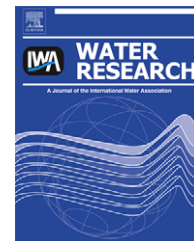


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Enhancement of biogas production by addition of hemicellulolytic bacteria immobilised on activated zeolite

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ARTICLE INFO

Article history:

Received 6 August 2009

Received in revised form

25 November 2009

Accepted 26 November 2009

Available online xxx

Keywords:

Biogas

Zeolite

Xylanase

Hemicellulose degradation

Community analysis

ABSTRACT

Biogas from agricultural biomass and residues is a valuable source of renewable energy. However, recalcitrant plant cell structures represent a barrier in the fermentative biodegradation process in single- and two-stage reactors. Therefore, approaches concerning a more efficient de-polymerisation of cellulose and hemicellulose to monomeric sugars are required amongst others in order to optimise the fermentation efficiency and to increase methane yields. Here we show a new strategy for the enhancement of biogas production from hemicellulose-rich substrates. Hemicellulolytic populations from a common biogas fermenter consortium were successively enriched in batch-cultures using a synthetic medium containing xylan powder as single carbon source under anaerobic mesophilic conditions. Enriched hemicellulolytic bacteria were immobilised on trace metal activated zeolite to ensure a stable storage and easy application. Xylanase activity increased continuously during subsequent enrichment cycles by up to 162%. In batch-culture experiments we were able to observe an increase of methane by 53% compared to controls without additionally introduced microorganisms immobilised on zeolite. Specific enrichment of hemicellulolytic bacteria during the process was confirmed by using single strand conformation polymorphism (SSCP) analysis based on amplification of the eubacterial 16S rDNA fragments. Using sequence analysis conspicuous bands from SSCP patterns could be identified as belonging to the groups *Bacteroides* sp., *Azospira oryzae* (*Dechlorosoma* sp.) as well as to a wide spectrum of diverse species within the order of Clostridiales (Firmicutes).

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1. Introduction

Previous studies have shown the assessment of various substrates ranging from organic household waste to more defined lignocellulosic substrates for biogas production (Held et al., 2002; Staubmann et al., 1997). Naturally occurring lignocellulosic plant biomass consists of 20–30% hemicellulosic materials which are heterogeneous polysaccharides

with xylan as major constituent found in association with cellulose. Xylans are heteropolysaccharides with a homopolymeric backbone chain of β -1,4-linked D-xylopyranose units. Common substituents found on the backbone are O-acetyl, α -L-arabinofuranosyl, α -1,2-linked glucuronic or 4-O-methylglucuronic acid. Especially O-acetyl groups at the positions C-2 and C-3 of xylosyl residues hinder xylanases from completely degrading acetyl xylan (Kulkarni et al., 1999). Therefore,

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¹ In memory of Remo Meincke (13 July 1978 to 1 October 2008), a keen and utmost promising scientist and adorable friend.

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doi:10.1016/j.watres.2009.11.048

a synergistic activity of several enzymes such as endo-1,4- β -xylanase, β -xylosidase, α -glucuronidase, α -L-arabinofuranosidase and acetyltransferase is essential for the complete breakdown of branched xylans. A universal obstacle for the anaerobic digestion of plant biomass is the structural heterogeneity and complexity of cell-wall constituents such as microfibrils and matrix polymers (Iiyama et al., 1994). At a molecular level, the crystalline cellulose core of cell-wall microfibrils is composed of precisely arranged cellodextrins with strong interchain hydrogen bonding between adjacent chains. The hydrophobic face provokes crystalline cellulose to be highly resistant to enzymatic hydrolysis (Matthews et al., 2006; Nishiyama et al., 2002). Additionally, hemicellulose restricts the access to crystalline cellulose cores of microfibrils by coating them (Ding and Himmel, 2006). Since it is known that the removal of hemicellulose increases the mean pore size of cell-wall structures which in turn enhances the enzymatic hydrolysis of cellulose in plant biomass substrates (Gretlein, 1985), we focused on hemicellulolytic bacteria producing xylan-degrading enzymes in this study.

Whereas mechanical and chemical pre-treatment methods are well studied and commonly used in practice to increase methane yields from recalcitrant biomass, little is known about the prospects of a biological pre-treatment for anaerobic digestion processes (Hendriks and Zeeman, 2009). However, enzymatic pre-treatment is quite expensive and demands strict control of reaction conditions (Zhang and Lynd, 2004), but the use of vital microorganisms is probably more dynamic and efficient due to their ability of regeneration and concomitant production of diverse enzymes responding to the given substrate. Bagi et al. (2007) demonstrated an increase of biogas formation by about 60–70% due to inoculation of biogas reactors up to 5 m³ size with external hydrogen-producing bacteria with cellulolytic activity (e.g. *Caldicellulosyruptor saccharolyticus*) in long-term experiments. In the present study, inocula of mixed bacteria were obtained from natural occurring consortia present in biogas plant second-stage sludge in order to enhance the hydrolytic activity by re-introduction of selectively enriched xylanolytic bacteria to the fermentation process. Expectable hydrolytic species resulting from the cultivation on xylan as mono-substrate belong to the genera *Clostridium*, *Bacillus*, *Bacteroides* and *Pseudomonas* sp. (Klocke et al., 2007). The use of second-stage sludge as inoculum for the fermentation of organic materials and cultivation of specific bacterial fractions delivers several major advantages compared to artificial inocula (Friedmann et al., 2004), that is: (i) providing a bioecosis accommodated to several organic substrates, (ii) vital bacteria cells held under starvation, responding sensitively to newly introduced substrates by metabolic adjustments and specific enzyme expression.

Zeolites are aluminosilicates with well-defined crystalline structures that contain aluminium, silicon and oxygen in their regulatory framework. The nanoporous crystal structure retains cations, water and has shown a great capacity for ammonia nitrogen (NH₄-N) and heavy metal adsorption (e.g. Cu, Cd, Pb and Zn), thus removing molecules toxic to microorganisms in anaerobic and aerobic digestion processes (Tada et al., 2005; Green et al., 1996). Moreover, zeolites can be purposefully modified by loading trace metal elements

(Fe, Mg, Ni and Co) in variable concentrations on their surface which favours methanogenic and acidogenic bacteria to be grouped in small micro-colonies, supplying co-factors for enzyme biosynthesis (Fernández et al., 2007). Consequently, zeolites are used for the immobilisation of microorganisms in anaerobic reactors to stabilise and optimise the process efficiency (Milán et al., 2003). Here we describe how immobilising hemicellulolytic bacteria populations on zeolite leads to an increase of methane yields in batch-culture experiments.

2. Materials and methods

2.1. Cultivation and enrichment

As inoculum for the enrichment of hemicellulolytic microorganisms, second-stage sludge from a biogas plant (Fürstenfeld, Austria) primarily digesting maize silage was chosen. The sludge was composed of 2.31% dry matter (DM), 1.7% organic dry matter (ODM), and the ODM fraction of DM (ODM/DM) was 73.64%. Cultivation was carried out using a minimal medium for the determination of total anaerobic degradability of organic compounds in wastewater and digestion slurry (DIN EN ISO 11734 L47, 1998) containing the following compounds in g l⁻¹: CaCl₂·2H₂O, 0.075; FeCl₂·4H₂O, 0.02; KH₂PO₄, 0.27; MgCl₂·6H₂O, 0.1; Na₂HPO₄·2H₂O, 0.56; Na₂S·1H₂O, 0.04; NH₄Cl, 0.53; resazurin, 0.001. After autoclaving, 10 ml of a mineral base (DIN EN ISO 11734, 1998) with the following composition in g l⁻¹ was added: CuCl₂, 0.003; H₃BO₃, 0.005; MnCl₂·4H₂O, 0.05; Na₂MoO₄·2H₂O, 0.001; ZnCl₂, 0.005. Before use, the medium was flushed with oxygen-free nitrogen gas for 20 min l⁻¹ to obtain anaerobic conditions. The cultivation experiment was carried out under mesophilic conditions at 35 °C in 1000 ml ground flasks with a maximum loading volume of 800 ml wherein 10% inoculum (v/v) was introduced. Insoluble powder of xylan from birchwood (Roth, Karlsruhe, Germany) was then added in a concentration of 0.1% (w/v) as sole carbon source. As blank pure second-stage sludge without any addition of xylan was used to estimate enzymatic auto-activity. To establish steady anaerobic conditions, an anaerobic system was used, following Louis Pasteur's bottle en col de cygne (around 1862), which allows gases to pass off whilst external air is prevented from entering via a water seal. After 5 days of cultivation 10% (v/v) were transferred into fresh L47 medium. These cycles of cultivation and re-inoculation were repeated for 13 times. Analysis data were obtained from the average of triplicate experiments. Samples were collected at 3–5-day intervals.

2.2. Immobilisation

For immobilisation of enriched xylanolytic bacteria, the zeolite product IPUS meth-max[®] was used and kindly provided by IPUS GmbH (Rottenmann, Austria). It consists of a natural zeolitic tuff containing >85% clinoptilolite, which was milled to a grain size below 100 μ m. Loaded with several trace metal elements in variable concentrations it is able to enhance microbial activity (Holper et al., 2005) and was therefore used as operational environment for xylanolytic

bacteria. Subsequent to the cultivation in L47 medium, vital cells were collected by centrifugation for 10 min at $16,000 \times g$ for cell mass determination. Ten percent of dry cell mass was loaded on 5 g of zeolite by resuspension in 100 ml of L47 medium (thoroughly mixed) followed by another centrifugation step. Thereafter immobilised material was air dried for 2 days at room temperature.

2.3. Batch-culture

Laboratory batch experiments were carried out following the guidelines for “Fermentation of organic materials” (Friedmann et al., 2004) and modified according to DIN DEV 38414 S8 (1985). Ground flasks (1000 ml) were used as reaction vessels with a total volume of 600 ml. The fermentation mixture contained 30% (v/v) of seeding sludge from an anaerobic treatment plant (origin and characteristics as described above) adjusted to 1.5% ODM with deionised H_2O and 0.7% (w/v) of insoluble powder of xylan from birchwood as substrate. Then 0.2% (w/v) of immobilised material was added introducing 0.02% DM of xylanolytic bacteria to the total volume. Controls were charged with 0.2% (w/v) of pure activated zeolite. To estimate the auto-activity of pure seeding sludge, blanks had no addition of substrate or zeolite. Resulting methane yields were used for blank subtraction. Before starting the incubation under mesophilic conditions at $35^\circ C$, the flasks were flushed with oxygen-free nitrogen gas for 20 min. To remove CO_2 , generated biogas was washed with 2 M NaOH solution. NH_4^+ and H_2S were eliminated using an acidic solution containing $Na_2SO_4 \cdot 10 H_2O$, $200 g l^{-1}$ and 98% H_2SO_4 , $30 ml l^{-1}$. However, trace gases cause an error ratio of round about 1%. The parameter of measurement is the amount of CH_4 in litres per kilogram of ODM at standard ambient temperature and pressure (STP), calculated from displaced acidic solution volumes following VDI 4630 (Friedmann et al., 2004). Analysis data were obtained from the average of triplicate samples collected daily over a total fermentation period of 34 days whereupon methane production was measured cumulatively.

2.4. Analytical methods

2.4.1. Enzyme activity assay

Xylanase activity was measured using a 3-amino,5-nitrosalicylic acid (DNS) assay which is based on the quantification of reducing sugars released with DNS as described previously by Bailey et al. (1992). The DNS reagent contained (in $g l^{-1}$): 3,5-dinitrosalicylic acid ($C_7H_4N_2O_7$), 7.48; NaOH, 13.98; sodium potassium tartrate ($C_4H_4KNaO_6 \cdot 4H_2O$), 216.1; phenol (C_6H_6O), 5.155; sodium metabisulfite ($Na_2O_5S_2$), 5.86. D-(+)-Xylose standards in $mg ml^{-1}$: 2.5, 1.25, 0.625, 0.313, 0.156. Composition of samples and sample blanks in μl : 1% xylan solution, 180; sample, 20; DNS solution, 300. Before adding the DNS solution, samples were incubated at $35^\circ C$ for 30 min. The photometric absorption was determined at 540 nm in 96-well plates (Greiner, Frickenhausen, Germany) using a plate reader (Tecan Infinite 200M, Männedorf, Switzerland). Xylanase activity is stated as units of enzyme activity per litre ($U l^{-1}$). An enzyme unit is defined as the conversion of $1 \mu mol$

D-(+)-xylose per minute under the given conditions above. One unit corresponds to 16.67 nanokatal.

2.4.2. Cell counting

Bacterial growth was estimated by microscopic determination of cell numbers using a counting chamber (Neubauer improved: $16 \times 0.0025 mm^2$, 0.01 mm depth; LO Laboroptik, Friedrichsdorf, Germany). Cell counts were determined fourfold from 8 squares for each sample and sample dilution. A pre-treatment step was necessary to separate cell clusters and cells from other organic and inorganic particles: Samples were washed in 1 ml phosphate buffered saline (PBS, pH 7.0) twice and centrifuged for 5 min at $16,750 \times g$. The pellet was resuspended in 1 ml PBS and then treated in an ultrasonic bath for 10 min at 100% intensity (Elma Transsonic Digital S, Schalltec, Mörfelden-Walldorf, Germany).

2.4.3. High performance liquid chromatography (HPLC) analysis

An HP 1100 HPLC system (Hewlett Packard, Palo Alto, California) equipped with an ICsep ION-300 column ($7.8 \times 300 mm$) (Transgenomic, Omaha, USA) and an ICsep GC-801/C guard cartridge ($4 \times 20 mm$) (Transgenomic, Omaha, USA) was used for quantification of organic acids. Sulphuric acid (5 mM) was used as mobile phase. Sample injection volumes were $40 \mu l$; flow rate of $0.5 ml min^{-1}$; at a temperature of $42^\circ C$. Compounds were detected with an HP 1047A refractive index detector (Hewlett Packard, Palo Alto, California). Standards of acetic acid, butyric acid, citric acid, isobutyric acid, isovaleric acid, lactic acid, propionic acid and valeric acid were injected for identification. Samples were prepared as follows. Carrez protein precipitation was carried out using $K_4[Fe(CN)_6] \cdot 3H_2O$ and $ZnSO_4 \cdot 3H_2O$ as previously described by Carrez (1912). Subsequently, samples were diluted with double deionised H_2O to a final dilution of 1:40.

2.5. PCR-based community analysis

2.5.1. 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 $16,750 \times g$. 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 $2.00 \mu m$ in diameter were added to crush cell structures using FastPrep Instrument (Qbiogene, Heidelberg, Germany) for $2 \times 30 s$ including cooling steps on ice in between for 2 min. After centrifugation for 1 min at $16,750 \times g$, the supernatant was mixed with 5 M sodium acetate, $100 \mu l$ and incubated on ice for 15 min for protein precipitation. Following 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 isopropanol 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 μ l of 10 mM Tris-HCl buffer (pH 8.0).

2.5.2. 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') according to Lieber et al. (2002), 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 MgCl₂ (Finnzyme, Espoo, Finland); 0.5 mM forward primer; 0.5 mM reverse primer; 20 ng template DNA (1 μ l) and double deionised H₂O to fill up to a final volume of 20 μ l for template DNA extracted from SSCP gels and 60 μ l for template DNA extracted from second-stage sludge and L47 culture samples respectively. Negative control PCR contained no template DNA. The cycling conditions 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, (6) hold at 4 °C. Steps (2) to (4) were repeated 35 times. For the purification of PCR generated DNA products, Gene Clean Turbo Kit (Qbiogene, Heidelberg, Germany) was used following the manufacturer's recommendations.

2.5.3. Single strand conformation polymorphism (SSCP) analysis

SSCP analysis of amplified bacterial 16S rRNA gene fragments was carried out according to Schwieger and Tebbe (1998). For single strand formation 10 μ l of purified PCR products, adjusted to 50 ng dsDNA content were used. Exonuclease digestion was performed with λ -exonuclease, 12 U (New England Biolabs, Frankfurt, Germany) at 37 °C for 1 h, followed by 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 \times Tris-borate-EDTA buffer (TBE) at 26 °C for 26 h 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, constantly dominant or variably emerging SSCP bands were excised and sequenced. DNA was eluted from gel slices through incubation in sterile elution buffer (5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), pH 8.0) at 37 °C for 5 h following Sambrook et al. (1989). Afterwards, eluted DNA was re-amplified using the same primer pairs as for the SSCP analysis as described above.

2.5.4. Computer-assisted cluster analysis

In order to compare SSCP fingerprints of microbial communities, a computer-assisted cluster analysis was carried out using the GelCompar[®] 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[®] 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 according to Dice (1945). 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 according to Kropf et al. (2004).

2.5.5. 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 (Altschul et al., 1997; <http://www.ncbi.nlm.nih.gov/blast/>).

3. Results and discussion

3.1. Cultivation and enrichment

In a first stage, the effect of xylan as single carbon source on xylanase production by anaerobic populations was studied. As shown in Fig. 1, xylanase activity in cultures containing xylan was significantly higher ($P < 0.05$) at day 3 to day 6 and day 10 of cultivation compared to blanks. A maximum activity of 335 U l⁻¹ in the mean was measured around day 5 representing a rise of 260 U l⁻¹ compared to blanks (following Fig. 1, standard deviations are indicated as error bars). The subsequent decrease of xylanase activity could be due to lower activity of xylanolytic organisms because of a complete consumption of all xylan followed by proteolytic degradation of xylanases. It is known that anaerobic cellulolytic species (e.g. those of the genera *Fibrobacter* and *Clostridium*) are limited in their carbohydrate range, growing well on cellulose and its hydrolytic products but often not on corresponding mono-, oligo- and polysaccharides (Lynd et al., 2002). Furthermore, increasing xylanase activities were accompanied by decreasing pH values, starting with pH 7.00 \pm 0.01 (adjusted through L47 medium) at day 1, decreasing to a minimum pH of 4.95 on average with a standard deviation of 0.07 at day 4 (data not shown). At day 5, where a maximum xylanase activity was observed, a pH of 5.03 \pm 0.08 appeared. Optimum pH conditions for xylanases have been previously reported to be at a pH of 5.2 for example for *Clostridium acetobutylicum* (Lee et al., 1985). In general, xylanases from different organisms show stabilities over a wide pH range with optima between pH 4 and 7 (Kulkarni et al., 1999).

Characteristics of enzymatic activity levels related to bacterial growth determined by cell counts simultaneously during the cultivation in minimal medium are also shown in Fig. 1. Focusing on day 3 of cultivation (2.0 ± 0.2) $\times 10^8$ ml⁻¹ cell counts were observable. Followed by an exponential growth phase, increasing cell counts were accompanied by an increase of pH values and decreasing xylanase activities. At day 7 a pH of 5.40 \pm 0.20 on average and (5.6 ± 1.3) $\times 10^8$ ml⁻¹ cell counts were observed. Both parameters increased from

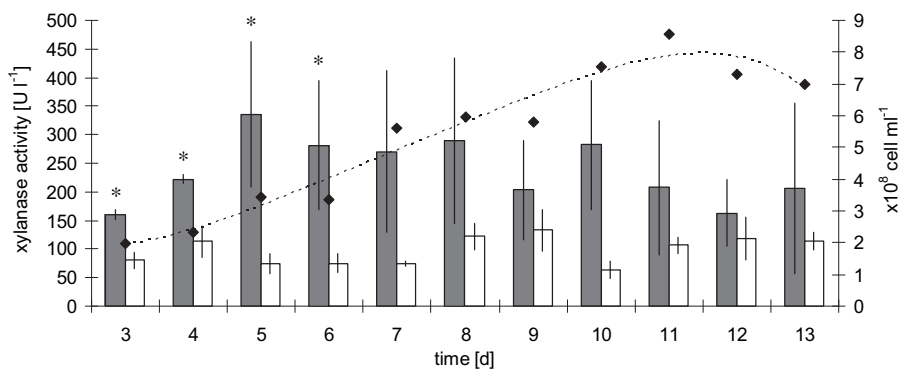


Fig. 1 – Xylanase activity determined during cultivation of an anaerobic populations in L47 medium on xylan as mono-substrate (grey columns) and in second-stage sludge without xylan as blank (white columns) over 13 days. Significance differences are defined as $P < 0.05$ and marked with an asterisk in the diagram. Cell counts ml^{-1} (line) represent the bacterial growth during a single cultivation cycle over 13 days in L47 medium on xylan.

this point on, reaching a pH value of 6.39 ± 0.09 and a maximum cell number of $(8.6 \pm 1.5) \times 10^8 \text{ ml}^{-1}$ at day 11, representing the end of the exponential growth phase. These observations for xylanolytic bacteria, where pH values were not controlled, match with former findings concerning the degradation of cellulose. Cellulolytic bacteria are known not to grow at pH below 6.0, but at the same time cellulose removal in some anaerobic mixed cultures is observed at pH as low as 4.5, having an optimum around pH 5 (Chyi and Dague, 1994). Most fermentative microbes grow within a fairly narrow pH range, but in some habitats pH fluctuations permit cellulose degradation to occur at pH values below those actually supporting bacterial growth (Lynd et al., 2002). Ruminant bacteria for example, once adhered to cellulose, having synthesised a glycocalyx, pH drops below 6.0 where substantial cellulose hydrolysis occurs, but growth has already stopped (Mourino et al., 2001). However, as maximum enzymatic activities were measured around day 5 during the exponential bacterial growth phase, cultures at days 4–6 were then considered as inoculum for subsequent cultivation steps depending on actual enzyme activity levels.

With respect to increasing bacteria cell counts resulting from co-cultivated non-xylanolytic populations, xylanase activity of successive enrichment cycles was related to total cell numbers to obtain a factor specific for xylanolytic populations. Cellulose and hemicellulose degradation in anaerobic processes have been primarily ascribed to the activity of multi-enzyme complexes, namely cellulosomes and xylanosomes, as well as to extracellular enzymes such as endoglucanase (carboxymethylcellulase), cellulase, xylanase and aryl- β -xylosidase produced constitutively (Schwarz, 2002; Thomson, 1993). Thus, a higher specific xylanase activity in culture liquids should indicate an enrichment of hemicellulolytic organisms within the mixed microbial community. However, this specific xylanase activity of successive cultivation cycles increased significantly until the fourth cultivation cycle and varied later with only a slight increase up to the thirteenth cultivation cycle as can be seen in Fig. 2.

Corresponding to the second (II), fourth (III) and thirteenth (IV) cultivation cycle, the following maximum values were stated at day 5 respectively: $495 \pm 150 \text{ U l}^{-1}$ to $(3.8 \pm 0.2) \times 10^8$, $509 \pm 18 \text{ U l}^{-1}$ to $(8.6 \pm 1.7) \times 10^8$ and $544 \pm 56 \text{ U l}^{-1}$ to $(1.4 \pm 0.4) \times 10^8 \text{ cell counts ml}^{-1}$. Compared to the values

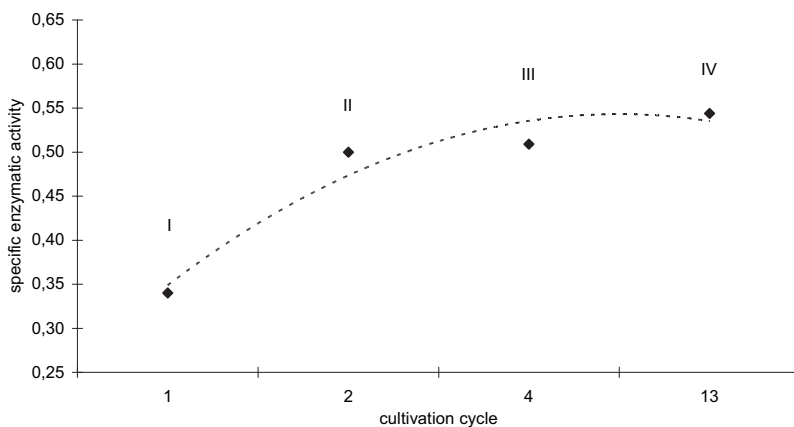


Fig. 2 – Enzymatic activity of xylanase (U l^{-1}) related to the bacterial growth ($\text{cell counts ml}^{-1}$) generates a community specific enzyme activity shown for the first (I), second (II), fourth (III) and thirteenth (IV) cultivation cycle in L47 medium on xylan as mono-substrate. Data were obtained at day 5 in respect to maximum enzymatic activity levels within exponential growth rate periods of each cultivation cycle.

obtained from the first cultivation cycle, a maximum increase of +60% in xylanase activity was measured after 13 cultivation cycles due to high enzymatic activity corresponding to relatively low cell counts at a pH of 4.53 ± 0.02 , while a value of 50% was almost reached after the fourth enrichment cycle at a pH of 5.72 ± 0.09 showing mildly lower enzymatic activity (-35 U l^{-1} on average) related to even more cells counted ($+7.2 \cdot 10^8$ on average) compared to cultivation cycle 13.

Overall, the increase in specific xylanase activity in successive enrichment cycles was limited and indicates that the anaerobic community from maize silage digestion was already well adapted to xylan as substrate, since whole maize plants show high contents of cellulose and hemicelluloses of about 29% and 32% on average respectively (Amon et al., 2003). Also obligate syntrophic relationships between hemicellulolytic bacteria and acidogenic or acetogenic bacteria in further converting mono- and oligomeric metabolites (e.g. xylose, xylobiose) might contribute to this effect by affecting the measurement of reducing sugars. In general particular enrichment of xylanolytic bacteria in synthetic medium L47 on xylan as carbon source was successful and is in agreement with previous reports (Poutanen et al., 1987; Lee et al., 1985). Even though *Bacillus* sp. strains show comparatively higher xylanase activities in pure cultures (Subramaniyan and Prema, 2002), enzymatic activities in this study represent a cumulative effect derived from different species contributing to a mixed culture, where, for example, interspecific stress must be considered. On the other hand, selective enrichment of certain species from naturally balanced communities, for example, biogas-producing community, entails the risk of interfering vital syntrophic relationships of hydrolytic, acidogenic, acetogenic and methanogenic partners (Schink, 2006). Moreover, enrichment in continuous batch or fed-batch reactors under controlled pH conditions might lead to even more specialised populations. However, as the specific xylanase activity has reached three paramount maxima (Fig. 2: II, III, IV), SSCP and sequencing analysis was carried out to identify responsible species.

3.2. Community profile

The identification of changes within bacterial community organisation during the cultivation on xylan as mono-substrate was achieved by SSCP analysis using 16S rDNA as bacteria-specific target for the PCR amplification. Fig. 3 shows an exemplary SSCP gel from samples of the cultivation in L47 medium compared to the bacterial community naturally occurring in second-stage sludge of a biogas plant mainly operated with mono-maize silage revealing distinguishable band patterns.

In summary, 29 bands for the total bacterial community in second-stage sludge were detected. In view of the total bacterial community in L47 medium at the third and fourth enrichment cycle, 19–24 bands with variations in dominance appeared. Boxes a, b and c demonstrate intensifications of specific band patterns of certain replicates, whereas boxes d and e reveal a weakening over the periods of cultivation. Enforcing a certain catabolic pathway constitutes a breach in syntrophic association of methane-producing bacteria and

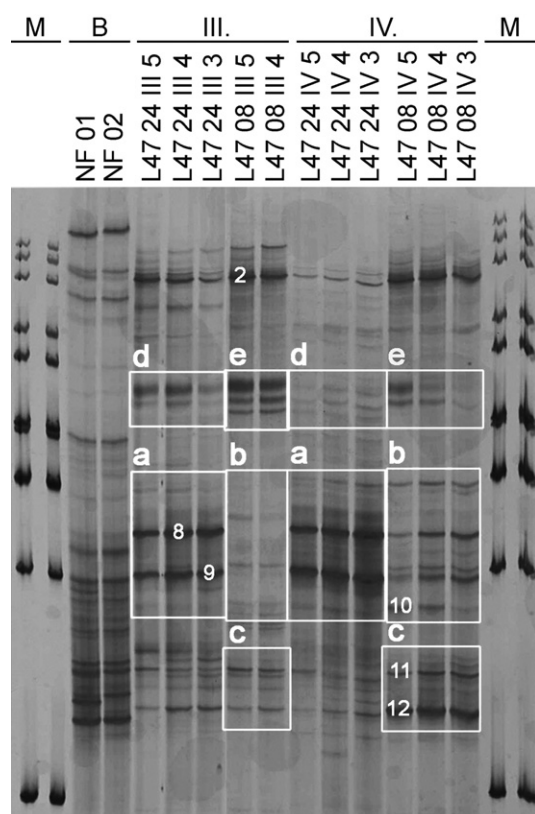


Fig. 3 – Exemplary non-denaturing polyacrylamide gel from the SSCP analysis based on 16S rDNA fragments focusing on the bacterial community cultured in L47 medium and from second-stage sludge: M, 1 kb DNA-ladder marker lanes; B, the blank, that is, pure second-stage sludge (NF 01/02); III and IV, corresponding cultivation periods in L47 medium on xylan as mono-substrate. Sample code: L47, medium; 08/24, replicate number; III/IV, cultivation cycle; 3/4/5, day of current cultivation. Boxes a, b and c show the intensification, boxes d and e the weakening of specific band patterns indicating shifts in bacterial community organisation during the cultivation in L47 medium. Boxes 2, 8, 9, 10, 11 and 12 represent specific bands selected for DNA extraction and sequencing analysis.

can therefore be assumed as causal reason for the vanishing of species.

Hence cultivation-dependent formation of specific band patterns indicates shifts within bacterial community structure; SSCP patterns were taken as operational taxonomic units (OTU). The cultivation in synthetic medium led to OTUs distinguishable from those found in second-stage sludge. To analyse the shifts in bacterial community composition, Pearson's correlation coefficients were compared by UPGMA. As shown in Fig. 4 all cultivation cycles in L47 medium analysed corporately formed one cluster significantly discriminatory ($P < 0.05$) from another one formed by pure second-stage sludge samples (blank) corroborating an assumed shift, potentially towards a distinctive enriched hydrolytic bacterial community. The corresponding similarity

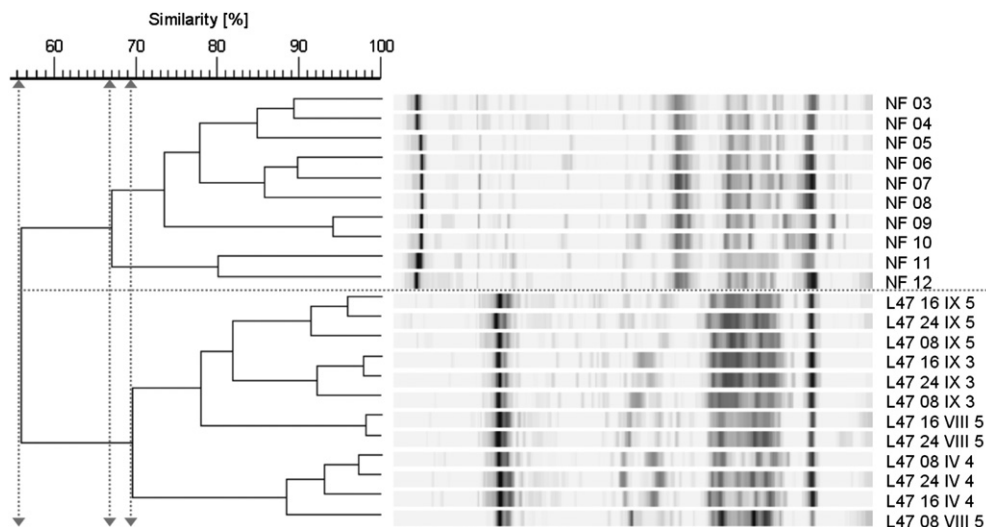


Fig. 4 – Dendrogram based on amplified 16S rDNA fragments of bacterial cultures in L47 medium and pure second-stage sludge (NF 03-12) respectively obtained by using eubacterial primers and separated by SSCP. Band patterns were grouped by UPGMA. Double-headed vertical arrows indicate the similarity for the groupings. Sample code: L47, medium; 08/16/24, replicate number; IV/VIII/IX, cultivation cycle; 3/4/5, day of cultivation and sample collection.

between OTUs of blank samples (NF 03 to NF 12) and the OTUs that have arisen from the cultivation on xylan (cycle 4, 8 and 9) was under 57%, whereas similarities between OTUs within one cluster were up to 67% for pure second-stage sludge samples and >69% for samples from the cultivation on xylan in L47 medium respectively.

Furthermore, a change of the eubacterial community structure was observed during the cultivation in L47 medium. Derived patterns differentiate significantly ($P < 0.05$) from cultivation cycle 3 to 4, forming separated OTUs at a similarity of <67–68% depending on triplicates observed. Triplicates participating in cultivation cycle 3 form one distinct OTU at a similarity of >74% as it can be seen in Fig. 5. Thus, increasing xylanase activity in successive enrichment cycles correlated with the observation of correspondingly differentiated community profiles.

Sequence analysis of DNA extracted from single bands representing specific species was then used as a holistic approach for further community characterisation (Schwieger and Tebbe, 1998). Sequence analyses of bands exposed in shown in Fig. 3 reveal three affiliations within the phylum Bacteroidetes (i.e. *Bacteroides* sp.) and one with the class Betaproteobacteria (i.e. *Azospira oryzae*), each with an identity value of 98%. *Bacteroides* sp. are well known plant cell structure degraders, while, for example, *B. succinogenes* together with *B. ruminicola* are among the most important cellulolytic bacteria in the rumen environment with endoglucanase (carboxymethylcellulase), cellulase, xylanase and aryl- β -xylosidase activities (Forsberg et al., 1981). Heylen et al. (2006) isolated species from the genus *Azospira* sp. from activated sludge samples derived from municipal wastewater treatment plants, characterising a denitrifying potential.

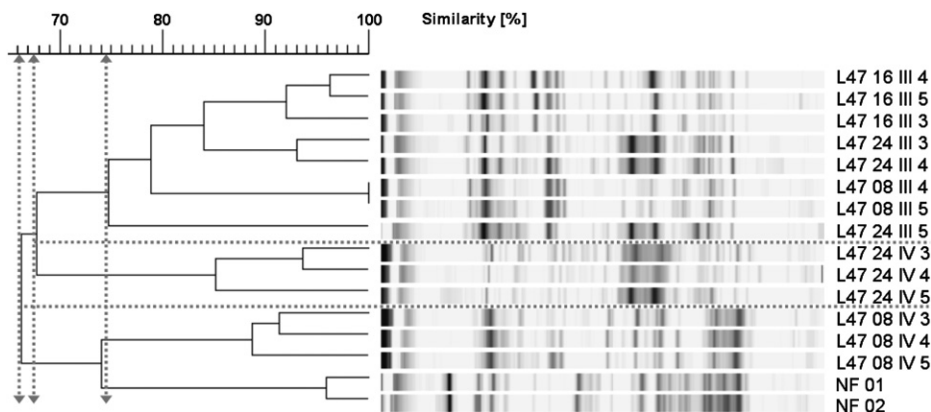


Fig. 5 – Dendrogram based on amplified 16S rDNA fragments of bacterial cultures in L47 medium and pure second-stage sludge (NF 01/02) respectively obtained by using eubacterial primers and separated by SSCP. Band patterns were grouped by UPGMA. Double-headed vertical arrows indicate the similarity for the groupings. Sample code: L47, medium; 08/16/24, replicate number; III/IV, cultivation cycle; 3/4/5, day of cultivation and sample collection.

Since ammonia has potential inhibitory effects on the anaerobic-digestion microbial consortia (Heinrichs et al., 1990; Sprott and Patel, 1986), for example, inhibition of methanogenic growth and acetate uptake (Poggi-Varaldo et al., 1991), the elimination of dissolved nitrogen by members of the microbial community would also have a positive effect on the production of biogas.

DNA extraction from bands represented in Fig. 3, that is, 11 and 12 reveal a wide spectrum of diverse species related to the phylum Firmicutes with a close relationship to different members of the order Clostridiales, that is, *Clostridium beijerinckii*, *C. butyricum*, *C. chromoreductans*, *C. corinoformum*, *C. diolis*, *C. favosporum*, *C. puniceum*, *C. roseum*, *C. saccaroperbutylacetonicum* with identity values of 97–98%. *Clostridium beijerinckii*, for example, is an exceptional bacterium for studies on crucial hemicellulose and cellulose degradation (Schwarz, 2002). Besides having a major hydrolysing activity, Clostridia are distinct hydrogen-producing bacteria, especially *C. butyricum* and *C. diolis* have shown specifically high hydrogenase activities in activated sludge samples (Wang et al., 2007). Although biogas formation only takes place below a certain threshold hydrogen concentration, the biological activity of methanogens requires hydrogen to carry out redox-reactions, for example, acetate reduction to methane and is therefore syntrophically related to acetogenic species via an interspecies hydrogen transfer (Fang et al., 2002; Wolin, 1975; Zeikus et al., 1975).

Intermittent dominant bands marked with 10 in Fig. 3 reveal sequences corresponding to *Bacteroides* sp. with an identity value of 98%, whereas bands marked with 2, which were dominant throughout all cultivation cycles, represent uncultured bacteria of unknown origin. Members of *Bacteroides* and *Clostridium* are known to be principally responsible for the bioconversion of renewable raw material to biogas, that is, the degradation of major plant structural polysaccharides forming acetate, formate, lactate amongst other fatty acids (Klocke et al., 2007).

A favourable change towards a more specialised and xylanolytically active community structure could be

confirmed by sequence analyses. Thus, our findings emphasise a hydrolytic potential of xylan-grown populations, which impact on the methanogenic biogas-producing process was studied as follows.

3.3. Influence of zeolite-immobilised hemicellulolytic microorganisms on the biogas process

To investigate the effect of hemicellulolytic microorganisms on biogas formation, enriched xylanolytic cultures were immobilised on trace metal activated zeolite and then introduced in a comparative batch-culture experiment under mesophilic temperature conditions (i.e. 35 °C). Fig. 6 shows a comparison of batch-cultures with and without addition of immobilised bacteria on activated zeolite, that is, their effects on corresponding methane yields cumulatively measured over a total fermentation period of 34 days (standard deviations are indicated as error bars in the diagram).

Small differences in measured methane amounts of about $\Delta 13.91 \text{ l CH}_4 \text{ kg}^{-1} \text{ ODM}$ at STP on average were already observed around day 9 of the fermentation, indicating a slightly better initiation of the fermentation process by introducing immobilised material. These first minor differences expanded from day 21 on, revealing a much higher biogas production rate in the presence of hemicellulolytic bacteria immobilised on activated zeolite when compared to controls supported with trace metal activated zeolite only. Methane yields were increased by about $64.1 \text{ l CH}_4 \text{ kg}^{-1} \text{ ODM}$ at STP on average which equals an increase of 53% in the mean. At day 26 of the fermentation a maximum discrepancy of $\Delta 73.2 \text{ l CH}_4 \text{ kg}^{-1} \text{ ODM}$ at STP was noticed which is equivalent to an increase of 121%.

Furthermore, HPLC analysis revealed a correlation between increased biogas productivity upon addition of xylanolytic bacteria immobilised on activated zeolite and an increase of acetic acid in corresponding replicates as presented in Fig. 7.

With day 7 of the fermentation process the concentration of acetic acid was starting to increase by 9.11 mM in

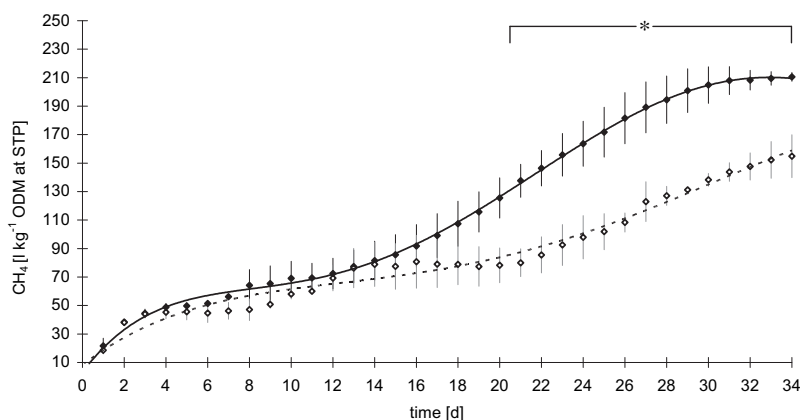


Fig. 6 – Cumulative biogas production, that is, CH_4 in $\text{l kg}^{-1} \text{ ODM}$ at STP during discontinuous fermentation in the presence of xylanolytic bacteria immobilised on activated zeolite (solid line) and controls with activated zeolite but without immobilised microorganisms (dotted line) at 35 °C over a total period of 34 days. From day 21 of the fermentation on differences in biogas amounts were significant. Significant differences are defined as $P < 0.05$ and are marked with an asterisk in the figure.

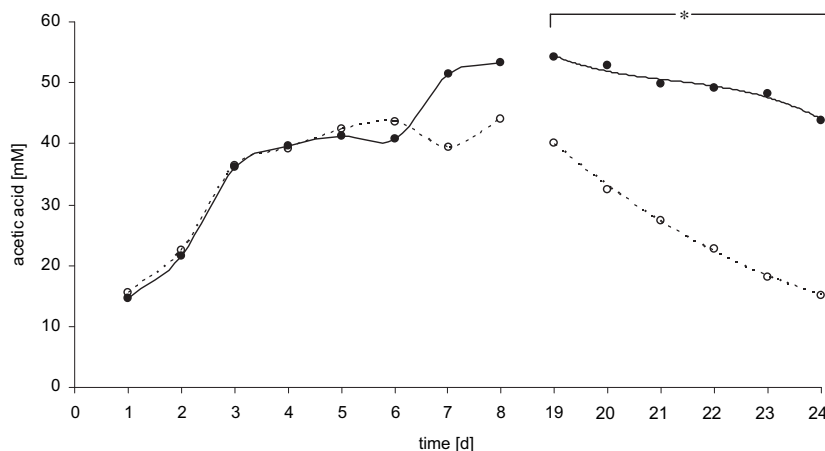


Fig. 7 – Acetic acid concentrations obtained from single measurements during discontinuous fermentation at 35 °C in the presence of xylanolytic bacteria immobilised on activated zeolite (solid line) and controls with activated zeolite but without immobilised microorganisms (dotted line). From day 19 on, acetic acid concentrations differ significantly. Significant differences are defined as $P < 0.05$ and marked with an asterisk in the figure.

comparison to control replicates without zeolite-immobilised hemicellulolytic microorganisms, correlating with initially increased amounts of methane as shown in Fig. 6 around day 9. However, from day 19 on a significant increase of 14.03 mM acetic acid (+35%) was observed, reaching a maximum at day 24 with 28.62 mM which is equivalent to an increase of 189%. Overall, acetic acid concentrations increased by 20.4 mM on average which equals 88% in the mean, correlating with simultaneously increased methane amounts observed between day 21 and day 26 of the total fermentation time (see Fig. 6). Thus, it is assumable that acetoclastic methanogenic species, producing a major part of the total methane yield during the methanogenesis, were favoured due to the increased availability of their exclusive substrate acetate (Jetten et al., 1992; Zeikus et al., 1975). A clarification could be achieved by DNA microarrays, which have been used for bacterial detection, as well as for microbial community analysis, while further functional gene arrays are able to verify the expression of specific enzymes in processes such as methanogenesis (Saleh-Lakha et al., 2005).

4. Conclusion

In summary we have shown that hemicellulolytic populations within mixed fermentative microbial communities can be enforced by substrate determination. Since the interaction of bacteria cells with certain substrates predisposes their growth and regulates the biosynthesis of their enzymes, xylan powder proved to be a capable carbon source for the induction of xylanase activity in mixed fermentative populations. As exposed by SSCP and sequence analyses, higher enzymatic activities were due to the development of a hemicellulolytic population, significantly distinctive from the natural bacteria community composition, revealing participation of *Bacteroides* sp., *Azospira* sp. among a wide spectrum of diverse Clostridia species in xylan degradation. In laboratory batch experiments methane yields in discontinuous fermentation of xylan

powder were increased significantly upon the addition of hemicellulolytic microorganisms when immobilised on trace metal activated zeolite. Increased methane production correlated with simultaneously increased concentrations of acetic acid, suggesting the acetogenic fermentation phase to be enhanced through higher hydrolytical activity derived from pre-cultured xylanolytic bacteria providing an enforced conversion of xylan. This strategy demonstrates a scheme easy to apply in principle to biogas processes. Although further studies in semi-continuous and continuous laboratory and large scale experiments are required, including considerations of the degradation of crystalline cellulose and lignocellulose, the use of supplemental introduced immobilised bacteria might either reduce the need for, for example, chemical pre-treatment approaches or make recalcitrant agricultural biomass and residues more economic substrates for the production of biogas. The understanding of cell adhesion to the surface of zeolites and its influence on growth and enzyme synthesis as an operational environment during subsequent fermentation stages are part of future studies.

Acknowledgements

This work has been kindly supported by IPUS GmbH, Department of Research and Development. The authors would also like to send many thanks to the Center for Medical Research at the Medical University of Graz.

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