Chemosphere 80 (2010) 829–836

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Characterization of an anaerobic population digesting a model substrate for maize in the presence of trace metals

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ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 6 June 2010
Accepted 7 June 2010
Available online 7 July 2010

Keywords:
Anaerobic digestion
Biogas
Cellulose
Nickel
SSCP

ABSTRACT

The influence of a defined trace metal solution and additionally Ni^{2+} on anaerobic digestion of biomass was investigated. A novel synthetic model substrate was designed consisting of cellulose, starch and urea as carbon and nitrogen source in a ratio mimicking the basic composition of maize silage. Two independent batch fermentations were carried out over 21 d with the synthetic model substrate in the presence of the trace metal solution. Particularly an increase in nickel concentrations (17 and 34 μM) enhanced methane formation by up to 20%. This increased activity was also corroborated by fluorescence microscopy measurements based on cofactor F_{420}. The eubacterial and methanogenic population was characterized with the single strand conformational polymorphism analysis and the amplified 16S rDNA restriction analysis of 16S rRNA genes amplified by different primer systems. Nearly the half of the analyzed bacteria were identified as *Firmicutes* while 70% in this phylum belonged to the class of Clostridiales and 30% to the class of Bacilli. *Bacteroides* and uncultured bacteria represented each a quarter of the analyzed community. *Methanogenic archaea* were investigated with ARDRA, too. The hydrogenotrophic *Methanoculleus* sp. was the dominant genus which is commonly described for maize digestion thus confirming the value of the model substrate.

1. Introduction

The environmental impact of anaerobic digestion and methane formation is related to its ecological role, on the one hand accumulation and effect as a greenhouse gas, on the other hand an application for conversion of biomass agricultural residues and biowaste into energy (Chynoweth, 1996). Production of biogas from a variety of organic substrates is a carbon dioxide neutral and versatile renewable energy source. Especially maize silage from the whole maize plant as energy crop has a high potential for biogas production. Anaerobic degradation of organic matter and methane formation is a multi step process carried out by a consortium of microorganisms. The production of methane requires a trophic chain of at least three interacting metabolic groups of strictly anaerobic microbes (Ferry, 1997). First of all the hetero-fermentative group reduce cellulose and other complex molecules into volatile fatty acids (e.g. acetate, propionate), CO_2, and H_2. The syntrophic bacteria further metabolize the alcohols and fatty acids into acetate, H_2, and CO_2, in which acetate, H_2, and CO_2 finally serve as substrates for the methanogenic archaea (Guedon et al., 2002). A detailed knowledge about the composition of these microbial communities participating in the degradation process of biomass will be essential to understanding and improve the biogas-forming process (Klocke et al., 2007). In addition to microbial community studies it is known from previous investigations that various trace metals play an important role in growth and biochemical processes of the methane producing microorganisms. (Takashima and Speece, 1989; Goodwin et al., 1990). Specific trace metals such as cobalt, nickel, tungsten or molybdenum serve as cofactors in enzymes which are involved in the biochemistry of methane formation (Zandvoort et al., 2006). For example, all methanogenic pathways converge to the enzymatic reduction of methyl-coenzyme M to methane. This reduction is catalyzed by the methyl-coenzyme M reductase complex, which includes a nickel containing cofactor called F_{430} (Friedman et al., 1990). Nevertheless, availability of essential trace elements in optimal amounts is still a problem when single substrates rather than complex mixtures of organic matter are used for biogas production. The bioavailability of trace elements for metabolic pathways of the archaeal and bacterial community is in most cases not related to the total amount measured in the medium since only a fraction is present in solution (Oleszkiewicz and Sharma, 1990). Shifts in pH-value or temperature may lead to metal precipitation and/or chelation which induce to a reduced bioavailability of these trace metals. A limitation of essential trace metals can lead to reduced methane yields and to considerable problems due to increasing process instability (Mosey et al., 1971; Speece, 1996; Zandvoort et al., 2005).

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doi:10.1016/j.chemosphere.2010.06.011

The objective of the present study was to characterize an anaerobic microbial community digesting a cellulose/starch based model substrate in the presence of trace elements. Both the replacement of real substrates (i.e. maize) with synthetic components as well as addition of trace elements could have an impact of microbial populations which has not been studied yet in detail. We realised this molecular biological investigations by means of a combination of single strand conformational polymorphism analysis (SSCP) and amplified 16S rDNA restriction analysis (ARDRA) to illustrate the population developing under these conditions. SSCP analysis is a semi-quantitative method while each band on the gel should represent one species. For this a 400 bp long fragment of the variable region V4–V5 (Schmalenberger et al., 2001) of the bacterial 16S rDNA was amplified. ARDRA analysis based on a fingerprinting of restriction patterns of the 16S rDNA.

2. Materials and methods

2.1. Liquid and solid media

The used LB (lysogeny broth) medium consisted of (in $g\,L^{-1}$) peptone 16, yeast extract 10, NaCl 5, and the 2×TY medium peptone 10, yeast extract 5 and NaCl 5. For the LBA agar, LB medium was added with $14\,g\,L^{-1}$ agar and $100\,\mu g\,mL^{-1}$ ampicillin. The LBA/X-Gal-agar was assembled like LBA agar with additionally $40\,\mu g\,mL^{-1}$ X-Gal (bromo-chloro-indolyl-galactopyranoside). All media were prepared with deionised water and autoclaved. Ampicillin solution was sterile filtrated. All chemicals were purchased by Roth and Merck (Germany).

2.2. Batch tests

2.2.1. Model substrate and fermentation medium

A new synthetic model substrate (dS) was developed following the composition of maize. Microcrystalline cellulose and starch from maize were used as carbon source in a mix of 50% cellulose and 46% starch, besides urea as a nitrogen source accounting for the remaining 4%. In addition phosphorus was supplied with a 0.1 M potassium phosphate buffer pH 7.2 resulting in a C:N:P ratio of 125:5:1. All chemicals are purchased by Roth Chemicals, Germany. The batch reactors were fed with a fermentation medium comprising 1% ODM (organic dry matter) from the defined model substrate and 1.5% ODM from an inoculum sludge (biogas plant Fürstenfeld, Styria). Trace metals were added as trace element solution ($1\,mL\,L^{-1}$ fermentation medium). The trace element solution contained (μM) Fe^{2+} 7.5 ($FeCl_2{\cdot}4H_2O$), Zn^{2+} 0.5 ($ZnCl_2$), Mn^{2+} 0.5 ($MnCl_2{\cdot}4H_2O$), B^{3+} 0.1 (H_3BO_3), Co^{2+} 0.8 ($CoCl_2{\cdot}6H_2O$), Cu^{2+} 0.01 ($CuCl_2{\cdot}2H_2O$), Ni^{2+} 0.1 ($NiCl_2{\cdot}6H_20$), Se^{6+} 1.0 ($Na_2SeO_3{\cdot}5H_2O$), Mo^{6+} 0.15 ($Na_2MoO_4{\cdot}2H_2O$), W^{6+} 0.1 ($Na_2WO_4{\cdot}2H_2O$). Additional nickel was also applied as $NiCl_2{\cdot}6H_20$ in concentrations as indicated below. Batch tests were started with a pH-value of 7.8 ± 0.2.

2.2.2. Experimental set up

Batch tests were conducted according to Friedmann et al. (2004) as also described in DIN DEV 38414 S8 (1985). The batch fermentations were performed in 2 L glass reactors with a volume of 1 L of the fermentation medium. Carbon dioxide was removed from the biogas with a 3 M sodium hydroxide solution. Ammonia and hydrogen sulphide were eliminated by an acidic solution containing 0.6 M $Na_2SO_4{\cdot}10\,H_2O$ and 0.5 M H_2SO_4. The displaced acidic solution was measured in a graduated cylinder and calculated to the produced methane. The reactors were mixed with a magnetic stirring system for 15 min every 3 h. The batch trials were carried out in triplicates at 35 °C for a time period of 21 d. Results from control reactor (including only the defined model substrate and inoculum sludge) were compared with reactors inclusive of an appropriate amount of trace metals.

2.3. Analytical measurements

ICP-OES, Spectro Ciros Vision, Germany was used for the quantification of macronutrients and trace metals in the inoculum sludge and the defined model substrate. TKN (total Kjeldahl nitrogen) was measured according to the method of Kjeldahl with a Vapodest Vap 50, Gerhardt; Germany (data not shown). The pH-readings were conducted with a WTW pH 540 GLP pH-meter. The DM (dry matter) was determined in a dryer from Heraeus HANAU, Germany, at 105 °C. The ODM was determined at 550 °C in a muffle furnace from Heraeus HANAU, Germany. Volatile fatty acids (VFAs) were measured by HPLC. Thus 1 g of sludge from batch fermentation was centrifuged for 10 min at 16,000*g* and the supernatant was collected. An aliquot of the supernatant was pre-treated according to Carrez precipitation to remove proteins and fat components. For HPLC performance a Hewlett Packard HPLC System 1100 was used. The system was equipped with aTRANSGENOMIC, ICSep ION-300, Art Nr. ICE-99–9850 column and WAGNER LÖFFLER, ICSep ION-300, Art Nr. CH0–0800 column respectively. As pre-column TRANSGENOMIC, GC801/C, Art Nr. ICE-99–2364 and WAGNER LÖFFLER, Interaction Replacement Cart. GC-801/C, 24∗4, 0 mm, Art Nr. CH0–0831 respectively were used. Measurements were carried out at following operating conditions: 5 mM H_2SO_4 as mobile phase and a flow rate of $1\,mL\,min^{-1}$, injection volume 40 μL, column temperature 42 °C. For reporting of chromatograms special software was used (HP chemstation).

2.4. DNA extraction and PCR-amplification

Samples of sludge from batch reactors were obtained on days 1, 7, 14, and 21 for gene-based analyses. Therefore in each case 1 g of the biogas sludge were centrifuged for 15 min at 16,000*g* and 4 °C with a Hermle Z300 K (Hermle Labortechnik, Germany). The supernatant was discarded and the pellet dissolved in 1 mL extraction buffer consisting of 100 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% Polyvinylpyrrolidone and 2% Sodium Dodecyl Sulfate (SDS). Total genomic DNA was actually extracted according to the protocol of Martin-Laurent et al. (2001). Bacterial 16S rRNA gene target for SSCP analysis was amplified with forward primer Com 1 (5′-CAGCAGCCGCGGTAATAC-3′; (Schwieger and Tebbe, 1998) and reverse primer 927r (5′-CCCGTCAATTYMTTTGAGTT-3′; Lieber et al., 2002). Primer 927r was phosphorylated for single strand digestion. The 50 μL reaction mixture consists of 10 μL Taq&Go (MP Biomedicals Europe), 1.67 μL 50 mM $MgCl_2$, 2 μL of forward and reverse primer respectively (5 μM), 1 μL template DNA, 33.3 μL H_2O (Roth, Germany). PCR (polymerase chain reaction) was performed with an initial denaturation at 94 °C for 3 min, 35 amplification cycles (60 s at 94 °C, 60 s at 50 °C, and 70 s at 72 °C), and a final elongation at 72 °C for 5 min.

Archaeal and bacterial 16S rRNA genes for ARDRA analysis were amplified with forward primer 21F (5′-TTCCGGTTGATCCYGCCGGA-3′; De Long, 1992) and reverse primer 958R (5′-YCCGGCGTTGAMTCCAATT-3′; De Long, 1992), and forward primer 27F (5′-GAGTTTGATCCTGGCTCAG-3′; Liesack et al., 1991) and reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′; Lane, 1991), respectively. In each case, a 20 μL reaction mixture for archael and bacterial DNA PCR-amplification was conducted contained 4 μL Taq&Go (MP Biomedicals Europe), 1 μL 50 mM $MgCl_2$, 1 μL of archael and bacterial forward and reverse primer respectively (5 μM), 1 μL template DNA, 13 μL H_2O (Roth, Germany). The PCR program for archael DNA amplification consisted of a initial denaturation at 95 °C for 5 min, 29 cycles (30 s at 95 °C, 30 s at 55 °C and 90 s at 72 °C), and a final elongation at 72 °C for 10 min.

Further PCR for bacterial DNA amplification consisted of initial denaturation at 95 °C for 5 min, nine cycles (30 s at 95 °C, 30 s at 52 °C and 100 s at 72 °C), 19 cycles (30 s at 95 °C, 30 s at 52 °C and annealing at 72 °C for 30 s with an increase of 10 s every cycle) and a final elongation at 72 °C for 5 min.

The Colony-PCR of positive clones was performed with the universal sequencing primers rsp (5′-CAGGAAACAGCTATGACC-3′) and usp (5′-GTAAAACGACGGCCAGT-3′). PCR reaction mix was done in a volume of 20 μL as described for archael and bacterial DNA amplification. The PCR program consisted of a initial denaturation at 95 °C for 5 min, 29 cycles (30 s at 95 °C, 30 s at 54 °C and 60 s at 72 °C), and a final elongation at 72 °C for 10 min.

The products from all PCRs were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega). Furthermore all PCR products were examined by electrophoresis on 0.8% agarose gels, viewing on a UV transillumination table to determine correct amplicon size.

2.5. SSCP and ARDRA analysis

SSCP analysis of amplified bacterial 16S rRNA gene fragments was realised according to Schwieger and Tebbe (1998). Exonuclease digestion of purified PCR products was performed with a λ-Exonuclease, 12 U (New England Biolabs, Germany) at 37 °C for 1 h, followed by an 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 regeneration step on ice for 5 min. Electrophoretic separation of DNA single strands was performed on a 8% polyacrylamide gel and a 1× TBE (Tris/Borate/EDTA) buffer pH 8.0 (89 mM TRIS, 89 mM boric acid, 2 mM Na_2EDTA) for 26 h at 26 °C, 400 V and 50 mA, using a TGGE MAXI system (Biometra). After silver-staining according to Bassam et al. (1991), gels were digitalised using a transillumination scanner and bands of interest were excised with a sterile scalpel for following sequence analysis. DNA was eluted from gel slices through incubation in sterile elution buffer pH 8.0 (5 M ammonium acetate, 10 mM magnesium acetate, 1 mM Na_2EDTA, 0.1% SDS) at 37 °C for 5 h with following freezing at −70 °C over night. After thawing of the samples the eluted DNA was amplified again using the same primer pairs for the SSCP analysis as described above. The sequencing was done at Eurofins MWG Operon, Germany. A minimum DNA concentration of 3 nM was used.

ARDRA analysis of amplified bacterial and archael 16S rRNA gene fragments was performed according to Vaneechoutte et al. (1992). The obtained PCR amplicons from PCR with archael and bacterial primers respectively (2.4.) were ligated into the pGEM®-T vector system I (Promega) and used to transform elcetrocompetent *E. coli* DH5α cells with a Bio-Rad MicroPulser™ by following the manufacturer's instructions. Clone libraries were generated, by growing regenerated cells undiluted and 10^{-1} diluted on LBA/X-Gal plates (2.1.) and incubated over night at 37 °C. A Colony-PCR as described above was done from positive, white clones. Amplification products were controlled on a 0.8% agarose gel to determine the concentration of the DNA for following digestion with restriction endonuclease *Hha*I (Fermentas) at 37 °C for 3 h. Resultant DNA fragments were separated electrophoretically for 3 h and 120 V on 1.5% 0.5 TBE agarose gels and afterwards stained in a ethidium bromide solution for 20 min. Operational taxonomic units (OTUs) were identified manually and additionally the classification was done with GelCompare© (Applied Maths, version 4.2) based on restriction cleavage patterns and clones representing the OTUs selected for sequencing. The sequencing was also done at Eurofins MWG Operon, Germany.

2.6. Fluorescence microscopy

Fluorescent microscopic analyses were carried out to determine the activity of methanogenic bacteria. Samples were taken on day 1, 7, 14 and 21 and investigated immediately under a Leitz Laborlux S fluorescence microscope. A 100 W high-pressure mercury lamp was used with a 460 nm longpass filter for blue excitation. 10 μL of each sample were fixed on a microscope slide. The samples were observed with a 1000-fold magnification. For documentation a Nikon D70 camera and the software Camera control Pro were used.

3. Results and discussion

3.1. Performance of anaerobic batch tests

Two independent batch fermentations with a novel model substrate for maize silage and addition of trace metals and supplementary nickel were conducted. A basic nickel concentration of 2 μM was used in the batch fermentations from the utilized inoculum sludge. Additionally nickel concentrations from 5–17 μM (batch 1) and 8.5–34 μM (batch 2) were analyzed (Table 1). Addition of the trace metal solution increased methane production by 4% in batch 1 and 6% in batch 2 respectively. Addition of 5 μM nickel to fermentations in batch 1 induced a methane enhancement of 8% compared to trials exclusive of defined substrate and which increased until a plateau at 17 μM nickel. Therefore, a second series of batch fermentations was conducted to evaluate higher nickel concentrations. However, additions of up to 34 μM nickel did not further increase methane production (Table 1). The obtained results from the conducted batch fermentations demonstrated the best performance and methane production at a nickel dosage of 17 μM in batch 1 with a methane increase of 14% and batch 2 up to 20%. These increases are in a similar range as previously reported for a xylan based substrate (Pobeheim et al., 2010). However, in this previous study the optimum effect was reached at a slightly lower nickel concentration of 10.6 μM. Similarly, in a study of Williams et al. (1986) an addition of 10 μM nickel to a chicken manure digester significantly stimulated biogas production, while nickel was present in the effluent (253 μM) before supplementary "fresh" nickel was added.

Moreover we determined formation and degradation of VFAs. Samples after 1, 7, 14 and 21 d of incubation were taken and measurements from acetic and propionic acid were analyzed (Table 2). At day 7 an increase of acetic acid between 3187 mg kg^{-1} fresh mass (FM), (reactor 7), and 2440 mg kg^{-1} FM (reactor 8) and propionic acid between 1635 mg kg^{-1} FM (reactor 5) and 1377 mg kg^{-1} FM (reactor 8) were consistent with the drop of the start pH-value

Table 1
Effect of nickel on methane yield in anaerobic digestion of a maize model substrate.

Reactor	Substrates	Batch 1		Batch 2	
		NL[c] CH4	Methane yield (%)	NL CH4	Methane yield (%)
1	dS[a]	225 ± 10	100	247 ± 2	100
2	dS + TES[b]	235 ± 12	104	262 ± 5	106
3	dS + TES + 5.0 μM	243 ± 10	108		
4	dS + TES + 8.5 μM	244 ± 8	108	275 ± 8	111
5	dS + TES + 13 μM	253 ± 17	113	270 ± 11	109
6	dS + TES + 17 μM	255 ± 5	114	297 ± 4	120
7	dS + TES + 26 μM			281 ± 10	113
8	dS + TES + 34 μM			277 ± 12	112

Note: 5.0–34 μM additionally nickel to anaerobic batch trials.
[a] Defined model substrate based on cellulose and starch.
[b] Trace element solution with 0.1 μM nickel.
[c] [L kg^{-1} ODM at STP].

Table 2
Acetic and propionic acid production during anaerobic digestion of a maize model substrate and different nickel concentrations over 21 d.

Reactor	Sample (days)	1	7	14	21
	Substrates	Acetic acid (mg kg^{-1} FM[c])			
1	dS[a]	124	2241	3236	908
2	dS + TES[b]	131	2377	3488	819
4	dS + TES + 8.5 μM	121	2648	3337	579
5	dS + TES + 13 μM	127	2641	3156	347
6	dS + TES + 17 μM	126	2576	3065	640
7	dS + TES + 26 μM	134	3187	172	35
8	dS + TES + 34 μM	134	2440	2108	467
		Propionic acid (mg kg^{-1} FM)			
1	dS	0	1545	1461	1152
2	dS + TES	0	1509	1478	795
4	dS + TES + 8.5 μM	0	1503	1493	449
5	dS + TES + 13 μM	0	1635	1649	1547
6	dS + TES + 17 μM	0	1546	1313	146
7	dS + TES + 26 μM	0	1532	1650	1642
8	dS + TES + 34 μM	0	1377	1387	769

[a] Defined model substrate.
[b] Trace element solution with 0.1 μM nickel.
[c] Fresh mass of fermentation medium.

from 7.8 ± 0.2 to 6.8 ± 0.1. Batch trials with addition of 17 μM nickel (reactor 6) showed a constant degradation of acetic acid to 640 mg kg^{-1} FM and lowest amount of propionic acid (146 mg kg^{-1} FM) after 21 d of incubation (Table 2).

Furthermore a pH-value of 7.6 in relation to remaining trials with pH-values between 7.35 and 7.5 were measured. Accumulation of propionic acid is an indication for process instability and could result from limited activity of synthrophic and/or methane bacteria. Alternatively inhibition of propionate degradation by propionate and acetate could happen due to the undissociated acid forms of these compounds as observed by Fukuzaki et al. (1990).

3.2. Analysis of eubacterial community by SSCP

SSCP analysis of 16S rRNA genes was used to characterize the anaerobic bacterial community developed on the maize model substrates in the presence and absence of trace elements. A couple of diverse bands were excised from SSCP gels, re-amplified and sequenced.

From the first batch fermentation with nickel concentrations from 5 to 17 μM 6 bands could be sequenced and assigned (Fig. 1a). As reported in Table 3 bands 1 and 2 were assigned as uncultured bacterium, bands 3 and 5 as *Clostridium* sp., band 4 as *Arcobacter* sp. and band 6 as *Sedimentibacter* sp. *Clostridia* are Gram-positive, rod-shaped, anaerobic and endospore-forming bacteria. Saccharolytic *Clostridia* are known to degrade polysaccharides like cellulose and starch (where the substrate in this study is based on) leading to monosaccharides (Zhang et al., 1994). One strain belonging to *Clostridia* (B3) started as a weak band and became dominant after day 7 (Fig. 1a). Another representative band (B5) showed the strongest appearance after 7 d, too. Obviously these hydrolytic bacteria degraded the polysaccharide substrates yielding intermediates for methane production. This is also indicated by a drop down of the pH-value and increased VFA concentrations at day 7 (Table 2). *Arcobacter* spp. belongs to the family of Campylobacteriaceae. They are Gram-negative, rod-shaped bacteria and are able to grow under aerobic and anaerobic conditions. These bacteria are common in poultry meat and manure (Ho et al., 2006). This bacterium was detected at the beginning of the fermentation (band 4), while the corresponding band became weaker at day 7 and disappeared after day 14. This nicely demonstrates the power of this analysis method since indeed poultry manure was used as inoculum sludge for the batch fermentations. However, the disappearance of these bacteria indicates that they play no dominant role in the degradation of polysaccharides and were displaced after 1 week by other hydrolytic bacteria.

Sedimentibacter sp. is counted to the order of Clostridiales. They are Gram-positive, anaerobic, endospore-forming bacteria. These bacteria utilize amino acids and pyruvate as substrates and metabolise acetate and butyrate. They do not produce hydrogen and carbohydrates are not fermented. The genus has a high similarity to Clostridia (Breitenstein et al., 2002). The corresponding band in Fig. 1a was dominant at day 14 when butyric and isobutyric acids were present at their highest amount with 80 mg kg^{-1} FM and 100 mg kg^{-1} FM for butyric and isobutyric acid respectively.

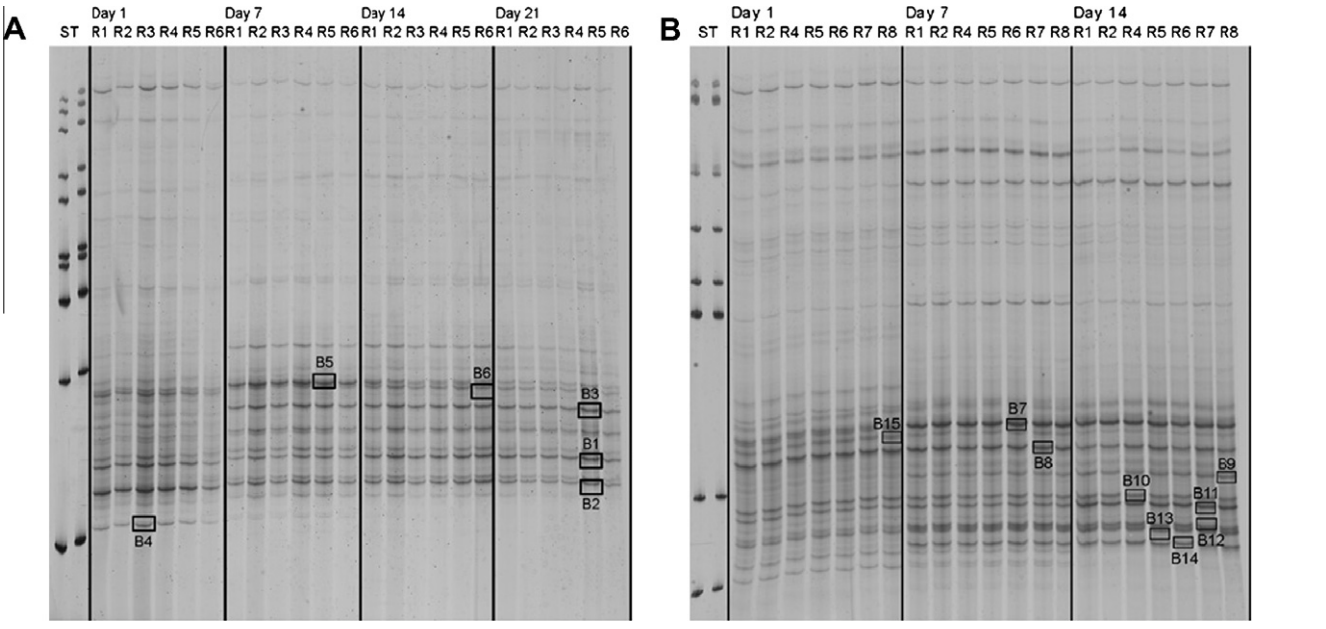

Fig. 1. SSCP gels of fragments of the 16S rDNA extracted from a batch anaerobic fermentation of a maize model substrate (a) with additionally nickel concentrations from 5–17 μM and second fermentation (b) from 8.5–34 μM. The framed bands were cut out and DNA was amplified for sequencing (Table 3). Band patterns show a similar behaviour on both gels. 1 kb standard (ST) was used.

Table 3
Summary of sequences from excised SSCP gel bands identified by searching the NCBI BLAST database.

Band	Sampling point	Species	Accession number
Batch 1			
1	R 5 day 21	Unc. Bacterium	AB248650.1
2	R 5 day 21	Unc. Bacterium	FJ205858.1
3	R 5 day 21	*Clostridium* sp.	DQ168250.1
4	R 1 day 1	Unc. *Arcobacter* sp.	EU403949.1
5	R 5 day 7	*Clostridium* sp.	AY330125.1
6	R 6 day 14	*Sedimentibacter* sp.	EF464626.1
Batch 2			
7	R 6 day 7	*Clostridium* sp.	AY330125.1
8	R 7 day 7	*Clostridium* sp.	DQ168269.1
9	R 8 day 14	*Clostridium* sp.	DQ168299.1
10	R 4 day 14	*Clostridium* sp.	AB286232.1
11	R 7 day 14	Unc. Bacterium	AB248650.1
12	R 7 day 14	*Proteiniphilum acetatigenes*	AY742226.1
13	R 5 day 14	Unc. Clostridiales	EU741667.1
14	R 6 day 14	Unc. Bacterium	FJ205864.1
15	R 8 day 1	*Clostridium* sp.	AB288660.1

Moreover 9 bands were considered for sequencing from the second batch fermentation (8.5–34 μM nickel). Thereof 5 bands corresponded to *Clostridium* sp., 2 bands to uncultured bacteria, one to uncultured Clostridiales and *Proteiniphilum acetatigenes* respectively and one band was identified as an uncultured Bacteroidetes (Table 3). *Proteiniphilum* are Gram-positive, anaerobic, non-spore forming bacteria. They utilize yeast extract, peptone, pyruvate, glycine and L-arginine as carbon and energy sources. Previously, representatives were isolated from a triculture degrading propionate to acetate and methane. They are symbiotic bacteria of the acetogenesis which is an important process to degrade the produced volatile fatty acids to acetate for the following methanogenesis (Chen and Dong, 2005). The corresponding SSCP gel band (B 12) showed a weak appearance on day 1 and became dominant at day 7 (Fig. 1b). Members of *Clostridium* sp. are important bacteria in hydrolysis. Corresponding bands showed different appearances over time (Fig. 1a and b). For example band 8 was dominant at the beginning and got weaker till day 21. The detected *Clostridium* sp. in band 10 seems constant over time. This could mean that individual representatives have a different substrate specificity ranging from polymers to smaller molecules.

In summary, manual analysis of the SSCP gels obtained over the duration of the batch fermentations was carried out. The presence of certain organisms deducted from the evolvement or diminishment of different bands nicely reflected their role in the microbial process. However, a clear influence of different nickel concentrations was not seen.

Additionally, a clustering of the band patterns from the first batch fermentation was done with GelCompare© (Applied Maths, version 4.2). The clustering method was Unweighted Pair Group Method with Arithmetic mean (UPGMA) and clustering correlation. With this method the program compares the position and the intensity of the bands.

The program generated three major clusters which show at least 87% similarity (Fig. 2). The first cluster consists of batch trials from day 1. These represent the community of the seeding sludge hence no significant difference can be seen in the band patterns. The second cluster represents all batch trials of day 7 inclusive of R 1 day 14. This means that there has been a change in the community within the first 7 d. The third cluster represents the batch fermentations of day 14 and day 21. They have a similarity of 95%. This indicates that no major change in the bacterial community appeared after day 14. The reason for this could be that the substrate is already consumed and the major community of hydrolyzing Clostridia is stable. R 6 day 21 and blank day 21 were not classified in a distinct cluster. Further no significant changes in bacterial abundance between different nickel concentrations could be investigated.

3.3. Eubacterial and methanogenic population dynamics investigation by ARDRA analysis

In addition to the SSCP analysis for the first anaerobic batch fermentation an ARDRA analysis from the trial with addition of 17 μM

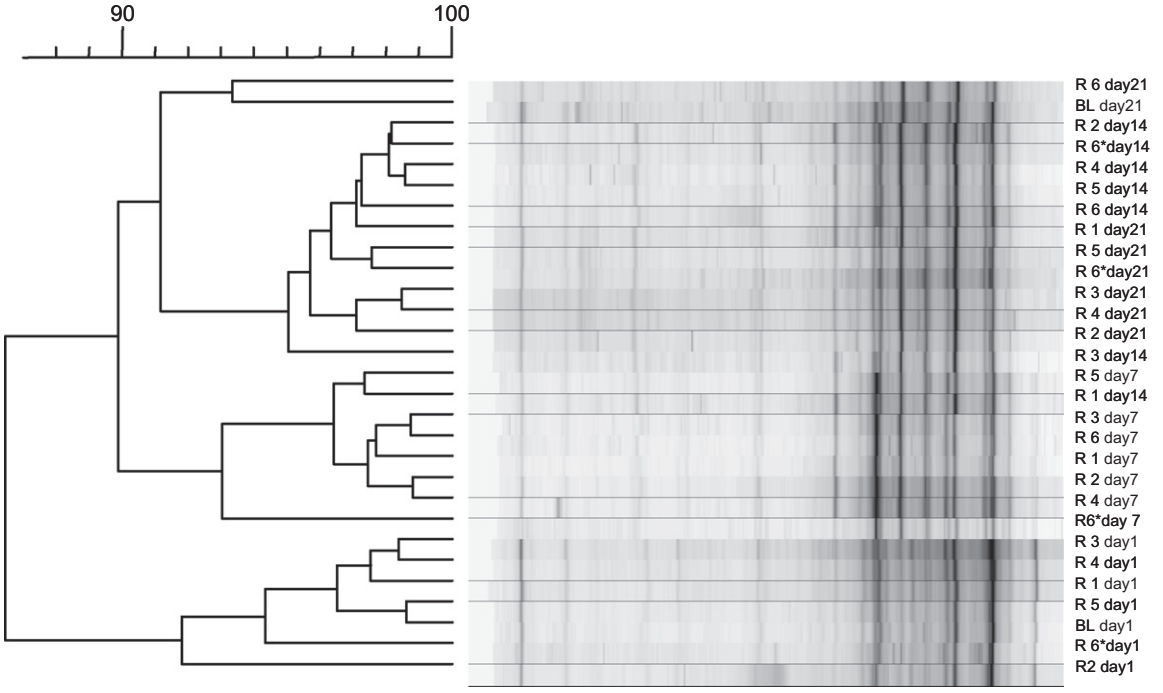

Fig. 2. Clustering of band patterns from the first batch anaerobic fermentation of a maize model substrate in the presence of nickel concentrations from 5 to 17 μM. The similarity of different clusters are indicated on the bar. BL means fermentations with inoculum sludge only, *R** are parallel reactor (R 6) with 17 μM nickel.

nickel was done from samples of day 1 and day 21. The community DNA was amplified with bacterial and archaeal primers, respectively. For each of the two sampling points 100 clones were used. For day 1 just 68 and for day 21 90 clones showed a positive result in the following PCR-amplification.

The results of the ARDRA analysis showed that nearly half (46%) of the bacterial population can be assigned to the phylum Firmicutes. 30% belongs to the class of Clostridia, 15% to the class of Bacilli. The phylum Bacteroidetes and so far uncultured bacteria appeared 25% each of the whole community. The remaining 4% were analyzed as Proteobacteria (Table 4).

The most abundant bacteria in the phylum Firmicutes were *Clostridium* sp. One species was found to be *Caloramator* sp. For example, the assigned *Caloramator* sp. ferments glucose and some other sugars to acetate, isobutyrate, isovalerate, valerate, lactate, and ethanol and is also able to degrade xylan but not cellulose (Collins et al., 1994). In the order Clostridiales the species *Butyrivibrio* sp. ferments glucose to formate, butyrate, lactate, succinate and ethanol and plays an important role in the protein degradation (Kopecny et al., 2003). Further a *Sedimentibacter* sp. was found besides three members of the Peptococcaceae namely *Desulfotomaculum*, *Pelotomaculum* and *Desulfosporosinus* sp. The also detected *Symbiobacterium* is a gram-negative bacterium which lives in symbiosis with a *Bacillus* strain. The bacterium was found to have its temperature optimum between 45 and 65 °C. However, it was also postulated that it could live in symbiosis with other bacteria than *Bacillus* at lower temperatures (Ohno et al., 2000; Ueda et al., 2004).

Among Bacilli there were found three different species. *Lactobacillus leuconostoc* produces acids from glucose, xylose, cellobiose and other compounds (Farrow et al., 1989). Representatives of the genus *Bacillus* and *Lysinibacillus* are Gram-positive, facultative anaerobic, endospore-forming bacteria. Two different species could be sequenced of the phylum Bacteroidetes. All other representatives were uncultured. Both species found, *Bacteroides nordii* and *Bacteroides acidifaciens*, belong to the order Bacteriales. *Bacteroides* are Gram-negative; obligate anaerobic, non-spore forming, rod-shaped bacteria. The major end products of glucose, cellobiose or xylose fermentation are acetic acid and in minor amounts isovaleric acid, propionic acid, and formic acid. Both species can be distinguished via the 16S rDNA (Miyamoto and Itoh, 2000; Song et al., 2004).

The phylum of Proteobacteria was represented by a beta- and an epsilon-proteobacterium. *Achromobacter* belonging to the order of Burkholderiales (beta-Proteobacteria). The epsilon-Proteobacterium was sequenced as *Arcobacter* which belongs to the order of Campylobacterales. This bacterium was already found in the SSCP analysis and appears in poultry manure.

It could be seen that different OTUs were sequenced as the same genus. This means that highly similar bacteria can appear as distinct OTUs. However, very low changes in the 16S rDNA can give a completely different band pattern. The ARDRA analysis was done additionally to the SSCP analysis. By means of this method it is possible to determine a statistical appearance of species (OTUs) in the eubacterial and archeal community, respectively. On the SSCP gels it could be seen that most of the sequenced dominant bands belonged to *Clostridium* sp. These results indicate that Clostridia are dominant in the whole population. A similar result was obtained with the ARDRA analysis. There Clostridiales and *Clostridium* sp. were also dominant in the bacterial population. The class of

Table 4
Characterisation of an anaerobic microbial population during degradation of a maize model substrate: Occurrence and specification of the different OTUs according to ARDRA analysis.

OTU	day 1 (%)	day 21 (%)	Phylum	Class	Order	Family	Genus	Species
1	19.1	11.1						Unc. Bacterium
2	2.9	1.1	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Butyrivibrio*	
3	5.9	15.6	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*	
4	2.9	4.4	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	
5	4.4	1.1	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium* sp.
6	0.0	2.2	Firmicutes	Clostridia	Clostridiales	Incertae sedis XI	*Sedimentibacter*	
7	1.5	2.2	Firmicutes					
8	8.8	4.4	Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	*Tannerella*	
9	1.5	5.6						Unc. Bacterium
10	0.0	1.1	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Bacillus* sp.
11	5.9	12.2	Unc. Bacteroidetes					
12	4.4	2.2						Unc. Bacterium
13	4.4	4.4	Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	*Bacteroides*	*Bacteroides acidifaciens*
14	0.0	2.2	Unc. Bacteroidetes					
15	7.4	2.2	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	
16	4.4	2.2	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae Peptococcaceae	*Pelospora* *Desulfotomaculum* *Pelotomaculum*	
17	0.0	1.1	Firmicutes	Clostridia	Clostridiales	Incertae sedis XVIII	*Symbiobacterium*	*Symbiobacterium* sp.
18	5.9	3.3	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Caloramator*	*Caloramator* sp.
19	0.0	1.1	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium* sp.
20	0.0	5.6	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	
21	0.0	5.6	Proteobacteria	Beta-Proteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*	
22	2.9	2.2	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	*Desulfotomaculum*	
23	2.9	1.1						Unc. Bacterium
24	2.9	2.2	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Lysinibacillus*	*Lysinibacillus fusiformis*
25	1.5	2.2	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium* sp.
26	2.9	0.0	Unc. Bacteroidetes					
27	1.5	0.0	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	*Desulfosporosinus*	
28	1.5	0.0	Proteobacteria	Epsilon-Proteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	*Arcobacter* sp

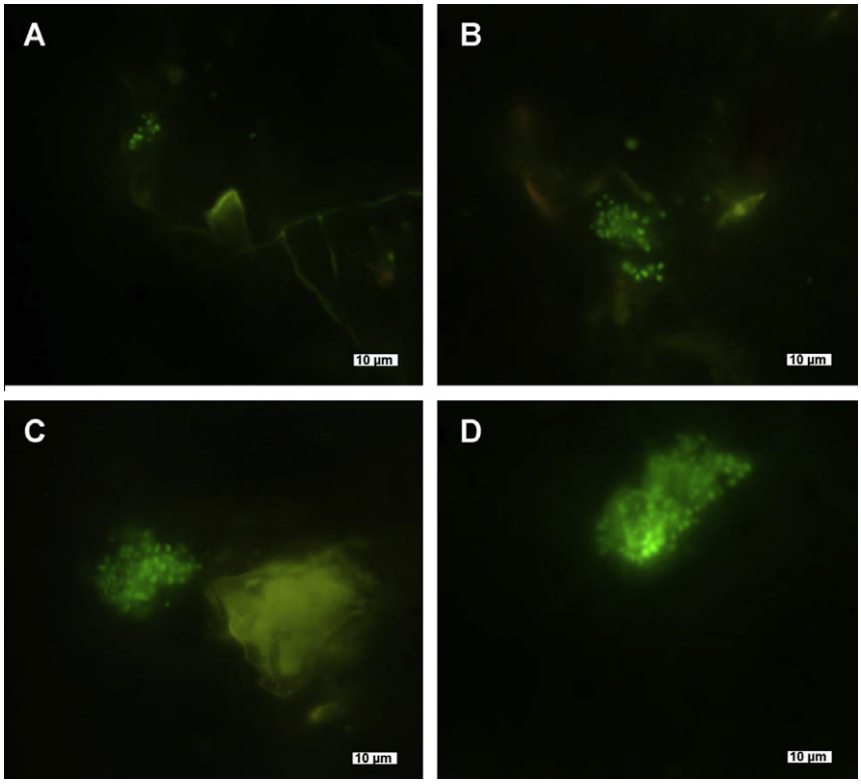

Fig. 3. Microscopic characterization of an anaerobic microbial population during degradation of maize model substrate. Fluorescence micrographs of community samples of the second batch fermentation. The samples were taken on after 21 d from the blank (A), dS (B), dS + TES (C) and dS + TES + 17 µM nickel (D). It can be seen that rod-shaped cells form clusters which got bigger and appeared more often with additional trace elements.

Clostridia represented one third of all sequenced bacteria. Compared to SSCP, employing ARDRA analysis a higher number of different species was found. The advantage of the ARDRA analysis is the detection of less dominant species due to statistical probability. Actually with SSCP weak bands on the gel are difficult to amplify for the following sequencing hence only dominant bands could be analyzed.

ARDRA for methanogenic Archaea displayed a dominance of *Methanoculleus* sp. Results from R 6 day 1 indicated two different band patterns on the agarose gel (data not shown). Both represented the species *Methanoculleus*. The same result was achieved with the samples of day 21. This species belongs to the order of Methanomicrobiales. Their major substrate is H_2–CO_2 and formate to produce methane (Maestrojuan et al., 1990). Furthermore in previous studies (Krause et al., 2008; Schlueter et al., 2008) it could be seen that in reactors fed with primary maize silage and low amounts of chicken manure *Methanoculleus* was the dominant species of the archaeal community while Clostridiales were dominant in the eubacterial community.

Here we found the same organisms when using a defined soluble model substrate for maize silage indicating the value for mechanistic investigations of anaerobic digestion of maize silage.

3.4. Methanogenic activity measurement using fluorescent microscopy

Fluorescence microscopic analyses based on factor F_{420} from samples taken after 21 d mainly displayed irregular coccoid cells (Fig. 3). Maestrojuan et al. (1990) described in their study that *Methanoculleus* sp. are irregular coccoids and cells are 1–2 µm in diameter and occur singly or in pairs. Furthermore some cells appear to have flagella, but motility has not been observed. This is in accordance to the dominant species *Methanoculleus* detected in ARDRA analyses. Further clusters of these cells were seen which appeared larger in size in the presence of trace elements (Fig. 3C). Moreover Fig. 3D demonstrates a considerable increase in size and appearance of cell clusters in batch trials with an extra addition of 17 µM nickel which correlates to an increase of methane production and conversion of VFA's in batch trials (Tables 1 and 2). These results indicate in the presence of nickel the methanogenic bacteria grow faster and build more biomass. As described by Friedman et al. (1990) all methanogenic pathways converge to the enzymatic reduction of methyl-coenzyme M to methane. This reduction is catalyzed by the Methyl-coenzyme M reductase complex, which includes a nickel containing cofactor called F_{430}.

4. Conclusion

In this study an increase of biogas yields in batch fermentations upon addition of trace elements and extra nickel was demonstrated for a cellulose and starch based model substrate. Molecular biological techniques were used to characterize microbial communities during anaerobic digestion of a maize model substrate. Using this tool, it was shown that the developed novel synthetic model substrate was able to mimic maize silage since a similar microbial population was observed (e.g. *Methanoculleus* most prominent methanogen). SSCP analysis, ARDRA and fluorescent microscopy results were in agreement regarding the most prominent species found. It was also shown, that certain species such as *Arcobacter* sp. from the inoculum are significantly reduced over the duration of the batch fermentation. Future experiments should involve continuous reactors where the influence of trace elements on the microbial community should be easier to analyze first due to longer retention times and secondly due to the possibility to dilute out background concentrations of trace elements resulting from the inoculum.

Acknowledgement

The authors gratefully acknowledge the Schaumann BioEnergy GmbH Austria for the financial support and the constructive cooperation.

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