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Bioconversion of wastewater to single cell protein by methanotrophic bacteria

Xiao Zha[a,b], Panagiotis Tsapekos[a,*], Xinyu Zhu[a], Benyamin Khoshnevisan[c], Xiwu Lu[b], Irini Angelidaki[a]

[a]Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby, DK-2800, Denmark

[b]School of Energy and Environment, Southeast University, No. 2 Sipailou Road, Nanjing, 210096, China

[c]Key Laboratory of Non-point Source Pollution Control, Ministry of Agriculture, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China

[*]Corresponding author: Panagiotis Tsapekos, Department of Environmental Engineering, Technical University of Denmark, Bld 115, 2800 Lyngby, Denmark.

E-mail address: ptsa@env.dtu.dk, Tel.: +45 45251404

Abstract

Single cell protein (SCP) provides an alternative protein source to partially replace the conventional agricultural resources and support the increased nutritional needs. Inexpensive feeding source is one of the key limiting factors for the expansion of SCP production. The present study examined the valorization of biogas derived from the anaerobic digestion (AD) of sewage sludge and the discarded effluent as nutrients source to produce SCP using methanotrophic bacteria. Results indicated that the mixed methanotrophic culture can grow well on the pasteurized AD supernatant and biogas, succeeding in promising dry weight (DW) yield (0.66±0.01 g-DW/g-CH_4 and 11.54±0.12 g-DW/g-NH_4^+). *Methylomonas* (56.26%) and *Methylophilus* (24.60%) spp. were the two main representatives of the mixed culture. The produced dried biomass had a protein content higher than 41% w/w, including essential amino acids like histidine, valine, phenylalanine, isoleucine, leucine, threonine and lysine. The cultivated SCP shows potential utilization as protein source for animal diets.

1 Introduction

Global food security and environmental sustainability are threatened by emerging trends in climate change, population growth, and resources scarcity (Beddington et al., 2012). Further increases will even stress many fields, including nitrogen and phosphorus needs for fertilization, amount of irrigated cropland, and land cultivation, leading to dramatic impacts on society and ecosystem (Tilman, 1999).

Interestingly, agriculture was previously indicated as the largest freshwater user (Mekonnen and Hoekstra, 2014) and important contributor to global anthropogenic greenhouse gas emissions (Vermeulen et al., 2012). The demand for proteins is increasing due to the change of food consumption patterns (Boland et al., 2013). However, the reliance on animal and dairy production to meet the growing demand for proteins is ultimately unsustainable (Ritala et al., 2017). Hence, alternative sources to supply food, especially proteins, for humans and animals should be explored.

Producing protein supplement for animal feed from nitrogen-rich waste streams is a promising alternative which avoids all the inherent losses of crops (Matassa et al., 2015). On this topic, single cell proteins (SCP) which are mainly edible unicellular microorganisms could potentially be provided to humans or animals. SCP has shown a big advantage because its independence of climate, soil characteristics and available land (Hülsen et al., 2018). Moreover, microbial protein has already tested at several monogastric species (Øverland et al., 2010), is approved by the European Union to be used at livestock (EC, 2017) and has already been commercially used as animal feed (Hülsen et al., 2018; Strong et al., 2015).

Among others, algae, fungi, yeast and bacteria can be utilized for SCP production (Ritala et al., 2017) having their own advantages and disadvantages (Khoshnevisan et al., 2019). Among them, bacteria possess higher growth rates, higher protein content, and more sulfur-containing amino acids (Khoshnevisan et al., 2019; Rudravaram et al., 2009). From an industrial perspective, methane oxidizing

bacteria (MOB) is the most advanced and market-ready bacteria for SCP production (Strong et al., 2016).

While the SCP production by methanotrophs has been studied since 1960s and was even developed in industrial production, the wide commercial production was terminated due to economic constraints (Øverland et al., 2010). Nevertheless, SCP production by MOB returns nowadays to the public view due to the increasing protein demand. Methane can be the sole carbon and energy source for MOB when assimilating nitrogen for protein production (Khoshnevisan et al., 2019) and can be connected with extra cost saving if found cheap and abundant (Øverland et al., 2010; Strong et al., 2015). Indeed, reducing the cost of SCP production by MOB is essential for commercial production expansion. Biogas supplies a cheap and abundant source for CH_4. For example, anaerobic digestion (AD) plays a key role in waste water treatment plants (WWTPs), to reduce the organic matter before disposal and recover energy, producing biogas which commonly contains 55-65% methane and 35-44% carbon dioxide. Apart from methane source, the composition of mineral medium also strongly affects the SCP production from MOB (Dedysh and Dunfield, 2014). Indeed, the effluent of AD systems can be used as a source of micro- and macro-nutrients (Tambone et al., 2010; Tsapekos et al., 2019). The supernatant of sewage sludge-based AD has high ammonium content, ranges from hundreds to thousands (Barua et al., 2019; Tan et al., 2016), which is a good nitrogen source for MOB as it can be directly assimilated into cell biomass (Tays et al., 2018). Nowadays, digested

sewage sludge is underutilized as it is typically incinerated after dewatering or spread as fertilizer after pasteurization to remove pathogens. Several techniques have been reported to upcycle the supernatant of sewage sludge-based AD, as for example via struvite formation and ammonia stripping. However, the wide commercial development of these technologies is still limited due to high economic constraints (Wu et al., 2017). Hence, the exploitation of digested and hygenized AD supernatant for SCP production could offer an alternative industrial perspective in terms of resource recovery. However, risks related to pathogens, pollutants, heavy metals and nucleic acids should be carefully addressed. In addition, the social acceptance of microbial protein produced from residual resources should be increased before further exploitation. If the final products have low social acceptance, action plans should be developed to ease product's social acceptance. Nevertheless, the exploitation of microbial protein produced from residual resources is nowadays focused on animal feed and not human food.

Despite pure cultures were previously employed for commercial SCP production, the potential risks (e.g. contamination, low tolerance to inhibitors) limit the commercial expansion. On the contrary, a robust bacterial consortium can support high biomass growth in long term operation (Bothe et al., 2002). A robust bacterial consortium can neglect the demand for strictly sterile conditions, which would reduce the operational costs (Tsapekos et al., 2019). Furthermore, the nutrients and feeding requirements for mixed cultures are also reduced accordingly (Nunes et al., 2016).

The present study explored for the first time to valorize the underutilized products generated at traditional WWTPs (i.e. biogas and pasteurized supernatant of sewage sludge-based AD) for SCP production. The microbial community structure was revealed at different growing conditions to achieve deeper biological insights. In addition, the production of MOB that could be used as an alternative source of proteins for animal feeding was evaluated based on biomass growth and amino acid profile.

2 Materials and methods

2.1 Batch tests

A mixed methanotrophic culture collected from a chemostat lab-scale fermenter was used as MOB seed. The seed was fed with pure synthetic methane and oxygen (Air Liquid A/S, Taastrup, Denmark) and diluted ammonium mineral salt (dAMS) (Khoshnevisan et al., 2019). Sterilized serum bottles with a total volume of 250 mL were used for seed growing. The bottles were filled with 2 mL mixed methanotrophic culture seed and 98 mL of sterilized dAMS (28 mg NH_4^+/L). The headspace was replaced by mixed composition gas (2:1 O_2:CH_4 v/v). The pressure in the bottles was kept at atmospheric pressure replacing the headspace with fresh gas mixture twice a day. The seed culture was cultivated in a shaken incubator (25 °C, 150 rpm).

Similar to seed cultivation, 250 mL sterilized serum bottles were employed for the batch assays following the procedure described above. A lab-scale thermophilic

AD reactor was occupied to provide the AD supernatant for nutrients provision and biogas for feeding. The substrate fed to the reactor was collected from Lynetten Wastewater Treatment plant (Copenhagen, Denmark) and was a mixture of primary and secondary sludge at 1:1 (v/v). After AD, the effluent was centrifuged at 17,000×g for 10 min and filtered through 0.2 μm filters. Then, the supernatant was pasteurized at 70°C for 1 hour and the ammonium concentration was measured. As a final step, the supernatant was diluted to the same-level of ammonium concentration with dAMS medium (i.e. 28 mgNH_4^+/L). The final centrifuged-filtered-pasteurized-diluted supernatant is named AD supernatant in the present research. Biogas was also collected from the same AD reactor and mixed with pure oxygen to achieve the desired O_2:CH_4 ratio (2:1 v/v) (Khoshnevisan et al., 2019; Tsapekos et al., 2019). The examined combinations are summarized in Table 1. All treatments were prepared in triplicate bottles.

2.2 Analytical methods

Biomass growth was tracked by optical density (OD) twice every day using a spectrophotometer (Hatch DR 3900) at 600 nm. To avoid measurement errors due to the presence of macro and micro-nutrients, the AD supernatant was used in order to calibrate the spectrophotometer. The gas composition (i.e. CH_4, O_2, CO_2) was monitored before and after refilling with mixed gases using a gas-chromatograph (GC-TRACE 1310, Thermo Scientific). Ammonium concentration levels at the

beginning and end of batch assays were measured using a photometric test kit (114752 Merck, USA). The calculation of generated dry weight (DW), gases, nitrogen consumption, biomass yields, ammonium assimilation efficiencies and biomass growth rate were previously described (Khoshnevisan et al., 2019). Macro elemental analysis of biomass (C, N, O, S) was analyzed using a vario MACRO cube analyzer. Amino acids analysis was performing according to method describing in literature (D'Este et al., 2017).

2.3 DNA extraction and sequencing

Triplicate bottles in every group, were mixed at the end of batch cultivation for further analysis. Samples were extracted in triplicate with PowerSoil® DNA Isolation Kit (QIAGEN Bioinformatics, Germany) following manufacture's instruction. The quality and quantity of DNA extracts were evaluated with NanoDrop (Thermo Scientific). The V4 regions of microbial 16S rRNA genes were amplified using 515F/806R primers and sequenced by Illumina MiSeq platform. 16S rRNA raw data were analyzed using CLC Workbench software (V.8.0.2) with Microbial genomics module plug in (QIAGEN) (Kougias et al., 2017). OTUs with relative abundance higher than 0.5% with respect to the total number of sequences were discussed in current study. The taxonomic assignment of sequences was verified in comparison with NCBI 16S rRNA database. The raw reads were uploaded in Sequence Read Archive (SRA) database with the accession BioProject number PRJNA644997.

2.4 Statistical analysis

Descriptive statistics, mean values and standard deviations were calculated using Excel software (Office 2013, Microsoft). STAMP software was used to identify significantly abundance differences for each microbe among the samples. Statistical differences between the mean values of relative abundance for the biological replicates were assessed using two groups comparison t-test (equal variance) setting the p-value to 0.05.

3 Results and discussion

3.1 Cultivation on different methane and nitrogen sources

3.1.1 Biomass growth

A comparative experimental batch set was conducted for investigating the biomass cultivation alternating the feeding supply. The mixed culture could grow under all examined conditions and the OD_{600} of all treatments reached stationary phase after 70-hour cultivation. Among treatments, MOB fed with synthetic methane and synthetic medium (i.e. group Control) achieved the highest biomass yield. In contrast, the group fed with AD supernatant and biogas (i.e. group ADs-B) achieved the lowest biomass yield. The highest growth rate (0.08 1/h) was achieved at group control, while group ADs-B achieved the lowest growth rate (0.06±0.001 1/h) (Fig. 1). Similar growth rate was reported in the literature using a mixed methanotrophic

culture, enriched in *Methylococcales* and *Methylophilales* orders, grown on digested urban biowaste (0.1 1/h) (Khoshnevisan et al., 2019). Moreover, the growth rates of *Gammaproteobacteria*, *Alphaproteobacteria* and *Verrucomicrobia* were reported at 0.45 1/h, 0.09 1/h and 0.085 1/h, respectively (Kalyuzhnaya, 2016). The growth rates in this study were close to literature range of *Alphaproteobacterial* and *Verrucomicrobia* but lower than the values of *Gammaproteobacteria*. However, growth rates can vary even for the same MOB species. For example, the maximum growth rate of type II (*Alphaproteobacteria*) MOB pure cultures ranged from 0.018 to 0.34 1/h (AlSayed et al., 2018). The growth rate can be influenced by the operational conditions, such as the nutrient, gas conditions and the biomass density.

Apart from biomass growth, ammonium assimilation efficiency and SCP yield on methane are also appropriate indicators for process evaluation (Table 2). In accordance with biomass growth, the group fed with AD supernatant and biogas achieved the lowest SCP yield on NH_4^+ (8.51±0.09 g-DW/g-NH_4^+). Groups fed with AD supernatant as their nitrogen source had lower SCP yield on CH_4 than the group fed with dAMS and biogas. Biogas feeding led to higher SCP yield, compared to treatments fed with pure methane (Table 2). Likewise, it was recently indicated that CO_2 contributed to better SCP yield on CH_4 (Khoshnevisan et al., 2019). Similarly, CO_2 encouraged the growth of the mixed methanotrophic consortium in pilot-scale biofilters (Karthikeyan et al., 2017). Both type I (*gammaproteobacteria*) and type II (*alphaproteobacteria*) methanotrophs have PEP-carboxylase for CO_2 assimilation

(Karthikeyan et al., 2015). Cell biomass derived from CO_2 as carbon sources, was 5-15% of all biomass formed, when use type I methanotrophs, whereas was up to 50% when use type II methanotrophs (Karthikeyan et al., 2015). Thus, it can be inferred that the higher SCP yield in the group fed with biogas was attributed to the presence of CO_2.

Similar to the applied experimental conditions, biomass yields of 0.99 g-DW/g-CH_4 for *Methylosinus trichosporium* (OB3b) and 1.05 g-DW/g-CH_4 for *Methylocystis parvus* (OBBP) were observed at 0.4 oxygen partial pressure (Rostkowski et al., 2013). Furthermore, biomass yields of other known MOBs were also reported, such as 0.4-1.03 g-DW/g-CH_4 for *Methylococcus capsulatus*; 0.60-1.03 g-DW/g-CH_4 for *M. alcaliphilum 20Z*^{R} (Harwood and Pirt, 1972; Henard et al., 2018). Similar results were also detected during the cultivation of mixed methanotrophic culture. Specifically, a biomass yield of 0.8 g-DW/g-CH_4 was reported using a mixed culture (Wilkinson et al., 1974). Besides, 0.71-0.88 g-DW/g-CH_4 yield was reported using a mixed culture (enriched in *Methylophilus* sp., *Methylomonas* sp., and *Comamonadaceae* sp.) cultivated on different nitrogen sources (Khoshnevisan et al., 2020). In addition, the cultivation of type I methanotrophs was reported with 0.59 g-DW/g-CH_4 (AlSayed et al., 2018), while for type II a higher value of 0.82 g-DW/g-CH_4 was reported (Fergala et al., 2018). Hence, the biomass yields on methane achieved in the present study (0.61-0.76 g-DW/g-CH_4) are within the literature range (Khoshnevisan et al., 2020).

Moreover, the calculated yields are close to the outcome of a recent study in which MOB were cultivated on the products of urban waste AD (Khoshnevisan et al., 2019). Though the substrates of AD were different between the two case studies, similar microbial seed inoculum and oxygen partial pressure were used. Nevertheless, in both studies the culture had lower performance when the gaseous and liquid AD streams were used. One of the reasons was that the raw biogas contained H_2S that could partially inhibit the methanogenic growth (Tsapekos et al., 2019). For practical applications, the H_2S obstacle can be easily overcome applying well-established desulfurization technologies suitable for biogas purification (Khoshnevisan et al., 2017). Apart from H_2S obstacle, the most important challenge is the micro- and macro-nutrients composition of digested samples. Control group was always grown at nutrient rich environment in contrast to the AD supernatant -related tests which were not supplemented with the potentially missing elements that are crucial to achieve high CH_4 oxidation. Hence, the limited availability of trace elements could hinder the biomass growth. Despite the lower growth, the present study clearly shows the potential of growing MOB on sewage sludge-based AD supernatant. However, process optimization is still necessary and on this topic, adjusting the concentration of macro- and micro-nutrients (e.g. nitrogen, phosphorus, iron, copper) in the AD effluent, upgrading the biogas for H_2S and CO_2 removal, optimizing the gas feeding composition and enhancing gas–liquid mass transfer can markedly boost the biomass growth (Khoshnevisan et al., 2019; Tsapekos et al., 2019). Regarding the biomass

yield on NH_4^+, values between 8.51 and 11.50 g-DW/g NH_4^+ (Table 2) were detected and were at the same level with a previous work at similar conditions (Khoshnevisan et al., 2019). The NH_4^+ assimilation efficiency was high; however, a high yield means lower nitrogen accumulation which will then result in lower protein content.

3.1.2 Macromolecular composition

Elemental content of the produced biomass was examined for all treatments grown on biogas and AD supernatant. The elemental analysis suggested that nitrogen content of the groups ranged from 6.06% to 7.04% of biomass dry weight. Based on theoretical relationship, the biomass can be presented as $CH_{1.90}O_{0.61}N_{0.14}$, $CH_{1.59}O_{0.82}N_{0.13}$ and $CH_{1.60}O_{0.73}N_{0.13}$ for dA-B, ADs-S and ADs-B, respectively. Nitrogen contents were lower and oxygen compositions were higher, compared to the typical value of biomass ($CH_{1.80}O_{0.5}N_{0.20}$). In the present study excess of O_2 was provided (2:1 O_2:CH_4 v/v) to ensure availability. However, 1.45 mol O_2 is theoretically needed to bio-oxidize 1 mol CH_4 (Nielsen and Villadsen, 1994). The stoichiometry for the biooxidation is:

$$CH_4 + 0.104NH_3 + 1.45O_2 \rightarrow 0.52CH_{0.18}O_{0.5}N_{0.2} + 0.48CO_2 + 1.69H_2O$$

Regarding the theoretically calculated protein content based on nitrogen content, group dA-B, ADs-S and ADs-B contained 46±2.3%, 40±2.0% and 41±2.0% w/w of DW, respectively. The statistical analysis showed that the difference observed was not significant ($p > 0.05$), meaning that the produced biomass under different treatments

had almost equal protein content. Interestingly, the detected protein contents were higher compared to MOB grown at digested urban biowaste, which reportedly ranged from 17% to 21% (Khoshnevisan et al., 2019). In the cited work, the seed inocula were grown in nutrient rich media and fed with pure gases. On the contrary, the seed inoculum used in the present study was sub-cultured and grown for multiple generations at biogas and different slurries. Thus, the microbes have evolved mechanisms to partially adapt to single or multiple nutrients (e.g. Iron, Copper) scarcity when digested organic matter was used as growing medium. However, the protein contents were lower than the initially targeted values. For example, the protein content of the model MOB *Methylococcus capsulatus* ranges between 53-81% (Øverland et al., 2010; Rasouli et al., 2018). To succeed in protein content comparable to pure cultures either a stepwise adaptation to low-nutrient environment or the supplementation with the scarce trace elements can contribute on growing a more proteinaceous biomass.

3.2 Microbial analysis and product quality

3.2.1 Microbial shifts

The compositions of mixed methanotrophic cultures at different growing conditions are illustrated in Fig. 2. At control operation, *Methylophilus* sp.1 (36.91%); *Methylomonas* sp.1 (20.07%) were the most abundant OTUs. Microbes within the aerobic methanol-utilizing genus of *Methylophilus* (i.e. *Methylophilus methylotrophus*)

were previously utilized to produce SCP assimilating ammonia (Windass et al., 1980). In industrial perspective, chemical industries (e.g. Pruteen) developed SCP production by *M. methylotrophus* for animal feed containing up to 70% protein (Ritala et al., 2017). There was no significant change in abundance of *Methylophilus* sp.1 when replacing the gas feeding with biogas or replacing the liquid feeding with the supernatant of sewage sludge-based AD. However, the abundance of *Methylophilus* sp.1 was decreased to 24.60% by 0.59-fold ($p < 0.05$) (Fig. 3) when both AD supernatant and biogas were used (ADs-B). Regarding the secondly dominant strain, *Methylomonas* is also widely used for SCP production (Kalyuzhnaya et al., 2015), reaching 69.3% w/w of DW protein when fed with natural gas (Yazdian and Hajizadeh, 2005). Compared to control group, *Methylomonas* sp.1 was decreased by 0.94-fold ($p < 0.05$) when fed with biogas and increased by 1.18-fold ($p < 0.05$) when cultivated on AD supernatant. When fed with both biogas and AD supernatant, the abundance of *Methylomonas* sp.1 reached to 56.26%, which increased by 1.49-fold ($p < 0.05$). *Methylomonas* is one of the typical type I MOB that have higher growth rates and higher methane affinity compared to other MOB types (AlSayed et al., 2018). The ability of *Methylomonas* strains to fixate CO_2 is well-known (Puhar et al., 1983) and this characteristic can help the mixed culture to grow on real biogas. Besides, it was also reported that *Methylomonas* strains expressed either sMMO or pMMO, which is conducive to adapting to different ion concentrations (e.g. Cu^{2+}) (Van Der Ha et al., 2013). Thus, the increased abundance in AD supernatant feeding groups can

be explained. Heterotrophic ammonia oxidizing microbes similar to *Ohtaekwangia* and *Emticicia* were detected in all groups (Fig. 2). Their presence may have negative impacts on microbial protein production due to extended ammonia oxidation (Berman et al., 2014). It may also contribute to the lower nitrogen content in the biomass, as mentioned above. However, the heterotrophs could have been essential to MOB and possibly related to vital processes such as toxic compounds removal (Van Der Ha et al., 2013).

Overall, the relative abundance of *Methylomonas* was higher in groups grown on AD supernatant compared to groups grown on dAMS. Due to the high protein content, the enrichment of *Methylomonas* is beneficial for SCP production. According to the metabolic pathways of MOB, methanol is the intermediate by the key enzymes (Kalyuzhnaya et al., 2015). The secondly dominant *Methylophilus* strains can grow on methanol to produce high content protein. *Methylomonas* and *Methylophilus* spp. were enriched together and could have growing sytrophically providing advantages for each other. The role of *Methylomonas* was to oxidize methane to methanol while the role of *Methylophilus* was indicated as assimilating the accumulated metabolites (i.e. methanol) in order to avoid the inhibition to *Methylomonas* (Tsapekos et al., 2020). The two species were found as the dominant ones in all different groups. Hence, the cultivation with biogas and the supernatant of sewage sludge-based AD can lead to the establishment of a specialized community dominated by *Methylomonas* and *Methylophilus* strains to produce SCP. Furthermore, the

sequencing analysis did not reveal any common pathogens presented in digestate (e.g. *Salmonella* or *Escherichia coli*) (Coelho et al., 2018). However, the pathogens should be more closely monitored prior to real-life applications and their absence should be ensured before usage of biomass as feed ingredient.

3.2.2 Amino acid profile

The amino acid profile on MOB grown on biogas and AD supernatant (ADs-B) was also explored (Fig. 3). Essential amino acids as histidine (4.2% w/w of DW), valine (1.2% w/w of DW), phenylalanine (1.4% w/w of DW), isoleucine (1.9% w/w of DW), leucine (2.8 w/w of DW), threonine (3.9 % w/w of DW), and lysine (0.8% w/w of DW) were detected in the biomass. The biomass contained all the amino acids for soya and fish meal replacement expect methionine and tryptophan (Yazdian and Hajizadeh, 2005). Although methionine and tryptophan were detected in former studies, they were also observed at low levels in relevant literature for SCP production by MOB (Hülsen et al., 2018; Khoshnevisan et al., 2019). Histidine, which is used in the biosynthesis of proteins, was 1.67 and 2.09 times higher than soybean and fish meal, respectively (Yazdian and Hajizadeh, 2005). Histidine is also important for fish as it strongly influence animal growth and structural integrity of gills (Jiang et al., 2016). Moreover, threonine was also detected at high levels reaching same level values with soybean and fish meal (Yazdian and Hajizadeh, 2005). Overall, the essential amino acids content provides potential utilization at animal diets.

4 Conclusion

The present study demonstrated that a wild methanotrophic culture, dominated by *Methylomonas* and *Methylophilus* spp. growing on syntrophic basis, can be efficiently cultivated on gas and liquid products of sewage sludge anaerobic digestion. The detected protein content was higher than 41% indicating biomass of relatively high protein content. The SCP contained a number of essential amino acids, such as histidine, valine, phenylalanine, isoleucine, leucine, threonine and lysine. The produced biomass could stand as alternative to partially replace soya and fish meal for protein supply in animal diets.

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Table List

Table 1. Liquid and gas feeding sources for batch assays

Group name	Control	dA-B	ADs-S	ADs -B
Nutrient source	dAMS	dAMS	AD supernatant	AD supernatant
Gas source	Synthetic CH_4 + O_2	Biogas + O_2	Synthetic CH_4 + O_2	Biogas + O_2

* In groups names: dA refers dAMS, ADs refers the supernatant of sludge-based AD, B refers biogas and S refers synthetic gas.

Table 2. Biomass yields and ammonium assimilation efficiencies in groups

group	Y_{SCP}/NH_4^+ (g-DW/g-NH_4^+)	Y_{SCP}/CH_4 (g-DW/g-CH_4)	NH_4^+-N assimilation (%)
Control	8.95	0.59	90
dA-B	11.50±0.18	0.76±0.01	96
ADs-S	9.79±0.21	0.61±0.01	93
ADs-B	8.51±0.09	0.66±0.01	97

Figure Captions

Fig. 1. Biomass growth monitoring

Fig. 2. Composition of mixed methanotrophic cultures at different growing conditions

Fig. 3. Amino acids profile of MOB grown on biogas and sewage sludge-based AD supernatant