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Enhanced production of propionic acid through acidic hydrolysis by choice of inoculum

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Abstract

BACKGROUND: In this study, the enhancement of propionic acid production from a model feedstock mimicking kitchen waste was investigated. For that purpose, two operational runs of a semicontinuous anaerobic hydrolysis reactor were carried out at pH 6.0 ± 0.1 and mesophilic (30 °C) temperature. Two different types of inocula, a mixed microbial culture selected over 24 months for growth on cellulose and a culture contained in goat cheese were compared.

RESULTS: The results show that the goat cheese inoculum was significantly more efficient for propionic acid (PA) production. The highest propionic acid concentration achieved amounted to 139 mmol L^{-1} at a yield of 23.3 mg g^{-1} volatile solids (*VS*), which was 55% greater than what was achieved with the mixed culture. Furthermore, it was observed that propionic acid production was enhanced by a combination of high hydraulic retention time (HRT) with low organic loading rate (OLR), ensuring sufficient time for complete processing of the complex organic substrates. The fermentation could be kept in a stable process of propionic acid production at HRT of 20 days and a rather low OLR of 11.1 g L^{-1} day^{-1} *VS*.

CONCLUSION: Our results give a better understanding of PA production in semicontinuous mode, applying optimized process parameters and selecting the adequate microbial community for inoculation. This study provides important information for the improvement of PA production from complex substrates for future industrial application.

Keywords: propionic acid; acidic hydrolysis; complex substrate; goat cheese

INTRODUCTION

Propionic acid (PA) and its salts are widely used in industries including agricultural, pharmaceutical and food as antifungal agents.[1,2] It also can be employed as precursor for the biotechnological production of value-added compounds, such as acetoin[3] and, thus, has been listed as an important platform chemical since the early 2000s.[4] Currently, most of the PA production around the world is by chemical synthesis through the oxidation of petrochemical raw materials such as propane or propionaldehyde.[5] Acidic hydrolysis is an alternative method that has gained greater attention for PA production from available renewable sources, such as organic waste. It is increasingly being applied with a focus on biohydrogen production, a process known as dark fermentation, in which organic waste is utilized to generate renewable energy.[3] However, the separation of single volatile fatty acids (VFA) from complex effluents such as the fermentation broth remains a challenge, owing to the complex nature and the presence of various organics.[6] Techniques such as electrodialysis,[7] reactive extraction,[8] reverse osmosis,[9] nanofiltration[10] and adsorption[11] have been investigated to separate and concentrate these acids from aqueous solution and fermentation broth. This downstream processing has to be considered carefully to make the hydrolytic process comparable to petrochemical synthesis in terms of commercial feasibility. As a first step, however, it is necessary generally to increase the portion of PA in the total VFA usually produced in acidic hydrolysis. Accordingly, this paper focuses on the optimization of PA production from a model organic kitchen waste.

In general, organic waste has shown great potential as feedstock for VFA and (bio)hydrogen production because of its constant availability, and high carbohydrate content.[12] Some researchers have

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used food waste as feedstock for successful VFA and hydrogen production via dark fermentation, applying anaerobic sludge as inoculum.[13,14] However, the concentrations of PA produced were quite low. In general, process parameters such as pH value, temperature, hydraulic retention time, organic loading rate and inoculum type are known to have strong impacts on PA production.[15]

Native PA-producing bacteria have been the primary candidates for the development of a biotechnological process, and several types of pure cultures and mixed cultures have been investigated. Species from the genera *Propionibacterium*, namely *P. acidipropionici* and *P. freudenreichii* are the most studied pure cultures for PA production from simple substrates such as glucose,[2,16] lactose[1] and glycerol.[16,17] Limited studies have been reported that applied anaerobic sludge as mixed culture inocula for PA production from glycerol[18] or crude glycerol.[19]

During the fermentation process of waste, some types of lactic acid (LA) bacteria (e.g. *Lactobacilli*) have an important function in breaking down carbohydrates, amino acids and monosaccharides into lactate, which is used by, for example, *propionibacteria* to produce PA as metabolic end-product.[20–22] The action of both microorganism types was reported to be important to increase the overall yield of PA.[23] Therefore, addition of a mixed culture of LA- and PA-producing microbial strains to the process seems to be promising. Few researchers investigated the species interaction in PA production in detail, but none of their studies shows the impact of these microorganisms on the breakdown of complex substrates (e.g. kitchen waste).Tyree *et al.*[24] used a mixed culture of *Lactobacillus* spp. and *Propionibacterium shermanii* to produce PA from simple substrates such as lactate, glucose and xylose. Border *et al.*[25] also produced PA from wheat flour with a mixed culture of *Propionibacterium*, *Lactobacillus* and *Streptococcus*.

Acidic hydrolysis of complex substrates with a special focus on and optimization of PA production has not been reported yet. Our aim in this work is to explore the efficacy of a mixed culture inoculum for PA production from food waste. We chose soft goat cheese as inoculum because it is naturally rich with LA and PA bacteria. For comparison, we also operated a reactor with a mixed microbial culture selected over 24 months for growth on cellulose.

MATERIALS AND METHODS

Substrate characteristics

In Table 1, the characteristics of the vegan, grain-free dog food (DF) used in this study are compared with organic waste sources applied in other studies. Total solids (TS), volatile solids (*VS*), carbon (C) and hydrogen (H) contents of the dog food on a dry-weight basis were within the sometimes broad ranges of the kitchen waste (KW)[26] and food waste (FW).[27] Only nitrogen (N) content is slightly higher and, thus, C:N ratio is a bit lower. However, the composition of kitchen and food waste will certainly vary from location of production to season. Kim *et al* (2003)[28] and Nagasaki *et al.* (2014),[29] who also used dog food as substrate in their studies, stated that it has similar ingredients to those in kitchen waste. Additionally, it provides reproducible experimental conditions because of its standardized composition. The vegan dog food used in this study was composed of dried potato, pea flour, potato protein, sunflower oil, beet and apple fibre, hydrolyzed vegetable protein, ground chicory root, herbs, fruits and dried algae.

Reactor configuration

A cylindrical stirred-tank reactor (BTP2, UIT Umwelt- und Ingenieurtechnik GmbH, Dresden, Germany) was operated in this study (Fig. 1). The reactor was made of glass and had a total volume of 15 L (12 L working volume). The temperature was maintained at 30 °C by means of an electrical heating control unit, and the pH value was automatically controlled at 6 ± 0.1 (by adding 5 mol L^{-1} NaOH or 3 mol L^{-1} HCl solutions). The substrate was fed manually through a feeding funnel located at the top of the reactor. For biogas production rate measurement a gas counter (MilliGascounter, Dr.-Ing. RITTER Apparatebau GmbH & Co. KG, Bochum, Germany) was connected to the top of the reactor to measure the biogas production rate. The gas produced during the fermentation process was sampled periodically by collection in a gasbag. The reactor was equipped with an internal agitator, which consisted of two parts: an upper U-shaped anchor-stirrer and a lower propeller shaped stirrer. The stirrer speed was set to 100 rpm to ensure homogeneous mixing of the digestate.

Inoculation and operation of the reactor

In this study, two operational runs of the reactor are compared where temperature (30 °C) and pH (6 ± 0.1) were fixed, but which differed with regard to the type of inoculum, organic loading rate, retention time, and substrate:water ratio of the feed. The reactor was operated for approximately 100 days.

In Run 1, the reactor was inoculated with a mixed microbial population that was selected for 24 months for growth on cellulose. We chose this inoculum because cellulose will be the major carbohydrate in the C source of kitchen waste. Additionally, it had been noted that the culture produced significant levels of propionic acid from cellulose. To remove cellulose particles from the former feed of the culture, the inoculum was filtered through paper filter with 25-µm pore size. The reactor was initially fed with 4 kg dried dog food (3560 g *VS*; equivalent to 1760 g total carbon (TC)) mixed with 4 L bacterial culture and 4 L tap water corresponding to 297 g L^{-1} *VS* in total. Thus, the substrate:water ratio was 1:2. Within the first 14 days, the reactor was operated in batch mode. After that, the operation mode was switched to three consecutive repeated fed-batch (semicontinuous) phases; where the reactor was fed daily with an organic loading rate (*OLR*) of 12.3 g L^{-1} day^{-1} *VS* for 27 days, then 17.8 g L^{-1} day^{-1} *VS* for 30 days and finally

Table 1 Characteristics of the DF compared to KW and FW

Parameter	DF	KW[26]	FW[27]
pH value	5.43	4.48	4.1
TS	95.9 ± 0.1 (%)	201.70 ± 3.41 g L^{-1}	29.4 (%)
VS	93.4 ± 0.2 (% TS)	194.50 ± 2.73 g L^{-1}	95.1 (% TS)
C (% TS)	44.4	49.94 ± 0.02	49.6
N (% TS)	4.5	2.14 ± 0.06	3.5
H (% TS)	7.2	6.97 ± 0.01	7.3
C:N ratio	10	23	14

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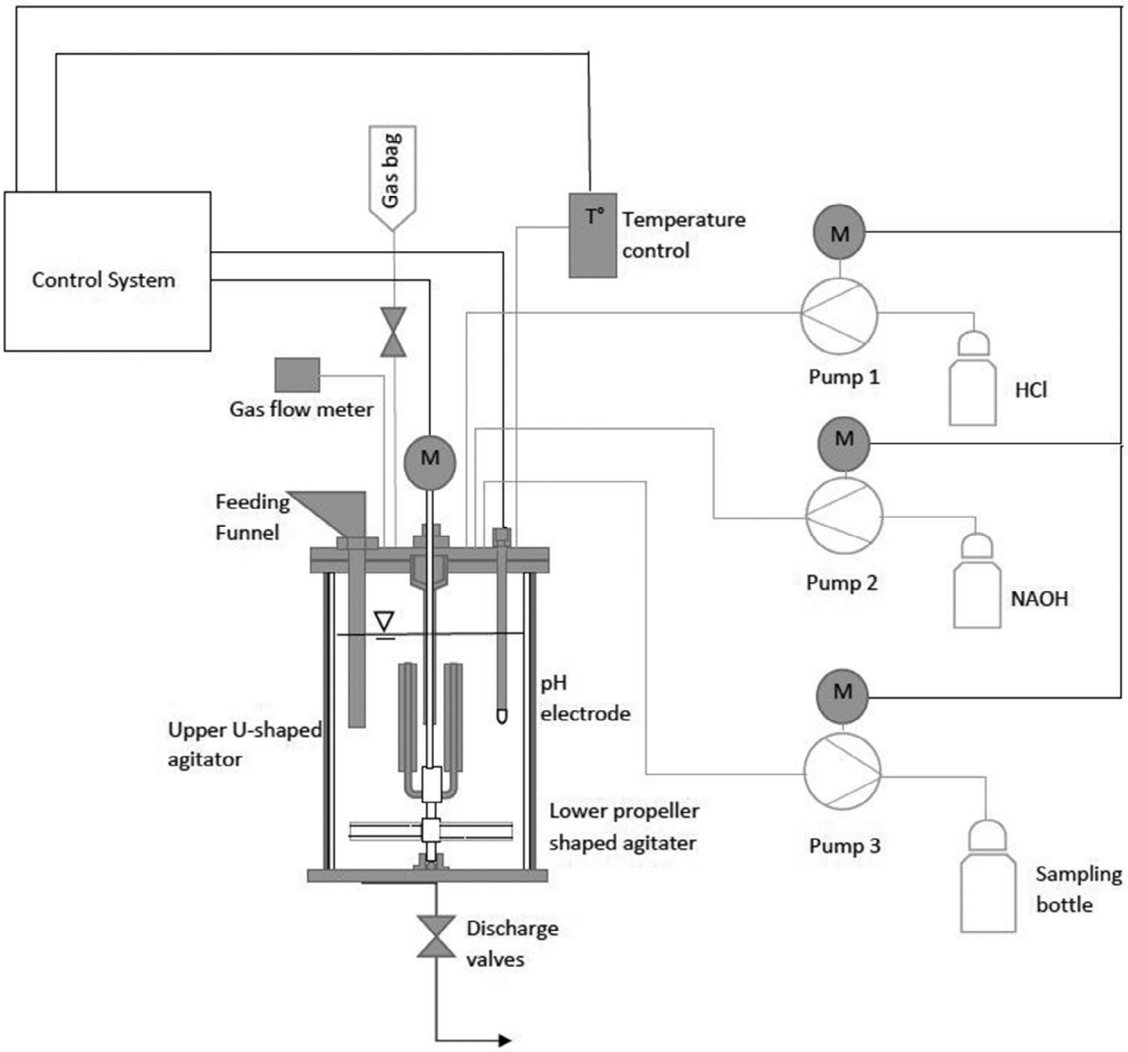

Figure 1 Schematic diagram of the reactor. M, motor.

29.7 g L^{-1} day^{-1} *VS* for 29 days (equivalent to 6.1, 8.8 and 14.7 g L^{-1} day^{-1} TC), respectively. This corresponds to hydraulic retention times (*HRT*) of 24, 16 and 10 days.

In Run 2, the reactor was inoculated with soft goat cheese ground into small particles using an iron grater. The reactor was initially fed with 3 kg dried dog food (2670 g *VS*; equivalent to 1320 g TC) mixed with 1 kg cheese (480 g *VS*) and 8 L tap water, corresponding to 260 g L^{-1}*VS* in total with a substrate:water ratio of 1:3. It also was started as batch for 10 days. As in Run 1, the operation mode was then switched to semicontinuous where the reactor was fed every second day with an *OLR* of 11.1 g L^{-1} day^{-1} *VS* (5.5 g L^{-1} day^{-1} TC) for another 90 days. The *HRT* was maintained at 20 days. The substrate:water ratios of the feed remained unchanged during both reactor runs.

It should be noted that the reactor was not operated under axenic conditions. Hence, the two starter communities from cellulose degradation and cheese production, respectively, are an addition to the community that naturally develops in the reactor.

Analytical methods

Samples were taken every two to three days to measure the concentrations of VFA, dissolved organic carbon (DOC), total solids (TS) and voltile solids (*VS*). Before the quantitative analysis, the samples were pretreated by centrifugation for 10 min at 8000 rpm, and then the supernatant was filtered through a 0.45-µm polyethersulfone (PES) membrane filter. The amount of VFAs was determined by ion chromatography analysis (Metrohm 881 Compact Pro, Herisau, Switzerland) using a Metrosep Organic Acids 250/7.8 column. DOC concentration was measured with a Shimadzu TOC-L_{CPH} analyzer (Duisburg, Germany). TS and *VS* measurements were carried out according to the DIN 38414.[30] Gas samples were collected every two to three days for composition analysis using gas chromatography (Agilent 490 micro GC, Santa Clara, United States).

DNA extraction and 16S Illumina MiSeq sequencing

The bacterial diversity in the reactor was assessed via amplicon sequencing using the Bact_341F/Bact_805R primer pair.[31] To this end, we took 200–300 mg samples at different time points and extracted genomic DNA by applying the innuSPEED Soil DNA Kit (Analytic Jena) according to the manufacturer's instructions. The microbial diversity was assessed via Illumina MiSeq sequencing (paired-end, 2 × 250 bp reads) conducted by IMGM Laboratories GmbH (Martinsried, Germany). The bioinformatic analysis was

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conducted with the CLC Genomic Workbench software 12.0.3 using the microbial genomic module 3.0 (Qiagen, Hilden, Germany) as described previously.[32]

RESULTS AND DISCUSSION

VFA concentration and composition

Time courses of VFA concentrations for runs 1 and 2, which were inoculated with a mixed bacterial culture and soft goat cheese are depicted in Fig. 2(a) and (b), respectively. The main products of the fermentation in both runs were LA, acetic and butyric acid, and the target acid of this study, PA. It cannot be ruled out, that longer-chain fatty acids such as caproic or enanthic acids also were produced, yet in low amounts. We detected formic acid, iso-butyric and valeric acid at very low concentrations. This is in line with what was reported in other studies on acidic hydrolysis of several substrates such as dog food,[28] organic waste,[33] landfill leachate[34] and food waste.[35] However, the concentrations reached in the broth were highly variable over time. Lactic acid was the main acid produced during the start-up batch period of Run 1 as the first detectable intermediate with a maximum concentration of 310 mmol L^{-1} at Day 9. The concentration subsequently had decreased significantly already towards the end of the batch phase, and resumed increasing once per adjusted *HRT* with maximum peaks being reached in intervals of approximately 23 days.

In general, there is a clear sequence of VFA appearance in the reactor broth. After LA, concentrations of butyrate, propionate and acetate peak although at different maximum values. Acetic and butyric acid reach maximum concentrations in the range of 325 to 340 mmol L^{-1}, whereas maximum PA concentrations reached only 77 mmol L^{-1}. It is noticeable that PA concentrations, which were ≈39 mmol L^{-1} on average, did not vary as much as the concentrations of the other acids. In addition, acetic acid, showing only one big peak during the course of the reactor run, remained at rather low but fairly constant concentrations of ≈27 mmol L^{-1} on average from Day 55 onwards. An important finding from the results of Run 1 was that a direct link between *HRT*/*OLR* and VFA concentrations could not be stated. Rather, it appears that the course of concentrations reached by one acid often is more dependent on the courses of the other acids, which act as precursors or develop as daughter products. The latter can, for example, be the result of a process called chain elongation which entails a reverse b-oxidation that enables the partial usage of the substrate for energy generation.[36] Chain elongation was, for instance, described for the conversion of ethanol and acetate

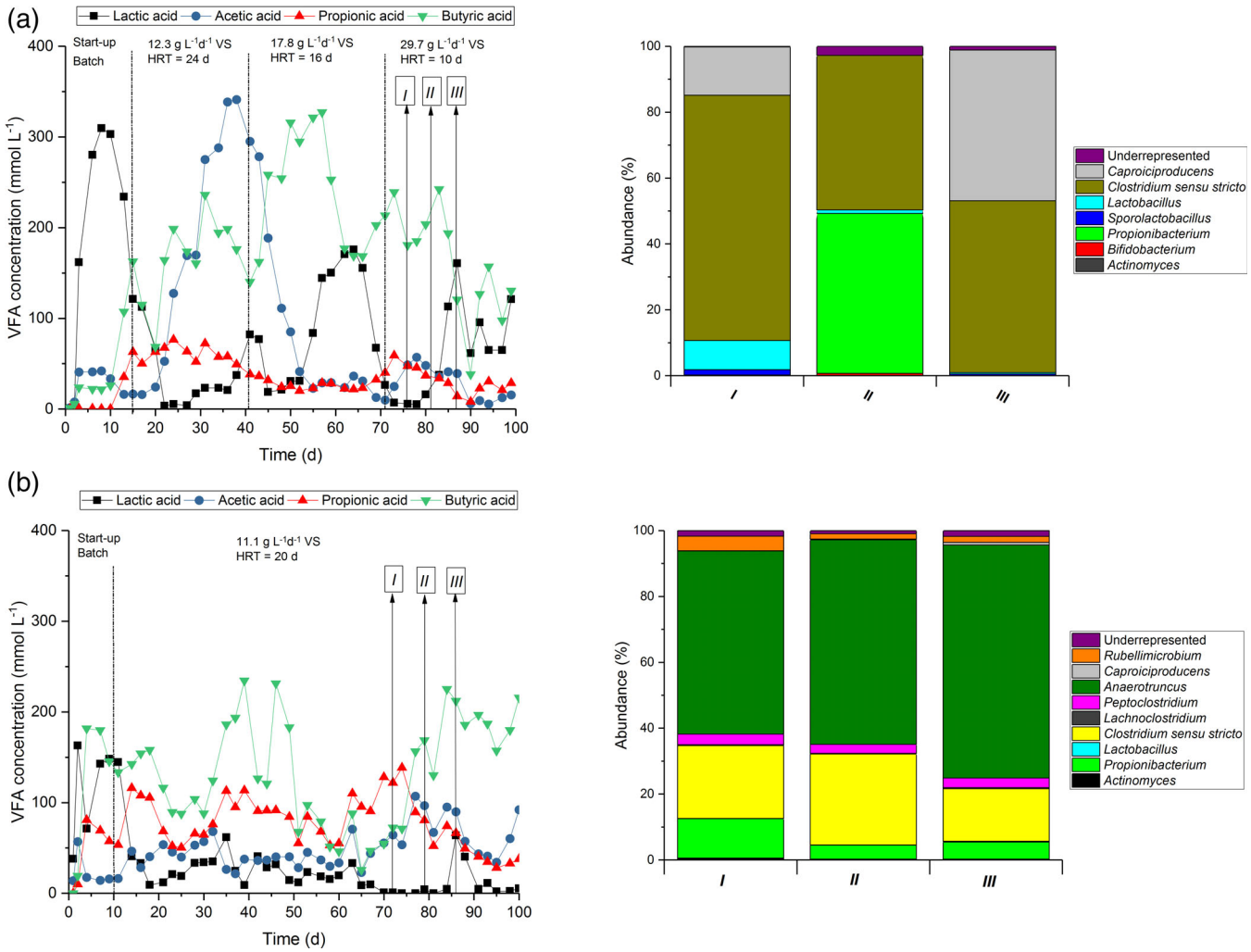

Figure 2 Courses of VFA concentrations during (a) reactor Run 1, inoculated with a mixed culture, and (b) Run 2, inoculated with soft goat cheese. In each run, three samples (I, II and III) were taken for 16S analysis (marked by arrows). The results of the relative abundance of genera are shown on the right.

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or lactate and acetate to butyrate and could probably explain the depletion of acetate and production of butyrate between dayd 35 and 50. It also appears that peaks in propionate production always occur after an increase in lactate productivity. The latter would be a logical consequence of secondary fermentation catalyzed by propionic acid bacteria. Using amplicon sequencing, we aimed to verify this and took samples from the reactor at days 76, 82 and 87, which correlate with a peak and following decrease in propionate concentration [Fig. 2(a)]. The data reveal the abundance of *Propionibacteria* but also emphasize the instability and the high variability of the microbial composition in the system. This high degree of instability is apparent in the fact that *Propionibacteria* were not detectable at days 76 and 87, yet 40% of the amplicon counts could be assigned to these organisms at Day 82. Moreover, LA bacteria were detectable only at Day 76. The concentration of these organisms was probably higher at earlier time points of the run corresponding to the lactate peak at Day 64.

Although the occurrence of LA consumption, propionic acid production and *Propionibacteria* is highly indicative of a Wood–Werkman cycle-based fermentation of lactate to propionate, it should not be forgotten that lactate is not the only substrate for *Propionibacteria*. Sugars and alcohols are used as well, and other fermentation pathways leading to propionate also exist in other microorganisms.[37] Still, thermodynamically the Wood–Werkman cycle is the most efficient fermentation pathway known so far.[38] Other organisms known to produce propionate fermentatively belong typically to the genera *Clostridium, Bacteroidetes, Veilionella, Propionigenum, Selenomonas, Megasphera* and *Salmonella*. Some of these produce propionate also from lactate and other substrates including succinate, sugars, glycerol, amino acids and propanediol.[38] Unfortunately, the phylogenetic diversity analysis conducted in the present study does not reveal whether these other organisms and their fermentation pathways might play a role as well.

Results of Run 1 show that lower *OLR*s might be beneficial for PA production. The detected PA concentration at an *OLR* of 12.3 g L^{-1} day^{-1} *VS* was higher compared to the concentration at other *OLR*s of 17.8 g L^{-1} day^{-1} *VS* and 29.7 g L^{-1} day^{-1} *VS*, respectively. Consequently, Run 2 was operated with a rather low OLR.

In Run 2, which was inoculated with the soft goat cheese, acid concentrations generally showed smaller amplitudes at much lower average concentrations than in Run 1. Lactic acid, for example reached a maximum of 163 mmol L^{-1} during the start-up phase, which is ≈50% of the value reported for Run 1. However, butyric acid showed both highest variability over time (between 136 and 235 mmol L^{-1}) and the highest concentrations compared to all other acids. Interestingly, PA was produced several days earlier than in Run 1 and reached the second highest concentrations of maximum 139 mmol L^{-1} and 78 mmol L^{-1} on average. This was twice as much as in Run 1. Accordingly, also the PA: VFA concentration ratio was significantly higher ranging from 10% to 62% (26% on average), whereas in Run 1 the range was between 4% and 26% (10% on average). This result also is corroborated with 16S rRNA gene diversity data for three days at the end of reactor operation (days 72, 79 and 86) [Fig. 2(b)]. The community seems to be more stable and *Propionibacteria* were detectable in all samples. Organisms belonging to the *Clostridium sensu stricto* group were not as common as in Run 1, whereas *Anaerotruncus* was the most abundant genus. Although the information regarding these organisms is sparse, it seems that they acid produce acetic and butyric as main fermentation end-products.[39] The same is true for organisms belonging to the *Peptoclostridium* group, although LA also was revealed to be a fermentation end-product.[40] It is not clear what the fermentation end-products are in organisms belonging to the genus *Rubellimicrobium*. Interestingly, a very low abundance of *Lactobacilli* of <1% was observed in the three samples, which might suggest that the *Propionibacteria* thrive to a main extent on a different substrate than lactate. In order to put our results into context, Table 2 lists achieved concentrations of PA as reported in literature. Only those studies were considered, where food waste was used as feed and operation conditions were similar to our study. As can be seen from Table 2, the concentrations of PA obtained in this study, especially in Run 2, are significantly higher than those obtained in other studies using mainly anaerobic sludge as inoculum. This indicates that the microbial communities contained in the soft goat cheese in Run 2 might have played an important role in improving PA production throughout the fermentation period. However, in comparison to studies that use synthetic medium as substrate and a pure culture of a PA-producing bacterial strain as inoculum, the PA production in our cultivations was rather low. For example, Liu *et al.*[41] achieved a maximum concentration of ≈1000 mmol L^{-1} PA during the batch fermentation of concentrated glucose solution (≈600 g L^{-1}) inoculated with a high density culture of *Propionibacterium acidipropionici* ATCC 4875. Chen *et al.*[2] obtained an even higher PA concentration of ≈1836 mmol L^{-1} in a fed batch fermentation of glucose (40 g L^{-1} as initial concentration) by using *Propionibacterium freudenreichii* CCTCC M207015 isolated from cheese.

Impact of *OLR* and *HRT* on PA production and yield

For comparison of VFA production in dependence on the operation conditions, VFA production rates and yields were calculated. This was only justified for the target product PA, because fluctuations of the concentration were much lower than for the rest of the acids, especially in Run 1, and trends of stable, increasing or decreasing concentrations were deducible from the data for the single combinations of *HRT/OLR* (compare Fig. 3). Moreover, concentrations of PA do not seem to be significantly dependent on the concentrations of the other acids. We consider these facts as prerequisites for the determination of a production rate that can be linked to the corresponding operation phases.

The average PA production rate P_{PA} (mg L^{-1} day^{-1}) was calculated by the following equation [Eqn (1)]:

$$P_{PA} = (dc_{PA})/dt + Q/V \cdot c_{PA,\ avg} \quad (1)$$

where the gradient dc_{PA}/dt represents the change of PA concentrations with time for the time period of a single operation phase (*OLR* and *HRT*), *Q* represents the volumetric flow rate in L day^{-1} (given as the liquid reactor volume *V* divided by the *HRT*), and $c_{PA,\ avg}$ is the average PA concentration of the corresponding operation phase.

Additionally, the corresponding yields of PA, Y_{PA}, given as mg g^{-1} PA per VS_{added}, were calculated as average P_{PA} per corresponding *OLR* [Eqn (2)]:

$$Y_{PA} = P_{PA}/OLR \quad (2)$$

The resulting PA production rates and yields calculated for the different operation phases are given in Table 3. Because the *VS* concentration of the feed solution was constant in Run 1, values of *HRT* and *OLR* are complementary in the semicontinuous feeding mode; an increase in the *OLR* is accompanied by a corresponding decrease in *HRT*.

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Table 2 Comparison with other fermentation processes using FW as substrate. In our study, vegan DF was applied as model of kitchen waste. Results from batch cultivations are representing the final concentrations reached. (W:S, water:substrate ratio)

Working volume (L)	Reactor operation mode	Duration of experiment	Substrate	W:S ratio	Inoculum	Working pH and temperature	Initial load (g L^{-1} *VS*)	*OLR* (g L^{-1} day^{-1} *VS*)	Propionic acid concentration (mmol L^{-1})	Reference
2	Batch mode	50 h	FW	2.5:1[a]	Anaerobic activated sludge	6 39 °C	40.0 70.2	—	50 40	13
10	Batch mode	14 days	FW	2.3:1.5	Thermophilic anaerobic sludge	6 50 °C	306.7[a]	—	15[a]	50
6	Semicontinuous feeding mode	96 days	FW	2:1.5[a]	Anaerobic sludge	6 37 °C	2432[a]	2	Ranged between 3 and 43[a]	14
20	Fed-batch mode	54 h	FW	_	Anaerobic culture from a bioreactor	Uncontrolled (6–6.5) 30 °C	_	_	≈12[a]	51
12	Semicontinuous feeding mode	100 days	DF	2:1	Mixed culture	6 30 °C	297	Batch 12.3 17.8 29.7	13 ± 24 59 ± 11 28 ± 6 31 ± 14	Present study Run 1
12	Semi continuous feeding mode	100 days	DF	3:1	Soft goat cheese	6 30 °C	260	Batch 11.1	54 ± 27 77 ± 30	Present study Run 2

aCalculated from the data published. Results include only the experiments that were conducted at pH 6.

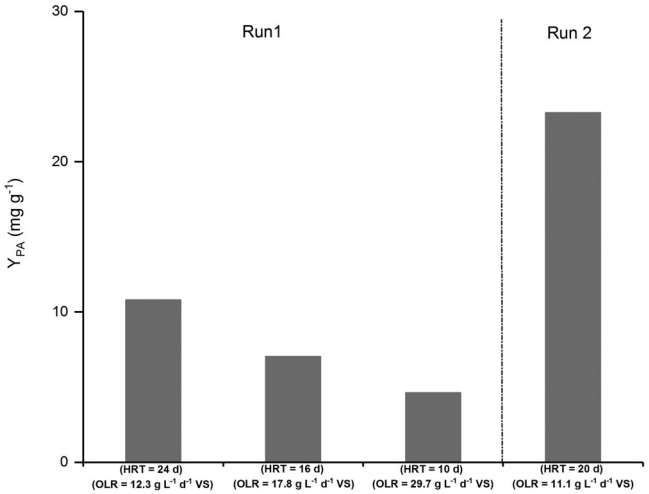

Figure 3 Yields of propionic acid Y_{PA} per *VS* added for different *HRT* and *OLR* in the semicontinuous operation mode.

The first finding that can be deduced from these values is that average PA production rates were fairly constant during Run 1, and obviously not solely dependent on either *HRT* or *OLR* but their combination. Here, with regard to the production rate, a lower *HRT* seems to be compensated by a higher *OLR* within the ranges of *HRT* and *OLR* investigated.

However, the Y_{PA} per volatile solid added listed in Table 3 and plotted in Fig. 3 indicate a much stronger dependency on the *HRT*, where the exploitation of the raw substrate gets worse with decreasing *HRT* and, thus, more substrate leaves the reactor before it can be converted to PA. The consequences of lowering the *HRT* are clear: slow-growing microorganisms might be washed out, and, thus, a shift in species composition and correspondingly the metabolic pathways realized by the biocoenosis will occur. At the same time, concentrations of intermediate products acting as precursors for *VFA* production might be affected.

According to many studies, applying longer *HRT* in general leads to increasing VFA production as the microorganisms have more time to consume the substrate and process intermediate products. For example, Lim *et al.*[42] obtained increasing total VFAs concentrations with increasing *HRT* in acidic fermentation of food waste. Bolaji and Dionisi[43] reported similar results for the fermentation of vegetable waste. They found an increase of 13.3% in propionate production by changing the *HRT* from 10 to 20 days. By contrast, other studies reported that increasing the loading rate at a certain point by decreasing the *HRT* could increase the VFA production by inhibiting the activities of H_2- and methane (CH_4)-producing microorganisms, resulting in the accumulation of VFAs.[44, 45] Thus, the impact of *OLR* and *HRT* seems to depend significantly on the consortium of microorganisms at work and their specific growth and production rates.

By considering the Y_{PA} obtained from the first run, in Run 2 it was decided to operate the reactor at a *HRT* of 20 days while choosing a rather low *OLR* of 11.1 g L^{-1} day^{-1} VS_{added}, which means that a significantly lower substrate concentration was offered to the reactor compared to Run 1. Thus, basically the time available for acidification was increased, considering especially slower metabolic pathways including several intermediate products. As can be seen from Table 3, and during the semicontinuous operation mode of Run 2, higher PA production rate was achieved of ≈258.5 mg L^{-1} day^{-1}. The impact on the PA yield is even more pronounced (compare Fig. 3), it amounted to 23.3 mg g^{-1} VS_{added}, which was the highest value achieved in this study. Thus, the comparison with the findings for Run 1 reveals that the PA production rates and yields of acidic hydrolysis of the synthetic kitchen waste (vegan dogfood) cannot generally be predicted from either the single parameters of *OLR* and HRT, or their combination. This might indicate that the biocoenosis itself has a critical role in the ultimate performance of the reactor in this study, and that the PA production might depend to a larger extent on the inoculum than on operation conditions.

Gas production and composition

The gas produced in this study was comprised of mainly H_2 and carbon dioxide (CO_2) with a very low concentration of N_2, whereas CH_4 was not detected in both reactor runs.

The total volumetric production rate of gaseous compounds ranged between 0.5 and 21 NL day^{-1} in Run 2, whereas it was not quantified in Run 1. The H_2:CO_2 ratio in the produced gas was similar between both runs. The highest content of H_2 in the gas phase was 52% (28% on average) and 45% (27% on average) in runs 1 and 2, respectively. The CO_2 contents amounted to a maximum of 94% (68% on average) in Run 1 and 84% (65% on average) in Run 2.

The production of butyric acid and/or acetic acid are usually accompanied by H_2 production under controlled laboratory conditions (e.g. use of a monoculture and glucose as substrate), whereas PA production consumes H. Thus, it is often reported that the increase in H_2 concentration stimulates PA production.[46-48] By contrast, the accumulation of PA was not always linked to the production rate of H_2 in anaerobic treatment of wastewater as stated by Wang *et al.*[48] Similar results also were observed by Inanc *et al.*[49] showing that a lower H_2 pressure did not affect the accumulation of PA and other VFA. However, no obvious correlation was observed between H_2 and PA or other VFA production in this study, probably as a consequence of the variations in the composition and performance of the microbial communities and the wide metabolic diversity associated with the different species.

Acidification yield

Acidification yield is an important indicator of how much soluble organic matter is converted into VFA and, thus, how successful

Table 3 Average P_{PA} and Y_{PA} at different *HRT*s and *OLR*s in both reactor runs

	HRT (d)	*OLR* (g L^{-1} day^{-1})	P_{PA} (mg L^{-1} day^{-1})	Y_{PA} (mg g^{-1})
	24	12.3	133	10.8
Run 1	16	17.8	126	7.1
	10	29.7	139	4.7
Run 2	20	11.1	259	23.3

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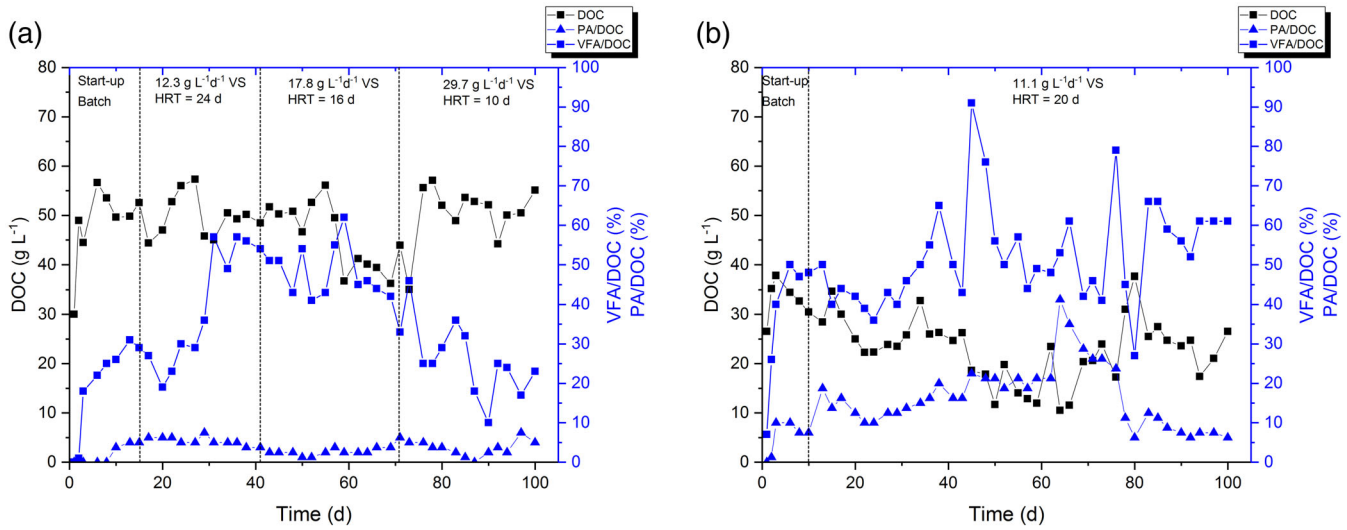

Figure 4 Variation of the average DOC concentration and VFAs:DOC ratio at different *OLR*s and *HRT*s for (a) Run 1 and (b) Run 2.

the VFA production process is. The acidification yield was calculated as the VFA:DOC average concentration ratio.

The variation of the average DOC concentrations in the reactors, the VFA:DOC ratios as well as the PA:DOC ratios achieved at different *HRT* and *OLR*s are shown in Fig. 4(a) and (b) for both reactor runs. In Run 1, it can be seen that the average DOC concentration was 48 g L^{-1}, and rather constant despite different *OLR*s. In Run 2, the values fluctuated more and only reached 24 g L^{-1} DOC on average. The latter was expected due to the lower *OLR*.

The higher DOC concentrations found in Run 1 indicate that much of the organic matter originating from the dog food released high levels of DOC and supplied an adequate amount of organic substrates to produce VFAs. However, the acidification attained by Run 1 was lower compared to Run 2. The highest values ranged between 33% and 62% at *HRT* of 16 days. By decreasing the *HRT* to 10 days, the VFA:DOC ratio was the lowest and ranged between 10% and 46%, which showed that the fermentation was to some extent delayed at this HRT owing to the higher *OLR*.

Although, the ratio was also low at *HRT* of Day 24, it seems probable that the acidification might not have been completed by the end of this phase of the fermentation, and it could have been increased further by maintaining the retention time at 24 days. As can be seen in Fig. 4(a), the VFA:DOC ratio increased to 60% in last few days of the fermentation at this *HRT*.

The same is true for Run 2 in which the longer *HRT* of 20 days led to a higher acidification yield. The highest VFA conversion ratio ranged between 40% and 90% (55% on average) and was observed during the semicontinuous feeding mode. More importantly, a high PA:DOC ratio of 14% on average was observed in Run 2 compared to 4% on average in Run 1 at the similar *OLR*. This indicates that the microbial community in Run 2 was more productive in acidification and, thus, achieved a higher yield per DOC offered.

CONCLUSION

Soft goat cheese was successfully used as inoculum to drive the PA production fermentation process. A maximum PA concentration of 139 mmol L^{-1} at a yield of 23.3 mg g^{-1} *VS* was obtained, which was 55% greater than what was achieved with the mixed culture. The fermenter could be kept in a stable process of propionic acid production at a *HRT* of 20 days and a rather low *OLR* of 11.1 g L^{-1} day^{-1} *VS*. The different inocula proved to have a significant impact on the absolute and relative production of the individual VFAs, which could be supported by microbial community analysis. 16S rRNA gene diversity data showed that the community was more stable in the run inoculated with goat cheese, in which *Propionibacteria* were detectable in all samples, even after 86 days of cultivation (corresponding to 3.6 times the *HRT*). Results show that a high PA production is possible, applying optimized process parameters and selecting the adequate microbial community for inoculation. A thorough characterization of the microbial community in the goat cheese has to be performed in order to understand their interactions and design a defined mixed culture for future implementation at large scale.

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