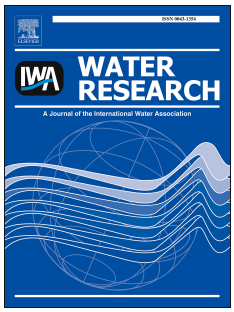
Journal Pre-proof



Sulfide restrains the growth of *Methylocapsa acidiphila* converting renewable biogas to single cell protein

Mingyi Xu, Huihui Zhou, Xiaoyong Yang, Irini Angelidaki, Yifeng Zhang

PII: S0043-1354(20)30675-8

DOI: https://doi.org/10.1016/j.watres.2020.116138

Reference: WR 116138

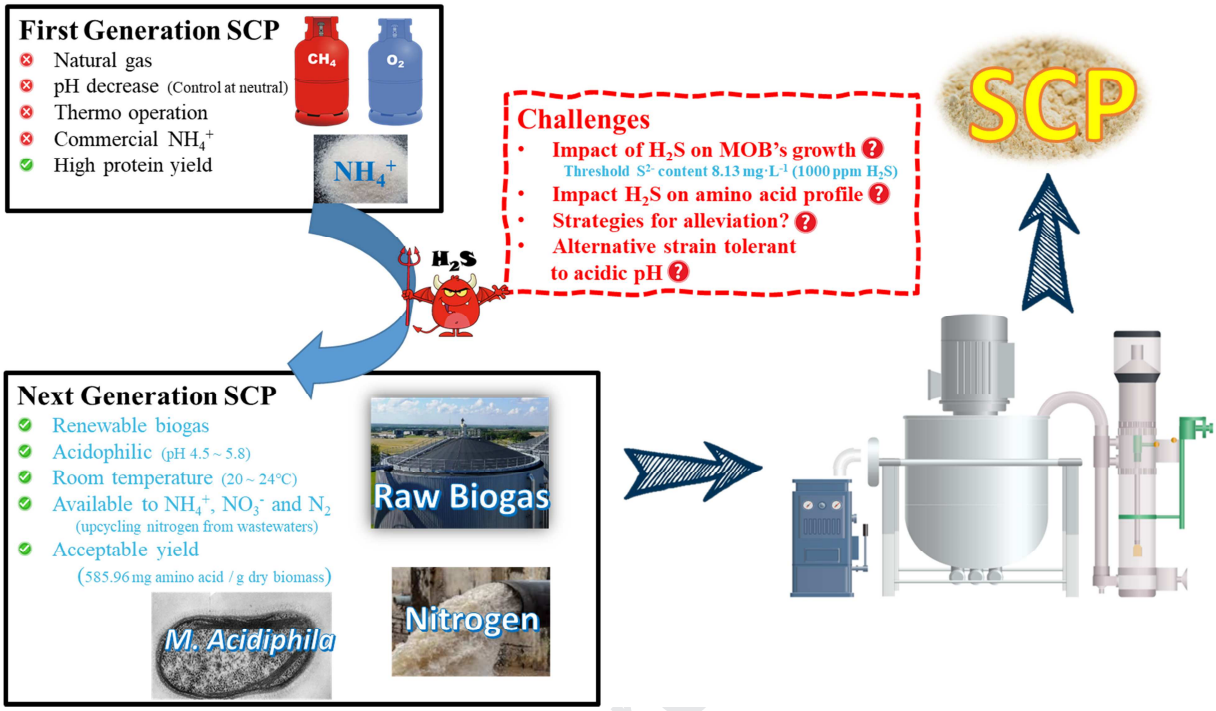
To appear in: *Water Research*

Received Date: 8 February 2020

Revised Date: 29 June 2020

Accepted Date: 30 June 2020

Please cite this article as: Xu, M., Zhou, H., Yang, X., Angelidaki, I., Zhang, Y., Sulfide restrains the growth of *Methylocapsa acidiphila* converting renewable biogas to single cell protein, *Water Research* (2020), doi: https://doi.org/10.1016/j.watres.2020.116138.



Sulfide restrains the growth of *Methylocapsa acidiphila* converting renewable biogas to single cell protein

Mingyi Xu[a], Huihui Zhou[a, b], Xiaoyong Yang[a], Irini Angelidaki[a], Yifeng Zhang[a,*]

a. Department of Environmental Engineering, Technical University of Denmark, DK-2800, Kongens Lyngby, Denmark

b. State Key Laboratory of Urban Water Resources and Environment, School of Environment, Harbin Institute of Technology, Harbin 150090, China

[*] **Corresponding author:**

Dr. Yifeng Zhang

Tel: (+45) 45251410; Fax: (+45) 45933850; E-mail address: yifz@env.dtu.dk; yifzmfc@gmail.com

Abstract

Methane-oxidizing bacteria (MOB) that can use biogas and recycled nitrogen from wastewater as a sustainable feedstock for single cell protein (SCP) synthesis are receiving increasing attention. Though promising, limited knowledge is available on the alternative strains especially the ones that can tolerant to strict environments such as acidic conditions. Furthermore, how would the hydrogen sulfide affect the MOB (especially the alternative strains) for SCP synthesis when crude biogas is used as feedstock is still unknown. In this study, the capability of an acidic-tolerant methanotrophic bacterium *Methylocapsa acidiphila* for SCP production using raw biogas and the associated inhibitory effect of sulfide on the bioconversion was for the first time investigated. Results showed that the inhibitory effect of sulfide on the growth of *M. acidiphila* was observed starting from 8.13 $mg \cdot L^{-1}$ Na_2S (equivalent to approximately 1000 ppm of H_2S in crude biogas). The total amino acid content in the dry biomass decreased more than two times due to sulfide inhibition compared with the control samples without the presence of sulfide (585.96 mg/g dry biomass), while the proportion of essential amino acids in the total amino acid was not affected when the concentration of Na_2S was lower than 5.73 $mg \cdot L^{-1}$. The performance of *M. acidiphila* in a sulfide-rich environment was further studied at different operational conditions. The feeding gas with a CH_4/O_2 ratio of 6:4 could help to alleviate the sulfide inhibition, compared with other ratios (4:6 and 8:2). Moreover, the sequential supply of the feed gas could also alleviate sulfide inhibition. In addition, the MOB's growth rate was higher when applying a higher mixing rate of 120 rpm, compared with 70 rpm and 0, due to a better gas-liquid mass transfer. The inoculum size of 20% and 10% resulted in a faster growth rate compared with the 5%. Furthermore, *M. acidiphila* could assimilate either NH_4^+ or NO_3^- as nitrogen source with a similar growth rate, giving it the potential to recycle nitrogen from a wide range of wastewaters.

The results will not only create new knowledge for better understanding the role of hydrogen sulfide in the assimilation of raw biogas by acid-tolerant *M. acidiphila* but also provide technical insights into the development of an efficient and robust process for the waste-to-protein conversion.

Keywords: Methane oxidizing bacteria; Single cell protein; Sulfide inhibition; Raw biogas; Amino acids; Nitrogen upcycling

1. Introduction

Increasing growth in the global population has put heavy pressure on our current food system (Godfray et al. 2010). The UN Food and Agriculture Organization (UNFAO) estimates that global food production should be doubled by 2050 to feed the population (Alexandratos 2009, Graham-Rowe 2011). Particularly, proteins are important and increasingly sought-after sources of feed and human nutrition (Boland et al. 2013). However, the conventional agricultural methods for protein production are not environmentally friendly because of the large arable land occupation, potential pesticide pollution, and greenhouse gas emission (Guerci et al. 2013, Tuomisto et al. 2012). Besides, overgrazing and over-farming may also lead to the destruction of grass and rainforest, and the loss of biodiversity (Abu Hammad and Tumeizi 2012). Therefore, it will be of great benefit if we can succeed in developing alternative protein sources or production technologies with a minimal footprint on climate, environment, and nature. In this context, single cell protein (SCP), which are dried cells of protein-rich microorganisms, has been considered as a promising protein source in the future (Ritala et al. 2017). Especially, the SCP derived from bacterial cells is the most feasible one, in light of its high protein production rate, moderate growth conditions, and high nutrient content (Chumpol et al. 2018, Matassa et al. 2016). Recently, methanotrophs, which are also known as aerobic methane-oxidizing bacteria (MOB), have been successfully commercialized for SCP production (Rasouli et al. 2018, Strong et al. 2015).

Currently, the main CH_4 source of MOB for industrial SCP production is still from natural gas (Hwang et al. 2018, Petersen et al. 2017). Thus, the renewable biogas from anaerobic digestion of organic wastes could be an alternative and renewable methane source of MOB for higher-

valuable SCP production (Strong et al. 2016). However, there are still several challenges in this process. For example, during the anaerobic digestion process, the organic wastes usually contain sulfate, which can be reduced into hydrogen sulfide by sulfate-reducing microorganisms along with the biogas generation (Angelidaki et al. 2018, Ge et al. 2014). The concentration of H_2S in crude biogas is usually 500 ~ 1000 ppm, but sometimes even as high as 5000 ppm (Cherosky and Li 2013). Biogas upgrading is normally required before injection into the gas grid to reduce the content of H_2S since it is both toxic and extremely corrosive. It could also be applied before using it as the feedstock of MOB for SCP production. However, the biogas upgrading process would significantly increase the overall cost of SCP production. Furthermore, the biogas after the H_2S cleaning unit can still contain H_2S (usually lower than 200 ppm H_2S) (Gasquet et al. 2020, Muñoz et al. 2015). Thus, using raw biogas as the feedstock for SCP production is more attractive from the economic and sustainability perspective. In this context, it is of utmost importance to study the effect of toxic compounds (especially H_2S) in the raw biogas on the growth of MOB for SCP production. The existence of sulfide may have a negative impact on bacterial activities by blocking cell respiration (Forte and Giuffrè 2016). To date, a systematic study of the impact of sulfide on the growth of MOB and biomass composition for the microbial protein production is still missing. Especially, it is still a key question to be answered how they would respond to the sulfide toxicity when raw biogas is used as a feedstock. Furthermore, most of the studies for SCP production from MOB were conducted with mixed cultures dominated by *Methylococcus capsulatus* (Jiang et al. 2016, Petersen et al. 2019), while alternative capable MOB strains that may adapt to different operating conditions (e.g., low pH) for SCP synthesis are rarely reported. In general, during the process of aerobic methane oxidation, the medium would turn to be acidic without pH control due to the generation of CO_2. It has been reported that

the growth of *M. capsulatus* was significantly suppressed when the pH of its medium was lower than 7.0 (Kolmert and Johnson 2001). In this context, *Methylocapsa acidiphila*, which prefers to grow at pH 4.5~5.8 at 20~24□ (Dedysh 2002), could be a promising alternative strain for SCP production under acidic conditions. It would greatly reduce the chemical costs for pH neutralization during SCP synthesis, compared to *M. capsulatus*. Thus, understanding the capability of *M. acidiphila* for SCP production from raw biogas, especially when using acidic streams as a medium, is of utmost importance and urgent.

In this study, the capability of a pure acidophilic strain *Methylocapsa acidiphila* for microbial protein synthesis and its response to sulfide toxicity were systematically investigated. The effect of different sulfide concentrations on protein production was first studied. Then the acclimatization competence of the strain against the sulfide inhibition was tested, followed by the analysis of system performance under different operational conditions in a sulfide-rich environment, including feed gas ratio, feeding frequency, mixing rate, inoculum size, and nitrogen source.

2. Materials and Methods

2.1. Strain, medium and substrates

Methylocapsa acidiphila DSM-13967 was purchased from the DSMZ (Leibniz Institut - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). *M. acidiphila* is an aerobic, Gram-negative, colorless bacterium, with curved coccoid morphotype, which possesses particulate methane monooxygenase (pMMO) and belongs to *Alphaproteobacteria* (Dedysh et al. 2002). It was grown on nitrate mineral salts medium DSMZ-Medium 922, including 100 $mg \cdot L^{-1}$ KNO_3, 100 $mg \cdot L^{-1}$ KH_2PO_4, 50 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 10 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2\ H_2O$, 5 $mg \cdot L^{-1}$ EDTA, 0.1 $mg \cdot L^{-1}$ $CuCl_2 \cdot 5\ H_2O$, 2 $mg \cdot L^{-1}$ $FeSO_4 \cdot 7\ H_2O$, 0.1 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7\ H_2O$, 0.02 $mg \cdot L^{-1}$

139 $NiCl_2 \cdot 6\ H_2O$, 0.2 mg·L^{-1} $CoCl_2 \cdot 6\ H_2O$ and 0.03 mg·L^{-1} Na_2MoO_4, without any organic nutrients.
140 Phosphate-buffered saline, with the final concentration of 0.33 g·L^{-1} $Na_2HPO_4 \cdot 12\ H_2O$ and 1.71
141 g·L^{-1} $NaH_2PO_4 \cdot 2\ H_2O$, was additionally added to the medium to maintain the pH during
142 experiments. The initial pH was adjusted at 5.7 ± 0.1 by adding phosphoric acid after autoclaving.
143 The methane content in the gas phase was maintained at 10% ~ 30% by adding a mixed gas of
144 60% CH_4 and 40% O_2 every 4 days, subject to the changes of conditions in each test run.

145 **2.2. Experimental setup**

146 **2.2.1. The effect of different sulfide concentrations on the SCP production**

147 The experiments were conducted in closed serum bottles in batch mode in triplicate. The total
148 volume of each bottle was 255 mL, with a working volume of 55 mL and a headspace of 200 mL.
149 All the reactors were flushed with N_2 gas to ensure equal starting conditions. Subsequently, 60
150 mL of the gas in the headspace was exchanged with the feed gas (60% CH_4 and 40% O_2). After
151 sterilization by autoclaving, eight concentrations of sulfide from 0 to 10.28 mg·L^{-1} (Group S-1 to
152 S-8, shown in Table 1) were prepared by adding sodium sulfide solution into the corresponding
153 vials. Afterward, all the reactors were inoculated (except for the Blank group) with an inoculum
154 size of 10%. The initial OD_{410} (optical density at the wavelength of 410 nm) after inoculation
155 was 0.12 ± 0.03. Thereafter the vials were placed in an incubator at 24 ± 1□. On day 4, 20 mL of
156 the same feed gas was additionally injected in all the reactors to maintain sufficient methane and
157 oxygen supply. The experimental period was 12 days.

158 **Table 1 is here**

159 **2.2.2. Acclimatization experiment**

160 To investigate whether the long lag phase caused by sulfide toxicity could be shortened after
161 acclimatization, three subsequent batches cultivations using five concentrations of sulfide

(referred to the sulfide concentration of Group S1 to S5) were conducted. For the acclimatization test, samples collected from the end (beginning of the stationary phase) of one batch were used as inocula for starting up new batches. The initial OD was controlled as 0.12 ± 0.03 for each batch. The else operational conditions were the same as 2.2.1.

2.2.3. The effect of different operation conditions on SCP production under sulfide inhibition

In this set of experiments, we focused on five strategies to alleviate sulfide inhibition. All the experiments were conducted in triplicate at 24 ± 1□. As the sulfide content in crude, not cleaned, biogas is around 500 ~ 800 ppm, a Na_2S concentration of 5.7 $mg \cdot L^{-1}$ (same as the value for Group S-5) was adopted in all experiments related to the five parameters. 1) The ratio of CH_4 and O_2 in the feed gas: three different CH_4/O_2 ratios of 4:6, 6:4, and 8:2 in the feed gases were individually applied to three groups. 60 mL of the feed gas was initially added in the bottles and an additional 20 mL of the corresponding feed gas was resupplied to all of the bottles on the 4^{th} day. 2) The feed gas resupply: three re-feeding approaches (i.e., no extra gas feeding, 20 mL every 2 days, 20 mL on the 4^{th} day) were tested to investigate whether gas feeding would influence sulfide inhibition. The gas-feeding period was eight days but the monitoring of the experiment lasted for twelve days. 3) Shaking speed: three shaking speeds of 0 rpm, 70 rpm, and 120 rpm were adopted to evaluate their impact on the MOB's growth and protein production under sulfide inhibition condition. The shaking radius was 15 mm. 4) Inoculum size: three ratios of 5%, 10%, and 20% were tested to consider the possibilities to counteract the sulfide inhibition by a better start-up related to inoculation. 5) Nitrogen source: ammonium and nitrate (13.85 mg $N \cdot L^{-1}$from chemical NH_4Cl and KNO_3, respectively) were used to study the impact of different nitrogen sources on SCP production under the sulfide inhibition.

2.2.4. Starvation experiments and the effect of the presence of CO_2 under sulfide inhibition

To better understand the individual effect of the two feed gases (CH_4 and O_2) on MOB's growth under sulfide inhibition and the corresponding starvation response, the starvation experiments were conducted with an insufficient initial feed gas applied and the additional CH_4 or O_2 successively added to the system. In addition, since real biogas from anaerobic digestion plants usually contains a certain amount of CO_2, synthetic biogas samples with different CO_2 contents (100% CH_4, 80% CH_4 and 20% CO_2, 60% CH_4 and 40% CO_2) were tested to study the influence of CO_2 in crude biogas. In this experiment, all the reactors were first purged with N_2 gas. Afterwards, 60 mL of the gas from the reactor was extracted and exchanged with 24 mL of pure O_2 (40% of the initial feed gas) and 36 mL of the synthetic biogas (60% of the initial feed gas). During the operation, 20 mL of an additional corresponding simulated biogas was added to the system on the 4^{th} day, followed by 20 mL of an additional pure O_2 being added on the 8^{th} day and 20 mL of an additional corresponding simulated biogas again on the 12^{th} day. A blank set without MOB inoculated (with the same feed gas content and gas supplement strategy as the group of 80% CH_4 and 20% CO_2) and a control group without feed gas supply were included. All the experimental groups were conducted in triplicate and started up with a sodium sulfide concentration of 5.7 $mg \cdot L^{-1}$.

2.3. Analytical methods and calculation

2.3.1. Sampling and analytical methods

Gas samples were collected from the headspace in each reactor every 4 days, and the content of CH_4, O_2, and CO_2 in the gas was analyzed by gas chromatography (GC-TRACE 1310, Thermo Scientific®) (Khoshnevisan et al. 2018). Meanwhile, liquid samples were taken every 2 days for optical density (OD), pH, and sulfide concentration measurement. The OD_{410} was determined by

UV-Visible spectrophotometer (Varian Cary® 50 Bio) at the wavelength of 410 nm. The concentration of S^{2-}-S was quantified by Methylene Blue Kit (HACH®), following the Method 8131 for 5 ~ 800 $\mu g \cdot L^{-1}$ S^{2-} described in HACH® manual, using a portable spectrophotometer (Model: DR3900, HACH Lange®) at a wavelength of 665 nm. The samples were measured immediately after sampling to prevent oxidation. At the end of each batch, the rest of the biomass samples were collected to measure the biomass yield and amino acid profile. Biomass yield was determined by the net weight of biomass powder from a certain volume of the liquid sample after the pretreatments. The pretreatments of samples included the sample concentration via centrifugation for 10 min at 4700 rpm, discard of the upper liquid then three times rinse of the biomass with distilled water, and freeze-drying. 5 mg of the dry biomass power was subsequently used for amino acid profile analysis. The samples were pretreated by microwave-assisted hydrolysis (3000 SOLV, Anton-Paar®) using 300 µL 6N HCl. The temperature in the hydrolysis vessel was raised to 130 □ at 5□·min^{-1} and held for 30 min. The vessels were flushed with Ar before hydrolysis. The samples were analyzed for individual amino acids by LC-MS-MS (1290 Infinity II 6470 QQQ, Agilent Technologies). Chromatographic separation was achieved on an InfinityLab Poroshell 120 HILIC-Z 100 mm × 2.1 mm, 2.7 µm (Agilent Technologies) column with a gradient of 20 mmol ammonium formate in water (Eluent A, pH 3) and 20 mmol ammonium formate in Acetonitrile (Eluent B, pH 3). The starting conditions were 100 % Eluent B with an increase of Eluent A to 30% over 10 minutes. The column flow was kept at 0.8 $mL \cdot min^{-1}$ and the column compartment at 30□. The MS-MS parameters were positive electrospray ionization, gas temperature 300 °C, gas flow 7.0 $L \cdot min^{-1}$, nebulizer 45 psi, sheath gas temperature 400 °C, sheath gas flow 11 $L \cdot min^{-1}$ with the CID and Fragmentor value optimized for each amino acid. The MS-MS was operated in dynamic MRM mode. The unit of

the amino acid amount was converted to $mg_{(amino\ acid)}/g_{(dry\ biomass)}$. The ultimate concentration of protein produced was quantified by summing up the masses of all amino acids. All the sampling and analysis were conducted in triplicate.

2.3.2. Determination of sulfide concentration

As hydrogen sulfide gas will easily be dissolved in water or evaporate from the reactor, inorganic sulfide salts are commonly used as H_2S equivalents for accurately quantifying it in experiments (Dan et al. 2020, Zhao et al. 2014). In this study, 8 concentrations of sodium sulfide solution were chosen as the initial sulfide level to simulate the invasion of H_2S gas from raw biogas. The concentration range of Na_2S was determined through the reverse inference of H_2S gas dissolution and dissolved H_2S dissociation equilibrium. The theoretical relationship between the hydrogen sulfide gas content in biogas $H_2S_{(Biogas)}$ and the dissolved sulfide ions parts from it such as $H_2S_{(aq.)}$, $HS^-_{(aq.)}$, $S^{2-}_{(aq.)}$ and the corresponding equivalent concentration of $Na_2S_{(aq.)}$ based on the dissolved sulfide were calculated (The calculation shows in **Appendices**) (Suleimenov and Krupp 1994, Suleimenov and Seward 1997, Sun et al. 2008). In this experiment, 8 different concentrations of sodium sulfide (assigned as S-1 to S-8, **Table 1)** were used to study the influence of sulfide on the growth of *M.acidiphila*. The concentrations studied covers the scenarios with 200 ~ 1400 ppm H_2S in crude biogas.

2.3.3. Growth performance and methane assimilation efficiency

The growth curve of *M. acidiphila* was made based on the change of OD_{410} over time. The maximum growth rate was derived from the slope of a semilogarithmic plot (the linear part) of the batch growth curve. The significant difference analysis was conducted by the software IBM® SPSS Statistics using the one-way ANOVA method with Post Hoc multiple comparisons of S-N-K (Levine 2013).

3. Results and discussions

3.1. Performance of MOB's growth for SCP synthesis under different sulfide concentrations

The effect of different sulfide concentrations on *M. acidiphila* for SCP production was studied. Results showed that a higher concentration of sulfide led to a progressively stronger inhibitory effect on the growth of *M. acidiphila* (**Fig. 1a**). Compared to the control group (Group S-1), all the groups with sulfide exhibited a lower growth rate in the initial six days. According to the ANOVA analysis, the biomass concentrations (represented by OD_{410}) between the group without sulfide and the groups with high sulfide concentrations during the exponential phase (2^{nd} ~ 6^{th} day) were of significant variation, where *p* values between Group S-1 and Group S-7 / Group S-8 were 0.047 and 0.033 respectively. Comparatively, the differences between Group S-1 and other groups were not significant (see **Table S1**). Thus, the inhibitory effect of Na_2S on the growth of *M. acidiphila* was 8.13 mg·L^{-1} (i.e.1000 ppm of H_2S in the biogas). The maximum growth rates (μ_{max}) of all the groups were calculated and summarized in **Table 1**. Results showed that the rates of the groups with sulfide were all obviously lower than the uninhibited group (Group S-1). In addition, the half-maximal inhibitory concentration was also taken into consideration. The relationship between the Ki ($\frac{OD_{with\ sulfide}}{OD_{without\ sulfide}} \times 100\%$) and the sulfide concentration was plotted and the curve was fitted in a Growth / Sigmoidal model. During the exponential phase (2^{nd} ~ 6^{th} day), the Logistic Fit estimated that when the Ki was 50%, the corresponding sulfide concentration would be 6.04 mg·L^{-1}. During the stationary phase (8^{th} ~ 12^{th} day), the corresponding sulfide concentration increased to 20.95 mg·L^{-1}.

Fig.1 is here

The change of CH_4 content in the headspace with time is shown in **Fig. 1b**. The same amount of additional feed gases were applied to all groups on the 4th day to ensure methane and oxygen amount for the bacteria growth were sufficient. The methane content in the gas phase showed that methane was consumed by the bacteria. The methane depletion rate in the vials without sulfide (Group S-1) was faster compared to all other groups. The total amount of methane consumption during the whole batch period was 21.6 mL. The methane consumption from Group S-7 and S-8 were obviously lower compared to other groups due to the inhibitory effect of sulfide on bacterial activity. The total methane consumption was only 13.7 mL in Group S-8.

The real-time concentrations of sulfide (converted from $\mu g \cdot L^{-1}$ S^{2-}-S to $mg \cdot L^{-1}$ Na_2S) in the medium were also measured. The results showed that the sulfide concentrations kept decreasing during the operation (**Fig. 1c)**. After the 8th day, the sulfide concentrations in all the groups dropped down to approximately 0 $mg \cdot L^{-1}$. As the detection method (USEPA Methylene Blue Method) measured all the dissolved sulfide forms including H_2S, HS^- and S^{2-}, the missing sulfide might be oxidized into sulfite or sulfate or assimilated into the cell. In response to the MOB's growth shown in **Fig. 1a**, on the 2nd day, the samples from Group S-2 to S-6 started to grow where the Na_2S concentrations were from 0.11 to 1.75 $mg \cdot L^{-1}$. Meanwhile, the sulfide concentrations in Group S-7 and S-8 were 3.38 $mg \cdot L^{-1}$ and 5.50 $mg \cdot L^{-1}$ respectively, which led to inhibition and caused the statistically significant difference in the MOB's growth compared with other groups. However, the sulfide concentrations were no longer higher than 1.26 $mg \cdot L^{-1}$ after the 6th day, which could be one of the reasons that the MOB's growth in Group S-7 and S-8 revived at a faster rate.

The amino acid profiles of the samples from Group S-1 to Group S-5 were summarized in **Fig. 1d**, and the detailed data were presented in **Table S2**. The samples without any sulfide (Group S-

1) showed the highest total amino acid content (58.60 ± 7.17% of dry biomass), while the presence of sulfide in the medium significantly reduced the amino acid content. The total amino acid content in Group S-2, S-3, S-4, and S-5 was only 39.74 ± 7.91%, 39.10 ± 4.83%, 38.94 ± 4.17%, and 27.69 ± 2.92%, respectively. As the final dry biomass content in these groups was quite similar, it shows that sulfide alters the biomass composition and thereby reducing protein content. The protein content decreased by more than 2 times if the sulfide concentration attained 5.73 $mg \cdot L^{-1}$. Forming agglomerates could be one of the reasons for biomass content alteration, which is a very common phenomenon when growing MOBs in a strict environment, i.e. low nitrogen source, high oxygen content, etc. Under such circumstances, higher content of extracellular polysaccharide matrix might be produced to protect the cells (Dedysh et al. 2002, Linton et al. 1986, Wei et al. 2015, Wilshusen et al. 2004). Thus, *M. acidiphila* was able to tolerate a certain level of sulfide for amino acid synthesis, which implies the threshold of sulfide concentration during the bioconversion.

The amino acid composition of the biomass from *M. acidiphila* was abundant and balanced, covering a wide range of essential amino acids. Comparatively, Glutamine/Glutamic acid and Asparagine/Aspartic acid were higher than other amino acids, which accorded with other MOBs, i.e. *Methylococcus capsulatus* (Rasouli et al. 2018, Skrede et al. 2009). In terms of the essential amino acids, *M. acidiphila* produced higher content of Leucine and Valine (41.82 ± 5.60 mg/g dry biomass and 38.04 ± 5.59 mg/g dry biomass, respectively), compared to *Methylococcus capsulatus* (39.5 $mg/g_{(dry\ biomass)}$ and 28 $mg/g_{(dry\ biomass)}$, respectively) (Rasouli et al. 2018). Leucine is one of the three branched-chain amino acids, which is beneficial for protein synthesis, muscle repair, blood sugar levels regulation, and it also helps in healing wounds (Kato et al. 2016). Valine is another branched-chain amino acids, which plays a major role in stimulating the

322 growth of muscle mass, increasing the synthesis rate of human protein, and producing energy
323 (Jackman et al. 2017) . The amino acid composition was not significantly altered by sulfide
324 inhibition. Interestingly, though the production of total protein was inhibited under the high
325 sulfide environment, the proportion of essential amino acids in total amino acids was slightly
326 higher than that in the control experiment. The proportion of essential amino acids in total amino
327 acids in Group S-1 to Group S-5 was 33.65%, 33.10%, 33.73%, 34.32%, and 37.69%
328 respectively. It could be due to that the sulfide in the medium contributed to the synthesis of
329 Cysteine and Methionine (Ferla and Patrick 2014, Grundy and Henkin 1998).
330 In general, the results indicated that the quality of protein produced, referred to the amino acid
331 composition and the proportion of essential amino acids, was not affected when the sulfide
332 concentration was lower than 5.73 $mg \cdot L^{-1}$. However, the amount of protein was significantly
333 reduced when sulfide was over this threshold concentration.

334 **3.2. Performance of acclimatization competence**

335 As shown in **Fig. 2**, the bacterium did not exhibit a higher final yield after the acclimatization.
336 The sulfide still restrained the growth of the culture in the groups with sulfide (S-2 to S-5) from
337 obtaining a maximum OD as high as the control group (Group S-1). For example, the maximum
338 OD of Group S-5 in batch 1, 2, and 3 were 1.09, 1.01, and 0.93, respectively, while the results of
339 Group S-1 were 1.21, 1.11, and 1.19. The growth gaps between the sulfide groups and the
340 control group in the first 6 days, resulting from the adaptation to the sulfide environment, still
341 existed in all batches. For example, on the 4th day in each batch, the value of OD_{S-5}/OD_{S-1} was
342 37.1%, 46.5%, and 56.4% respectively. However, it can also be noticed that the gaps were
343 slightly narrowed in batch 2 and batch 3 compared with batch 1, which could be confirmed by
344 the increase of the OD_{S-5}/OD_{S-1} value on the 4th day in the sequential batches. The maximum

345 growth rates in the three batches (in **Table 2**) also showed an increasing trend as the batches
346 went. For example, the maximum growth rates of Group S-5 slightly increased from 0.41 d^{-1} in
347 batch 1 to 0.48 d^{-1}.

348 Therefore, the short-term acclimatization process, to some extent, can assist the bacterium to
349 adapt to a strict environment but it cannot generally eliminate the negative effect of sulfide on
350 the growth of *M. acidiphila*. The reason why *M. acidiphila* failed to adapt to or metabolize
351 sulfide might be due to lacking related enzymes or genes. The key enzymes for sulfide oxidation
352 are Sulfide:quinone oxidoreductase (SQOR) or Flavocytochrome c sulfide dehydrogenase
353 (FCSD), neither of which were found in *M. acidiphila* according to the NCBI Protein Table.

354 **Fig.2 and Table 2 are here**

355 **3.3. Performance of MOB's growth with different operational conditions under sulfide**
356 **inhibition**

357 **3.3.1. Different ratios of CH_4 and O_2**

358 In the actual condition, the sulfide concentration in the raw biogas may vary during the time.
359 Thus, for practical application, it is necessary to control the up limits concentration of H_2S in the
360 reactor for MOB growth. To maintain a constant H_2S concentration (e.g., the threshold
361 concentration starting inhibition) in the reactor producing SCP, the CH_4 and O_2 ratio would
362 subject to change upon the original H_2S concentration in the raw biogas. In this context, it is
363 important to investigate how the different CH_4 and O_2 ratios at a constant H_2S concentration
364 would affect the MOB growth for SCP production. Thus, the effect of three different ratios of
365 CH_4/O_2 in the headspace on the SCP production under sulfide inhibition was further investigated.
366 Group FR-$1_{\text{-}4:6}$ (CH_4/O_2 of 4:6) was assumed as at the optimum ratio considering that the
367 reported preferable O_2 content in the headspace was usually 1.45 to 2 times higher than the CH_4

content when using MOBs to produce SCP, as well as the possible O_2 loss due to the oxidation of sulfide (Khoshnevisan et al. 2019). However, the results from **Fig.3a** showed that the final OD (0.93 ± 0.02) from this group was lower than the OD (1.10 ± 0.01) from Group FR-2$_{-6:4}$ (CH_4/O_2 of 6:4). The reason could be the insufficiency of CH_4. According to **Fig. 4a** and **Fig. 4b**, the methane content in Group FR-1$_{-4:6}$ was lower than 11.5% after adding the supplement gas on the 4th day, and the final concentration was lower than 5.5%, while it was 14.2% on the 4th day and 7.0% at end of batch in Group FR-2$_{-6:4}$. 10% ~ 30% was reported as the appropriate CH_4 content in the headspace to grow *M. acidiphila* (Dedysh et al. 2001, Ricke et al. 2005). This amount was consistent with the conventional MOB studies using other strains and natural gas where methane is the key limiting factor (Reitner and Thiel 2011, Roslev and King 1995). Especially, it was also reported that type □ methanotrophs (*Alphaproteobacteria*) could be more dominant under higher CH_4 concentration than type I methanotrophs (Amaral and Knowles 1995). Therefore, given the biomass production performance of Group FR-1$_{-4:6}$ after the 6th day, we could conclude that methane content below 10% may lead to a negative effect on the growth of MOB under the sulfide inhibition. When a higher content of CH_4 (Group FR-3$_{-8:2}$, CH_4/O_2 ratio = 8:2) was applied, the bacterium grew well concerning growth rates in the first 6 days, which was similar to Group FR-2$_{-6:4}$. However, after the 6th day, the MOB's growth stopped increasing and it reached a lower level compared to the other applied ratios with a final OD of only 0.73 ± 0.01. From **Fig. 4a** and **Fig. 4b**, it was noticed that the MOB's growth was not interfered with when the initial methane content was over 19%. However, the oxygen content in this group was lower than the others during the whole batch. The final O_2 content in the headspace was only 1.9 ± 0.8%. It was reported that 4%~12% of O_2 content should be necessary for type □ methanotrophs to accumulate biomass (Bewersdorff and Dostálek 1971, Rostkowski et al. 2013). Therefore, the

low O_2 availability (lower than 4%) could be the reason for Group FR-3$_{-8:2}$ achieving relatively lower OD at the end.

The consumption rates of methane (r_{CH_4}) and oxygen(r_{O_2}) were calculated individually and shown in **Table 3**. The period of growth was divided into two stages (before or after adding supplement gases). In Group FR-2$_{-6:4}$, the stoichiometric relationship between r_{CH_4} and r_{O_2} was approximately 1:1, which indicated that this gas ratio should be the optimum for growing MOB with the presence of sulfide. In Group FR-1$_{-4:6}$, r_{O_2} was relatively higher than r_{CH_4} due to its higher solubility in the liquid phase resulting from the high O_2 partial pressure. In Group FR-3$_{-8:2}$, r_{CH_4} was high in stage I, which contributed to the higher growth rate, while it was low in stage II due to lack of O_2.

Fig.3, Fig.4 and Table 3 are here

3.3.2. Different frequencies of gas-supplement.

The effect of frequencies of resupplying the gas-feed, on the SCP production in the sulfide-rich environment was also investigated. During the tests, 20 mL of the feed gas (CH_4/O_2 ratio of 6:4) was re-injected in the vials three times every second day (Group FF-1$_{-3\ times}$), once on the 4th day (Group FF-2$_{-1\ time}$), or none (Group FF-3$_{-none}$). As shown in **Fig. 3b**, better performance was achieved in Group FF-2$_{-1\ time}$ over other groups. In Group FF-3$_{-none}$, the MOB's growth ceased after the 8th day, with a final OD of 0.74, which could be due to the starvation phenomenon as a result of the lack of feed gas. As shown in **Fig. 4c**, the CH_4 was consumed during the time and the final content was as low as 6.3%. Interestingly, a higher frequency of gas supplement did not assist the growth of the bacterium. The final OD of Group FF-1$_{-3\ times}$ was 1.04 with a large deviation. In **Fig. 4d**, the oxygen content was higher than that of the other groups with a maximum content of 13.1%. Assuming that high CH_4 content will not have a negative effect on

414 the growth of MOB, the oxidative stress in cells as a result of the excessive O_2, therefore, could
415 be the reason for the deterioration of the MOB growth performance (Baez and Shiloach 2014).
416 Excessive aeration was also reported that toxically affected the growth of MOBs (Chu and
417 Alvarez-Cohen 1999). In addition, it was also noticed that both r_{CH_4} and r_{O_2} of Group FF-1$_{\text{-3 times}}$
418 were much higher than that of the other two groups (**Table 3**). However, the high consumption
419 rate did not contribute to the MOB biomass accumulation. It could be due to that there were
420 additional limiting factors than CH_4 and O_2 (i.e. lack of specific micronutrients).

421 **3.3.3. Different shaking speeds**

422 As shown in **Fig. 3c**, it was observed that the increased shaking speed obviously facilitated the
423 MOB's growth for SCP production. In the condition without shaking (Group SS-1), the final OD
424 of the samples only reached to 0.62. In addition, there was a large deviation in the MOB's
425 growth for the triplicate experiments under this condition, which was probably because MOB
426 can easily aggregate into clusters during the cultivation, especially under the environmental
427 stresses such as sulfide inhibition in this study. This would seriously hamper the cellular
428 assimilation of methane and further inhibit the growth of the bacterium. Besides, the gas-liquid
429 mass transfer capacity can be significantly improved by increasing the shaking frequency, which
430 would permit better contact of the gases and the bacterium (Maier and Büchs 2001). Therefore,
431 the MOB's growth increased with the increasing of shaking speed.

432 **3.3.4. Different inoculum sizes**

433 The MOB's growth was further studied with different inoculum sizes, which could be one of the
434 key parameters for the start-up of the system under severe conditions (i.e. high sulfide
435 concentration environment). **Fig. 3d** shows that the inoculum size higher than 5% increased the
436 MOB's growth rates, while a further increase of the inoculum addition neither had an impact on

the growth rates nor the final achieved OD. All inoculum sizes tested ended with the same final MOB's growth as indicated by the same level of OD. Thus, considering the economic benefit, an inoculum size of 10% could be selected for the following experiments.

3.3.5. Different nitrogen sources

The nitrogen sources for the MOB can be ammonium, nitrate, and even nitrogen gas. The MOBs may follow different metabolic pathways with varied nitrogen sources. **Fig. 3e** shows the growth curve of *M. acidiphila* fed with ammonium or nitrate with an equivalent amount of nitrogen. The results showed that there was no significant difference between ammonium and nitrate for *M. acidiphila* growth. Thus, *M. acidiphila* can assimilate both nitrate and ammonia at a similar consumption rate, regardless of the sulfide influence.

3.4. Impact of intermittence gas supply and the presence of CO_2 on *M. acidiphila* growth

Fig. 5a shows the performance of the MOB's growth under the starvation condition in the sulfide-rich environment. All the groups exhibited a diauxic growth curve with limited feed gas supply. On the 4^{th} day, 20 mL of the simulated biogas was added to all the groups, but the performance of the MOB's growth was not improved. However, after adding 20 mL of pure O_2 on the 8^{th} day, the MOB started growing immediately and the OD increased from 0.3 to 0.8 in 2 days. This indicated that the limited initial O_2 amount deteriorated the system performance under sulfide inhibition as it could be potentially consumed by sulfide. Therefore, when starting up the system under the sulfide-rich environment, a sufficient amount of O_2 would be of higher importance than CH_4 due to the competitive relationship between the reactions of sulfide oxidation and bacterial metabolism. The results of O_2 content in the headspace (**Fig. 5c**) supported this conclusion. After the 10^{th} day, the MOB stopped growing again but it revived after adding 20 mL of the simulated biogas on the 12^{th} day. The OD increased from 0.8 to 1.2 in

2 days then turned to be stable. This indicated that the main limiting factor changed from O_2 to CH_4 during the exponential phase of MOB growth, as the consumption of CH_4 could be much faster due to its roles as both carbon source and energy source. The change of CH_4 content in the headspace (**Fig. 5b**) also supported this conclusion that this second restriction was due to the lack of CH_4. Efficient and safe O_2 and CH_4 supply appears a bottleneck for high-rate methanotrophs cultivation, which can limit protein production and nutritional quality. The ratio of CH_4 and O_2 may also affect the flammability of the mixture gas and lead to the safety issue. Diffusion via hydrophobic hollow fibers membranes will allow efficient and safe gas supply, solubilizing them in the liquid phase without compromising safety issues (Valverde Pérez et al. 2020).

Fig.5 is here

Fig.5a also shows the performance of the MOB's growth with different ratios of CO_2 added in the feed gas. The results indicated that the presence of CO_2 would not impede the growth of the MOB. The performance of the group with 50% CH_4 and 50% CO_2 was slightly worse than the other two groups. The reason for that was probably due to the relatively lower content of CH_4 in the headspace.

3.5. Significance and perspectives

This study for the first time demonstrates the potential of acid-tolerant *M. acidophila* for the conversion of sulfide-rich raw biogas to SCP production, which integrated the strengths of renewable energy and aerobic methane oxidation-based biosynthesis. The results revealed the impact of H_2S and other associated operating conditions on the growth and protein production of *M. acidophila* and thereby filling the gap between fundamental science and applied research. All these together could provide solid fundamental ground for the further integration of anaerobic digestion, nitrogen recovery and recycling from wastewater, and aerobic methane oxidation for

food and animal feed production. The comparison of total amino acid production from *M. acidophila* with other methanotrophic bacteria was summarized in **Table 4**. *M. acidophila* with 58.6% of amino acids in the dry mass could be an acceptable alternative SCP producer, considering the results were obtained under unoptimized conditions (e.g., limited nitrogen supply) in batch mode. This yield was even slightly higher than the frequently used *Methylococcus capsulatus* when it was cultivated as a pure culture) (Rasouli et al. 2018). Besides, the amino acid content achieved by *M. acidophila* was also comparable to other commercial bacterial protein products (Øverland et al. 2010, Schøyen et al. 2005, Skrede et al. 1998, Skrede et al. 2009). The amino acid content in some common protein-rich Food was also summarized in **Table 4** according to FAO Food Policy and Food Science Service Nutrition Division (1970). With a higher and more balanced amino acid yield, *M. acidophila* is competent and feasible to be used as a protein source for livestock and aquaculture instead of soybean or fish meal.

Table 4 is here.

The successful SCP production by the new strain *M. acidophila* will also bring additional benefits to different fields. Firstly, the acidic wastewaters such as the fermentation leachate and broth from raw food waste (pH as low as 4~5) can be sustainable options as medium and nitrogen source (Kolmert and Johnson 2001). In this case, the costly synthetic medium and the pH neutralization towards the problem of pH decrease resulted from the generation of CO_2 could no longer be necessary. Secondly, as *M. acidophila* can use different types of nitrogen, the used nitrogen from a wide range of wastewaters could be first recovered and then reused as a nitrogen source of MOB for SCP production. Thus, wastewater could be a very important nitrogen source for the growth of methanotrophs and the production of SCP. The advances in MOB based SCP production will add value to the wastewater treatment industry. Thirdly, the MOB fermentation

could be integrated with microbial electrosynthesis systems (MES) to achieve power-to-protein conversion. Furthermore, it has been reported that *M. acidiphila* is capable of fixing the gaseous dinitrogen, which offers it more potentials in practical application (DeLong et al. 2014).

Though promising, there are still several aspects that need to be investigated in the future. Firstly, an efficient and cost-effective approach needs to be developed to mitigate the sulfide inhibition on MOB for SCP production. The bioaugmentation of sulfide-oxidizing bacteria (SOB) has been reported as an efficient way to lower the H_2S concentration in biogas reactors (Marín and Arahal 2014). It may help to mitigate the risk of sulfide to MOB when they grow together in the mixed culture system. Thus, the synergistic interactions between MOB and SOB should be studied in detail in the future. Besides, the process should be further optimized to improve biomass production. The biomass concentration of *M. acidiphila* observed during the sulfide toxicity experiment (65.0 ~ 91.4 $mg \cdot L^{-1}$, Figure 1) was comparable to that reported by other lab-scale studies using the commercialized strain *M. capsulatus* or other mixed cultures (Rasouli et al. 2018, Valverde Pérez et al. 2020). The biomass samples were collected at the end of each batch. The strain without sulfide inhibition could obtain a higher yield on the 5^{th} ~ 6^{th} day than the 12^{th} day because part of the biomass would be degraded during the bacterial death phase. The current biomass concentration could also be limited by the nitrogen concentration adopted for the tests. All these limitations could be addressed in future work to increase biomass and protein production. In general, biomass concentration obtained in the lab-scale is always several orders lower than that in the large-scale operation. For instance, *M. capsulatus* can just produce 263 $mg \cdot L^{-1}$ biomass in the lab, while the yield from Norferm's SCP production facility at Tjeldbergodden, Norway was reported as high as 20 $g \cdot L^{-1}$ (Olsen et al. 2010). As the protein portion in biomass and biomass yield on methane (or nitrogen) were also comparable to the

widely studied MOB cultures, *M. acidiphila* could also have the applicability and economic viability if the operational conditions (e.g., feedstocks concentrations, continuous mode, gas-liquid mass transfer) are optimized. Furthermore, the downstream process for SCP harvesting is a costly step, which applies to all the conventional and emerging microbial protein technologies. One potential solution is to grow live feeds for fish or crustacean larvae in the culture suspension, which could simplify and reduce the costs for SCP harvesting.

4. Conclusion

This study systematically elucidated factors affecting the growth of *M. acidiphila* for microbial protein production in a sulfide-rich environment. It was found here that 8.13 $mg \cdot L^{-1}$ Na_2S which is equivalent to approximately 1000 ppm of H_2S in crude biogas was the threshold concentration over which inhibition of cell growth and protein synthesis was observed. Besides, the amino acids produced by *M. acidiphila* were significantly influenced by sulfide. The total amino acid content in the dry biomass decreased more than two times with sulfide inhibition compared with the control samples without the presence of sulfide, while the ratio of essential amino acids was not affected when the concentration of Na_2S was lower than 5.73 $mg \cdot L^{-1}$. Furthermore, cell growth was affected by the CH_4/O_2 ratio, gas supplement frequency, mixing rate, and inoculum size. In addition, *M. acidiphila* can assimilate both NH_4^+ and NO_3^- under the sulfide-rich environment with a similar growth rate. The presence of CO_2 in the feed gas did not significantly influence the MOB's growth if the amount of CH_4 and O_2 were sufficient. This study could provide solid fundamental ground for the further integration of anaerobic digestion, nitrogen recovery and recycling from wastewater, and aerobic methane oxidation for food and animal feed production.

Acknowledgment

This research was sponsored by the Novo Nordisk Foundation (NNF16OC0021568). The authors would like to acknowledge the financial support from the China Scholarship Council and the Otto Mønsted Fond. The authors would like to thank Hector Gracia and Sinh Hy Nguyen for the experimental assistance. The authors would also appreciate the technical assistance by Ru Shang from Hach Company.

Reference

Abu Hammad, A. and Tumeizi, A. (2012) Land degradation: Socioeconomic and environmental causes and consequences in the eastern mediterranean. Land Degradation and Development 23(3), 216-226.

Alexandratos, N. (2009) World food and agriculture to 2030/50, Highlights and views from mid-2009. Expert Meeting on How to feed the World in 2050. http://www.fao.org/wsfs/forum2050/wsfs-background-documents/wsfs-expert-papers/en/.

Amaral, J.A. and Knowles, R. (1995) Growth of methanotrophs in methane and oxygen counter gradients. FEMS Microbiology Letters 126(3), 215-220.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H. and Kougias, P.G. (2018) Biogas upgrading and utilization: Current status and perspectives. Biotechnology Advances 36(2), 452-466.

Baez, A. and Shiloach, J. (2014) Effect of elevated oxygen concentration on bacteria, yeasts, and cells propagated for production of biological compounds. Microbial Cell Factories 13(1), 181.

Bewersdorff, M. and Dostálek, M. (1971) The use of methane for production of bacterial protein. Biotechnology and Bioengineering 13(1), 49-62.

574 Boland, M.J., Rae, A.N., Vereijken, J.M., Meuwissen, M.P.M., Fischer, A.R.H., van Boekel,
575 M.A.J.S., Rutherfurd, S.M., Gruppen, H., Moughan, P.J. and Hendriks, W.H. (2013) The
576 future supply of animal-derived protein for human consumption. Trends in Food Science &
577 Technology 29(1), 62-73.
578 Cherosky, P. and Li, Y. (2013) Hydrogen sulfide removal from biogas by bio-based iron sponge.
579 Biosystems Engineering 114(1), 55-59.
580 Chu, K.-H. and Alvarez-Cohen, L. (1999) Evaluation of Toxic Effects of Aeration and
581 Trichloroethylene Oxidation on Methanotrophic Bacteria Grown with Different Nitrogen
582 Sources. Applied and Environmental Microbiology 65(2), 766-772.
583 Chumpol, S., Kantachote, D., Nitoda, T. and Kanzaki, H. (2018) Administration of purple
584 nonsulfur bacteria as single cell protein by mixing with shrimp feed to enhance growth,
585 immune response and survival in white shrimp (Litopenaeus vannamei) cultivation.
586 Aquaculture 489, 85-95.
587 Dan, M., Yu, S., Li, Y., Wei, S., Xiang, J. and Zhou, Y. (2020) Hydrogen sulfide conversion:
588 How to capture hydrogen and sulfur by photocatalysis. Journal of Photochemistry and
589 Photobiology C: Photochemistry Reviews 42, 100339.
590 Dedysh, S.N. (2002) Methanotrophic Bacteria of Acidic Sphagnum Peat Bogs. Microbiology
591 71(6), 638-650.
592 Dedysh, S.N., Horz, H.-P., Dunfield, P.F. and Liesack, W. (2001) A novel pmoA lineage
593 represented by the acidophilic methanotrophic bacterium Methylocapsa acidophila B2.
594 Archives of Microbiology 177(1), 117-121.
595 Dedysh, S.N., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Liesack, W. and
596 Tiedje, J.M. (2002) Methylocapsa acidiphila gen. nov., sp. nov., a novel methane-oxidizing

and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. International Journal of Systematic and Evolutionary Microbiology 52(1), 251-261.

DeLong, E.F., Lory, S., Stackebrandt, E. and Thompson, F. (2014) The prokaryotes: alphaproteobacteria and betaproteobacteria, Springer Berlin Heidelberg.

FAO Food Policy and Food Science Service Nutrition Division (1970) Amino-acid Content of Foods and Biological Data on Proteins, Food and agriculture organization of the United Nations.

Ferla, M.P. and Patrick, W.M. (2014) Bacterial methionine biosynthesis. Microbiology 160(8), 1571-1584.

Forte, E. and Giuffrè, A. (2016) How bacteria breathe in hydrogen sulfide-rich environments. Biochemist 38(5), 8-11.

Gasquet, V., Kim, B., Sigot, L. and Benbelkacem, H. (2020) H2S Adsorption from Biogas with Thermal Treatment Residues. Waste and Biomass Valorization, 1-11.

Ge, X., Yang, L., Sheets, J.P., Yu, Z. and Li, Y. (2014) Biological conversion of methane to liquid fuels: Status and opportunities. Biotechnology Advances 32(8), 1460-1475.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. and Toulmin, C. (2010) Food Security: The Challenge of Feeding 9 Billion People. Science 327(5967), 812-818.

Graham-Rowe, D. (2011) Agriculture: Beyond food versus fuel. Nature 474(7352), S6-S8.

Grundy, F.J. and Henkin, T.M. (1998) The S box regulon: a new global transcription termination control system for methionine and cysteine biosynthesis genes in Gram-positive bacteria. Molecular Microbiology 30(4), 737-749.

Guerci, M., Knudsen, M.T., Bava, L., Zucali, M., Schönbach, P. and Kristensen, T. (2013) Parameters affecting the environmental impact of a range of dairy farming systems in Denmark, Germany and Italy. Journal of Cleaner Production 54, 133-141.

Hwang, I.Y., Nguyen, A.D., Nguyen, T.T., Nguyen, L.T., Lee, O.K. and Lee, E.Y. (2018) Biological conversion of methane to chemicals and fuels: technical challenges and issues. Applied Microbiology and Biotechnology 102(7), 3071-3080.

Jackman, S.R., Witard, O.C., Philp, A., Wallis, G.A., Baar, K. and Tipton, K.D. (2017) Branched-Chain Amino Acid Ingestion Stimulates Muscle Myofibrillar Protein Synthesis following Resistance Exercise in Humans. Frontiers in Physiology 8(390).

Jiang, H., Duan, C., Jiang, P., Liu, M., Luo, M. and Xing, X.-H. (2016) Characteristics of scale-up fermentation of mixed methane-oxidizing bacteria. Biochemical Engineering Journal 109, 112-117.

Kato, H., Miura, K., Nakano, S., Suzuki, K., Bannai, M. and Inoue, Y. (2016) Leucine-enriched essential amino acids attenuate inflammation in rat muscle and enhance muscle repair after eccentric contraction. Amino Acids 48(9), 2145-2155.

Khoshnevisan, B., Tsapekos, P., Alvarado-Morales, M. and Angelidaki, I. (2018) Process performance and modelling of anaerobic digestion using source-sorted organic household waste. Bioresource Technology 247, 486-495.

Khoshnevisan, B., Tsapekos, P., Zhang, Y., Valverde-Pérez, B. and Angelidaki, I. (2019) Urban biowaste valorization by coupling anaerobic digestion and single cell protein production. Bioresource Technology 290, 121743.

640 Kolmert, Å. and Johnson, D.B. (2001) Remediation of acidic waste waters using immobilised,
641 acidophilic sulfate-reducing bacteria. Journal of Chemical Technology & Biotechnology
642 76(8), 836-843.
643 Levine, G. (2013) A guide to SPSS for analysis of variance, Psychology Press.
644 Linton, J.D., Watts, P.D., Austin, R.M., Haugh, D.E. and Niekus, H.G.D. (1986) The Energetics
645 and Kinetics of Extracellular Polysaccharide Production From Methanol by Micro-
646 organisms Possessing Different Pathways of C1 Assimilation. Microbiology 132(3), 779-
647 788.
648 Maier, U. and Büchs, J. (2001) Characterisation of the gas–liquid mass transfer in shaking
649 bioreactors. Biochemical Engineering Journal 7(2), 99-106.
650 Marín, I. and Arahal, D.R. (2014) The family beijerinckiaceae. The prokaryotes, 115-133.
651 Matassa, S., Boon, N., Pikaar, I. and Verstraete, W. (2016) Microbial protein: future sustainable
652 food supply route with low environmental footprint. Microbial Biotechnology 9(5), 568-575.
653 Muñoz, R., Meier, L., Diaz, I. and Jeison, D. (2015) A review on the state-of-the-art of
654 physical/chemical and biological technologies for biogas upgrading. Reviews in
655 Environmental Science and Bio/Technology 14(4), 727-759.
656 Olsen, D.F., Jørgensen, J.B., Villadsen, J. and Jørgensen, S.B. (2010) Modeling and Simulation
657 of Single Cell Protein Production. IFAC Proceedings Volumes 43(6), 502-507.
658 Øverland, M., Tauson, A.-H., Shearer, K. and Skrede, A. (2010) Evaluation of methane-utilising
659 bacteria products as feed ingredients for monogastric animals. Archives of Animal Nutrition
660 64(3), 171-189.

661 Petersen, L.A., Villadsen, J., Jørgensen, S.B. and Gernaey, K.V. (2017) Mixing and mass
662 transfer in a pilot scale U‐loop bioreactor. Biotechnology and Bioengineering 114(2), 344-
663 354.
664 Petersen, L.A.H., Lieven, C., Nandy, S.K., Villadsen, J., Jørgensen, S.B., Christensen, I. and
665 Gernaey, K.V. (2019) Dynamic investigation and modeling of the nitrogen cometabolism in
666 Methylococcus capsulatus (Bath). Biotechnology and Bioengineering 116(11), 2884-2895.
667 Rasouli, Z., Valverde-Pérez, B., D'Este, M., De Francisci, D. and Angelidaki, I. (2018) Nutrient
668 recovery from industrial wastewater as single cell protein by a co-culture of green
669 microalgae and methanotrophs. Biochemical Engineering Journal 134, 129-135.
670 Reitner, J. and Thiel, V. (2011) Encyclopedia of geobiology, Springer Amsterdam.
671 Ricke, P., Kube, M., Nakagawa, S., Erkel, C., Reinhardt, R. and Liesack, W. (2005) First
672 Genome Data from Uncultured Upland Soil Cluster Alpha Methanotrophs Provide Further
673 Evidence for a Close Phylogenetic Relationship to Methylocapsa acidiphila B2 and for
674 High-Affinity Methanotrophy Involving Particulate Methane Monooxygenase. Applied and
675 Environmental Microbiology 71(11), 7472-7482.
676 Ritala, A., Häkkinen, S.T., Toivari, M. and Wiebe, M.G. (2017) Single Cell Protein—State-of-
677 the-Art, Industrial Landscape and Patents 2001–2016. Frontiers in Microbiology 8(2009).
678 Roslev, P. and King, G.M. (1995) Aerobic and anaerobic starvation metabolism in
679 methanotrophic bacteria. Applied and Environmental Microbiology 61(4), 1563-1570.
680 Rostkowski, K.H., Pfluger, A.R. and Criddle, C.S. (2013) Stoichiometry and kinetics of the
681 PHB-producing Type II methanotrophs Methylosinus trichosporium OB3b and
682 Methylocystis parvus OBBP. Bioresource Technology 132, 71-77.

683 Schøyen, H.F., Frøyland, J.R.K., Sahlström, S., Knutsen, S.H. and Skrede, A. (2005) Effects of
684 autolysis and hydrolysis of bacterial protein meal grown on natural gas on chemical
685 characterization and amino acid digestibility. Aquaculture 248(1), 27-33.
686 Skrede, A., Berge, G.M., Storebakken, T., Herstad, O., Aarstad, K.G. and Sundstøl, F. (1998)
687 Digestibility of bacterial protein grown on natural gas in mink, pigs, chicken and Atlantic
688 salmon. Animal Feed Science and Technology 76(1), 103-116.
689 Skrede, A., Mydland, L. and Øverland, M. (2009) Effects of growth substrate and partial
690 removal of nucleic acids in the production of bacterial protein meal on amino acid profile
691 and digestibility in mink. Journal of Animal and Feed Sciences 18(4).
692 Strong, P.J., Kalyuzhnaya, M., Silverman, J. and Clarke, W.P. (2016) A methanotroph-based
693 biorefinery: Potential scenarios for generating multiple products from a single fermentation.
694 Bioresource Technology 215, 314-323.
695 Strong, P.J., Xie, S. and Clarke, W.P. (2015) Methane as a Resource: Can the Methanotrophs
696 Add Value? Environmental Science & Technology 49(7), 4001-4018.
697 Suleimenov, O.M. and Krupp, R.E. (1994) Solubility of hydrogen sulfide in pure water and in
698 NaCl solutions, from 20 to 320°C and at saturation pressures. Geochimica et Cosmochimica
699 Acta 58(11), 2433-2444.
700 Suleimenov, O.M. and Seward, T.M. (1997) A spectrophotometric study of hydrogen sulphide
701 ionisation in aqueous solutions to 350°C. Geochimica et Cosmochimica Acta 61(24), 5187-
702 5198.
703 Sun, W., Nešić, S., Young, D. and Woollam, R.C. (2008) Equilibrium Expressions Related to the
704 Solubility of the Sour Corrosion Product Mackinawite. Industrial & Engineering Chemistry
705 Research 47(5), 1738-1742.

706 Tuomisto, H.L., Hodge, I.D., Riordan, P. and Macdonald, D.W. (2012) Comparing energy
707 balances, greenhouse gas balances and biodiversity impacts of contrasting farming systems
708 with alternative land uses. Agricultural Systems 108, 42-49.
709 Valverde Pérez, B., Xing, W., Zachariae, A.A., Skadborg, M.M., Kjeldgaard, A.F., Palomo, A.
710 and Smets, B.F. (2020) Cultivation of methanotrophic bacteria in a novel bubble-free
711 membrane bioreactor for microbial protein production. Bioresource Technology 310,
712 123388.
713 Wei, X., Su, Y., Zhang, H., Chen, M. and He, R. (2015) Responses of methanotrophic activity,
714 community and EPS production to CH4 and O2 concentrations in waste biocover soils.
715 Waste Management 42, 118-127.
716 Wilshusen, J.H., Hettiaratchi, J.P.A. and Stein, V.B. (2004) Long-term behavior of passively
717 aerated compost methanotrophic biofilter columns. Waste Management 24(7), 643-653.
718 Zhao, Y., Biggs, T.D. and Xian, M. (2014) Hydrogen sulfide (H2S) releasing agents: chemistry
719 and biological applications. Chem Commun (Camb) 50(80), 11788-11805.
720

Table 1. The maximum growth rate of *Methylocapsa acidiphila* DSM 13967 under eight different initial sulfide concentrations, and the corresponding calculated equivalent H_2S content in biogas

Group	$Na_2S_{(aq)}$, $mg \cdot L^{-1}$	Equivalent $H_2S_{(biogas)}$, ppm	μ_{max}, d^{-1}
S-1	0.00	0.00	0.51 ± 0.02
S-2	1.30 ± 0.02	164.72 ± 2.18	0.36 ± 0.04
S-3	2.71 ± 0.02	343.31 ± 2.17	0.38 ± 0.02
S-4	3.81 ± 0.19	481.86 ± 23.95	0.41 ± 0.07
S-5	5.73 ± 0.22	725.10 ± 28.30	0.41 ± 0.03
S-6	8.13 ± 0.13	1028.38 ± 17.42	0.35 ± 0.00
S-7	9.40 ± 0.03	1188.48 ± 4.35	0.44 ± 0.01
S-8	10.28 ± 0.26	1300.48 ± 33.20	0.38 ± 0.00

Table 2. Performance of acclimatization competence of *Methylocapsa acidiphila* DSM 13967 towards sulfide inhibition: a comparison of the maximum growth rate with five sulfide concentration during three batches (Unit: d^{-1})

Group	Batch 1	Batch 2	Batch 3
μ_{max} S-1	0.51 ± 0.02	0.40 ± 0.0	0.48 ± 0.01
μ_{max} S-2	0.36 ± 0.04	0.38 ± 0.02	0.50 ± 0.03
μ_{max} S-3	0.38 ± 0.02	0.45 ± 0.00	0.50 ± 0.12
μ_{max} S-4	0.41 ± 0.07	0.41 ± 0.02	0.56 ± 0.13
μ_{max} S-5	0.41 ± 0.03	0.44 ± 0.02	0.48 ± 0.13

Table 3 Average consumption rate of CH_4 and O_2 in the feed-gas-related conditions experiments (Unit: r_{gas}, mL·d^{-1})

Feed gas	Period	Feed gas ratio CH_4:O_2			Period	Feed gas adding frequency		
		4:6	6:4	8:2		every 2 d	every 4 d	never
CH_4	Stage I	2.35 ± 0.14	1.53 ± 0.06	3.14 ± 0.16	Stage I-1	3.31 ± 0.23	1.53 ± 0.06	1.06 ± 0.08
					Stage I-2	6.21 ± 0.42		
	Stage II	3.01 ± 0.58	3.62 ± 0.30	1.30 ± 0.05	Stage II-1	8.42 ± 0.20	3.62 ± 0.30	2.45 ± 0.07
					Stage II-2	2.47 ± 0.47		
	Total	2.68	2.58	2.22	Total	5.10	2.58	1.75
O_2	Stage I	3.84 ± 0.13	1.65 ± 0.07	0.77 ± 0.17	Stage I-1	0.74 ± 0.04	1.65 ± 0.07	1.75 ± 0.15
					Stage I-2	4.68 ± 0.06		
	Stage II	4.35 ± 0.08	3.43 ± 0.34	1.66 ± 0.55	Stage II-1	5.66 ± 0.42	3.43 ± 0.34	1.57 ± 0.10
					Stage II-2	7.48 ± 1.98		
	Total	4.09	2.54	1.21	Total	4.64	2.54	1.66

Table 4. Comparison of total amino acid production and operation conditions in this study with methanotrophic single cell protein producers reported in other studies and a summary of total amino acid content in common protein-rich food

Methanotrophic SCP producers	Total amino acid (% Dry Mass)	Operational conditions			Feedstock		Reference	Protein-rich food	Total amino acid□ (% Dry Mass)
		Temperature (□)	pH	Operation	Carbon source	Nitrogen source			
M. acidiphila	58.6 ± 0.7	24 ± 1	5.7 ± 0.1	Batch in bottle	CH_4	NO_3^- or NH_4^+ (available to N_2)	This work	Fresh fish	70.81
Methylococcus capsulatus (Bath)	53	37	7	Batch in bottle	CH_4	NH_4^+	(Rasouli et al. 2018)	Chicken	53.54
Mix MOB dominated by *Methylomonas sp.*	65.2	32	6.8~7.0	Batch in bubble column reactor	CH_4	NO_3^-, NH_4^+ or Urea	(Yazdian et al. 2005)	Egg	49.09
BPM□	62.90	45	7	Industrial continuous aerobic fermentor	CH_4	NH_4^+	(Schøyen et al. 2005, Skrede et al. 1998, Skrede et al. 2009)	Beef	44.01
BPMM□	63.36	N.A.	N.A.	Laboratory scale fermentor	Methanol	NH_4^+	(Skrede et al. 2009)	Brewer's Yeast	38.29
PRUTEEN®□	62.09	37	7.0	Continuous fermentor	Methanol	NH_4^+	(Øverland et al. 2010)	Fish meal	33.73

□ BPM referred to Bacterial Protein Meal, a commercial product that was produced and supplied by Dansk Bioprotein A/S (Odense, Denmark). The bacteria culture consisted of Methylococcus capsulatus (Bath) (88%), Alcaligenes acidovorans (12%), Bacillus brevis (0.3%), and Bacillus firmus (0.2%). The percentage of total amino acid was calculated as the average according to the results from three publications.

□ BPMM was the BPM (□) grown on methanol in a lab-scale fermentor.

□ PRUTEEN® is a commercial SCP product produce by Imperial Chemical Industries Ltd (Billingham, Cleveland, Great Britain). The bacteria culture mainly consisted of *Methylophilus methylotrophus*. The percentage of total amino acid is an average of the results from three publications calculated by Øverland et al. (2010). The temperature and pH referred to Wyborn et al. (1994).

□ The data were gathered from the FAO Food and Nutrition Series(FAO Food Policy and Food Science Service Nutrition Division 1970).

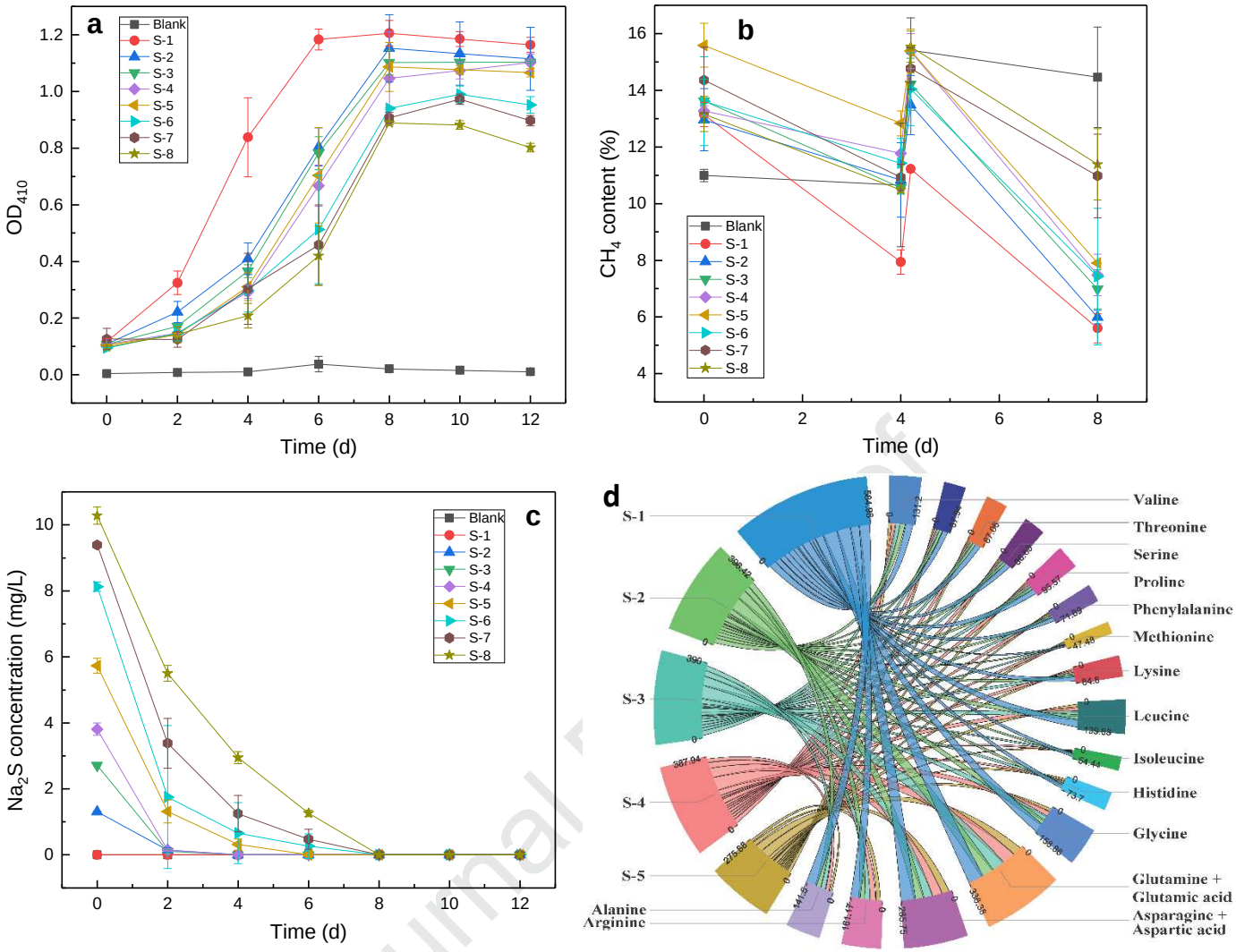


Fig. 1. Inhibitory effect of sulfide concentrations on the growth of *Methylocapsa acidiphila DSM 13967*: a) the change of OD_{410} over time; b) the change of CH_4 content in the headspace over time; c) the change of sulfide concentration in the liquid phase over time; d) the final amino acid profile analysis of the samples from Group S-1 to S-5 (unit: percentage of dry biomass). Sample S-1 to S-8 represented the S^{2-} concentration of 0, 1.30, 2.71, 3.81, 5.73, 8.13, 9.40, 10.28 mg·L^{-1}, respectively.

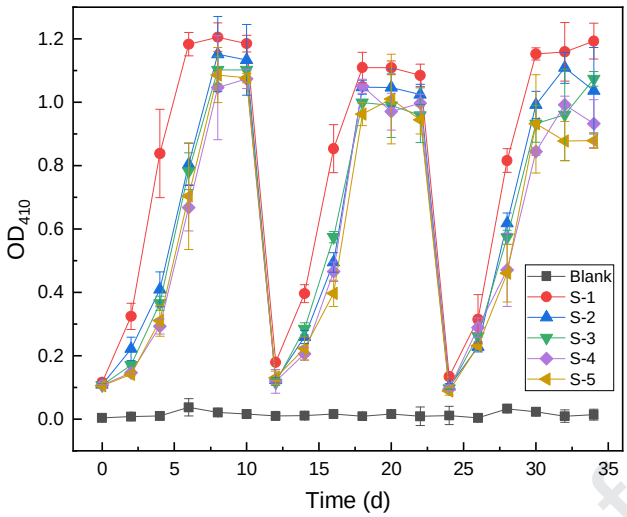


Fig. 2. Acclimatization performance of *Methylocapsa acidiphila* DSM 13967 in sulfide-rich environment in three sequential batches

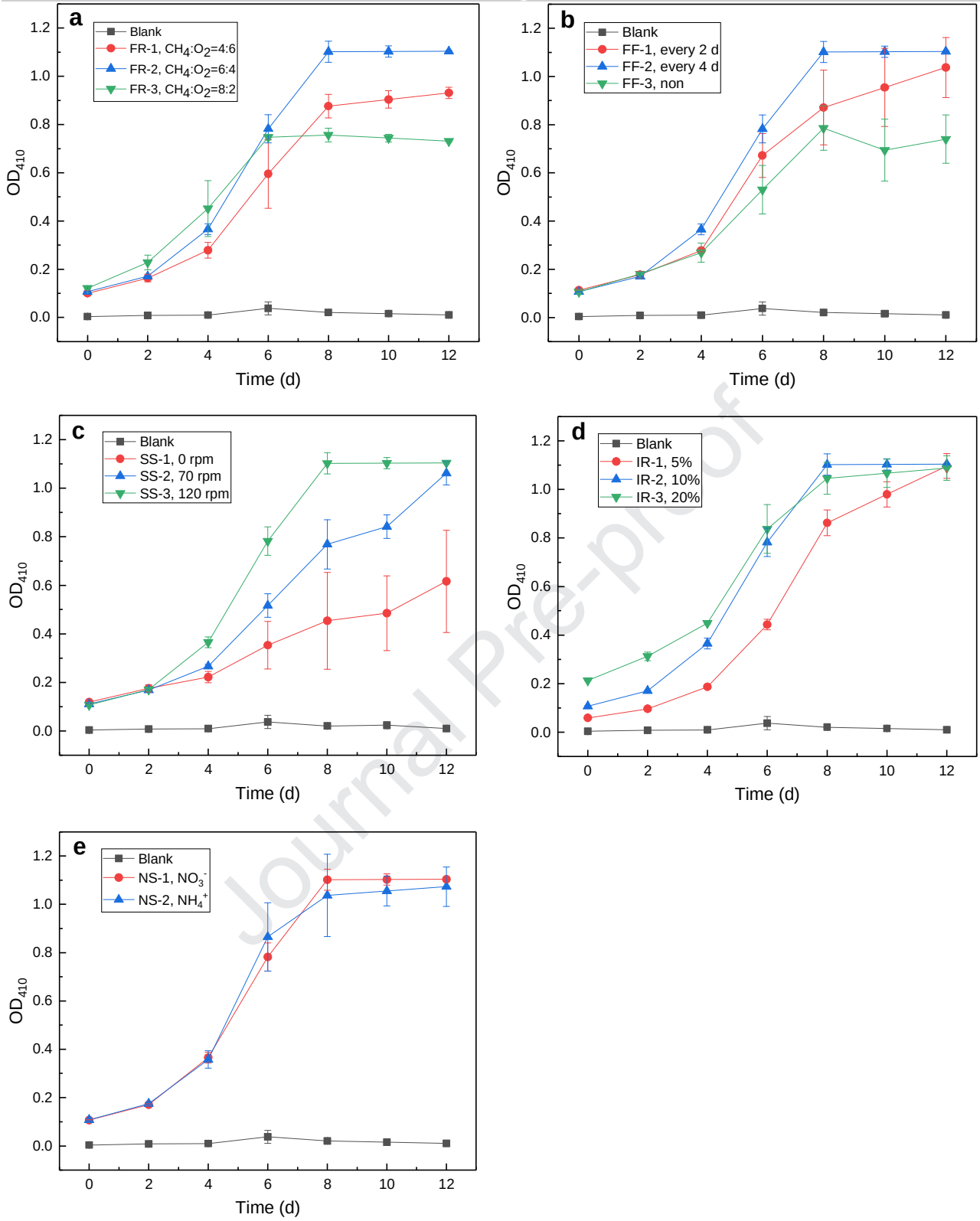


Fig. 3. The impact of different operational conditions on the growth performance (OD_{410} change over time) of *Methylocapsa acidiphila* DSM 13967 at 5.7 mg·L^{-1} Na_2S: a) feed gas ratio - CH_4:O_2 of 4:6 (FR-$1_{\text{-4:6}}$), 6:4 (FR-$2_{\text{-6:4}}$) and 8:2 (FR-$3_{\text{-8:2}}$); b) frequency of the additional 20 mL feed gas resupply – 3 times feeding on every 2 days (FF-$1_{\text{-3 times}}$), once feeding on the 4th day (FF-$2_{\text{-1 time}}$), and never feeding (FF-$3_{\text{-none}}$); c) shaking speed – 0 rpm (SS-1), 70 rpm (SS-2) and 120 rpm (SS-3); d) initial inoculation ratio – 5% (IR-1), 10% (IR-1) and 20 % (IR-1); e) nitrogen source – ammonium (NS-1) and nitrate (NS-2).

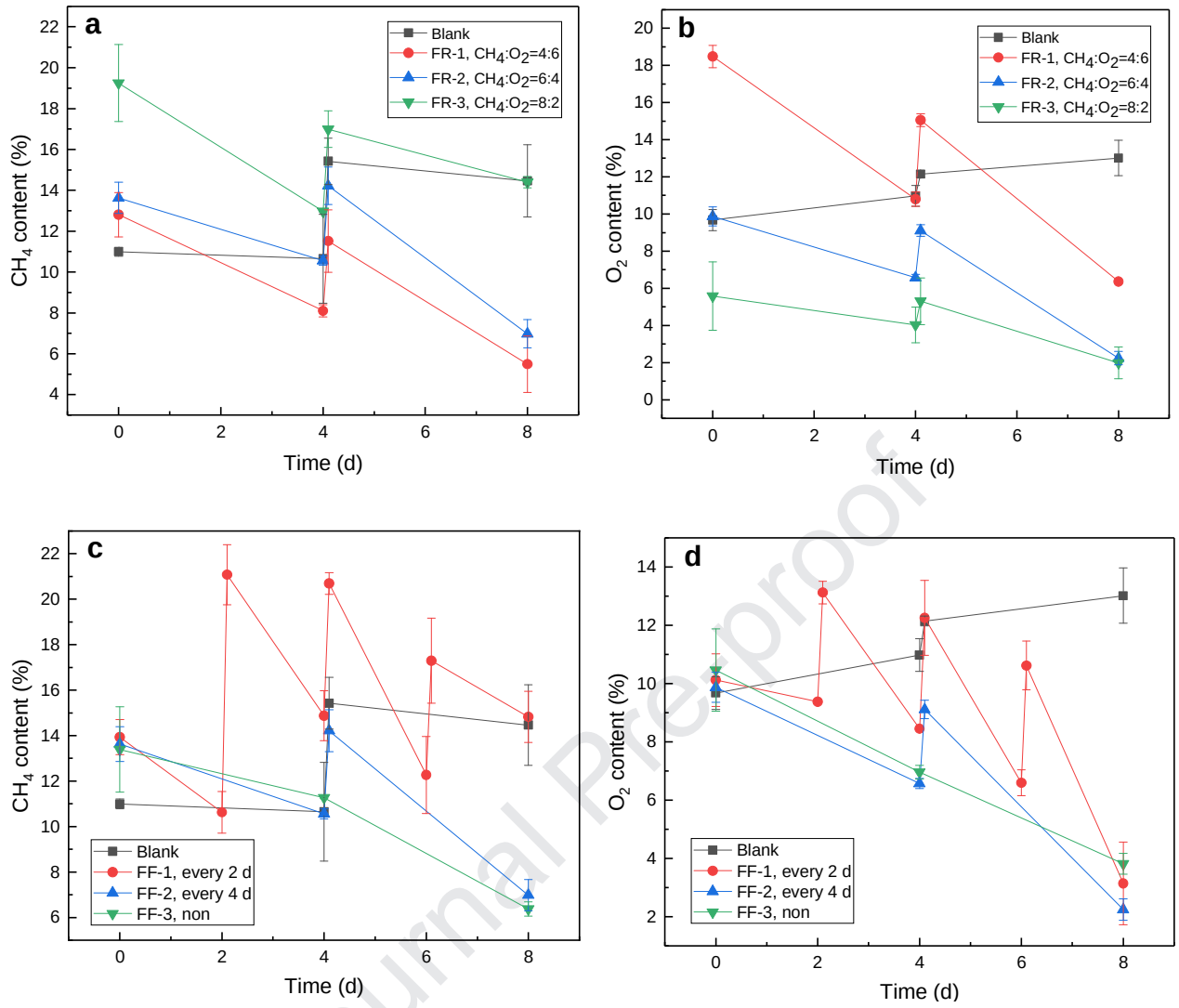


Fig. 4. The corresponding results of gas composition and content in the headspace of the samples in the experiments with different operational conditions: a) change of CH_4 content over time under different feed gas ratio - CH_4:O_2 of 4:6 (FR-$1_{\text{-4:6}}$), 6:4 (FR-$2_{\text{-6:4}}$) and 8:2 (FR-$3_{\text{-8:2}}$); b) change of O_2 content over time under different feed gas ratio; c) change of CH_4 content over time under different feed gas supply frequency - 3 times feeding on every 2 days (FF-$1_{\text{-3 times}}$), once feeding on the 4th day (FF-$2_{\text{-1 time}}$), and never feeding (FF-$3_{\text{-none}}$); d) change of O_2 content over time under different feed gas supply frequency.

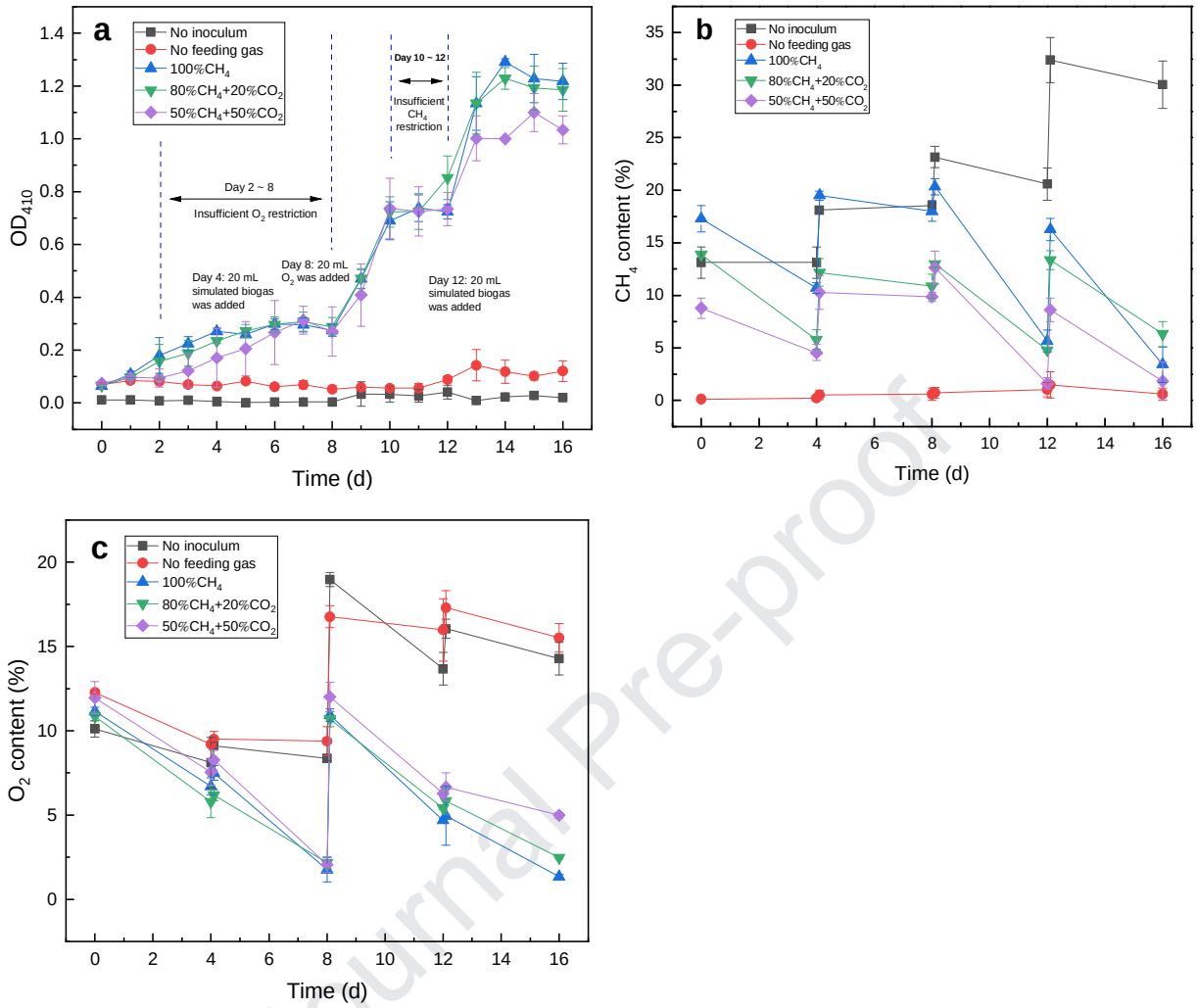


Fig. 5. The impact of different contents of CO_2 in feeding gas and the system performance under the starvation condition in sulfide-rich environment: a) performance of MOB's growth over time; b) change of CH_4 content in the headspace over time; c) change of O_2 content in the headspace over time.

Highlights

- Protein synthesis from raw biogas first time reported by *Methylocapsa acidiphila*.
- The first evidence of H_2S toxicity on *M. acidiphila* converting raw biogas.
- The H_2S inhibition started from 8.13 mg·L^{-1} Na_2S (1000 ppm H_2S).
- Cells underwent inhibition had at least 2 times less protein content in the dry biomass.
- The essential amino acid synthesis was not affected when the concentration of Na_2S was lower than 5.73 mg·L^{-1}.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: