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Research Article

Carboxylic acid production from ensiled crops in anaerobic solid-state fermentation - trace elements as pH controlling agents support microbial chain elongation with lactic acid

Background: For the production of carboxylic acid platform chemicals like medium-chain fatty acids (MCFA) by anaerobic fermentation, pH control is required. However, adding buffer solutions is ineffective in leach-bed reactors. Aim: In order to increase the MCFA production by maize silage fermentation and to engineer the process we investigated the effect of solid alkaline iron and manganese additives on the process performance and microbial community dynamics. Results: Without additives, the pH dropped to 3.9 and lactic acid bacteria were favored. Total product yields of 207 ± 5.4 g organic acids (C_2-C_6) and alcohols per kg volatile solids were reached. The addition of trace elements increased the pH value and the product spectrum and yields changed. With a commercial iron additive, the product yields were higher (293 ± 15.2 g/$kg_{volatile\ solids}$) and supposedly clostridia used lactic acid for microbial chain elongation of acetic acid producing *n*-butyric acid. With the addition of pure $Fe(OH)_3$ or $Mn(OH)_2$, the total product yields were lower than in the other reactors. However, increased production of MCFA and the occurrence of distinct bacterial taxa (*Lachnospiraceae*, *Ruminococcaceae* and *Megasphaera*) related to this metabolic function were observed.Conclusions: The application of alkaline trace metal additives as pH stabilizing agents can mitigate spatial metabolic heterogeneities when trace metal deficient substrates like specific crops or residues thereof are applied.

Keywords: Iron / Leach-bed / Manganese / MCFA / Medium-chain fatty acids

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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1 Introduction

Biorefinery processes with combined production of chemicals and fuels become more and more important. Thereby, the production of bio-based chemicals is achieved while energy carriers are gained from fermentation residues in a cascaded utilization process where biomass is degraded stepwise. Cascaded production of medium-chain fatty acids (MCFA, herein defined as carboxylic acids with a carbon chain length of 5–6 C-atoms) and biogas can be conducted by two-phasic anaerobic digestion (AD) processes, where hydrolysis/acidogenesis and acetogenesis/methanogenesis occur in separate reactors [1]. For the utilization of solid biomass, leach-bed processes are state of the art. Such reactor systems operated at high total solids (TS) conditions offer the advantages of reduced reactor size and lower energy consumption for reactor heating. Furthermore, solid-liquid separation in a leach-bed reactor simplifies product extraction when MCFA shall be produced. For maximum product yields in the anaerobic fermentation (AF) step, control of process parameters including pH value is essential. Compared to stirred reactors or liquid substrates, effective pH control in leach-bed

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Abbreviations: AD, anaerobic digestion; **AF**, anaerobic fermentation; **DGμ**, FerroSorp® DGμ; **LAB**, lactic acid bacteria; **MCFA**, medium-chain fatty acids; **NMDS**, nonmetric multidimensional scaling; **TE**, trace element; **T-RF**, terminal restriction fragment; **T-RFLP**, terminal restriction fragment length polymorphism; **TS**, total solids; **VFA**, volatile fatty acid; **VS**, volatile solids

Compiled in honour of the 80th birthday of Professor Wolfgang Babel.

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reactors with solid substrates is challenging. The pH value of the liquid phase can be directly controlled by automated addition of alkaline solutions. However, the pH value in the solid phase depends on the penetration and distribution of the process liquid which is often unbalanced in large scale reactors due to spatial heterogeneities [2].

For biogas production, the addition of trace elements (TE) is advisable when the inherent TE concentration in the substrate is too low for an adequate supply of the microorganisms. TE limitations lead to decreased methane production and thus to suboptimal reactor performance. Therefore, TE are supplemented to biogas processes of various types and scales and with various substrates. Several commercial supplements for biogas reactors providing TE are available as liquids or solids. Iron-dominated TE mixtures are used frequently as they additionally help precipitate toxic heavy metals as sulfide [3]. Trace metals like cobalt, nickel, iron, molybdenum, and tungsten are known as essential TE specifically for acetogenic and methanogenic microorganisms, which play the main role in the last AD steps [4,5] and thus in the second phase of two-phasic AD systems. A depletion of these trace metals can affect process performance as well as microbial community composition and activities and lead to process instabilities such as acidification [6–9]. Acidogenic bacteria in anaerobic processes have been scarcely investigated with respect to their TE requirement, probably due to their limited response to concentration changes of such TE that are known as decisive factors affecting the methanogenic community [10,11].

Whereas MCFA production from liquid substrates is comparably well investigated [12], solid substrates are less studied in this regard. Ensiled dedicated crops like maize silage are particularly pertinent substrates for MCFA production due to their high lactic acid content. Lactic acid can be exploited for MCFA production during acidogenesis by microbial chain elongation of short-chain fatty acids [13,14]. However, these crops are typically TE-deficient [6,15–17]. Thus, TE limitations are frequently observed when crops are used as a sole substrate in AD. In the case of co-digestion with agricultural waste products like manure, sufficient TE amounts may be provided by the co-substrate depending on type and share of the co-substrate in the substrate mixture [18]. For the use of dedicated crops in combined MCFA/biogas production, a mixture of the crops with co-substrates lacking lactic acid would imply a dilution of this chain elongation educt. As TE addition is therefore necessary, alkaline solid TE additives might be used as pH-stabilizing agents in the leach-bed AF step, thus saving consumables and equipment for pH control. The additives can be mixed with the solid substrate and stabilize the pH value within the solid substrate by dissolving in the process liquid over time. After being leached out from the solid phase together with the process liquid, the TE end up in the subsequent methanogenesis step.

The aim of this study was to engineer and optimize the production of carboxylic acids by leach-bed AF of maize silage with a microbiome. The effects of alkaline TE additives on the metabolic pathways, involved fermenting bacteria and the yields and composition of fermentation products were investigated. Different alkaline TE additives, i.e. the commercial TE compound FerroSorp® DGμ, iron hydroxide ($Fe(OH)_3$), and manganese hydroxide ($Mn(OH)_2$) were added and the effect of these supplements on the AF process was investigated. Besides process performance, the bacterial community dynamics during AF were analyzed. Community composition was monitored by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of bacterial 16S rRNA genes. Correlations between the abiotic process parameters and the community profiles were revealed by multivariate data analysis.

2 Materials and methods

2.1 Trace elements

FerroSorp® DGμ (P.U.S. GmbH, Lauta, Germany), $Fe(OH)_3$, and $Mn(OH)_2$ were used. DGμ contained 334 g Fe and 4.8 g Mn per kg_{TS} as determined by ICP-OES according to DIN EN 16170 [19], whereupon manganese and iron were primarily available as hydroxides (personal communication of Dr. Leiker, P.U.S. GmbH). Further DGμ components are listed in the Supporting Information 1.

Pure $Fe(OH)_3$ and $Mn(OH)_2$ were prepared by dissolving $FeCl_3 \times 6\ H_2O$ (reagent grade ≥ 99%; Fluka, Buchs, Switzerland) and $MnCl_2 \times 4\ H_2O$ (reagent grade ≥ 99%; Sigma-Aldrich, Steinheim, Germany), respectively, in deionized water and precipitated with a spillover of 50% KOH solution (reagent grade ≥ 99%; VEB Chemie Kombinat Bitterfeld, Germany). They were filtered using ashless hardened cotton plain filter paper 390 (Ahlstrom GmbH, Bärenstein, Germany), washed with deionized water and dried at 105 ± 5°C for 24 h. Purity grades of 95% for $Fe(OH)_3$ and $Mn(OH)_2$ were determined by ICP-OES according to DIN EN 16170 [19] and DIN EN 12457-4 [20] (Supporting Information 2).

2.2 Reactor operation

Eight leach-bed reactors were operated at 38°C for the batch-wise AF of maize silage in lab-scale with and without the addition of TE. Each reactor (inner height, 91.5 cm; inner diameter, 25 cm; total volume, approx. 45 L) was made of polyvinyl chloride and contained two chambers, separated by a horizontal perforated plate (4 mm thick, one 2-mm-hole per 2 cm^2), and an integrated percolation system (Fig. 1). The upper chamber was filled with substrate whereas the lower chamber served as a storage tank for the process liquid, i.e. percolate. Two pipes (inner diameter, 20 mm) connected the chambers. One pipe was used for pressure balance during the reactor operation, whereas the other pipe served as an emergency connector between the chambers in the case of blockage of the perforated plate by substrate particles. Thus, overflow of the reactor was prevented. A pump (SN 1011–478; Medorex, Nörten-Hardenberg, Germany) was used to circulate the percolate from the lower to the upper chamber. The water-jacketed reactors were heated using a water bath (Lauda 2000; Lauda, Germany).

Two lots of maize silage obtained from Bioenergiezentrum Westewitz GmbH (Großweitzschen, Germany) were used as substrate. For the experiments without any TE addition and with DGμ, maize silage with a TS content of 35.4% and a volatile solids (VS) content of 95.4%$_{TS}$ was used. For the experiments

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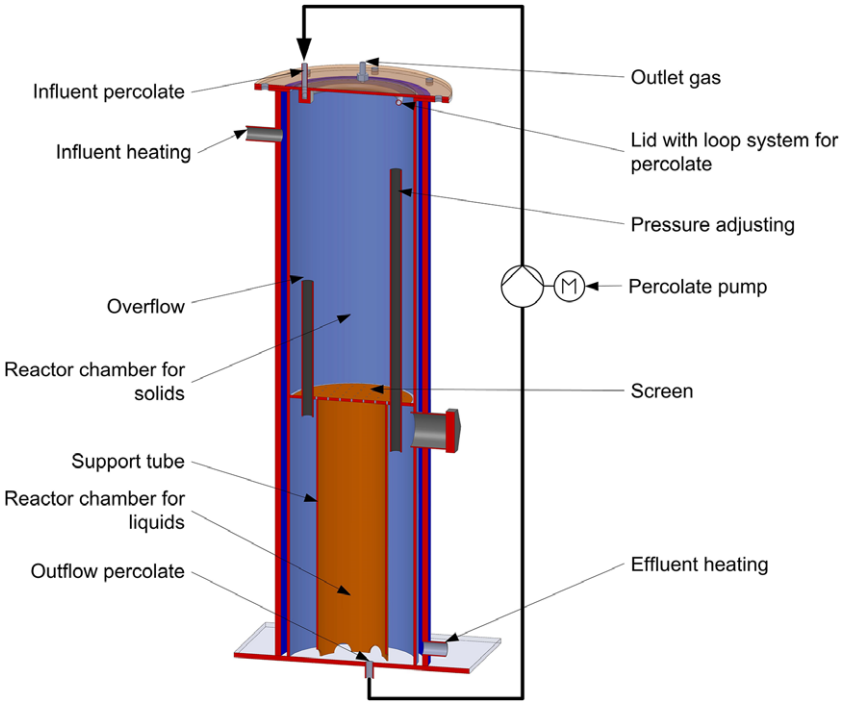

Figure 1. Schematic setup of the laboratory-scale batch leach-bed digester. (Source: Zechendorf/Pröter/Stur ©2016).

with $Fe(OH)_3$ or $Mn(OH)_2$, maize silage with TS = 36.3% and VS = 95.6%$_{TS}$ was used. Two kg$_{Fresh\ Mass}$ maize silage was filled into the upper chamber of each reactor. Prior to that, the substrate was mixed with 20 g DGμ according to [21], 15 g $Fe(OH)_3$, or 15 g $Mn(OH)_2$, respectively. Assays without any trace element addition served as references. Five kg deionized water used as a basis for the process liquid in every reactor was poured over the substrate. As an inoculum, 1 kg process liquid from a two-phase biogas plant located in eastern Saxony (Germany) with maize silage mono-digestion was added to each reactor. After checking the gas-tightness of the reactors, the reactors were flushed with 3 bar overpressure N_2 for 1.5 h. Afterwards, sequential percolation (average liquid flow, 300 mL every 30 min) was carried out throughout the whole experimental period. An automatic pH control was not applied. All experiments were run in duplicates to ensure reproducibility of the data.

2.3 Analysis of substrate and process parameters

TS and VS contents of substrate or solid digestate were analyzed as reported previously [22]. Crude protein, crude fat, nitrogen-free extractives, cellulose, and hemicellulose content of maize silage and solid digestate were determined according to standard procedures [23,24]. Conversion degrees (in %) were determined as described by Sträuber et al. [22]. The average percolate volume measured after AF without TE addition was used for calculation of conversion degrees of all experiments.

The pH values of liquid samples were measured using a pH meter (model 3310; WTW, Weilheim, Germany) equipped with a pH electrode Sentix 41 (WTW). For chemical analyses, 35 mL samples were centrifuged for 10 min at 10 000 × *g* and 10°C (CT 15RE; Hitachi Koki, Tokyo, Japan). The pellet was washed with 1 M phosphate buffered saline (PBS) pH 7.0 or 1 M Tris/HCl buffer pH 8.5 and frozen at −20°C until DNA extraction. The supernatant was used for chemical analyses. The concentrations of volatile fatty acids (VFA; acetic, propionic, *n*-butyric, *iso*-butyric, *n*-valeric, *iso*-valeric, and *n*-caproic acid) were determined in triplicate by gas chromatography (7890 A, Agilent Technologies) as previously described [25]. Lactic acid, ethanol, 1-propanol and 1,2-propanediol were analyzed by high-performance liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector RID-10A and a HiPlex H column with a pre-column (Agilent Technologies). The HPLC measurements were conducted as described by Sträuber et al. [22]. Prior to that, the supernatant was filtered using Nanosep® MF filter units with modified nylon membranes (pore size, 0.2 μm). The ammonia nitrogen concentration was determined according to Lv et al. [26].

Volume of the produced gas was measured using Milligas-counters MGC-1 V3.1 (Ritter Apparatebau, Bochum, Germany). The measured gas volume was normalized to standard pressure and standard temperature conditions using the following equation:

$$V_N = \frac{\left(p - 10^{\left(7.19621 \frac{1730.63}{233.426+T}\right)} + 0.1\ \text{kPa}\right) \times 273.15\ \text{K}}{101.325\ \text{kPa} \times (273.15+T)\ \text{K}} \times V_{gas}$$

where V_N is the gas volume at standard atmospheric pressure of 101.325 kPa and standard temperature of 273.15 K (mL), p is the ambient pressure (kPa), T is the ambient temperature (°C), and V_{gas} is the volume of the raw gas (mL). The gas composition (hydrogen, carbon dioxide, nitrogen, oxygen, methane) was measured by gas chromatography as described by Sträuber et al. [25]. Since molecular nitrogen and oxygen were not micro-

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bially produced during the process, the detected concentrations of hydrogen plus carbon dioxide were set to 100%. Methane was not detected.

2.4 Microbial community analysis

The composition of the bacterial communities was analyzed by T-RFLP fingerprinting of 16S rRNA genes in combination with a clone library. Total DNA was extracted from frozen cell pellets using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's protocol. DNA concentration and quality were determined photometrically using a NanoDrop® ND-1000 UV-Vis spectral photometer (Thermo Fisher Scientific Inc., Waltham, USA) and by agarose gel electrophoresis. The polymerase chain reaction (PCR) was carried out as described by Ziganshin et al. [27] with the exception that only 12.5 μL reaction mixture containing 2 ng template DNA, 3.5 pmol each of the Bacteria-specific primers 27F and 1492R [28], 1 μL of 99.5% dimethyl sulfoxide, 2.85 μL of nuclease free water, and 6.25 μL of *Taq* Master Mix (Qiagen, Hilden, Germany) were used. PCR products were purified and quantified as described previously [25]. T-RFLP analysis was done as described by Sträuber et al. [22]. Ten units of *Msp*I (New England Biolabs, Frankfurt/Main, Germany) were used as restriction enzyme to digest 20 ng of purified PCR product. Fragment analysis by capillary electrophoresis and raw data analysis were done according to Sträuber et al. [25]. Taxonomic assignment of terminal restriction fragments (T-RF) was done based on calculated and experimental T-RF lengths of cloned 16S rRNA amplicons (Supporting Information 3). Cloning and sequencing were performed as described by Ziganshin et al. [27] with few modifications. PCR products were purified as specified by Sträuber et al. [22], and for assembling of single sequence reads, the program Sequencher 5.3 (Gene Codes Corp., Ann Arbor, USA) was used. Clone sequences were checked for chimeras using DECIPHER [29]. The determined 16S rRNA gene sequences were deposited in the GenBank database under the accession numbers KT456558 - KT456621.

2.5 Statistical analysis

Multivariate statistical analysis of the normalized sample-peak tables was performed with the R package "vegan" [30, 31] in R Version 3.0.1 by nonmetric multidimensional scaling (NMDS) as described by Sträuber et al. [1]. The major process parameters as well as single T-RF determining the community composition were fitted using the "envfit" algorithm provided within the "vegan" package. Vectors of T-RF without taxonomic assignment were removed from the plot for the sake of clarity. The significance of the fitted single process parameters and the NMDS results were tested using a Monte-Carlo test with 1,000 permutations. Furthermore, significant correlations between T-RF assigned to lactic acid bacteria (LAB) and other bacterial T-RF were examined based on the Spearman's rank correlation coefficient by using the R package "corrplot" with its embedded significance test (level at $p < 0.05$) [32].

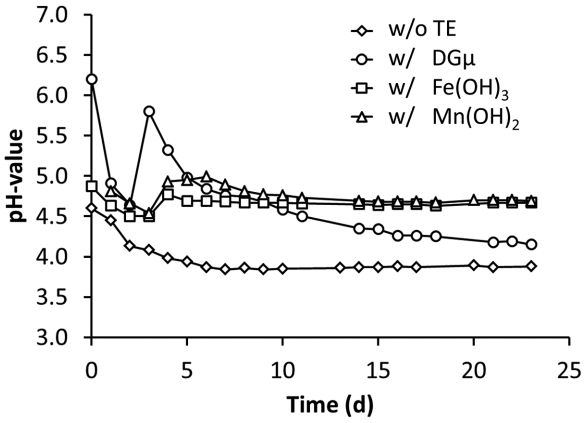

Figure 2. Development of the pH values during the AF of maize silage. Without TE additions (w/o TE), with DGμ (w/ DGμ), with $Fe(OH)_3$ (w/ $Fe(OH)_3$), with $Mn(OH)_2$ (w/ $Mn(OH)_2$).

3 Results

3.1 Effect of trace element addition on the reactor performance

The fermentation of maize silage in leach-bed reactors with the addition of DGμ, $Fe(OH)_3$, or $Mn(OH)_2$ was monitored for 23 days. As a reference system, maize silage was used as a substrate without any TE addition. The TE supplementation resulted in an increase of the initial pH values (Fig. 2) ranging from pH 4.6 (reference reactor) to pH 6.2 (experiment with DGμ). The development of the pH values in the replicate experiments is shown in the Supporting information 4. Independently of the initial pH, the pH values in all experiments dropped during the fermentation below 5.0. In the experiments with TE supplementation, an intermittent pH increase up to maximum 5.8 at day 3 and 4 was observed, whereas this was not detected in the experiment without TE addition. The lowest final pH value of 3.9 was measured in the experiment without TE addition.

An 11 to 36% higher final product concentration including organic acids and alcohols was obtained when DGμ was added compared to the reference experiment without TE addition (Table 1). This was owing to the higher VS conversion degree of 36 $\pm$ 1.7% compared to 25 $\pm$ 1.6% in the reactors without TE addition. Mainly the substrate fractions nitrogen-free extractives and hemicellulose were considerably better converted when DGμ was added (complete analytic results of substrate and solid digestates as well as conversion degrees are shown in the Supporting Information 5). For comparison, when $Fe(OH)_3$ or $Mn(OH)_2$ was added, 28–36% or 15–35% less organic acids and alcohols were produced, respectively (Table 1). The ammonia nitrogen (NH_4^+-N) contents in the reactors were between 0.25 and 0.49 g/L and only small differences between the experiments were observed (Supporting Information 6).

In all fermentation experiments, maize silage was degraded to typical fermentation products. No methane production was detected. The organic acids and alcohols detected at the beginning of the experiments (Fig. 3, data of the replicate experiments are depicted in the Supporting Information 7) originated from

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Table 1. Product concentrations and yields from the anaerobic maize silage fermentation without TE addition, with DGμ, with $Fe(OH)_3$, and with $Mn(OH)_2$

Experiment	Product concentration ($g_{product}/L$)	Product yield ($g_{product}/kg_{VS}$)	Lactic acid yield ($g_{lactic\ acid}/kg_{VS}$)	*n*-Butyric acid yield ($g_{n\text{-butyric acid}}/kg_{VS}$)	*n*-Caproic acid yield ($g_{n\text{-caproic acid}}/kg_{VS}$)
w/o TE	23.9 (±1.1)	207 (±5)	84 (±5)[a)]	13 (±4)	0.1 (±0.0)
w/DGμ	29.4 (±1.6)	293 (±15)	110 (±12)	131 (±0)	1.6 (±1.1)
w/$Fe(OH)_3$	16.3 (±0.3)	146 (±6)	7 (±7)	96 (±3)	8.1 (±2)
w/$Mn(OH)_2$	17.8 (±1.6)	156 (±15)	0.08 (±0.08)	70 (±43)	5.3 (±1.3)

[a)] based on lactic acid concentrations on day 10.
The sum of all measured organic acids and alcohols was termed as product. Averages were calculated from values of all experiments (including the replicate experiments). The differences of average values from minimum and maximum are indicated in parentheses. Yields were related to the added substrate VS amount.

the ensilage process and were leached out from the substrate during percolation. Without TE addition, lactic acid fermentation dominated during the experimental time (Fig. 3A). The maximum lactic acid concentration of 10.2 g/L was reached after 10 days corresponding to a yield of 84 ± 5 g/kg_{VS}. Afterwards, lactic acid was slowly degraded. Besides, acetic acid and 1-propanol were produced in increasing concentrations up to 7.1 and 2.7 g/L, respectively. Propionic acid, ethanol, and *n*-butyric acid were produced in minor concentrations up to 2.2, 1.7, and 0.8 g/L, respectively. Almost no *n*-valeric and *n*-caproic acid (final concentration of both compounds: 0.01 g/L) were detected. The concentrations of the branched-chain VFA and 1,2-propanediol were lower than those of the unbranched VFA (Supporting Information 8). In total, 16.77 L gas was produced (see gas composition in the Supporting Information 9). The microbially produced gas share was composed of 4.1% hydrogen and 95.9% carbon dioxide. A maximum oxygen content of 3.3% was detected.

When TE were added to the system, the fermentation characteristics changed. With DGμ, the fermentation started in a similar way with mainly lactic and acetic acid fermentation (Fig. 3B). However, after two days lactic and acetic acid were degraded while up to 13.7 g/L *n*-butyric acid was produced. The final *n*-butyric acid yield was 131 ± 0 g/kg_{VS}. The lactic acid concentration increased again after day 8 up to a yield of 110 ± 12 g/kg_{VS}. The concentrations of all other products remained below 1.9 g/L. With a gas amount of 36.08 L produced until day 10, the gas production was more than doubled compared to the reference experiment. The produced gas contained more hydrogen (12.3%) and accordingly less carbon dioxide (87.7%) (the complete gas composition is shown in the Supporting Information 9). Afterward, low pressure was observed. An increase of the oxygen content up to 13.8 % was measured, probably caused by the development of leakages.

In the experiments with $Fe(OH)_3$ or $Mn(OH)_2$, the fermentation characteristics differed only slightly (Fig. 3C and D). At the

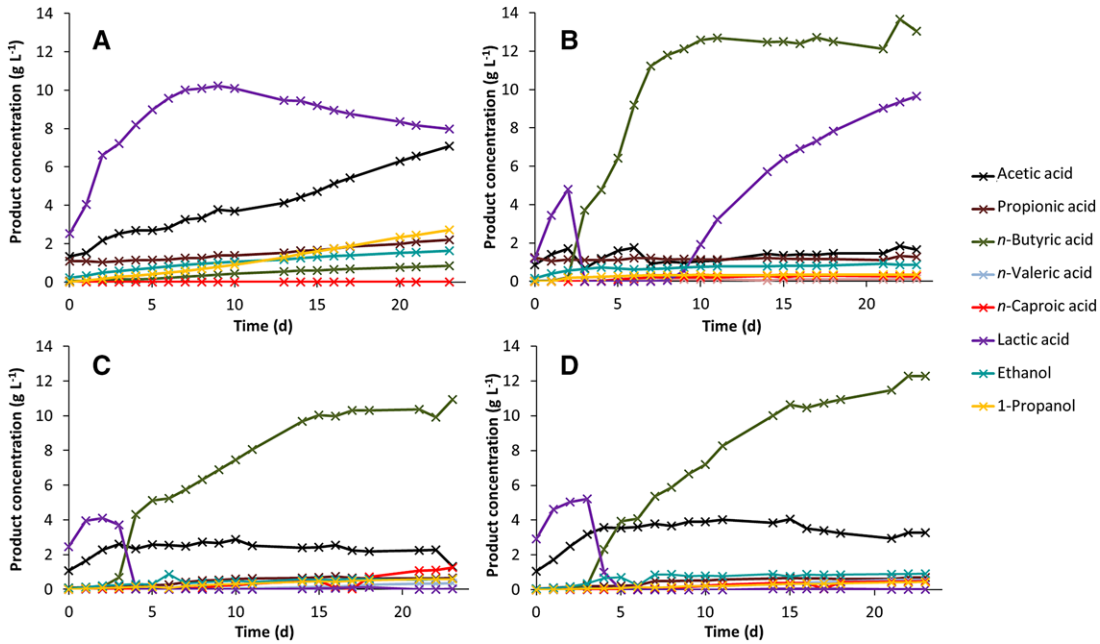

Figure 3. Concentrations of organic acids and alcohols in the AF of maize silage. (A) Without TE additions, (B) with DGμ, (C) with $Fe(OH)_3$, (D) with $Mn(OH)_2$.

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beginning, lactic acid was produced and subsequently degraded as it was similarly observed in the experiment with DGμ. Afterwards, *n*-butyric and acetic acid fermentation predominated. After day 4, the lactic acid concentration was throughout below 0.15 g/L and no re-accumulation of lactic acid was observed. With $Fe(OH)_3$, the final concentrations of the main products *n*-butyric and acetic acid were slightly lower compared to the experiment with $Mn(OH)_2$. However, this difference was not confirmed to be significant when the replicate experiments were taken into account (Supporting Information 8). The highest *n*-caproic acid concentrations were reached at 1.2 g/L (with $Fe(OH)_3$, Fig. 3C) and 1.4 g/L (replicate experiment with $Mn(OH)_2$, Supporting Information 8). This corresponded to *n*-caproic acid yields of 8.1 ± 2 g/kg$_{VS}$ (with $Fe(OH)_3$) and 5.3 ± 1.3 g/kg$_{VS}$ (with $Mn(OH)_2$). The gas production in the experiment with $Fe(OH)_3$ was with 19.78 L higher than in the experiment with $Mn(OH)_2$ (15.40 L), but lower than with DGμ. The produced gas of the experiment with $Fe(OH)_3$ contained with 22.4% the highest hydrogen content (carbon dioxide: 77.6%), whereas the hydrogen content of the gas originating from the experiment with $Mn(OH)_2$ was only 10.8% (carbon dioxide: 89.2%) (see the Supporting information 9 for complete gas composition). Maximum oxygen contents in the second half of the experiments were 6.7% (with $Fe(OH)_3$) and 16.1% (with $Mn(OH)_2$). In all experiments, the concentrations of *iso*-butyric and *iso*-valeric acid were 0.31 g/L or less, while the final concentration of 1,2-propanediol was not higher than 0.4 g/L.

3.2 Composition and dynamics of the bacterial communities

T-RFLP profiles showing the dynamics of the bacterial communities in the AF experiments are depicted in Fig. 4. Except at the starting point, the bacterial communities in the reference reactors (without TE addition) were dominated by LAB comprising more than 60%. The T-RF 178, 573, and 582 assigned to different *Lactobacillus* phylotypes predominated. Other abundant bacterial phylotypes were *Acetobacter* 440 (the number after the taxon name always indicates the T-RF length in bp) (up to 20% relative abundance) and *Paenibacillus* 131 (up to 7%). Phylotypes affiliated with the *Clostridia* were detected at the beginning (day 0) and only occasionally and in low abundances during the progress of the experiment. The bacterial community composition was relatively stable during the experimental time. The proportions of single phylotypes shifted slightly, but only few new T-RF emerged during the fermentation, e.g. *Lactobacillus* 563 emerged from day 16 on and reached a relative abundance of 13% on day 23.

In the experiment with DGμ addition, the bacterial community was more dynamic. Additionally, the richness was with 50 T-RF higher compared to the experiment without TE addition (24 T-RF; data from day 0 not considered, respectively). The proportion of LAB decreased from more than 85% on day 2 to about 31% at the end of the experiment. First, the LAB proportion dropped significantly from day 2 to day 3. Then, although the lactic acid concentration decreased, the LAB proportion increased again and reached more than 80% of the total bacterial community on day 9. Afterwards, the LAB share decreased in an erratic manner, whereas the lactic acid concentration increased steadily. Meanwhile, the abundance of bacterial phylotypes belonging to *Clostridiales* increased up to 55–65%. T-RF 506, 516, 517, and 520 assigned to *Clostridium sensu stricto* (*s.s.*) predominated. Furthermore, *Acetobacter* 440 was detected, but in smaller proportions than in all other experiments.

In the experiments with $Fe(OH)_3$ or $Mn(OH)_2$, the LAB sub-communities were similar in their composition as was the reactor performance. The proportions of the LAB sub-communities decreased during the fermentation from about 73% (day 1, experiment with $Fe(OH)_3$) to less than 21% (day 23, experiment with $Fe(OH)_3$), and to 11% (day 23, experiment with $Mn(OH)_2$) as similarly observed in the experiment with DGμ. In contrast, with $Fe(OH)_3$ or $Mn(OH)_2$ the reorganization of the LAB sub-communities was not that all-encompassing. *Lactobacillus* 573, which was abundant at the beginning of the experiment, was overgrown quickly. Besides *Lactobacillus* 178, *Lactobacillus* 175 additionally predominated in these experiments, whereas the latter was not at all detected in the reference experiment or with DGμ. The clostridial sub-communities showed a relatively high diversity and dynamics. *Clostridium s.s.* 516, 517 and 519 (together up to 38% relative abundance) as well as *Ruminococcaceae* 286 (up to 11%) and *Lachnospiraceae* 296 (up to 16%) predominated in distinct time ranges. Besides, *Megasphaera* 297 and 298 (together up to 50%), *Veillonellaceae* 303 (up to 8%), *Acetobacter* 440 (up to 17%), *Ureibacillus* 164 (up to 23%), and two different T-RFs assigned to *Prevotella* (T-RF 86 and 90, together up to 28%) were detected with high relative abundances. However, their proportions differed in the replicate experiments.

The dynamics and similarity of the bacterial communities based on their T-RFLP profiles and their correlations with process parameters and single T-RF are visualized in NMDS plots shown in Fig. 5. The strong shifts of the bacterial communities from the beginning to the end of the experiments are clearly shown. The data points of the reference experiment clustered indicating the high similarity of the bacterial communities therein. The community composition in this experiment was shaped by the formation of lactic and acetic acid as well as by the occurrence of *Lactobacillus* 582, 573, 178 and 580 as indicated by vectors in the NMDS plots. In contrast, the scattered distribution of the data points of the other experiments (with TE addition) showed the higher dynamics of the respective bacterial communities. Additionally, relative dissimilarities of the bacterial communities of the DGμ experiment compared to those of the experiments with $Fe(OH)_3$ or $Mn(OH)_2$ were indicated by the distance of the respective data points. The communities of the DGμ experiments were associated with the production of ethanol as well as with the development of *Clostridium s.s.* 506 and 517. Additionally, the occurrence of distinct LAB shaped the communities of this experiment: *Lactobacillus* 574 and 563 as well as *Olsenella* 53. The production of the MCFA *n*-valeric and and *n*-caproic acid as well as the *iso*-forms of butyric and valeric acid was characteristic for the communities of the experiments with $Fe(OH)_3$ and $Mn(OH)_2$. Furthermore, T-RF assigned to *Clostridiales* (T-RF 286, 296) and *Megasphaera* T-RF 298 influenced the development of these communities. Although the lactic acid concentration was very low in these experiments, one LAB T-RF was

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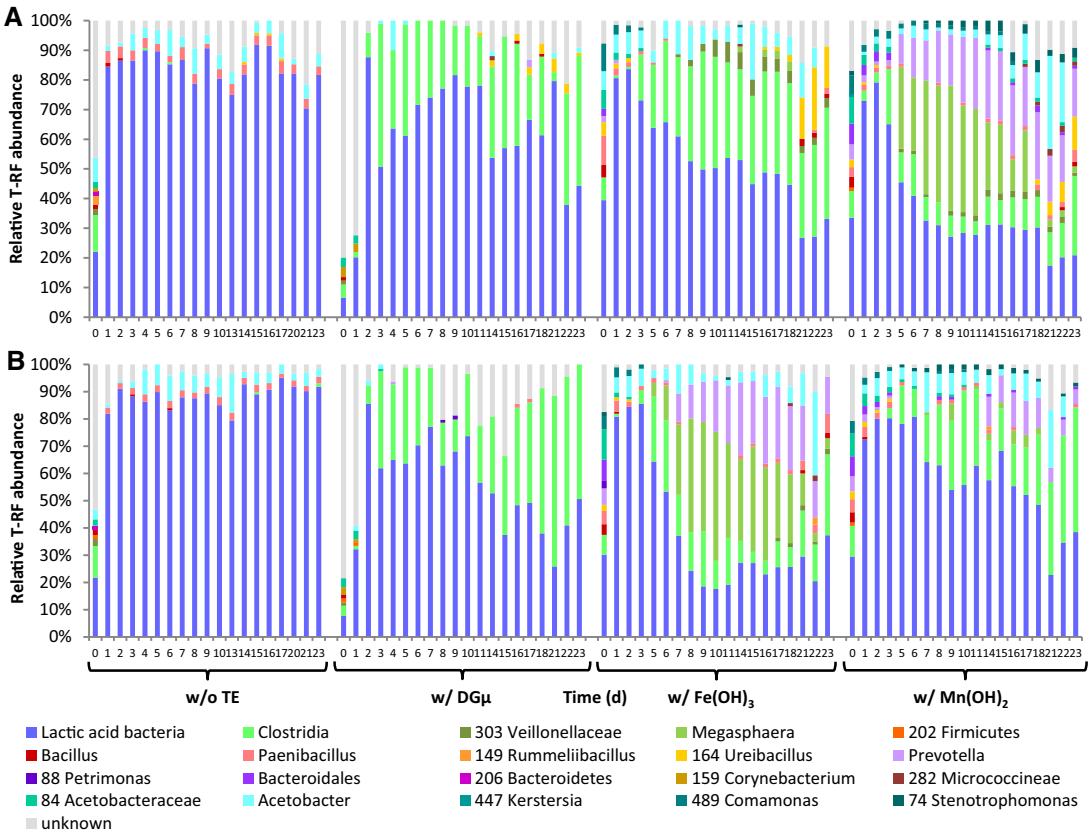

Figure 4. Bacterial community dynamics during the AF of maize silage without TE additions (w/o TE), with DGμ (w/ DGμ), with $Fe(OH)_3$ (w/ $Fe(OH)_3$), and with $Mn(OH)_2$ (w/ $Mn(OH)_2$). (A) and (B) represent replicate experiments. "Lactic acid bacteria" comprised 14 different T-RF assigned to the genus *Lactobacillus*, T-RF 53 (*Olsenella*), as well as T-RF 60 and 125 (*Coriobacteriaceae*). "Clostridia" comprised different T-RF assigned to the *Ruminococcaceae*, seven different *Clostridium* T-RF, four different T-RF assigned to the *Lachnospiraceae* as well as T-RF 323 and 483 (*Clostridiales*). "Megasphaera" comprised T-RF 297 and 298 assigned to the genus *Megasphaera*. "Bacillus" comprised three different T-RF assigned to the genus *Bacillus*. "Paenibacillus", "Prevotella", "Bacteroidales" and "Acetobacter" comprised each of two T-RF assigned to the respective taxon. "Unknown" indicates 60 different T-RF that could not be identified.

found shaping the communities of the $Fe(OH)_3$ and $Mn(OH)_2$ experiments, i.e. *Lactobacillus* 175.

Correlation analyses were performed to investigate in more detail the functional interrelationships between LAB and clostridial fermenters or *Megasphaera* (Supporting Information 10). In total, 13 negative correlations between LAB and clostridia/*Megasphaera* with correlation factors (r_S) between -0.41 and -0.59 were detected. *Lactobacillus* 573 and 582, which predominated in the reference experiment and partly at the beginning of the other experiments, were always negatively correlated to various clostridia or *Megasphaera*. This was also observed for *Lactobacillus* 178 which predominated in all experiments and in the DGμ experiment particularly in the end. In total, 18 positive correlations of LAB to clostridia or *Megasphaera* with r_S between 0.40 and 0.82 were observed. *Lactobacillus* 563 and 574, which predominated in the DGμ experiment, were positively correlated to *Clostridium s.s.* 506 and 517. *Lactobacillus* 175, which predominated in the experiments with $Fe(OH)_3$ or $Mn(OH)_2$, was positively correlated to *Ruminococcaceae* 286 as well as *Megasphaera* 297 and 298 and *Veillonellaceae* 303.

4 Discussion

4.1 The pH effect of the TE supplements on the AF

The addition of alkaline TE supplements increased the pH values in the reactors, resulting in varying acidogenic fermentation performance and different bacterial communities. Without addition of any TE supplements, the substrate-caused low pH value dropped even further and lactic acid production dominated, but low yields of other organic acids resulted probably due to low hydrolysis rates [33]. The respective bacterial catalysts, i.e. LAB, were highly abundant. Lactic acid is produced mainly by LAB via homolactic or heterolactic fermentation and represents a characteristic fermentation product from ensiled substrates [1, 22, 34]. LAB can resist oxidative and acid stress [35] as well as low pH values [36].

When TEs were added, mixtures of LAB and clostridial strains dominated resulting in an extended product spectrum. These products are comprised of considerable concentrations of VFA like acetic and *n*-butyric acid, besides smaller concentrations of MCFA and other compounds. The abundance of LAB was

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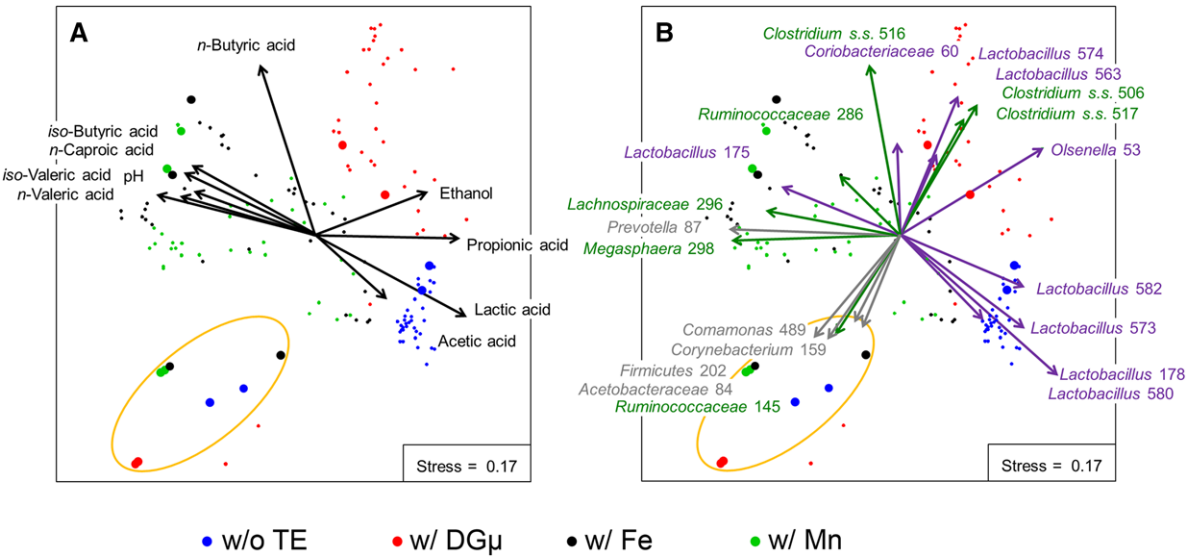

Figure 5. NMDS plots of T-RFLP profiles of bacterial 16S rRNA gene amplicons of the AF experiments. Without TE additions (blue), with DGμ (red), with $Fe(OH)_3$ (black), and with $Mn(OH)_2$ (green data points). Community similarity is based on the Bray-Curtis index, which includes the presence and relative abundance of T-RF. Starting and end points of the respective experiments are indicated by bigger data points, while the hull encloses the communities of the starting points. (A) Black arrows indicate vectors of process parameters (significance factors $p < 0.05$). (B) Green, lilac and grey arrows indicate vectors of single clostridial, LAB and other T-RF, respectively, which shaped the communities most (significance factors $p < 0.001$).

surprisingly high in periods where lactic acid concentration was below the detection limit, suggesting that LAB could compete with the clostridial strains and probably even profited from their hydrolytic activity. Starch can be hydrolyzed by LAB alone [37, 38], but cellulose hydrolysis by LAB has not been reported so far [39]. The relation of pH and lactic as well as butyric acid production has been reported previously [40, 41]. Temporary oxic conditions in the reactors may have further favored LAB because LAB tolerate oxygen better than the strictly anaerobic clostridia. The occurrence of *Acetobacter*, *Rummeliibacillus* and *Ureibacillus*, which are strict aerobes, additionally indicated that oxygen influenced the community development [42–44]. Oxygen in the reactors resulted from substrate input and from low pressure probably causing reactor leakages as the lab equipment was not designed for such pressure conditions. It can be assumed that oxygen plays a role also in large scale leach-bed reactors. This is caused by frequent addition of large amounts of substrate, which is often saturated with oxygen.

4.2 Role of iron and manganese in anaerobic fermentation and MCFA production

Besides the pH effect caused by the addition of the alkaline supplements, the effect of the metallic cations on the microorganisms has to be considered. Generally, clostridia are supported by iron addition due to their comparably high demand. It is part of their cytochromes and of various iron-sulfur proteins which are involved in energy metabolism [45]. In contrast to most clostridia, LAB are devoid of cytochromes [46]. Moreover, enzymes of the Embden-Meyerhof-Parnas (EMP) pathway used by LAB do not need iron as a cofactor. Thus, iron-deficient conditions systematically favor lactic acid fermentation in mixed cultures [47, 48], and LAB overgrew clostridial competitors in the reactors without TE addition. Furthermore, the pyruvate ferredoxine oxidoreductase catalyzing the decarboxylation of pyruvate to acetyl-CoA, which is necessary for *n*-butyric acid fermentation, needs iron as a cofactor [48], and also clostridial hydrogenases are Fe-dependent. Accordingly, highest hydrogen contents in the gas were measured in the experiments with iron addition. Iron- or manganese-dependent superoxide dismutases (SOD) play a key role in adaptation of aerobic and facultative anaerobic bacteria to oxidative stress [49]. The oxygen content in the reactors led probably to oxidative stress on bacteria. Some microorganisms can freely exchange the iron in the SOD by manganese [50, 51]. Optimal conditions for such bacteria helped reduce the oxygen content in the reactors, thus providing anaerobic conditions for the more oxygen-sensitive bacteria. High manganese concentrations particularly supported LAB because the NAD-dependent lactate dehydrogenase needs Mn for its function [52]. Furthermore, LAB are able to form non-proteinaceous manganese antioxidants to combat oxidative damage [49], which can be accumulated intracellularly in high concentrations [53, 54].

4.3 Carbon chain elongation of short-chain fatty acids with lactic acid

In periods with low lactic acid concentrations, most likely lactic acid was produced but instantly consumed by secondary processes. Lactic acid can be converted to acetic and propionic acid by microbes using the acrylyl-CoA or the methylmalonyl-CoA pathway [55] or solely to acetic acid by heterotrophic

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homoacetogens exploiting lactate dehydrogenase, pyruvate decarboxylase and the Wood-Ljungdahl pathway [56]. Alternatively, the reduction to 1,2-propanediol and ethanol is possible [57]. However, the predominant pathway in our system seemed to be the conversion of lactic to *n*-butyric acid. This was obvious in periods between day 2 and 4 in the experiments with TE addition and was similarly observed in a previous study [21]. Various bacteria are known to catalyze this reaction, e.g. *Clostridium acetobutylicum* [58], *Clostridium tyrobutyricum* [59] and *Megasphaera elsdenii* [60,61]. Several phylotypes assigned to the genus *Clostridium* as well as members of the genus *Megasphaera* were detected as abundant T-RF in the experiments with DGμ and $Fe(OH)_3$ or $Mn(OH)_2$, respectively. Some abundant T-RF can be potentially affiliated to *C. tyrobutyricum* (*Clostridium* 517, 99% BLAST identity) or to strain MB9-9 (*Clostridium* 506, 100% BLAST identity), which is related to *C. tyrobutyricum* [62] (Supporting Information 3). This adumbrates a related metabolic potential of these phylotypes. Based on the positive correlations of clostridial phylotypes with LAB, we speculate that there was a direct relationship between lactic acid produced by LAB and its subsequent conversion to *n*-butyric acid by *Clostridium* (T-RF 506 and 517) and *Megasphaera*.

In the experiment with DGμ, accumulation of lactic acid was observed after day 9, while *n*-butyric acid production stagnated. Oxic or micro-oxic conditions caused by leakages at this time point may have supported this development. Additionally, the pH value was with 4.8 very low and decreased even further. The environmental conditions were obviously not suited for complete lactic acid conversion at this low pH value. The comprehensive reorganization of the LAB community in the DGμ experiment may be explained by the limited hydrolysis capability of LAB as described above. Nevertheless, the highest product yields were reached with DGμ based on the mutual support of LAB and clostridia (hydrolysis and chain elongation). In the experiments with $Fe(OH)_3$ and $Mn(OH)_2$, the *n*-butyric acid production was slower compared to the DGμ experiment. Furthermore, with only $Fe(OH)_3$ or $Mn(OH)_2$ the production stopped after 15 days, although the pH values at this time point were higher compared to those of the DGμ experiment. Additionally, the lactic acid production discontinued leading finally to much lower product yields (referring to the sum of all fermentation products) in the $Fe(OH)_3$/$Mn(OH)_2$ experiments compared to the DGμ experiment. Probably, not only the pH-increasing effect of the TE supplements influenced the AF. The complex composition of DGμ additionally supported the bacteria with TEs other than iron, indicating that the TEs originating from the substrate or the inoculum were not sufficient for optimal performance.

The experiments differed in their MCFA production performance. With $Fe(OH)_3$ or $Mn(OH)_2$, more MCFA, i.e. valeric and caproic acid, were produced than with DGμ, where the pH was lower. The pH value in the $Fe(OH)_3$/$Mn(OH)_2$ experiments was between 4.5 and 5.0, at which also Kucek et al. [14] obtained the highest caproic acid productivities from chain elongation of butyric with lactic acid. At lower pH values, the inhibitory effect of undissociated hydrophobic carboxylic acids on bacteria probably prevailed. For the effective selection of acid-tolerant MCFA producers, semi-continuous conditions, e.g. by time-delayed operation of replicate leach-bed reactors, have to be established. The bacterial communities in the $Fe(OH)_3$/$Mn(OH)_2$ experiments were linked to MCFA production. Furthermore, distinct phylotypes (*Lachnospiraceae* 296, *Ruminococcaceae* 286 and *Megasphaera* 298) were related to these metabolic functions. *Megasphaera elsdenii* is a known caproic acid producer [63]. It can catalyze carbon chain elongation processes and uses lactic acid as a growth substrate [60,61].

5 Concluding remarks

The use of chemicals like NaOH and HCl for adjusting pH in the AF is one of the main parameters affecting the life cycle impact of carboxylic acid production, e.g. caproic acid [64]. The addition of solid alkaline trace element supplements such as residues from drinking water treatment can support the pH control required in solid-state AF. Thus higher total product yields can be reached. Careful application of the supplements in the reactor is essential to avoid environmental pollution by the fermentation residues. For the production of selected MCFA, an additional automatic pH control in the AF reactor is recommended to support bacterial chain elongation processes. By alkaline trace element addition, pH controlling chemicals can be saved and spatial metabolic heterogeneities in the solid material are mitigated. Hence, substrates like crop silages or the organic fraction of municipal solid waste can be effectively utilized for the production of chemicals. Such substrates contain high amounts of lactic acid, which can be used for chain elongation and is a substitute for ethanol. The in-situ lactic acid formation during AF supports the chain elongation further. Potential strategies that stimulate this process may be developed in future studies.

Practical application

For biogas production from solid biomass, leach-bed reactors are state of the art. After minor technical modifications, such reactors might alternatively be used for the production of organic acids which are platform chemicals with a wide application range or can be utilized for biogas production in a demand-driven mode. Medium-chain fatty acids from anaerobic fermentation processes are particularly well suited for the production of lubricants, detergents or fuels. They can be applied as mixtures with less cost-intensive down-streaming for purification and refining compared to the production of pure chemicals. Refitting existing biogas plants to biorefineries for the combined production of chemicals and energy carriers from biomass would open new perspectives and additional income for biogas plant owners and facilitate their flexibility.

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