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Increase of methane formation by ethanol addition during continuous fermentation of biogas sludge

Sarah Refai · Kati Wassmann · Sebastian van Helmont · Stefanie Berger · Uwe Deppenmeier

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Abstract Very recently, it was shown that the addition of acetate or ethanol led to enhanced biogas formation rates during an observation period of 24 h. To determine if increased methane production rates due to ethanol addition can be maintained over longer time periods, continuous reactors filled with biogas sludge were developed which were fed with the same substrates as the full-scale reactor from which the sludge was derived. These reactors are well reflected conditions of a full-scale biogas plant during a period of 14 days. When the fermenters were pulsed with 50–100 mM ethanol, biomethanation increased by 50–150 %, depending on the composition of the biogas sludge. It was also possible to increase methane formation significantly when 10–20 mM pure ethanol or ethanolic solutions (e.g. beer) were added daily. In summary, the experiments revealed that "normal" methane production continued to take place, but ethanol led to production of additional methane.

Keywords Biogas plant · Aceticlastic methanogenesis · Syntrophic bacteria · Anaerobic digestion · Methane production

Introduction

Biogas, which is formed during the degradation of organic material, is one of the most important renewable energy sources and is used for the generation of electric power and heat. In general, agricultural biogas plants (referred to as NawaRo biogas plants) typically use energy crops and animal manure as a fermentation substrate. In addition, anaerobic degradation also represents a suitable method for waste and wastewater treatment. The market for biogas plants and biogas production is constantly increasing because of the need to facilitate a sustainable development of energy supply and to reduce greenhouse gas emissions. More and more countries create the necessary framework conditions for a fast growth of the biogas industry. It is expected that the worldwide installed capacity will increase between 2012 and 2016 from 4,700 MWel to about 7,400 MWel [33]. Currently, more than two-thirds of the world's 10,000 operational biogas plants are located in Germany [23]. In 2012, the total installed electric capacity of these power plants was 3,352 MW and 22.84 TWh with a market value of 7.3 billion Euros were produced [9]. Because of the enormous economical importance of biogas production numerous studies have been performed to find ways for process optimization. There are plenty of approaches described in the literature to increase the biomethanation processes in biogas plants [3, 10, 12, 15, 16, 20, 25, 27].

For future perspectives of biogas production, one of the most critical issues is to understand the biological processes and to identify metabolic bottlenecks during the fermentation process. Biogas is formed in the course of anaerobic fermentation and consists mainly of methane (45–75 %) and carbon dioxide (25–55 %) [8, 26]. The decomposition of organic material during the fermentation process in a biogas plant is conducted by many groups of microorganisms. Renewable organic polymers are first degraded by enzymatic hydrolysis generating monomers such as sugars, amino acids, purines, pyrimidines, fatty acids and glycerol. In the second step, referred to as acidogenesis,

S. Refai and K. Wassmann authors contributed equally to this work.

S. Refai · K. Wassmann · S. van Helmont · S. Berger · U. Deppenmeier (✉)
University of Bonn, Institute for Microbiology and Biotechnology, Meckenheimer Allee 168, 53115 Bonn, Germany
e-mail: udeppen@uni-bonn.de

these intermediates are converted to the short organic acids butyric acid, propionic acid and acetic acid and to a lesser extent to ethanol and propanol. Byproducts are hydrogen and carbon dioxide. Subsequently, organic acids and alcohols are converted by syntrophic bacteria to acetic acid, carbon dioxide and hydrogen (syntrophic acetogenesis). The last step comprises the process of methane formation (methanogenesis). Two different metabolic groups of archaea are responsible for methane production. The hydrogenotrophic methanogens generate methane from hydrogen and carbon dioxide, whereas aceticlastic methanogens cleave acetate and form CH_4 and CO_2.

As described above, the anaerobic digestion process is a sequential, complex biochemical process, in which organic compounds are mineralized to biogas. The slowest reaction of the overall degradation acts as the rate-limiting step and determines the overall performance of biogas plants. It has been suggested that the rate-limiting factor of biomethanation is either the activity of exoenzymes that hydrolyze large polymeric substrates [6, 35] or the process of methanogenesis [22]. A third possibility is that the conversion of propionic acid and butyric acid during syntrophic acetogenesis is the bottleneck because these volatile acids are the most important intermediates in an anaerobic digestion, and their degradation is extremely complicated because of thermodynamic restrictions [2].

Very recently, Refai et al. [28] demonstrated that aceticlastic methanogenesis is not the limiting factor during biogas production when biogas sludge is supplemented with acetate or ethanol. Since these results are only based on 24 h batch fermentations, it was not possible to draw conclusions on the long-term effects of ethanol and its effect on process stability in biogas plants. Here, we present data on the effect of ethanol on methanogenesis during continuous incubation of biogas sludge under real process conditions using a small-scale (200 g biogas sludge) and a laboratory-scale (8 L biogas sludge) system which copy the conditions of a full-operating biogas plant.

Materials and methods

Unless otherwise noted, the biogas sludge used for experiments in this work was obtained from a commercially operating full-scale biogas plant (in the following referred to as full-scale reactor), which is located near Cologne (Germany). The operation temperature of the full-scale reactor was 40 °C and maize silage, cattle manure and poultry dry manure served as substrates resulting in a constant power of 540–580 kWh. Samples were collected between September 2013 and February 2014 and were used to analyse the effects of ethanol on methane formation. Biogas sludge was stored at 4 °C up to 2 weeks in sealed plastic bottles until use.

Preparation of small-scale continuous reactors

Small-scale reactors were set up in an anaerobic chamber (98 % N_2/2 % H_2) using screw top transfusion glass bottles (1,000 mL, Müller-Krempel, Bülach, Switzerland) which were filled with 200 g of original biogas plant sludge, sealed with a butyl rubber stopper and locked with an aluminium screw cap that had a round opening to allow the insertion of needles. The cultures were subsequently gassed with N_2/CO_2 (50/50 %) for 10 min. Incubation took place in a shaking incubator at 40 °C. Biogas production was measured every day by correlation of overpressure and concentration of CH_4 in the head space of the reactors. Overpressure was determined by means of water replacement in an upside down measuring cylinder and the respective methane concentration was determined by taking 30 µL samples from the head space of the reactor which were then analysed by gas chromatography (GC, Perkin Elmer Clarus® 480, Rascon FFAP column 25 m 0.25 µm, Perkin Elmer, Waltham, USA) with an FID detector. Measurements were performed with a column temperature of 120 °C, an injector temperature of 150 °C and a detector temperature of 250 °C with N_2 as carrier gas. For methane yield calculations, a 10 % methane standard (90 % argon) was analysed before and after every series of measurements. The values were normalized to standard conditions and were used to calculate the methane formation rates which are specified as µmol CH_4 per g sludge per h (µmol g^{-1} h^{-1}).

Feeding and sampling procedures of small-scale reactors

Samples (2 g) were taken every day under N_2-aeration and served for the determination of organic dry weight (oDM), pH and fatty acid concentration. After sampling, the reactors were fed with 2.5 g premixed and shredded substrates (8.6 g/L maize silage, 2.7 g/L cattle manure and 1.4 g/L dry chicken faeces) and 0.4 mL recirculate (supernatant of centrifuged biogas plant sludge) which resulted in an organic loading rate of 4.1 g oDM d^{-1} L^{-1}. Feeding was the same as in the full-scale reactor from which the sludge was derived. Ethanol was added after feeding as indicated. Fermenters were kept in a preheated water bath (40 °C) during the daily feeding and measuring procedures. Finally, fermenters were gassed with N_2/CO_2 (50/50 %) for 10 min and then incubated in shaking incubators at 40 °C for 24 h before the feeding and sampling procedures were repeated. In addition, beer was tested as supplement. To determine whether beer shows the same effect on methane production as pure ethanol, small-scale reactors were fed as described above and were mixed with beer (Paulaner, unfiltered "Hefe-Weißbier", alcohol content of 5.5 % v/v) to a final ethanol concentration of 10 mM. To reveal a possible effect of beer ingredients other than alcohol on biogas production,

alcohol was eliminated from the beer by lyophilisation. The lyophilisate was suspended in water and added to control fermenters. Fermenters supplemented with pure ethanol or with lyophilisate complemented with pure ethanol served as further controls.

Analysis of volatile acids and ethanol concentrations

For determination of acetate and ethanol content in small-scale reactors, 1 g biogas sludge was centrifuged for 2 min at 12,840*g*. 250 µL of the supernatant was mixed with 50 µL Carrez solution I (300 g/L zinc sulphate heptahydrate), 50 µL Carrez solution II (150 g/L potassium hexacyanoferrate(II) trihydrate) [7], and 150 µL H_2O and 50 µL 2 N HCl. The mixture was incubated at room temperature for 10 min and subsequently centrifuged for 2 min at 12,840*g*. The supernatant was filtered through a 0.45 µm nitrocellulose filter (Carl Roth, Karlsruhe, Germany) and 1 µL was injected to a GC (Shimadzu GC-14A, Shimadzu, Duisburg, Germany with Agilent Chromosorb 101 column, Agilent Technologies, Santa Clara, USA) with N_2 as carrier gas and a constant column temperature of 170 °C. Injector and FID-detector temperature was set to 220 °C, respectively. Calibration curves were generated with sodium acetate or ethanol standard solutions, which were processed according to the sludge samples. Determination of organic dry mass (oDM) was performed as described in German Standard DIN 12879.

Setup of lab-scale continuous reactors

To ensure the technical viability of enhanced biogas formation as a consequence of ethanol supplementation, upscale experiments were performed. Therefore, acryl glass vessels with a capacity of 9 L (ATB Potsdam) and stirring devices (Stirring devices: IKA RW 20, Heidolph RZR 2051, controlling device: Conrad Electronics) were used (from here on referred to as lab-scale reactors). The double walled reactors were connected to a 39 °C water bath. To eliminate loss of heat and to protect the fermenter content from light, reactors were isolated with foam plastics. The reactors were filled with 8 L of 100 % microbial active digestate from a NawaRo plant and fed with maize silage and cattle slurry every day. The amount of substrates that were fed every day was increased over 4 weeks until an organic loading rate of 3.5 g oDM d^{-1} L^{-1} was reached, which was then maintained during the remaining time of the experiment. Addition of substrate was carried out once a day and 200 mL of the fermenter content was removed. Generated biogas was collected in gas collection bags (Tecobag, Fa. Tesseraux Spezialverpackungen GmbH, Bürstadt, Germany). Methane content was measured as described above. Ethanol (95 %) was added up to a final concentration of 50 mM on incubation days 42–44, 53–54 and 63–65. For the determination of FOS/TAC values, which represent the proportion of volatile fatty acids (FOS or VFA) and carbonate buffer capacity in terms of total inorganic carbon (TAC), the method according to Nordmann [24] was used.

Results

Development of small-scale continuous reactors

For evaluation of the biological process within NawaRo biogas plants usually laboratory-scale continuous fermenters are used that are typically filled with several liters of effluent from existing anaerobic digester facilities. Usually, the reactors are technically complex and a start-up process is necessary to increase the organic loading rate until stable biogas production is observed. This procedure is time consuming, the number of fermenters is limited and there is the danger that the experimental conditions do not directly reflect the conditions in biogas plants. In contrast, batch fermenters are technically more easy to handle and dozens of reactors can be analysed at a time. However, batch cultures do not allow the investigation of biogas production over a longer period and the long-term effects of additives on biogas production cannot be analysed. Therefore, there is a need for simple procedures to mimic the process of methane production in full-scale biogas plants using simple and space saving continuous reactors that can be run with authentic biogas sludge over days without changing the physico-chemical parameters. Therefore, we developed a small-scale continuous reactor system that was filled with 200 g biogas sludge from the model biogas plant. The fermenters were incubated at 40 °C for 14 days, and feeding and analysis of different process parameters were conducted daily as described in materials and methods. Methane production rates, oDM and volatile acid concentration were chosen as parameters to determine the stability of the continuous test system. All three parameters stayed constant in the daily fed fermenters (Fig. 1a, b) and were in line with parameters measured in the full-scale biogas plant from which the sludge was obtained. The full-scale biogas plant comprised a fermenter volume of 2,800 m^3 and produced approximately 140 m^3 methane per hour. This corresponded to a methane production rate of 2.2 µmol g^{-1} h^{-1} and was similar to the rate of 2.16 ± 0.07 µmol g^{-1} h^{-1} observed in the small-scale continuous reactors over a period of 14 days (Fig. 1a). Interestingly, the methane formation rate stayed constant even over a period of 30 days with daily feeding of the small-scale reactors (not shown). oDM was approximately 90–100 g per kg biogas sludge in the small-scale reactors from day 1–14 and varied between 83 and 105 g kg^{-1} in

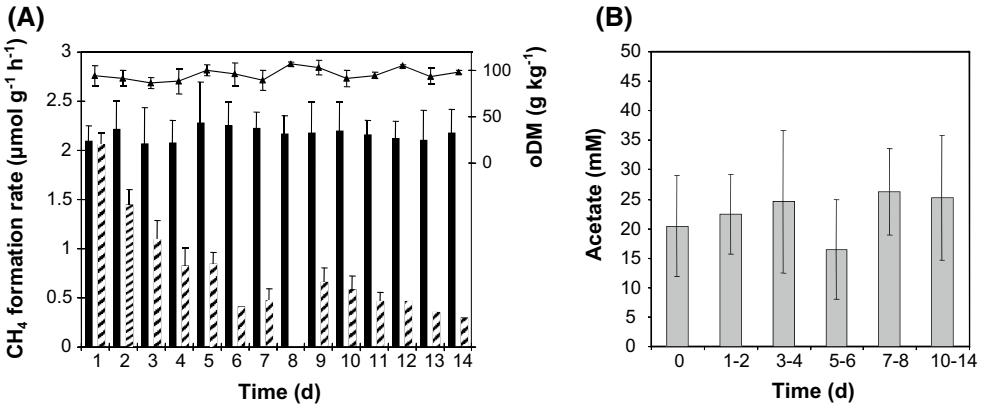

Fig. 1 Performance and stability of small-scale reactors. All reactors were daily fed with maize silage, cattle manure and dry chicken faeces as indicated in materials and methods. **a** *Black bars*, methane formation rate of reactors with daily feeding. *Dashed bars*, methane formation rate of reactors without feeding. (*filled triangle*) oDM in daily fed fermenters. **b** Acetate concentration in small-scale reactors with daily feeding. The experiments were conducted in duplicate and repeated at least five times using different sludge samples. The respective standard deviations are indicated by *error bars*

the full-scale plant. Acetate concentrations of 14–30 mM were determined in the full-scale biogas plant and were 16–26 mM in the small-scale reactors (Fig. 1b). Also, propionate concentrations were similar with 1.7–2.8 mM in the full-scale plant and 2–4 mM in the small-scale reactors (not shown). Butyrate concentrations were always below detection limit. Fermenters, which were not fed, served as control and results clearly showed that feeding was necessary to maintain constant biogas formation. Without the addition of substrate, methane production rates decreased from 2.1 to 0.3 µmol g^{-1} h^{-1} within 14 days (Fig. 1a). From these results, it became evident that the continuous fermentation in small-scale reactors is a suitable system to observe the effect of different conditions or additives on biogas formation within a period of 14 days.

Effect of ethanol supplementation on methane formation in small-scale continuous reactors

Experiments in small batch cultures pointed to potential bottlenecks during biogas formation from organic matter [28] and it was shown that neither ethanol oxidizing bacteria nor aceticlastic methanogenic archaea are involved in the limitation of methane production. Furthermore, methane production increased as a consequence of ethanol addition without interfering with the normal digestion processes during a period of 24 h [28]. However, investigation of long-term effects of ethanol additions is indispensable before possible biotechnological applications can be taken into consideration. Therefore, the effect of ethanol supplementation in the above-mentioned small-scale continuous reactors was studied with normal feeding over a time period of 14 days. In Fig. 2, the relative change of methane production following the addition of 100 mM

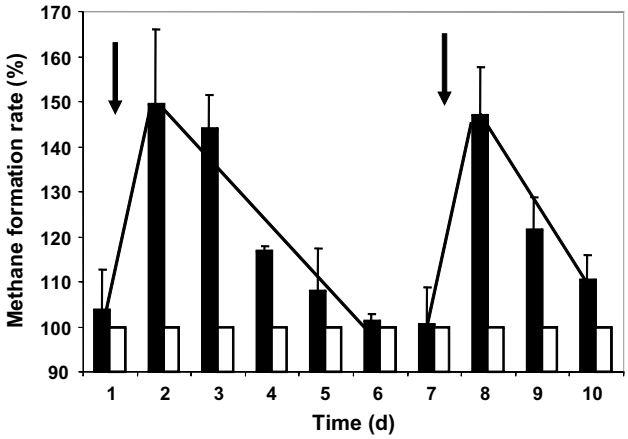

Fig. 2 Increase of methane formation rate after ethanol pulses in small-scale reactors. *Bars* represent the average methane production rates from three independent experiments performed in triplicates. Standard deviations are indicated by *error bars*. *Black bars*, fermenters with ethanol pulses (100 mM final concentration). *White bars*, control fermenters. *Black arrows* indicate addition of ethanol. All reactors were daily fed as indicated in materials and methods

ethanol in the small-scale reactors after 1 day and 7 days is shown, respectively. Already 24 h after the first ethanol pulse, the methane production rate reached its maximum and was enhanced by approximately 50 % in comparison to the control. During the following days, methane production slowly decreased until the rate of the control fermenter was reached again. When a second 100 mM ethanol pulse was applied after 168 h, methane formation increased again by about 50 % and slowly aligned to that of control fermenters after a few days. Furthermore, the basic methane formation rate of 100 % in the control fermenters was determined to an average of 1.93 ± 0.22 µmol g^{-1} h^{-1} over

14 days and was similar to the full-scale operating biogas plant productivity with an average of 2.2 μmol g^{-1} h^{-1}. The maximum methane production rate in ethanol fermenters was 2.83 ± 0.27 μmol g^{-1} h^{-1} and pH, acetate concentration and oDM were constant and comparable to control fermenters during the time of fermentation (not shown).

Effect of ethanol supplementation on methane formation in 9-L continuous lab-scale fermenters

To investigate whether the ethanol effect was also prominent in lab-scale reactors for a time period which is similar to the hydraulic retention time of full-scale biogas plants, 9 L acryl glass reactors were filled with 8 L of microbial active digestate from a NawaRo biogas plant and were fed with increasing amounts of maize silage and cattle manure every day. After 4 weeks, an organic loading rate of 3.5 g oDM d^{-1} L^{-1} was reached and maintained. Ethanol was added up to a final concentration of 50 mM on incubation days 42–44, 53–54 and 63–65. The impact on methane formation is depicted in Fig. 3a. Every ethanol pulse resulted in an immediate increase in methane production by nearly 150 % compared to control fermenters. In each case, maximum values were reached 2–3 days after supplementation. Subsequently, methane production of ethanol fermenters progressively aligned with that of control fermenters until they almost matched after 8–10 days. The additional production of methane after ethanol supplementation was directly linked to the activity of ethanol oxidizing bacteria, which is well reflected in slightly enhanced acetate concentrations after each ethanol pulse. Acetate concentrations increased from initially 5 to 10–20 mM within 2 days after the addition of ethanol and decreased to the initial concentration during the following 4 days (Fig. 3b). However, determination of pH values revealed that the buffer capacity of the biogas sludge was high enough to countervail acidification as a consequence of slightly increased acetate concentrations. The pH values of control and ethanol fermenters equalled each other during the whole time of incubation and averaged between 7.7 and 8.0 (Fig. 3c). Moreover, the oDM values of control and ethanol fermenters were similar and ranged from 55 to 68 g kg^{-1}. The FOS/TAC value represents the proportion of volatile fatty acids (FOS or VFA) and carbonate buffer capacity in terms of total inorganic carbon (TAC). This proportion is an important stability parameter during the anaerobic digestion in biogas plants. FOS/TAC is mainly depending on substrate composition and values between 0.2 and 0.4 have proven to be optimal [14]. As shown in Fig. 3e, FOS/TAC varied between 0.21 and 0.4 in the large-scale continuous fermenters (with a VFA of 3–5 HAceq L^{-1} and a TAC of 13–16 g $CaCO_3$ L^{-1}) and was therefore in accordance with the recommended value for full-scale plants. With these results, it became evident that ethanol does not only enhance biogas formation in small scale during a short observation period in batch fermenters but also when realistic conditions of full-scale biogas plants are imitated. Since this effect could be observed in both, the small-scale and the lab-scale continuous fermentation, these test systems can be regarded as comparably suitable to study long-term effects of ethanol on biogas formation. Hence, in terms of saving time, space and costs, further investigations were conducted in the more convenient small-scale reactors.

Influence of daily addition of ethanol on methanogenesis in biogas sludge

Besides the addition of pure ethanol every 7 days (pulsed supplementation) as shown above, the effect of a continuous supplementation with diluted ethanolic solutions was of great interest with regard to possible future biotechnological applications. Therefore, small-scale continuous reactors were fed as described in materials and methods and supplemented with 10 and 20 mM ethanol every 24 h, respectively. Methane formation rates of control fermenters without addition of ethanol were set to 100 % corresponding to methane formation rates of 2.0 ± 0.3 μmol g^{-1} h^{-1}. When the ethanol concentration was adjusted to 20 mM once a day, methane production increased in average by 30 % after 1 day and 60 % after 6 days, respectively, and revealed a constant rate of 3.2 ± 0.3 μmol CH_4 g^{-1} h^{-1} till the end of the experiment. The addition of 10 mM ethanol per day enhanced methane production by 30 % compared to control reactors and reached a methane formation rate of 2.6 ± 0.3 μmol g^{-1} h^{-1}. The values are in agreement with the theoretically expected increase of methane formation. The final concentration of 10 mM ethanol for example corresponded to 2 mmol of ethanol that was consumed every 24 h in the fermenters with a sludge content of 200 g (equals about 200 mL). Keeping in mind that 3 mmol of methane is formed from 2 mmol of ethanol, the amount of methane produced from ethanol should have been in the range of 0.6 μmol g^{-1} h^{-1}. The rate of the ethanol-supplemented fermenter was 2.6 μmol CH_4 g^{-1} h^{-1} indicating that 2.0 μmol CH_4 g^{-1} h^{-1} was generated from normal feeding (as in the control fermenters) and 0.6 μmol CH_4 g^{-1} h^{-1} from ethanol. Important physico-chemical parameters such as pH values, oDM and acetate concentration stayed constant over the whole period of incubation and no ethanol remained when 10 or 20 mM was added daily. Hence, ethanol was completely metabolized to methane in a range of 10–20 mM within 24 h. Therefore, the continuous addition of ethanol did not disturb processes, which normally occur in the biogas plant. The "normal" methane formation from digestion of the daily fed substrates continued to take place, indicating the possibility to constantly increase biogas formation by ethanol supplementation.

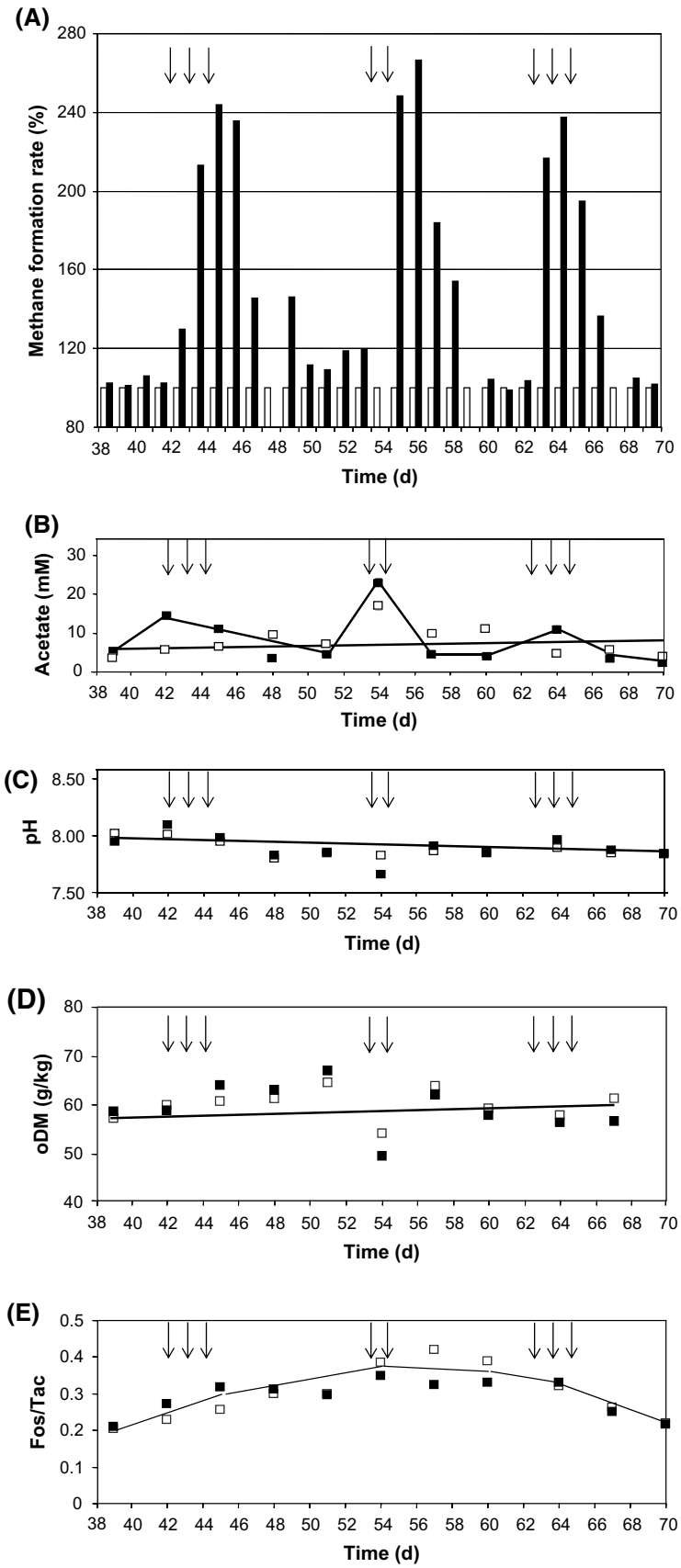

Fig. 3 Effect of ethanol on CH_4-formation in 8 L lab-scale reactors. Feeding of the reactors was performed daily as described in materials and methods. *Arrows* indicate ethanol (95 %) addition to a final concentration of 50 mM each on incubation days 42–44, 53–54 and 63–65. **a** Methane formation rate. *Black bars*, fermenters with ethanol pulses. *White bars*, control fermenters. **b** Acetate concentration (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses. **c** pH values, (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses. **d** oDM, (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses. **e** FOS/TAC value, (*open square*) control fermenters, (*filled square*) fermenters with ethanol pulses

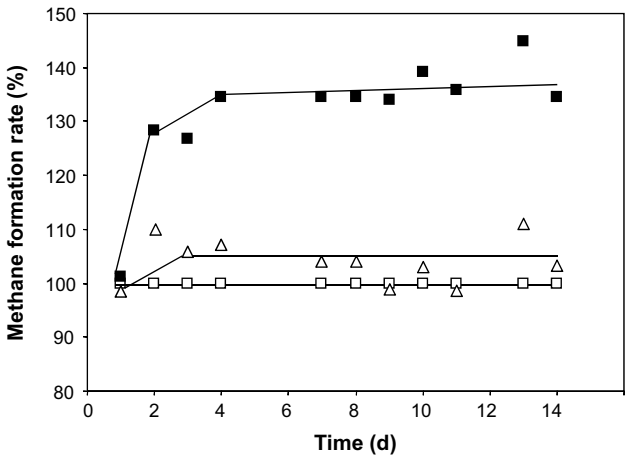

Fig. 4 Effect of beer on methane formation in continuous small-scale reactors. All reactors were daily fed with maize silage, cattle slurry and dry chicken faeces as indicated in materials and methods. The methane formation rate of control fermenters was 1.8 ± 0.3 µmol g^{-1} h^{-1} (*open square*) and was set to 100 %, (*filled square*) daily addition of beer to a final ethanol concentration of 10 mM ethanol, (*open triangle*) daily addition of ethanol-free beer. Data of one representative experiment out of three independent experiments are shown. Average methane production was calculated from two repeats for each condition

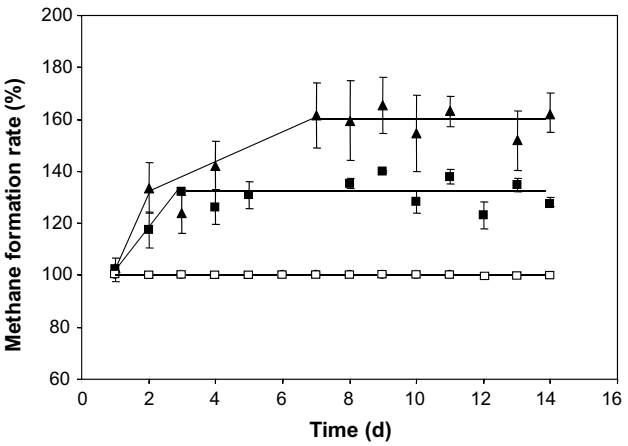

Fig. 5 Effect of daily addition of ethanol on methane formation. A substrate mixture of maize silage, cattle slurry and dry chicken faeces was fed to all reactors daily as indicated in materials and methods. The methane formation rate of control fermenters was 2.0 ± 0.3 µmol g^{-1} h^{-1} (*open square*) and was set to 100 %, (*filled square*) daily addition of 10 mM ethanol (final concentration), (*filled triangle*) daily addition of 20 mM ethanol (final concentration). Data represent the average activities of sludge from three different samples with two replicates of the full-scale biogas plant. *Error bars* indicate standard deviations

However, with respect to economic issues, the use of ethanolic solution produced by alcoholic fermentation of organic material instead of pure ethanol is preferable. In this work, beer served as an example for such alcoholic fermented substrates. With an ethanol content of 5.5 %, beer reasonably represents a diluted complex ethanol containing solution. To investigate the potential of beer to increase methane formation, small-scale continuous reactors were supplemented with 2 mL beer (Paulaner, unfiltered "Hefe-Weißbier", alcohol content of 5.5 % v/v) per day to a final ethanol concentration of 10 mM in addition to the normal feeding. This resulted in a constant increase in methane formation by approx. 35 % (Fig. 4). Similar to fermenters, which were supplemented with pure ethanol, no ethanol could be determined after 24 h of incubation. With an oDM of beer of ~40 g kg^{-1}, the additional input of fermentable organic substrates was slightly increased by ~0.4 g kg^{-1} per day in fermenters supplemented with beer, which corresponds to an increase of total oDM in the fermenter by ~5 %. Therefore, methane production rates resulting from supplementation with beer were naturally slightly higher than those observed with pure ethanol (Fig. 5). This was also in accordance with methane production of control fermenters, which were supplemented with beer without ethanol. Methane production rates were approximately 5 % higher than those of non-supplemented control fermenters. From these results, it can be calculated that the use of beer with a lower percentage of ethanol would lead to a lower increase in methane production. But even a beer with an oDM of ~40 g kg^{-1} and an ethanol content of 1 % (corresponds to 1.8 mM final concentration of ethanol in the 200 mL fermenters) would theoretically lead to an enhanced methane production of about 11 % in our small-scale continuous fermenters when 2 mL of beer is fed every day. In summary, not only pure ethanol but also reasonably priced complex alcoholic fermented substrates with low alcohol content are suitable to raise methane production. This finding supported the biotechnological relevance and practicability of the addition of ethanol to enhance biogas formation.

Discussion

Recently, a test system for the analysis of the performance of biogas sludge using small-scale batch fermenters was established which facilitated the investigation of effects of ethanol and acetate supplementation on methane formation during 24 h incubation. The convenience to receive statistically firm data within a short period of time due to the potential to run a multitude of small-scale batch fermenters simultaneously was one advantage of the batch experiments. However, batch cultures did not necessarily reflect conditions of full-scale reactors. To determine if increased methane production rates due to ethanol addition can be maintained over a longer time period, it was necessary to establish a reliable and convenient test system that allowed

a continuous and stable biogas production. We demonstrated that conditions of the full-scale biogas plant were well reflected during 14 days of observation in our small-scale continuous reactors. Hence, it can be concluded that the experimental setup allowed the simulation of realistic operation conditions of biogas plants. It is worth to mention that the application of small-scale continuous fermenters for analysis of full-scale biogas plants has a number of advantages. There is no need for a time-consuming start-up phase which is characterized by a gradually increase of oDM before steady state conditions are reached [4]. Our system allows a direct use of active biogas sludge from a running system and steady state conditions are already reached after a few hours [28]. The small size of the reactors enables handling in an anaerobic hood preventing possible inhibitory effects of oxygen during inoculation or feeding.

When small-scale or lab-scale continuous fermenters were pulsed with 50–100 mM ethanol, biomethanation increased by 50–150 %, depending on the consistency and composition of the biogas sludge. It was also possible to increase methane formation by 30–60 % in the small-scale reactors when pure ethanol or ethanolic solution (e.g. beer) was added daily to a final ethanol concentration of 10–20 mM. Furthermore, different important process parameters such as FOS/TAC, organic dry mass (oDM), acetate concentration and pH in the control and ethanol-supplemented fermenters were in the range of the model biogas plant. In summary, the experiments revealed that methane production, which normally proceeds in a biogas plant, is not inhibited by the addition of ethanol. This means that basic methane production continues to take place (100 % efficiency), but ethanol leads to the production of additional methane in the biogas plant. In principal, an increase in organic loading rate was achieved without influencing normal fermentation processes.

Multiple renewable organic materials can be applied to 'NawaRo'-biogas plants; however, most often maize is the dominant substrate, which is usually combined with grass silage and cattle or pig manure [8]. Depending on the type of substrate, a wide range of values can be obtained for the substrate-specific methane yield. In addition, system-dependent parameters such as volume load or hydraulic retention time play a role for the resulting substrate-specific methane yields [13]. It was observed that increased loading rates often led to acidification and to a breakdown of the methanation process [17]. Therefore, an improvement of efficiency of biogas plants by increased feeding is obviously very difficult or even impossible. The synthesis of methane depends on a variety of microorganisms and includes a huge number of biochemical reactions that form a reaction chain for the conversion of biopolymers into CH_4. However, the "weakest link of the chain" determines the performance and the speed of the overall system. Previous experiments and thermodynamic considerations already gave a clue about this "weakest link of the chain": it was assumed that anaerobic fatty acid oxidation constitutes one bottleneck in the process of biogas formation [28]. This hypothesis is based on the fact that many bacteria grow in obligate syntrophy with methanogens on substrates that are not fermentable under standard conditions. In these cases, methanogens are essential to reduce the concentrations of hydrogen to make the reaction sufficiently exergonic to support energy conservation, cell maintenance and growth. In fact, hydrogen partial pressures below ca. 10^{-4} and 10^{-3} atm are necessary for degradation of propionate and butyrate (Eqs. 1, 2), respectively [1, 5, 31]. Such low hydrogen partial pressures in methanogenic systems are achieved by interspecies transfer of molecular hydrogen or formate from syntrophic bacteria to hydrogen-oxidizing methanogens [19, 21, 30, 32].

$$\text{Propionic acid} + 2\,H_2O \rightarrow \text{Acetic acid} + CO_2 + 3\,H_2 \quad \Delta G^{o\prime} = +76\ \text{kJ/mol} \tag{1}$$

$$\text{Butyric acid} + 2\,H_2O \rightarrow 2\ \text{Acetic acid} + 2\,H_2 \quad \Delta G^{o\prime} = +48\ \text{kJ/mol} \tag{2}$$

$$\text{Ethanol} + H_2O \rightarrow \text{Acetic acid} + 2\,H_2 \quad \Delta G^{o\prime} = +9.6\ \text{kJ/mol} \tag{3}$$

$$CO_2 + 4\,H_2 \rightarrow CH_4 \quad \Delta G^{o\prime} = -130\ \text{kJ/mol} \tag{4}$$

In this respect it is to note that biogas sludge is not a homogenous material and consists of particles, granules, cell aggregates and biofilms with different composition and diameters, which can be defined as micro-scale habitats or microenvironments. It is tempting to speculate that the actual H_2 pressure is not always the same in these microenvironments because of mass transfer imbalances and different physico-chemical conditions (e.g. pH, substrate and product concentration). Hence, only a part of the microenvironments may possess the proper thermodynamic conditions to allow fatty acid oxidation by syntrophic bacteria. The consequence would be a fluctuation of active and inactive microenvironments depending on the mass transfer and activity of hydrogenotrophic methanogens. Therefore, the overall performance of a biogas plant might depend on the number of active microenvironments that are able to perform butyrate and propionate oxidation to form acetate. The average of active microenvironments in turn depends on the overall H_2 pressure, which varies between 10 and 1,000 ppm in a normally operating biogas plant [18]. The lower the overall hydrogen concentration the more microenvironments can degrade butyrate and propionate and the higher the H_2 concentration the less microenvironments are active in short fatty acids oxidation to acetate. However, ethanol oxidation (Eq. 3) already turns to an exergonic reaction at a

H_2 pressure of about 10^{-2} atm. That means the H_2 concentration in ethanol oxidation can be much higher compared to butyrate/propionate oxidation. Taking together these facts, the oxidation of ethanol to acetate can be performed by the majority of microenvironments found in the biogas plant. It can also take place in those microenvironments that are temporally inactive with respect to propionate and butyrate oxidation. Thus, our hypothesis is that the addition of ethanol circumvents the butyrate/propionate bottleneck and leads to an increase in the velocity of methane production. Furthermore, it allows getting around the rate-limiting step in biogas production leading to an optimized methane formation and an increase of the overall throughput and the electricity yield per time. In this way, the entire capacity and the full potential of the biogas plant can be exploited. Therefore, the great advantage of supplementation with ethanol is the fact that ethanol added to the fermenter is directly channelled into methanogenesis so that volatile fatty acids such as propionate or butyrate cannot be formed from ethanol. Thus, the risk to head toward acidification after ethanol addition in biogas plants is eliminated.

With this knowledge, ethanol seems to be suitable to be applied to full-scale reactors to enhance biogas formation without disturbing normally occurring fermentation processes. The following factors are important for possible technical applications of ethanol with respect to increase in biogas formation:

(i) Cost-efficient production of ethanolic solutions: despite the fact that the addition of pure ethanol to a full-scale biogas plant will presumably enhance methane formation similarly to the effects we observed in our laboratory-scale fermenters, economic relevance is not given due to high ethanol costs. A cost-efficient alternative to increase biomethanation by the addition of ethanol is the application of diluted ethanol, whereas its origin can be diverse. To test whether complex diluted ethanolic substrates generally affect biomethanation positively, biogas sludge was supplemented with beer to a final ethanol concentration of 10 mM in addition to the usual daily feeding and methane formation was determined over 14 days. Methane production rates were increased by approximately 35 % which is in line with results of continuous fermentations with 10 mM pure ethanol and theoretical values. To reach an ethanol concentration of 10 mM in the fermenters, ~2 mL beer was added daily. With an oDM of beer of ~40 g kg^{-1}, the input of additional oDM per day was very low so that a continuation of normal feeding was possible. From these results, one can conclude that complex substrates with relatively low ethanol contents deriving from alcoholic fermentations are just as well applicable to improve biogas formation as pure ethanol. This opens up potential for biotechnological application. The addition of not saleable alcoholic drinks, e.g. because of exceeding expiration dates, is one possibility to increase cost effectiveness. A further industrially practicable opportunity is the setup of a pre fermenter, which allows an alcoholic fermentation of renewable vegetable raw material. The alcoholic fermented digestate of the pre fermenter could be added stepwise to the main fermentation vessel. Considering a final ethanol content of 10 % v/v in the pre fermenter, its volume could amount to only 10 % of the main fermentation vessel, but production of methane would be enhanced to a considerable degree. However, the principle idea of setting up a pre fermenter for alcoholic fermentation of organic material is not new [34]. But in contrast to other inventions, ethanol is not meant to be removed before the digestate is transferred to the main fermenter.

(ii) Adjustment of methane production to fluctuant power demands: biogas formation increases directly after the addition of ethanol. As shown in previous experiments in small-scale batch fermenters, a significant increase in methane production already occurs within 2 h after supplementation with ethanol [28]. In case of a future industrial application, this outstanding feature facilitates the adjustment of methane production to fluctuant demands and to secure power supply at any time. Addition of ethanol to the biogas plant can be used to ensure power supply in times of peak loads or temporary occurring maximum demands in the electric supply network arising from time of the day or season.

(iii) Increased methane concentration: Though the increase in biogas formation is not the only advantage, ethanol addition entails. Before biogas is fed into the natural gas grid, it has to be upgraded to concentrate methane in the gas mixture [11, 26, 29]. With the conversion of ethanol to methane, the methane content in the biogas is increased compared to "normal" biogas formation. A higher methane content involves a higher quality of the biogas and alleviates the gas reprocessing to natural gas quality.

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References

1. Ahring BK, Westermann P (1988) Product inhibition of butyrate metabolism by acetate and hydrogen in a thermophilic coculture. Appl Environ Microbiol 54:2393–2397

2. Amani T, Nosrati M, Mousavi SM, Kermanshahi RK (2010) Study of syntrophic anaerobic digestion of volatile fatty acids using enriched cultures at mesophilic conditions. IJEST 8:83–96
3. Bordeleau ÉL, Droste RL (2011) Comprehensive review and compilation of pretreatments for mesophilic and thermophilic anaerobic digestion. Water Sci Technol 63:291–296
4. Brambilla M, Araldi F, Marchesi M, Bertazzoni B, Zagni M, Navarotto P (2012) Monitoring of the startup phase of one continuous anaerobic digester at pilot scale level. Biomass Bioenergy 36:439–446
5. Bryant MP, Wolin EA, Wolin MJ, Wolfe RS (1967) *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch Microbiol 59:20–31
6. Busch G (2013) Biogas technology. In: Yang ST, Enshasy HE, Thongchul N (eds) Bioprocessing technologies. Wiley, New York, pp 279–292
7. Carrez PMC (1908) Le ferrocyanure de potassium et l'acétate de zinc comme agents de défécation des urines. Annales de chimie analytique 13:97–101
8. Deublein D, Steinhauser A (2008) Biogas from waste and renewable resources: an introduction. Wiley, Weinheim
9. Fachverband Biogas e.V. (2012) Branchenzahlen 2011 und Prognose der Branchenentwicklung 2012/2013. http://www.biogas.org/edcom/webfvb.nsf/id/DE_PM-29-12/$file/12-11-16_Biogas%20Branchenzahlen%202011-2012-2013.pdf Accessed 10 Jul 2014
10. Frac M, Ziemiński K (2012) Methane fermentation process for utilization of organic waste. Int Agrophy 26:317–330
11. Hagen M, Polman E, Jensen J, Myken A, Jönsson O, Dahl A (2001) Adding gas from biomass to the gas grid. Swedish Gas Center, Malmö
12. Hahn H, Krautkremer B, Hartmann K (2014) Review of concepts for a demand-driven biogas supply for flexible power generation. Renew Sust Energ Rev 29:383–393
13. Hashimoto AG (1982) Methane from cattle waste: effect of temperature, hydraulic retention time, and influent substrate concentration on kinetic parameter. Biotechnol Bioeng 14:2039–2052
14. Hölker U (2013) Eine ständig aktualisierte und erweiterte Beschreibung von über 1.600 Biogasanlagen. http://www.biogaswissen.de. Accessed 10 Jul 2014
15. Krishania M, Kumar V, Vijay VK (2013) Analysis of different techniques used for improvement of biomethanation process. Fuel 106:1–9
16. Krishania M, Kumar V, Vijay VK (2012) Opportunities for improvement of process technology for biomethanation processes. Green process Synt 1:49–59
17. Lerm S, Kleyböcker A, Miethling-Graff R, Alawi M, Kasina M, Liebrich M, Würdemann H (2012) Archaeal community composition affects the function of anaerobic co-digesters in response to organic overload. Waste Manag 32:389–399
18. McInerney MJ and Bryant MP (1981) In: Wise DL (ed) Fuel gas production from biomass. Chemical Rubber Co. Press Inc. West Palm Beach, pp 26–40
19. McInerney MJ, Sieber JR, Gunsalus RP (2009) Syntrophy in anaerobic global carbon cycles. Curr Opin Biotechnol 20:623–632
20. Morita M, Sasaki K (2012) Factors influencing the degradation of garbage in methanogenic bioreactors and impacts on biogas formation. Appl Microbiol Biotechnol 94:575–582
21. Müller N, Worm P, Schink B, Stams AJM, Plugge CM (2010) Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms. Environ Microbiol Rep 2:489–499
22. Munk B, Bauer C, Gronauer A, Lebuhn M (2012) A metabolic quotient for methanogenic Archaea. Water Sci Technol 66:2311–2317
23. Munk B, Lebuhn M (2014) Process diagnosis using methanogenic Archaea in maize-fed, trace element depleted fermenters. Anaerobe. doi:10.1016/j.anaerobe.2014.04.002
24. Nordmann W (1977) Die Überwachung der Schlammfaulung. Korrespondenz Abwasser 3
25. Parawira W (2012) Enzyme research and applications in biotechnological intensification of biogas production. Crit Rev Biotechnol 32:173–186
26. Persson M, Jönsson O, Wellinger A (2006) Biogas upgrading to vehicle fuel standards and grid injection. Brochure of IEA Task 37 "Energy from Biogas and Landfill Gas"
27. Rajagopal R, Masse D, Singh G (2013) A critical review on inhibition of anaerobic digestion process by excess ammonia. Bioresour Technol 143:632–641
28. Refai S, Wassmann K, Deppenmeier U (2014) Short term effect of acetate and ethanol on methane formation in biogas sludge. Appl Microbiol Biotechnol 98:7271–7280
29. Ryckebosch E, Drouillon M, Vervaeren H (2011) Techniques for Transformation of Biogas to Biomethane. Biomass Bioenergy 35:1633–1645
30. Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262–280
31. Schmidt JE, Ahring (1993) Effects of hydrogen and formate on the degradation of propionate and butyrate in thermophilic granules from an upflow anaerobic sludge blanket reactor. Appl Environ Microbiol 59:2546–2551
32. Sieber JR, McInerney MJ, Gunsalus RP (2012) Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. Annu Rev Microbiol 66:429–452
33. Whitlock R (2012) German biogas market slumps in contrast to Europe http://www.renewableenergymagazine.com/
34. Wilkie AC, Riedesel KJ, Owens JM (2000) Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstock. Biomass Bioenergy 19:63–102
35. Wirth R, Kovács E, Maróti G, Bagi Z, Rákhely G, Kovács KL (2012) Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnol Biofuels 5:41