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Identification of methanogenesis and syntrophy as important microbial metabolic processes for optimal thermophilic anaerobic digestion of energy cane thin stillage

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ABSTRACT

The aim of this research was to identify key microorganisms for thermophilic (55 °C) anaerobic digestion of thin stillage derived from hydrolysis and ethanol fermentation of energy cane in a conventional stirred tank reactor with a 10-day hydraulic retention time. Efficient thermophilic anaerobic digestion with a specific methane production of 0.43 Lmethane/gtCOD used/d and biogas containing around 56% methane was accomplished. Due to an overnight temperature perturbation the specific methane production decreased to 0.16 Lmethane/gtCOD used/d. Analysis of the microbial community showed the importance of methanogenic Archaea belonging to *Methanosarcina* and *Methanothermobacter* as well as syntrophic Bacteria related to *Thermacetogenium*, *Tepidanaerobacter* and *Anaerobaculum*. This indicates that retention of biomass maintaining syntrophy and methanogenesis more efficiently may be useful for thermophilic anaerobic digestion of thin stillage derived from the production of energy cane ethanol.

1. Introduction

Lignocellulosic biomass is milled, pretreated, hydrolyzed and fermented prior distillation to retrieve bioethanol. Roughly one third of the carbon is converted to bioethanol, one third to carbon dioxide and the remainder to dissolved and suspended carbon in whole stillage. Dried distiller's grains can be produced from whole stillage by evaporation and drying. Remaining thin stillage can be directly recycled as process water. Energy investment for bioethanol production was markedly decreased when applying anaerobic digestion of thin stillage and recovery of methane as biofuel. Using thermophilic anaerobic sequencing batch reactors as well as thermophilic and mesophilic semi-continuously stirred tank reactors, natural gas consumption could be lowered by 51%, 43–59% and 54%, respectively (Agler et al., 2008; Lee et al., 2011; Schaefer and Sung, 2008).

For efficient anaerobic digestion, integration of microbial ecology by selecting appropriate microbial communities for fermentation is important (Wang et al., 2013). A few studies on anaerobic digestion of thin stillage have applied microbial community analysis. For example, a follow-up study showed stability when predominantly aceticlastic methanogenesis was present in a mesophilic fixed bed reactor (high relative abundances of *Methanosaeta* and *Methanospirillum*) and less stability when both hydrogenotrophic and aceticlastic methanogenesis were present in mesophilic UASB and anaerobic sequencing batch reactors (high abundance of *Methanoculleus* and *Methanosarcina*) (Ziganshin et al., 2016). This indicates the importance of aceticlastic methanogenesis and may show that this pathway should be optimized to improve bioreactor performance under mesophilic conditions. A similar study involving batch reactors under thermophilic conditions (55 °C) showed predominance of hydrogenotrophic methanogenesis (*Methanoculleus bourgensis*) during anaerobic digestion of thin stillage (Town et al., 2014b). However, methanogenesis collapsed in these reactors, which was not observed in a subsequent batch study where the dominance of both hydrogenotrophic and aceticlastic methanogens (*Methanothermobacter marburgensis* and *Methanosarcina barkeri*) was proposed to provide a more robust microbial community (Town et al.,

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2014a). In a lab-scale CSTR (continuously stirred tank reactor) fed with thin stillage that was changed from 38° to 44 °C abundance of *Tepidanaerobacter acetatoxydans*, Methanomicrobiales and Methanosarcinaceae increased with temperature indicating more syntrophic acetate oxidation (Moestedt et al., 2014). Syntrophic acetate oxidation (with *Tepidanaerobacter syntrophicus* plus *Methanothermobacter thermautotrophicus*) was also observed in a CSTR operated at 55 °C and fed with thin stillage (Sabra et al., 2015). Based on this previous work the processes of methanogenesis and syntrophy could be important for increased robustness of performance under thermophilic conditions.

In this study, we aimed to identify signature microorganisms that offer stability to thermophilic (55 °C) anaerobic digestion of thin stillage derived from a pilot plant producing bio-ethanol from energy cane. The importance of syntrophy and methanogenesis under these test conditions was hypothesized and studied. To achieve the aim, a CSTR was set up and underwent inoculation, adaptation and stabilization. Further, an additional temperature perturbation was used to study the reactor performance stability and the response of its microbial community in more detail. Chiefly chemical composition of bioreactor effluent and gas production were used as measures of performance. Furthermore, pyrosequencing analysis was used to study the reactor microbial community.

2. Materials and methods

2.1. Seed sludges and thin stillage

A mixture of sludges from five different anaerobic thermophilic digesters from wastewater treatment plants using a temperature-phased anaerobic digestion system was used as inoculum. These five digesters were located in Delafield (WI), Oswego (WI, two digesters), Sturgeon Bay (WI) and in Duluth (MN). 100-mL samples of the five different sludges were mixed and 450 mL (15% of total working volume) of this mixture was added into a CSTR that contained 2.55 L of energy cane stillage (7.5 g COD L^{-1}), pH 7. A detailed description of storage conditions and energy cane stillage properties including its chemical composition has been provided previously (Oosterkamp et al., 2016). Briefly, energy cane stillage was derived from the BP Advanced Biofuel Demonstration Plant located in Jennings (LA). Thin stillage was transported in 55-gallon drums under refrigeration and aliquoted in a total amount of about 35 sealed buckets that were stored at 4 °C.

2.2. CSTR running conditions and operation

The New Brunswick Microferm fermentor series MF-105 CSTR with 5-L vessels (New Brunswick, Edison, NJ, USA) was used with a working volume of 3 L, temperature of 55 °C and mixing at 200 rpm. At start an inoculation or batch condition was used with a 10.8 g COD L^{-1} feed from inoculation on day 0 until day 5 (a 0.5 HRT period). Subsequently, the reactor was run semi-continuously with a hydraulic retention time (HRT) of ten days. Adaptation involved feeding firstly with 10.8 g COD L^{-1} thin stillage from day 6 until day 11 (0.6 HRT) and secondly with 21.7 g COD L^{-1} stillage from day 12 until day 33 (2.2 HRT). Stabilization was achieved with 43.4 g COD L^{-1} stillage from day 34 until day 99 (6.6 HRT). Temperature perturbation that cooled the reactor to ambient temperature at day 99 was further studied by monitoring the reactor when thermophilic temperature was restored from day 100 to day 154 (5.5 HRT). An overview of the operating conditions of the reactor is provided (Table 1).

2.3. Methane percentage in total gas and specific methane production

Biogas production was monitored by a Milli GasCounter (MGC-10, Ritter, Bochum, Germany). Methane percentage and specific methane production were calculated according to a previously described procedure (Oosterkamp et al., 2016). Briefly, the total volume of gas produced, the methane percentage (determined using a gas chromatograph) and the COD (chemical oxygen demand) used per day were measured. Gas production and methane percentage were determined on a daily basis. Average and standard deviations of daily values reached during the different conditions were calculated and shown using GraphPad prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Table 1
The five different operating conditions of the thermophilic CSTR producing methane from thin stillage derived from the production of energy cane ethanol.

Condition	Time (days)	HRT (days)	Feed COD (g tCOD L^{-1})	OLR (g tCOD L^{-1} day^{-1})
Inoculation	1 to 5	Batch	10.8	–
Adaptation 1	6 to 11	10.0	10.8	1.1
Adaptation 2	12 to 33	10.0	21.7	2.2
Stabilization	34 to 99	10.0	43.4	4.3
Perturbation	100 to 154	10.0	43.4	4.3

2.4. COD analysis

COD was determined using the COD2 Mercury-free reagent (Hach, Loveland, CO, USA) according to the manufacturers' instructions. COD used by the digester was based on the difference between influent and effluent COD. Total COD (tCOD) as well as soluble COD (sCOD) determined on the supernatant after centrifugation at 10,000 rpm for 5 min. COD measurements were taken at day 1, 4 and 5 for inoculation, day 8, 10, 11, 21, 26 and 32 for adaptation, day 43, 58, 70, 82 and 96 for stabilization plus day 111, 125, 133, 146 and 154 for perturbation. The averages and standard deviations were determined using GraphPad prism 6.0 (GraphPad Software, Inc.).

2.5. pH measurement

The pH of bioreactor effluent was determined using an Accumet AB15 pH meter (Fisher Scientific, Pittsburgh, PA, USA) on a daily basis. The average and standard deviation of daily pH measurements for each of the different conditions were shown using GraphPad Prism v. 6.0 (GraphPad Software, Inc.).

2.6. Liquid chromatography

Anions and organic acids were analyzed by hydroxide-selective anion exchange chromatography, monosaccharides by anion exchange chromatography and glycerol, ethanol, 5-HMF and furfural using liquid chromatography as described previously (Oosterkamp et al., 2016). Samples from day 1 and 5 were analyzed for inoculation, samples from day 6, 10, 21 and 32 for adaptation, day 82 and 96 for stabilization plus 111 and 154 for perturbation. Averages from technical duplicate measurements of the samples were used, and the average and standard deviation between the biological duplicates (samples from the different days) were calculated and shown using GraphPad Prism v. 6.0 (GraphPad Software, Inc.).

2.7. Pyrosequencing analysis

For pyrosequencing analysis, sludge samples were harvested during inoculation at day 0, from stabilization at day 82, perturbation at day 111 and at the end on day 154. DNA was isolated from the samples using the FastDNA SPIN kit for soil (Qiagen, Hilden, Germany) according to the instructions from the manufacturer. To verify that no contamination was introduced from the kit a negative control to which no sample material was added followed the similar DNA isolation procedure and this control showed no detectible DNA. The integrity of the isolated DNA was checked on 1% agarose gel and the DNA

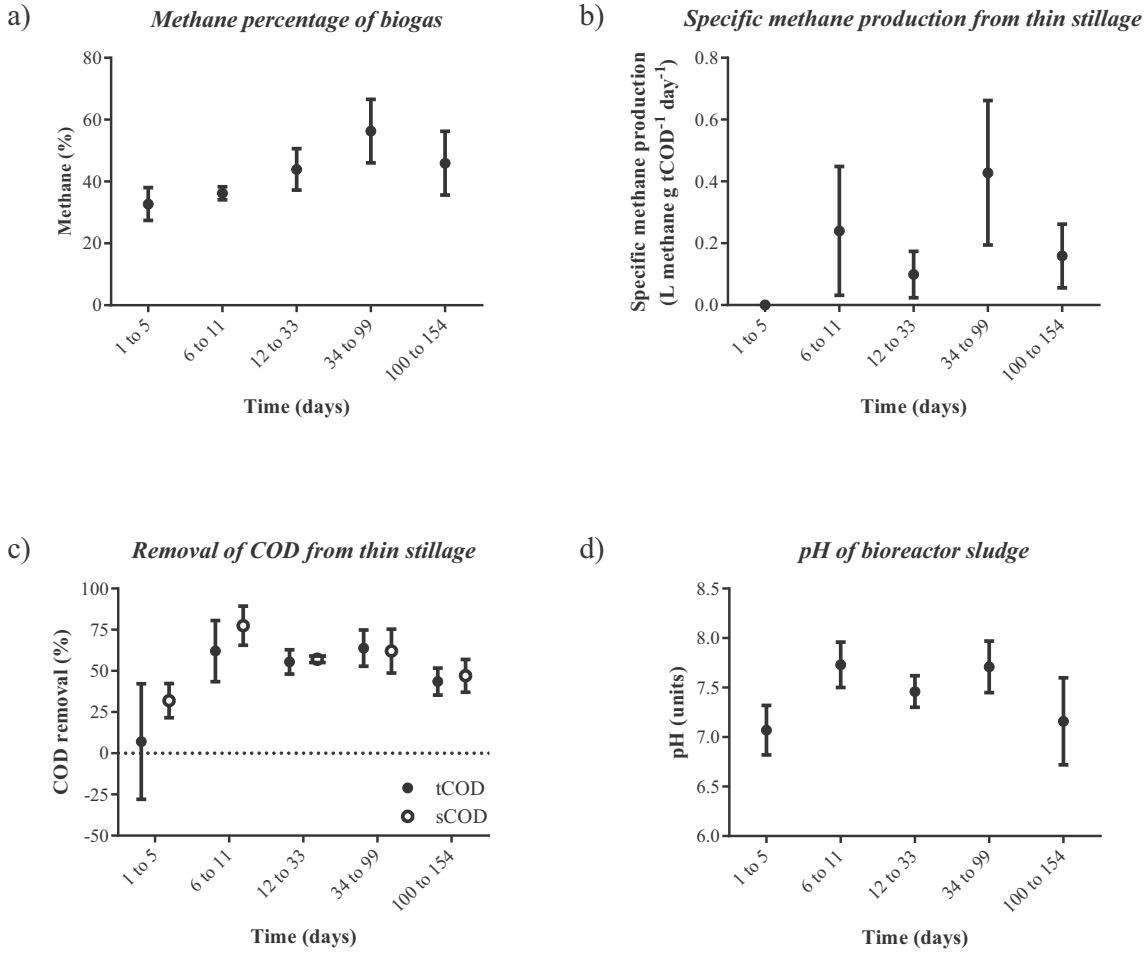

Fig. 1. Bioreactor performance of a CSTR producing methane from thin stillage derived from the production of bioethanol from energy cane. The percentage of methane in the biogas produced (a), specific methane production (b), removal of COD from thin stillage (c) and pH of the bioreactor sludge (d) are shown for each of the five different run stages.

concentration was measured using Nanodrop (ND 2000, Thermo Fisher Scientific, Waltham, MA). The DNA was used to amplify the V4 region of the 16S rRNA using PCR and with 515F and 806R primers. Primer constructs, PCR conditions and the PCR program were as previously described (Oosterkamp et al., 2016). All reactions were performed in triplicate and checked on 2.5% agarose gel. As a PCR negative control reactions were included to which no DNA template were added and these showed no visible amplified DNA fragment. The PCR amplification was also performed using DNA from a soil microbial community. The soil microbial community was checked with pyrosequencing to contain a low relative abundance of archaeal representatives (Bates et al., 2011). Other controls used for pyrosequencing included the PCR negative as well as the DNA isolation negative control. Triplicate PCR reactions were combined in preparation for pyrosequencing. Combined PCR reactions were purified using the Zymo DNA clean and concentrator kit (Zymo Research, Irvine, CA) and the Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, CA) was used for quantification. The quantified PCR fragments were pooled in equimolar ratios. A final quantification of the PCR fragment pools (Qubit dsDNA BR assay kit) followed. Quality control and pyrosequencing were performed at the Keck center (University of Illinois at Urbana-Champaign). Briefly, quality control included a qPCR and High Sensitivity DNA chip (Agilent, Santa Clara, CA). Emulsion PCR was performed using the Roche emPCR method (Roche Group, Basel, Switzerland) and 454 pyrosequencing was using the Roche GS FLX+ system, v2.9, flow pattern A. Sequences obtained were analyzed through amplicon signal processing using Roche software version 2.9 (Roche Group, Basel, Switzerland).

2.8. Pyrosequencing data analysis

The pyrosequencing data were analyzed using the QIIME pipeline (Caporaso et al. 2010b). Reads were excluded when the length was below 200 bp and/or the quality score < 25. No mismatches were allowed in the forward primer. The sequences were denoised and binned into operational taxonomic units (OTU) at a cut-off of 97% similarity using uclust (Edgar, 2010). The cluster seed was used as representative sequences. Chimeric sequences were detected with Chimera Slayer and excluded (Haas et al., 2011). Subsequently, the sequences were aligned with PyNAST using the Greengenes core set alignment as reference (Caporaso et al., 2010a; DeSantis et al., 2006). Taxonomy was assigned by comparing to the database of the Ribosomal Database Project (Cole et al., 2009). An OTU table was prepared and phylogeny was constructed using raxml (Stamatakis, 2006). Taxonomy results were plotted using the gplots package in the R environment (R Core Team, 2014). Further processing of the data involved rarefaction analysis to remove heterogeneity in the amount of sequences per sample and the calculation of within (Alpha) variations using Chao-1 and Shannon indices within the QIIME pipeline. For the Beta diversity samples were plotted together with most abundant microorganisms on L6 level and in a three-dimensional plot visualized in Emperor (Vazquez-Baeza et al., 2013). We used PCoA plots, prepared and visualized with ggplot2, biom, phyloseq and seqinr packages in R (R Core Team, 2014). Non-parametric multidimensional scaling of genera abundance relative to

bioreactor samples plus of bioreactor performance parameters and metabolite concentration relative to bioreactor samples was performed using Primer-E v. 7 software (www.primer-E.com). The genera abundance plot was performed with square-root transformed data and S17 Bray-Curtis similarity. The bioreactor performance and metabolite plot contained log(X + 1) transformed data that was normalized and with D1 Euclidean distance similarity. Vectors shown in both plots were correlating with values > 0.8 Pearson to the spatial distribution of the bioreactor samples.

3. Results and discussion

3.1. Performance of a thin stillage fermenting CSTR

A thermophilic (55 °C) lab-scale CSTR with a working volume of 3 L anaerobically digested energy cane stillage under different conditions shown in Table 1. During inoculation and start-up the reactor was kept in batch condition and in this period the percentage of methane in the bioreactor gas phase was around 33%. There was no gas or methane production, low removal of COD from thin stillage (around 7% of the tCOD and 32% of the sCOD) and the pH was adjusted and remained around 7 (Fig. 1). For adaptation, the reactor was fed semi-continuously with low strength 10.8 g COD L^{-1} energy cane stillage from day 6 to 11. The methane percentage of the biogas was not significantly different from the percentage in the headspace during inoculation (up to 36%, see Fig. 1a and the statistical analysis in the Supplementary data). However, during this condition methane was produced and specifically around 0.24 Lmethane/g tCOD used/d. The COD removal increased (tCOD to 62% and sCOD to 77%). The pH was not controlled and significantly increased to around 7.7 (Fig. 1, statistical analysis in Supplementary data). During the next adaptation step, medium strength 21.7 g COD L^{-1} energy cane stillage was fed on days 12 to 33. Compared to the first adaptation step, the methane percentage of biogas increased significantly to 44%, biogas production was similar and around 0.10 L methane/g tCOD used/d. COD removal was around 55% of tCOD plus 57% of sCOD and the pH significantly decreased to around 7.5 (Fig. 1, statistical analysis in Supplementary data). Successively, during stabilization the feed was changed to 43.4 g COD L^{-1} and full strength thin stillage from day 34 to 99. Compared to the adaptation stage the bioreactor was performing with higher and around 56% methane in the biogas and with higher methane production of around 0.43 Lmethane/g tCOD used/d (Fig. 1a, b, statistical analysis in Supplementary data). The tCOD removal of 64% and sCOD removal of 62% were not statistically different from the adaptation stage (Fig. 1c, statistical analysis in Supplementary data). The pH increased significantly to 7.7 (Fig. 1, statistical analysis in Supplementary data). This pH condition is within the optimal range for methanogenesis (6.6–7.8 (Lay et al., 1997)). Also compared to a range of different reactor types using various feedstocks and running conditions, the thermophilic energy cane digesting CSTR performed well during the stabilization stage (Muhammad Nasir et al., 2012; Nasir et al., 2012). In a thermophilic hybrid reactor that was operated with the same substrate under similar conditions, but with a shorter HRT of 2.5 days, the COD removal and pH were similar, but methane percentage and production were lower (with around 40% methane and 0.1 to 0.2 Lmethane/g tCOD used/d) (Oosterkamp et al., 2016). This indicates the CSTR was more efficiently converting energy cane stillage to methane compared to the anaerobic hybrid reactor under thermophilic conditions. An advantage of the anaerobic hybrid reactor may be that this reactor can be run with shorter hydraulic retention times and similar bioreactor performance where this is more difficult to accomplish for the CSTR because of biomass washout with short HRT. Overall, the performance data showed that during the adaptation stage with low-strength energy cane stillage and during the stabilization stage, the performance was most optimal. During the second step of adaptation the performance was relatively low, as in this step the organic load was increased for the first time the lower performance can be due to acclimation to this condition.

Because of an overnight thermostat failure, the bioreactor cooled to ambient temperature. The reactor was re-heated and in the following perturbation stage operation was similar to that during the stabilization stage. The perturbation stage started from day 100 and finalized at day 154. All bioreactor performance parameters significantly decreased during perturbation, the methane percentage of biogas decreased to 46%, the methane production to around 0.16 Lmethane/g tCOD used/d, tCOD removal to 44%, sCOD removal to 47% and the pH to 7.2 (Fig. 1, statistical analysis in Supplementary data). Therefore, although the thermophilic CSTR performed stably, this drastic effect of thermostat failure on bioreactor performance indicates that this reactor system is temperature sensitive. Although this was not indicated for thermophilic anaerobic digestion of thin stillage, sensitivity of thermophilic anaerobic digesters treating wastewater was reported (van Lier, 1996). Furthermore, other reactor systems such as high-rate reactors with immobilized biomass and plug-flow systems have been indicated as a better alternative to CSTR reactors for wastewater treatment. Biomass immobilization was proposed to provide buffering capacity in case of sudden temperature decreases and plug-flow systems to lower toxicity effects of high VFA, sulfide and ammonia (van Lier, 1996). Very few studies on thermophilic anaerobic digestion of thin stillage have been reported. A comparison of bioreactor performance parameters suggests lower methane percentage in total gas produced, lower COD removal, but higher specific methane production in CSTR compared to high-rate hybrid reactor and anaerobic sequencing batch reactors (Supplementary data).

3.2. Metabolic profile of the bioreactor

Organic acids, sugars and inorganic compounds were studied in the bioreactor effluent (Fig. 2). During the inoculation and start-up phase, organic acid concentrations were high while sugar and inorganic compound concentrations were low. The main organic acids measured were acetic acid, butyric acid and formic acid. In the energy cane stillage (undiluted) feed, acetic acid was not detected while butyric and formic acid concentration was not reported (Oosterkamp et al., 2016). The concentration of acetic and butyric acid during inoculation was 29 and 28 mM (Fig. 2a), which are in the same range as the acetic and butyric acid concentrations of previously run anaerobic hybrid reactors that were kept in batch condition using energy cane stillage under similar conditions (Oosterkamp et al., 2016). No methane production was observed under batch conditions in the CSTR and this may be due to these high VFA concentrations. As previously indicated VFA toxicity is a limitation in thermophilic anaerobic digestion (van Lier, 1996). On the other hand, very low concentrations of the sugars xylose, galactose, glucose and fructose were detected, 0.001, 0.0004, 0.0008 and 0.0007 mM, respectively (Fig. 2b). Concentrations of sugars in the energy cane stillage were very high and the very low concentrations of sugars in the CSTR indicate that sugar utilization was highly effective. Chloride plus sulfate were the main inorganic compounds detected (3.7 and 1.2 mM, Fig. 2c). These compounds were also present in high concentrations in the energy cane stillage.

During adaptation, concentrations of organic and inorganic compounds were relatively low (Fig. 2). The concentrations of acetic acid, butyric acid and formic acid decreased to 17.5, 1.8 and 4.2 mM, respectively. This is comparable to VFA concentrations in a thermophilic anaerobic hybrid reactor ran under similar conditions with 2.5-day HRT (Oosterkamp et al., 2016). Of the sugars, only glucose could be detected (at the low concentration of 0.0009 mM). Sugar concentrations were not significantly different compared to during inoculation and start-up (statistical analysis in Supplementary data). Because of the lower VFA concentrations the toxicity of these compounds was likely reduced as methane production from methanogenic microorganisms was observed (Fig. 1). Of the inorganic compounds, sulfate concentration decreased significantly from 1.2 to 0.4 mM (Fig. 2c, statistical analysis in

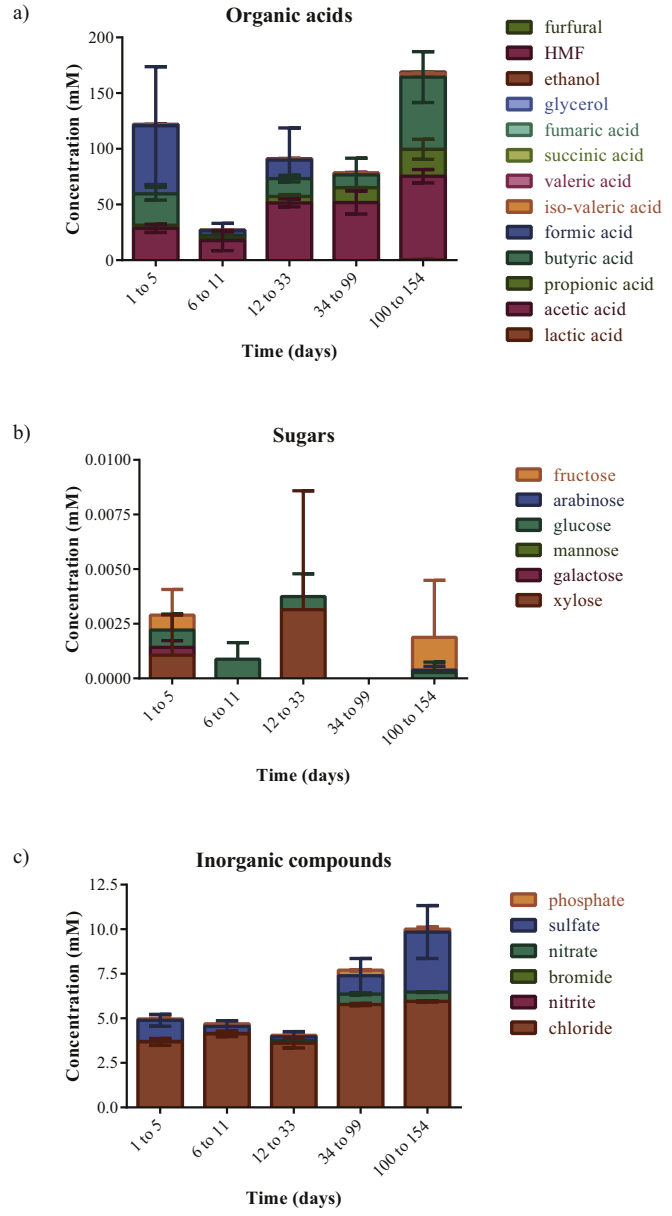

Fig. 2. Concentration of organic acids (a), sugars (b) and inorganic compounds (c) at each of the five run stages of a CSTR that produces methane from thin stillage derived from the production of energy cane ethanol.

Supplementary data). This may indicate higher activity of the sulfate-reducing microorganisms. Sulfate-reducing bacteria compete with methane-forming archaea for the substrates acetate and dihydrogen, which can significantly reduce methane production in the bioreactor (Kristjansson et al., 1982). An effort was made to separate hydrolysis/acidogenesis and sulfidogenesis in a two-stage system where growth of sulfate-reducing bacteria in the second stage was stimulated by making use of their high tolerance to higher volatile fatty acid concentrations and lower pH and possibility to proliferate with shorter hydraulic retention time and higher organic loading rate compared to methanogenic archaea (Moestedt et al., 2016). As also postulated in the study, altering the COD:sulfate ratio seems so far the most promising strategy that allows methanogens to compete successfully with sulfate-reducing bacteria for acetate and dihydrogen. The COD:sulfate ratio in our CSTR digesting energy cane stillage was high with 19.5 (g/g), therefore successful competition by methanogens should be possible. This allows development of a microbial community with optimal structure for the anaerobic digestion of the energy cane stillage.

Increasing the organic loading rate during the adaptation stage resulted in an accumulation of organic compounds. The concentration of acetic acid increased significantly to 51 mM, butyric acid to 16 mM and propionic acid to 6 mM (Fig. 2a, statistical analysis in Supplementary data). This similar increase of organic acids was also observed in thermophilic hybrid reactors with a 2.5-day HRT (Oosterkamp et al., 2016). The sugar and sulfate concentrations did not differ significantly in the adaptation stage. Because of the higher organic acid concentrations methane production may not have increased further during adaptation (Fig. 1b).

When the bioreactor was stabilized and operated semi-continuously with full strength thin stillage, sugars were not detected (Fig. 2). Propionic acid concentration increased significantly to 13 mM, valeric acid concentration increased to 0.8 mM, total organic acid concentrations slightly decreased and pH slightly increased (Figs. 1d, 2a, statistical analysis in Supplementary data). Organic acid concentrations were in a similar range as in an anaerobic hybrid reactor treating cane stillage (Oosterkamp et al., 2016). During stabilization, methanogenic population may have adapted to the high organic acid concentration and overcome toxicity.

After the temperature perturbation, the concentrations of organic acids markedly increased and more specifically the concentrations of acetic acid, butyric acid and isovaleric acid significantly increased (statistical analysis in Supplementary data). Acetic acid increased from 52 to 75 mM, butyric acid from 12 to 65 mM and isovaleric acid increased slightly from 1 to 3 mM (Fig. 2a). Furthermore, low concentrations of the sugars fructose, arabinose and glucose (0.002, 0.0001 and 0.0003 mM) were detected and these did not differ significantly compared to the stabilization stage (Fig. 2b, statistical analysis in Supplementary data). Overall, the metabolic profile and especially the organic acid concentrations of a bioreactor give insight into the metabolic activity of its microbial community. Therefore, and as postulated previously, organic acids can indeed be used as indicators for process imbalance (Ahring et al., 1995). In the CSTR this suggested that before stepping up the organic loading rate during adaptation and during stabilization the CSTR microbial community was performing well.

3.3. Microbial diversity analysis

In order to analyze the microbial community of the CSTR in detail, samples from the CSTR were used for DNA isolation and next generation sequencing. Based on the sequencing data obtained the microbial community diversity was analyzed. Alpha (within community) diversity was determined for the inoculation, stabilization and perturbation stages. As a comparison, we used the diversity of the five different thermophilic WWTP digesters that were used as inoculum. Samples from the WWTP digesters as well as from the CSTR during inoculation showed relatively high alpha diversity index values. This was observed for both species richness with the Chao-1 index in the range of 410 to 1091 and evenness with the Shannon index from 1.5 to 6.0. As a comparison, the index values during stabilization and perturbation were lower with Chao-1 between 268 and 290 and Shannon index from 3.7 to 4.1 (Fig. 3a, b). This indicates that the CSTR microbial community had changed after inoculation, likely due to adaptation. Furthermore, the data also show that microbial diversity had not changed drastically during perturbation as during stabilization and perturbation the Chao-1 and Shannon index showed relatively similar values (Fig. 3a, b).

Based on two-dimensional beta (or between-sample) diversity analysis, samples of the CSTR cluster separately from samples of WWTP digesters (Fig. 3c). This is in agreement with observations from the alpha diversity. In addition, samples of the stabilization and perturbation clustered relatively closely together. Altogether these data show that the microbial community composition of the WWTP digesters is different from the energy cane stillage treating CSTR, that selective

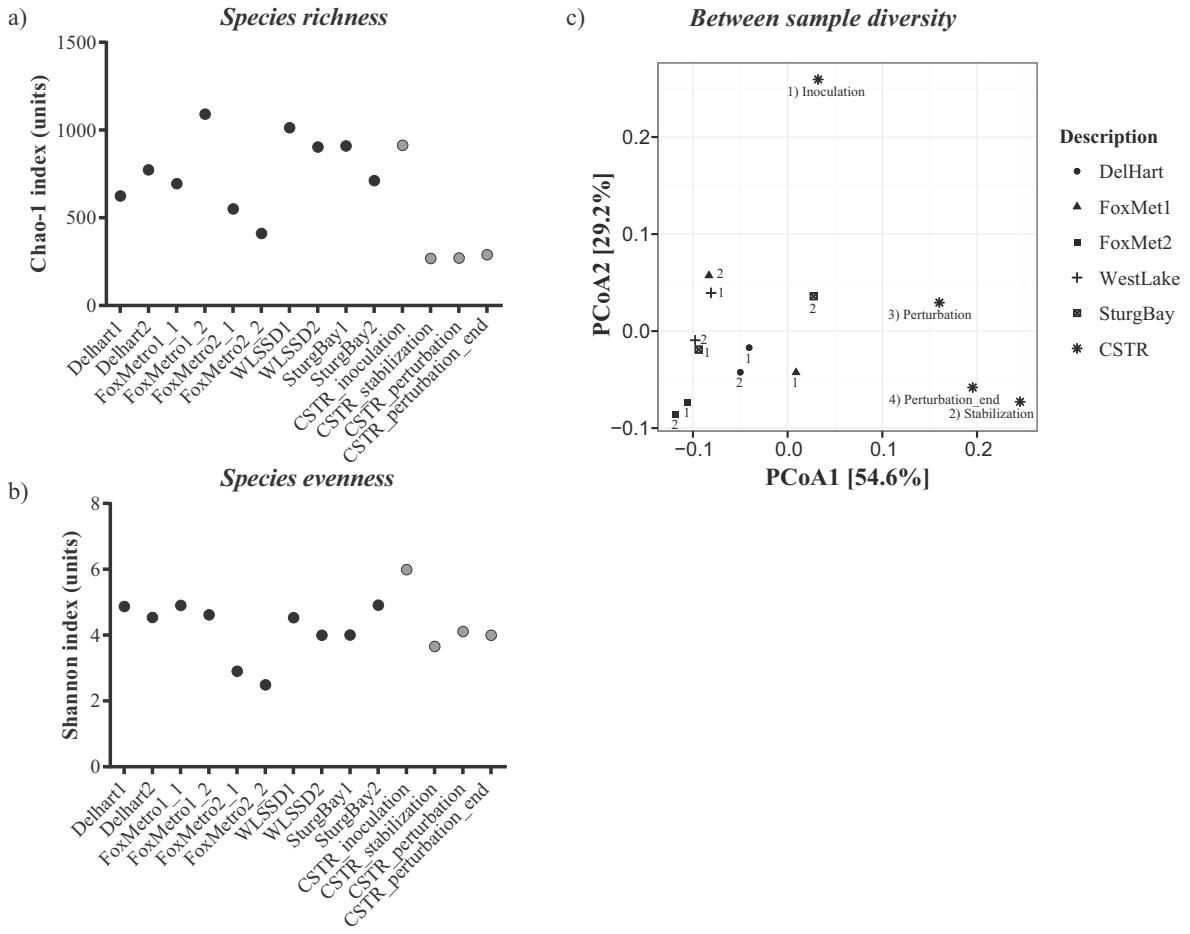

Fig. 3. Microbial community analysis of duplicate samples (1, 2) of five different thermophilic digesters of wastewater treatment plants and of samples taken during the inoculation, adaptation, stabilization and (temperature) perturbation of a thermophilic CSTR that degrades thin stillage derived from energy cane bioethanol production. Species richness using the Chao-1 index (a), species evenness using the Shannon index (b) and between sample diversity using PCoA analysis (c) is shown. WWTP have been indicated as follows: Delafield (Delhart1/2), Oswego (digester 1: FoxMetro1_1/2; digester 2: FoxMetro2_1/2), Sturgeon bay (SturgBay1/2) and Duluth (WLSSD1/2).

enrichment of appropriate microorganisms took place in the CSTR and that perturbation did not very drastically alter the microbial community diversity.

3.4. Composition of the microbial community in the CSTR

A three-dimensional plot of beta diversity that also indicated the presence and location of the most abundant microorganisms showed more clearly the observed differences between the samples from CSTR and WWTP digesters. More specifically there were differences between microorganisms that were present in the thin stillage treating CSTR during inoculation, stabilization plus perturbation and in the digesters of the selected WWTP treating municipal wastewater (Fig. 4). Among the most abundant microorganisms some were more abundant in the CSTR as they were located more closely to the CSTR samples in the three-dimensional plot. Among these microorganisms were bacteria belonging to the genera/families *Thermacetogenium*, Thermoanaerobacterales and *Anaerobaculum*. Both *Thermacetogenium* and Thermoanaerobacterales are syntrophic bacteria and *Anaerobaculum* species are peptide fermenting bacteria that also have been shown to be syntrophic (Maune and Tanner, 2012; Sekiguchi et al., 2006; Hattori et al., 2000). During stabilization, the relative abundance of *Thermacetogenium* was 22%, Thermoanaerobacterales < 1% and *Anaerobaculum* 15% (the microbial community composition at the Genus level is shown in the Supplementary data). This indicates the higher contribution of *Thermacetogenium* and *Anaerobaculum* in the digestion process. Their specific abundance in the CSTR may indicate the importance of syntrophy for thin stillage treatment. Other microorganisms present in higher abundance in CSTR compared to WWTP digester samples include members of the Christensenellaceae. Christensenellaceae are saccharolytic fermentative anaerobes mainly known to impact the metabolism of its human host (Goodrich et al., 2014). Furthermore, bacteria related to the uncultured genus S1 that were also more abundant in the CSTR are members of the Thermotogaceae, this family is composed of thermophilic and hyperthermophilic species (Bhandari and Gupta, 2014). Other more CSTR-associated members of the OP9 lineage or 'Atribacteria' have been shown to lack respiratory metabolism and to be involved in fermentation (Nobu et al., 2016). These microorganisms were not present in a very high abundance with < 1% for Christensenellaceae, 3% for S1 and for OP9-related genus when the CSTR was in stabilization (microbial community composition can be found in the Supplementary data). Finally, methanogens belonging to the *Methanosarcina* (relative abundance in stabilization 15%) and *Methanothermobacter* (22%) genera were also more abundant in CSTR samples compared to WWTP digester samples. *Methanosarcina* includes the most metabolically diverse methanogens that can use CO_2 and H_2, methanol, methylated amines, dimethylsulfide and acetate to produce methane (Welander and Metcalf, 2005), whereas strains belonging to the *Methanothermobacter* are facultative syntrophic and more restricted to hydrogenotrophic methanogenesis (for example Enoki et al., 2011; Kato et al., 2009; Luo et al., 2002). Both of these types of methanogens were probably involved in methanogenesis during anaerobic digestion

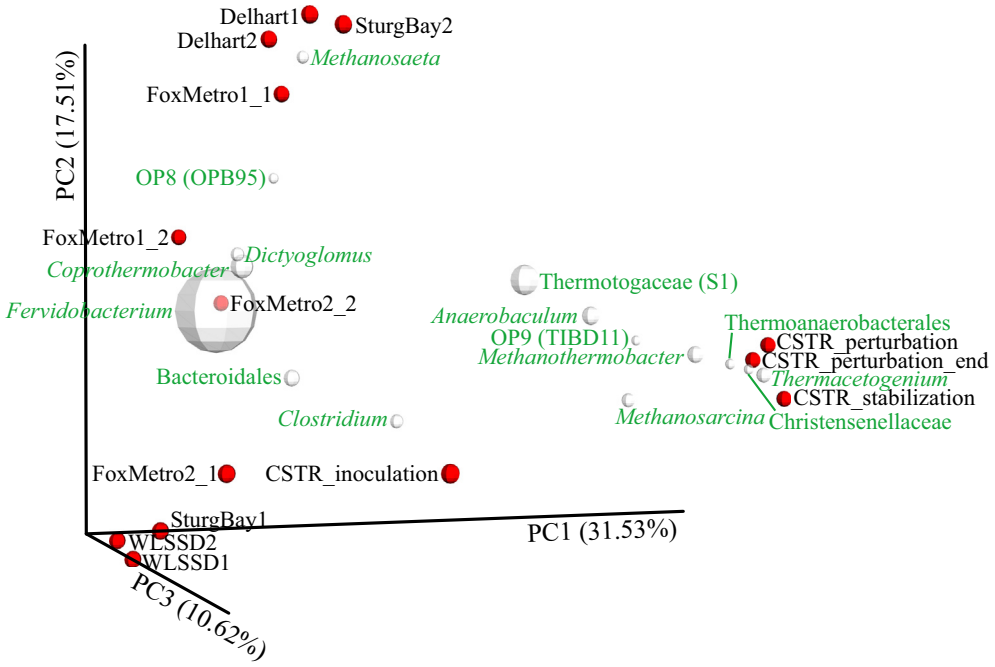

Fig. 4. Three-dimensional plot indicating spatial distribution of beta-diversity of samples from the CSTR degrading energy cane stillage and the thermophilic digester of different WWTP together with the abundance (sphere size) and presence (location) of most abundant genera. Principal coordinates (PC1, PC2, PC3) with the percentage of the variation that can be explained by the respective component (between brackets) are indicated along the axes. WWTP have been indicated as follows: Delafield (Delhart1/2), Oswego (digester 1: FoxMetro1_1/2; digester 2: FoxMetro2_1/2), Sturgeon bay (SturgBay1/2) and Duluth (WLSSD1/2).

of energy cane stillage.

Abundant microorganisms located more closely to samples from the WWTP digesters include *Clostridium* species, members of the Bacteroidales, *Fervidobacterium*, *Coprothermobacter*, *Dictyoglomus* species, members of the OP8 lineage and *Methanosaeta* species (Fig. 4). These bacteria were found to be related to digestion processes, polysaccharide utilization, degradation of complex compounds such as cellulose, keratin and biopolymers under thermophilic conditions or were metabolically diverse and able to thrive under harsh conditions (Farag et al., 2014; Flint et al., 2008; Friedrich and Antranikian, 1996; Gagliano et al., 2015; Lee et al., 2015; Minton et al., 2016; Nishida et al., 2011; Podosokorskaya et al., 2011). This is in agreement with the anaerobic degradation in the WWTP digesters as thermophilic conditions plus mixed, complex and differential types of substrates were likely presented. The more abundant methanogens belong to the *Methanosaeta* that produce methane from acetate and generally occur at low acetate concentrations which were probably present in the WWTP digesters (Qu et al., 2009).

Based on the three-dimensional plot the CSTR sample from the inoculation stage clustered more close to samples from the WWTP digesters. The other CSTR samples from the stabilization and perturbation stages were clustering together very closely which is in agreement with the two-dimensional beta diversity analysis (Figs. 3c, 4). The microbial community composition of the WWTP digesters and CSTR is highly complex. In addition, based on the composition profiles, the microbial communities from the WWTP digester and the CSTR clearly differ. This is observed at the different levels of phylogeny, for example at the phylum, family as well as the genus level (the microbial community composition on these levels is shown in the Supplementary data). The microbial community in the CSTR originated from the WWTP digesters and was treating energy cane stillage instead of municipal wastewater. The microbial community composition changed in the CSTR and more abundant microorganisms were for example involved in syntrophy and methanogenesis. In thermophilic anaerobic hybrid reactors treating energy cane stillage *Methanothermobacter*, *Coprothermobacter* and *Thermacetogenium* were abundant and *Anaerobaculum*, OP9 and Thermotogaceae could be related to the thermophilic condition (Oosterkamp et al., 2016). The abundance of these microorganisms in hybrid reactors supports the importance of syntrophy and methanogenesis in the functioning of these reactors. As described in the Introduction section, syntrophy and methanogenesis were likely important processes for the degradation of energy cane stillage under thermophilic conditions and this research provides evidence to support this hypothesis.

3.5. Constraint analysis to further study the perturbation stage

The effect of the perturbation event in the CSTR that was treating energy cane stillage under thermophilic conditions was analyzed in more detail. To this end, constraint analysis was performed taking into account highly abundant microorganisms in the CSTR samples. Twelve genera were > 1% abundant in the CSTR at least under one condition and present under all conditions studied. Using nonmetric multidimensional scaling, the different CSTR samples were plotted with the abundance of these twelve genera (Fig. 5a). In agreement with the diversity analyses, the constraint analysis shows that the twelve genera of microorganisms in the CSTR just after inoculation are different from when the CSTR was adapted, when it was stable, and in the perturbation stage (Figs. 3c, 4, 5a). However, using the nonmetric multidimensional scaling plot of the constraint analysis, specific genera could be attributed to specific CSTR samples. Just after inoculation, *Clostridium*, *Fervidobacterium*, *Moorella*, *Methanosaeta*, *Syntrophomonas*, *Coprothermobacter* and *Ruminococcus* are more abundant. Of these genera *Clostridium*, *Fervidobacterium*, *Methanosaeta* and *Coprothermobacter* were found to be more important for anaerobic degradation in the thermophilic WWTP digester according to the beta diversity analysis (Fig. 4). These microorganisms are likely present just after inoculation when the microbial community had not yet specialized to be more suitable for thin stillage digestion. Other genera attributed to the inoculation stage included *Moorella* species that are acidogenic bacteria (Alves et al., 2013; Slobodkin et al., 1997). Further, *Syntrophomonas* species are syntrophic bacteria and *Ruminococcus* species are mainly known to degrade plant cell wall polysaccharides in the gut (Cann et al., 2016; McInerney et al., 1981; Morrison and Miron, 2000; Sousa et al., 2007; Wu et al., 2007). Genera correlated to the stabilization and perturbation stages were syntrophic *Anaerobaculum*, *Thermacetogenium*, *Tepidanaerobacter* as well as methanogenic *Methanothermobacter* and *Methanosarcina*. More specifically, of these genera *Anaerobaculum* and *Methanothermobacter* were strongly correlated to the perturbation stage indicating that these genera prevail under the harsh perturbation event and could provide a robust core for the process of thermophilic thin

Fig. 5. Non-parametric multidimensional scaling plots showing the twelve most highly abundant genera (a) as well as bioreactor parameters plus compound concentrations (b) relative to spatial distribution of bioreactor samples from four different time points. Vectors shown correlate with values > 0.8 Pearson to the spatial distribution of the bioreactor samples.

stillage treatment. The other genera, which are the synthropic *Thermacetogenium*, *Tepidanaerobacter* and methanogenic *Methanosarcina* likely were related to the further improvement of the process during the stabilization stages indicating the additional importance of these three species for thermophilic anaerobic digestion of energy cane stillage.

3.6. Bioreactor parameters related to CSTR microbial community

In order to understand better the correlation of bioreactor parameters, organic compounds and anions relative to the bioreactor samples nonparametric multidimensional scaling was applied and plotted (Fig. 5b). In this plot, the sample taken from the CSTR just after inoculation separated clearly from the other CSTR samples. Just after inoculation the CSTR had low values of all bioreactor parameters and high glucose and galactose concentrations. This is likely related to the low activity and not yet optimal anaerobic digestion just after inoculation. The chloride concentration was clearly higher in the other CSTR samples compared to just after inoculation. During anaerobic digestion abiotic and biotic pathways can co-occur (Walter et al., 2012). Chloride formation may be part of an abiotic pathway, for example due to dissociation of salts. Furthermore, during bioreactor perturbation, acetic acid and propionic acid concentration were clearly higher compared to the other CSTR samples. This association of high concentration of acetic acid and propionic acid concentration to bioreactor perturbation also indicates the importance of monitoring organic acid concentrations and that they are useful as indicators for bioreactor condition and performance. During adaptation and stabilization, the methane percentage in the biogas, sCOD removal, tCOD removal and phosphate concentration were higher compared to just after inoculation and during perturbation. Based on a Bioenv or BEST analysis in Primer-E software, the pH, methane percentage in total gas and the chloride concentration correspond best with the twelve genera of microorganisms present in the CSTR ($\rho = 0.943$). The correlation of pH and methane, in particular, with that of abundant species such as synthrophic *Anaerobaculum*, *Thermacetogenium*, *Tepidanaerobacter* as well as methanogenic *Methanothermobacter* and *Methanosarcina* during the optimal operation phases suggests that these listed microbial genera are those playing important roles in optimal thermophilic anaerobic digestion of energy cane thin stillage.

4. Conclusions

Thin stillage from energy-cane-to-ethanol fermentation was digested in a CSTR under thermophilic (55 °C) conditions with a 10-day hydraulic retention time. Biogas produced contained 56% methane, with methane production of 0.43 Lmethane/gtCOD used/d, tCOD

removal of 64%, sCOD removal of 62% and pH 7.7. Temperature perturbation reduced bioreactor performance, with 46% methane in biogas, methane production 0.16 Lmethane/gtCOD used/d, tCOD removal 44%, sCOD removal 47% and pH 7.2. Microbial community diversity was high and composition differed for each condition. Detailed analysis showed importance of methanogens (*Methanothermobacter* and *Methanosarcina*) and syntrophic bacteria (*Tepidanaerobacter*, *Thermacetogenium* and *Anaerobaculum*) for thermophilic digestion of thin stillage.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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