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Augmented biogas production from protein-rich substrates and associated metagenomic changes

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HIGHLIGHTS

- After appropriate acclimation, sustainable biogas production is achievable from low C/N substrates.
- Metagenomic analysis reveals alterations in the microbial community.
- High biogas yields in anaerobic degradation (AD) of protein-rich monosubstrates.
- Addition of selected protein-degrading strains leads to effective AD.
- Selected strains lead to sustainable AD without acclimation.

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ABSTRACT

This study demonstrates that appropriate adaptation of the microbial community to protein-rich biomass can lead to sustainable biogas production. The process of acclimation to these unusual mono-substrates was controlled by the protease activity of the microbial community. Meat extract (C/N = 3.32) and kitchen waste (C/N = 12.43) were used as biogas substrates. Metagenome analysis highlighted several mesophilic strains that displayed a preference for protein degradation. *Bacillus coagulans*, *Bacillus subtilis* and *Pseudomonas fluorescens* were chosen for detailed investigation. Pure cultures were added to biogas reactors fed solely with protein-rich substrates. The bioaugmentation resulted in a 50% increase in CH_4 production even without any acclimation. The survival and biological activity of the added bacteria were followed in fed-batch fermenters by qPCR. Stable biogas production was observed for an extended period of time in laboratory CSTR reactors fed with biomass of low C/N.

1. Introduction

The consumption of energy is rapidly increasing, and the related environmental pollution and climate changes are approaching critical stage requiring sustainable solutions. The human population worldwide uses around 220 EJ per year (US-EIA, 2013), 78% of this being supplied by fossil energy carriers. The virtual depletion of economically recoverable fossil resources is predicted in the foreseeable future, and the search for new opportunities is therefore warranted. Renewable energy production is expected to increase by nearly 50% in the next 35 years, whereas it is predicted that biomass-derived energy will contribute only 3 EJ yr^{-1} (US-EIA, 2013). Biomass plants and the use of green biomass are currently attracting increased interest in view of their CO_2-neutral nature (Tafdrup, 1995), these technologies therefore not contributing to global environmental changes.

The European Union (EU) has earmarked a joint effort to reduce the emissions of greenhouse gases by 20% and 20% of the energy produced should be supplied from renewable sources by 2020 (Directive, 2009).

Anaerobic degradation (AD) and the accompanying biogas production is a particularly attractive way to generate renewable energy, as the disposal of organic waste from various sources is

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associated with energy carrier production. In addition to waste management and carbon-neutral renewable power generation, the technology helps return essential nutrients to the soil (Tafdrup, 1995).

After cleaning, biogas offers various opportunities for use as an energy carrier, as bio-CH_4 it becomes equivalent to fossil natural gas and the two CH_4 streams are interchangeable in all applications.

More than 12,000 biogas plants are currently operating in the EU, but none of them process primarily protein-rich waste, despite huge amount of such materials being generated continuously.

At least 30% of the weight of animals processed for food ends up as slaughter-house protein-rich waste. The world's annual meat production is around 300 million t, topped up with some 180 million t of milk production, which yields well over 100 million t of protein-rich waste produced annually by pig, cattle, sheep, fish and bird breeding worldwide (FAO Statistics Division, 2009), and a significant proportion of this type of biomass is classified as hazardous waste in many countries. This protein-rich biomass would be extremely valuable as biogas substrate if the basic questions concerning its AD could be solved. Besides the advantages of the biogas produced, the environmentally harmless fermentation residue is an excellent fertilizer for agriculture.

Proteins are decomposed through hydrolysis by proteases. Amino acids are metabolized via two main routes: pairs of amino acids can be decomposed through the Stickland reaction; and single amino acids can be degraded in the presence of H_2-utilizing bacteria (Ramsay and Pullammanappallil, 2001). Stickland reactions are usually faster than uncoupled amino acid fermentation (Ramsay and Pullammanappallil, 2001).

AD involves the concerted action of many microbes from various taxonomic groups, each performing a special role in the overall degradation process (Ahring, 2003; Wirth et al., 2012; Kovács et al., 2014). CH_4 formation is influenced by a number of environmental factors, including the NH_3 concentration (van Velsen, 1979). In AD, NH_3 is produced through degradation of the N-containing biomass present in the feedstock, mostly in the form of proteins (Kayhanian et al., 1999). NH_3 can enter cells in the unprotonated form, which readily diffuses across the cell membrane, equilibrating the intracellular and extracellular NH_3 concentrations. On the other hand, the NH_4^+ ion does not readily diffuse through lipid membranes (Kleiner, 1993). At least two possible mechanisms of NH_3 toxicity have been postulated: (i) NH_3 could inhibit the activity of cytosolic enzymes directly, or (ii) NH_4^+ accumulated inside cells might be toxic through its effect on the intracellular pH (Sprott et al., 1984) or on the concentrations of other cations such as K^+ (Sprott and Patel, 1986). Ca^{2+} or Mg^{2+} triggers antagonistic effects to NH_4^+. In the absence of terminal electron acceptors such as NO_3^-, O_2 or SO_4^{2-}, the methanogenic conversion of organic matter is an essential feature of many ecosystems (Conrad et al., 1989). The optimal carbon/nitrogen/phosphorus (C/N/P) ratio for a high CH_4 yield is around 100:3:1 (Conrad et al., 1989). In AD, the acidogens and methanogens differ in their physiology, nutritional needs, growth kinetics and sensitivities to the environmental conditions (Kayhanian, 1994). Failure to sustain the balance between these two groups is the main cause of process instability (Demirel and Yenigun, 2002).

The introduction of energy-rich proteinaceous waste products in large quantities into the AD process is not recommended in view of the increased risk of inhibition by NH_3 (Ahring, 2003). The quantity of NH_3 generated from AD of an organic substrate can be estimated (Nielsen and Angelidaki, 2008). The fraction of NH_3 relative to the total (NH_3 + NH_4^+)-nitrogen (TAN) is dependent on pH and temperature (Hansen et al., 1996). Inhibitory thresholds for NH_3 have been reported in a number of studies, but the limiting concentrations varied significantly (Kroeker et al., 1979; Angelidaki and Ahring, 1993; Banks and Wang, 1999, Hansen et al., 1996).van Velsen (1979) concluded that a TAN between 0.2 and 1.5 g L^{-1} has no effect on CH_4-forming microbes. Koster and Lettinga (1984) reported that the maximum methanogenic activity under mesophilic conditions was unaffected at a TAN concentration of 0.68 g L^{-1}. However, a TAN concentration between 1.5 and 3 g L^{-1} was found to be inhibitory at pH > 7.4 (van Velsen, 1979). A TAN concentration of 4 g L^{-1} or more inhibited thermophilic digestion of cattle manure (Angelidaki and Ahring, 1993). The inhibitory TAN concentrations mentioned in different studies are seldom comparable, unless the pH and temperature conditions are also stated.

Microbial adaptation to higher NH_3 concentrations and cation antagonism effects (Chen et al., 2008) may also contribute to the broad range of inhibitory NH_3 concentration thresholds reported in the literature. Higher tolerances can be achieved by acclimation and NH_3-adapted anaerobic consortia were observed to be inhibited at an NH_3 concentration of 0.7–1.1 g NH_3-N L^{-1} (Angelidaki and Ahring, 1993, Hansen et al., 1996). Hashimoto (1986) demonstrated that NH_3 inhibition began at about TAN concentrations of 2.5 g L^{-1} and 4 g N L^{-1} for unacclimated and acclimated thermophilic methanogens, respectively. A tolerance of up to 3–4 g NH_4^+-N L^{-1} for an adapted process has also been described (Angelidaki and Ahring, 1993).

Edström et al. (2003) used blood, stomach and intestinal content and food waste in co-fermentation with animal manure. Feedstock mixtures containing 8–15% of animal waste products could be co-digested under stable conditions at a total concentration of 4.5–5.0 g NH_4^+-N L^{-1}.

It is generally acknowledged that, of all the microorganisms involved in AD, the methanogens are the least tolerant to inhibitors and the most likely to cease growth due to NH_3 inhibition (Kayhanian, 1994). Elevated NH_3 levels cause changes in microbial communities; the shift from aceticlastic methanogenesis to syntrophic acetate oxidation is a consequence of the effect of the inhibition by NH_3 (Westerholm et al., 2012). Tests of NH_3 toxicity on the acetate- and hydrogen-utilizing populations reveal a higher sensitivity of the aceticlastic relative to the hydrogenotrophic methanogens; the specific growth rate for the aceticlastic methanogens was halved at an NH_3 concentration of 3.5 g N L^{-1}, as compared with 7 g N L^{-1} for the hydrogenotrophic methanogens (Angelidaki and Ahring, 1993). A lower biogas yield and/or CH_4 yield was observed, however, in the case of an elevated NH_3 load (Koster and Lettinga, 1988).

A common feature of NH_3 adaptation attempts was that the substrate with a high N content was fed together with a substantial amount of C-rich materials in order to approach the recommended C/N/P ratio.

The goal of the present study was to extend the C/N range of protein-rich materials suitable for effective and sustainable AD and biogas production following a controlled adaptation of the microbial community to the unusual substrates. In addition the possibility to eliminate the adaptation period by adding selected protein degrading microbes in pure culture was tested. These bacteria were expected to help the community to cope with the stressful task of utilizing the protein-rich substrates. The practical implications of this strategy for industrial scale biogas production are evident.

2. Methods

2.1. Substrates, inoculum and bacterial strains

Meat extract was purchased from Sigma, and vegetable-rich kitchen waste was made by mixing various fruits (apples, melons, pears and plums) and vegetables (potatoes and tomatoes) in a blender, and the homogenized material was stored in aliquots at −20 °C. The parameters of the biogas substrates are to be seen in

Table 1
Properties of the substrates used in this study.

Parameters	Unit	Meat extract	Vegetable waste
Organic total solids (oTS)	% of total organic solids	87.56	92.71
Total solids (TS)	%	94.16	16.60
Water content	%	5.84	83.40
Density	kg/m^3	1.47	1.06
Carbon to nitrogen ratio		3.32	12.43
Lipid content	% of total organic solids	<1	<1
Protein content	% of total organic solids	68.20	7.20
Carbohydrate content	% of total organic solids	30.50	68.70

Table 1. Inoculum was taken from the effluent of a mesophilic industrial biogas facility using pig slurry and a mixture of maize and sweet sorghum silages (Wirth et al., 2012). The bacterial strains *Bacillus subtilis* (DSM10), *Bacillus coagulans* (DSM1) and *Pseudomonas fluorescens* (DSM50090) were obtained from Deutsche Sammlung von Microorganismen und Zellculturen GmbH (DSMZ, Braunschweig, Germany), and were cultivated in the media recommended by DSMZ. The bacterial biomass was collected by centrifugation and counted, and the wet biomass was added to the reactors when needed.

2.2. Determination of protease activity

Relative protease activity was determined by measuring the release of acid-soluble material from azocasein (Sigma). 200 μL of filtered sample was added to 50 μL of phosphate buffer (6.7×10^{-2} M, at pH 7.0) and 200 μL of 1% (w/v) azocasein. Following incubation at 37 °C for 1 h, 700 μL of ice-cold 5% (w/v) trichloroacetic acid was added to stop proteolysis, with simultaneous vortexing. The sample was placed on ice for 10 min before centrifugation at 13,000 rpm for 10 min. The quantity of acid-soluble material in the supernatant was measured via the absorbance at 440 nm (Kovács et al., 2013b).

2.3. Measurement of biogas production and gas composition

Laboratory-scale 5-L AD reactors, described in detail elsewhere (Kovács et al., 2013a), were used in fed-batch operational mode at mesophilic temperature (37 ± 0.5 °C). The reactors were fed with a pig manure and energy plant silage mixture (Wirth et al., 2012) until the operation and biogas production became stabilized prior to the start of protein feeding. All fermentations were carried out in triplicate (Kovács et al., 2013b). Batch biogas yields were determined according to the Verein Deuthscher Ingenieure standard protocol (VDI, 2006). The composition of the evolved biogas was measured by taking 100-μL aliquots from the headspace and injecting them into 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) and a thermal conductivity detector. N_2 was used as carrier gas. Additional details of biogas analysis have been reported earlier (Kovács et al., 2013a,b; Wirth et al., 2012).

2.4. Analytical methods

2.4.1. pH

A Radelkis OP-211/2 digital pH-meter was used to measure the pH of samples.

2.4.2. Volatile organic acids/alkaline buffer capacity (VOAs/TAC)

5 g of fermenter substrate was taken for analysis and diluted to 20 g with distilled water. The subsequent titration process was fully automatic (Pronova FOS/TAC 2000 Version 812-09.2008). The results for VOAs, TAC and VOAs/TAC are displayed after a few minutes.

2.4.3. Volatile fatty acids (VFAs)

VFAs were determined by high-performance liquid chromatography (a Hitachi Elite instrument, equipped with an ICSep ICE-COREGEL 64H column and a refractive index detector L2490) with the following parameters: 0.05 M H_2SO_4 as solvent, a flow rate of 0.8 mL min^{-1}, a column temperature of 50 °C, and a detector temperature of 41 °C.

2.4.4. NH_4^+-N

This was determined by the Merck Spectroquant Ammonium test (1.00683.0001).

2.4.5. H_2S

The H_2S content of the evolved gas was measured with the Hydrogen sulfide 100/a test tube from Dräger (CH 29101).

2.4.6. Organic total solid (oTS)

The dry matter content was quantified by drying the biomass at 105 °C overnight and weighing the residue. Further heating of this residue at 550 °C in a furnace until its weight did not change yielded the organic total solid content.

2.4.7. C/N

To determine C/N, an Elementar Analyzer Vario MAX CN was employed. This works on the principle of catalytic tube combustion under an O_2 supply at high temperatures (combustion temperature: 900 °C, postcombustion 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. He served as flushing and carrier gas.

2.5. Metagenome analysis

2.5.1. DNA extraction

2-mL liquid fermentation samples were collected for total community DNA isolation by applying a cetyltrimethylammonium bromide-based DNA extraction buffer (Miller et al., 1999, Wirth et al., 2012). Cell lysis was carried out at 55 °C overnight. Phenol:chloroform (1:1) was used to extract contamination, and the genomic DNA was precipitated with ethanol (90%). The DNA pellet was resuspended in 100 μL of Tris–EDTA buffer. The DNA content was determined in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies); the DNA purity was tested by agarose gelelectrophoresis. This method yielded a pure ($A_{260}/A_{280} \geqslant 1.8$) and sufficient amount of total DNA (200–800 ng/μL).

2.5.2. DNA sequencing and data handling

Sequencing was performed with the Life Tech SOLiD™ V4 sequencing platform. 30 million reads (average read length 50 nt) were generated for each sample. Primary data analysis was carried out with the software provided by the supplier (base-calling). The 50 nucleotide reads were analyzed, quality values for each nucleotide were determined, and the reads were assembled into contigs through use of the CLC Bio Genomics Workbench 4.6 program. The *de novo* contig assembly was followed by MG-RAST analysis. Details of the bioinformatic evaluation and statistical analyses were published previously (Wirth et al., 2012).

2.6. Quantitative PCR (qPCR)

For identification of *B. coagulans*, BcoFW (5′ CGACATGGACAT GATCCAGAATA 3′) and BcoREV (5′ GAAATTCGGGCTCTGGTTTG 3′) primers were used. For *B. subtilis*, the forward and reverse primers were BsuFW (5′ GCTGCCGTGATCTTGGTGAA 3′) and BsuREV (5′ GGCGAACAGCCTCAACGATA 3′). For *P. fluorescens*, they were PflFW (5′ CAACGACGCCGCAATCTC 3′) and PflREV (5′ GCCAGATGCACC GTGAACT 3′). qPCR was performed on a 7500 Fast Real Timer PCR system using the SYBR Green PCR Master Mix (Applied Biosystems). Cycling was carried out under the default conditions of the 7500 Software v2.0.5: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. All qPCR assays involved three technical replications and template-less control.

3. Results and discussion

3.1. AD of meat extract and kitchen waste

Efficient AD of blood protein and milk protein wastes were demonstrated previously (Kovács et al., 2013b). In light of these results it was intriguing to test if protein-rich AD substrates of different chemical compositions could be subjected to efficient AD and the protease activity based technology could therefore be generalized. In order to extend the application of this approach additional two potential AD substrates were examined, which differed in both chemical composition and C/N content. To facilitate comparisons with previous results the most important operational parameters and arrangements, i.e. mesophilic, fed-batch AD in identical laboratory CSTR reactors, were the same as reported in Kovács et al., 2013b. The reactors were supplied with each of the protein-rich substrates weekly. Following stabilization of the reactor performance and biogas production from the pig slurry and maize silage mixture (4–5 weeks, data are not shown), the acclimation to the protein-rich materials started at low biomass load for 5 weeks. In the case of the meat extract (Fig. 1), elevation of the biogas production was already observed at week 2–3 in this period, indicating a relatively rapid and positive response to the new biogas substrate. The protein loading was then increased in a stepwise manner. Until week 12–13, the biogas production rose together with continuing protein loading. When the weekly protein load reached 25 g oTD, the system started to show signs of operational failure: the biogas production decreased significantly, the acetate accumulation exceeded the 3 g L^{-1} level, and the FOS/TAC value started to rise, although it remained below the recommended upper limit (0.6) until week 16–17 (data not shown). It is noteworthy noting that the NH_4^+-N concentration followed a pattern distinct from those of the volatile fatty acids and biogas production. Both the NH_4^+-N and H_2S contents increased almost monotonously from the start of the adaptation period, and NH_4^+-N reached the critical level of 4 g L^{-1} at the end of the adaptation period. During the intensive biogas production stage, the NH_4^+-N level increased still further, which suggests a correlation between the active biogas production by the microbial community and the VFAs level rather than with NH_4^+-N (Fig. 1). H_2S started at the moderate concentration of 250 ppm and reached the toxic level of 1700 ppm by the end of the experimental period.

Although the C/N ratio of the mixed vegetable waste far way below the optimal value of 30, it behaved completely differently from that of the meat extract (Fig. 2). Immediately after the change of the feedstuff to the vegetable waste, intensive biogas production was monitored making the adaptation period unnecessary. The substrate load was elevated bi-weekly. The specific biogas production started to decrease in week 5–6, and the decrease continued during the next 8 weeks. The volatile fatty acid, NH_4^+-N and H_2S concentrations did not change at an alarming level. The likely cause of the system failure at high substrate loading was the low buffering capacity of the reactor content, indicated by the pH drop from the initial 8.1 to 6.9, in spite of the addition of a weak base from week 8.

3.2. Changes in the biogas-producing microbial community

3.2.1. Initial community structure

At the start of the experiments, the biogas-producing microbial community exhibited essentially the same composition as described earlier for AD reactors fed with animal manure and maize silage (Wirth et al., 2012). Members of the classes *Clostridia* and *Bacilli* accounted for the majority of the bacteria, comprising 49–50% and 12–14% of the total bacterial population, respectively. An uncultivated strain, *Candidatus Cloacamonas acidaminovorans*, was found to be most abundant similarly as in earlier studies (Pelletier et al., 2008), followed by *Clostridium thermocellum*. Other prominent *Clostridia* included *Clostridium kluyveri*, *C. cellulolyticum* and *C. phytofermentans*, reflecting the predominance of this taxonomic group. There were two *Alkaliphilus* strains among the most abundant species. These strains have been noted for their ability to reduce metals (*Alkaliphilus metalliredigens*) or arsenic (*A. oremlandii*), but in the biogas community they are likely to exploit their additional metabolic versatility (Ye et al., 2004). The composition of the microbial community at the beginning of the experiments was very similar to those reported in earlier studies (Klocke et al., 2007; Krause et al., 2008; Jaenicke et al., 2011; Wirth et al., 2012). The inoculum used to startup and stabilize the operation of the reactors came from an industrial AD plant fed with pig slurry and maize silage, and this microbial distribution therefore was not surprising.

3.2.2. Microbial community alterations in response to meat extract feeding

Substantial changes in the microbial community were found during and after the adaptation to the protein-rich substrate. In general, these changes were similar to those observed earlier in fed-batch experiments in which casein or pig blood protein was used (Kovács et al., 2013b). Similarly to most biogas-producing communities, the phylum *Firmicutes* predominated in the community. As in the case of the previously studied monosubstrates (Kovács et al., 2013b), their relative abundance decreased upon switching from the conventional substrate composition to feeding with the meat extract alone but by the end of the process their overall number had increased again (Fig. 3). At higher taxonomic resolution, it became obvious that there had been a significant rearrangement within the members of the *Firmicutes*, i.e. the primarily cellulolytic *Clostridia* had been replaced by the primarily proteolytic members of the taxon. The population belonging in the class *Bacilli* within phylum *Firmicutes* displayed distinct behavior: their relative number decreased significantly when the reactors were fed with meat extract. A very similar response to the protein-rich substrate was noted with other protein-rich biomasses earlier (Kovács et al., 2013b). The *Proteobacteria* adapted poorly to the changing environment: they were present in substantial numbers at the start of the experiment, but were significantly reduced by the end of the AD of the meat extract. A similar fate was experienced by *Candidatus Cloacamonas acidaminovorans*, which was eradicated by the protein-rich substrate, similarly as observed for other protein-rich substrates (Kovács et al., 2013b). The phylum *Thermatogae* could evidently adapt successfully to the new substrate. It was hardly detectable in the starting community, whereas its relative abundance increased continuously during AD on the meat extract, though it still remained a minor group in the community. The bacteria belonging in the phylum *Bacteroidetes* were present in low numbers at the beginning of the experiments,

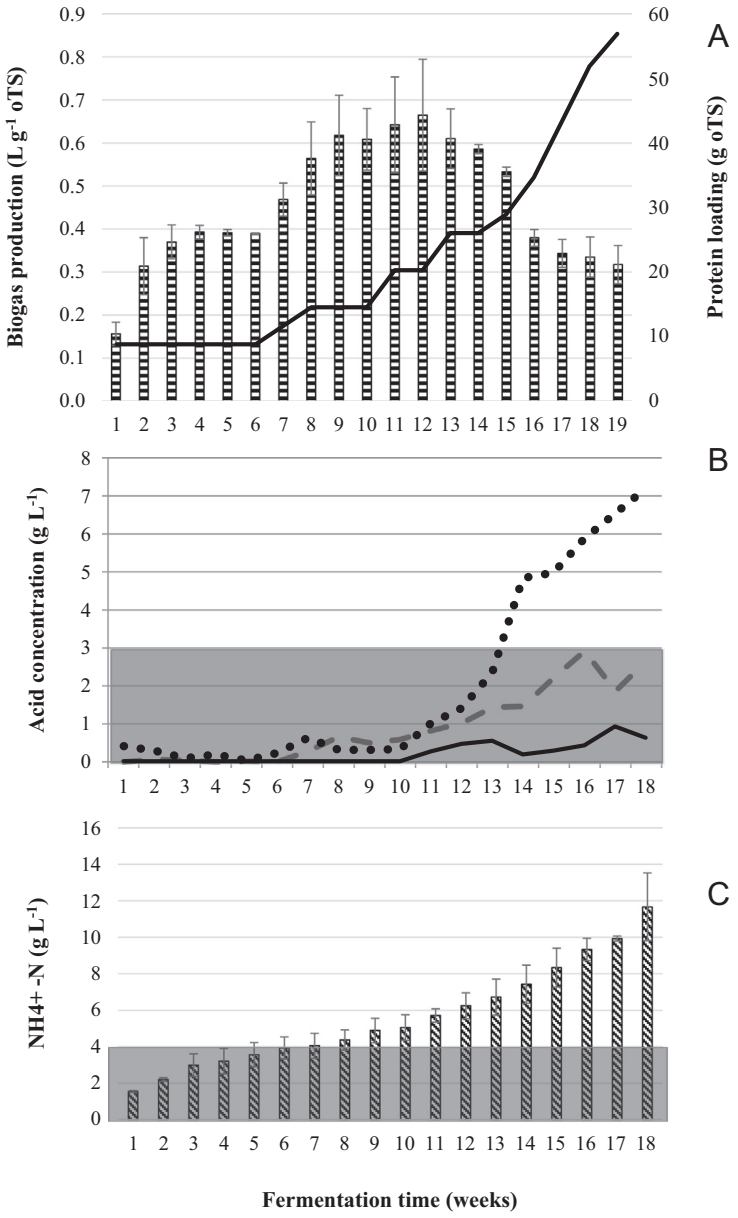

Fig. 1. Biogas production from meat extract (A), volatile fatty acid accumulation (B) and NH_4^+-N generation (C) during the fed-batch AD of the monosubstrate. (A) Weakly biogas production is shown by the columns, protein loading is indicated by the continuous line. (B) Acetate (dotted line), propionate (dashed line) and butyrate (continuous) weekly build-up is shown. The grey area indicates the level not inhibitory to the process according to previous studies. (C) Columns show the NH_4^+-N concentrations measured every week, the grey area indicates the recommended range of this parameter.

but their numbers soared in response to protein feeding, but exhibited a decreasing richness as the AD fermentation came to an end.

The Archaea domain did not display striking changes, in line with earlier findings on other protein-rich substrates (Kovács et al., 2013b). The most important development was the dramatic decrease in the abundance of the class *Methanomicrobia* as the protein-based AD progressed.

3.2.3. Microbial community alterations in response to vegetable waste feeding

The mixed vegetable kitchen waste biomass is substantially different from the protein-rich substrate meat extract for AD, though its C/N ratio is still greatly below the optimal value recommended for efficient biogas production. Characteristic changes occurred in the microbial community. Members of the phylum *Bacteroidetes* appeared to favor this substrate because their originally low representation multiplied spectacularly during the feeding of the fed-batch AD reactors with the vegetable waste mixture. Even though it still outnumbered other taxa, the phylum *Firmicutes* suffered severely from the feeding with the mixed vegetable biomass. The class *Clostridia* and order *Clostridiales* were the greatest losers. The phylum *Bacteroidetes* drastically increased its relative abundance in the community and its vigorous representation was obvious at all taxonomic levels (Fig. 4). The phylum *Thermatogae* could not cope with the stressful task of becoming adapted to the protein-rich monosubstrate, and its relative abundance dropped during the experiment. As concerns the archaeal members of the community, the main tendency did not change, the phylum *Euryarchaeota* remaining predominant. Higher taxonomic resolution revealed some remarkable responses to the vegetable diet. The class *Methanomicrobia* and order *Methanomicrobiales* responded very poorly to the new substrate, whereas the class *Thermococci* and order

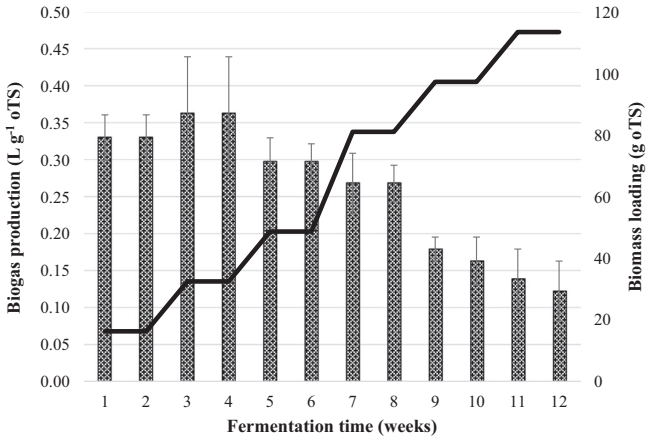

Fig. 2. Biogas production from vegetable waste in fed-batch AD. Weekly biogas evolution is indicated with the columns, biomass loading is shown by the continuous line.

Thermococcales increased their representation in the community, as did a number of smaller taxa, including the orders *Methanosarcinales*, *Archeoglobales* and *Methanobacteriales* and the genus *Halobacterium*. One of the most abundant members of the community, *Candidatus Cloacamonas acidaminovorans*, disappeared indicating its sensitivity to the low C/N ratio, regardless of the specific composition of the substrate. The members of the community that gained in abundance belonged in the order *Bacteroidales*, with a significant increase in *Lactobacillales*.

3.3. Sustainable biogas production from meat extract

The preliminary experiments led to the choice of a meat extract concentration that was not yet deleterious to the AD process in order to test the sustainability of biogas production from the protein-rich monosubstrate. This loading value was 14.4 g oTS $week^{-1}$. During the adaptation period at a lower loading rate for 5 weeks, as applied earlier, the weekly biogas production (Fig. 5) and protease activity (data not shown) gradually increased, indicating the adaptation of the microbial community to the substrate. The pH increased from the initial 7.8 to 8.4, and remained stable thereafter. The FOS/TAC ratio persisted in the acceptable range 0.14–0.20 range throughout the experiments. The CH_4 content of the produced biogas was 50–54% but the level of H_2S rose from the initial 200 ppm to 850 ppm by the end of the tests. The NH_4^+-N level also increased, but stabilized at 4.5–5 g NH_4^+-N L^{-1} in the course of the 15-week experimental period. Biogas production at this level persisted for an extended period of time, i.e. more than 3 months, without signs of system failure (Fig. 5).

3.4. Bioaugmentation of biogas production from protein-rich substrates

Several species were identified as excellent protein degraders in the sustained biogas-producing AD microbial community fed with protein-rich monosubstrates in these and previous studies (Kovács et al., 2013b). Three strains that displayed robust survival in the protein-fed biogas reactors, *P. fluorescens*, *B. coagulans* and *B. subtilis* species, were chosen for further bioaugmentation investigations. Strain selection was based on the proteolytic activity and the survival capability in the AD community. Easy cultivation conditions and fast growth on inexpensive substrates were additional considerations in the selection of these particular strains. It is recognized, however, that the selection is not necessarily the optimal and best combination. The experiments reported here serve as "proof of principle" only. Additional and detailed optimization studies are under way. 3×10^8 cells mL^{-1} reactor volume of each bacterium was added to the test reactors at the beginning of the AD process. It is noteworthy that no adaptation period at low protein loading was utilized in these experiments. As in previous fermentations,

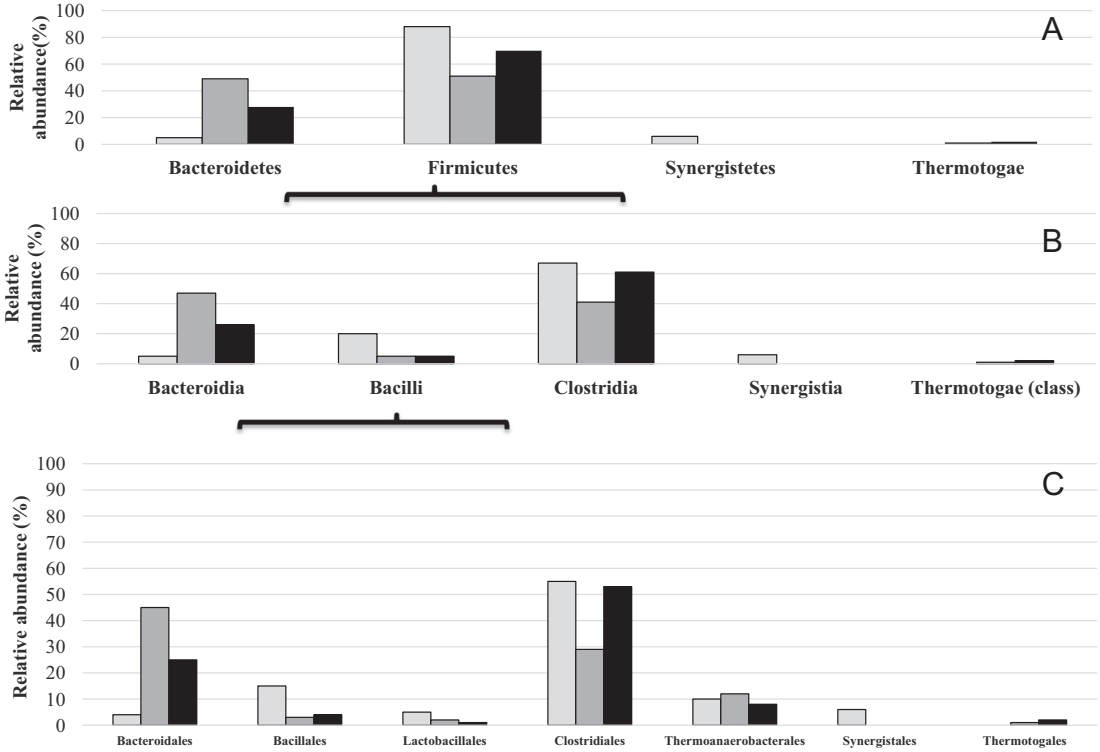

Fig. 3. The composition of the major bacterial taxa at phylum (A), class (B) and order (C) resolution as derived from metagenomic analysis of the AD community fed with meat extract exclusively. Samples of the community were taken at week 2 (light columns), week 10 (darker columns) and week 18 (black columns).

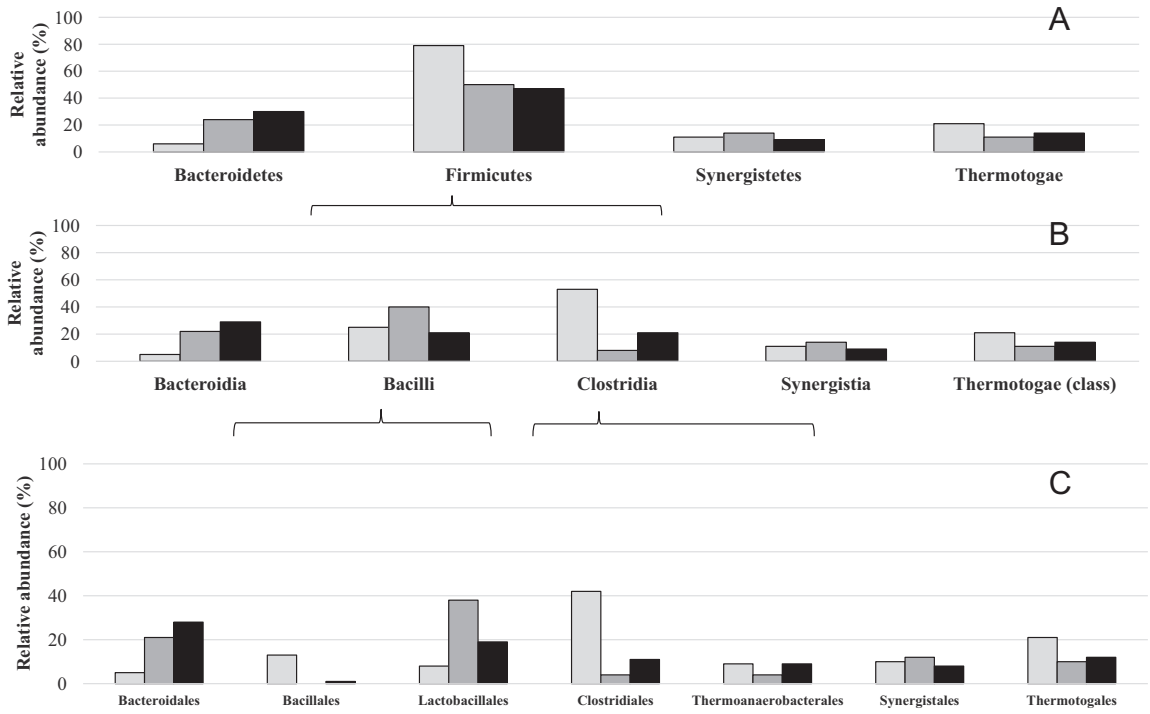

Fig. 4. The composition of the major bacterial taxa at phylum (A), class (B) and order (C) resolution as derived from metagenomic analysis of the AD community fed with vegetable waste exclusively. Samples of the community were taken at week 2 (light columns), week 6 (darker columns) and week 11 (black columns).

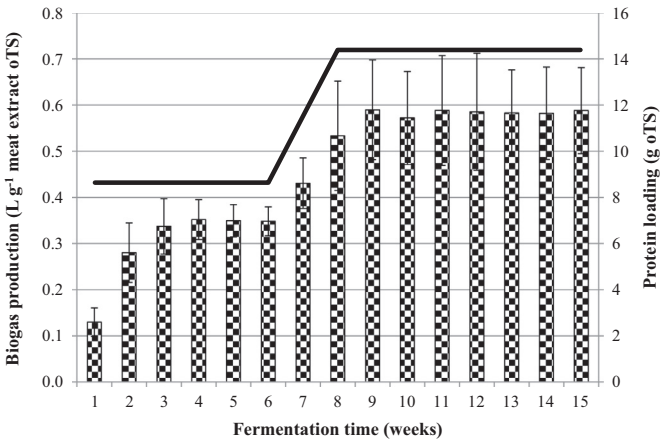

Fig. 5. Sustainable biogas production from meat extract monosubstrate in fed-batch laboratory AD fermentations. Columns show the weekly biogas productions, continuous line indicates the weekly protein loading.

the fed-batch operational mode was carried out under mesophilic conditions, and both control and test reactors were fed with meat extract (11.5 g oTS) once a week.

In the control reactors the CH_4 content of the biogas was 53% and the average biogas yield was 0.34 L g^{-1} oTS, i.e. the calculated CH_4 yield was 0.18 L g^{-1} oTS. When the mixture of the three selected bacteria was added, the CH_4 content of the biogas rose to 58% and the biogas yield was on average 0.46 L g^{-1} oTS (Fig. 6). From these data the calculated CH_4 yield was 0.27 L g^{-1} oTS. The increase in the biogas yield was 35% and the CH_4 yield improved by 50%. The mixture of the necessary strains, their dosage, the inoculation frequency and other parameters still require thorough optimization. Nevertheless, the data already clearly demonstrate that the efficient AD of protein-rich wastes is possible if certain important members of the microbial community are boosted, and the system can operate in a sustainable manner without an acclimation period.

The total organic acid content, the buffering capacity, the pH and the NH_4^+-N and VFA levels were in the normal ranges, indicating a balanced fermentation. The protease activity increased sharply following the addition of the proteolytic bacteria, and remained elevated throughout the 3-month experiment. In the control fermenters a gradual increase in protease activity was observed, approaching that in the test reactors at the end of the experimental period.

In order to ascribe the beneficial effects to the added bacteria, their presence in the reactors was screened by qPCR. The reactors were sampled every 4 days and the amounts of DNA corresponding to the selected strains were determined. Cell numbers were calibrated by using pure cultures. Following a transient upsurge the number of *P. fluorescens* cells stabilized at 3×10^8 cells mL^{-1}. The levels of *B. subtilis* and *B. coagulans* decreased and stabilized at 1×10^8 cells mL^{-1}.

Both substrate sources proved suitable for stable AD below a critical loading rate, although the two materials were digested in different manners. Above a critical weekly loading rate, the system collapsed, as expected. This process was apparently correlated with the accumulation of volatile fatty acids rather than with the elevated NH_4^+-N level.

Sustainable biogas production was maintained for 3 months without any sign of system failure when the meat extract was applied as the sole substrate at a loading rate below the critical level. Metagenomic analysis revealed characteristic change in the composition of the biogas-producing communities. The theoretical maximum biogas yields calculated for meat extract is 0.469 NL g oTS^{-1}, and for vegetable waste 0.326 NL g oTS^{-1}, respectively (Symons and Buswell, 1933).

The main message for the biogas industry is that with appropriate process control actions protein-rich substrates can be safely and effectively introduced into the AD technology.

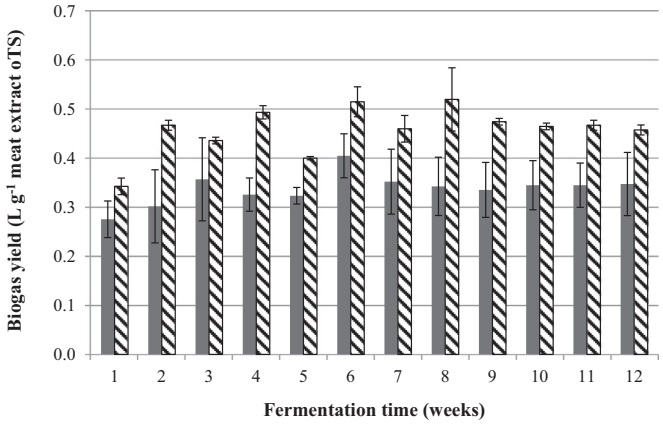

Fig. 6. Bioaugmentation with selected protein degrading mixture of mixed bacteria. Controls are shown in black columns. Weekly biogas productions under identical conditions but with added bacterial mixture are shown with the cross-hatched columns.

4. Conclusions

A meat extract (C/N = 3.32) and vegetable waste (C/N = 12.43) were successfully used as monosubstrates for the continuously stirred mesophilic laboratory biogas reactors in fed-batch operational mode. In the case of the meat extract, an acclimation period at low levels of the substrate was necessary to allow adequate adaptation of the microbial community.

From a consideration of the metagenomic data, 3 strains were selected for bioaugmentation tests, and the survival of the added bacteria was followed by qPCR. Although the system requires further optimization, the preliminary data clearly demonstrate that it is possible to avoid the time consuming and risky acclimation procedure and improve biogas productivity by adding bacteria which exert strong proteolytic activity.

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