).

PHB is a critical component of lipid in MOB. The results indicate a minimum PHB content (0.02 g/g biomass) on day 3 in the presence of adequate ammonium, whereas a tendency towards accumulation (0.05 g/g biomass) under nitrogen deficiency was observed (Fig. 23b). Even though the accumulation trend is in line with the findings obtained from pure MOB cultivation, the content of PHB was found to be relatively low in the current coculture, which was likely

- attributed to the presence of non-MOB microorganisms in the mixed culture. The change in glycogen content was opposite to that of protein. The trend of increased glycogen content was observed when ammonium was depleted after day 3. The results are in line with previous research, which reported that nitrogen limitation is one of the most effective methods for shifting protein production to carbohydrates production in MG (Chen, S., Xie, J. & Wen, Z., 2021. In: Li, Y., Zhou, W. (Eds.), *Advances in Bioenergy*. Elsevier, pp. 165–198) and is a necessary condition for carbohydrate and starch formation in *Chlorella* (Cheng, D. *et al.*, *Biotechnol. Biofuels* **2017**, 10, 75; and Kaur, A. *et al.*, *Bioenergy Res.* **2022**, 15, 253–263).
- It is worth noting that the level of DON released in the system increased over time (Fig. 23a). Moreover, it could be expected that DOC also increased as the operating conditions of N-7d were similar to those of Fe-7d (Fig. 18). These intermediate metabolites, in addition to nitrogen limitation, may therefore influence kinetics and compositions of the coculture. Nonetheless, previous study asserted that the release of dissolved microalgal organics is primarily triggered by nitrogen insufficiency (Cheng, T. *et al.*, *Bioresour. Technol.* **2017**, 241, 360–368). In contrast, carbon and iron may have a minimal impact on the system as they remained sufficient during the test. Without wishing to be bound by theory, in a comprehensive view, the primary factor causing these observed changes should be nitrogen insufficiency.
- In the present disclosure, we aimed to elucidate the role of iron and nitrogen availability in regulating the growth and biomass compositions of MOB-MG coculture and provide valuable insights into the optimization of a MOB-MG coculture system for efficient utilization of biogas. To this end, a coculture of MOB and MG were enriched from waste activated sludge using synthetic biogas. The transformation of CH₄ and CO₂ by this coculture was explored by manipulating the availability of iron and nitrogen in the growth medium. To assess the effects of nutrient availability, the composition of biomass was profiled and compared across different experimental treatments. Furthermore, the microbial community and dynamic performance of the coculture were monitored, and the secretion of intermediates by the coculture was examined.

Example 10. The effects of VOCs and H₂S under optimal growth condition

- Through the previous batch experiment and long-term parent reactor operation in the above examples, the optimal growth conditions were identified. It was also found that H₂S may have a different effect on the culture when the culture conditions are different. An additional batch experiment was set up under Fe and nitrogen sufficient conditions. The operation of reactors RB and SBS were resumed with the same operation conditions stated in Example 8 for reactor

SB. The cycle duration was 2 days. In addition, the sampling interval was also the same as the 2-day experiment described in Example 8. The performance of RB and SBS was compared with that of SB reactor to further understand the effects of H₂S.

5 Results and discussion

Under the optimal nutrient conditions, the biomass yield, substrate consumption rate, and specific growth rate, of the three reactors (Table 11) were all significantly higher compared with the study in Example 7.

Table 11. Kinetics data and liquid parameters in three reactors.

Reactors	Biomass Yield (mg TSS/mg C)	Specific Substrate Utilization Rate (mg C/mg TSS per day)	Specific Growth Rate (day ⁻¹)	TSS (g/ L)
SB	2.42±0.52	0.27±0.05	0.64±0.02	0.67±0.07
RB	2.64±0.05	0.29±0.03	0.76±0.09	0.70±0.04
SBS	2.10±0.24	0.28±0.03	0.6±0.07	0.53±0.03

Table 12. Macromolecular compound composition in three reactors.

Reactor	Protein	Glycogen	Lipid	Sum
SB	0.51±0.07	0.17±0.06	0.14±0.01	0.81±0
RB	0.49±0.03	0.21±0.04	0.14±0.01	0.84±0.03
SBS	0.62±0.02	0.07±0.02	0.17±0.02	0.87±0

However, a comparison between three gas supplements under optimal condition showed that when the optimal condition was provided, the VOCs in RB reactor might stimulate the system. The real biogas increased 8% while the presence of H₂S led to 13% lower in biomass yield compared with reactor SB. In addition, the RB reactor showed the highest specific growth rate and biomass concentration. The H₂S effect still existed even with optimal condition provided, which led to a significant drop in biomass concentration ($p < 0.05$).

The presence of 500 ppm H₂S did not significantly alter lipid content (Table 12). It is worth noting that the protein content was significantly higher in the SBS reactor (0.62 ± 0.02 g/g of CDW) than that in the other two reactors. In contrast, the glycogen metabolite ways may be suppressed when H₂S was present, leading to significant lower glycogen content of 0.07 ± 0.02 g/g compared with 0.17 ± 0.06 g/g of CDW under synthetic biogas. It was reported that NaHS ≤ 0.5 mM enhanced glycolysis in microalgae (Cheng, J. *et al.*, *ACS Sustain. Chem. Eng.* **2020**, *8*, 2481-2489). Past research has demonstrated that H₂S is a crucial gas transmitter with multiple physiological roles (Giuffrè, A. & Vicente, J. B., *Oxid. Med. Cell. Longev.* **2018**, *2018*, 6290931). Compared to synthetic biogas, the VOCs in the real biogas did not impact the protein and glycogen content when the optimal condition was provided.

The bacteria community in the three reactors are compared in Fig. 25. SB had the highest methanotrophs abundance. RB had a higher percentage of *Chthonobacter* and *Ferrovibrio*, which may be due to the higher organic content in the system, which can be used as substrate. The dominant three heterotrophs remained as *Ferrovibrio*, *Chthonobacter*, and *Terrimonas*, which are aerobic heterotrophs same as in Example 8. SBS had a specific genus *Parvibaculum* which is related to desulfonation reactions and has a high salt tolerance.

To summarize, the mixed culture of microalgae and methanotrophs is robust to real biogas. In contrast, the presence of H₂S reduced biomass concentration, biomass yield, specific growth rate and glycogen percentage in CDW, corresponding with an increase in protein content.

Example 11. Impact of H₂S concentration in biogas on the microbial community composition

A 5L commercial fermenter (working volume: 3L) was inoculated with a non-axenic methanotroph-microalgae coculture, previously enriched from waste activated sludge (Jurong Water Reclamation Plant, Singapore) using a lab-scale bioreactor (described in Example 1, see Fig. 2). The system was operated continuously (hydraulic retention time and sludge retention time: 14 days) and supplemented was a modified ammonium mineral salts medium.

Synthetic biogas (40% CO₂, 60% CH₄) was used as the sole carbon source, stored in a 10-liter gas bag, and fed into the reactor via a peristaltic pump (100 rpm). Excess biogas was vented and recirculated back into the gas bag, which was replaced every 7 days or when nearly depleted. The fermenter was maintained at 27 ± 1 °C, illuminated with a 6000k LED lamp, and the pH was automatically controlled at 6.8 (1M HCl or 1M NaOH). An overhead mixer (150 rpm) ensured uniform mixing.

After 64 days, H₂S was introduced into the biogas to achieve final concentration as 1000 ppm (group '1000) and the reactor was operated for an additional 74 days. Subsequently, H₂S was increased to 5000 ppm and the reactor was operated for more than two months. The control group was designated "control," while the 5000 ppm H₂S treatment, which exhibited distinct performance characteristics during the first half and second half treatment period of exposure, the group was further divided into "5000_1" and "5000_2," respectively. For start and end periods were distinguished using "_s" and "_e" suffixes respectively. For instance, "1000_s" denotes the start period of the 1000 ppm H₂S treatment, while "5000_2_e" represents the end period of the second half period under 5000 ppm H₂S treatment.

The impact of hydrogen sulfide (H₂S) concentration in biogas on the microbial community composition was assessed by analyzing the relative abundance of bacterial genera in a control group and groups subjected to two H₂S concentrations: 1000 ppm and 5000 ppm. Comparisons were drawn between the control group and systems exposed to 1000 ppm H₂S at both the commencement and conclusion of the experiment, as well as with systems treated with 5000 ppm H₂S at two distinct periods – initially and at the end of the first 40 days, followed by another assessment at the beginning and conclusion of the subsequent 40 days.

Results and discussion

The microbial community composition reveals differential abundance patterns across the treatment conditions (Fig. 26).

The dominance of *Leptolyngbya* in the control group, constituting 52.58% of the microbial population, was noted. Under the 1000 ppm H₂S treatment, a slight reduction in *Leptolyngbya*'s abundance was observed, decreasing from 47.28% at the experiment's start to 46.46% at its end. The 5000 ppm H₂S treatment resulted in a more pronounced decrease to 42.29%, before experiencing a significant rise to 62.20% at the second interval's start, followed by a decline to 41.67% at its conclusion. *Leptolyngbya*, a genus often associated with photosynthetic capabilities, showed a decrease in abundance with the introduction of 1000 ppm H₂S. This decline might be attributed to the sensitivity of photosynthetic organisms to H₂S,

which can disrupt photosynthetic processes or signal a shift in metabolic priorities under sulfide stress.

For *Methylocystis*, the difference between the control group (13.45%) and the 1000 ppm H₂S treatment (13.80%) was minimal, indicating no clear trend. This may be because *Methylocystis* possesses *sqr* gene which is involved in the oxidation of sulfide in organisms. SQR catalyzes the oxidation of sulfide to sulfane sulfur, transferring electrons to ubiquinone in the respiratory chain. This process is crucial for sulfide detoxification and energy production in environments with high sulfide concentrations. However, under the 5000 ppm H₂S treatment, *Methylocystis* showed a notable decrease, falling to 7.55% at the start and further to 4.61% by the end of the second stage. This finding is in line with a previous finding that the threshold of H₂S on *Methylocystis* is around 1000 ppm.

Lentimicrobium, comprising 10.14% of the control population, increased to 14.77% at the start and 22.99% at the end of the 1000 ppm treatment. This genus exhibited a more variable pattern under the 5000 ppm treatment, with an initial increase to 27.76%, followed by a decrease to 3.75% at the start of the second interval, and a subsequent increase to 28.11% at the end. The increase in *Lentimicrobium* suggests that members of this genus either have metabolic pathways that allow them to tolerate or possibly utilize H₂S, or they may benefit from the decrease in competitive pressure from other genera like *Leptolyngbya*.

In addition to the high-abundance populations, other low-abundance genera such as *Melioribacter*, *Rhodoplanes*, and *Sulfurisoma* exhibited an increasing trend under H₂S treatment, potentially due to their ability to metabolize sulfur-related compounds. Notably, *Methylococcus*, a type I methanotroph, increased from negligible to 5% at the conclusion of the 5000 ppm H₂S treatment. This transition among methanotrophs reveals their differing tolerances and preferences for H₂S.

The data indicate that the microbial community in a methanotroph-microalgae coculture reactor exhibits a differential response to varying concentrations of H₂S. At a lower concentration of 1000 ppm, changes within the microbial community are relatively subdued, suggesting that most genera possess a certain level of tolerance or resilience to H₂S at this concentration. However, the observed decrease in the relative abundance of *Leptolyngbya* and the concurrent increase in *Lentimicrobium* imply that even subtle changes in H₂S levels can affect the community structure. At a higher concentration of 5000 ppm H₂S, the microbial community exhibits more pronounced changes, particularly in the second month of exposure. This response indicates a time-dependent adaptation or acclimatization process. The initial

changes in 5000_2_s, followed by a moderate recovery suggests a selection pressure favoring H₂S-resistant strains or a community shift towards organisms with metabolic capabilities aligned with high-H₂S environments. This adjustment period might reflect a community-level shift towards a more stable state that can persist under continued H₂S stress.

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In the control group (cont), *Chlorella* is the most abundant genus, constituting 82.41% of the population (Fig. 27). It was followed by *Ochromonas* at 16.91%, while *Trichoderma*, unclassified Eukaryota, and *Naegleria* constituted minor proportions of 0.6%, 0.6%, and 0.3%, respectively. Exposure to 1000 ppm H₂S resulted in the relative stability of the abundance of all dominant genera, with fluctuations confined within a 2% margin. However, at a higher concentration of 5000 ppm H₂S, a significant shift was observed. The abundance of *Chlorella* decreased to 75.18% at the start of the first 30-day interval and further declined to 56.26% by the end, continuing to drop to 35.84% at the start of the second interval, with a modest recovery to 52.27% by its end. Conversely, *Ochromonas* exhibited an inverse trend, initially increasing to 18.26% at the beginning of the first period and then markedly to 37.96% by its end, followed by a rise to 60.10% at the commencement of the second period and a decrease to 36.00% at its later phase.

These results indicate that a low concentration of H₂S does not significantly affect the eukaryotic population. However, a high concentration of H₂S shifts the dominance within this co-culture system. Despite some initial fluctuations during the second stage of 5000 ppm H₂S treatment, which appeared to disrupt the trend, the population ultimately attained a steady state, emerging from this chaos.

In the absence of H₂S, the consumption of CO₂ and CH₄ by the coculture system exhibited a pattern of relative stability over time, suggesting a balanced microbial activity (Fig. 28). The introduction of 1000 ppm H₂S didn't result in notable changes. However, at the transition from 1000 to 5000 ppm H₂S, an unexpected rise in consumption rate was observed followed by a gradual decrease, indicating a time-dependent response of the coculture to high H₂S concentration. The subsequent decrease indicated that this response is not sustainable, as the inhibitory effects of high H₂S concentrations begin to dominate, leading to reduced metabolic activity. Furthermore, after a period of continuous declines spanning a month, a sudden and significant rise in the consumption rates was observed. With the rate of consumption increasing from its initial level of approximately 100 to 300 mg C/mg VSS per day.

Nutrient supply is important for maintaining a methanotroph and microalgae (MOB-MG) system for biogas valorization. However, there is a lack of understanding regarding how key elements regulate the growth of a MOB-MG coculture. Herein, a non-axenic MOB-MG coculture system with high protein content (0.47 g/g biomass) was established from waste activated sludge using synthetic biogas without the needs of sterile operation in the present disclosure. The eukaryotic population was predominantly represented by the genus *Chlorella*, while *Methylococcus* and *Methylocystis* dominated the MOB populations before and after iron addition, respectively. An increase in iron availability substantially stimulated the specific growth rate (from 0.18 to 0.62 day⁻¹) and biogas conversion rate (from 26.81 to 106.57 mg-C L⁻¹ day⁻¹) of the coculture. Moreover, the protein content remained high (0.51 g/g biomass), and the total lipid content increased (from 0.09 to 0.14 g/g biomass). Nitrogen limitation apparently constrained the specific growth rate (from 0.64 to 0.28 day⁻¹) and largely reduced the protein content (from 0.51 to 0.31 g/g biomass) of the coculture. Intriguingly, the lipid content remained unchanged after nitrogen was depleted. The eukaryotic community was consistently dominated by MG belonging to *Chlorella*, while the populations of MOB shifted from *Methylococcus*/*Methylosinus* to *Methylocystis* due to iron and nitrogen amendment. In addition, diverse non-methanotrophic heterotrophs were present in the community. Their presence neither compromised the performance of the coculture system nor affected the protein content of the biomass. However, these heterotrophs may contribute to high carbon conversion efficiency by utilizing the dissolved organic carbon released by MOB and MG. Overall, the findings highlight the vital roles of iron and nitrogen in achieving sustainable and efficient conversion of biogas, fast growth of cells, and optimal biomass composition in a MOB-MG coculture system.

The main findings of this study are as follows:

- 1) the addition of external iron significantly enhanced various performance parameters of the coculture, including carbon assimilation rate, biomass yield, specific growth rate, specific substrate utilization rate, and total lipid content; and
- 2) the availability of ammonium was found to be crucial for regulating the protein and glycogen content in the coculture.

Overall, the present disclosure highlights essential roles of key nutrients on MG and MOB and their interactions, which can be further adopted to develop a potential strategy for optimal biogas transformation and quality SCP products.

Claims

1. A method of generating a biomass mixture, comprising:
 - (a) providing a co-culture comprising microalgae and methanotrophs obtained from a waste activated sludge in a reaction vessel; and
 - (b) operating the reaction vessel under conditions that comprise feeding a gas comprising CO₂ and CH₄ that is depleted of oxygen to the co-culture and subjecting the co-culture to light to generate the biomass mixture, wherein
the biomass mixture comprises proteins, lipids and glycogen in addition to the microalgae and methanotrophs.
2. The method according to Claim 1, wherein one or more of the following apply:
 - a protein content of the biomass mixture is from 40 to 60 wt%, such as from 44 to 56 wt%, such as about 50 wt%, of a cellular dry weight of the biomass mixture;
 - an omega-6 fatty acid content of the biomass mixture is from 2 to 5 wt%, such as about 2.7 wt%, of a cellular dry weight of the biomass mixture; and
 - a glycogen content of the biomass mixture is from 22 to 35 wt%, such as about 28 wt%, of a cellular dry weight of the biomass mixture.
3. The method according to Claim 1 or Claim 2, wherein the co-culture further comprises heterotrophs, optionally wherein the heterotrophs comprise one or more of *Ferrovibrio*, *Terimonas*, and *Chthonobacter*.
4. The method according to any one of the preceding claims, wherein the methanotrophs comprise one or more prokaryotes selected from the group consisting of *Methylosinus*, *Methylocystis*, *Leptolyngbya*, *Lentimicrobium*, *Melioribacter*, *Rhodoplanes*, *Methylococcus*, and *Sulfurisoma*, optionally wherein the methanotrophs comprise one or more prokaryotes selected from the group consisting of *Methylosinus* and *Methylocystis*.
5. The method according to any one of the preceding claims, wherein the microalgae comprise one or more eukaryotes selected from the group consisting of *Chlorella*, *Ochromonas*, and *Trichoderma*, optionally wherein the microalgae comprise one or more eukaryotes selected from the group consisting of *Chlorella sorokiniana*.
6. The method according to any one of the preceding claims, wherein one or more of the following operating conditions are applied:
 - (ai) a pH within a range of from 6.5 to 7, such as from 6.7 to 6.8;

- (aii) a temperature of from 25 to 30 °C, such as from 26 to 30 °C;
- (aiii) a hydraulic retention time (HRT) and/or a sludge retention time (SRT) of from 5 to 19 days, such as from 7 to 14 days in a continuously running process;
- (aiv) an ammonium mineral salt medium comprising ammonium chloride, magnesium sulfate (e.g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), disodium hydrogen phosphate (e.g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), potassium dihydrogen phosphate, calcium chloride (e.g. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$), and ethylenediaminetetraacetic acid (e.g. as the free acid and/or as the ferric-sodium salt thereof, optionally wherein the free acid is used initially and the ferric-sodium salt is used after continual operation of 100 days or more).

7. The method according to any one of the preceding claims, wherein the gas further comprises H_2S at a concentration of greater than or equal to 500 ppm.

8. The method according to Claim 7, wherein the H_2S concentration is from 750 to 10,000 ppm, such as from 1,000 to 5,000 ppm.

9. The method according to any one of the preceding claims, wherein the volume to volume ratio of CH_4 to CO_2 is from 1.2:1 to 1.7:1, such as from 1.26:1 to 1.67:1, such as about 1.5:1.

10. The method according to any one of the preceding claims, wherein the gas further comprises one or both of nitrogen gas and volatile organic compounds.

11. The method according to any one of the preceding claims, wherein the co-culture comprising microalgae and methanotrophs obtained from a waste activated sludge is obtained by a method comprising the steps of:

- (bi) providing a waste activated sludge in a precursor reaction vessel;
- (bii) operating the precursor reaction vessel under conditions that comprise feeding in a gas comprising CO_2 and CH_4 that is depleted of oxygen to the waste activated sludge for a first period of time;
- (biii) removing a biomass suspension after the first period of time and subjecting the biomass suspension to conditions that comprise feeding in a gas comprising CO_2 and CH_4 that is depleted of oxygen to the biomass suspension for a second period of time; and
- (biv) repeating step (biii) from 2 to 10 times until a co-culture comprising microalgae and methanotrophs is obtained.