



Investigation of microorganisms colonising activated zeolites during anaerobic biogas production from grass silage

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ABSTRACT

The colonisation of activated zeolites (i.e. clinoptilolites) as carriers for microorganisms involved in the biogas process was investigated. Zeolite particle sizes of 1.0–2.5 mm were introduced to anaerobic laboratory batch-cultures and to continuously operated bioreactors during biogas production from grass silage. Incubation over 5–84 days led to the colonisation of zeolite surfaces in small batch-cultures (500 ml) and even in larger scaled and flow-through disturbed bioreactors (28 l). Morphological insights were obtained by using scanning electron microscopy (SEM). Single strand conformation polymorphism (SSCP) analysis based on amplification of bacterial and archaeal 16S rRNA fragments demonstrated structurally distinct populations preferring zeolite as operational environment. via sequence analysis conspicuous bands from SSCP patterns were identified. Populations immobilised on zeolite (e.g. *Ruminofilibacter xylanolyticum*) showed pronounced hydrolytic enzyme activity (xylanase) shortly after re-incubation in sterilised sludge on model substrate. In addition, the presence of methanogenic archaea on zeolite particles was demonstrated.

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1. Introduction

In numerous studies grass silage has been recommended as an excellent substrate for biomethane production resulting from high energy yields and low energy input demand (Prochnow et al., 2009). The high potential of methane production from grass silage was confirmed both in batch and semi-continuous experiments and batch leach bed processes (Lehtomäki et al., 2008). In practice, grass silage is the most important substrate for agricultural biogas production following maize silage in Germany (Rösch et al., 2007). Though grass silage may be less energetically productive compared to maize silage, it still offers a good energy balance and environmental advantages (Gerin et al., 2008). The formation of inhibitory NH₃ and high chemical oxygen demand (COD) values in the course of grass biomethane production are drawbacks, which may be counteracted by the high ammonia binding capacity of zeolites as reported by Montalvo et al. (2005).

The addition of support materials like magnesium or aluminium silicates to anaerobic digestion processes has been reported to lead to higher methane yields or better gas quality (Pande and Fabiani, 1989). Furthermore, the immobilisation of microorganisms on various zeolite types was claimed to be beneficial, expanding the pos-

sibilities to support the process (Murray and van den Berg, 1981). Among natural and synthetic zeolites, clinoptilolite has a superior CO₂ adsorption capacity, regenerability and stability through several adsorption–desorption cycles, upgrading the quality of biogas by adjusting the CO₂/CH₄ ratio (Alonso-Vicario et al., 2010). Moreover, clinoptilolite has been shown to be suitable as operational environment for microorganisms in biogas production processes (Milán et al., 2003). Recently, we have demonstrated that the degradation of recalcitrant cell wall components can be enhanced by addition of hydrolytic bacterial populations immobilised on activated zeolite to the biogas process (Weiß et al., 2010). However, our understanding of the specificity of zeolites regarding the immobilisation of certain microbial populations is still poor. Therefore, we characterised the microbial populations colonising trace metal activated clinoptilolite during anaerobic digestion of grass silage and of a model substrate in this study.

2. Methods

2.1. Batch-culture and continuously operated bioreactor experiments with zeolite

The zeolite used (IPUS GmbH, Rottenmann, Austria) consisted of a natural zeolitic tuff containing >85% clinoptilolite, which was milled to a grain size below 2.5 mm. The material was loaded with

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Fe, Ni, Co, Mo, Se, Cu and Zn as trace metal elements to enhance microbial activity (Holper et al., 2005). To investigate the capability of zeolite surfaces as a functional colonisation area, particles of 1.0–2.5 mm were introduced to batch experiments (0.01 g ml^{-1}) carried out in 1000 ml ground flasks with a total volume of 500 ml of seeding sludge with the following properties: dry matter content (DM) of 9.50%; organic dry matter content (ODM) of 6.45% and an ODM fraction of DM (ODM/DM) of 67.39%. The seeding sludge was obtained from fermenters long-term mono-digesting grass silage (360 d) at increasing organic loading rates of 0.5–3.0 kg ODM $\text{m}^{-3} \text{ d}^{-1}$ and mesophilic conditions (Andrade et al., 2009). Before use, the sludge was flushed with oxygen-free nitrogen gas for 20 min l^{-1} to obtain anaerobic conditions. Two different substrates were used: (i) Grass silage (DM content of 56.13%), grained to fibrous wool with a blender and (ii) a model substrate which properties resemble natural grass silage in principle (DM ratio adapted from Wiseloge et al. (1996): Carboxymethyl cellulose sodium salt (Fluka, St. Gallen, Schweiz), 16.7%; xylan from Birchwood (Roth, Karlsruhe, Germany), 25.8%; lignin with low sulfonate content (Sigma–Aldrich, St. Louis, Missouri, USA) 7.2% and amylopectin (Roth), 0.5%. 1% DM (w/v) of substrate (i) or (ii) were alternatively introduced to the total reaction volume, which was then digested under mesophilic conditions at 35 °C for community and enzymatic analyses or at 45 °C for morphological studies using scanning electron microscopy. Batch-cultivation was carried out over a total time period of 6 weeks. Alternatively, six fractions of a 0.4 g zeolite were introduced to previously described single-stage flow-through reactors of 28 l operating volume (Lebuhn et al., 2008), continuously mono-digesting grass silage at an organic loading rate of 1–2 kg ODM $\text{m}^{-3} \text{ d}^{-1}$ during gradual adjustment of the temperature (1°C d^{-1}) from 37 °C to 45 °C. The seeding sludge was obtained from fermenters mono-digesting grass silage at mesophilic conditions (Andrade et al., 2009). The zeolite was entrapped in 0.5 mm mesh size polyamide bags (“*in sacco*”).

2.2. Enzyme activity assay

Xylanase activity was measured after re-incubation of zeolite particles exposed to batch-culture experiments before, using in-activated inoculum sludge as full medium or synthetic medium as control (DIN EN ISO 11734 L47, “Evaluation of the ultimate anaerobic biodegradability of organic compounds in digested sludge”, 1998). Inoculum sludge was in-activated by autoclaving at 121 °C for 20 min, heating to 95 °C for 5 min, followed by another autoclaving cycle. Re-incubation was carried out at 35 °C for up to 62 h. Zeolite particles were rinsed thrice with $1 \times$ PBS (phosphate-buffered saline; pH 7.4) before activities were determined. The 3-amino, 5-nitrosalicylic acid (DNS) assay which is based on the quantification of reducing sugars released with DNS was used as described previously by Bailey et al. (1992). The DNS reagent contained (in g l^{-1}): 3,5-dinitrosalicylic acid ($\text{C}_7\text{H}_4\text{N}_2\text{O}_7$), 7.48; NaOH, 13.98; sodium potassium tartrate ($\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4 \text{H}_2\text{O}$), 216.1; phenol ($\text{C}_6\text{H}_6\text{O}$), 5.155; sodium metabisulfite ($\text{Na}_2\text{O}_5\text{S}_2$), 5.86. For the determination of xylanase activity, D-(+)-xylose standards were prepared (in mg ml^{-1}): 2.50, 1.25, 0.63, 0.31 and 0.16. Samples and sample blanks contained (in μl): 1% xylan solution, 180; sample, 20; DNS solution, 300. Before adding the DNS solution, samples were incubated at 35 °C for 30 min. Sample blanks were not incubated before addition of DNS solution to suppress enzymatic reactions. The photometric absorption was determined at 540 nm in 96 well plates (Greiner, Frickenhausen, Germany) using a plate reader (Tecan Infinite 200 M, Männedorf, Switzerland). An enzyme unit is defined as the conversion of 1 μmol substrate per minute, in which 1 U corresponds to 16.67 nanokatal.

2.3. PCR-based community analysis

2.3.1. DNA extraction

The total bacterial community DNA was extracted as described by Martin-Laurent et al. (2001). To collect microorganisms, 1 g of sample material was centrifuged for 15 min at 16,750g. The pellet was then resuspended in 1 ml of extraction buffer containing (in g l^{-1}): ethylenediaminetetraacetic acid (EDTA), 37.25; NaCl, 5.85; polyvinylpyrrolidone (PVP), 10; Tris–HCl, 12 and 20% (v/v) sodium dodecyl sulphate (SDS), 100 ml l^{-1} . To ensure complete cell lysis, glass beads from 0.15 to 2.00 μm in diameter were added to crush cell structures using FastPrep Instrument (Qbiogene, Heidelberg, Germany) for $2 \times 30 \text{ s}$ including a cooling step on ice in between for 2 min. After centrifugation for 1 min at 16,750g, the supernatant was mixed with 5 M sodium acetate, 100 μl and incubated on ice for 15 min for protein precipitation. Succeeding another centrifugation step for 5 min, an equal volume of chloroform-phenol-isoamylalcohol mixture (15:24:1) was added to the supernatant. Subsequently, the genomic DNA was precipitated by adding an equal volume of ice-cold iso-propanol to the upper phase. The precipitated DNA was recovered by centrifugation for 10 min, washed once with 70% (v/v) ethanol and resuspended in a total volume of 50 μl of 10 mM Tris–HCl buffer (pH 8.0). Alternatively a DNA extraction kit for soil samples was used (FastDNA® Spin Kit for Soil, MP Biomedicals, Solon, Ohio, USA) following the manufacturer's recommendations.

2.3.2. Polymerase chain reaction (PCR) amplification

Amplification of bacterial 16S rRNA gene fragments was carried out using the eubacterial primer pair Unibac-II-515f (5'-GTG CCA GCA GCC GC-3') and Unibac-II-927rP (5'-CCC GTC AAT TYM TTT GAG TT-3') for an amplicon size of 412 bp according to Lieber et al. (2002). Amplification of archaeal 16S rRNA gene fragments was performed using the primer pair ARC-787f (5'-ATTAG ATACC CSBGT AGTCC-3') and ARC-1059r (5'-GCCAT GCACC WCCTC T-3') for an amplicon size of 273 following Yu et al. (2005), using a Biometra T personal/gradient system (Biometra, Göttingen, Germany). The reaction mixture was set up on ice and contained: $1 \times$ Taq & Go (Qbiogene, Heidelberg, Germany); 3.0 mM MgCl_2 (Finnzyme, Espoo, Finland); 0.5 mM forward primer; 0.5 mM reverse primer; 20 ng template DNA (1–2 μl) and double deionised H_2O to fill up to a final volume of 20 or 60 μl for template DNA extracted from polyacrylamide SSCP-Gels and template DNA extracted from digestion sludge samples respectively. Negative control PCR contained no template DNA. The cycling conditions for eubacterial templates were as follows: (1) denaturation at 94 °C for 4 min, (2) denaturation at 94 °C for 20 s, (3) annealing at 53 °C for 30 s, (4) extension at 72 °C for 60 s, (5) final extension at 72 °C for 10 min, (six) hold at 4 °C. Steps (2) till (4) were repeated 30 times. For archaeal templates the annealing temperature was set to 55–50 °C (touch down: 0.6 °C per cycle). For the purification of PCR generated DNA products, Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA) was used following the manufacturer's recommendations. For DNA-content determination 1 μl of purified PCR-products were analysed using a micro-volume spectrophotometer for nucleic acid and protein quantitation (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, Delaware, USA).

2.3.3. Single strand conformation polymorphism (SSCP) analysis and DNA elution from gel slices

SSCP analysis of amplified bacterial and archaeal *rrs* (16S rRNA gene) fragments was carried out according to Schwieger and Tebbe (1998). For single strand formation 10 μl of purified PCR products, adjusted to 150 ng dsDNA-content were used. Exonuclease digestion was performed with a λ -Exonuclease of 12 U initial concentration (New England Biolabs, Frankfurt, Germany) at 37 °C for 1 h,

followed by the addition of 50% (v/v) loading buffer (95% deionised formamide, 10 mM NaOH, 0.025% (w/v) bromophenol blue), a denaturation step at 98 °C for 3 min and a re-folding step on ice for 5 min. Separation of folded ssDNA was achieved by electrophoresis in 5× Tris–borate–EDTA buffer (TBE) using a polyacrylamide gel (8% for eubacterial DNA, 9% for archaeal DNA) at 26 °C for 17 h (archaeal DNA) and 26 h (eubacterial DNA) respectively using a TGGE MAXI system (Biometra). After silver-staining according to Bassam et al. (1991), gels were digitalised using a transillumination scanner. For further characterisation of microbial communities, i.e. sequence analysis, constantly dominant or variably emerging SSCP bands were excised with a scalpel and transferred into 1.5 ml reaction tubes for an incubation time of 12 h at –70 °C. For DNA elution from gel slices, a method previously described by Sambrook et al. (1989) was modified. One-fifty µl sterile elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulphate [SDS], pH 8.0) were added to each gel slice sample and subsequently incubated for 15 min at –70 °C, 5 min at 50 °C, 5 min at 90 °C and finally at 37 °C for 3 h with 500 rpm shaking speed (Thermomixer, Eppendorf, Hamburg, Germany). After 3 days of incubation at –20 °C, a centrifugation step at 14,500g and 4 °C for 15 min was used to eliminate gel slice debris. 120 µl of the supernatant were taken and mixed with 300 µl 70 % ethanol for protein precipitation at –20 °C for 3 h. DNA was pelletized by a final centrifugation step at 14,500g for 30 min, then air dried and dissolved in 20 µl 10 mM Tris–HCl (pH 8.0). Afterwards, eluted DNA was re-amplified using the same primer pairs as for the PCR (Section 2.3.2).

2.3.4. Computer-assisted cluster analysis

In order to compare SSCP fingerprints of microbial communities, a computer-assisted cluster analysis was carried out using the GelCompar® II software (Applied Math, Kortrijk, Belgium). DNA standards were loaded onto each gel allowing a post-run correction of gel-specific differences between several gel runs using the normalisation function of the GelCompar® II software. After background subtraction and normalisation of digitalised images of the SSCP gels, similarities between the SSCP fingerprints were calculated using the band-based Dice similarity coefficient. Afterwards, the fingerprints were grouped according to their similarity using the hierarchical cluster method: *unweighted pairwise grouping method using arithmetic means* (UPGMA). Differences between clusters within software generated dendrograms were statistically verified by permutation significance tests.

2.3.5. Sequence analysis

About 60 ng of each PCR product from bands excised from PA gels were used for sequencing reactions, which were performed by the ZMF laboratory of the Medical University of Graz (Graz, Austria). To identify similar sequences that are available in the NCBI Genbank, sequences were used in BLASTn searches (<http://www.ncbi.nlm.nih.gov/blast/>).

2.4. Scanning electron microscopy

The scanning electron microscope (SEM) Zeiss ULTRA 55 was used to investigate zeolite particles with 1.0–2.5 mm in diameter. The biological material was imaged with the high efficiency “In-lens SE detector” using secondary electrons (SE), which deliver topographic contrast (Goldstein et al., 2003). Additionally imaging with an angle selective backscattered electron (AsB) detector was performed. It detects backscattered electrons (BSE) and delivers compositional contrast, which is determined by the differences in the local chemical composition of an investigated specimen (Goldstein et al., 2003). In the case of SE imaging 5 kV acceleration voltage of the primary electrons was applied with the exception of one

micrograph which was imaged simultaneously with the AsB detector at an acceleration voltage of 15 kV. Before application fresh samples were fixed in glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 7.2), dehydrated in a graded ethanol series with a final step in propylene oxide and ultimately dried by lyophilisation (Labconco Freeze Dry System FreeZone 4.5, Kansas City, Missouri, USA) or critical point drying (CO₂; Bal-Tec CPD, Balzers, Fürstentum Liechtenstein). For observation, prepared samples were mounted on aluminum stubs using double-sided carbon tape and moreover a carbon film was applied onto the specimens' surfaces by high-vacuum evaporation.

3. Results and discussion

3.1. Scanning electron microscopy imaging

For morphological studies, zeolite particles were introduced to batch-cultures fed with model substrate at 45 °C in order to allow the colonisation. Scanning electron microscopy (SEM) was chosen to visualise how the surface of zeolite particles is colonised as micro-geographical relief. During batch-wise cultivation over 5 days, rod-shaped microorganisms with dimensions of approximately 0.4 µm in diameter and ranging from 1.6 to >5.0 µm in length were seen on zeolite particles (S1A–D). For colonisation, cragged and pitted areas were preferred. Coccoid organisms were not seen which is in contrast to previous studies reporting large accumulation of coccoid type microorganisms besides bacillary, filamentous shaped bacteria in the interior of ruggedness and superficial zones of zeolite particles (Fernández et al., 2007). However, these authors investigated digestion of distillery wastewater with pig sludge inoculation in contrast to the model substrate used in this study mimicking the cell wall composition of grass silage. Furthermore, the inoculum used in the present study was well adapted to grass silage as substrate. As a novel aspect, bacteria/archaea-borne fibrous structures could be seen. Two drying methods, i.e. critical point drying (CPD) and lyophilisation were compared regarding the stability of biological structures as cell morphology. There were no significant differences, but fibrous structures were conserved slightly better using CPD. Pilus-like appendages (S1B, D) appeared to reach out from bacterial/archaeal cells directly either to the zeolite's surface or to neighbouring cells. The pili-dimensions were around 45 nm in width and ranging from 300 nm up to 3 µm in length. This would be within the interspecies distance for both, syntrophic oxidation of propionate, ethanol and iso-propanol between methanogenic consortia members (Ishii et al., 2005) and for elongated polycellulosomal protuberance fibres between substrate and the cell surface as described for *Clostridium thermocellum* (Shoham et al., 1999). However, these structures could have different functions: (i) adhesion fibres for attachment, supporting growth and nutrition for substrate-cellulosome-bacteria interactions (Shoham et al., 1999), (ii) conductive nanowires for interspecies hydrogen or electron transfer like demonstrated for syntrophic co-cultures of hydrogen-consuming methanogenic *Methanothermobacter thermautotrophicus* and propionate-oxidizing *Pelotomaculum thermopropionicum* (Gorby et al., 2006; Ishii et al., 2006; Reguera et al., 2005), (iii) non-conductive nanowires as a foremost structural factor for cell aggregation and biofilm formation as described for pili of metal-reducing bacteria, e.g. *Geobacter sulfurreducens* (Reguera et al., 2007).

In a second approach zeolite particles from a continuously operated 28 l single-stage flow-through reactor fed on grass silage were depicted (S2a–d). After a total incubation time of 12 weeks, microorganisms similar in morphology and dimensions as for batch-wise operated reactors had colonised the surface of zeolite particles. This finding confirms the capability of zeolites to act as a carrier both in batch and in larger scale flow-through disturbed fermenta-

tion processes. Micrographs imaged simultaneously using In-lens and AsB detectors at an acceleration voltage of 15 kV showed topographic and compositional contrasts from one sample site in comparison (S2c, d). The compositional contrast gives the opportunity to distinguish between carbon-based biological material (i.e. bacteria/archaea cells) and inorganic matter and thus clearly confirms the biological origin of bacillary and fibrous structures as they disappeared in comparison to the inorganic zeolite background.

3.2. Enzymatic activity from colonised zeolites

To investigate functionality of bacterial populations immobilised on zeolite surfaces, the activity of xylanase was determined in zeolite particles taken from batch-cultures fed with model substrate at 35 °C. This enzyme is responsible for the hydrolysis of xylan as a major cell wall component of grass silage (Nizami et al., 2009). Furthermore, solubilisation of xylan triggers the accessibility of cellulose (Hendriks and Zeeman, 2009). Plant cell wall polymer degradation should naturally depend on the presence of grass silage fibres. Even so, xylanase activity was detected on zeolite fractions obtained from batch-cultivation on model substrate at 35 °C over 14 d. The pH value was decreasing from 8.7 to 7.6 during this fermentation time. Colonised zeolite particles were then re-incubated in autoclaved fermentation broth as well as in synthetic minimal medium as a control (Fig. 1). After 38 h of re-incubation of washed zeolite a maximum xylanase activity of $116 \pm 57 \text{ U l}^{-1}$ was measured. When washing before re-incubation was omitted, a higher activity of $190 \pm 26 \text{ U l}^{-1}$ was seen which is most likely due to the release of adsorbed enzymes. In both cases, enzyme activity decreased after 62 h. A considerably lower activity was determined after re-incubation of washed zeolite in minimal medium ($25 \pm 4 \text{ U l}^{-1}$ after 38 h) indicating that xylanase production was related to growth of immobilised microorganisms and probably is induced by medium components (e.g. xylo-oligosaccharides). However, a slight increase in activity over re-incubation time still demonstrates that microbes slowly grew in minimal medium probably utilizing adsorbed biomass as carbon source. In contrast, when zeolite particles were autoclaved too, no significant enzyme activity was observed during re-incubation at 35 °C over 62 h. These findings clearly indicate that hydrolytic microorganisms were adherent to zeolite particles.

3.3. Community profile and sequencing analysis

3.3.1. Bacteria screening

For further characterisation of microbial populations immobilised on zeolite, PCR-based SSCP-analysis of amplified bacterial

rrs (16S rRNA gene) fragments was carried out with samples from batch-cultivation on grass silage or model substrate at 35 °C for comparison. Banding profiles from the batch-fermentations clearly formed three major clusters as stated in Fig. 2. Bacteria attached to the surface of grass silage fibres formed one cluster with band patterns of >60% similarity. Bacteria attached to the surface of zeolite particles formed a second cluster with a similarity of >66%, combining samples from incubation upon model substrate and grass silage. This cluster was separated distinctively ($P < 0.01$) from grass silage surfaces, as indicated by a similarity value of only 7%. A third cluster was formed by the supernatant samples regardless of the substrates used. Here the homologous band patterns were forming an exclusive cluster at a similarity of >82%, which is significantly differing ($P < 0.01$) from both surface generated clusters. These findings suggest that different sample sites are colonised by distinct bacterial populations. Functional populations developed on zeolites, producing the hemicelluloses degrading enzyme xylanase (Section 3.2), were different from populations in the liquid phase and from those present on grass silage fibres. Thus, zeolites are not only providing more surface as support, but obviously allow the development of specific functional populations which are essential in the biogas process (e.g. hemicellulase producers).

Sequence analysis of DNA extracted from single bands representing specific species was then used for further analysis of community members (Schwieger and Tebbe, 1998). Databank searches with obtained sequences (in total 44 reads) foremost led to entries for uncultured bacteria. Bands chosen for sequencing analyses are labelled in Fig. 3. Top hits found for surface sample sequences (bands 9, 13 and 13b) were typical for fermentation processes (FN436026, CU919914, FN436026). Amongst them were bacteria sequences from thermophilic methanogenic sludge (AB530683) that match ours with 98% identity (band 2) and sequences from uncultured bacteria of the class Thermotogae (CU924654). After 28 d of fermentation, the populations on zeolite surfaces were affiliated with the phylum Bacteroidetes, i.e. *Ruminofilibacter xylanolyticum* (DQ141183, EU551120) within the family Rikenellaceae with 98% identity (band 7). This band also appeared from surface samples of grass silage fibres (band 7b). After 42 d of fermentation, the close relationship to *Ruminofilibacter xylanolyticum* was confirmed with an identity value of 99%. This species has been isolated from the rumen in other studies where growth of *Ruminococcus* spp. and other bacteria with cellulolytic, pectinolytic and hemicellulolytic activities (*Bacteroides* spp. and *Clostridium* spp.) was increased by high levels of dietary fibre (Metzler and Mosenthin, 2008). In addition, *R. xylanolyticum* was also found in a production-scale biogas plant fed with maize silage, green rye and liquid manure (Kröber et al., 2009).

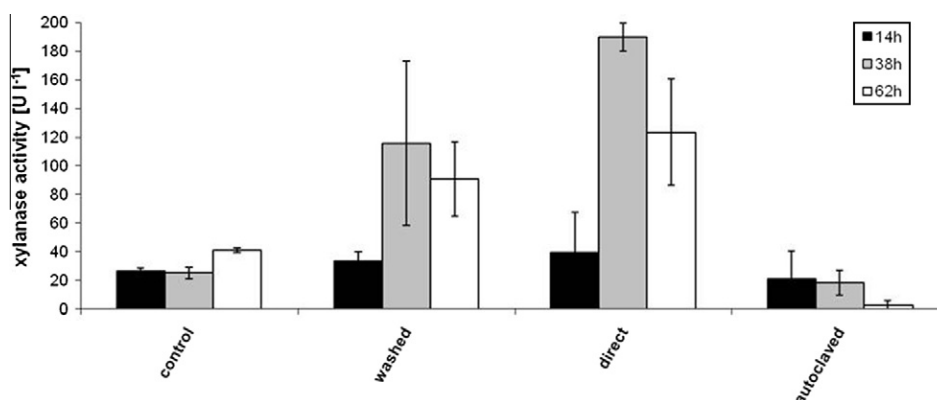


Fig. 1. Xylanase activity determined after re-incubation of zeolite particles in synthetic minimal medium (control), full medium (washed; carefully rinsed with $1 \times$ PBS; direct: not rinsed; autoclaved: in-activated zeolite fraction) for 14 h (black columns), 38 h (grey columns) and 62 h (white columns) at 35 °C. Zeolite samples were taken from anaerobic batch-cultivation on model substrate after 14 d at 35 °C.

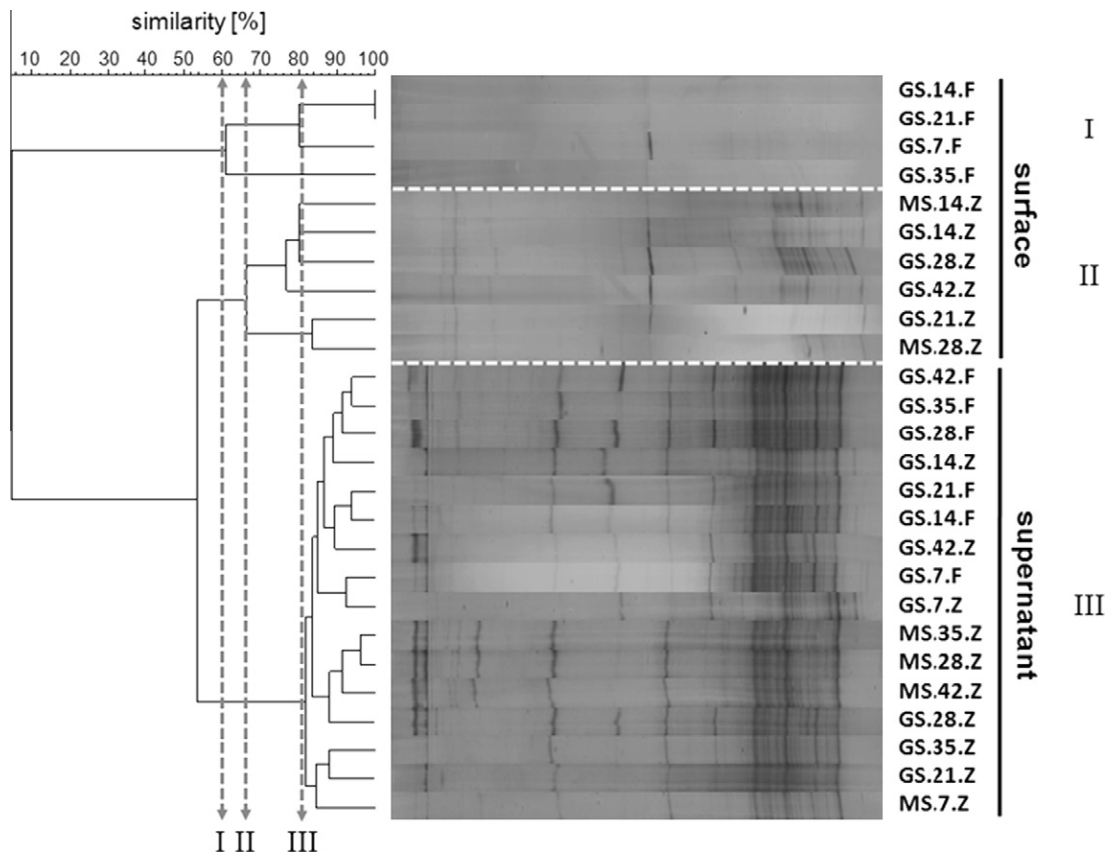


Fig. 2. Dendrogram based on SSCP separated *rrs* (16S rRNA) amplicons of batch-cultures fed with grass silage or model substrate for comparison. Band patterns were grouped by UPGMA. Double-headed vertical arrows indicate the similarity for the groupings. The dendrogram reveals three major clusters depending on the sample site: (I) surface of grass silage fibres, (II) surface of zeolite particles and (III) supernatant samples forming one cluster. Sample code: sample site; batch-identity (MS = model substrate, GS = grass silage, 7–42 = fermentation time in days, F = grass silage fibres, Z = zeolite).

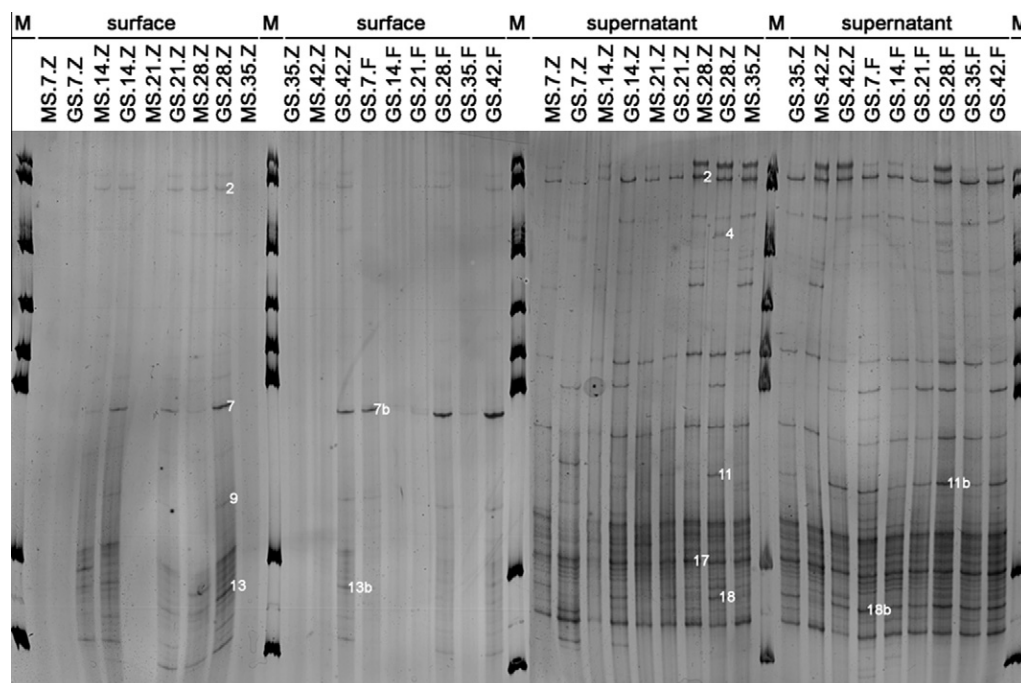


Fig. 3. Non-denaturing polyacrylamide gel from the SSCP analysis of batch-cultures fed with grass silage or model substrate for comparison based on bacterial *rrs* (16S rRNA gene) fragments. Sample code: M, 1 kb DNA-ladder marker lanes; sample site; batch-identity (MS = model substrate, GS = grass silage, 7–42 = fermentation time in days, F = grass silage fibres, Z = zeolite).

In the present study, there were multiple hits for two bands (2 and 17) of supernatant samples from batch-cultures fed with model substrate. These were similar to a sequence from an uncultured bacterium found in a large-scale thermophilic municipal biogas plant (EU878320) and to sequences from thermophilic anaerobic solid biowaste digestors (AM947527, EF558972) with identity values of 97%. Furthermore, there were again affiliations to Thermotogae (CU918433) and to an uncultured member of the class Bacteroidetes (CU919517) with 98% identity. In contrast, sequence of band 4 from grass silage batch-culture supernatant after 28 days of fermentation revealed a relationship to the phylum Firmicutes, i.e. *Bacillus* sp. (AF548884) with 97% identity. Sequences obtained from bands 11, 11b, 18 and 18b were moderately similar (92% identity) to uncultured *rrs* (16S rRNA gene) sequences from bacterial community analyses of mesophilic bioreactors (FJ825461), cassava pulp and pig manure co-digestion (GQ458213) and bacteria associated with the leachate of a closed municipal solid waste landfill (AJ853654).

3.3.2. Archaea screening

The second important taxonomic group investigated was the domain of archaea. Members of this domain are responsible for methanogenesis. PCR-based SSCP-analysis of amplified archaeal *rrs* (16S rRNA) fragments was carried out with samples from batch-cultivation on grass silage or model substrate at 35 °C for comparison similar to Section 3.3.1. Amplicons were obtained for supernatants and zeolite surfaces, but not for grass silage fibres surfaces. SSCP analysis revealed two foremost double-banded pattern fields (Fig. 4), in which the upper one was intense in samples from zeolite surfaces over the total fermentation time (7–42 days), utilising model substrate. Here, bands 1 and 2 were excised for sequencing analysis. In contrast, supernatants from the same batch-cultures showed less intense bands in this field, but slightly stronger double-bands in the lower area. Supernatants from batch-cultures fed with grass silage (right side in Fig. 4) showed a weakening of bands in the upper field from day 21 on (bands 1b and 2b

were excised). Possibly the corresponding archaea were diminished, eventually outcompeted by the archaea corresponding to the lower bands which apparently increased (see below), or they moved from the supernatant to the zeolite's surface. However, zeolite surfaces appeared to be suitable for colonisation by methanogenic archaea (see below).

Bands appearing from samples of zeolite surfaces incubated at 35 °C in batch-cultures fed with model-substrate (bands 1–6 in Fig. 4) were most similar to uncultured *rrs* (16S rRNA gene) sequences from archaeal community analyses of anaerobic digestion in biogas digestors (AB479397, DQ262609, FJ205782, HM066950) with 89–99% identity. Bands marked with 1 and 2 were present in all batch-cultures regardless of the substrate used. Sequences of band 1 affiliated with Methanomicrobiales (AB479397) with 94% identity. The amplicon of band 2 was moderately similar (89% identity) to *Methanosarcina barkeri* (AF028692) within the order Methanosarcinales. *M. barkeri*, known for its metabolic diversity, is able to use methanol, H₂/CO₂ and acetate for methanogenesis (Meurer et al., 2002). Furthermore, *Methanosarcina* spp. were previously identified to be part of the predominant genera on zeolite surfaces (Fernández et al., 2007). Band 5 appeared at day 21 of cultivation on model substrate and grass silage. Sequences were most similar to *Methanoculleus* sp. (AF107105, AJ550158) and *M. bourgensis* (AY196674, DQ150254) with moderate identity values of 91% indicating the presence of the order Methanomicrobiales. However, members of the genus *Methanoculleus* spp. were to be found the dominant archaea in the methanogenic microbial community of an agricultural biogas plant (Kröber et al., 2009). Certain representatives of the order Methanomicrobiales (obligatory hydrogenotrophic), were recently proposed to be useful as bio-indicators for process disturbance through acidification in methanogenesis (Lebuhn et al., 2009).

Sequences from supernatant samples of batch-cultures fed with grass silage (bands 1b–6b in Fig. 4) were most related to databank entries originating from biogas production (AB494258, FJ205785, HM066955) and included origins such as landfill sites and com-

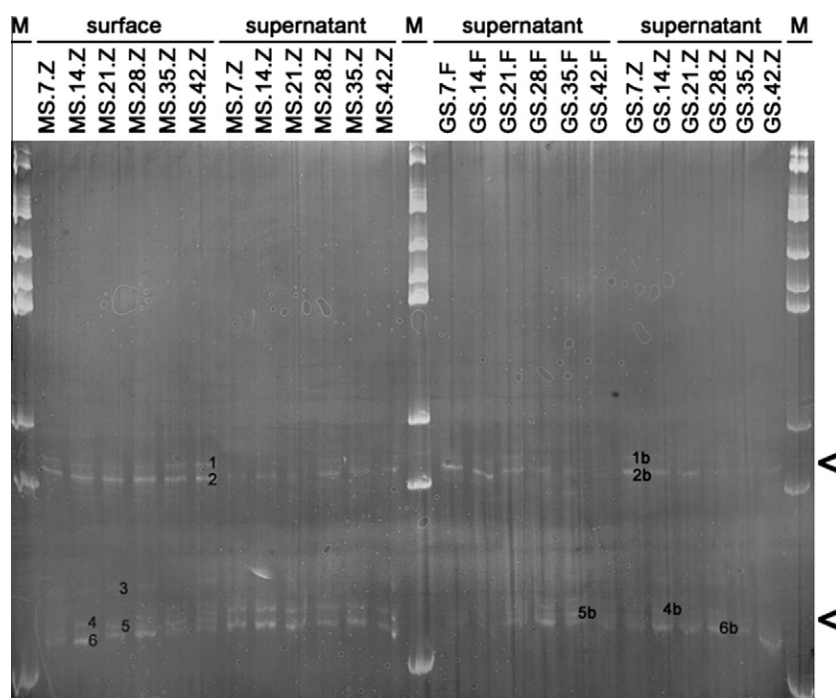


Fig. 4. Non-denaturing polyacrylamide gel from the SSCP analysis of batch-cultures fed with grass silage or model substrate for comparison based on archaeal *rrs* (16S rRNA gene) fragments. Sample code: M, 1 kb DNA-ladder marker lanes; sample site; batch-identity (MS = model substrate, GS = grass silage, 7–42 = fermentation time in days, F = grass silage fibres, Z = zeolite).

posting processes (AB541745, AJ576208, AY062220) with identity values of 94–99%. Amplicons of bands 4b–6b which appeared later (at day 14, 28 and 35) were most similar to *Methanoculleus* spp. (AF107103, AF107106, AJ550158) with identity values of 97% and 94%, respectively.

4. Conclusion

Colonisation of clinoptilolite particles by microorganisms was demonstrated, revealing spontaneous adhesion to preferentially sheltered areas as pits. The microbial abundance ranged from single cells to dense biofilm-like aggregations on the cratered zeolite's surface. Two dominant cell morphologies were observed, i.e. long rod-shaped and shorter bacillus-like types. Colonies adherent to zeolite surfaces were composed of bacterial representatives with confirmed hemicellulolytic activity and methanogenic archaea known to be predominant in biogas-producing communities. This neighbouring existence argues for a syntrophic relationship between members of both domains, naturally co-cultured and spontaneously co-aggregated on zeolite as an exclusive operational environment in the biogas production process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.12.076.

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