Journal of Biotechnology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Metagenome changes in the mesophilic biogas-producing community during fermentation of the green alga *Scenedesmus obliquus*

Roland Wirth[a], Gergely Lakatos[b], Tamás Böjti[a], Gergely Maróti[b], Zoltán Bagi[a], Mihály Kis[c], Attila Kovács[d], Norbert Ács[a], Gábor Rákhely[a,e], Kornél L. Kovács[a,e,f,*]

[a] Department of Biotechnology, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary
[b] Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Temesvári krt. 62, H-6726 Szeged, Hungary
[c] Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Temesvári krt. 62, H-6726 Szeged, Hungary
[d] Phytoplankton and Macrophyte Research Team, Balaton Limnological Institute, Klebersberg Kuno 3, H-8237 Tihany, Hungary
[e] Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, Temesvári krt. 62, H-6726 Szeged, Hungary
[f] Department of Oral Biology and Experimental Dental Research, University of Szeged, Tisza L. krt. 64, H-6720 Szeged, Hungary

ARTICLE INFO

Article history:
Received 12 December 2014
Received in revised form 8 June 2015
Accepted 12 June 2015
Available online xxx

Keywords:
Microalgae
Scenedesmus obliquus
Biogas
Microbial community
Next-generation sequencing
Metagenomics

ABSTRACT

A microalgal biomass offers a potential alternative to the maize silage commonly used in biogas technology. In this study, photoautotrophically grown *Scenedesmus obliquus* was used as biogas substrate. This microalga has a low C/N ratio of 8.5 relative to the optimum 20–30. A significant increase in the ammonium ion content was not observed. The methane content of the biogas generated from *Sc. obliquus* proved to be higher than that from maize silage, but the specific biogas yield was lower. Semi-continuous steady biogas production lasted for 2 months. Because of the thick cell wall of *Sc. obliquus*, the biomass-degrading microorganisms require additional time to digest its biomass. The methane concentration in the biogas was also high, in co-digestion (i.e., 52–56%) as in alga-fed anaerobic digestion (i.e., 55–62%). These results may be related to the relative predominance of the order *Clostridiales* in co-digestion and to the more balanced C/N ratio of the mixed algal–maize biomass. Predominance of the order *Methanosarcinales* was observed in the domain Archaea, which supported the diversity of metabolic pathways in the process.

1. Introduction

The increasing global demand for energy heavily depends on fossil fuels such as oil, coal, and natural gas. With the anticipation of fossil fuels becoming exhausted in the foreseeable future, novel strategies need to be discovered for alternative energy generation. Photosynthetic biomass-based fuels are widely regarded as sustainable alternatives to fossil fuels. Biofuels and other forms of bioenergy are currently produced from terrestrial plants (Schenk et al., 2008). Microalgae may represent an alternative to terrestrial crops because they have higher photosynthetic efficiencies and higher growth rates, and can be grown in saline waters and marginal land areas (Posten and Schaub, 2009; Dębowski et al., 2013). Microalgae can be harvested practically all year round, which results in enhanced biomass-production efficacy. Cultivation can be carried out in closed photobioreactors or in open ponds. Open systems are usually considered economical, whereas closed systems are more efficient from the aspects of biomass production and control of the cultivation parameters (Edward, 2009; Edward, 2009), so that either concept may be competitive in the various applications (Guccione et al., 2014).

Microalgal biomass is of potential for anaerobic digestion (AD) as it can have high contents of lipids, carbohydrates, and proteins, and does not contain recalcitrant lignin (Chen et al., 2009; González-Delgado and Kafarov, 2011; Yen et al., 2013; Ward et al., 2014). With regard to the enormous biodiversity of microalgae and the recent developments in genetic engineering, this group of organisms is clearly one of the most promising sources for new-generation biofuels. Research on the AD of algal biomass goes back more than 50 years (Golueke et al., 1957). That early study made a comparison of sewage sludge and green algae (*Scenedesmus* sp. and *Chlorella* sp.). Following such pioneering experiments, relatively few investigations dealt with the anaerobic fermentation of microalgae (Uziel et al., 1974; Keenan, 1977; Binot et al., 1977; Samson and LeDuyt,

* Corresponding author at: Department of Biotechnology, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary.
E-mail addresses: roland.w@freemail.hu (R. Wirth), lakatos.gergely@brc.mta.hu (G. Lakatos), tamas.bojti@gmail.com (T. Böjti), maroti.gergely@brc.mta.hu (G. Maróti), bagiz@brc.hu (Z. Bagi), kis.mihaly@brc.mta.hu (M. Kis), kovacs.attila@okologia.mta.hu (A. Kovács), acsn@brc.hu (N. Ács), rakhely@brc.hu (G. Rákhely), kovacs.kornel@brc.mta.hu (K.L. Kovács).

http://dx.doi.org/10.1016/j.jbiotec.2015.06.396
0168-1656/

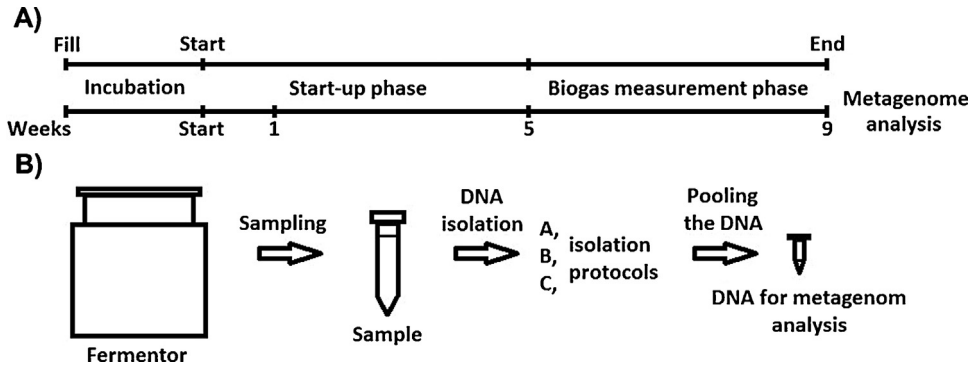

Fig. 1. The scheme and timeline of the experimental set-up. A. the time course of the various stages of the experiment. First a 2–3 weeks long "incubation" period indicates the set-up phase of the reactors. During the "start-up" phase the reactors, already producing biogas from a mixture of pig slurry and maize silage, were fed with the selected substrates, i.e., algal biomass, maize silage or a 1:1 mixture thereof. This lasted about 4 weeks. In the "biogas measurement" stage biogas yield from the selected substrates and metagenomic changes were monitored. B: The sample preparation steps for metagenomic studies. Note: the individual steps do not correspond to the time scale indicated in A.

1982; Becker, 1983; Hernández and Córdoba, 1993) until recently. Various freshwater and salt water algal strains were compared under mesophilic conditions (Mussgnug et al., 2010) and the biogas potential proved to depend strongly on the species and on the thickness of the cell wall. One noteworthy feature was that the CH_4 content of the biogas from the microalgae was 7–13% higher than that from maize silage (Mussgnug et al., 2010).

Intensive studies of the microbial communities of maize silage-fed anaerobic digesters (Schlüter et al., 2008; Krause et al., 2008; Kröber et al., 2009; Jaenicke et al., 2011; Wirth et al., 2012; Stantscheff et al., 2014; Ziganshina et al., 2014) have demonstrated that, although the anaerobic fermentation conditions (fermenter size, feedstock composition, and origin, mixing, inoculum composition, etc.) differed somewhat, but the substrates were essentially the same (maize silage and pig manure) and coherent data sets could be collected. Members of the phyla *Firmicutes* and *Bacteroidetes* played the most important roles in the hydrolysis of the plant biomass and in the secondary fermentation. In particular, many *Clostridium* species were identified which possess cellulolytic and H_2-producing activities, both properties probably being essential for the efficient degradation of the biomass. *Methanomicrobiales*, the most abundant order in the domain Archaea in large scale AD process, uses CO_2 as a carbon source and H_2 as an electron donor for methanogenesis. The general features of the community structure in the domain Bacteria appeared similar in the various studies, but alterations were noted in the domain Archaea. The most sensitive element in the microbiological food chain yielding biogas is the methanogenic group, changes in which may be associated with seasonal fluctuations or the variation of specific fermentation conditions (Rastogi et al., 2008; Lee et al., 2009). As an example, acetoclastic tend to predominate in biogas fermenters operated with wastewater sludge, while reactor communities fed with more diverse substrates prefer hydrogenotrophic methanogenesis (Sundberg et al., 2013).

Little is known about the microbial community of an anaerobic digester sustained with algal biomass. Ellis et al. (2012) employed 454 pyrosequencing to study the archaeal community during microalgal fermentation following the PCR amplification of *mcrA* gene regions. In alga-fed mesophilic AD inoculated with wastewater sludge, the majority of annotated *mcrA* sequences were assigned to the genus *Methanosaeta*. That investigation did not extend to the composition of the bacteria in the substrate algal biomass or within the anaerobic digester, although heavy bacterial representation could be expected in algal biomass cultivated in open ponds filled with wastewater. A more recent study (Wirth et al., 2014) analyzed the complete microbial community of a laboratory-scale AD fed with an algal–bacterial co-culture. A large proportion of bacteria belonging to the genera *Rhizobium* and *Burkholderia* lived in apparent syntrophic community together with the microalgal biomass, which changed the bacterial community composition significantly. This effect obscured the changes in the domain Bacteria as a result of the algal feedstock. The pronounced alterations observed in the domain Bacteria did not affect the microbial composition of the domain Archaea (Wirth et al., 2015).

Scenedesmus obliquus is a common freshwater microalga which can accumulate high amount of oil (Breuer et al., 2014; Mandal and Mallick, 2009) and starch (Batista et al., 2014). It can grow in various industrial wastewaters (Mata et al., 2013; Hodaifa et al., 2008) in a relatively wide temperature range (Xu et al., 2012). We report here an AD process involving the use of a photoautotrophically grown *Sc. obliquus* microalgal biomass with the aim of determining the response of the biogas producing microbial community to the novel substrate. The microbial community was monitored during the process by using high-throughput sequencing technology. The AD parameters and microbial community in an anaerobic reactor fed with *Sc. obliquus* and a co-digestion of maize silage and algal biomass were compared with the corresponding data on maize silage alone as control.

2. Materials and methods

2.1. Sc. obliquus biomass production

For biomass production, a culture of *Sc. obliquus* obtained from the culture collection of algae and protozoa (catalog no. CAAP276/72) was cultivated under natural light illumination at ambient temperature in a 4000 L tubular photobioreactor by first Hungarian Algatechnic Ltd. (ELMAT). BG11 medium was used (Stainer et al., 1971; Rippka et al., 1979). The biomass yield was approximately 2 g L^{-1}. The harvested biomass was stored at −20 °C until utilization.

2.2. Anaerobic fermentation and biogas analysis

Anaerobic fermentations were carried out in 5 L continuously-stirred tank reactors (CSTR) (Kovács et al., 2013a) in fed-batch operational mode. The experimental design and time course are summarized in Fig. 1. The reactors were operated with a pig manure + maize silage mixture (Wirth et al., 2012) until the biogas yield became stable prior to the commencement of feeding, i.e., start-up phase with the algal/maize silage substrates. The three reactors were fed with distinct substrates from the beginning of the start-up phase. One fermenter was fed with *Sc. obliquus* biomass at a loading rate of 1 g oDM L^{-1} day^{-1} (oDM = organic dry mat-

Table 1
Substrates used in the experiments.

Substrate	Wet mass N (mg/g)	Wet mass C (mg/g)	C/N ratio	TS (%)	oDM (%)
Maize silage	4.35	196.86	45.3:1	41.19	94.59
Sc. obliquus	8.10	72.22	8.9:1	16.99	97.71

N = nitrogen content; C = carbon content; TS = total solid content; oDM = organic dry matter content.

ter), while parallel fermenters were supplied with a mixture of *Sc. obliquus* + maize silage (each 0.5 g oDM L^{-1} day^{-1}) or with maize silage (1 g oDM L^{-1} day^{-1}). Temperature was maintained constant at 37 ± 1.0 °C by an electronically heated jacket which surrounded the cylindrical apparatus. The pH was kept between 7 and 8, and the redox potential was less than −500 mV. After the 1-month start-up phase (weeks 1–4), the gas generated and its quality were measured daily. Gas volumes were measured with thermal mass flow devices (DMFC SLA5860S, Brooks) attached to each gas exit port. The composition of the evolved biogas was measured with a gas chromatograph (6890N Network GC System, Agilent Technologies) equipped with a 5 Å molecular sieve column (length 30 m, I.D. 0.53 megabore, film 25 μm). Ultrapure N_2 was used as carrier gas.

2.3. Determination of fermentation parameters

Organic dry matter (oDM): The dry matter content was determined by drying the biomass at 105 °C overnight and weighing the residue. Further, heating of this residue at 550 °C provided the organic total solids content.

Density measurement: Sample density was measured by an automatic density meter (Grabner Instruments, MINIDENS)

C/N: To determine C/N, an Elementar Analyzer Vario MAX CN was employed. This works on the principle of catalytic tube combustion under a supply of O_2 at high temperatures (combustion temperature: 900 °C, post-combustion temperature: 900 °C, reduction temperature: 830 °C, column temperature: 250 °C). The desired components were separated from each other with the aid of specific adsorption columns (containing Sicapent, in CN mode) and determined in succession with a thermal conductivity detector. Helium served as flushing and carrier gas.

NH_4^+-N: For the determination of NH_4^+ content, the Merck Spectroquant Ammonium test (1.00683.0001) was used. At the beginning of the experiment the samples contained 1400 mg NH_4^+-N L^{-1}.

VOA/TIC (Volatile organic acids/Total inorganic carbon): 5 g of fermenter sample was taken for analysis and diluted to 20 g with distilled water. The subsequent process was carried out with a Pronova FOS/TAC 2000 Version 812-09.2008 automatic titrator. At the beginning of the experiment the VOA/TIC ratio was 0.2.

2.4. Substrate composition

The characteristics of the algal biomass and maize silage substrates are presented in Table 1.

2.5. DNA isolation for metagenomic studies

2 mL samples from the reactors were used for total-community DNA isolation. The extractions were carried out with a slightly modified version of the Zymo Research kit (Zymo Research, D6010). Parallel samples from each reactor were lysed with three different lysis mixtures (Table 2). After lysation and bead beating, the Zymo Research kit protocol was followed. The quantity of DNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and a Qubit 2.0 Fluorometer (Life Technologies). DNA integrity was tested by agarose gel electrophoresis and with Agilent 2200 Tape Station (Agilent Technologies).

2.6. Next-generation DNA sequencing and data handling

The sample preparation for total metagenome sequencing of the pooled samples was carried out following the recommendations of the Ion Torrent PGM sequencing platform (Life Technologies). Sequencing was performed using Ion Torrent PGM 316 chips. The reads were analyzed and quality values were determined for each nucleotide. An average of 152,909 reads containing more than 31 million bp were identified. The average read length was 201 bp (Table 3). The individual sequences were further analyzed by using the MG-RAST software package (Meyer et al., 2008), which is a modified version of RAST (Rapid annotations based on subsystem technology). The MG-RAST server computes results against several reference datasets (protein and ribosomal databases) (MG-RAST, 2015). The acceptable percentage of identity was set to be >70%, the read length was >50 bp and the *e*-value cut-off was $<10^{-6}$. The generated matches of external databases were used to compute the derived data (Wirth et al., 2012; Kovács et al., 2013b; MG-RAST, 2015). The sequence data have been uploaded on the NCBI database, accession number SRA271138 and can be found on MG_RAST under project name "*Scenedesmus* fermentation".

3. Results and discussion

3.1. Biogas yield from Sc. obliquus

The amounts of biogas produced from the biomass substrates in the CSTRs were determined after a one-month start-up phase, i.e., in weeks 1–4 of the experiment (Fig. 1). During this preliminary period, the reactors were fed with the chosen substrate to ensure that all the remaining and digestible biomass from the inoculum (containing pig slurry + maize silage) had been degraded and did

Table 2
Lysis conditions for total-community DNA preparation.

	Lysozyme[a] (μL)	10% CTAB[b] (μL)	Genomic CTAB lysis buffer[c] (μL)	Qiagen buffer[d] (μL)	Zymo buffer[e] (μL)
A	–	100	–	100	550
B	250	100	–	100	300
C	250	–	300	200	–

[a] 100 mg/mL (Applychem).
[b] Cetyltrimethylammonium bromide (w/v).
[c] 1 M Tris–HCl 100 mL, 500 mM EDTA 50 mL, 5 M NaCl 300 mL, 10% CTAB, 20% SDS, pH 8 (Wirth et al., 2012).
[d] ASL buffer from Qiagen QIAamp DNA Stool miniprep kit (Qiagen, 51,504).
[e] From Zymo Research Fecal DNA kit (Zymo Research, D6010).

Please cite this article in press as: Wirth, R., et al., Metagenome changes in the mesophilic biogas-producing community during fermentation of the green alga *Scenedesmus obliquus*. J. Biotechnol. (2015), http://dx.doi.org/10.1016/j.jbiotec.2015.06.396

Table 3
Sequencing statistics.

AD	Bases	≥Q20[a]	Reads	Mean read lenght (bp)
Start point	35,892,397	30,596,563	182,567	196
Sc. obliquus week 1	30,457,018	26,703,086	155,532	195
Sc. obliquus week 5	28,436,345	24,543,128	136,052	209
Sc. obliquus week 9	30,357,877	26,253,819	146,689	206
Co-fermentation week 1	39,261,836	33,955,385	194,460	201
Co-fermentation week 5	39,555,893	34,418,139	197,742	200
Co-fermentation week 9	33,463,221	28,819,903	170,369	200
Maize silage week 1	25,257,622	21,699,918	124,637	202
Maize silage week 5	28,438,018	24,579,600	140,986	201
Maize silage week 9	26,403,818	22,626,256	126,530	208

[a] Predicted quality (Q20): Predicted error rate of one percent.

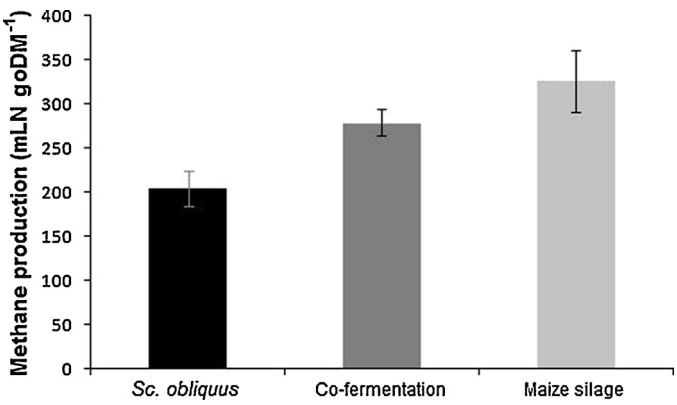

Fig. 2. Specific CH_4 production from the various biomasses.

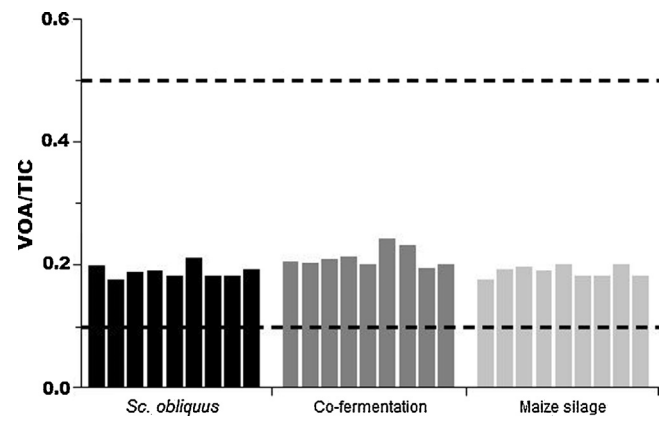

Fig. 3. Weekly measured VOA/TIC ratios. The area between the dashed lines indicates the optimum range.

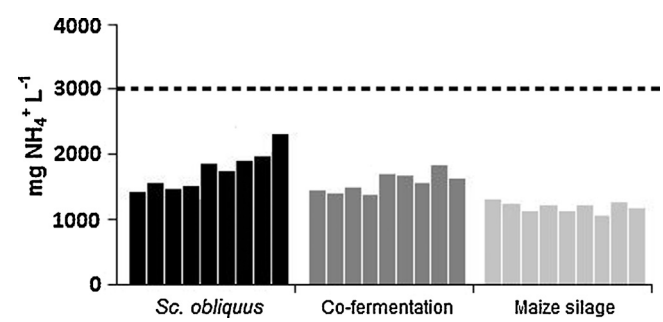

Fig. 4. Weekly measured NH_4^+ concentrations. The dashed line indicates the highest value recommended by the various studies.

not contribute to the biogas yield. The proper length of the start-up phase was determined in separate (unpublished) experiments. Gas production data were collected during weeks 5–9. The extent of biogas generation from the *Sc. obliquus* biomass was compared with that from co-digestion of the algal biomass and maize silage; reactors fed with maize silage alone were used as controls. The CH_4 concentration in the gas from the *Sc. obliquus* substrate proved to be 55–62%, which was comparable with previous findings (Mussgnug et al., 2010), although the average CH_4 content was somewhat lower in our 5 L fermenter. The biogas CH_4 concentration from the maize silage alone was 50–52%, as found previously (Amon et al., 2010). Co-digestion of the *Sc. obliquus* biomass + maize silage in a ratio of 1:1 yielded a CH_4 content of 52–56%, a value intermediate between those for the maize silage and the algal biomass. The daily average generated biogas volumes were as follows: from the maize silage 3.20 L day^{-1}, from the co-digestion 2.61 L day^{-1}, and from the *Sc. obliquus* biomass 1.79 L day^{-1}. Fig. 2 shows the specific average CH_4 production levels in normal mL (mLN) calculated in (g oDM^{-1}).

In biodiesel production, pure algal cultures are used to avoid contamination, which makes the production process expensive (Singh and Gu, 2010). The cost of the process can be reduced by using the algal residue in AD and the by-product biomass from biodiesel production is suitable for biogas generation (Sialve et al., 2009; Razon and Tan, 2011; Harun et al., 2011). Our results corroborated these findings. It is noteworthy that in biohydrogen production, pure algal cultures are not needed and this reduces the biomass cultivation price (Lakatos et al., 2014), while the algal–bacterial biomass remaining after biohydrogen production can be used for biogas yield (Wirth et al., 2015).

3.2. Process parameters during the AD fermentations

A constant value of VOA/TIC (Volatile organic acids/Total inorganic carbon) is a reliable indicator of a stable fermentation process (McGhee 1968; Nordmann, 1977). During the different AD processes, the average VOA was 2 g L^{-1} and the average inorganic carbon was 10–12 g $CaCO_3$ L^{-1} in all cases. Fig. 3 displays the weekly VOA/TIC ratios. Because of the low loading rate, the VOA/TIC ratios were on the low side, which allowed balanced operations.

The amount of NH_4^+ formed from nitrogen containing compounds present in the aqueous medium is an indicator of a stable biogas-forming process (Alexander, 1985). Theoretically, levels above 3000 mg NH_4^+ L^{-1} may have a negative effect on the methanogenic community, which is the most sensitive group of microbes in the AD process (Chen et al., 2008; Nielsen and Angelidaki, 2008).

As a result of the low C/N ratio (8.9:1) of the *Sc. obliquus* biomass, the NH_4^+ content steadily increased during the experiments, but remained under the recommended upper limit of 3000 mg NH_4^+ L^{-1} (Fig. 4). *Sc obliquus* develops a thick cell wall (Stainer et al., 1971), and the ammonification of AD may therefore be retarded. In

the co-digestion, the higher C/N ratio of the maize silage balanced the increasing NH_4^+ level.

3.3. Microbial community changes during the AD processes

The composition of the microbial community was investigated four times during the AD processes: at the beginning of feeding with the selected substrate (start), one week later (week 1), when the system was working at full capacity (week 5), and at the end of the process (week 9, see Sections 2.5 and 2.6).

3.3.1. Microbiological compositions of the substrates

The microflora of the maize silage consisted primarily of representatives of the genera *Lactobacillus* and *Acetobacter* (Fig. 5). Members of the genus *Lactobacillus* are to be found in the intestinal flora and they also thrive on degrading plant biomass. These microorganisms produce lactic acid from mono- and disaccharides (Makarova et al., 2006). Members of the genus *Acetobacter* are acetate producers (Yamada and Yukphan, 2008).

Sc. obliquus was cultivated in an industrial-scale tubular photobioreactor. The algal biomass was contaminated with a very low amount (1%) of bacterial cells. These bacterial species belong predominantly in the genus *Rhizobium*. The interactions of Rhizobia and plants are well known and similar mutualism has also been observed in the case of several microalgal species (Keshtacher-Liebso et al., 1995; Watanabe et al., 2005; Nikolaev et al., 2008; Amin et al., 2009; Kazamia et al., 2012; Xie et al., 2013; Kim et al., 2014; Wirth et al., 2015). These interactions facilitate the growth of algae and improve their resistance to environmental stresses.

3.3.2. Biogas-producing microbial community

The composition of the biogas-producing microbial community at the start of the experiment was very similar to that found in earlier studies in reactors fed with pig manure + maize silage (Wirth et al., 2012); it may therefore be regarded as an internal control with which to validate the metagenome sequencing method. In the detailed discussion of the metagenomic results, the unidentified sequences are disregarded.

3.3.2.1. Microbial community of maize silage fermentation (Bacteria domain). During the fermentation of maize silage, the dominant taxa were preserved and only small changes occurred in the composition of the taxa (Fig. 6). This was not surprising in view of the fact that the fermentation process had been maintained on maize silage supplemented with pig slurry prior to the start of the experiment. The members of the phyla *Firmicutes* and *Bacteroidetes* predominated. In the phylum *Firmicutes*, the order *Clostridiales* prevailed, followed by *Bacillales*, while in the phylum *Bacteroidetes* the order *Bacteroidales* was found most commonly.

3.3.2.2. Microbial community in the co-digestion (domain Bacteria). The microbial composition of the reactor fed with the *Sc. obliquus*

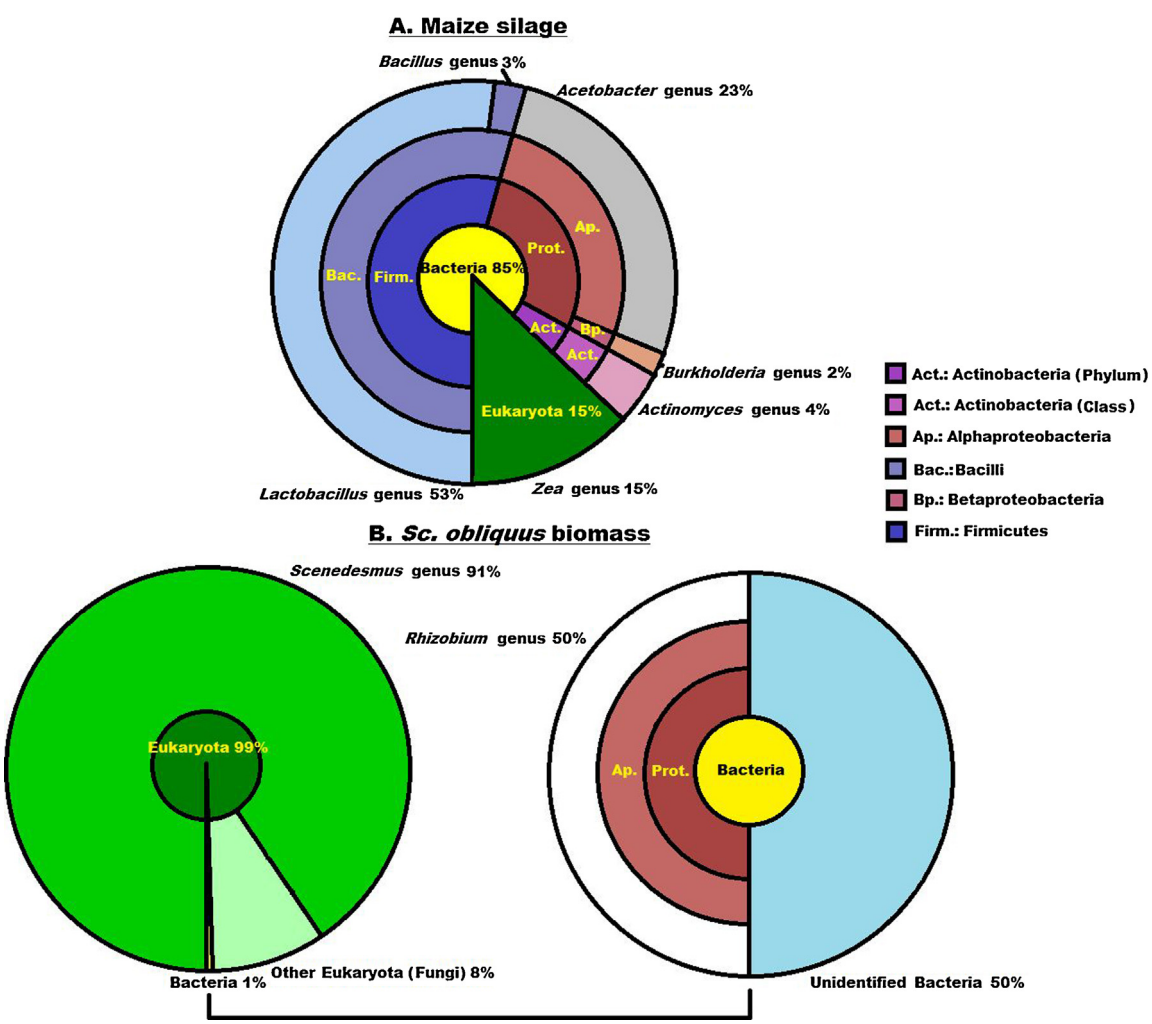

Fig. 5. Composition of the microbial community associated with the substrates: A. Maize silage, B. *Sc. obliquus* biomass. The communities are indicated at domain, phylum, class, and genus levels. The diagram on the right side of Fig. 4B shows the composition of the bacteria (total abundance 1%) in the algal biomass.

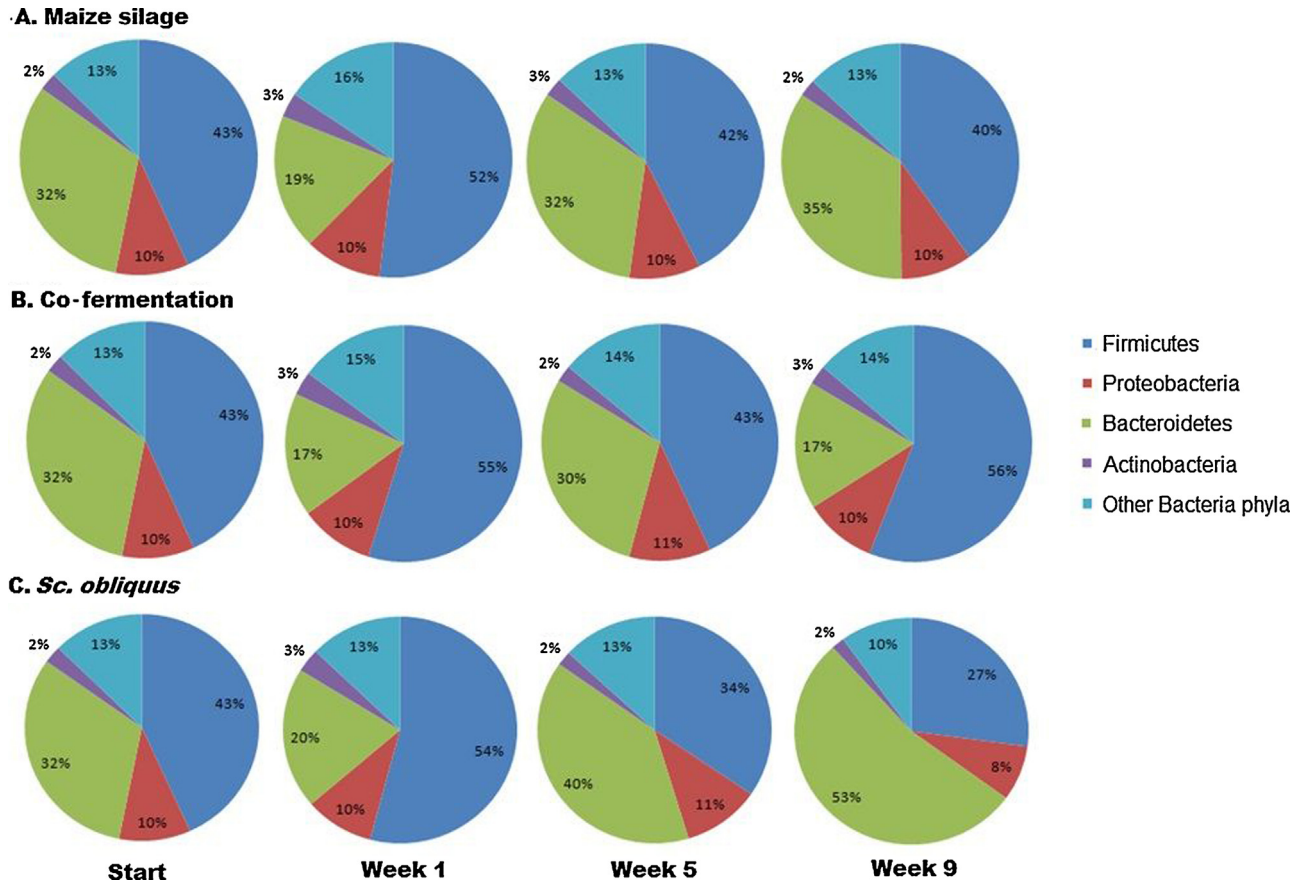

Fig. 6. Changes in the domain Bacteria of the microbial community at a phylum level.

algal biomass and maize silage displayed minor changes relative to that in the case of maize silage alone. The representatives of the phylum *Firmicutes* predominated in the bacterial community (Fig. 6). Within this taxon, the members of the order *Clostridiales* were found in great number. *Clostridiales* are well known as efficient cellulose-degrading bacteria (Schwarz, 2001); their thriving in the co-digestion is justified by their cellulase activity in the case of the presence of the maize silage. It should be noted that polysaccharide degrading metabolisms are significantly increased in the case of co-digestion (Fig. 7). This finding may be related to the observation that the co-digestion of microalgal biomass with waste paper improved the fermentation process in consequence of the higher C/N ratio of the mixed substrate and the induction of cellulase biosynthesis (Yen and Brune, 2007). The elevated cellulase activity may have contributed to the faster breakdown of the algal cell wall and the efficient release of valuable nutrients from the algal biomass, thereby increasing the biogas yield.

3.3.2.3. Microbial community in the Sc. obliquus *AD (domain Bacteria).* A pronounced shift in the biogas-producing microbial community was seen when the only substrate was the *Sc. obliquus* biomass. Because of the low bacterium contamination (1%) of the algal biomass the changes could readily be observed. Within the phylum *Bacteroidetes*, a predominance of the order *Bacteroidales* developed, with the concomitant decline of the representatives of the *Clostridiales* (Figs. 6 and 8), because of which the digestion of the microalgal cell wall was probably not as effective as in the co-digestion. This affected the subsequent steps in the biogas microbial food chain, influencing the biogas yield. Although *Bacteriodales*, which can degrade cellulose have been found in biogas reactors

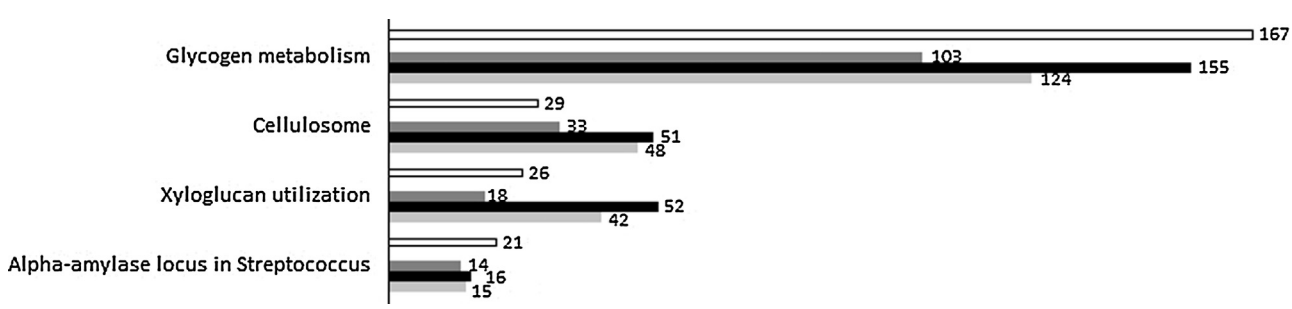

Fig. 7. Distribution of identified polysaccharide degrading and metabolism functions at week 5. Open column: at "start", light grey: maize silage, darker grey: *Sc. obliquus*, black: co-digestion.

Please cite this article in press as: Wirth, R., et al., Metagenome changes in the mesophilic biogas-producing community during fermentation of the green alga *Scenedesmus obliquus*. J. Biotechnol. (2015), http://dx.doi.org/10.1016/j.jbiotec.2015.06.396

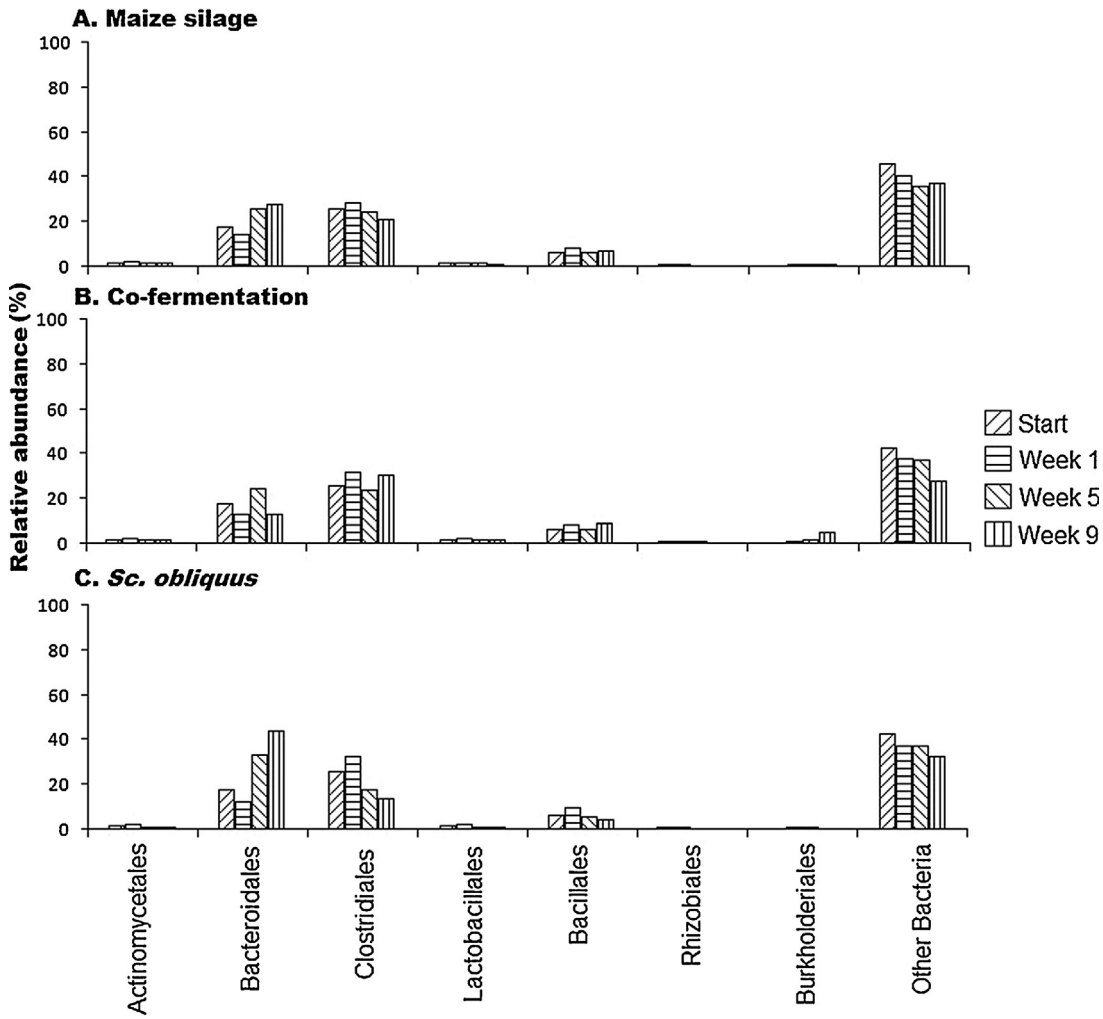

Fig. 8. Changes in the domain Bacteria of the microbial community at the order level.

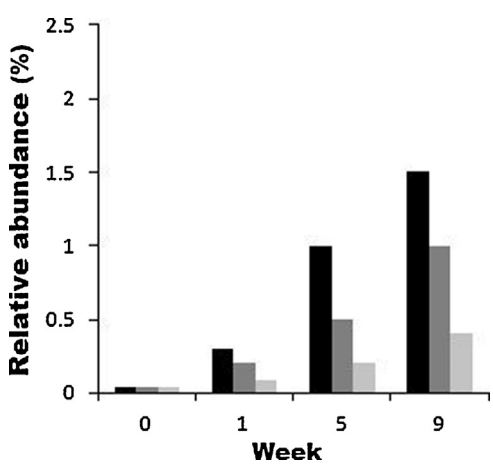

Fig. 9. Eukaryotic sequences in the reactors. Black: of *Sc. obliquus*, Grey: co-digestion, light grey: maize silage.

(Betian et al., 1977; Bjursel et al., 2006; Wirth et al., 2012), this order has primarily been considered to be a major participant in the secondary fermentation (Delbès et al., 2001; Hanreich et al., 2013). The ineffective degradation of the *Sc. obliquus* biomass is reflected in the abundance of eukaryotic DNA sequences in the reactors (Fig. 9). The eukaryotic DNA content in the samples taken from the algal biomass AD was three times higher than that in the case of the maize silage-fed digester, suggesting that the algal cell wall was more recalcitrant than the lignocellulosic material of the maize silage to microbial degradation.

3.3.2.4. The domain Archaea. The microbial composition of the domain Archaea was somewhat different from those in several previously studied mesophilic fermenters fed with maize (Schlüter et al., 2008; Krause et al., 2008; Kröber et al., 2009). Seasonal or uncontrolled factors may be involved in the background of this phenomenon (Rastogi et al., 2008; Lee et al., 2009). The order *Methanosarcinales* predominated in the Archaeal community throughout the entire study, practically independently of the substrate used, and their number even increased in time in the reactors containing the algal biomass. In an earlier study, in which the specific methanogen marker gene *mcrA* was monitored by next generation sequencing, the order *Methanosarcinales* was found in greatest abundance, and within this taxon the strictly acetoclastic genus *Methanosaeta* was predominant (Ellis et al., 2012). Interestingly, this genus was present in our fermentors too, though in less abundance. Within the order *Methanosarcinales*, the genus *Methanosarcina* was found in high abundance (Fig. 10). The main difference between the two genera of *Methanosarcinales* is that the members of the genus *Methanosarcina* are able to carry out all three pathways of methanogenesis, i.e., hydrogenotrophic, acetoclastic, and methylotrophic (Sirohi et al., 2010), while *Methanosaeta* can function only in the acetoclastic mode. Similarly, to our previous

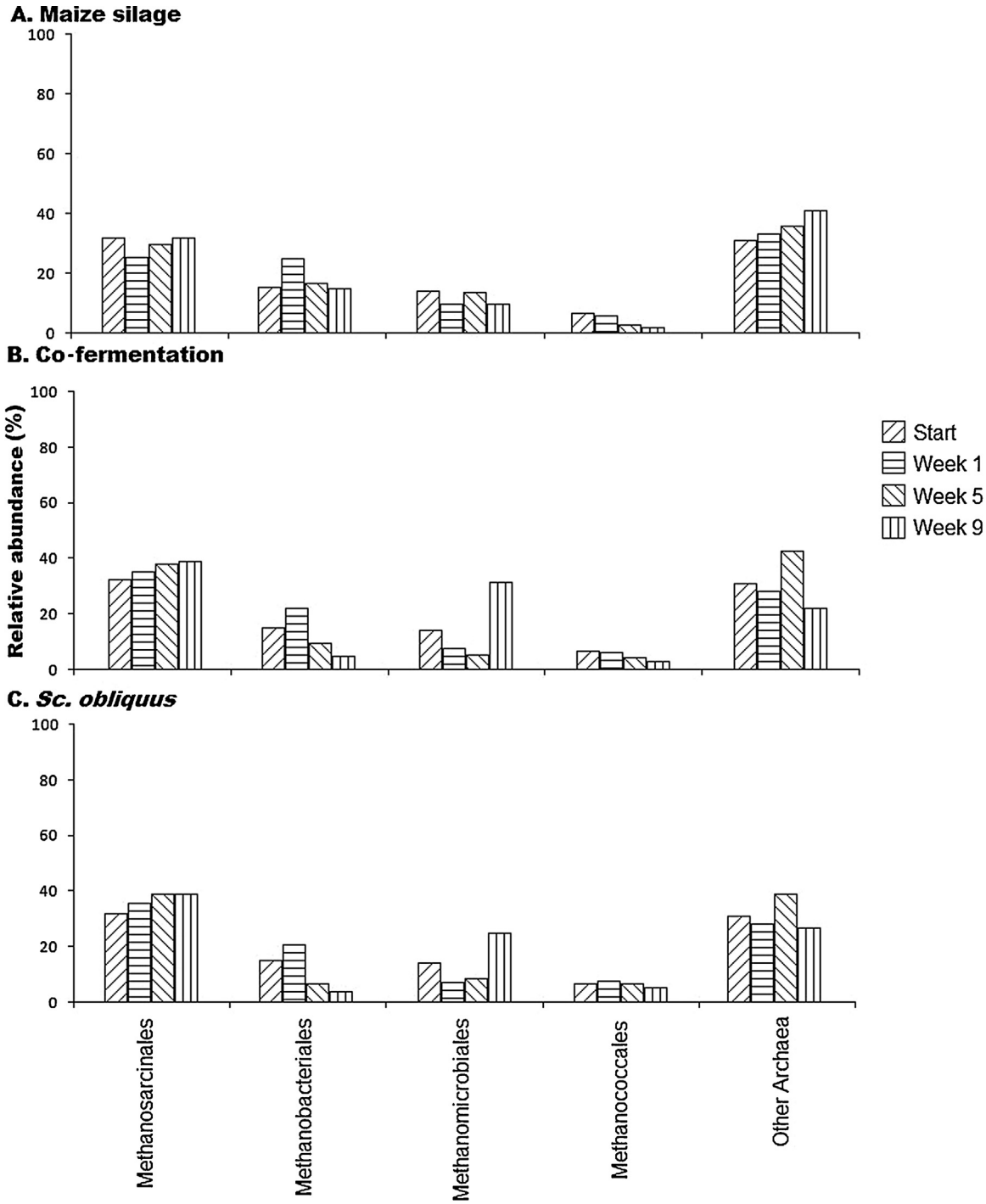

Fig. 10. Changes in the domain Archaea of the microbial community at the order level.

results (Wirth et al., 2015), the feeding with the microalgal biomass did not cause any appreciable changes in the methanogenic community.

4. Conclusions

The diversity of microalgal species allows their use in various ways. With the help of microalgae, biohydrogen, biodiesel, biogas or other valuable products can be produced. For the production of biodiesel and other valuable commodities, microbiologically pure algal biomass is needed, which increases the biomass production costs. In a biorefinery approach, the microalgal biomass that remains after the extraction of various chemicals is also a good substrate for biogas generation (Sialve et al., 2009; Lakaniemi et al., 2011). We have demonstrated in a separate study that biohydrogen generation is feasible by means of algal-bacterial co-culturing (Lakatos et al., 2014), and the biomass used is also an appropriate substrate for biogas generation in a two-step process (Wirth et al., 2015).

In the present study, a *Sc. obliquus* algal biomass was tested. The CH_4 content of the produced biogas (55–62%) proved to be higher than that from the commonly used maize silage (50–52%), but the biogas yield estimated on the basis of the organic material input was lower than that from maize silage. Stable operation was

achieved during the 2-month duration of the experiment. No sign of any upcoming process failure was observed, in spite of the low C/N ratio (8.9:1) of the *Sc. obliquus* algal substrate. *Sc. obliquus* has a thick cell wall, and a slow, though steady increase in ammonium ion content was therefore observed. Because of the delay in the attainment of efficient degradation of the algal biomass, a longer retention time is needed than the conventional retention times based on maize silage fermentation. In co-digestion, the maize silage added to the algal biomass increased the C/N ratio considerably and improved the digestibility of the microbial biomass and balanced the operation.

Metagenome analysis demonstrated that the composition of the microbial communities present in the AD reactors fed with the various substrate combinations changed as a result of the microalgal biomass. During the microalgal AD process, members of the order *Bacteroidales* became predominant. When the algal and maize biomasses were mixed in 1:1 oDM ratio, thereby increasing the C/N ratio of the substrate, the predominance of the order *Clostridiales* was maintained. The members of this order are noteworthy in the degradation of cellulose-containing materials; and they therefore appear important for microalgal AD processes too. The high cellulase activities of the members of the *Clostridiales* and the balanced C/N ratio led to co-digestion proving an efficient way to use algal biomass. In the control reactors fed with maize silage alone, the microbial taxa belonging to the phyla *Firmicutes* and *Bacteroidetes* persisted.

The pronounced changes observed in the domain Bacteria did not take place in the Archaeal community. The order *Methanosarcinales*, and within it the representatives of the metabolically versatile genus *Methanosarcina* predominated, regardless of the substrate composition.

Author contributions

RW, ZB, and NÁ developed the DNA extraction protocol, designed and performed the experiments and contributed to the evaluation of the metagenomic data. GL and TB took part in the execution of the anaerobic fermentation process and measurements. MK and AK arranged the large-scale algal fermentation with ELMAT Ltd., GM organized and performed the DNA sequencing work and participated in the evaluation of the data. KLK and ZB conceived the project, participated in its design and in the evaluation of the data. RW and KLK compiled the manuscript. GR supervised the operation and participated in the writing of the manuscript. All the authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

Acknowledgments

The authors thank Ms. Netta Bozóki for technical assistance and ELMAT Ltd. for the algal biomass cultivation. This work was supported by the domestic grants GOP-1.1.1-11-2012-0128 and PIAC_13-1-2013-0145 provided by the Hungarian Government, and financed by the Research and Technology Innovation Fund. Support from the EU project H2020-LCE-2014-2015-646533 BIOSURF is gratefully acknowledged.

References

Alexander, M., 1985. Biodegradation of organic chemicals. Env. Sci. Technol. 19, 106–111.
Amin, S.A., Green, D.H., Hort, M.C., Küpper, F.C., Sunda, W.G., Carrano, J.C., 2009. Photolysis of ion–siderophore chelates promotes bacteria–algal mutualism. Proc. Natl. Acad. Sci. U. S. A. 106, 17.071–17076.
Amon, T., Gruber, W., Hoffstede, U., Jäger, P., Jäkel, K., Kaiser, F., Keymer, U., Linke, B., Berettig-Bruns, U., Niebaum, A., Öchsner, H., Reinhold, G., Schwab, M., Telschow, D., Weiland, P., Welsch, W., Wesolowski, S., 2010. Gasausbeute in landwirtschaftichen biogasanlagen. KTBL, ISBN: 978-3-941583-49-9.
Batista, A.P., Moura, P., Marques, P.A.S.S., Ortigueia, J., Alves, J., Gouveia, L., 2014. *Scenedesmus obliquus* as feedstock for biohydrogen production by *Enterobacter aerogenes* and *Clostridium butyricum*. Fuel 117, 537–543.
Becker, E.W., 1983. The production of microalgae a source of biomass. Biomass Util. 67, 205.
Betian, H.G., Linehan, B.A., Bryant, M.P., Holdeman, L.V., 1977. Isolation of *Bacteroides* sp. from human feces. Appl. Environ. Microbiol. 33, 1009–1010.
Binot, R., Martin, D., Nyns, E.J., Naveau, H., 1977. Digestion anaerobic d'algues cultivees dans les aux de refroidissement industrielles. In: In: Proc. Heliosynthese Aquaculture Semin, Martigues, France, September, 20.
Bjursel, M.K., Martens, E.C., Gordon, J.I., 2006. Functional genomic and metabolic studies of the adaptations of a prominent adult human gut symbiont, *Bacteroides thetaiotaomicron*, to the suckling period. J. Biol. Chem. 281, 36269–36279.
Breuer, G., de Jaeger, L., Artus, V.P.G., Martens, D.E., Springer, J., Draaisma, R.B., Eggink, G., Wiffels, R.H., Lamers, P.P., 2014. Superior triacylglycerol (TAG) accumulation in starchless mutants of *Scenedesmus obliquus*: (II) evaluation of TAG yield and productivity in controlled photobioreactors. Biotech. Biofuels 7, 70.
Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Biores. Technol. 99, 4044–4064.
Chen, P., Min, M., Chen, Y., Wang, L., Li, Y., Chen, Q., Wang, C., Wan, Y., Wang, X., Cheng, Y., Deng, S., Hennessy, K., Lin, X., Liu, Y., Wang, Y., Martinez, B., Ruan, R., 2009. Review of biological and engineering aspects of algae to fuels approach. Int. J. Agric. Biol. Eng. 2, 1.
Dębowski, M., Zieliński, M., Grala, A., Dudek, M., 2013. Algae biomass as an alternative substrate in biogas production technologies-review. Renew. Sustain. Energy Rev. 27, 596–604.
Delbès, C., Moletta, R., Godon, J.-J., 2001. Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digester ecosystem. FEMS Lett. 35, 19–26.
Edward, M. 2009. The algal industry survey–a white paper by Dr. Mark Edward & Centre for management technology.
Ellis, J.T., Tramp, C., Sims, R.C., Miller, C.D., 2012. Characterization of a methanogenic community within an algal fed anaerobic digester. ISRN Microbiol., http://dx.doi.org/10.5402/2012/753892
Golueke, C.G., Oswald, W.J., Gotaas, H.B., 1957. Anaerobic digestion of algae. Appl. Microbiol. 5, 47–55.
González-Delgado, A.D., Kafarov, V., 2011. Microalgae based biorefinery: issues to consider. a review. CT&F – Ciencia. Tecnología Futuro 4, 5–22.
Guccione, A., Biondi, A., Sampietro, G., Rodolfi, L., Bassi, N., Tredici, M.R., 2014. Chlorella for protein and biofuels: from strain selection to outdoor cultivation in Green Wall Panel photobioreactor. Biotechnol. Biofuels 7, http://dx.doi.org/10.1186/1754-6834-7-84
Hanreich, A., Schimpf, U., Zakrewski, M., Schlüter, A., Benndorf, D., Heyer, R., Rapp, E., Pühler, A., Reichl, U., Klocke, M., 2013. Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation. Syst. Appl. Microbiol. 36, 330–338.
Harun, R., Davidson, M., Doyle, M., Gopiray, R., Danquah, M., Forde, G., 2011. Technoeconomic analysis of an integrated microalgae photobioreactor, biodiesel and biogas production facility. Biomass Bioenergy 35, 741–747.
Hernández, E.P.S., Córdoba, L.T., 1993. Anaerobic digestion of *Chlorella vulgaris* for energy production. Res. Con. Recyc. 9, 127–132.
Hodaifa, G., Martínez, E., Sanchez, S., 2008. Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*. Biores. Technol. 99, 1111–1117.
Jaenicke, S., Ander, C., Bekel, T., Bisdorf, R., Dröge, M., Gartemann, K.-H., Jünemann, S., Kaiser, O., Krause, L., Tille, F., Zakrzewski, F., Pühler, A., Schlüter, A., Goesmann, A., 2011. Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454-pyrosequencing. PLoS One 6, e14519.
Kazamia, E., Czesnick, H., Nguyen, T.T.V., Croft, M.T., Sherwood, E., Sasso, S., Hodson, S.J., Warren, M.J., Smith, A.G., 2012. Mutualistic interaction between vitamin B-12 dependent algae and heterotrophic bacteria exhibit regulation. Environ. Microbiol. 14, 1466–1476.
Keenan, J.D., 1977. Bioconversion of solar energy to methane. Energy 2, 365.
Keshtacher-Liebso, E., Hadar, Y., Chen, Y., 1995. Oligotrophic bacteria enhance algal growth under ion-deficient conditions. Am. Soc. Microbiol. 61, 2411–2439.
Kim, B.-H., Ramanan, R., Cho, D.-H., Oh, H.-M., Kim, H.-S., 2014. Role of *Rhizobium*, a plant growth promoting bacterium, in enhancing algal biomass through mutualistic interaction. Biomass Bioenergy 69, 95–105.
Kovács, E., Wirth, R., Maróti, G., Bagi, Z., Rákhely, G., Kovács, K.L., 2013a. Biogas production from protein-rich biomass: fed- batch anaerobic fermentation of casein and pig blood and associated changes in microbial community composition. PLoS One 8, e77265.
Kovács, K.L., Ács, N., Kovács, E., Wirth, R., Rákhely, G., Strang, O., Herbel, Z., Bagi, Z., 2013b. Improvement of biogas production by bioaugmentation. BioMed. Res. Int., http://dx.doi.org/10.1155/2013/482653
Krause, L., Diaz, N.N., Edwards, R.A., Gartemann, K.-H., Krömeke, H., Neuwger, H., Pühler, A., Runte, K.J., Schlüter, A., Stoye, J., Szczepanowski, R., Tauch, A., Goesmann, A., 2008. Taxonomic composition and gene content of a

methane-producing microbial community isolated from a biogas reactor. J. Biotechnol. 136, 91–101.
Kröber, M., Bekel, T., Diaz, N.N., Goesmann, A., Sebastian, J., 2009. Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. J. Biotechnol. 142, 38–49.
Lakaniemi, A.-M., Hulatt, C.J., Thomas, D.N., Tuovinen, O.H., Puhakka, J.A., 2011. Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. Biotechnol. Biofuels 4, 34.
Lakatos, G., Deák, Z., Vass, I., Rétfalvi, T., Rozgonyi, S., Rákhely, G., Ördög, V., Kondorosi, É., Maróti, G., 2014. Bacterial synbionts enhance photo-fermentative hydrogen evolution of Chlamydomonas algae. Green Chem., http://dx.doi.org/10.1039/C4GC00745J
Lee, C., Kim, J., Hwang, K., O'Flaherty, V., Hwang, S., 2009. Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters. Water Res. 43, 157–165.
Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.-H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acid. Sci. U. S. A. 103, 15611–15616.
Mandal, S., Mallick, N., 2009. Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. Appl. Microbiol. Biotechnol. 84, 281–291.
Mata, M.T., Meloa, A.C., Meirelesb, S., Mendes, A.M., Martins, A.A., Caetano, N.S., 2013. Potential of microalgea *Scenedesmus obliquus* grown in brewery wastewater for biodiesel production. Chem. Eng. Trans. 32, 901–906.
McGhee, T.J., 1968. A method for approximation of the volatile acid concentrations in anaerobic digesters. Water Sewage Works 115, 162–166.
Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J., Edwards, R.A., 2008. The metagenomics RAST server -a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinf. 9, 386.
MG-RAST manual for version 3.3.6 revision 9. ftp://ftp.metagenomics.anl.gov/data/manual/gmg-rast-manual.pdf
Mussgnug, J.H., Klassen, V., Schlüter, A., Kruse, O., 2010. Microalgae as a substrates for fermentative biogas production in a combined biorefinery concept. J. Biotechnol. 150, 51–56.
Nielsen, H.B., Angelidaki, I., 2008. Strategies for optimizing recovery of the biogas process following ammonia inhibition. Biores. Technol. 99, 7995–8001.
Nikolaev, Y.A., Plakunov, Y.K., Voronina, N.A., Nemtseva, N.V., Platnikov, A.O., Gogoleva, O.A., Murav'eva, M.E., Ovechkina, G.V., 2008. Effect of bacterial satellites on *Chlamydomonas reinhardtii* in an algo-bacterial community. Microbiology 77, 78–83.
Nordmann, W., 1977. Die Überwachtung der Schlammfaulunk. KA-Informationen für das Betriebspersonal, Beilage zur Korrespondenz Abwasser 3/77.
Posten, C., Schaub, G., 2009. Microalgae and terrestrial biomass as source for fuels-a process view. J. Biotechnol. 142, 64–69.
Rastogi, G., Ranade, D.R., Yeole, T.Y., Patole, M.S., Houche, Y.S., 2008. Investigation of methanogen population structure in biogas reactor by molecular characterization of methyl-coenzyme M reducrase A (*mcrA*) genes. Biores. Technol. 99, 5317–5326.
Razon, L.F., Tan, R.R., 2011. Net energy analysis of the production of biodiesel and biogas from the microalgae: *Haematococcus pluvialis* and *Nannochloropsis*. Appl. Energy 88, 3507–3514.
Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Staier, R.Y., 1979. Generic assessments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1–61.
Samson, R., LeDuyt, A., 1982. Detailed study of anaerobic digestion of *Spirulina maxima* algal biomass. Biotechnol. Bioeng. 24, 1919.
Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B., 2008. Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Res. 1, 20–43.
Schlüter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., Mussgnug, J.H., Neuweger, H., Niehaus, K., Pühler, A., Runte, K.J., Szczepanpwski, R., Tauch, A., Tilker, A., Viehöver, P., Goesmann, A., 2008. The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analyzed by the 454-pyrosequencing technology. J. Biotechnol. 136, 77–90.
Schwarz, W.H., 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. 56, 634–649.
Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol. Adv., http://dx.doi.org/10.1016/j.biotechadv.2009.03.001
Singh, J., Gu, S., 2010. Commercialization potential of microalgae for biofuels production. Renew. Sustain. Energy Rev. 14, 2596–2610.
Sirohi, S.K., Pandey, N., Singh, B., Puniya, A.K., 2010. Rumen methanogens: a review. Indian J. Microbiol. 50, 253–262.
Stainer, R.Y., Kunisawa, R., Mandel, M., Cohen-Bazire, G., 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol. Rev. 35, 171–205.
Stantscheff, R., Kuever, J., Rabenstein, A., Seyfarth, K., Dröge, S., König, H., 2014. Isolation and differentation of methanogenic *Archaea* from mesophilic corn-fed on-farm biogas plants with special emphasis ont he genus *Methanobacterium*. Appl. Environ. Biotechnol. 98, 5719–5735.
Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svenson, B.H., Sørensen, S.J., Karlsson, A., 2013. 454 pyrosequencing analysis of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol. Ecol. 85, 612–626.
Uziel, M., Oswald, W.J., Golueke, C.G., 1974. Solar energyfixation and conversion with algal?bacterial system. In: U.S. National Science Foundation Report. No. NSF-RA-N-74-195. NSF, Washington, D.C, pp. 1974.
Watanabe, K., Takihana, N., Aoyagi, H., Hanada, S., Watanabe, Y., Ohmura, N., Saiki, H., Tanaka, H., 2005. Symbiotic association in *Chlorella* culture. FEMS Microbiol. Ecol. 51, 187–196.
Ward, A.J., Lewis, D.M., Green, F.B., 2014. Anaerobic digestion of algae biomass: a review. Algal Res., http://dx.doi.org/10.1016/j.algal.2014.02.001
Wirth, R., Kovács, E., Maróti, G., Bagi, Z., Rakhely, G., Kovács, K.L., 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnol. Biofuels 5, 1–16.
Wirth, R., Lakatos, G., Maróti, G., Bagi, Z., Minárovics, J., Nagy, K., Kondorosi, É., Rákhely, G., Kovács, K.L., 2015. Exploitation of algal–bacterial associations in a two-stage biohydrogen and biogas-generation process. Biotechnol. Biofuels 8, 1–14.
Xie, B., Bishop, S., Stessman, D., Wright, D., Spalding, M.H., Halverson, L.J., 2013. *Chlamydomonas reinhardtii* thermal tolerance enhancement mediated by mutualistic interaction with vitamin B_{12}-producing bacteria. ISME J. 7, 1544–1555.
Xu, K., Jang, H., Juneau, P., Qiu, B., 2012. Comperative studies on the photosyntethic responses of three freshwater phytoplancton species to temperature and light regiments. J. Appl. Phycol., http://dx.doi.org/10.1007/s10811-011-9741-9
Yamada, Y., Yukphan, P., 2008. Genera and species in acetic acid bacteria. Int. J. Food Microbiol. 125, 15–24.
Yen, H.-W., Brune, D.E., 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. Biores. Technol. 98, 130–134.
Yen, H.-W., Hu, I.-C., Chen, C.-Y., Ho, S.-H., Lee, D.-J., Chang, S., 2013. Microalgae based biorefinery- from biofuels to natural products. Biores. Technol. 135, 166–174.
Ziganshina, E.E., Bagmanova, A.R., Khilyas, I.V., Ziganshin, A.M., 2014. Assesment of biogas-generating microbial community in a pilot-scale anaerobic reactor. J. Biosci. Bioeng. 117, 730–736.