Stepwise pH control to promote synergy of chemical and biological processes for augmenting short-chain fatty acid production from anaerobic sludge fermentation

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Abstract

Although sludge-converted short-chain fatty acids (SCFAs) are promising feedstocks for biorefineries, it remains challenging to maximise SCFA production by enhancing synergies between chemical/biological hydrolysis and acidogenesis processes while employing a balanced composition of microbial communities to counteract methanogenesis. Herein, stepwise control of fermentation pH and chemical/microbiological composition analysis of fermented sludge were used to probe the underlying mechanisms of SCFA production. Fermentation at pH 11 during the first three days promoted both chemical and microbial hydrolysis of sludge proteins and provided a niche for *Anaerobrancaceae sp.* to transform soluble protein into SCFAs. When pH was decreased from 11 to 9, *Acinetobacter*, *Proteiniborus*, *Proteiniclasticum*, and other acetogens became predominant and stayed significantly more active than during first-stage fermentation at pH 11, which benefited the acidification of hydrolysed substrates. Further assays indicated that early-stage sludge fermentation at pH 11 decreased the total amount of methanogenic archaea and hence reduced the amount of SCFAs consumed for methane production. Thus, the use of stepwise pH control for sludge fermentation allowed one to establish process synergies, facilitate chemical and biological hydrolysis, inhibit methanogens, and promote the growth of acidifying bacterial communities, which resulted in efficient SCFA production from sludge.

Keywords: waste activated sludge, fermentation, short-chain fatty acids, stepwise pH control, microbial community structure, conceptual model

1. Introduction

Waste-derived short-chain fatty acids (SCFAs) are attractive feedstocks for renewable energy and biopolymer production (Pagliano et al. 2017). The genesis of SCFAs from sludge is an anaerobic fermentation process involving the steps of hydrolysis, acidogenesis, and methanogenesis, among others. Among these steps, the rate-limiting one corresponds to sludge hydrolysis, while competition between methanogenic archaea (responsible for SCFA consumption) and acidogenic bacteria determines whether methane or SCFAs are formed as main fermentative products (Zhang et al. 2009). Consequently, the maximisation of SCFA yield and realisation of optimum SCFA composition for downstream applications are tasks of high practical significance.

Previous studied revealed that the yield of SCFAs produced by anaerobic sludge fermentation is influenced by fermentation pH, temperature, and retention time, with most of these parameters also affecting SCFA composition (Arslan et al. 2016, Jankowska et al. 2018). Moreover, it was found that SCFA yield can be augmented through the application of different sludge pre-treatments (Yu et al. 2018) and the usage of additives such as free ammonia during fermentation (Wang et al. 2019, Wang et al. 2018, Zhao et al. 2018a). Based on these findings, a number of approaches to enhance the fermentative conversion of sludge into SCFAs have been proposed, implemented, and verified (Lee et al. 2014). However, most of these approaches are commonly designed to address one factor at a time, as exemplified by studies on the enhancement of sludge particulate hydrolysis (Chen and Chang 2017), improvement

of acidogenesis (Wang et al. 2017), or the inhibition of methanogenesis (Wilson et al. 2012). Currently, it remains unclear whether and how an individual approach can have counteracting effects on the chemical and biological processes involved in anaerobic sludge fermentation and thereby reduce the yield of the thus produced SCFAs.

Fermentation pH is an important parameter that can influence anaerobic sludge fermentation in a number of ways. Alkaline pH is believed to enhance the anaerobic conversion of sludge into SCFAs, e.g., Yuan et al. (2006) documented that SCFA production at alkaline pH (10–11) significantly surpassed that observed at acidic or neutral pH. Additionally, Wang et al. (2016) revealed that alkaline sludge pre-treatment (pH 12) causes the fragmentation and denaturation of proteins, improving their susceptibility to subsequent proteolytic cleavage and hydrolysis and hence augmenting SCFA production via anaerobic fermentation at pH 9. Moreover, Zheng et al. (2013) reported that alkaline fermentation hampers methanogenesis and benefits SCFA accumulation. However, some researchers claimed that even though alkaline conditions can enhance SCFA production, they concomitantly inhibit the ability of acidogenic bacteria to transform hydrolysed organic substrates into SCFAs, thereby decreasing the potential of SCFA yield maximisation (Li et al. 2017, Lin and Li 2018, Yuan et al. 2015). Others also claimed that the use of neutral pH generates a niche for microbial populations involved in acidification but fails to enhance the hydrolysis of organic particulates, resulting in low substrate availability for the biological generation of SCFAs (Ma et al. 2016). Notably, the above and similar studies revealed that fermentation pH usually remains relatively constant over the entire process of

anaerobic sludge fermentation, implying that the obtained results may not reflect the optimum composition of microbial communities and process synergy maximisation. To address this challenge, recent research efforts have focused on dividing the process of anaerobic sludge fermentation into two or more stages so that different pH values can be applied at each stage to maximise chemical hydrolysis at early stages while substantially enhancing biological hydrolysis and acidogenesis at later stages for accumulative SCFA yield (Chen et al. 2017, Zhao et al. 2018b). However, these works lack a systematic approach and do not provide a clear mechanistic understanding required to increase process synergies and gain a balanced composition of microbial communities for a further enhancement of SCFA production.

Herein, six anaerobic fermentation reactors were supplied with the same feed sludge and operated using one-step pH (pH 11), stepwise pH control (pH 11, plus pH 10, 9, 8, or 7), and uncontrolled pH approaches. Temporal dynamics of substrate availability, carbon matter balance, and microbial community assembly were monitored over 25 days and linked to SCFA production. Further, the underlying mechanisms of augmented SCFA production under the optimal conditions of stepwise pH fermentation (pH 11, plus pH 9) were investigated by analysing the key microorganisms involved in biological hydrolysis, acidification, and methanogenesis. Moreover, real-time quantitative polymerase chain reaction (q-PCR) assays were applied to determine the total number of bacteria and archaea and thus reveal the competition between acidifying bacteria and methanogens in fermentation reactors. A conceptual paradigm with two possible scenarios was also proposed to (i) advance our

understanding of how augmented SCFA yield and related microbial community assembly respond to pH adjustment throughout the complete process and (ii) provide insights into the potential role of environmental driving forces (e.g., pH) on the enhanced yield of SCFAs produced by anaerobic sludge fermentation.

2. Materials and Methods

2.1 Sludge Source and Characteristics

Sludge was obtained from the primary clarifier of a municipal wastewater treatment plant in northern China operated using the conventional process of biological nutrient removal. Sludge was concentrated by allowing it to settle at 4 °C for 24 h, with its main characteristics presented in Table S1. The seed inoculum was obtained from an anaerobic sludge digester at the same plant. Details of sludge sample and seed inoculum preparation procedures are provided in Supplementary Information S1.

2.2 Operation of Sludge Fermentation Reactors

Sludge samples containing the seed inoculum were mixed to afford feed sludge that was then transferred into six identical 2.5-L fermentation reactors at 8.6 g/L (transferred volume = 2 L) to test different methods of fermentative pH control. The test period was at least 20-day long, a typical duration for SCFA production by anaerobic sludge fermentation (Wu et al. 2010, Yuan et al. 2006), and it was assumed that the degree of hydrolysis can be maximised within the first 72 h under high-pH conditions (Wang et al. 2016). Consequently, during the first three days (Phase I) of

the experimental process, five fermentation reactors (R1–R5) were maintained at a constant pH of 11 by addition of 2 M NaOH. During the next fermentation phase (Phase II), the pH values of these five reactors were adjusted to and maintained at 11, 10, 9, 8, and 7, respectively, via the addition of 2 M HCl or 2 M NaOH. The remaining reactor (R0) was used as the control (uncontrolled pH) throughout the entire process. After feed sludge addition, all reactors were purged with nitrogen gas to remove oxygen, sealed, and kept at 34 ± 1 °C in an air-bath shaker (150 rpm).

2.3 Reactor Performance Monitoring

Sludge and biogas samples were collected from reactors every 24 h using previously described procedures (Cai et al. 2004, Wang et al. 2016) and subjected to chemical analysis (for details, see Section 2.6). In addition, sludge samples collected on days 3 (Phase I end) and 25 (Phase II end) were also used to analyse microbial populations in relation to hydrolysis, acidification, and methanogenesis. The acquired sludge samples were preserved in 10-mL centrifuge tubes at −20 °C.

2.4 DNA Extraction and Quantification of Total Bacteria and Archaea

Genomic DNA was extracted from sludge samples using the FastDNA SPIN Kit (MP Biomedicals, USA), and DNA concentration/purity was determined by microspectrophotometry (NanoDrop ND-1000, USA). Bacterial and archaeal genes were quantified by q-PCR conducted on a Rotor-Gene 6000 Series instrument employing Software 1.7 (QIAGEN, the Netherlands). The experimental procedures

are described in detail in Supplementary Information S2.

2.5 MiSeq Sequencing and Data Processing

The 16S rRNA gene was amplified for the MiSeq platform, with detailed molecular and bioinformatics procedures provided in Supplementary Information S3. Raw sequence data were deposited in the NCBI Short Red Archive (SRA) database under the accession number SRR151628 for both bacterial and archaeal sequences.

2.6 Analytical Methods

To measure the concentration of produced SCFAs, fermentation mixture samples withdrawn from each reactor were immediately filtered through a 0.45-μm membrane, and the resulting filtrates were analysed using a gas chromatograph (GC, Agilent 6890N, USA) equipped with a flame ionisation detector (FID) and a DB-FFAP column (30 m × 0.32 mm × 0.25 μm). Injection port, detector, and column temperatures were set at 240, 260, and 210 °C, respectively, and carrier gas (nitrogen) was supplied at a flow rate of 30 mL/min. The total SCFA concentration was calculated as the sum of acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, and isovaleric acid concentrations. Methane and carbon dioxide were determined by the same GC equipped with a thermal conductivity detector (TCD) and a 2-m stainless column filled with Porapak T (80–100 mesh). In this case, injection port, column, and detector temperatures were set at 110, 60, and 200 °C, respectively, and carrier gas (helium) was supplied at a flow rate of 40 mL/min. Hydrogen gas was quantified

using the abovementioned GC-TCD equipped with a 2-m stainless steel column filled with activated carbon (60–80 mesh) and employing nitrogen (30 mL/min) as a carrier gas. Biogas in the headspace of serum bottles was sampled with a 0.1-mL gastight syringe and analysed by comparison with standard CH_4/CO_2 or standard H_2 samples. Thereafter, the yields of CH_4, CO_2, and H_2 were calculated and expressed in the units of mL per g volatile suspended solids (VSS) based on the steady state at 25 °C. Protein and carbohydrate contents were determined in filtrates obtained by passing samples through a 0.45-μm membrane. Protein concentration was determined by the Lowry method using bovine albumin as the standard (Lowry et al. 1951), while carbohydrate concentration was measured by the phenol-sulphuric acid method using glucose as the standard (Herbert et al. 1971). Chemical oxygen demand (COD), soluble chemical oxygen demand (SCOD), total suspended solids (TSS), and VSS were quantified in line with standard methods (APHA 1998). Carbonaceous-substrate-to-COD conversion coefficients (g COD/g substrate) were determined as 1.07, 1.51, 1.82, 2.04, 1.50, and 1.07 for acetic acid, propionic acid, butyric (or isobutyric) acid, valeric (or isovaleric) acid, protein, and carbohydrate, respectively (Cokgor et al. 2009, Sophonsiri and Morgenroth 2004).

2.7 Statistical Analysis

All tests were performed in triplicate. Analysis of variance was used to test the significance of results, and *p* values of less than 0.05 were considered to represent statistically significant differences. Pearson's linear correlation analysis was

conducted to explore relationships between SCFA production yield and substrate parameters (protein/carbohydrate contents and their combination) using IBM SPSS Statistics v. 21. Principle component analysis (PCA), based on the microbial community composition obtained by MiSeq, was performed employing weighted Unifrac distances (considering both species phylogeny and relative abundance) to determine changes in microbial diversity under different conditions, and the obtained results were visualised in three dimensions using R v 3.6.0. Significant differences were defined as those with $p < 0.05$.

3. Results and Discussion

3.1 Effects of Fermentation pH Approach on Substrate Availability and SCFA Production

In all fermentation reactors with controlled operational pH, the solubilised substrates (mainly protein and carbohydrate) quickly became available, and SCFA production substantially increased after the first three days; however, concentration changes after 25 days were insignificant ($p > 0.05$). Thus, Fig. 1 only shows variations of soluble protein, soluble carbohydrate, and SCFA concentrations in fermentation reactors for uncontrolled pH (R0), one-step pH (R1), and stepwise pH alternatives (R2, R3, R4, and R5) during the fermentation time of 25 days.

As shown in Figs. 1A and B, the use of controlled fermentation pH (pH 11) during the early period resulted in increased availability of soluble protein and carbohydrate in comparison to the control (soluble protein: 272 ± 7 vs. 19 ± 2 mg

COD/g VSS; soluble carbohydrate: 13 ± 1 vs. 2 ± 1 mg COD/g VSS; on day three). However, significant differences were observed for trends of soluble protein and carbohydrate concentrations under different pH conditions over the second fermentation period. Soluble protein concentration decreased in all pH-controlled reactors after three days, with the maximum concentration observed in R1, which indicated that alkaline pH–induced solubilisation of particulate protein was most pronounced during the early phase of fermentation, while one-step fermentation at pH 11 favoured the accumulation of soluble protein during the next phase (Fig. 1A, dark green curve). Furthermore, soluble carbohydrate accumulation behaviour was also observed in R1 and R2 after three days (Fig. 1B, green curves).

It should be noted (Fig. 1C) that the average end concentration of SCFAs in fermentation reactors with stepwise pH control (R1–R5) equalled 238 mg COD/g VSS, whereas those in one-step alkaline pH (R1) and uncontrolled pH (R0) reactors equalled 267 ± 11 and 86 ± 3 mg COD/g VSS, respectively. Apparently, both one-step alkaline pH and stepwise pH fermentation approaches significantly augmented the accumulation of SCFAs compared to the case of uncontrolled pH, whereas the end production of SCFAs was highest in R3 (324 ± 12 mg COD/g VSS). This finding indicated that the use of stepwise pH fermentation, particularly when pH was held constant at pH 11 in Phase I and then altered to pH 9 during Phase II, can enhance the production of SCFAs from soluble sludge substrates.

Pearson's linear correlation analysis indicated that in fermentation reactors where pH was maintained at pH 11 during Phase I, SCFA production was positively

correlated to protein/carbohydrate concentration and their combination (Fig. 1D, grids in red and orange). On the contrary, these correlations became weak as fermentation reactors moved into Phase II (Fig. 1D, grids in yellow and pale green), which indicated that driving factors other than substrate availability may be responsible for augmented SCFA production in the late fermentation period.

3.2 Compound effects of fermentation pH adjustment on sludge hydrolysis, acidogenesis, and methanogenesis

In general, the extent of hydrolysis represents the percentage of large organic compounds solubilised to smaller organics, whereas the extent of acidogenesis is defined as the biodegradability of soluble substrates, which can be further converted into SCFAs or biogas by acidogens or methanogens, respectively. Figure 2 represents performance variations for pH-differentiated reactors, including the parameters of SCOD, SCFA amount, biogas amount, and the SCFA content/SCOD ratio.

On Day 3 (Fig. 2, blue dotted ellipse), reactors with pH 11 (R1–R5) featured SCOD values higher than those of R0 with uncontrolled pH (365 vs. 80 mg COD/g VSS, respectively), i.e., pH 11 favoured the hydrolysis of large organic particulates within a short time period. This finding agreed with the observation that the SCFA-to-SCOD ratio achieved in R1–R5 was lower than that in R0 (0.21 vs. 0.74), while SCFA yield varied only slightly between these two systems (70 vs. 59 mg COD/g VSS, respectively).

The effects of fermentation pH control in Phase II were more complex (Fig. 2,

purple dotted ellipse) because of the counteracting biological processes involved in SCFA production. When fermentation pH was changed from alkaline (pH 11) to neutral (pH 7), R5 exhibited much lower SCOD (134 vs. 484 mg COD/g VSS, respectively) and SCFA (66 vs. 267 mg COD/g VSS, respectively) levels than R1 (where pH 11 was maintained) while featuring a much more pronounced biogas production (83 vs. 0.12 mL/g VSS, respectively; detailed ratio of CH_4 to CO_2 given in Table S2). These findings agreed with previous observations that at neutral pH, strong methanogenesis is coexistent with decreased hydrolysis and acidogenesis efficiency (Ma et al. 2016, Zhou et al. 2016). Given that fermentation pH in R0 (uncontrolled pH) varied within the narrow range of pH 6.8–7.1, it is not surprising that performance variation between R0 and R5 was not significant ($p > 0.05$). At fermentation pH ≥ 9, methane production was substantially inhibited, while hydrolysis was greatly enhanced compared to the case of neutral pH, which indicated that alkaline conditions might favour acidogenesis during the second fermentation stage. Moreover, acidogenesis was greatly augmented at fermentation pH 9. Specifically, when fermentation pH was decreased to pH 9 and maintained at this value during Phase II, R3 featured the maximum SCFA yield (324 mg COD/g VSS) along with the greatest SCFA-to-SCOD ratio of 0.79 on day 25, although the capability of this reactor to promote the hydrolysis of organics and inhibit methanogenesis was not competitively higher than that of the one-step pH reactor (pH 11, R1).

Carbon mass balance in reactors was studied to better understand the composition

of carbonaceous substances produced during sludge fermentation (Fig. 3). Notably, sludge hydrolysis was quickly initiated within the first three days, and in controlled-pH reactors (R1–R5) on day 3, sludge hydrolysis efficiency reached 25.6%, which was nearly three-fold higher than that obtained in the case of uncontrolled pH (R0). However, only a small proportion of hydrolysis-produced soluble organics was converted to SCFAs (5.3%). Although in R1, the extent of hydrolysis was further augmented on day 25 and exceeded the value observed for R3 (34.1% vs. 28.6%; Fig. 3), the efficiency of solubilised organic substance transformation into SCFAs was higher in the latter reactor (22.1% vs. 18.8%), i.e., the stepwise decrease in pH (pH 11 → pH 9) favoured the augmented conversion of hydrolysed substrates to fermentative SCFAs, in agreement with previous results (Zhao et al. 2018b). This phenomenon may be attributable to the inhibitory effect of strongly alkaline conditions on acidogens (Huang et al. 2016, Ma et al. 2016). To better understand this process, the origin of SCFA production variation under different fermentation conditions (i.e., in one-step pH (R1), optimum stepwise pH (R3), and uncontrolled pH (R0) reactors) was probed by analysis of functional bacterial and archaeal populations.

3.3 Overall Effects of Stepwise pH Decreases on Microbial Community Structure

In emphasised fermentation reactors (R0, R1, and R3) on days 3 and 25, microbial community structures were comparatively explored using 16S rRNA gene sequencing, and microorganism diversity was characterised using microbial α-diversity metrics (Table S3). Samples associated with the optimum stepwise pH approach (R3) were

found to exhibit less rich, even, and diverse bacterial and archaeal communities than those associated with other approaches (R0 or R1).

Figure 4 presents the results of PCA-based characterisation of microbial community structure linked to R0, R1, and R3 that was performed relying on phylotype abundance, demonstrating that the clearest clustering was observed for R1 (where pH 11 was maintained throughout the whole process). However, the sample collected from R3 on day 25 was distinct from that collected from R1 on the same day, particularly in terms of PC1 and PC2 values (Figs. 4A and B, respectively), which indicated that the stepwise decrease in fermentation pH during Phase II substantially influenced the microbial community, particularly the bacterial community.

3.4 Microbial Populations Involved in Biological Hydrolysis, Acidification, and Methanogenesis

To characterise the differences in functional microorganism compositions between R1 and R3, the microbial populations of these fermentation reactors on days 3 and 25 were analysed at the genus level. Figure 5A shows the relative abundance of key microbial populations in the acquired biomass samples. Specifically, the 25 most abundant operational taxonomic units (OTUs) affiliated with bacterial populations across all samples could be grouped into hydrolysers (solubilising organic particulates into low-molecular-weight molecules including soluble organic matter), acetogens (degrading soluble organic matter to SCFAs and H_2), syntrophs (converting SCFAs to

332 acetic acid and H_2), and other unclassified bacteria, while the 10 most abundant OTUs
333 among archaea were identified in relation to methanogens.

334 Hydrolysis of complex organic matter is the rate-limiting step of anaerobic sludge
335 fermentation. Previous works reported that *Pseudomonas sp.* can easily produce and
336 secrete alkaline protease and elastase (Guzzo et al. 1991), which were identified as the
337 most significant enzymes to undertake the hydrolysis of sludge protein, in agreement
338 with more recent findings indicating that high abundances of protein-hydrolysing
339 bacteria (particularly *Pseudomonas sp.*) in alkaline fermentation reactors benefit the
340 biological hydrolysis of particulate protein in sludge (Zheng et al. 2013). However,
341 we observed that rare bacterial microorganisms were capable of hydrolysing
342 particulate organics in R1 over time (Fig. 5A), except for a *Firmicutes* phylum–
343 affiliated bacterial strain (OTU296) that might have the potential to solubilise
344 particulate protein and simultaneously get involved in the acidification process (Xia et
345 al. 2008). In fact, sludge hydrolysis could be enhanced by both chemical and
346 biological processes at high pH, and our previous work demonstrated that heat-
347 alkaline environments can stimulate protein fragmentation and improve the chemical
348 hydrolysis efficiency of sludge protein by inducing conformational changes indicative
349 of protein unfolding (Wang et al. 2016). Thus, the enhanced solubilisation of
350 particulate protein in the fermentation reactor with pH 11 could be partly attributed to
351 the synergies of chemical and biological hydrolysis processes. However, as the
352 fermentation pH was altered to pH 9 during Phase II in R3, the total abundance of
353 hydrolysers therein decreased from nearly 30% to less than 8%, which implied that

354 pH adjustment can greatly affect the process of biological hydrolysis.

355 Large numbers of bacterial populations are involved in the acidification process
356 of anaerobic sludge fermentation. In this study, *Anaerobranca* (OTU296) was
357 identified as the most abundant genus (~40%) in R1 on both days 3 and 25 (Fig. 5A).
358 As documented in previous studies (Gorlenko et al. 2004, Prowe and Antranikian
359 2001), *Anaerobranca* comprises only several species of anaerobic microorganisms
360 that have the capability to degrade protein into SCFAs as primary end products under
361 alkaline conditions and at meso-thermophilic temperatures. Notably, species of the
362 above genus require sodium ions for their metabolism (Engle et al. 1995). Since 2 M
363 NaOH was used herein to maintain fermentation pH at a value of 11, Na^+ ions were
364 present in both reactors, which could partly explain why *Anaerobranca* (OTU296)
365 was traced throughout the one-step fermentation at pH 11.

366 As fermentation pH was decreased to pH 9 from pH 11 in R3 during Phase II, the
367 populations of acidogenic microbes became much diverse, and their total abundance
368 greatly increased from 34.5 to 56.1%. This result agreed with the augmented
369 production of SCFAs in R3 on day 25 (Fig. 1). Thus, the higher abundances of
370 *Acinetobacter* and *Proteiniborus* in the stepwise pH fermentation reactor could benefit
371 the conversion of soluble organics into SCFAs, which agreed with the fact that many
372 species affiliated with the phyla *Proteobacteria* and *Firmicutes* were linked with the
373 fermentative production of volatile organic acids (Dai et al. 2016, Ma et al. 2015). In
374 addition, *VadinBC27 sp.* was reported to undertake the anaerobic degradation of
375 aromatic hydrocarbons (Corteselli et al. 2017, Liu et al. 2016, Pereira et al. 2015),

whereas the relative abundance of a similar bacterium (*VadinBC27 sp.* (OTU967)) on day 25 in R3 was more than 100 times that of R1 (Fig. 4), which further favoured the fermentative production of SCFAs from sludge.

It is well known that fermentation-produced SCFAs can be consumed in the subsequent methanogenesis stage. As seen in Figs. 2 and 3, methane production was inhibited when sludge fermentation was conducted under either alkaline pH (pH 11) or stepwise pH increase (pH 11 → pH 9) conditions. Therefore, Fig. 5A also presents the genus-level distributions of archaeal populations in R1 and R3, revealing that the relative abundances of *Methanobacterium*, *Methanobrevibacter*, and *Methanosarcina* in R1 on day 25 equalled 40.9, 27.1, and 14.9%, respectively, while the most abundant archaeal microbes in R3 at the same fermentation time belonged to the genera of *Methanomassiliicoccus* (49.4%) and *Methanobacterium* (35.5%). In general, the genera *Methanobacterium* and *Methanobrevibacter* are known to be obligate hydrogenotrophic methanogens preferring to utilise H_2 and CO_2 rather than acetic acid (Leclerc et al. 2004), which may the key reason behind the relative abundance decrease observed for *Methanobacterium sp.* and *Methanobrevibacter sp.* in R3 on day 25. The total numbers of archaea in R1 and R3 on day 25 were determined as 550 and 606 copies/mg TSS, respectively (Fig. 5B), i.e., the change of fermentation pH from pH 11 to pH 9 could increase the number of total methanogenic archaea, which helps to explain why greater methane production was observed during sludge fermentation under stepwise pH decrease conditions (Fig. 3).

3.5 Conceptual Paradigm for Understanding Improved SCFA Production in Stepwise pH Fermentation

The above findings inspired us to propose a conceptual model describing how dynamics in SCFA production and microbial community assembly responded to fermentation pH adjustment during sludge fermentation (Fig. 6A) and providing insights into the potential roles of environmental filtering and selection on improved SCFA production by sludge fermentation (Fig. 6B).

Specifically, we hypothesised two possible scenarios for changes occurring during anaerobic sludge fermentation. In the first (business-as-usual) scenario, strongly alkaline pH at early fermentation stages was suggested to facilitate the chemical hydrolysis of organic particulates and benefit the growth of bacteria over archaea, offering a niche for acetogens to degrade hydrolysed matter. In subsequent stages, the maintenance of fermentation pH at a constant value resulted in an increased production of soluble protein (i.e., in enhanced biological hydrolysis) and the selection of more clustered bacterial populations (of the *Anaerobrancaceae* genus) capable of converting protein into SCFAs under alkaline conditions. As an alternative, we hypothesised that the second (pH-adjustment) scenario, where fermentation pH is controlled at a lower level at late fermentation stages, provides a niche for diverse acidogenic bacteria (mainly *Acinetobacter sp.*, *Proteiniborus sp.*, *VadinBC27 sp.*, and *Proteiniclasticum sp.*) and allows one to ferment a wider range of organic substances, facilitating the accumulation of SCFAs. This model provides a novel scheme that can be experimentally examined further in other stepwise pH fermentation systems.

420

421 **3.6 Implications of the Present Work**

422 SCFAs produced by anaerobic sludge fermentation are viewed as promising platform
423 molecules for the production of renewable energy (Hwang et al. 2018), biopolymers
424 (Frison et al. 2015), and medium-length fatty acids with high added value (Xu et al.
425 2018), while alkaline fermentation of sludge was reported to allow for highly efficient
426 SCFA production (Lee et al. 2014). However, the present work raises a critical
427 question of whether concerted efforts should be devoted to controlling fermentation
428 pH at constantly high levels throughout the entire fermentation process, as such
429 actions can result in counteracting effects on the interactions of hydrolysis,
430 acidification, and methanogenesis and consequently hinder SCFA production. Thus,
431 greater focus should be placed on solutions aimed at maximising process synergies for
432 augmented SCFA production. Herein, stepwise pH fermentation was found to be a
433 promising solution, since it ensured high efficiency of sludge particulate hydrolysis at
434 early stages, inhibited methanogenesis, enriched the microbial community in diverse
435 acetogens, and enhanced late-stage acidogenesis, which, taken together, resulted in an
436 optimised yield of SCFAs produced by anaerobic sludge fermentation. Despite these
437 positive findings, further validations and long-term monitoring are needed for larger-
438 scale sludge fermentation systems.

439 Table 1 indicates that the economic benefit provided by the stepwise pH control
440 strategy arises from the reduced cost of chemicals (mainly NaOH utilisation) and the
441 increased revenue from SCFA production. The cost of alkali dosage can be further

442 decreased if $Ca(OH)_2$ is applied instead of NaOH for pH adjustment, since the former
443 is much cheaper, less corrosive, more stable, and easier to store and transport than the
444 latter (Li et al. 2011). Since this preliminary economic analysis was conducted mainly
445 based on the laboratory-scale parameters of the present study, some uncertainties
446 might exist in the expected results, because full-scale systems can behave differently
447 in terms of SCFA production, chemical use, and downstream applications.
448 Consequently, further studies and more real-life data are necessary to interpret the
449 new-build strategy and identify the key aspects requiring cost-benefit optimisation.
450 Our results also highlight that environmental selective pressures (e.g., pH
451 adjustment) play a dominant role in guiding microbial community structure and
452 dynamics during the anaerobic conversion of sludge into SCFAs. Thus, our findings
453 agree with the niche-based theory that microbial community assembly is mainly
454 shaped by deterministic processes mostly including substrate availability, operating
455 conditions, niche differentiation, and interactions (Vanwonterghem et al. 2014), as has
456 been witnessed in both natural and engineered systems such as soil (Tripathi et al.
457 2018), lakes (Eiler et al. 2012), activated sludge (Ju et al. 2014), and anaerobic
458 digesters (Ju et al. 2017). However, the establishment of a neutral theory (as a null
459 hypothesis) neglecting the differences among species in their response to ecological
460 conditions and only considering stochastic processes such as birth, death, dispersal,
461 colonisation, and immigration (Ofiteru et al. 2010) along with state-of-the-art analytic
462 tools such as metagenomics- and metabolomics-based approaches (Aguiar-Pulido et
463 al. 2016, Beale et al. 2016) and mathematical models (Astals et al. 2013, McLeod et

al. 2015) should benefit the further interpretation of microbiome assemblies involved in the production of fermentative SCFAs. Overall, this study tests the potential of stepwise pH fermentation to enhance SCFA production from sewage sludge and investigates the resultant microbial community assembly and dynamics in response to pH adjustments.

4. Conclusions

Herein, we investigated a pH-mediated strategy to enhance SCFA production through anaerobic sludge fermentation by promoting the synergy between chemical and biological processes. Early-stage sludge fermentation (at pH 11) enhanced both chemical and microbial sludge hydrolysis, decreased the total amount of methanogenic archaea, and provided a niche for protein-utilising microbes to transform soluble protein into SCFAs, whereas subsequent pH reduction (to pH 9) facilitated the proliferation of diverse acetogens and benefited the acidification of hydrolysed substrates, promoting the efficient generation of SCFAs from sewage sludge. A conceptual model was developed to advance our understanding of how improved SCFA yield and related microbial community assembly respond to pH adjustment throughout the complete process.

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665 **Figure Captions**

666

667 **Fig. 1.** Comparisons of (A) protein and(B) carbohydrate solubilisation, (C) SCFA
668 accumulation, and (D) Pearson's correlation indices for SCFA production and soluble
669 protein/soluble carbohydrate content and their combination for different pH control
670 strategies. All results are expressed as mean values of triplicate tests.

671

672 **Fig. 2.** Performance differences of six fermentation reactors at days 3 (Phase 1 end)
673 and 25 (Phase II end).

674

675 **Fig. 3.** Accumulative VSS compositions in different fermentation reactors at days 3
676 (Phase I end) and 25 (Phase II end).

677

678 **Fig. 4.** Microbial community dynamics in fermentation reactors with uncontrolled pH,
679 alkaline pH, and stepwise pH decrease conditions at different time points. 3D-PCA
680 results showing microbial dynamics (0.97-OTU level) on the basis of the relative
681 abundance of (A) bacterial and (B) archaeal 16S rRNA gene sequences and separating
682 different sludge samples according to pH control methods. Circles indicate Phase I or
683 Phase II ends for reactors. Translucent colour shades indicate separated samples:
684 green = uncontrolled pH group, blue = alkaline pH group (pH 11); pink = stepwise pH
685 decrease group (pH 11, plus pH 9). Red circles denote raw feed samples prior to
686 fermentation.

687

688 **Fig. 5.** (A) Community compositions of main OTUs affiliated with the key functional
689 microorganisms and (B) copy numbers of total bacteria and archaea in fermentation
690 reactors with alkaline pH and stepwise pH decrease conditions at different time
691 points. (A): Circle sizes correspond to abundances, as shown at the bottom left of the
692 figure, while circle lines indicate statistical differences of OTU abundance between
693 days 0 and 3 or between pH 11 and 9 at day 25, based on Welch's test (p <0.05). (B):
694 Stack heights correspond to copy numbers of total bacteria (green) or archaea
695 (purple), as shown at the upper left of the figure. Asterisks represent microorganisms
696 that are both hydrolysers and acetogens.

697

698 **Fig. 6.** (A) Responses of microbial community assembly and performance to changes
699 in fermentation pH. (B) Conceptual model showing alternative interplays between
700 fermentation pH, microbial community structure, and SCFA yield alongside potential
701 shifts in the predominant roles of enhanced SCFA production.