Metabolic and microbial community dynamics during the anaerobic digestion of maize silage in a two-stage process

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Abstract
The dynamics in the anaerobic digestion of maize silage was investigated in a lab-scale two-stage reactor system. The first stage (hydrolysis/acidogenesis) was performed as solid state digestion with percolation. For the second stage (acetogenesis/methanogenesis), a continuous stirred tank reactor (CSTR) with biomass retention was used with the percolate of the first stage as substrate. The study aimed at the monitoring of process parameters like pH, gas production and concentration of organic acids in the first and second stage, respectively, as well as the involved microbial key players. Two alternating metabolic phases characterized by the production of different organic acids and gas amounts were observed in the first stage process. The microbial community dynamics, monitored by community fingerprinting of bacterial 16S rRNA genes, correlated with the production of the respective metabolites. In the L-phase, lactic acid fermentation dominated. Accordingly, phylotypes affiliated to the genus *Lactobacillus* were detected. In the G-phase, the fermentation pathways changed and volatile fatty acid fermentation dominated accompanied by a high gas production rate with high hydrogen content and a shift in bacterial community composition with phylotypes of *Clostridium* and *Ruminococcaceae* becoming dominant. The changing composition of the percolate significantly influenced the methane content of the resulting biogas in the second stage. The biogas produced during the G-phase contained more methane than the biogas of the L-phase indicating distinct secondary fermentation and/or methanogenesis pathways. The different biotic and abiotic process parameters of both phases were correlated with each other, respectively, and control strategies for biogas processes were proposed.

Keywords
Two-stage process; maize silage; hydrolysis; acidogenesis; lactic acid; volatile fatty acids; bacterial 16S rRNA genes; T-RFLP fingerprinting

INTRODUCTION

The metabolic degradation cascade of the biogas process leading to the desired product is complex and the understanding of the single microbially catalyzed steps and their linkages is scarce. Usually, volatile fatty acids (VFA) accumulate in significant amounts in the process representing the metabolic link between the bacterial primary fermenters and the syntrophic consortia of bacterial secondary fermenters and methanogenic archaea. Besides VFA, lactic acid plays a key role during the anaerobic digestion of ensiled energy crops like maize silage or waste of fermented vegetable food (Jo et al., 2007; Sträuber et al., 2012) as it is a substrate and key metabolite, simultaneously. Little is known about the effect of lactic acid or its producers, i.e. lactic acid bacteria (LAB), on the biogas process. In this study, the metabolic dynamics of acidogenic fermentation and the involved bacterial communities as well as their impact on biogas production in a semi-continuous two-stage process were investigated.

MATERIAL & METHODS

Reactor operation and analysis of process parameters

A mesophilic (37°C) two-stage fermentation system consisting of a hydrolytic/acidogenic first stage and an acetogenic/methanogenic second stage was established. The first stage, a solid state fermentation with percolation, consisted of four equal glass columns (in total 6.6 L). The percolate

of all four columns was collected in a storage tank. Maize silage served as sole substrate. No inoculum was added. Semi-continuous conditions were realized by periodically replacing solid digestate with fresh substrate in the columns in turn. The second stage of the two-stage fermentation system consisted of a 7.5 L CSTR with biomass retention system. Initially, digestate from a single stage biogas reactor fed with maize silage as mono fermentation was used as inoculum. The percolate of the first stage reactor was used as substrate for the second stage. Digestate from the second stage was recirculated into the percolate storage tank of the first stage. VFA concentrations in the percolate and liquid digestate as well as lactic acid in the percolate were determined by headspace GC and HPLC, respectively. The volumes of the produced hydrolysis gas (first stage) or biogas (second stage) were on-line measured using Milligascounters. The measured gas volume was normalized to standard conditions. The gas composition was determined by GC.

Molecular community analysis

Cells were harvested from the percolate by centrifugation and total DNA was extracted using the NucleoSpin® Soil Kit (Macherey-Nagel). PCR amplification and T-RFLP fingerprinting as well as cloning and sequencing of bacterial 16S rRNA genes were performed as described by Sträuber et al. (2012).

RESULTS & DISCUSSION

The anaerobic digestion of maize silage in a two-stage system was monitored for 112 days. In the first stage reactor, characteristic primary fermentation products were formed (Fig. 1A) indicating that only hydrolytic and acidogenic processes were active. No methane production was observed. The pH values of the first stage were in the acidic rage between 4.2 and 5.7, but no stable pH value developed (Fig. 1B). Composition and dynamics of the bacterial community were monitored by T-RFLP fingerprinting (Fig 1C).

During the experimental time, periodical fluctuations of the measured process parameters were observed in the first stage. Two different alternating phases were identified, named L-phase and G-phase. These phases were characterized by the occurrence of distinct metabolites and products as well as corresponding community dynamics, respectively. In the L-Phase, lactic acid production dominated accompanied by a comparably low pH value and a low specific gas production. Furthermore, the hydrogen content of the hydrolysis gas decreased during this phase as described for lactic acid fermentation (Hawkes et al., 2002; Noike et al., 2002). Phylotypes affiliated to the genus *Lactobacillus* were the dominant community members. Lactobacilli produce lactic acid as the major fermentation product from sugars (Stiles & Holzapfel, 1997).

In the alternating G-phase, the fermentation performance changed – while lactic acid production was diminished, the concentrations of propionic acid, butyric acid, *n*-valeric acid, and caproic acid increased as well as the concentration of acetic acid. The overall gas production and the hydrogen content were higher than in the L-phase, presumably caused by the increased butyric and acidic acid fermentation activity (Hawkes et al., 2002). The appearance of the distinct organic acids in the percolate was delayed in time partly. While the production of propionic, butyric and valeric acid coincided with the decrease of lactic acid, the highest concentrations of acetic and caproic acid as well as hydrogen were detected when propionic, butyric and valeric acid were diminished. This time-delayed effect was observed similarly by Jo and colleagues (Jo et al., 2007). In the G-phase, *Lactobacillus* strains were partly replaced by phylotypes affiliated to the genus *Clostridium* and the *Ruminococcaceae,* both belonging to the order Clostridiales. Clostridia represent the majority of the light-independent fermentative bacteria which have the ability to produce hydrogen (Das & Veziroglu, 2001). Lactic and acetic acid are typical products of the substrate conservation process of ensiling prior to biogas production. Hence, these substances as well as LAB were added semi-continuously during the feeding procedure. However, the rhythm of feeding did not coincide with the phase change. As a result, the feeding regime proved not to be the main reason for the

fluctuations in community composition and fermentation characteristics. Rather it is assumed, that the dynamics of the pH values led to the temporary dominance of LAB as their high acid tolerance give them a selective advantage over more acid-sensitive bacteria. Additionally, an inhibition of hydrogen producing strains of *Clostridium* by LAB was described and the production of bacteriocins by LAB assumed (Noike et al., 2002).

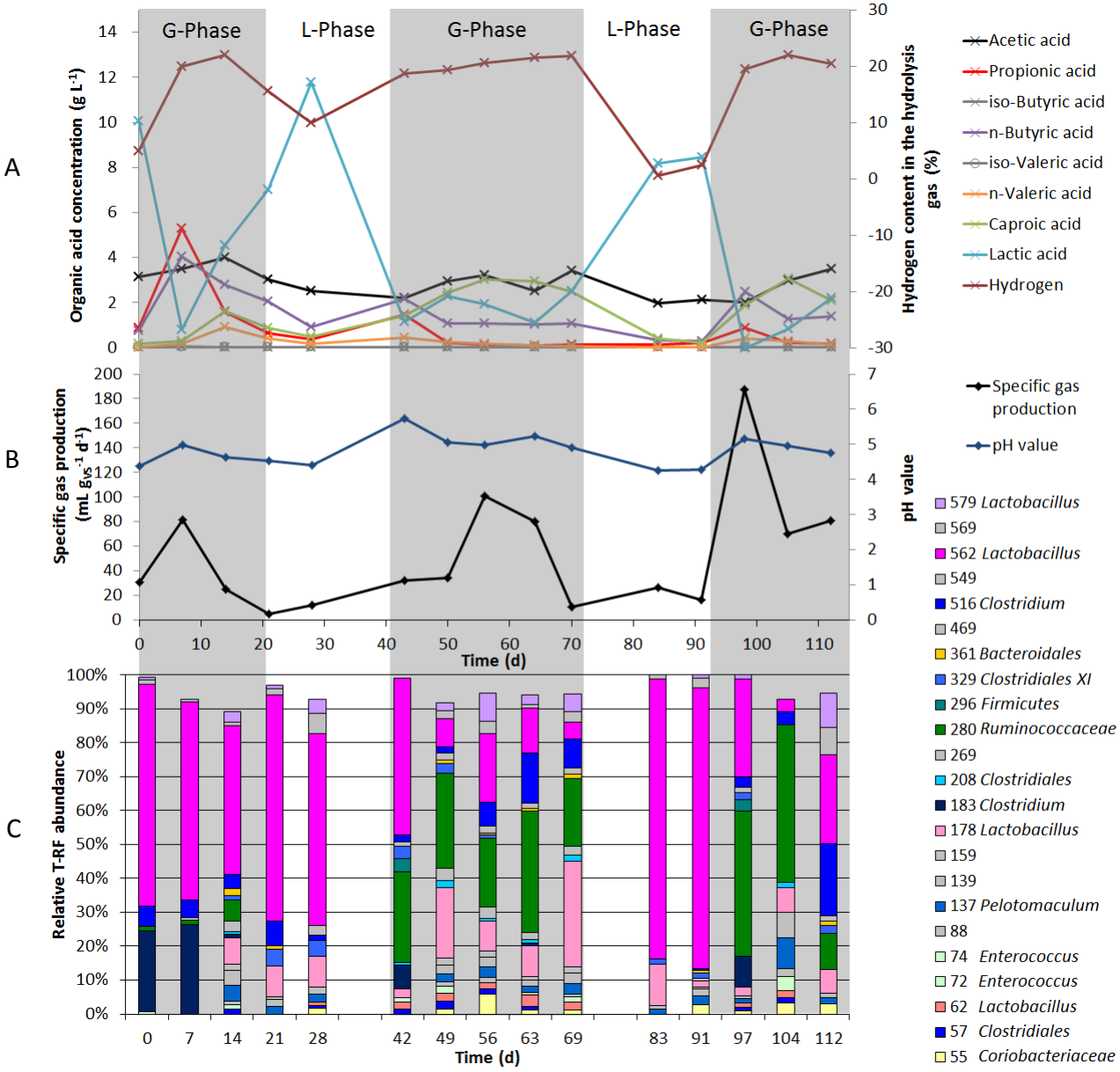

Figure 1. Metabolic phases of the anaerobic fermentation shown by the formation of soluble and gaseous metabolites (A) and the specific gas production and pH values (B) in the first stage. Community dynamics in the percolate of the first stage are shown by T-RFLP profiles of bacterial 16S rRNA amplicons (C). Only T-RF with a relative abundance of minimum 2% are shown. *MspI* was used as restriction enzyme. Metabolic phases are displayed by grey bars (G-phase) or no bars (L-phase) indicating the gradual adjustment of the metabolism.

In the second stage of the two-stage system, the organic acids produced in the first stage were degraded effectively and biogas was produced. Thus, the acidic pH values of the percolate were levelled to the neutral range between 7.3 and 8.0. The metabolic fluctuations in the first stage did not affect the VFA degradation degree or the amount of biogas daily produced in the second stage (not shown). The concentrations of the VFA in the digestate of the second stage never exceeded 94

mg L^{-1} in total (average value: 40.2 mg L^{-1}). In average, 4005 mL biogas was produced per day within the last third of the experimental time. The corresponding biogas yield was 648 mL g_{VS}^{-1}. In contrast to VFA concentration and biogas production, the variable chemical composition of the percolate influenced the composition of the resulting biogas. The methane yield increased to up to 580 mL g_{VS}^{-1} during the G-phase and decreased to ca. 390 mL g_{VS}^{-1} in the L-phase (Fig. 2). This effect was mirrored by the CH_4:CO_2 ratio that shifted from around 2 in the L-phase to more than 2.8 in the G-phase. Hence, the biogas of the second stage contained more valuable methane, when no lactic acid fermentation but other fermentation pathways dominated in the first stage. Furthermore, a varying amount of hydrogen in the ppm concentration range was detected during the biogas production. Whereas in the L-phase several hundred ppm of hydrogen were detected, the hydrogen content was usually lower or even not detectable in the G-phase. This gives a hint on different metabolization ways within the two phases during the digestion not only in the first, but also in the second stage. As the processes during the L-phase proved to be disadvantageous for the biogas production, the entire process should be controlled to favour the G-phase. This could be done e.g. by addition of trace elements as iron was shown to influence the fermentation pathways (Lee et al., 2009).

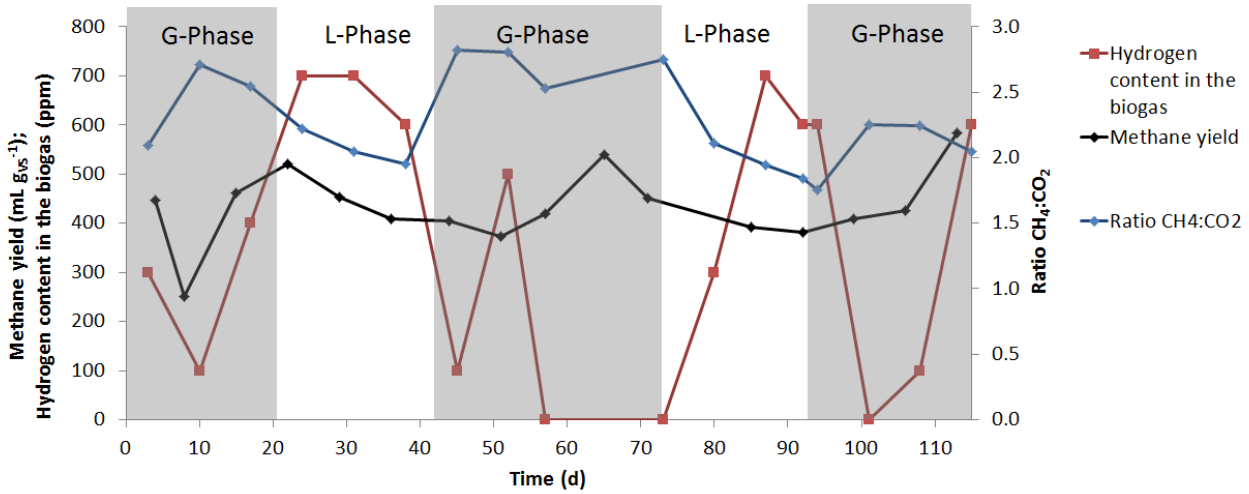

Figure 2. Metabolic phases of the anaerobic fermentation shown by methane yield, the ratio of CH_4 and CO_2 as well as the hydrogen content in the biogas of the second stage. Metabolic phases are displayed by grey bars (G-phase) or no bars (L-phase) indicating the gradual adjustment of the metabolism.

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