Metabolomics reveals stage-specific metabolic pathways of microbial communities in two-stage anaerobic fermentation of corn-stalk

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Abstract Analysis of intracellular metabolites is essential to delineate metabolic pathways of microbial communities for evaluation and optimization of anaerobic fermentation processes. The metabolomics are reported for a microbial community during two stages of anaerobic fermentation of corn stalk in a biogas digester using GC–MS. Acetonitrile/methanol/water (2:2:1, by vol) was the best extraction solvent for microbial community analysis because it yielded the largest number of peaks (>200), the highest mean summed value of identified metabolites (23) and the best reproducibility with a coefficient of variation of 30 % among four different extraction methods. Inter-

stage comparison of metabolite profiles showed increased levels of sugars and sugar alcohols during methanogenesis and fatty acids during acidogenesis. Identification of stage-specific metabolic pathways using metabolomics can therefore assist in monitoring and optimization of the microbial community for increased biogas production during anaerobic fermentation.

Keywords Anaerobic fermentation · Corn stalk · Metabolomics · Microbial communities

Introduction

Cellulosic biomass is used to produce biofuels by microbial fermentation (Levin et al. 2007; Gunaseelan 1997). In China about 700 million tons of cellulosic biomass are produced each year, of which around 100 million tons is corn stalk (Fan et al. 2008; Wang et al. 2010). Development of an advanced anaerobic fermentation process of corn stalk is under active study to improve its efficient utilization for methane production.

Metagenomic analysis of microbial communities have suggested that a range of metabolic genes are active in biogas digester processes (Handelsman 2004; Riesenfeld et al. 2004; Tringe et al. 2005). Although the presence of genes in microbial communities suggests possible metabolic trajectories, quantitative measurements of metabolites and proteins are required

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to reveal the metabolic pathways activated in different fermentation stages.

Metabolomics is the comprehensive, non-biased analysis of all the metabolites that contribute to the molecular interactions and metabolic pathways in biological systems (Bino et al. 2004; Fiehn 2002). Microbial communities have strong relationships between members of the community and their function rather than the environment during anaerobic digestion (Werner et al. 2011). Therefore, it is possible to explore the relationship between metabolism of a microbial community and its overall activity (e.g. methanogenic activity) using a metabolomics approach. To date, no metabolomics studies have been reported on the microbial community using corn stalks in an anaerobic fermentation.

The present study assessed four different extraction solvents that are the most widely used in microbial metabolomics studies. The optimized metabolomics method was then used to investigate two-stage corn stalk fermentation, a methane production method that separates the hydrolysis/acidogenesis stage from the methanogenesis stage, resulting in improved biogas yield compared with single-stage fermentation approach (Blonskaja et al. 2003; Liu et al. 2006; Lu et al. 2009). This approach should therefore reveal relationships between variations in metabolism and the different fermentation process improving our understanding of microbial community regulation and function.

Materials and methods

Parameters of the continuous stirred tank

A continuous stirred tank (CSTR) reactor with a working volume of 4 l was operated at 35 ± 1 °C. The reactor was stirred with a blender for 10 min every 3 h. At start-up, the reactor was filled with 35 g total solid (TS) l^{-1} cattle manure as the inoculum. The digester was fed with cornstalks (60 g TS l^{-1}) as substrate. The reactor performance (biogas–methane-production and pH) was monitored daily. A steady state was assumed when the variation of the monitoring parameters over time was less than 10 %. The daily biogas production rate was 0.98 days l^{-1} at a hydraulic retention time (HRT) of 20 days. Samples for optimization of metabolomics sample preparation

were obtained from an outlet pipe attached to the CSTR reactor during steady state operation.

Two-stage fermentation process

The sun-cured cornstalk was milled to 5 mm. The total solid (TS) and volatile solid (VS) contents of cornstalk were 94 and 85 %, respectively. The TS and VS of manure were 17 and 13 %, respectively. Prior to feeding to the reactors, both substrates were heated at 100 °C for 15 min to inhibit the activity of methanogenic and other pathogenic microbes and to enrich the H_2 -producing bacteria.

The two-stage process consisted of two reactors, an acidogenic reactor and a methanogenic reactor, with working volume of 1 and 2 l, respectively. Both reactors were maintained at 37 ± 1 °C. The acidogenic reactor was operated for 6 days in a batch mode. The pH and COD of the acidogenic liquid were 5.1 and 8.7 g l^{-1} , respectively. The acidogenic liquid was fed into the methanogenic reactor by a peristaltic pump. The pH was 7.2 ± 0.2 and the methane content was 60 ± 5 % with a HRT of 10 days in the methanogenic reactor. Six replicate samples were collected during steady state operation of the acidogenic and methanogenic fermentation stages for metabolomics analysis.

Quenching

Ten ml of fermentation broth was sprayed into 10 ml quenching solution (methanol/water, 3:2 v/v) at -50 °C (Winder et al. 2008). The solution was rapidly filtered through a membrane (30–50 μm) to remove corn stalk debris. The samples were centrifuged at $10,000 \times g$ for 5 min at 4 °C. Cell pellets were snap-frozen in liquid N_2 and stored at -80 °C for further extraction.

Extraction of intracellular metabolites

After quenching, the cell pellets were subjected to different metabolite extraction methods. Four different protocols for extraction of intracellular metabolites were tested using six technical replicate samples.

Cold methanol/water

We modified a procedure described Duportet et al. (2012). Briefly, the cell pellets were re-suspended in

2.5 ml methanol/water (1:1, v/v) at -20°C . Each sample was vortexed vigorously for 30 s. The mixed samples were then frozen at -80°C and subjected to three freeze–thaw cycles of 2 min for each cycle after which the samples were centrifuged at $10,000\times g$ at 4°C .

100 % methanol coupled to sonication

The cell pellets were re-suspended in 2.5 ml methanol at -20°C and vortexed for 30 s. The re-suspended samples were sonicated for 1 min in an ice bath with a sonicator operating at 20 kHz. The samples were then centrifuged at $10,000\times g$ at 4°C .

Acidic solvent mixtures

The cell pellets were extracted with 2.5 ml acetonitrile/methanol/water (2:2:1, by vol) or water/2-propanol/methanol (2:2:5, by vol) at -20°C according to Kim et al. (2013) and Shin et al. (2010). After centrifugation, all the extraction solution and the supernatant were concentrated to dryness in a vacuum concentrator. The residue was re-suspended in 500 μl acetonitrile/water (1:1, v/v) to remove complex lipids and waxes. After centrifugation at $16,000\times g$ for 5 min, the supernatant was concentrated to dryness again and kept at -80°C prior to derivatization.

Sample derivatization

The residue was dissolved in 20 μl 98 % methoxyamine hydrochloride (40 mg ml^{-1}) in pyridine and shaken at 30°C for 90 min. Then, 90 μl *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was added for trimethylsilylation of acidic protons and shaken at 37°C for 30 min.

GC–MS analysis

GC–MS analysis was carried out using a $30\text{ m}\times 0.25\text{ mm}\times 0.25\text{ }\mu\text{m}$ DB-5 MS column (J&W Scientific, CA, USA). Helium was used as carrier gas at 1 ml/min. Derivatized samples (1 μl) were injected at splitless mode. The oven was held at 100°C for 3 min, then ramped by $5^{\circ}\text{C min}^{-1}$ to 320°C , and held for 10 min. The ionization was processed in EI positive mode. Ion source and quadrupole temperature were set to 230 and 150°C , respectively. Mass spectra were

acquired in a scan range of 50–500 m/z . All samples were prepared and analyzed in random order.

Data processing

GC–MS data were exported from Agilent MSD ChemStation into netCDF format. The Automated Mass Spectral Deconvolution and Identification System (AMDIS) was used to deconvolute the netCDF files in batch mode. AMDIS deconvolution settings were as follows: resolution was medium, sensitivity was low, shape requirement was medium and component width was set at 10. The *.ELU files generated from AMDIS were uploaded to the SpectConnect online service (<http://spectconnect.mit.edu/>) to find the complement of peaks that are consistently detected in multiple chromatograms of one of the study design classes (e.g. per extraction method) (Barupal et al. 2010; Styczynski et al. 2007). After data was pre-processed by SpectConnect, four matrices, including RA, RT, IS and AM (for relative amount, retention time, integrated peak and base peak) can be acquired for further multivariate statistics. Peak identification was achieved in AMDIS to search the mass spectra against the NIST mass spectral library (consisting of NIST 08, Wiley, Replib and Fiehn Library) (Kind et al. 2009).

Multivariate statistical analysis was performed with a web-based analytical pipeline Metaboanalyst (Xia et al. 2012). One-way analysis of variance (ANOVA) was achieved by Statistica (Statsoft Tulsa). Metabolic networks were performed by MetaMapp (Barupal et al. 2012), which uses KEGG reaction pairs to build a metabolic network and then adds non-mapped compounds via Tanimoto similarity.

Results and discussion

Different metabolic profiles from different extraction methods

We observed different metabolite profiles were generated from the four extraction methods as confirmed by PCA analysis of the data matrix (see Fig. 1a). The differences in the metabolite profiles explained by the first principal component, 46.8 %. Samples from the same extraction methods were clustered together. PCA analysis showed that extraction with acetonitrile/

Fig. 1 Score plot shows the response of intracellular metabolites to four extraction methods using PCA **a** and the clustered heatmap of intracellular metabolites of four extraction methods (**b**). *Green* represents compounds detected at low concentration.

methanol/water mixture (AMW) and water/2-propanol/methanol (WIM) presented similar profiles but they were different from the profiles generated using methanol/water or 100 % methanol.

Optimization of extraction method

Extraction with AMW generated the highest numbers of peaks among the extraction methods tested (see Table 1). WIM generated the second highest number of peaks, followed by 100 % methanol while methanol/water gave the smallest number of peaks. The number of peaks detected was counted in the deconvolution data after processing by SpectConnect. Extracting the largest number and diversity of metabolites is the most important criterion for the extraction solvent, so AMW and WIM were the best extraction solvents because of the larger pool of metabolites detected compared with the other two solvents. Supplementary Fig. 1 shows a box plot of some representative metabolites based on ANOVA analysis showing specific trends in metabolite differences obtained with different extraction methods. A diverse range of metabolites, including xylitol, succinic acid, stearic acid, proline, acetic acid, inositol, glycerol

Red represents compounds detected at high concentrations. MW, methanol/water (1:1 v/v); PM, 100 % methanol; AMW, acetonitrile/methanol/water (2:2:1, by vol); WIM, water/2-propanol/methanol (2:2:5, by vol)

Table 1 Comparison of the number of peaks, mean summed value of identified metabolites and coefficient of variation when different extraction solvents were used

Extraction method	Number of peaks ^a	Mean summed value	CV ^b (%)
MW ^c	163.5 ± 13.9	−21.1	46.4
PM ^d	178 ± 12.8	−14.3	43.3
AMW ^e	212.3 ± 9.7	23	30
WIM ^f	206.8 ± 8.8	12.3	35.5

^a Six replicates were analyzed for each extraction method and expressed as the mean ± standard deviation

^b Coefficient of variation

^c Methanol/water (1:1, v/v)

^d Methanol (100 %)

^e Acetonitrile/methanol/water (2:2:1, by vol)

^f Water/2-propanol/methanol (2:2:5, by vol)

3-phosphate, mannose, and mannitol were efficiently extracted with AMW and WIM.

The second criterion for the selection of the optimal extraction method is the peak intensities of each metabolite. A heat-map of the intracellular metabolites obtained from each of four extraction methods is shown in Fig. 1b. WIM and AMW methods generated more and higher peaks of high concentration than the

other two methods. Peak intensity of each metabolite, normalized by autoscaling method (Shin et al. 2010; van den Berg et al. 2006), subtracted by its mean and then divided by its standard deviation, was used to evaluate the four extraction methods as the second criterion. The mean summed value of identified metabolites for methanol/water, methanol only, AMW and WIM were -21.1 , -14.3 , 23 and 12.3 (see Table 1), respectively. The reproducibility of each extraction method was assessed on the coefficient of variation (CV) of all metabolites. AMW showed the highest reproducibility for the total metabolites with a % CV of 30 (see Table 1), followed by WIM. Methanol/water had the highest median CV, indicating the lowest reproducibility with respect to the extraction yield of metabolites. Overall, the best performance, based on three criteria, was achieved with AMW, which yielded the largest number of peaks, the highest peak intensities of identified metabolites and the best reproducibility among four different extraction methods.

In previous microbial metabolomics studies, cold methanol was selected as the best extraction solvents for *Escherichia coli* (Winder et al. 2008), yeast (Villas-Bôas et al. 2005) and *Lactobacillus plantarum* (Faijes et al. 2007). Acidic acetonitrile/methanol/water mixture was also recommended for extraction of the *E. coli* metabolome (Rabinowitz and Kimball 2007). Acetonitrile/water (1:1, v/v) or boiling ethanol (75 %, v/v) was the most effective extraction solvent for *Saccharomyces cerevisiae* (Kim et al. 2013). In contrast, acidic acetonitrile/methanol, compared with boiling ethanol and chloroform/methanol, was not suitable for extracting the metabolome of yeast (Canelas et al. 2009). Therefore, the extraction method needs to be optimized for each microorganism and especially for microbial communities that contain different bacteria and archaea.

Metabolic shift in two-stage fermentation

We applied the optimized metabolomics method for the analysis of metabolic alterations in the two-stage

Fig. 2 Typical chromatograms of intracellular metabolites profile during acidogenesis and methanogenesis, and PCA analysis of metabolic response during the two-stage fermentation process

fermentation of corn stalk. A total of 238 peaks (86 knowns and 152 unknowns) were detected by GC–MS (Supplementary Table 1). Typical chromatograms of different fermentation stages showed different metabolite profiles (Fig. 2). The acidogenesis stage contained more peaks than the methanogenesis stage during the first 30 min, which meant that more small molecular weight metabolites were detected in the acidogenesis stage. GC–MS data were subjected to Metaboanalyst to perform PCA analysis by normalizing metabolite concentrations to their DNA content (see Supplementary Methods: $2 \pm 0.75 \mu\text{g ml}^{-1}$ for the acidogenesis stage and $6.86 \pm 1.45 \mu\text{g ml}^{-1}$ for the methanogenesis stage). The PCA score plot readily distinguished between acidogenesis and methanogenesis based on the metabolite profiles (Fig. 2).

Mapping metabolomics data is highlighted in Fig. 3 for the two-stage fermentation process, displaying significantly altered metabolites while not labeling compounds that were unchanged. Fatty acids and fatty acid esters were detected at higher concentration during acidogenesis stage, whereas sugars and sugar

alcohols were more abundant during methanogenesis than acidogenesis (Fig. 3). Surprisingly, most of the sugars and sugar alcohols were not detected during acidogenesis stage. Most amino acids and amines, except leucine and isoleucine, were detected at higher concentrations during acidogenesis but not methanogenesis.

In the acidogenesis stage, methanogenic archaea were inactivated by the heating procedure, therefore, the main microorganisms functioning during acidogenesis were hydrolytic and fermentative microorganisms digesting cellulose, protein and fat into sugar, amino acids, fatty acids and other small metabolites. Concentrations of fatty acids and amino acids increased during acidogenesis, validating the use of the metabolomics method of this study. The absence of sugars and sugar alcohols during acidogenesis suggested that microbial communities without archaea consumed sugars derived from cellulose. Because of the large amount of sugars and sugar alcohols in methanogenesis stage, we infer that the metabolic functions of the microbial community during methanogenesis

Fig. 3 MetaMapp visualization of metabolomics data in the two-stage fermentation process. Red edges denote KEGG reactant pair links; blue edges symbolize Tanimoto chemical similarity at $T > 700$; Blue nodes represent up-regulated

metabolites during acidogenesis; Red nodes represent up-regulated metabolites during methanogenesis; Nodes with borders represent metabolites detected only in one stage; Node size reflects fold change

differ from those during acidogenesis. During methanogenesis a new microbial community was formed with both anaerobic bacteria and archaea. The limited number of small molecular weight metabolites during methanogenesis reflects the fact that those were utilized by methanogenic archaea for methane production, while not all of the sugars and sugar alcohols were used at that time. This explains why we observed both sugars and small metabolites during methane production in the methanogenesis stage. Overall, the metabolic changes reflect the shift in composition of the microbial community in the different fermentation stages. Metabolomics was used to establish the relationship between metabolites and biogas production of the biogas reactor. Therefore, the metabolomics approach is a promising method to monitor the status of biogas reactors by analyzing the intracellular metabolites from microbial communities.

Conclusions

This is the first study of the metabolome of the microbial community during corn stalk anaerobic digestion. The strategy presented in this study may shed light on development of combination of metabolomics and metagenomics in microbial community research providing important information for improving biogas yield.

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