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Process diagnosis using methanogenic *Archaea* in maize-fed, trace element depleted fermenters

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ABSTRACT

A mesophilic maize-fed pilot-scale fermenter was severely acidified due to trace element (TE) deficiency. Mainly cobalt (0.07 mg * kg^{-1} fresh mass (FM)), selenium (0.007 mg * kg^{-1} FM) and sodium (13 mg * kg^{-1} FM) were depleted. From this inoculum, three lab-scale flow-through fermenters were operated to analyse micronutrient deficiencies and population dynamics in more detail. One fermenter was supplemented with selenium, one with cobalt, and one served as control. After starvation and recovery of the fermenters, the organic loading rate (OLR) was increased. In parallel, the concentration (Real-Time PCR) of methanogens and their population composition (amplicon sequencing) was determined at the DNA and mRNA level. The parameters Metabolic Quotient (MQ) and cDNA/DNA were calculated to assess the activity of the methanogens.

The control without TE supplementation acidified first at an OLR of 4.0 kg volatile solids (VS) * m^{-3} * d^{-1} while the singular addition of selenium and of cobalt positively influenced the fermenter stability up to an OLR of 4.5 or 5.0 kg VS * m^{-3} * d^{-1}, respectively. In the stable process, the methanogenic populations were dominated by probably residual hydrogenotrophic *Methanoculleus* sp. (DNA-level), but representatives of versatile *Methanosarcina* sp. were most active (cDNA-level). When the TE supplemented fermenters began to acidify, *Methanosarcina* spp. were dominant in the whole (DNA-level) and the active (cDNA-level) community. The acidified control fermenter was dominated by *Methanobacteriaceae* genus IV. Until acidification, the concentration of methanogens increased with higher OLRs. The MQ indicated stress metabolism approximately one month before the TVA/TIC ratio reached a critical level of 0.7, demonstrating its suitability as early warning parameter of process acidification. The development of the cDNA/DNA ratio also reflected the increasing methanogenic activity with higher OLRs. Highest cDNA/DNA values (ca. 2) were obtained at metabolic strain of the methanogens, at the onset of acidification.

1. Introduction

Biogas can contribute to mitigation of green house gas emissions. Currently, there are ca. 7720 biogas plants in Germany. Mainly renewable resources are used as substrate for higher energy recovery. Even if no manure is added, trace element (TE) deficiency can occur. Some micronutrients are essential for methanogenic *Archaea*. If they are not supplemented, the biogas process can collapse. Specific addition of trace elements can antagonise this problem [1–3].

Methanogens are responsible for the production of methane, which can be used for electricity generation by combined heat and power units, heat supply, in the traffic sector or for upgrading to natural gas quality and feed-in into the gas grid. So far, methanogenic *Archaea* are the only known microorganisms which are capable of producing significant amounts of methane. Since cultivating methanogens is difficult, molecular biological tools targeting a key enzyme of methanogenesis – *mcr*A/*mrt*A (methyl-coenzyme M reductase subunit A, EC: 2.8.4.1) – were used for the specific quantitative and qualitative analysis of methanogens.

One aim of this study was to determine the impact of cobalt and selenium deficiency on the performance and stability of lab-scale flow-through fermenters fed with maize-silage. In parallel, population shifts in the differently treated fermenters were analysed in order to define indicator organisms for trace element depletion.

Additionally, the recently developed Metabolic Quotient [5] was evaluated as a suitable early warning system of microbial strain, stress and process disturbance. This parameter is based on a standard for stably and efficiently running maize-fed fermenters, and

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gives information about the actual metabolic activity of the methanogens and their physiological state [5].

The cDNA/DNA ratio was measured to determine the actual metabolic transcriptional activity of the methanogens targeting *mcr*A/*mrt*A transcripts in relation to the actual gene concentration [5].

2. Material and methods

2.1. Fermenter management

A mesophilic maize-fed pilot-scale fermenter (SB1) with a working volume of 2.8 m^3 showed severe acidification after a shock-load experiment and subsequent dilution of the fermenter sludge with water. After feeding was stopped, the fermenter did not recover yet. Trace element (TE) concentrations were determined, and TE deficiency was found to be a possible reason for the acidification. Mainly cobalt (0.06 mg * kg^{-1} fresh matter, FM) and selenium (0.007 mg * kg^{-1} FM) were present only at very low concentration in the fermenter (Table 1). Besides, the sodium concentration (13 mg * kg^{-1} FM) was near the limit of ca. 10 mg * kg^{-1} FM, as defined recently [4]. Other important TEs like nickel (3.8 mg * kg^{-1} FM) and molybdenum (4.9 mg * kg^{-1} FM) were not in a critical range. The impact of TE deficiency on the stability of the biogas process was determined in three semi-continuously operated flow-through fermenters with a working volume of 28 L. These reactors were filled with SB1 sludge and fed daily with maize silage which had a dry matter content of 37% and relatively low cobalt (0.06 mg * kg^{-1} FM) and selenium (0.02 mg * kg^{-1} FM) concentrations.

Due to the severe acidification, the low total inorganic carbon (TIC) value and hence the low buffer capacity, the fermenters were treated with sodium carbonate (day 8–15, 40 g per fermenter) to increase the TIC value to ca. 10 g * kg^{-1} FM. Due to this treatment the sodium concentration was increased strongly in the fermenters, and the influence of sodium deficiency could not be determined during the experiment. After stabilisation of the TIC value, one fermenter (S1) served as control without TE addition, one fermenter (S2) was supplemented once (day 29) with selenium (as Na_2SeO_4) to 0.02 mg * kg^{-1} FM and one fermenter (S3) with cobalt (as $CoCl_2$) to 0.12 mg * kg^{-1} FM. At day 57, feeding was started by an organic loading rate (OLR) of 0.5 kg volatile solids (VS) * m^{-3} * d^{-1}, and in the following, the OLR was increased continuously to 5.0 kg VS * m^{-3} * d^{-1} (Fig. 1).

Gas production was measured online continuously with Milligascounters. Using an AWITE gas analyzer, the components CH_4 and CO_2 in the biogas were determined continuously by infrared gas analysis, and O_2, H_2 and H_2S by electrochemical sensors. Gas production was normalised to standard temperature pressure (STP). Samples for chemical and molecular biological analyses were taken irregularly at significant process stages. TEs were analysed from aqua regia extracts by ICP-OES or flameless atomic absorption (Co). Concentrations of short chain fatty acids (SCFA, C2–C7) were determined by gas chromatography and the pH value according to DIN 38404-C5 and DIN 12176 standard procedures using a pH-electrode. The ratio of total volatile acids/total inorganic or alkaline carbonate (TVA/TIC) was assessed as a measure of carbonate buffer capacity and process stability according to Nordmann (1977) [6].

Table 1
Development of the trace element concentrations in the differently treated fermenters.

Day	Se [µg * kg^{-1} FM]			Co [µg * kg^{-1} FM]		
	S1 – control	S2 – +Se	S3 – +Co	S1 – control	S2 – +Se	S3 – +Co
0	6.67	6.67	6.67	63.85	63.85	63.85
28	8.03	8.76	8.76	62.78	54.75	60.59
156	8.41	11.89	8.75	62.23	61.13	171.50
211	6.05	9.64	5.91	47.19	47.00	117.02
220	BDL	BDL	BDL	n.m.[a]	n.m.[a]	n.m.[a]
228	n.m.[a]	n.m.[a]	n.m.[a]	46.85	41.10	100.65

BDL: below detection limit.
[a] n.m.: not measured.

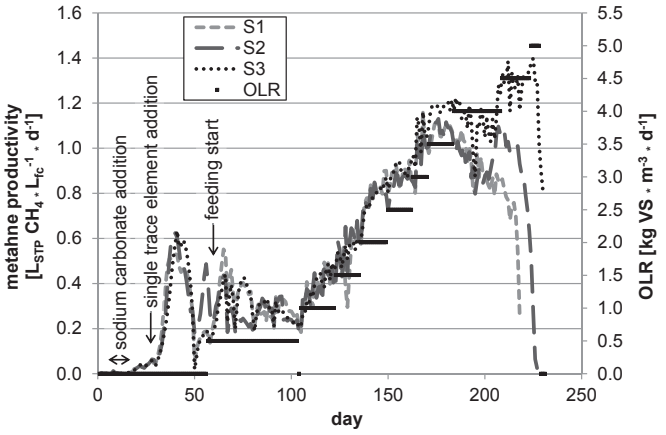

Fig. 1. Methane productivity and organic loading rate (OLR) of the control (S1), selenium supplemented (S2) and cobalt supplemented (S3) fermenter.

2.2. Nucleic acid extraction, qPCR and RT-qPCR

DNA and RNA were extracted following the optimised protocols described by Munk et al. (2010) [4] and Munk et al. (2012) [5], respectively. In brief, fermenter samples were washed twice in 0.85% potassium chloride. DNA and RNA was extracted from 40 µL washed sample using the FastDNA™ Spin Kit for Soil (MP Biomedicals) and the FastRNA® Pro Soil – Direct Kit (MP Biomedicals), respectively, according to the manufacturer's protocol except for a second washing step with SEWS-M buffer (DNA) or ethanol (RNA). Eluted RNA was immediately treated with DNase (TURBO DNA-*free*™ Kit, Ambion) to remove co-extracted DNA and stored at −80 °C.

cDNA was synthesised using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Invitrogen) according to the manufacturer's protocol for gene specific primers at 52.5 °C for 60 min.

The functional gene *mcr*A/*mrt*A was quantified in 1 µL of undiluted and 10-fold diluted DNA or cDNA solution by qPCR in triplicates using primers (MeA-i 1046f: 5′-TAYATGWSIGGHGGIGTIGGITTYAC-3′ and MeA-i 1435r: 5′-TGRTCYTGIARRTCRWAICCRWAGAAICC-3′) and an internal prequantified *mcr*A standard as described earlier [4].

2.3. Direct-PCR-cloning and plasmid sequencing

Each fermenter was sampled at a stable (day 129) and an acidified (day 211) process state. The detailed protocol of population analysis is described in Munk et al. (2010) [4]. In brief, the gene fragment of *mcr*A/*mrt*A was amplified using the above mentioned primers, ligated into the pCR®4 vector and transformed into OneShot® TOP10 chemically competent cells using the TOPO-TA® cloning kit (Invitrogen). Clones carrying a plasmid insert were selected on ampicillin-supplemented lysogeny broth agar, and inserts were checked by whole-cell PCR using M13 primers. For each sample, 16 clones with inserts of correct size were propagated.