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Effect of co-substrate feeding on methane yield of anaerobic digestion of *Chlorella vulgaris*

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Abstract Microalgal production has many advantages over the use of terrestrial plants; therefore, increases in the use of microalgae for energy production can be expected. Algal biomass can be processed anaerobically to methane; however, the unfavorable C/N ratio of the substrate may have an inhibitory effect. The impact of the application of used cooking oil, maize silage, and mill residue on anaerobic co-digestion of the microalgal *Chlorella vulgaris* was studied in semi-continuous, laboratory-scale digestion. During the full period of the trial involving anaerobic digestion of algae in the case of mono-digestion and co-digestion with used cooking oil, maize silage, and mill residue, the volumetric methane yields were 0.38 ± 0.07, 1.56 ± 0.26, 1.19 ± 0.18, and 1.16 ± 0.13 L L^{-1}, respectively. Trials were carried out to determine the long-term effect of the total solid (TS) content of substrates (co-digestion of *C. vulgaris* and used cooking oil at 3.8 and 7.2 % of TS, respectively). Both designs could be increased to 5.5 g VS L^{-1} d^{-1}, but a higher TS% resulted in increased methane production and a longer period of decline in the methane yield due to washout. The sharp decrease in methane content at the end of 90 days was accompanied by a reorganization of the methanogenic archaeal community.

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Introduction

In fossil fuel-poor countries, the use of biomass to meet energy demands poses a substantial challenge. The output of energy crops is limited by competition with primary food production (Rathmann et al. 2010). Microalgal production offers many advantages over the use of terrestrial plants, because microalgae have higher photosynthetic efficiencies and higher biomass yields, and non-conventional rural areas can be used for algal cultivation (Adenle et al. 2013; Mussgnug et al. 2010). The main energetic use of algal biomass can be highly variable; it is regarded as the source of gaseous (methane and biohydrogen) and liquid biofuels (biodiesel, ethanol, and butanol) (Amin 2009; Lakaniemi et al. 2011). In the case of non-pretreated microalgal biomass, the highest-energy yield with methanogenic digestion is very close to the value obtained for ethanol production (Lakaniemi et al. 2011). The anaerobic digestion (AD) process of algal biomass of low lipid content produces a significantly larger amount of energy compared with biodiesel alone (Bohutskyi et al. 2014b). The conversion rate of biomass depends greatly on the species of algae and the digestion and pretreatment method (Dębowski et al. 2013). In a non-defined mixed culture of freshwater algae, the methane concentration of biogas varies between 40 and 65 % (De Schamphelaire and Verstraete 2009). In the case of the microalgae *Chlorella* spp., the methane yield ranges from 174 to more than 400 mL g^{-1} (Lakaniemi et al. 2013). Bohutskyi et al. (2014a) found that thermochemical pretreatment improved methane yield by 30–40 % from certain species. Thermal hydrolysis can also enhance methane productivity by 46–62 % (Alzate et al. 2012).

The biochemical composition of algal biomass has a major impact on methane production potential (Bohutskyi and Bouwer 2013). Algae with high carbohydrate and protein contents are theoretically poorer substrates for methane production than lipid-rich algae (Bohutskyi et al. 2014a). The optimal C/N ratio (*w*/*w* %) for AD is 25–30, but this ratio is only 6.7 in *Chlorella* sp. and *Scenedesmus* sp. cultures (Costa et al. 2012; Zhong et al. 2012; Dębowski et al. 2013; Wang et al. 2013).

Due to the low C/N ratio of microalgal biomass, the addition of a carbon-rich co-substrate has benefits for biogas production (Kwietniewska and Tys 2014). Yen and Brune (2007) found that co-digestion of wastepaper and algal sludge could efficiently balance the feedstock carbon and nitrogen ratio, thus improving methane productivity. The addition of maize silage as a co-substrate is widely used in practice, particularly in the case of high-nitrogen media (Yangin-Gomec and Ozturk 2013), contrary to the restriction of ammonia toxicity (Prajapati et al. 2013).

The vulnerability of the biogas production system is based on the robustness of the microbial community. Kim et al. (2014) found that during digestion of *Ulva* biomass in batch bioreactors, the bacterial community structure varied continuously and dynamically while the archaeal composition changed little. A higher ammonia content is toxic to methanogens and is related to increased volatile fatty acid (VFA) concentrations (Chen et al. 2008).

Most methane production trials have been carried out by batch digestion (Passos et al. 2013; Zhao et al. 2014), but semi-continuous feeding reactors have provided more information on sustainable operation and reliable methane yield (Bohutskyi and Bouwer 2013; Kolbl et al. 2014).

The cost to harvest algae is significantly influenced by the total solid content (TS%) of the algal product. The effect of the TS% of the algal substrate on AD yields has not yet been clarified. Various digestion technologies based on wastewater treatment have been developed for use with material of different TS% (Zamalloa et al. 2011; Alzate et al. 2012).

The main objectives of this study were to investigate the effect of different co-substrates (maize silage, used cooking oil, and mill residue) on methane production in AD of *Chlorella vulgaris*. In addition, the effects of different substrate TS% on methane yield and alteration of metagenomic consortia were investigated.

Materials and methods

Cultivation, harvesting, and pretreatment of algae

Microalgal biomass of *Chlorella vulgaris* (MACC-755) derived from the Mosonmagyarovar Algal Culture Collection (MACC) was produced in tubular photobioreactors by Agro-Bioferment LLC in Hungary. The algal suspension was harvested using an Alfa Laval Clara 80 separator (Alfa Laval, Sweden), and the biomass was oven-dried at 40 °C. The dried biomass was ground in a porcelain mortar to break the algal cells. Twenty milligram of algal biomass was resuspended in 100 mL distilled water before and after the cells were disrupted. The numbers of intact cells in both samples were counted in a Bürcker chamber under a microscope to calculate the proportion of broken cells, which was 28 ± 4 %.

Biogas reactors and substrates

The anaerobic digesters were 1-L working volume bottles (2.50 L capacity threaded brown bottles; Merck, Germany). The digesters were incubated in a water bath (Memmert WNB 14 Basic, Memmert GmbH & Co.) at a constant temperature (38 °C). Anaerobic digester inoculum was obtained from a biogas plant (Sugar Factory, Kaposvár, Hungary) operating with sugar beet-pressed pulp (20 day hydraulic retention time (HRT)). The reactors were manually mixed three times per day. Biogas was collected in Tedlar gas sampling bags, and its volume was measured with Hamilton Gas Tight Syringe (Sigma-Aldrich). The biogas and methane yields were recalculated according to the standard conditions for pressure and temperature.

The *C. vulgaris* (MACC-755) raw material had the following composition: protein 60.2 ± 2.1 % of TS, carbohydrates 13.5 ± 0.6 %, and lipids 11.2 ± 0.4 %. Crude protein and lipids were analyzed according to the methods previously described by Ördög et al. (2012). Carbohydrate content was measured using a method based on the MSZ 6830/26 (Hungarian standard for determination of feed nutrient value and sugar content).

The algal solution [20 % TS and 18 % volatile solids (VSs)] was prepared by distilled water once a week and kept in a refrigerator until feeding. Methane yield can be increased by optimization of the C/N ratio using co-substrates instead of mono-digestion of *C. vulgaris*. Different types of co-substrates were used to determine the best method of raising the C/N ratio. The C/N ratios and the TS% and VS% values of substrates can be found in Table 1. Co-digestion was achieved by addition of co-substrates at 50 % VS content in all cases.

Table 1 C/N ratio, TS%, and VS% values of the substrates

Substrates	C/N ratio	TS%	VS%
Chlorella vulgaris	5	94	84
Used cooking oil	1000	100	99
Maize silage	28	30	29
Mill residue	17	88	81
C. vulgaris and used cooking oil	477	97	92
C. vulgaris and maize silage	16	62	57
C. vulgaris and mill residue	12	91	82
Optimal	20	–	–

Co-digestion with used cooking oil was repeated at 3.8 and 7.2 % of TS in the substrate mixture. Algal substrates were diluted in distilled water. The duration of all trials was 90 days.

In all trials, substrate overloading was employed to determine the achievable maximum organic loading rates (OLRs). Feeding was controlled and redesigned based on titrated VFA (tVFA) measurements. The daily feeding amount was weighed on an analytical balance and added to the bioreactor once a day through a stopcock. The use of additives is necessary for the maintenance of optimal digestion conditions (macronutrient and micronutrient) (Rétfalvi et al. 2011). A trace element supplement solution consisting of 10,875 mg manganese, 20,000 mg Co, 1625 mg Zn, 163 mg Cu, 138 mg Mo, 113 mg Se, and 93 mg Bo, in organic complex form, per liter of solution (42.2 % TS), was also used. Phosphoric acid was obtained from Sigma-Aldrich Co. and added as phosphorous supplement at 75 % (*v*/*v*).

Chemical analysis of sludge

Samples of sludge (10 mL) were taken for chemical analysis before feeding. Samples were centrifuged for 10 min at 3420×*g* (EBA 21, A. Hettich, Germany). From the resulting supernatant, 5 mL was used for the determination of tVFA levels using a potentiometric pH meter (EuTech PC 510, ThermoFisher Scientific) and calculated in acetic acid equivalents (Rétfalvi et al. 2011). The components of biogas were analyzed using an Ecoprobe 5-IR (RS Dynamics Ltd., Czech Republic). The TS contents of substrates and digestion sludge were determined by weight loss drying the samples at 105 °C. The TS% is a measure of the amount of material remaining after all of the water has been evaporated. The VS% was measured by weight loss ignition of the dried samples at 600 °C.

The C/N ratio was determined using an Elementar vario MAX CNS analyzer (Elementar Analysensysteme GmbH, Germany) coupled to a WLD detector. The measured mass was 80–100 mg, the burning temperature was 1140 °C, and the carrier gas was helium. Other chemical parameters (chemical oxygen demand (COD), total P, and soluble NH_4^+) were monitored weekly during the experiment to ensure stable operation. COD determination was carried out according to the Hungarian standard protocol (MSZ ISO 6060). The method was based on the oxidation of the oxidizable organic matter by an excess of potassium dichromate solution in the presence of $HgSO_4$ and a Ag catalyst. The excess potassium dichromate is titrated with ferrous ammonium sulfate. The COD value is calculated from the reduced amount of Cr^{3+}.

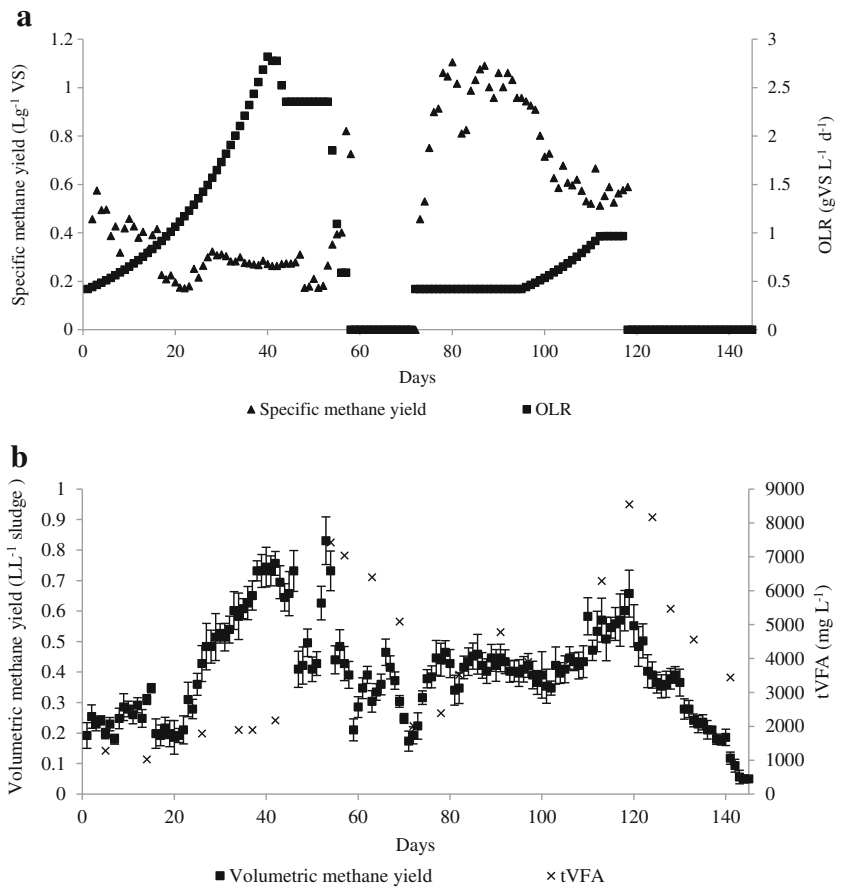

Fig. 1 **a** Alteration of specific methane yield with OLR during mono-digestion of *Chlorella* microalgae. **b** Alteration of tVFA levels with volumetric methane yield during mono-digestion of *Chlorella* microalgae

The determination of ammonium and total phosphorus was carried out according to the Hungarian standard protocols MSZ ISO 7150-1 and MSZ 488/18-77, respectively. The methods are based on a manual spectrophotometric measurement. In the case of ammonium measurement in the presence of nitroso-pentaciano-sodium-ferrate, ammonium reacts with salicylate and hypochlorite ion. The absorbance of the resulting blue compound can be measured by spectrophotometry.

Water-soluble orthophosphate was determined directly; all other forms of phosphorus were converted into orthophosphate by hydrolysis and destruction. Orthophosphate gave a blue color after reaction of molybdate in a sulfuric acid medium in the presence of antimony(III) ion.

Molecular characterization

Isolation of metagenomic DNA Total DNA was extracted from the three samples according to the method of Sharma et al. (2007) with minor modifications.

Samples (0.600 mL) were suspended in 0.650 mL of extraction buffer (100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 1.5 M NaCl and 100 mM sodium phosphate, pH 8.0; and 1 % CTAB) and 0.0035 mL proteinase K (20.2 mg mL^{-1}) and incubated horizontally at 37 °C for 45 min, after which time 0.080 mL of 20 % SDS was added. Samples were mixed by inversion several times with further incubation at 60 °C for 1 h. Samples in each tube were mixed thoroughly at 15-min intervals. The particles were collected by centrifugation (350×*g*) for 5 min.

The supernatant was transferred into clean tubes, mixed with equal quantities of phenol chloroform and isoamyl alcohol (25:24:1), and extracted two or three times. DNA was precipitated with 0.7 volumes of isopropanol, and the pellet was washed with 70 % ethanol. Crude DNA pellets were dried and dissolved in 0.050 mL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

Metagenomic DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen, USA). Half of the total metagenomic DNA from the parallel samples was pooled and stored at −20 °C for sequencing.

Metagenome sequencing Total environmental DNA was sequenced using the Ion Torrent PGM platform (Life Technologies, USA). Ion Torrent PGM fragment libraries of

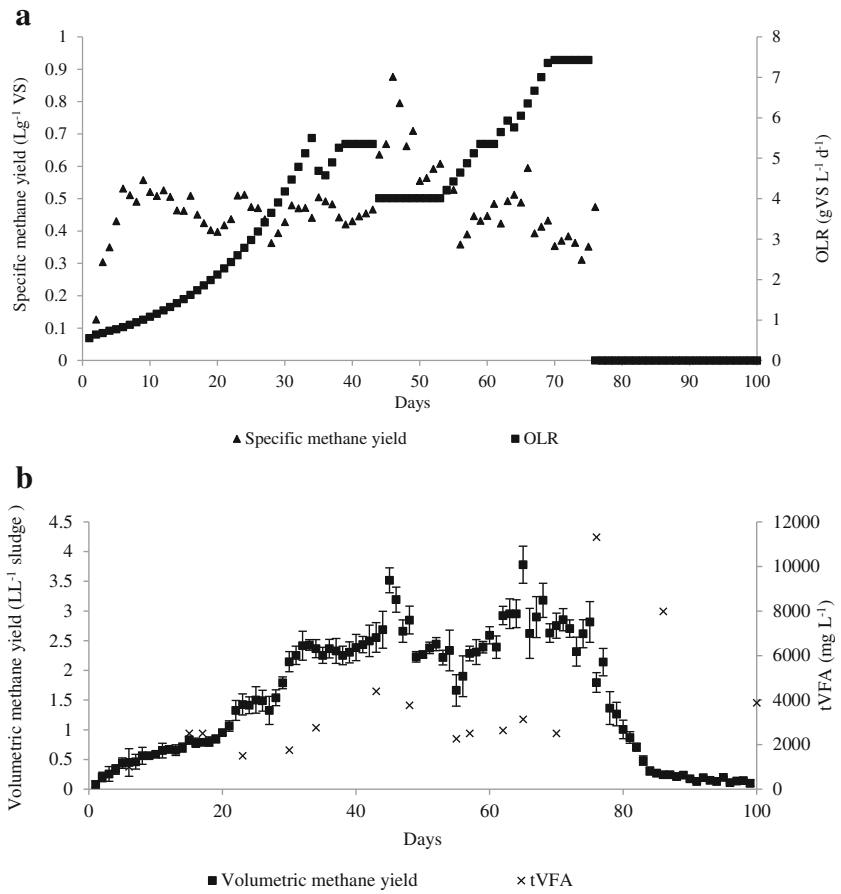

Fig. 2 **a** Alteration of specific methane yield with OLR during co-digestion of *Chlorella* microalgae with used cooking oil. **b** Alteration of tVFA levels with volumetric methane yield during co-digestion of *Chlorella* microalgae with used cooking oil

200 nt were generated according to the appropriate protocols. Metagenomic DNA (1 μg) pooled from each sample was used for library preparation. DNA shearing and end-repair were achieved using the Ion Xpress Plus Fragment Library Kit, and DNA was purified using a PureLink PCR Purification Kit.

Adapter ligation and nick translation were performed using the Ion Shear Plus Reagents Kit. Size selection was performed in a 2 % agarose gel to enrich the 300–350-nt fragments; then, library amplification was achieved using Platinum PCR SuperMix. Ion Library TaqMan qPCR was used for quantitation, and the Ion Xpress Template 200 ePCR Kit was used for emulsion PCR. Sequencing was performed on an Ion Torrent PGM using the Ion 316 chip. Sequencing resulted in 350,944 sequence reads for the initial sludge (first day), with an average read length of 198 ± 62 nt and 61,791,428 ≥ Q20 value; 383,162 sequence reads were obtained for the 3.8 % algal sample (90th day) with an average read length of 201 ± 59 nt and 68,634,313 ≥ Q20 value and 322,006 sequence reads for the 7.2 % algae sample (90th day) with an average read length of 190 ± 58 nt and 54,548,009 ≥ Q20 value.

Bioinformatic analysis An initial quality control step was followed by an automatic normalization of the FASTQ sequence data with a maximum *e*-value cutoff of 10–5, a minimum percentage identity cutoff of 80 %, and a minimum alignment length cutoff of 15 bp. Taxonomic assessments were performed after data filtering. For the taxonomic analyses, a metagenomic analysis server (MG-RAST) was employed using various ribosomal RNA databases.

Results

Mono- and co-digestion of algae

To compare the effect of co-substrates on OLR rate and methane yield, progressive loading experiments were carried out.

Mono-digestion of algae At the beginning of the mono-digestion trial, the OLR was raised by 5 % per day from 0.42 to 2.82 g VS L^{-1} d^{-1} (on day 40), then decreased by 16 % per day to 2.36 g VS L^{-1} d^{-1}, and held constant for 9 days (Fig. 1a). In association with the OLR, the daily specific methane yield reached its maximum on day 53 (0.83 L g^{-1} VS). This was presumably due to the effect of delayed decomposition of accumulated organic matter. During the overload period, tVFA levels increased slowly (from 1282 to 2176 mg L^{-1}; Fig. 1b) and dissolved NH_4^+

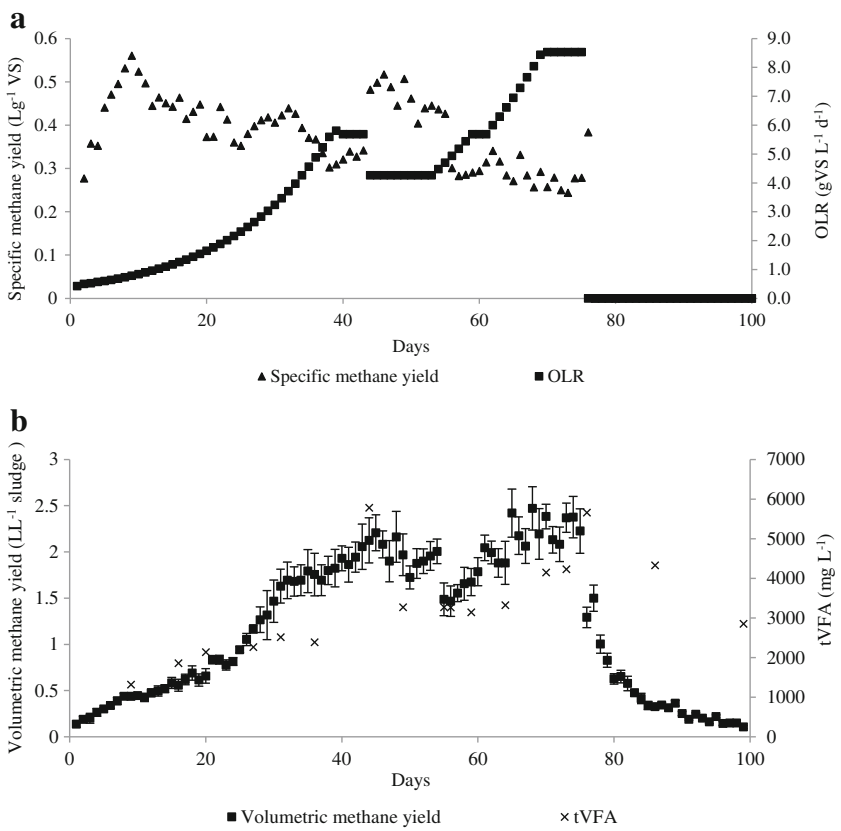

Fig. 3 **a** Alteration of specific methane yield with OLR during co-digestion of *Chlorella* microalgae with maize silage. **b** Alteration of tVFA levels with volumetric methane yield during co-digestion of *Chlorella* microalgae with maize silage

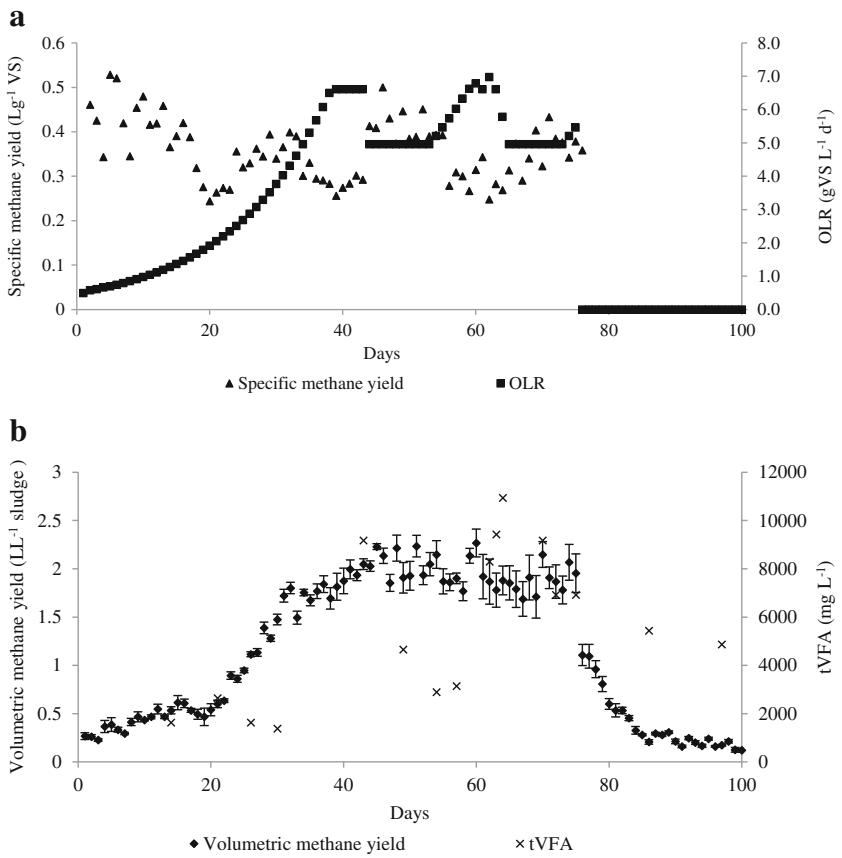

Fig. 4 **a** Alteration of specific methane yield with OLR during co-digestion of *Chlorella* microalgae with mill residue. **b** Alteration of tVFA levels with volumetric methane yield during co-digestion of *Chlorella* microalgae with mill residue

concentration increased 10-fold from 229 to 2302 mg L^{-1}. On day 54, the tVFA concentration increased drastically to 7424 mg L^{-1}, so the OLR was decreased to 0 g VS L^{-1} d^{-1} for 14 days (Fig. 1a). Due to the reduced OLR, all parameters (volumetric and specific methane yield, tVFA) had decreased to near their respective minimum levels.

The stable run of OLR at 0.42 g VS L^{-1} d^{-1} from days 72 to 95 resulted in an average daily volumetric methane yield of 0.40 L L^{-1} d^{-1} with increasing tVFA levels (up to 4779 mg L^{-1}). The increase in the NH_4^+ level to 4972 mg L^{-1} on day 97 (pH 8.49) and to 5547 mg L^{-1} on day 141 (pH 8.41) partially inhibited methanogenesis, causing tVFA levels to rise. This observation is consistent with the findings of Prajapati et al. (2013). In the reloading period (starting to feed the digesters again), OLR levels could not be increased above 0.97 g VS L^{-1} d^{-1} due to the high tVFA level (8551 mg L^{-1}); thus, the trial was ended by withdrawal of substrate.

The reduction in volumetric methane yield from 0.66 to 0.05 L L^{-1} d^{-1} lasted 26 days. The average volumetric methane yield during the experiment was 0.44 L L^{-1} d^{-1}, which was similar to the value of 0.57 L L^{-1} d^{-1} obtained by Yen and Brune (2007) for the digestion of algal biomass at a loading rate of 4 g VS L^{-1} d^{-1} (10-day HRT). Because of the biogas potential of the active inoculum, the methane yield over the first 20 days was not included in the average yields.

Algae-used cooking oil co-digestion During the upload period, the OLR was raised from 0.55 to 5.50 g VS L^{-1} d^{-1} (day 34; Fig. 2a) by 7 % per day, and then, substrate loading was reduced in two main steps to 4.01 g VS L^{-1} d^{-1}. Volumetric methane yield increased during the uploading period and stabilized at an average of 2.35 L L^{-1} d^{-1} in the stable loading period. tVFA levels increased slightly to 4401 mg L^{-1}, indicating overload (Fig. 2b). The NH_4^+ concentration increased drastically to 4087 mg L^{-1} and increased by 9 % during the stable run. Due to the decreased tVFA levels (2264 mg L^{-1}), the upload of the OLR was started from days 53 to 70 to 7.43 g L^{-1} d^{-1}. The specific methane yield reached the maximum level (0.88 L g^{-1} VS) on day 46 by the effect of decomposition of the accumulated VFA. In the reloading period, volumetric methane yield increased slowly, reaching an average of 2.86 L L^{-1} d^{-1}. A delayed increase in the tVFA concentration (maximum 11,318 mg L^{-1} on day 76) was observed when the NH_4^+ level increased to 4848 mg L^{-1}. When loading was stopped, the volumetric methane yield decayed by 90 % over 11 days, stabilizing at an average of 0.17 L L^{-1} d^{-1}.

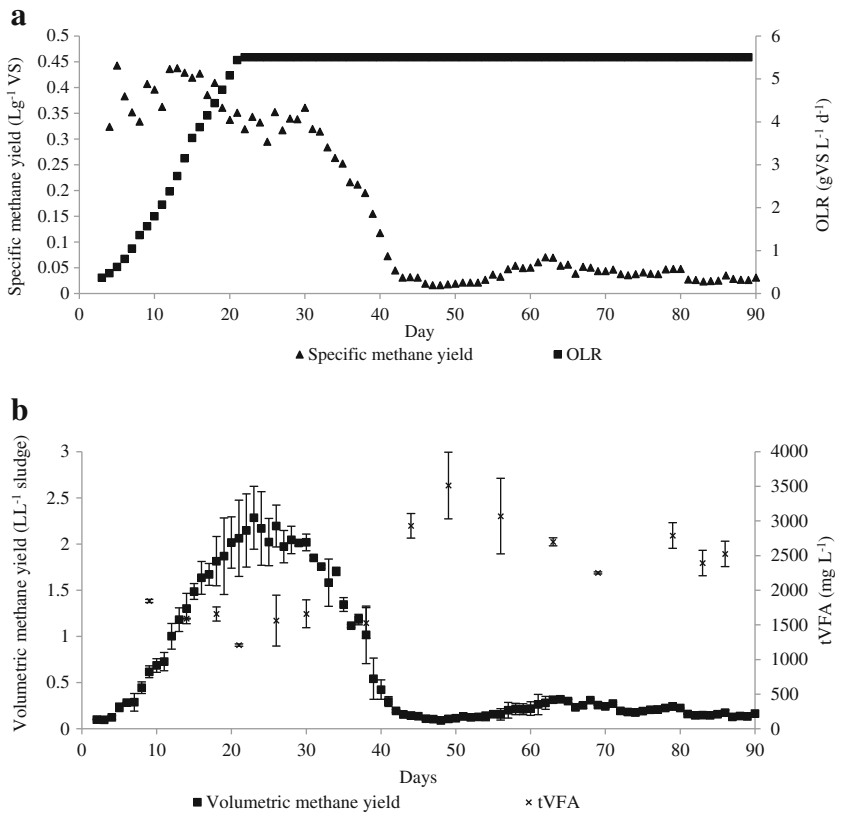

Fig. 5 **a** Alteration of specific methane yield with OLR with feeding with 3.8 % TS substrate mixture. **b** Alteration of tVFA levels with volumetric methane yield with feeding with 3.8 % TS substrate mixture

Algae-maize silage co-digestion During the uploading period, a maximum OLR was reached on day 39 at 5.81 g VS L^{-1} d^{-1} (Fig. 3a) in increments of 7 % per day. Alteration of the daily volumetric methane yield followed the OLR, reaching 1.82 L L^{-1} d^{-1}. The tVFA concentration did not exceeded 2515 mg L^{-1} during the overloading period (Fig. 3b). On day 44, the tVFA level jumped to 5785 mg L^{-1}, and the OLR was reduced to 4.27 g VS L^{-1} d^{-1}. In the stable run of 10 days, the tVFA level decreased by 43 %, while the average volumetric methane yield remained relatively high at 1.99 L L^{-1} d^{-1}. The short reloading period took 16 days with a maximum of 8.53 g VS L^{-1} d^{-1} (on day 70). The NH_4^+ concentration was relatively high during this period, reaching a maximum of 4766 mg L^{-1}. The tVFA level stabilized during the overloading period, but, similar to the first burden period, a delayed increase in the acid level (5659 mg L^{-1}) was observed. In addition, a decreasing tendency in the specific methane yield indicated the overloading effect. The decay period of the trial lasted 25 days, and the volumetric methane yield dropped to 0.11 L L^{-1} d^{-1}.

Algae-mill residue co-digestion During the upload period, OLR increased to 6.1 g VS L^{-1} d^{-1} by 7 % per day until day 39 (Fig. 4a). tVFA levels remained under 2640 mg L^{-1} during the first 31 days but jumped to 9180 mg L^{-1} on day 44 (Fig. 4b). The NH_4^+ concentration was 2858 mg L^{-1} on day 24. There was a significant increase in the NH_4^+ concentration between days 24 and 40 (to 5558 mg L^{-1}). With the reduced OLR (4.96 g VS L^{-1} d^{-1}), the tVFA levels decreased slowly to 2892 mg L^{-1}. Reloading the OLR to 6.97 g VS L^{-1} d^{-1} resulted in a significant increase of tVFA concentration to 10, 940 mg L^{-1}, indicating vulnerability of the anaerobic system. This statement is verified by the oscillation of specific methane yields during the overloading period. Volumetric methane yields were not sensitive to the effect of alteration of OLR between days 42 and 76, oscillating around an average of 1.96 L L^{-1} d^{-1}. The reduction of OLR and stopping feeding resulted in a continuous decline in the tVFA concentration, while volumetric methane yield decreased and then stabilized at an average of 0.22 L L^{-1} d^{-1} in the last 15 days of the experiment.

Effect of TS% on anaerobic co-digestion of algae and used cooking oil

TS% substrate mixture During the upload period, the maximum volumetric methane yield was registered on day 23, reaching 2.28 L L^{-1} d^{-1} at an OLR of 5.5 g VS L^{-1} d^{-1} (Fig. 5a, b). At steady load, volumetric methane yield decreased to under 0.30 L L^{-1} d^{-1} on day 42 and remained below 0.39 L L^{-1} d^{-1} during the rest of the experiment. The TS% of the inoculum was initially 7.14 % and was decreased to 2.87 %

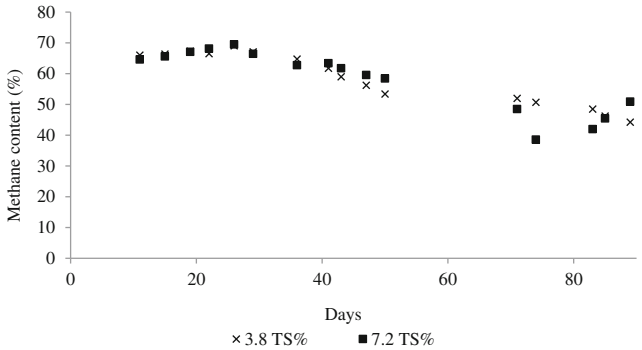

Fig. 6 Methane contents (%) of biogas during the 3.8 and 7.2 % TS feeding trials

by the end of the experiment. Alterations in tVFA levels indicated the disturbance caused by decreasing the TS% of sludge (Fig. 5b). In contrast to methane yield, tVFA levels decreased slightly during the upload period and increased at steady load. During the upload period, the lowest tVFA level was on day 21 (1208 mg L^{-1}), with a slight increase to day 38. tVFA increased significantly to day 49, then declined until day 69 with a minor increase in methane production between days 50 and 70 (Fig. 5b). The methane content of the biogas decreased continuously (Fig. 6) during the experiment. The specific methane yield decreased from 0.44 L g^{-1} VS (day 15), reached a minimum level (0.01 L g^{-1} VS) at day 47, and remained under 0.07 L g^{-1} VS during the rest of the experiment.

TS% substrate mixture During the upload period (5.5 g VS L^{-1} d^{-1} OLR), the maximum volumetric methane yield was 2.63 L L^{-1} d^{-1} (Fig. 7a, b). During the stable run (4.95 g VS L^{-1} d^{-1} OLR), volumetric methane production decreased to day 48 (0.39 L L^{-1} d^{-1}) and declined further throughout the rest of the trial (Fig. 7a, b). The TS% of the inoculum was 7.14 % at the beginning of trial and decreased to 6.18 % by the end of the experiment. The lowest tVFA concentration occurred on day 16 (1519 mg L^{-1}), increased slightly to day 29, reaching its highest concentration (6904 mg L^{-1}) on day 49 (Fig. 7b). The acid level remained relatively high until a significant decrease from day 81 to the end of the trial. The methane content of the biogas increased by 4.89 % from day 11 to day 26, reaching 69.49 % (Fig. 6). The methane content decreased slightly until day 50 (58.41 %), further decreased to day 74 (38.57 %), and increased again until day 89 (50.88 %; Fig. 6). The maximum specific methane production

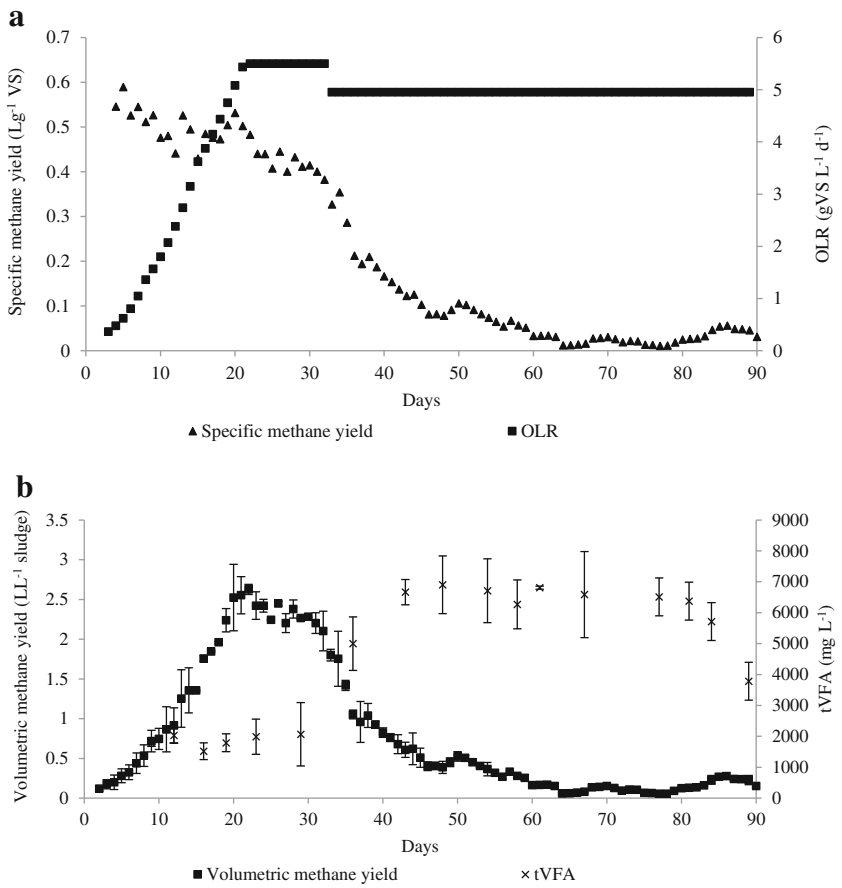

Fig. 7 **a** Alteration of specific methane yield with OLR with feeding with 7.2 % TS substrate mixture. **b** Alteration of tVFA levels with volumetric methane yield with feeding with 7.2 % TS substrate mixture

was 0.59 L L^{-1} d^{-1} (day 5) and decreased to 0.01 L L^{-1} d^{-1} (day 62), indicating a disturbance in AD.

Metagenomic analysis A sequencing-based metagenomic approach was used to follow the transformation of the microbial community structure in the laboratory-scale anaerobic fermenters. Two sampling times were selected for metagenomic analysis, a control sample taken from the initial sludge and samples taken from the 3.8 and 7.2 % TS experiments on day 90 (end points of the experiments).

Bacterial diversity was higher in the initial samples than in those taken at day 90. *Bacteroidetes* was the most abundant bacterial phylum in the initial sludge (Fig. 8a), but almost disappeared from the sludge in the 3.8 % TS experiment, representing only 0.6 % of the total bacterial community (Fig. 8b). The largest increase was in the *Firmicutes phylum* represented by the genus *Clostridium*, which increased from 13.4 to 44.0 % of the total bacterial community. A minor change was observed in the genus *Bacillus* (from 3.0 to 7.7 % of the total bacterial community), and the genus *Parabacteroides* was eliminated from the system.

In the 7.2 % TS system, the abundance of the phylum *Bacteroidetes* was halved compared with the initial sample, and the presence of the genus *Clostridium* was tripled from

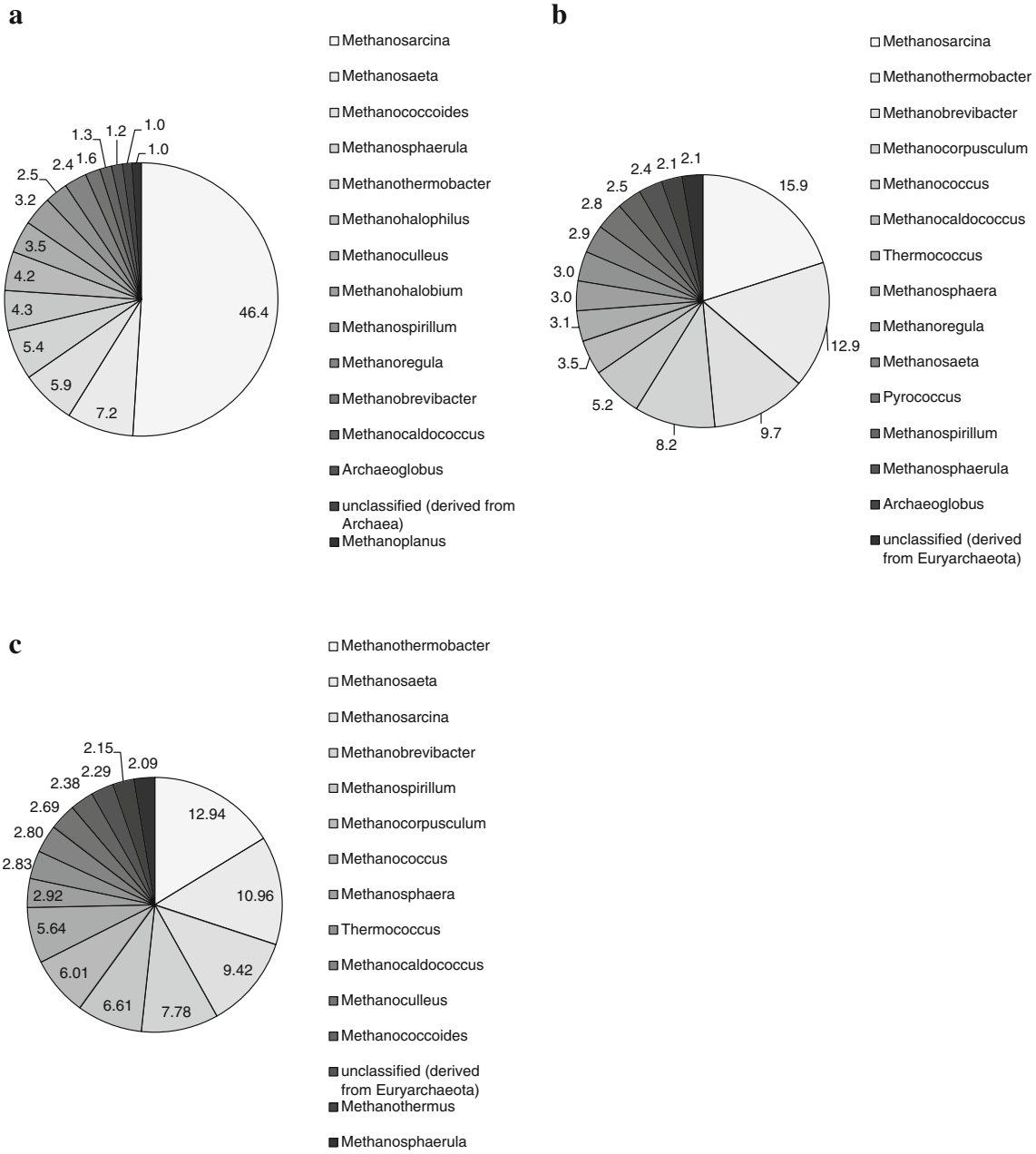

Fig. 8 Archaeal community structure of **a** initial sludge, **b** 90th day of the 3.8 % TS substrate feeding trial, and **c** 90th day of the 7.2 % TS substrate feeding trial

13.4 to 37.8 % of the total bacterial community (Fig. 9c). The genus *Parabacteroides* disappeared from the system, while the abundance of the phylum *Lactobacillales* increased from 0.9 to 6.3 % (Fig. 9c). The reliability values of the reads confirm the significance of the changes in the bacterial communities in both experiments.

Greater alterations in the abundance of the total bacterial community were found in the more diluted system (3.8 % TS).

We also investigated the abundance of the methanogenic archaeal community. In contrast to the decrease in diversity we observed in the bacterial community, the initial system was less diverse in methanogens compared with the system at the end point of the experiments (Fig. 9a). In the 3.8 % TS trial, a major decrease was found in the total abundance of the *Methanosarcina* (from 46.4 to 16.0 %). Similar reductions were observed in the case of the genera *Methanosaeta* and *Methanococcoides*, 7.2 to 2.9 % and 5.9 to 1.4 %, respectively (Fig. 9b). Interestingly, a large increase was detected in the abundance of the *Methanothermobacter* genus in the total archaeal community (from 4.3 to 12.9 %; Fig. 9b).

In the less diluted system, a major change was found in the *Methanosarcinaceae* family; the abundance decreased from 46.4 to 9.4 % (Fig. 9c). In this case, the presence of the genus *Methanosaeta* increased from 7.2 to 11.0 %, while that of the genus *Methanothermobacter* increased from 4.3 to 12.9 % (Fig. 9c). According to reliability values, significant changes were observed in abundance of the genus *Methanobrevibacter* (1.6 to 7.8 %) and that of the genus *Methanocorpusculum* (0.8 to 6.0 %) in the total archaeal community. In addition, the genus *Methanospirillum* increased in abundance from 2.5 to 6.6 % (Fig. 9c).

The greatly diminished methane content at the end of the experiments was accompanied by a reorganization of methanogenic archaeal members in both cases.

Discussion

Mono-digestion of *C. vulgaris* is not favorable due its low C/N ratio. To avoid the accumulation of ammonia during

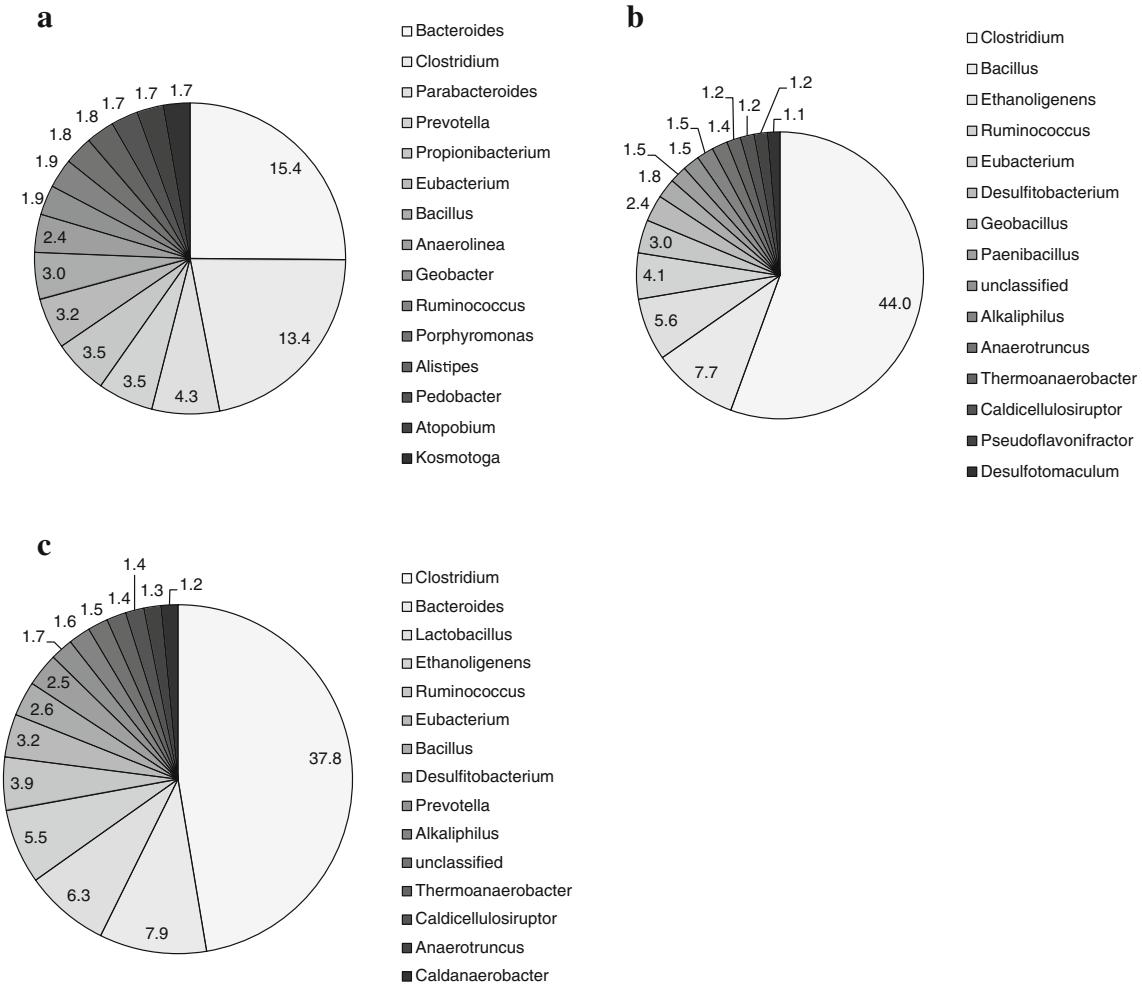

Fig. 9 Bacterial community structure of **a** initial sludge, **b** 90th day of the 3.8 % TS substrate feeding trial, and **c** 90th day of the 7.2 % TS substrate feeding trial

Table 2 VSR%, HRT, and OLR levels during mono-digestion and co-digestion experiments on *Chlorella* sp.

Experiment	VSR% (%)			HRT (days)			OLR (g VS L^{-1} d^{-1})		
	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average
Mono-digestion of algae	33	212	104	98	660	383	0.0	2.82	0.78
Algae-used cooking oil co-digestion	28	165	92	23	476	102	0.0	7.43	2.96
Algae-maize silage co-digestion	46	113	75	19	417	88	0.0	8.53	3.00
Algae–mill residue co-digestion	48	114	65	24	476	101	0.0	6.61	2.15

digestion, the use of co-substrates is preferred. To investigate the effect of co-digestion, semi-continuous feeding of a laboratory-scale fermentation was successfully employed with uploading and stable run periods. An AD batch process provides less information for studying the effect of accumulation of metabolites.

OLR values were regulated by tVFA levels, which is a generally used method (Chen et al. 2008) for the operation of an AD process. A drastic increase in tVFA concentration indicates a disturbance in methanogenesis. In the case of relatively high ammonia levels, significant alterations in tVFA levels do not influence the pH value of sludge.

The average volumetric methane yields during the loading period were 0.41 ± 0.06, 1.88 ± 0.31, 1.41 ± 0.1, and 1.39 ± 0.12 L L^{-1} for mono-digestion and co-digestion with used cooking oil, maize silage, and mill residue, respectively. During the full period, these values were 0.38 ± 0.07, 1.56 ± 0.26, 1.19 ± 0.18, and 1.16 ± 0.13 L L^{-1}, respectively, indicating the lowest loading rate (a maximum of 0.8 g VS L^{-1} d^{-1}) in the case of mono-digestion of algae.

Chlorella vulgaris in mono-digestion provided the best specific methane yield. The average specific methane yield in the loading period for mono-digestion was 0.53 ± 0.08 L g^{-1} VS, which exceeded the values of 0.47 ± 0.17, 0.38 ± 0.05, and 0.36 ± 0.04 L g^{-1} VS obtained in co-digestion with used cooking oil, maize silage, and mill residue, respectively. Over the full trial period, the average specific methane yield was 0.51 ± 0.12 L g^{-1} VS for mono-digestion and 0.52 ± 0.09 L g^{-1} VS for co-digestion with cooking oil, 0.39 ± 0.08 L g^{-1} VS with maize silage, and 0.39 ± 0.04 L g^{-1} VS with mill residue. A similar yield (0.47 L g^{-1} VS) was published by Ehimen et al. (2010) in the case of mono-digestion of *Chlorella* sp. in the biomethane potential assay. Yen and Brune (2007) found that the highest specific methane yield (0.29 L g^{-1} VS) could be reached in AD by adding wastepaper, as 50 % of VS, to algal sludge.

The conversion rate of substrate was highest in mono-digestion of algae based on VSR% levels (Table 2). Co-digestion with used cooking oil, maize silage, and mill residue originated with 12, 28, and 38 % lower VSR% values, respectively. These results suggested that, among the investigated substrates, *Chlorella* could also be degraded.

The specific methane yield is not always the most important operational parameter in industrial practice. Even if the specific methane yield is high in the case of algal mono-digestion, due to its high HRT and low volumetric methane yield, the industrial application is economically irrelevant. The HRT and volumetric methane yield could be improved using co-digestion, decreasing by 77 % and increasing 3.4–4.5-fold, respectively. Co-digestion with used cooking oil proved to be the best operational parameter, because of its high VS content.

The TS% (3.8 and 7.2 %) of the different substrates had an impact on digestion parameters. Both designs could be uploaded to 5.5 g VS L^{-1} d^{-1}, but a higher OLR resulted in increased methane production and a longer period of decline in methane yield caused by washout. A similar effect could explain the lower tVFA concentrations at 3.8 % TS. Based on the C/N ratios, the lower methane production could not be explained by nitrogen accumulation (Table 3). The lower C/N ratios obtained in the 7.2 % TS experiment confirmed a smaller degree of inhibition of washout and the higher calculated values for methane production. Decreasing rates of specific methane yields and methane content of biogas in both experiments indicated a disturbance in the AD processes.

The HRT of the stable run in the case of both experiments was too low to meet the reproductive needs of the methanogenic archaeal community (3.8 TS% at 6 days and 7.2 TS% at 12 days). This resulted tVFA accumulation (Figs. 5b and 7b) and low pH values (5.0–5.5). In this overdiluted system, a syntrophic interaction may have been lacking. Based on our measurements, the low HRT and pH values were the main inhibitory factors in this system.

Table 3 C/N ratios during the 3.8 and 7.2 % TS feeding trials

Day	3.8 TS%			7.2 TS%		
	C	N	C/N ratio	C	N	C/N ratio
12	29.89	3.38	8.84	30.29	3.46	8.75
25	56.05	1.44	38.92	47.35	2.81	16.85
43	71.96	0.86	83.67	64.08	2.02	31.72
60	65.17	0.92	70.84	66.84	3.27	20.44

In conclusion, the addition of maize silage, used cooking oil, and mill residue had beneficial effects on the digestion of the microalgal *C. vulgaris*. The highest specific methane yield (0.53 L g^{-1} VS) was measured in a mono-digestion culture, but from a practical point of view, the methane yield of digesters was the highest in the case of co-digestion of algae with used cooking oil (1.88 L L^{-1} d^{-1}). Digestion with diluted (3.8 and 7.2 % TS) substrate mixtures could not be stabilized in a laboratory-scale fermentor. Based on metagenomic analysis, the archaeal community decreased in abundance over time, resulting in a 20 % decrease in methane content.

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