Anaerobic digestion of wheat straw by alkaliphilic mixed cultures and their physiological and molecular characterization

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Abstract
Alkaline pre-treatment of lignocellulose is commonly used in bioethanol production and might also enhance the anaerobic digestion of lignocellulosic biomass to biogas. However, the elevated pH of the substrate may require an alkalitolerant microbial community for its effective digestion. Therefore, the aim of the present study was to enrich anaerobic, lignocellulolytic cultures batchwise from sediments of two soda lakes with wheat straw as substrate under alkaline (pH 9) and mesophilic (37°C) conditions. Two mixed cultures were characterized in detail. Their gas production ceased after four to five weeks and the gas was mainly composed of CO_2 and CH_4. Acetate and propionate were the main liquid intermediates, which were nearly completely consumed until the end of incubation. The physiological behaviour of the cultures was stable even after several transfers. The enrichment process was followed by molecular fingerprinting (T-RFLP) of the bacterial 16S rRNA gene and of the mcrA functional gene for methanogens. Additionally, the enriched microorganisms were identified by cloning and sequencing of the same genes. The main shift in the community compositions occurred between the sediment samples and the first enrichment, whereas the structure was stable in the following transfers. The two communities mainly consisted of Sphingobacteriales and Spirochaeta, but differed at genus level. *Methanosaeta* and *Methanosarcina* were the predominant methanogenic genera in cultures from Lake Velencei and Szarvas, respectively. Single lignocellulolytic microorganisms were isolated and identified as members of the alkaliphilic or alkalitolerant genera *Actinotalea, Alkaliflexus, Natronincola, Anaerovirgula, Alkaliphilus* and *Alkalitalea.* Nine strains could not be affiliated to any described genera. However, the most predominant members of the enrichment cultures were not obtained in pure cultures. The results of the present study show that anaerobic, alkaline habitats harbour diverse microbial communities, which can degrade lignocellulose effectively and are therefore a potential resource for improving anaerobic digestion by bioaugmentation.

Keywords
biogas process; alkaline pre-treatment; bioaugmentation potential; T-RFLP fingerprinting; sequencing; mcrA

INTRODUCTION

Lignocellulosic wastes have a high potential as substrate for biogas production by anaerobic digestion (Kumar et al., 2008). However, due to its lignin content it is difficult to degrade microbially under anoxic conditions and hence its hydrolysis is the rate limiting step (van Wyk, 2001; Hendriks & Zeeman, 2009). On the other side, lignocellulosic biomass is commonly used for bioethanol production and there its recalcitrant structure is broken up by different pre-treatment methods such as incubation with highly concentrated base (Hendriks & Zeeman, 2009). Potentially this method could be also applied for the biogas process, but it would require a microbial community adapted to elevated pH. Such communities can be found in different natural methanogenic and alkaline habitats such as in biomass-decaying sediments of soda lakes. The bioaugmentation of biogas plants with these communities could facilitate the anaerobic digestion of alkaline pre-treated lignocellulose. Therefore, the aim of the present study was to enrich anaerobic, alkaliphilic, lignocellulolytic microbial communities from soda lakes. The enrichment process was

followed by chemical and molecular methods and we further aimed to isolate single lignocellulolytic microorganisms as pure cultures.

MATERIAL & METHODS

Sampling, enrichment, physiological analysis, and isolation

Sediment samples were obtained from the littoral zone of the two soda lakes Szarvas and Velencei in Hungary. For the enrichment in batch systems the modified DSMZ medium 1036 (pH 9.0) with wheat straw as lignocellulose source was used. The produced gas volume was determined volumetrically (Rozzi & Remigi, 2004) and normalized to standard conditions. The gas composition was analyzed by GC-TCD and the liquid metabolites by HPLC-RID. The isolation of microorganisms was performed on agar plates consisting of the same medium as used for the enrichment with 1.5% (m/v) agar. As lignocellulose source 1.0% (m/v) wheat straw, 0.5% (m/v) crystalline cellulose, 0.2% (m/v) cellobiose or a mixture of wheat straw/crystalline cellulose or wheat straw/cellobiose was used. The plates and the enrichment cultures were incubated anaerobically at 37°C.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) and sequence analysis

DNA was isolated using the kit FAST DNA Spin for Soil. Bacterial 16S rRNA gene fragments were amplified using the primers 27F and 1492R, while the forward primer mlas and reverse primer mcrA-rev were used for the amplification of the mcrA gene. One of the primers was FAM-labelled when amplicons were further analysed by T-RFLP. Restriction digestion of the fluorescently labelled PCR products was performed either by the restriction endonuclease *HaeIII* or *MspI*. Fluorescently labelled terminal restriction fragments (T-RFs) were separated on an ABI PRISM 3130xl Genetic Analyzer. Fluorescent data for the range of 50-1000 bp were exported to R script, peak areas were normalized and noise filtering was applied. A multivariate cluster analysis with the Bray-Curtis similarity index was performed using the program PAST. PCR products from the transfer used for the strain isolation were cloned. After screening by T-RFLP selected clones were partially sequenced. Examination of phylogenetic relationships and taxonomic assignments was performed using the rRNA Classifier and the Sequence Match tools of the RDP-II and the BLASTN tool of the NCBI sequence database.

RESULTS & DISCUSSION

Physiology of enrichment cultures

During all transfers of the enrichment cultures obtained from two different soda lakes the cumulative gas production increased over time and ceased after four to five weeks (see Figure 1a as example for the third transfer). In the negative controls the gas volume increased only slightly in the beginning of incubation due to the temperature increase from room temperature to the incubation temperature. The gas composition of the negative control did not change over time and represented the gas composition of the anaerobic chamber (98% N_2, 2% H_2) in which the medium was filled into the culturing bottles. Additional CO_2 originated from the carbonate buffer in the medium. CH_4 was not detected in the controls. In the enrichment cultures the N_2 concentration decreased over time due to the production of CO_2 and CH_4 with an increasing CH_4:CO_2 ratio (Figure 1a). After a few days H_2 was not detected anymore in the gas phase of the cultures. In all transfers of the two cultures the main dissolved metabolites were acetate and propionate (Figure 1b). The maximum concentration of around 2 g/L of acetate was reached within the first two weeks, whereas the propionate concentration increased only up to 500 mg/L. Both acids were nearly completely consumed until the end of incubation.

Regarding the physiological data both alkaliphilic enrichment cultures behaved similarly and degraded the wheat straw successfully. A decrease in the straw particle size could also be observed

visually. However, the straw was not completely consumed within the incubation time of around five weeks, probably due to its lignin content. The formation and consumption of acetate as well as the production of CH_4 showed that the complete biogas production process occurred in the cultures (Weiland, 2010). The stability of the physiological parameters over the transfers indicated the functional stability of the cultures.

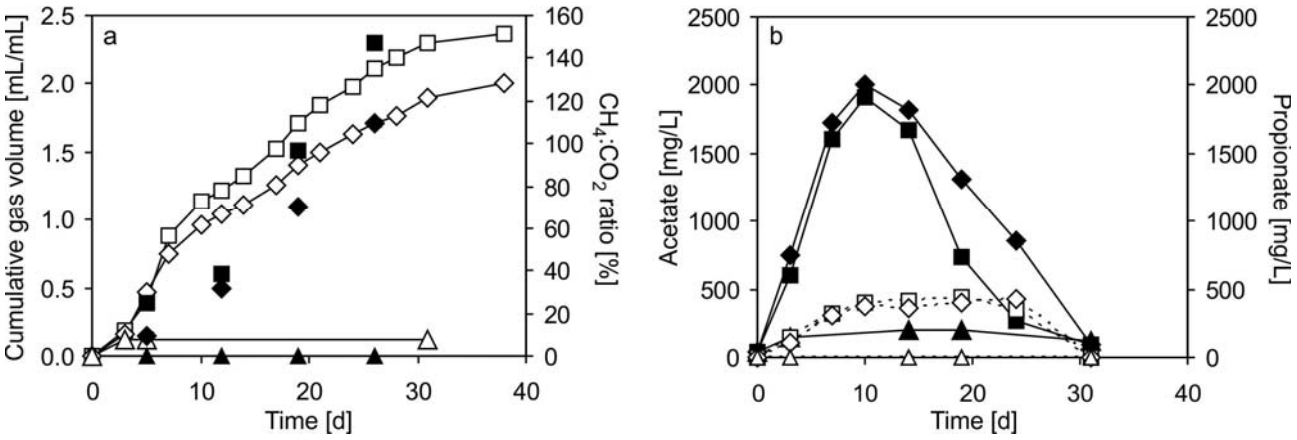

Figure 1. a) Normalized, cumulative gas production [$mL_{gas}/mL_{cultivation\ medium}$] of the third transfer of mixed alkaliphilic, wheat straw-degrading cultures obtained from the Szarvas (S37°C, □) and Velencei (V37°C, ◇) Lake, and of the negative control (NC, △), as well as the CH_4:CO_2 ratio of these setups (■ S37°C, ◆V37°C, ▲ NC). b) Acetate (■ S37°C, ◆V37°C, ▲ NC) and propionate (□ S37°C, ◇ V37°C, △ NC) concentration of the same setups as shown in a). In a) and b) values for S37°C and V37°C are the average of duplicates, values for NC were obtained from one parallel. Bars representing the difference between the minimum and maximum are smaller than the symbol size.

Microbial community dynamics and composition

Bacterial communities. Although the enrichment conditions were the same and the physiological characteristics of the two enrichment cultures were similar, completely different bacterial communities have been developed. The main shifts in the community composition occurred between the sediment samples and the first enrichment. During the following transfers the community structure was stable with only little overlap between the dominant T-RFs from the two cultures (Figure 2). The sequences obtained from the clone libraries showed low similarity (89-96%) to already described bacterial taxa and mostly represented novel species. However, phylogenetic assignments of the predominant T-RFs by sequence analysis and classification at higher taxonomical level showed that the two enrichment cultures were similar. In both cultures, the most abundant T-RFs affiliated to the order Sphingobacteriales and the family Spirochaetaceae. Members of the Clostridiaceae were also present in both cultures.

Isolates. Several attempts were made to isolate important lignocellulolytic members of the two communities in pure culture to investigate their physiological role in lignocellulose degradation in future. Overall 106 colonies obtained on agar plates were screened for purity and similarity by T-RFLP. Genus level identification was achieved with the strains from Velencei Lake affiliated to the *Anaerovirgula, Clostridium, Alkaliflexus, Alkaliphilus* genera, while strains from Szarvas Lake were affiliated to *Actinotalea, Alkaliflexus, Clostridium, Natronincola, Sphaerochaeta* and *Alkalitale*a, all of them being known as genera with alkaliphilic or alkalitolerant species. Classification of members from nine T-RF groups was not possible at genus level and they most probably represent novel taxa. However, based on the T-RFLP patterns of the colonies the isolation strategy on agar plates missed the most abundant members and perhaps the key-players of the liquid cultures.

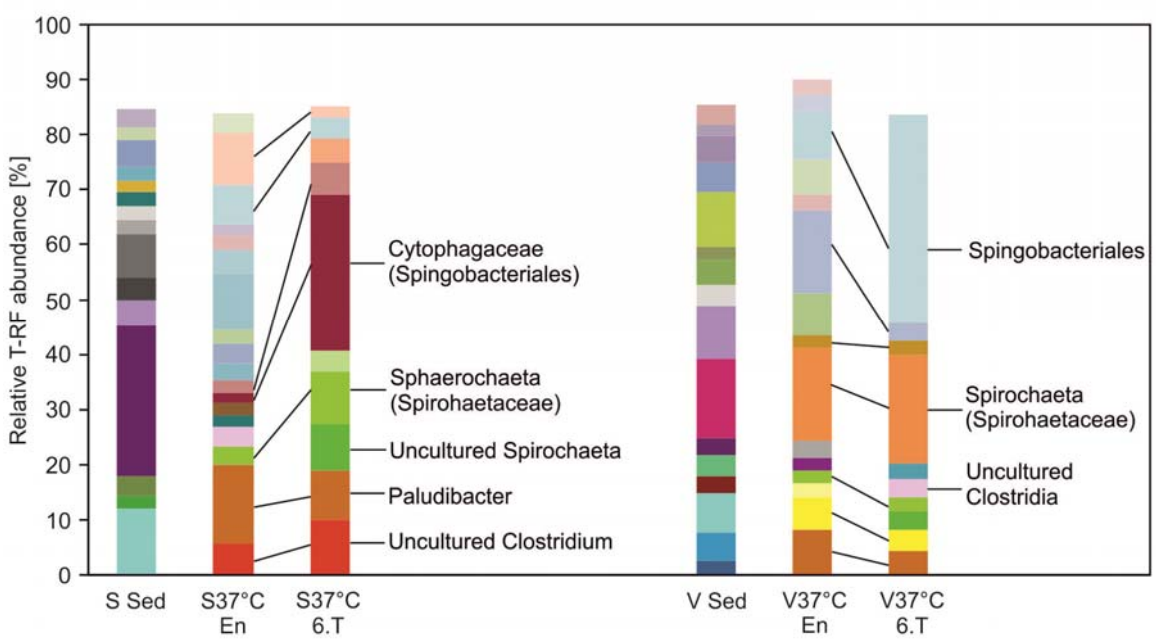

Figure 2. Relative T-RF abundance of the bacterial 16S rRNA gens digested with the restriction enzyme *MspI* of the sediment samples (Sed), first enrichment culture (En) and 6th transfer (6.T) of the mixed alkaliphilic, wheat straw-degrading cultures obtained from the Szarvas (S37°C) and Velencei (V37°C) Lake. T-RFs with an abundance <2% are not shown. Each colour represents one T-RF and T-RFs of the same length are connected by lines. Microorganisms producing certain T-RFs were identified by cloning and sequencing.

Methanogenic communities. Based on the mcrA T-RFLP patterns a less diverse methanogenic archaeal community was enriched compared to the original lake sediments. The culture derived from the Velencei Lake was dominated by strict aceticlastic *Methanosaeta* while the Szarvas Lake culture harboured a more diverse community with mixotrophic *Methanosarcina* as dominant methanogenic Archaea. Another abundant sequence had no cultivable representative yet, but related sequences were found frequently in lacustrine environmental samples.

Conclusions

From the two soda lakes different microbial communities were enriched which digested wheat straw anaerobically to biogas and consisted partly of so far unknown microorganisms. The results indicate the potential of the cultures to enhance the biogas production under alkaline conditions, and indeed first bioaugmentation experiments with the cultures showed an increase in biogas production from alkaline pre-treated wheat straw in batch systems.

ACKNOWLEDGEMENTS

This work was financially supported by the German Academic Exchange Service (DAAD) and Hungarian Scholarship Board Office (MÖB).

REFERENCES

Hendriks, A.T.W.M. 2009 Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology* **100**(1), 10-18.

Kumar, R., Singh, S., Singh, O.V. 2008 Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. *Journal of Industrial Microbiology and Biotechnology* **35**(5), 377-391.

Rozzi, A., Remigi, E. 2004 Methods of assessing microbial activity and inhibition under anaerobic conditions: A literature review. *Reviews in Environmental Science and Bio/Technology* **3**(2), 93-115.

Van Wyk, J.P.H. 2001 Biotechnology and the utilization of biowaste as a resource for bioproduct development. *Trends in Biotechnology* **19**(5), 172-177.

Weiland, P. 2010 Biogas production: Current state and perspective. *Applied Microbiology and Biotechnology* **85**(4), 849-860.