Analysis of the key enzymes of butyric and acetic acid fermentation in biogas reactors

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Summary
This study aimed at the investigation of the mechanisms of acidogenesis, which is a key process during anaerobic digestion. To expose possible bottlenecks, specific activities of the key enzymes of acidification, such as acetate kinase (Ack, 0.23–0.99 U mg⁻¹ protein), butyrate kinase (Buk, < 0.03 U mg⁻¹ protein) and butyryl-CoA:acetate-CoA transferase (But, 3.24–7.64 U mg⁻¹ protein), were determined in cell free extracts of biogas reactor content from three different biogas reactors. Furthermore, the detection of Ack was successful via Western blot analysis. Quantification of corresponding functional genes encoding Buk (buk) and But (but) was not feasible, although an amplification was possible. Thus, phylogenetic trees were constructed based on respective gene fragments. Four new clades of possible butyrate-producing bacteria were postulated, as well as bacteria of the genera Roseburia or Clostridium identified. The low Buk activity was in contrast to the high specific But activity in the analysed samples. Butyrate formation via Buk activity does barely occur in the investigated biogas reactor. Specific enzyme activities (Ack, Buk and But) in samples drawn from three different biogas reactors correlated with ammonia and ammonium concentrations (NH₃ and NH₄⁺-N), and a negative dependency can be postulated. Thus, high concentrations of NH₃ and NH₄⁺-N may lead to a bottleneck in acidogenesis due to decreased specific acidogenic enzyme activities.

Introduction
The biochemical process of methane formation in biogas reactors can be divided into four phases: (i) hydrolysis of complex polymers, (ii) acidogenesis, (iii) acetogenesis and (iv) methanation (Weiland, 2010). In the first and second steps, the main products sugars and amino acids are formed, as well as volatile fatty acids (VFA) such as acetate, butyrate and propionate. These VFAs play a key role in biogas reactors. If the hydrolysation of organic material occurs too fast, VFA are accumulating, which has an effect on the growth of aceticlastic and hydrogenotrophic methanogens and their ability to produce methane (McCarty and McKinney, 1961; Kroecker et al., 1979; Chen et al., 1980; Weiland, 2010). The most widespread acetate-producing pathway comprises the enzymes phosphotransacetylase (Pta) and acetate kinase (Ack), which convert acetyl-CoA to acetate (Ljungdahl, 1986). This pathway can be found in many different prokaryotes, e.g. Roseburia sp. (Duncan et al., 2002), Clostridium acetobutylicum (Winzer et al., 1997) or Methanosarcina thermophila (Garrell and Ferry, 2007). Butyrate-producing bacteria can be found, e.g., in marine sediments (Sørensen et al., 1981), and the human colon (Pryde et al., 2002; Louis et al., 2004; 2010), e.g. species related to Eubacterium, Roseburia or Faecalibacterium. These bacteria use one of the four major bacterial butyrate synthesis pathways – acetyl-CoA, glutarate, lysine and 4-aminobutyrate pathway – which all have in common crotonyl-CoA as an intermediate (Vital et al., 2014). By activity of butyryl-CoA dehydrogenase electron-transferring flavoprotein complex, butyryl-CoA is formed from crotonyl-CoA. Finally, butyryl-CoA is transformed to butyrate by a number of butyryl-CoA transferases, e.g. butyryl-CoA:acetate-CoA transferase (But), which uses acetate as co-substrate (Duncan et al., 2002). Butyryl-CoA can also be phosphorylated and transformed to butyrate by butyrate kinase (Buk) with simultaneous production of ATP (Diez-Gonzalez et al., 1999).

Here, we studied the specific activity of the enzymes Ack, Buk and But in three different biogas reactors. Specific activities can be used to determine the amount of the respective catalyst present in reactor content under different process conditions (Table 1). Furthermore, the presence and the quantity of the conserved genes buck and but were analysed in different samples of one biogas reactor.

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Results

Specific enzyme activities of the key enzymes Ack, Buk and But were measured photometrically by using cell free extracts of biogas reactor content obtained by ultrasonic pulsation (Table 2, Table S1). Specific activity of Ack was on average 0.41 Units (U) mg\(^{-1}\) protein in cell free extracts of samples A-G of the biogas reactor content of BR 1. Furthermore, only low specific Buk activity (<0.02 U mg\(^{-1}\) protein) was measurable in the same samples. In contrast, high specific But activity (on average, 5.92 U mg\(^{-1}\) protein) was determined in samples B-G of BR 1. In cell free extracts of biogas reactor content obtained from BR 2 and BR 3 (samples A1, B1, A2 and B2) showed the same tendency of specific enzymatic activities. Specific Buk activity was lowest (<0.03 U mg\(^{-1}\) protein), followed by specific Ack activity (0.23 and 0.99 mU mg\(^{-1}\) protein) and specific But activity (3.24 and 7.64 U mg\(^{-1}\) protein) respectively. Statistical analysis showed a correlation between specific enzyme activities and total ammonia and ammonium concentrations in reactor content of three different biogas reactors (Kendall’s rank coefficient \(\tau = -1\)).

Furthermore, cell free extract of biogas reactor content (sample B, BR1) and corresponding controls were loaded on an SDS-PAGE (Fig. 1A) for subsequent Western blot analysis, which was conducted for the detection of Ack in the analysed biogas reactor content of BR1 (Fig. 1B). In all dilutions of cell free extracts from biogas reactor content (lanes 2–4), the same signal was detected as in

![Fig. 1. Detection of Ack (∼46 kDa): (A) SDS-PAGE (10%, silver staining) – 1 C. glutamicum 13AK; 2, undiluted reactor content; 3, 1:2 diluted reactor content; 4, 1:5 diluted reactor content; 5–6, C. kluyveri (exponential and stationary growth phase respectively); M, protein standard (PageRuler™ prestained) (B) Western blot – 1, C. glutamicum 13AK; 2–4, reactor content; 5–6, C. kluyveri.](image-url)
cell free extracts of *C. kluyveri* DSM 555, which was used as positive control (lanes 5 and 6).

Quantification of the genes coding for Buk (*buk*) and But (*but*) was performed using genomic DNA from samples A–E from BR1 listed in Table 1. Neither *buk* nor *but* gene fragments were quantifiable in any sample by quantitative polymerase chain reaction (qPCR) assay because all used dilutions in the assay had the same crossing point (Cp) value (55.0 for *buk* and 42.6 for *but*). However, simultaneous quantification of the 16S rRNA used as reference gene was successful for all samples and every dilution used, in terms of sufficient calculation of standard curves.

Furthermore, possible transcription of the genes *buk* and *but* was checked using reverse transcriptase PCR (RT-PCR). No PCR product was amplified from respective cDNA for *buk* and *but* in the samples A and D, indicating that the transcription of both genes did not occur (Fig. S1).

The results suggest that amplification of both conserved genes is possible, although the quantification was not successful. By using a standard cloning approach of *buk* and *but* gene fragments, 218 and 301 clones were obtained for *buk* and *but* respectively. The corresponding gene fragments were translated into respective amino acid sequences. Only 17 translated and meaningful *buk* sequences from initially 218 picked and analysed clones could be finally used for the phylogenetic tree (Fig. 2), leading to an efficiency of 7.8%. Three hundred one *E. coli* clones were analysed for the phylogenetic tree of *but* gene fragments (Fig. 3) with 144 potentially useful sequences, adding up to a theoretical efficiency of 47.8%. Amino acid sequences that did not group with any...
reference sequences were designated as butyrate-producing bacteria using Buk or But (BBBuks or BBButs) respectively. Considering the phylogenetic tree of Buk, amino acid sequences of buk1 and buk2 cluster in the genus Clostridium. The amino acid sequences of Buk of C. celatum and a branched-chain fatty-acid kinase of C. ultunese have the highest sequence similarity to buk1 and buk2 respectively. The amino acid sequence of buk9 shows highest similarity to the reference sequence of a hypothetical protein of Proteiniphilum acetatigenes. BBUks A and BBBuks B do not group in any reference phyla, indicating two new clades of butyrate-producing bacteria.

Thirteen of the 28 amino acid sequences derived from but fragments cluster in the phylum Firmicutes and particularly in the order Clostridiales. Furthermore, six and five of these sequences group in the genera Roseburia and Clostridium respectively. Hence, but4 and but12 cannot be assigned to any reference genus and form a new clade BBButs B. The remaining 15 sequences form apparently also a new clade BBUks A, not belonging to the phylum Firmicutes.

**Discussion**

The aim of this study was to elucidate the activity and presence of acidogenic key enzymes Ack, Buk and But, and the corresponding genes buck and but, respectively, in the biogas process. Results of the specific enzyme activity assays of Ack, Buk and But indicate a stable substrate degradation within the sampled biogas reactor BR1, as there are hardly any fluctuations during the sampling period. Duncan and colleagues (2002) as well as Louis and colleagues (2004) also did report on specific enzyme activities of Ack, Buk and But using bacterial strains from the human large intestine. The determined specific enzyme activities were in a similar range as found in this study. The overall trend was that specific But activity was highest, followed by specific Ack activity, and specific Buk activity was lowest (Duncan et al., 2002; Louis et al.,...
Isolates from swine intestinal tract, which showed that the activity of But rather than the activity of Buk (Levine et al., 2013) because bacterial strains tested positively for But activity were at the same time negative for Buk activity. This can also be supported by metagenomic data drawn from stool samples, in which the pathway of butyrate production by but as terminal gene was favoured over buk (Vital et al., 2014). Both findings match with the results obtained in this study. Latest results of Klang and colleagues (2015) showed that there was a correlation between the ammonium (NH₄⁺-N) concentration and the diversity and composition of the microbial community. Similar findings were reported by Westerholm and colleagues (2011) to the effect that increasing ammonia concentration caused a shift in the acetogenic population in a mesophilic anaerobic digester. Ammonia concentration has a strong impact on the microbial community structure in anaerobic environments (Westerholm et al., 2011; Fotidis et al., 2013; Klang et al., 2015). Moreover, we show an influence of NH₄⁺-N concentration on the enzymatic activities within different biogas reactors. Based on the collected data, we postulate that high NH₄⁺-N and NH₃ concentrations in biogas reactor content cause low enzyme activities of acidogenesis, and consequently a low amount of respective catalyst is present.

Western blot analysis was successfully used for the detection of enzymes in a biogas reactor, in this case Ack (Fig. 1B). So far, an unsuccessful attempt has been reported to detect enzymes (exoglucanase CelS from \textit{C. thermocellum}) in samples from a biogas reactor (Köllmeier, 2013). Due to low Buk activity and lack of suitable antibody against But at the time of the study, Western blot analysis was neither performed using Buk nor But.

After determination of specific Buk and But activities in the sampled biogas reactor, the next step was the analysis of the quantity and the transcription of the corresponding gene fragments of Buk and But. Primer pairs targeting both mentioned gene fragments were introduced by Louis and colleagues (2004) and by Louis and Flint (2007) for butyrate-producing bacteria in the human colon. In this study, both qPCR and RT-PCR failed for the gene fragments buk as well as but. In the case of buk, the failed quantification on DNA level was not surprising due to the fact that specific Buk activity was barely measurable. In contrast, high specific But activity was found in all sampled biogas reactors. For this reason, quantification of but gene fragment was actually expected. Levine and colleagues (2013) also reported on butyrate-producing isolates from swine intestinal tract, which showed But enzyme activity. However, but genes could not be detected by using the same degenerate primer pair (BCoATscrF and BCoATscrR) used in this study. Furthermore, unsuccessful RT-PCR (Fig. S1) for both gene fragments buk and but indicates a restricted usage of the used primer pairs for genomic DNA or cDNA obtained from the sampled biogas reactor. Additionally, the used primer pairs need to be improved for this kind of samples and analyses. Anyhow, high Cp values in qPCR indicate an amplification of both gene fragments anyway, despite no possible quantification. Therefore, phylogenetic trees were constructed for either buk or but gene fragments to get an overview of butyrate-producing bacteria in the sampled biogas reactor (Figs 2 and 3). At the 16S rRNA and at the metagenome level, the presence of the phyla Firmicutes and Bacteroidetes was also previously reported for biogas reactors (Soudi et al., 2007; Schlüter et al., 2008; Liu et al., 2009; Kampmann et al., 2012; Bengelsdorf et al., 2013; Li et al., 2013; Stolze et al., 2015). In the reports at the 16S RNA level, both phyla were the predominant bacteria in biogas reactors, with up to 58.9 and 35.4% of all analysed sequences respectively. In the anaerobic environment of the human intestinal tract, bacteria of the genera \textit{Roseburia} and \textit{Clostridium} were also found as butyrate producers at the but gene level and at the 16S rRNA level respectively (Duncan et al., 2002; Pryde et al., 2002; Louis et al., 2004; 2010; Louis and Flint, 2007; Hippe et al., 2010). In this study, four new classes of possible butyrate-producing bacteria were identified. So far, BBBuks A and B, and BBButs A seem to form each an unidentified clade of bacteria. Assigning these clades to any known phylum is challenging as most reports on microbial diversity in biogas reactors are based on 16S rRNA analyses rather than analyses with special biomarkers, such as the buk or the but gene. For example, members of the phylum Spirochaetes are in possession of a gene encoding Buk (Mavromatis et al., 2010) and were also found in low percentages in biogas reactors (Soudi et al., 2007; Liu et al., 2009; Stolze et al., 2015). In some cases, Proteobacteria were found in small numbers in biogas reactors (Fang et al., 2002; Soudi et al., 2007; Stolze et al., 2015) and in larger fractions in gastrointestinal tracts of different animals (Metzler-Zebeli et al., 2010; Wüst et al., 2011; Van den Abbeele et al., 2013). Furthermore, some Proteobacteria, such as \textit{Burkholderia vietnamiensis} G4 (NCBI Gene ID: 4953143), \textit{Shewanella} sp. MR-7 (NCBI Gene ID: 4257334) or \textit{Sphingomonas wittichii} RW1 (NCBI Gene ID: 5198581), are actually carrying a gene encoding But. Apparently, BBButs B clustered in the phylum Firmicutes (Fig. 3). For example, the genera \textit{Faecalibacterium} or \textit{Coprococcus} were found in the human colon as butyrate producers using But (Duncan et al., 2002; Pryde et al., 2002; Hippe et al., 2010).

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4.2 mM Na₂HPO₄, 137 mM NaCl, 1.5 mM KCl) and stored at 50 ml reactor content were centrifuged (4000 × g, 15 min, 4°C), washed twice with 25 ml PBS buffer (1.5 mM KH₂PO₄, 4.2 mM Na₂HPO₄, 137 mM NaCl, 1.5 mM KCl) and stored at −20°C. For DNA and RNA studies, 25 ml ethanol (96%, v/v) was added to 25 ml reactor content and stored at −20°C. 

Experimental procedures

Biogas plant and sampling

A stably performing mesophilic full-scale biogas reactor fed with maize silage, cattle manure and poultry dry manure located near Bonn (Troisdorf, Germany) was chosen for protein and genome studies (biogas reactor 1, BR 1). The organic loading rate of the reactor was on average 4.8 kg VS m⁻³ d⁻¹ (VS, volatile solids) with a total solid feeding of 14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days. The methane content accounted to 53% (v/v) of the 330–14 800 kg per day and a hydraulic retention time of 85 days.

Determination of protein concentrations and enzyme activities

Protein concentrations of cell free extracts were determined by using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford IL, USA) following the manufacturer’s instructions. Samples A-G of BR 1 were used for the determination of the enzyme activities of Ack and Buk, and samples B-G of BR 1 were used for the determination of the enzyme activity of But. Both samples of BR 2 and BR 3 were used for all three enzymatic assays. In all cases, at least two dilutions of each sample were measured twice to assure linearity of enzyme activity. Enzyme assays were performed routinely in a cuvette at 30°C. Ack and Buk activity assays (Nakajima et al., 1978) comprised reaction buffer (100 mM Tris, pH 7.2, 5 mM MgCl₂), 0.5 mM NADP⁺, 3.0 mM ADP, 2.0 mM glucose, and 10 μl hexokinase/glucose-6-phosphate dehydrogenase (3 mg ml⁻¹; Roche Diagnostics, Filderstadt, Germany). In total volume of 1.0 ml, the reaction was initiated by the addition of 5 μM acetyl- or butyryl-phosphate for the estimation of specific Ack or Buk activity respectively. The increase of absorbance of NADPH was followed at 340 nm with an extinction coefficient ε = 0.063 M⁻¹ cm⁻¹. But activity assay (Buckel et al., 1981) comprised reaction buffer (100 mM potassium phosphate, pH 7.0), 200 mM sodium acetate, pH 7.0, 1.0 mM oxaloacetate, 1.0 mM DTNB, and 10 U citrate synthase (Sigma-Aldrich, Steinheim, Germany). In total volume of 1.0 ml, the reaction was initiated by the addition of 1.0 mM butyryl-CoA. The increase of absorbance of TNB⁻ was followed at 412 nm with an extinction coefficient ε = 14.2 M⁻¹ cm⁻¹.

Extraction of total protein from biogas reactor content

For the aerobic determination of specific enzyme activities of Ack, Buk and But, as well as Western blot analysis of Ack, samples stored at −20°C were thawed on ice by the addition of one volume of corresponding enzyme assay reaction buffer (see below). Homogenization of the cells was carried out by using an ultrasonic pulser (Sonifier 250, Branson, Dietzenbach, Germany) with the following adjustments: 30 s pulsing at 60–70% intensity, 30 s break, 15 cycles (modified; Refai et al., 2014). Cell debris was pelleted by centrifugation (4000 × g, 20 min, 4°C). The supernatant was filtrated using folded filters (particle retention 7 μm, 595.5 grade) and centrifuged (11 000 × g, 5 min, 4°C). The obtained cell free extract was used for enzyme assays and Western blot analysis.

Corynebacterium glutamicum ATCC 13032 13AK with defective Ack activity (Wendisch et al., 1997) and Clostridium kluyveri DSM 555 (DSMZ, Braunschweig, Germany) were used as negative and positive controls in the enzyme studies respectively. Both strains were cultivated to mid-exponential or stationary growth phase aerobically in CGXII minimal medium (Keilhauer et al., 1993) with 1% glucose or anaerobically in medium 52 (DSMZ) respectively. Harvested cells were washed twice with corresponding enzyme assay reaction buffer and stored at −20°C. Mechanical cracking of the cells was achieved by using a homogenizer (three cycles, 45 s, 6500 m s⁻¹), followed by centrifugation (11 000 × g, 30 min, 4°C) to obtain cell free extracts for further use.

Western blot

For Western blot analysis, equal amounts (200 μg) of cell free extracts (sample B of BR1, controls) and respective dilutions (1:2 and 1:5) were loaded onto an SDS-PAGE (10%, v/v) to
detect Ack in biogas reactor content. Protein was silver-stained or transferred onto nitrocellulose membranes by electro blotting and blocked with 2% BSA over night. Blots were incubated with primary antibody (Ack antibody; Biorbyt, Cambridge, UK) and with secondary antibody [polyclonal goat anti-rabbit (HRP, horse radish peroxidase) antibody; Dakocytomation, Glostrup, Denmark], each for 1 h. Signals were detected by enhanced chemiluminescence (Pierce ECL Western Blotting, Thermo Fisher Scientific).

**Extraction of total DNA and RNA from biogas fermenter content**

Analysis of the functional genes *buk* (encoding Buk), *but* (encoding But) and 16S rRNA as reference gene was achieved by qPCR, RT-PCR and PCR. Nucleic acids were obtained by using extraction kits for DNA (NucleoSpin® Soil, Macherey-Nagel GmbH & Co. KG, Düren, Germany) from samples A–E of BR1 (Table 1) and for RNA (ZR Soil/Fecal RNA MicroPrep™, Zymo Research Corp., Irvine CA, USA) from samples A and D of BR1, respectively, by following the manufacturer's instructions. Additionally, extracted total RNA was treated with DNase I (Invitrogen GmbH, Karlsruhe, Germany).

**qPCR, RT-PCR, PCR**

Primer sets used in this study for the analysis of the functional genes *buk*, *but* and 16S rRNA were recommended in the respective manual for tree reconstruction were used for the analyses. All used sequences for phylogenetic trees were submitted to GenBank under accession numbers KP136873 to KP136889 and KM594289 to KM594320 respectively.

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Conflict of interest

There is no conflict of interest.
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Supporting information
Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Detection of buk (A, 740 bp), but (B, 540 bp) and 16S rRNA gene (C, 450 bp) via RT-PCR: agarose gel (2%) of PCR products – Lane: M, 50 bp DNA ladder (GeneRuler™); 1 and 3, cDNA; 2 and 4, RNA; 5, positive control (DNA); 6, negative control (H2O).

Table S1. Specific enzyme activities in U mg−1 protein, enzymatic volume activity in mU g−1 oDS of Ack, Buk and But in cell free extracts of biogas reactor content BR 1, BR 2 and BR 3. Values of ammonia concentrations for NH4+ and NH3 in g l−1.