Effect of feed concentration and residence time on anaerobic fermentation in CSTR and SBR to produce short-chain organic acids

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Abstract

This study investigated the production of short-chain organic acids (SCOAs) and ethanol using anaerobic fermentation (AF) in semi-continuous CSTRs (continuously stirred tank reactors) with intermittent feed without solids separation and in SBRs (sequencing batch reactors) with solids separation. A model feedstock, which included the main classes of substances present in food waste, was used (24.7-394.6 gCOD L^{-1}). To improve process sustainability, conditions of uncontrolled pH and room temperature were used. The effect of feed concentration, HRT (hydraulic residence time), SRT (solids residence time) on product yield, concentration and productivity was investigated. In CSTRs (HRT=7.5-120 d), the highest product concentration was 113 g L^{-1}, which is amongst the highest values reported for these processes. The product yield was in the range 15-43% g COD

g COD^{-1} (as total COD of the products vs COD of the feed) and increased with the HRT. Productivity strongly increased for lower HRTs, with maximum of 9.7 g L^{-1} d^{-1}. SBR runs allowed to uncouple the HRT (2 d) from the SRT (2-20 d), improving process productivity for the most diluted feeds. For the most diluted feed, the productivity in SBR was over 5 times higher than the productivity in CSTR. Generally, the yield increased with the SRT. Lactic acid was the main product in all runs except in those with the lowest feed concentration. The analysis of the microbial community showed a strong and rapid selection towards the genus *Lactobacillus*.

Keywords

Anaerobic fermentation; short-chain organic acids (SCOA); CSTR; SBR; food waste; *Lactobacillus*.

1 Introduction

Anaerobic digestion (AD) of organic waste is an established process with open (undefined) mixed microbial cultures converting organic waste or biomass into methane, which is combusted to generate renewable energy. As an alternative to methane production, anaerobic digestion could be used to produce the liquid-phase intermediates, which are mainly short-chain organic acids (SCOAs, e.g. acetic, propionic, lactic acid) [1, 2]. When the main products are SCOAs rather than methane, AD is often referred to as anaerobic fermentation (AF), which is the terminology we use in this paper. SCOAs are valuable chemicals used for a wide variety of purposes, e.g. the innovative production of polyhydroxyalkanoates (PHAs) [3], and are produced at global rates of millions of tonnes per year mostly using fossil resources as feedstock [4]. Current production processes for SCOAs usually require high temperature and non-renewable metal catalysts, which negatively impact their sustainability [4]. On the other hand, the production of SCOAs by AF only requires organic waste as feedstock, uses temperatures close to ambient values and doesn't need the external addition of metal catalysts.

Although it has been shown that use of organic waste in biorefinery to produce organic acids and hydrogen can be more profitable than methane production [5] and there has been considerable research effort on the production of SCOAs with AF [6], to the best of our knowledge there are to date no commercial plants that use AF to produce SCOAs, mainly because of the high overall cost.

This study was aimed at investigating AF for SCOA production in a low-cost and environmentally friendly process operated at ambient temperature and at uncontrolled pH. The use of ambient temperature reduces the energy consumption of the process,

and the use of uncontrolled pH reduces the need for chemicals addition, both factors contributing to an improved sustainability of the process. This investigation focused on the SCOA yield, concentration and productivity, which are key process performance variables. Indeed, a successful AF process requires SCOAs to be produced at high yield to maximise the products produced per unit mass of feedstock, at high concentration to make the downstream separation easier, and at high productivity to maximise the products produced per unit volume of reactor and unit time. The following process design parameters were investigated in this study: feedstock concentration, hydraulic residence time (HRT), solids residence time (SRT). Two reactor configurations were used [7]: semi-continuous CSTR (continuously stirred tank reactors) with intermittent feed without solids separation and SBR (sequencing batch reactors) with solids separation by settling. In the CSTRs the HRT was the same as the SRT, while in the SBRs the HRT and SRT were decoupled, allowing the separate investigation of the effect of the two parameters. The CSTR is a simple reactor configuration and was used to investigate the effect of residence time on process performance. The SBR is a more complex reactor configuration and was used to investigate the possibility of achieving high productivity with high yield, by working at short HRT and long SRT.

This study aimed to fill several gaps in the literature, identified in a recent literature review [8]:

- High feedstock concentration: high feedstock concentration is essential to obtain high product concentration and high productivity. However, only 10 % of previous studies used a feedstock concentration higher than 127 gCOD L^{-1} (COD=Chemical Oxygen Demand). The highest substrate concentration used in this study (394.6 gCOD L^{-1}) is

close to the maximum concentration of undiluted food waste and is among the highest concentrations used so far in these studies.

- SBRs: only 3 % of previously reported studies were carried out in SBR or in systems with SRT different from the HRT. However, the very few SBR studies have generally reported higher SCOA concentration, yield and productivity than the much more numerous studies performed in CSTR and batch.

- Low process temperature and acidic pH: most previously reported studies were carried out at approximately neutral pH (range 6-8) and at mesophilic temperature (35-55°C). On the other hand, the relatively few studies that were carried out at low pH and low temperature (lower than 30°C) gave good performance, highlighting the need for more studies in this more environmentally friendly range of process variables.

This study extends the results of our previous study carried out with the same feedstock in batch reactors [9]. Our previous study obtained, with uncontrolled pH, SCOA yields in the range 15-22% g COD g COD^{-1}, product concentrations up to 61 g L^{-1} and productivities up to 1.5 g L^{-1} d^{-1}. The present study aims to improve process performance by using semi-continuous reactors. To improve our understanding of mixed-culture AF processes for SCOA production, this study also investigated the evolution of the composition of the microbial community in selected bioreactors.

2 Materials and methods

2.1 Substrate and inoculum

The feed to the reactors was a model substrate prepared in the lab using commercial chemicals as reported in our previous study [9]. The substrate was prepared by mixing wheatgrass (72.1 g L^{-1} for the most concentrated feed, feed A), yeast extract (80.0 g L^{-1}), soluble starch (45.7 g L^{-1}), peptone (26.0 g L^{-1}), sucrose (66.6 g L^{-1}), oleic acid (52.6 g L^{-1}) and distilled water. These concentrations were chosen in order to represent the total concentration of organic matter and the macronutrient composition (considering fats, proteins, fibre, total carbohydrates and sugars) of unavoidable food and drink waste produced in the UK, calculated using data on UK's waste [10] and information on typical food composition [11]. The substrate at the highest concentration (feed A) had a total COD of 394.6 g L^{-1},164.4 g VSS L^{-1} , 172 g TC L^{-1} (VSS=Volatile Suspended Solids, TC=total carbohydrates) and was diluted 1:2, 1:4, 1:8 and 1:16 to obtain four concentrations (feeds B, C, D, E respectively) at total COD of 197.3, 98.7, 49.3 and 24.7 g L^{-1}. In the feed, the soluble COD represented 63 % of the total COD and the soluble carbohydrates represented 62 % of the total carbohydrates. The pH of the feed was in the range 5.9 (for the most concentrated feed) to 6.4 (for the most diluted feed). The inoculum was an anaerobic mesophilic sludge, obtained from an anaerobic digester in Turriff, Aberdeenshire, Scotland, fed with pig slurry, fish, bakery and cow waste. After collection, it was stored at 4°C and filtered with a Buchner funnel to remove larger, undigested solids prior to use. The inoculum had VSS concentration of 19 g L^{-1}, pH of 8.5 and total COD of 43 g L^{-1}. The characterization of the substrate and of the inoculum are described in our previous work [9].

2.2 Experimental set-up

Two types of reactor runs were carried out (Table 1): semi-continuous runs without phase separation (CSTR runs) and sequencing batch reactors with settling of the suspended solids (SBR runs). Customised glass jacketed reactors with a working volume of 300 ml were used for all experiments. The reactor type and size were chosen in order to ensure good mixing while minimizing the volumes of feed required. While the reactor size was appropriate for the aims of this study, i.e. the investigation of the effect of residence time and feed concentration, further study at larger scale is required to investigate scale-up effects (e.g. mixing effectiveness, mass and heat transfer) before process transfer to commercial scale. They were closed at the top with PTFE lids and sealed via a ground glass flange with a fluorinated ethylene propylene coated O-ring. The lid was secured to the vessel through a stainless-steel quick release clamp. In CSTR runs with a residence time of 120 days, one port in the lid was used for manual discharging and feeding. The CSTR runs with residence times of 30 and 7.5 days had two ports for feed inlet and discharge outlet. In the SBR runs, a third port was also used to sample the reactor's content during stirring. Rubber bungs closed the unused ports.

All reactors were started up by filling with 285 ml of substrate and 15 ml of inoculum, then flushed with bubbling nitrogen for 10 minutes and immediately closed with a rubber bung. The reactors' content was continuously mixed using magnetic stirring (300-450 rpm). The reactors were operated at ambient temperature (21-25 °C) and without pH control.

The CSTRs were operated with intermittent feed with the following scheme: HRT 120 d, 52.5 mL every 21 d; HRT 30 d, 10 mL every 24 h; and HRT 7.5 d, 20 mL every 12 h.

Immediately before feeding, a volume equivalent to the volume of feed was discharged. Feed and discharge were performed manually (instant feed) at HRT 120 d and with peristaltic pumps (VELP Scientifica SP 311, feed length 1 min) at HRT 30 and 7.5 d. The pumps were activated through a Power Management System (Energenie ENER011) consisting of multiple sockets set via software.

The SBRs were operated with 6 cycles per day. SBR cycles consisted of a sequence of phases: feed (2 min), reaction (176 min), settling (60 min) and discharge (2 min). The phases were controlled by the same Power Management System used for the CSTRs. The pumps used for feeding and discharge were of the same type used for the CSTRs. In all SBRs, a volume of 25 mL was fed and discharged in each cycle. In contrast to the CSTRs, the SBRs agitation was stopped for part of the cycle (settling phase) to allow the suspended solids to settle. The discharge was done from the top of the liquid and therefore only included those suspended solids which didn't settle within the settling time. In the SBRs, no suspended solids were withdrawn except those removed for sampling and during the discharge phase, therefore the SRT was mainly determined by the settling behaviour of the biomass.

2.3 Analytical methods

The reactors were sampled once or twice per week for the measurement of the pH, TSS, VSS, soluble and total COD, soluble carbohydrates, and fermentation products. The products of interest were ethanol and SCOAs (lactic acid, acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid, iso-caproic acid and caproic acid). In the CSTRs samples were taken just before feeding. In the SBRs samples were taken at

the end of the reaction phase and, for the measurement of the effluent VSS only, from the discharged effluent.

For the measurement of VSS, soluble COD, soluble carbohydrates and fermentation products, the samples were filtered on glass microfibre filters grade GF/F, with porosity 0.6-0.8 μm. Samples of the reactors with substrate concentration A and B were diluted respectively 1:5 and 1:2 prior to filtration due to high viscosity and solids content. pH was manually measured with a pH probe (Sentek). TSS and VSS were measured according to standard methods [12]. COD was measured with cell test kits (Spectroquant COD cell test, from Merck Millipore). Carbohydrates were measured via colorimetric method using the anthrone reagent [13]. Fermentation products were measured by gas chromatography using a flame ionization detector (FID) and a TG-WAX MS A capillary column. The column temperature was held at 80 ^{O}C for 2 min, then increased to a final temperature of 200 ^{O}C at a rate of 10 ^{O}C min^{-1}. The final temperature of 200 ^{O}C was held for 1 min. The injector and detector temperatures were 250 ^{O}C. Further details on the gas chromatographic for the analysis of the fermentation products have been reported earlier [9, 14].

2.4 Calculations

Results are presented as the average values for each run. For the CSTR runs, where the SRT was equal to the HRT, the average value of each parameter was calculated from all the data points collected during each run, ignoring the data collected in the initial start-up phase of the run. The initial start-up phase was assumed to have the length of 1 HRT (e.g., in the runs with HRT 30 d, the data collected in the first 30 d were not included in the calculations). In the SBR runs, since the SRT was variable during each run and

different for different runs, the average value of each parameter was calculated from the data points collected in the final part of the run, ignoring the initial data points when the total product concentration was not approximately constant with time. The length of all runs was in all cases longer than 2 HRTs. Table 1 reports the length of each run and the number of data points used in each run to calculate the average values. The average values were reported with their standard error, calculated as the standard deviation divided by the number of data points used to calculate the averages. Figures S1-S4 in the Supplementary Materials report the time profiles of the total products in each run, showing the data points used for the calculations of the average values.

For both CSTR and SBR runs, product concentration was defined as the sum of SCOAs and ethanol and reported as g L^{-1}. Productivity and yield were calculated according to Eq (1) and Eq (2) respectively, and product composition was calculated in % (w/w). The removal of soluble carbohydrates (SC) was calculated according to Eq 3 where SC_{feed} and $SC_{reactor}$ represent the concentration of SC in the feed and in the reactor respectively.

$$Productivity\ (g\ L^{-1}d^{-1}) = \frac{g\ L^{-1}\ products}{HRT} \qquad \text{Eq 1}$$

$$Yield\ (\%\ g\ COD\ g\ COD^{-1}) = \frac{gCOD\ L^{-1}\ products}{gCOD\ L^{-1}\ feed} \cdot 100 \qquad \text{Eq 2}$$

$$SC\ removal(\%) = \frac{SC_{feed} - SC_{reactor}}{SC_{feed}} \cdot 100 \qquad \text{Eq 3}$$

In the SBR runs, the SRT was calculated from the measurement of VSS in the effluent and in the reactor (Eq 4):

$$SRT\ (d) = \frac{V \cdot VSS_{reactor}}{Q_{sampling} \cdot VSS_{reactor} + Q_{effluent} \cdot VSS_{effluent}} \qquad \text{Eq 4}$$

where V is the working volume of the reactor (0.3 L), $Q_{sampling}$ and $Q_{effluent}$ (L d^{-1}) are the volumetric flow rates of the samples and of the effluent, and $VSS_{reactor}$ and $VSS_{effluent}$ (g L^{-1}) the concentration of VSS in the mixed reactor and in the discharged effluent.

2.5 Statistical analysis

Statistical analyses were performed on the CSTR data using the software Minitab 20. The analyses were performed on the two design variables feed concentration and HRT, using a full quadratic design, for the three performance variables product concentration, yield, and productivity.

2.6 Microbial community analysis

Reactors CSTR 30 A, CSTR 30 B, CSTR 7.5 A and CSTR 7.5 B were sampled every 7 days for microbial composition analysis. The microbial composition of these reactors was analysed using 16S rRNA gene profiling. The inoculum was also sampled for this analysis. Samples were stored at -80°C prior to DNA extraction using the DNeasy PowerSoil Pro kit (Qiagen, Germany) following the manufacturer's instructions. Dual-indexed Illumina-compatible libraries were prepared from 2.5 μl extracted DNA and no template control, using 2 rounds of PCR to firstly amplify V1-V2 region of the 16S rRNA gene with primers containing a region specific sequence [15] and an Illumina compatible overhang (Forward primer: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGMGTTYGATYMTGGCTCAG 3'; Reverse primer: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCCTCCCGTAGGAGT 3'), followed by second round PCR to introduce barcodes and Illumina adapters, according to manufacturer's protocols for 16S metagenomic sequencing (Illumina, CA). Final libraries were quantified (Quant-IT, Thermo Fisher Scientific, UK and Tapestation 4200,

Agilent, CA), equimolar pooled, and sequenced on an Illumina MiSeq v2 Nano flowcell producing 250 bp paired end reads. The quality of the raw sequences was assessed using FastQC (version 0.11.8) [16]. The DADA2 package (version 1.14.0) for R (version 3.6.0) was used for trimming and filtering reads and for ASV (amplicon sequence variant) identification. All reads were hard trimmed to 220 base pairs to remove low quality bases (<Q30) at the 3' end [17]. Trimmed reads were filtered, with retention of reads having a maximum expected error of 1 [18]. Amplicon sequence variants (ASVs) were identified in the filtered reads using DADA2. The software uses error modelling to predict the likelihood of sequencing errors along the length of each read and uses this information to help distinguish between true unique sequence variants and sequencing errors. Taxonomic assignment was performed with the SILVA4 database (version 132) [19]. The resulting output is a sequence table containing the number of times each ASV occurred in each sample and a taxonomy table containing the taxon assigned to each ASV. There were 1792 unique ASVs identified. Following the removal of 8 singletons, 1784 ASVs were carried forward in the analysis. The mean read depth across samples was 11629 (min=1777, max=31970, median=10042). All samples were retained for analysis. The ASV table and taxonomy information were combined with metadata to create a phyloseq (version 1.32.0) object for further analysis [20]. Sequence variants from mitochondria or chloroplasts were removed leaving 1540 ASVs. Phyloseq and ggplot2 (version 3.3.4) [21] were used to produce plots of the 20 most abundant taxa at each taxonomic rank.

3 Results

3.1 CSTRs

Figure 1 shows the average results of the CSTRs. Product concentration (Figure 1a) increased with feed concentration as expected. The highest values of 113.4 ± 6.32 and 100.8 ± 8.91 g L^{-1} were achieved in CSTR 120 A and CSTR 30 A. The yield (Figure 1b) was in most cases in the range 15-35 % g COD g COD^{-1}. The highest value of 43.7 ± 1.47 % g COD g COD^{-1} was observed in CSTR 120 E, followed by CSTR 120 A with a yield of 32.2 ± 1.78 % g COD g COD^{-1}, which was very similar to the yield obtained in CSTR 120 B and 120 D. The productivity (Figure 1c) was higher for shorter HRT and for higher feed concentrations. CSTR 7.5 A had the highest productivity of 9.7 ± 0.64 g L^{-1} d^{-1}.

The pH (Figure 1d) was acidic (between 3.6 and 4.9) in reactors with feed A, B, C or D and feed E with 120d HRT. Reactors with feed E and shorter HRT had a higher pH value (6.9). The fraction of the total COD of the feed that was present as soluble COD (Figure 1e) was generally higher for higher feed concentrations and for longer HRT. The highest value of 94.6 ± 4.84 % SCOD $TCOD_{feed}^{-1}$ was observed in CSTR 120 A. A virtually complete removal of SC (Figure 1f) was observed in all reactors with feed E. The removal of SC was above 85 % for the reactors with feed B, C or D, except for a slightly lower value in CSTR 120 B. CSTR with feed A and 30 d HRT had a similar level of SC removal. However, SC removal was much lower for feed A at 7.5 and 120 d HRT at around 40 %.

By taking the averages of all feed concentrations, Figure 2 shows the yield (2a) and productivity per unit of feed concentration (2b) as a function of the HRT. It can be observed that the product yield increased and the productivity per unit feed concentration decreased as the HRT increased.

3.2 Statistical analysis

Using the results of the CSTR runs (section 3.1), Table 2 shows the analysis of variance of the response surface regression of the performance variables product concentration, yield and productivity versus the design parameters feed concentration and HRT. Product concentration is highly significantly affected by substrate concentration and HRT (p-values < 0.001 and 0.004, respectively). Interaction between substrate concentration and HRT is also significant. Yield is significantly affected by HRT, with a p-value of 0.020, and not significantly affected by the substrate concentration (p-value 0.895), however it is affected by the square term of substrate concentration with a slightly significant effect (p-value 0.046). The correlations between productivity and both substrate concentration and HRT are highly significant, with p-values of 0.001 and < 0.001, respectively. The squared HRT and the interaction between HRT and substrate concentration are also significant.

The model (Table 3) shows for the product concentration an adjusted R square of 95.80%, which is high and in reasonable agreement with the predicted R square, indicating that the model is a good fit of the data and shows a high level of correlation. For the yield the model shows an adjusted R square of 48.69%, which is low and far from the predicted value (14.94%), hence the model is not a good fit of the data. For the productivity, the model shows an adjusted R square of 85.56% and a predicted R square of 58.72%, indicating a modest model fit.

The obtained regression equations (in uncoded units) for product concentration, yield and productivity are shown in Eq. 5-7, where non-significant terms (p > 0.05) are removed. In Eq. 5-7 the HRT has the units of d and the feed concentration of gCOD L^{-1}.

$$Product\ concentration\ (g\ L^{-1}) = 2.11 + 0.0701\ Feed\ concentration + 0.050\ HRT + 0.000772\ Feed\ concentration * HRT \quad \text{Eq 5}$$

$$Yield\ (\%\ g\ COD\ g\ COD^{-1}) = 22.54 + 0.191 HRT + 0.000267 Feed\ concentration\text{^}2 \quad \text{Eq 6}$$

$$Productivity\ (g\ L^{-1} d^{-1}) = 1.718 + 0.01738\ Feed\ concentration - 0.1263\ HRT + 0.000959\ HRT\text{^}2 - 0.000165\ Feed\ concentration * HRT \quad \text{Eq 7}$$

3.3 SBRs

Figure 3 shows the average results of SBRs. In contrast to CSTR runs, in SBRs the SRT was uncoupled from the HRT and was determined by the settling of the suspended solids, i.e. by the ratio of the VSS in the effluent and in the reactor (Figure 3a). The VSS in the effluent were quite low for the most diluted feeds D and E, indicating good settling, but they increased significantly with the feed concentration for feeds C, B and A, indicating poorer settling due to the high feed concentration. Consequently, the SRT (Figure 3b) was low (5 d or lower) in runs with feeds A, B, C and was higher (in the range 15-20 d) for runs with feeds D and E.

The product concentration (Figure 3c) increased with feed concentration, although similar values were observed for SBRs A and B (18.1 ± 0.71 g L^{-1}; 18.9 ± 2.20 g L^{-1}). On the contrary, the yield (Figure 3d) was higher for the more diluted feeds. The highest yield of 29.9 ± 1.32 % g COD g COD^{-1} was achieved in SBR E. The productivity (Figure 3e), like the product concentration, increased with the feed concentration, and the highest values were observed in reactors with feeds A and B (respectively 9.0 ± 0.35 and 9.4 ± 1.10 g L^{-1} d^{-1}).

A pH of 3.9-4 was measured in all reactors except in SBR E, where it was slightly higher, with a value of 4.7 (Figure 3f). The ratio between soluble COD of the effluent and total COD of the feed (Figure 3g) was similar, with values between 51.7 and 55.3 %, in reactors with intermediate feed concentration. SBR E had a lower value, while reactor SBR A had the highest ratio: 62.2 ± 4.17 % $SCOD_{effluent}$ $TCOD_{feed}^{-1}$. SC removal (Figure 3h) was virtually total for reactors with feeds D (88.5 ± 1.72 %) and E (97.6 ± 0.41 %) and lower with feeds A and B.

The plot in Figure 4 indicates that the yield generally increased with the SRT. Figure 5 shows the ratio of the productivity in SBR (from Figure 3e) vs the productivity in CSTR (from Figure 1c) as a function of the feed concentration. For feed A (highest concentration) the productivity was similar in SBR and CSTR while for all other feeds the productivity was higher in SBR, the highest increase being observed for the most diluted feeds D and E.

3.4 Product composition

Figure 6 shows the product composition of CSTRs and SBRs. Lactic acid was the main product in experiments with feed A, B, C and D. It was generally followed by acetic acid and ethanol. CSTR 30 D has the highest percentage of lactic acid (85.6 %) with similar levels in CSTR 30 A (83.8 %) and 120 A (83.0 %). CSTR runs 30 A and 120 A also had the highest lactic acid concentration, 84.5 ± 8.91 and 94.1 ± 5.57 g L^{-1} respectively. Reactors with feed E showed a more diverse product composition than the other reactors, with higher percentages of acetic acid and some modest amounts of propionic (between 5.0-15.3 %) and butyric acids (between 7.6- 16.4 %). Small amounts of all other SCOAs were also present. In feeds E, ethanol was produced at very low amounts, between 1.1 and

3.7 %, and lactic acid was generally produced at lower percentages than in reactors with the other feeds (up to 50.1%). The highest percentage of acetic acid was observed in SBR E (38.6%), which had also the highest percentages of propionic (15.3%) and butyric (16.4%) acids. In Figure 7 the content of lactic acid and of the other SCOAs are shown as a function of the total product concentration: the general trend was an increase in lactic acid content and a decrease in the content of other SCOAs as the product concentration increased.

3.5 Microbial community

The analysis of the microbial community focused on the CSTR runs at high feed concentration and shorter HRT, where the highest productivity was observed, a critical output parameter for sustainable and economically attractive production. Figure 8 shows the composition of the microbial community at phylum and genus levels in the inoculum and from days 7 to 91 during runs CSTR 30 A, 30 B, 7.5 A, 7.5 B. In the analysed runs, the composition of the microbial community rapidly changed after start-up. While the inoculum included a diverse microbial community, the CSTR runs were dominated by microorganisms belonging to the Firmicutes genus *Lactobacillus*. The Bacteroidetes phylum virtually disappeared from an early timepoint, consistent with the high lactic acid and low levels of propionate and butyrate in the reactors. The community composition shifted rapidly in the first few weeks of the runs and approximately stabilised from day 49 onwards. In all reactors, after just 7 days of operation most of the genera present in the inoculum were not detectable or were present in small fractions. In the first few weeks of operation, in addition to the genus *Lactobacillus*, the genus *Pediococcus* increased in abundance compared to the inoculum, however *Pediococcus*

abundance later decreased to low or non-detectable levels. After day 42, *Lactobacillus* was virtually the only genus detected in CSTR runs 30A, 7.5A and 30B, while in run 7.5B some *Acetobacter* was also detected. Resolution to species level using 16S gene variable region profiling is typically poor, and therefore the majority of ASVs belonging to *Lactobacillus* were not assigned a species. Small amounts of *Lactobacillus brevis* were found in CSTRs 30 B, 7.5 A, 7.5 B, while *Lactobacillus acetotolerans* was present in CSTRs 30 A and 30 B.

4 Discussion

The results of our CSTRs and SBRs show that, for some of the reactors, product concentrations and productivities are among the highest of those reported previously [8], highlighting the benefits of working at high feedstock concentration. More remarkable are the highest SCOA plus ethanol concentrations (113.4 g L^{-1}) ever reached to our knowledge using mixed microbial cultures. Table 4 compares the best results obtained in this study with selected results from the literature.

To our knowledge, only few studies have investigated AF in semi-continuous mode with feed concentrations higher than 100 gCOD L^{-1} [8]. Yields previously reported were, in most cases, in the same range as the ones obtained in our CSTRs (15-35%), except in very few studies where higher yields (41-49 % g COD g COD^{-1}) could be attributed to control of pH to less acidic values than in our study (5.5-6) [31]. A less acidic pH may bring an increase in the SCOA yield as also observed in other studies e.g. [9, 32], however it brings the disadvantage of the need for either chemicals to control pH or use of more dilute feeds. High feed concentrations are rarely investigated because of the high substrate viscosity and difficulty in controlling the pH to neutral values [33]. However, this study has shown that high concentration of substrate can produce high concentrations of products with high productivity without the need of pH control, making the process more commercially attractive. There is therefore need for more studies at high feed concentration, including investigation of process stability and mixing effectiveness in scaled-up reactors.

As far as the effect of the residence time is concerned, our SBR results, obtained at the same HRT but at different SRT, indicate that it is the SRT rather than the HRT that

determines the product yield. Very few studies have been carried out in systems with SRT different from HRT. Karthikeyan et al. [34] used a mesh system to retain solids, managing to improve performance compared to similar experiments with HRT=SRT, achieving a yield of 70.9 % g COD g COD^{-1}. Park et al. [28] investigated the AF of diluted model kitchen waste in a reactor with HRT different from SRT, achieving a SCOA productivity of 55.5 g L^{-1} d^{-1} at HRT 1 d and SRT 2 d. This productivity is, to the best of our knowledge, the highest value reported in the literature for SCOA from complex organic waste. However, the concentration of SCOA obtained by Park et al. [28] was lower than in our study, up to 60 gCOD L^{-1}, and their investigation was carried out at 55°C and at the controlled pH of 6. These conditions are likely to improve the SCOA yield but, as stated earlier, have a cost in terms of process sustainability. The vast majority of AF studies have been done with HRT equal to the SRT. In these systems, Farouk et al. obtained their highest volatile fatty acids (VFA) concentration (40.2 g L^{-1}) with an intermediate HRT among the values investigated [35], similar to Cavinato et al. [36] and Luongo et al. [37]. Han et al. [25] observed an increase in production with increasing HRT up to 3 d. Increase of product concentration as the HRT increased was also observed in Jankowska et al., and Lim et al. [27, 38]. Overall, the SCOA yield is favoured by long SRT (at least in systems with no methanogenesis, e.g. due to the acidic pH), while SCOA productivity is favoured by short HRT. Hence, systems with SRT longer than the HRT, like SBR or membrane reactors, should be preferred. However, as we have seen in this study, solid-liquid separation can become difficult at high feedstock concentration preventing an efficient decoupling of the SRT from the HRT. From Figure 5, the benefits of SBRs over CSTRs are evident with diluted feeds D and E, where the relatively good settling allowed to achieve relatively long SRT, obtaining much higher SCOA productivities than with the

same feeds in CSTR mode. With more concentrated feeds and poorer settling properties, Figure 5 shows that the benefits of SBRs are less evident although the productivity for feeds B and C at intermediate concentration was still higher in SBR than in CSTR. In summary, the benefits of SBR are achieved when short HRT can be coupled with long SRT. More research needs to be done on achieving long SRT and short HRT in systems with high feedstock concentration.

Production of SCOAs in AF is also reported in many literature studies aimed at biohydrogen production (dark fermentation, DF) [39]. In DF studies, SCOAs are usually produced at lower concentrations than in AF studies specifically aimed at SCOAs. The composition of SCOAs from DF is usually dominated by acetic and butyric acids since hydrogen is mainly produced simultaneously to the production of these acids [40]. This SCOA composition is different from what we observed in most runs in this study, where the dominant product was lactic acid (see also the discussion in the next paragraph). In general, the conditions more favourable for biohydrogen production include relatively diluted feedstocks and moderately acidic pH. A recent review on DF reports SCOA production from various types of waste (e.g. food waste, sewage sludge, manure) at concentrations up to approximately 30 g L^{-1} with pH values in most cases of 5 or above [41].

The product composition in this study was dominated by lactic acid for most feedstock compositions, except for the most diluted (reactors with feed E). The evidence of higher concentration of lactic acid and lower concentration of other acids as the total product concentration increased (Figure 7) confirms and reinforces what we already observed in our earlier study in batch experiments [9]. This result can be explained based on acid

inhibition and on the pKa of these acids and on the pH tolerance of different microbial strains. It is known that the undissociated form of acids is the most inhibiting in anaerobic digestion. Since lactic acid is more acidic than any other SCOAs, at pH 4.0 most of lactic acid will be present in dissociated form while other acids would be present mostly in undissociated form. Therefore, in an acidic system when the feed concentration is high leading to high concentration of products, microorganisms such as *Lactobacillus* that can produce the less inhibiting acid, lactic acid, are favoured. When the feed concentration is relatively low leading to a low product concentration, microorganisms that can produce other acids, e.g. acetic, propionic and butyric, can survive and grow, because the concentration of the acids, even though they'll mainly be undissociated, will not reach toxic levels. Studies on the pH tolerance of pure microbial strains and of gut bacteria [42-44] also indicate that lactic acid producers are more tolerant of acidic pH than butyric and propionic acid producers. Since lactic acid producers are not known to be able to produce butyric or propionic acid, an acidic pH, which is typical of high-concentration feed without pH control, favours lactic acid predominance in the reaction products. In our earlier study in batch experiments with pH in the range 4-6 [9], lactic acid was the main product at high feed concentration, while acetic acid was the main product at low feed concentration in the whole pH range 4-6. Production of lactic acid from organic waste using AF can become commercially important as lactic acid has many applications, including its growing use for poly-lactic acid synthesis. The preferential production of lactic acid at high feedstock concentration may have consequences on the spectrum of products obtainable with mixed culture AF in a biorefinery context. Indeed, biochemistry shows that production of lactic acid via homofermentative or heterofermentative pathways is not associated with hydrogen

production. In a biorefinery context, it seems likely that AF of high concentration feedstocks at uncontrolled pH will mainly yield lactic acid with little or no hydrogen. If biohydrogen production is desired, diluted feedstocks or pH control to less acidic values should be considered, as already discussed above.

Since this study was targeted at the liquid-phase products, no measurements were taken about the possible production of biogas (methane, hydrogen and carbon dioxide) during the experiments. Therefore, it cannot be excluded that some of the COD of the feed was converted into hydrogen and/or methane. Methane production usually does not occur at the acidic pH (< 5) of most of the reactor runs presented in this study, however it may have occurred in some of the CSTR runs with the diluted feed E where the pH was approximately neutral. Further studies are needed to measure all the fermentation products, included those in the gas phase. This could be achieved by direct measurement of the biogas flow rate and composition and/or by measuring the COD of the digestate using analytical methods suitable for streams with high solids concentration. Measuring the COD of the digestate, together with measurement of the biogas produced, would also allow a full closure of the COD balance with identification of all the outlet routes for the COD of the feed.

The analysis of the microbial community in CSTR runs with feeds A and B indicates that the selected design conditions operated a very strong and rapid selection with only very few microorganisms, among the many microorganisms in the inoculum, being able to survive and grow. It is remarkable that the microbial community was already significantly changed just 7 days after start-up, in spite of the relatively long residence times of 7.5 and 30 d. The fact that many genera found in the inoculum were not found

after just 7 days of operation indicate that the chosen operating conditions caused the inactivation of most microorganisms. The composition of the microbial communities corresponds to the observed predominance of lactic acid among the produced SCOAs. The dominant genus found in our reactors, *Lactobacillus,* is known to dominate under acidic conditions [45, 46]. Considering the species that were identified in the CSTR runs, *Lactobacillus brevis* is a heterofermentative bacterium associated with the production of ethanol, lactic acid and acetic acid [47], not inhibited by high substrate concentrations [48] while *Lactobacillus acetotolerans* produces lactic acid homofermentatively but is tolerant to the presence of acetic acid [49].

In the broader context of biorefinery for waste valorisation, AF at uncontrolled pH and high feed concentration, as reported in this study, can represent a first stage of treatment. The liquid SCOA-rich phase from AF should be separated from the undigested suspended solids and sent to SCOA separation, concentration or conversion. The undigested suspended solids, which contain a significant fraction of the feed COD, should be converted to energy or chemicals via biological (anaerobic digestion carried out at more neutral pH and/or longer residence time than the AF stage) or chemical (e.g. pyrolysis or gasification) processes or should be spread on soils, where the undigested organic matter can have a beneficial effect on physical properties [50].

5 Conclusions

Overall, this study shows that AF of concentrated biomass at uncontrolled pH and ambient temperature is a promising strategy for the production of SCOAs at high concentration and high productivity. Very high SCOA concentrations of more than 100 g L^{-1} and very high productivities of almost 10 g L^{-1} d^{-1} were obtained in this study. The chosen operating conditions, characterised by high feed concentration and uncontrolled pH, led to lactic acid being the main product in most runs and imposed a strong and rapid selection in the microbial community which became dominated by *Lactobacillus*.

Compared to CSTRs, SBRs have shown to give higher productivity for less concentrated feeds, however at higher feed concentrations the effectiveness of SBRs was limited. More studies are needed on the practical operation of fermenters with concentrated feedstocks at larger scale and on finding innovative processes for the uncoupling of SRT and HRT to maximise both product concentration and productivity.

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Supplementary material

E-supplementary data can be found in online version of the paper.

Tables

Table 1. Summary of operating conditions and data analysis for CSTR and SBR runs

	Feed Concentration (gCOD L^{1})	HRT (d)	Organic load rate (OLR, gCOD L^{-1} d^{-1})	Run length (d)	Number of data points used to calculate the average values
CSTR 120 A	394.6	120	3.29	315	10
CSTR 120 B	197.3	120	1.64	315	10
CSTR 120 C	98.7	120	0.82	273	7
CSTR 120 D	49.3	120	0.41	315	10
CSTR 120 E	24.7	120	0.21	315	10
CSTR 30 A	394.6	30	13.15	90	9
CSTR 30 B	197.3	30	6.57	90	9
CSTR 30 C	98.7	30	3.29	84	14
CSTR 30 D	49.3	30	1.64	72	13
CSTR 30 E	24.7	30	0.82	84	8
CSTR 7.5 A	394.6	7.5	52.61	90	12
CSTR 7.5 B	197.3	7.5	26.30	90	12
CSTR 7.5 C	98.7	7.5	13.16	84	11
CSTR 7.5 D	49.3	7.5	6.57	84	11
CSTR 7.5 E	24.7	7.5	3.29	84	11
SBR A	394.6	2	197.30	63	4
SBR B	197.3	2	98.65	70	7
SBR C	98.7	2	49.35	122	15
SBR D	49.3	2	24.67	105	17
SBR E	24.7	2	12.3	92	8

Table 2. Response surface regression: product concentration, yield and productivity vs substrate concentration and HRT. Analysis of variance: degrees of freedom (DF), adjusted sum of squares (Adj SS), adjusted mean squares (Adj MS), F-value (variance of the group means/mean of the within group variances), and P-value (probability)

Source	Perfomance variable	DF	Adj SS	Adj MS	F-Value	P-Value
Model	Product concentration	5	17427.9	3485.6	64.86	0.000
	Yield	5	637.557	127.511	3.66	0.044
	Productivity	5	87.0629	17.4126	17.59	0.000
Linear	Product concentration	2	16528.4	8264.2	153.77	0.000
	Yield	2	279.762	139.881	4.01	0.057
	Productivity	2	72.9511	36.4755	36.85	0.000
Feed concentration	Product concentration	1	16234.3	16234.3	302.07	0.000
	Yield	1	0.640	0.640	0.02	0.895
	Productivity	1	26.5650	26.5650	26.84	0.001
HRT	Product concentration	1	767.4	767.4	14.28	0.004
	Yield	1	274.971	274.971	7.89	0.020
	Productivity	1	40.4207	40.4207	40.83	0.000
Square	Product concentration	2	262.6	131.3	2.44	0.142
	Yield	2	188.986	94.493	2.71	0.120
	Productivity	2	11.3966	5.6983	5.76	0.025
Feed concentration^2	Product concentration	1	261.1	261.1	4.86	0.055
	Yield	1	187.156	187.156	5.37	0.046
	Productivity	2	11.3966	5.6983	5.76	0.025
HRT^2	Product concentration	1	1.5	1.5	0.03	0.872
	Yield	1	1.830	1.830	0.05	0.824
	Productivity	1	11.2342	11.2342	11.35	0.008
2-Way Interaction	Product concentration	1	382.7	382.7	7.12	0.026
	Yield	1	25.244	25.244	0.72	0.417
	Productivity	1	17.4033	17.4033	17.58	0.002
Feed concentration*HRT	Product concentration	1	382.7	382.7	7.12	0.026
	Yield	1	25.244	25.244	0.72	0.417
	Productivity	1	17.4033	17.4033	17.58	0.002
Error	Product concentration	9	483.7	53.7		
	Yield	9	313.780	34.864		
	Productivity	9	8.9088	0.9899		
Total	Product concentration	14	17911.6			
	Yield	14	951.337			
	Productivity	14	95.9717			

Table 3. Response surface regression: product concentration, yield and productivity vs feed concentration and HRT. Model summary: standard deviation of the distance between the data values and the fitted values (S), percentage of variation in the response that is explained by the model (R-sq), R-sq adjusted (R-sq(adj)), and R-sq predicted (R-sq(pred)).

Performance variable	S	R-sq	R-sq(adj)	R-sq(pred)
Product concentration	7.33100	97.30%	95.80%	86.22%
Yield	5.90461	67.02%	48.69%	14.94%
Productivity	0.994923	90.72%	85.56%	58.72%

Table 4. Selected examples of product concentration, yield and productivity in anaerobic fermentation studies from the literature. The products refer to the total SCOAs and ethanol. In this table, the highest values for the concentration, yield and productivity obtained in each study are reported.

Feed	Reactor configuration	Product concentration (g L^{-1})	Yield (% g COD g COD^{-1})	Productivity (g L^{-1} d^{-1})	Ref.
Food waste (127 gCOD L^{-1})	CSTR	10	9	3.8	[22]
Food waste (130-163 gCOD L^{-1})	CSTR	25	26	3	[23]
Food waste (1.75-8.76 % TS)	CSTR	42	35	3.8	[24]
Food waste-recycling wastewater (128 gCOD L^{-1})	CSTR	25	32	19	[25]
Food waste (157 gCOD kg^{-1})	Batch, CSTR	30	40	5	[26]
Food waste (20-60 gVS L^{-1})	CSTR	25	39	3.8	[27]
Kitchen waste (3.5-10 % TS)	Batch, CSTR	35	33	30	[28]
Food waste (7 % TS)	Batch, CSTR	30	46	6.7	[29]
Fruit and vegetable waste (70-110 gTS L^{-1})	Batch, CSTR	16	33	1.6	[30]
Model food waste (25-395 gCOD L^{-1})	CSTR, SBR	113	43	9.7	This study

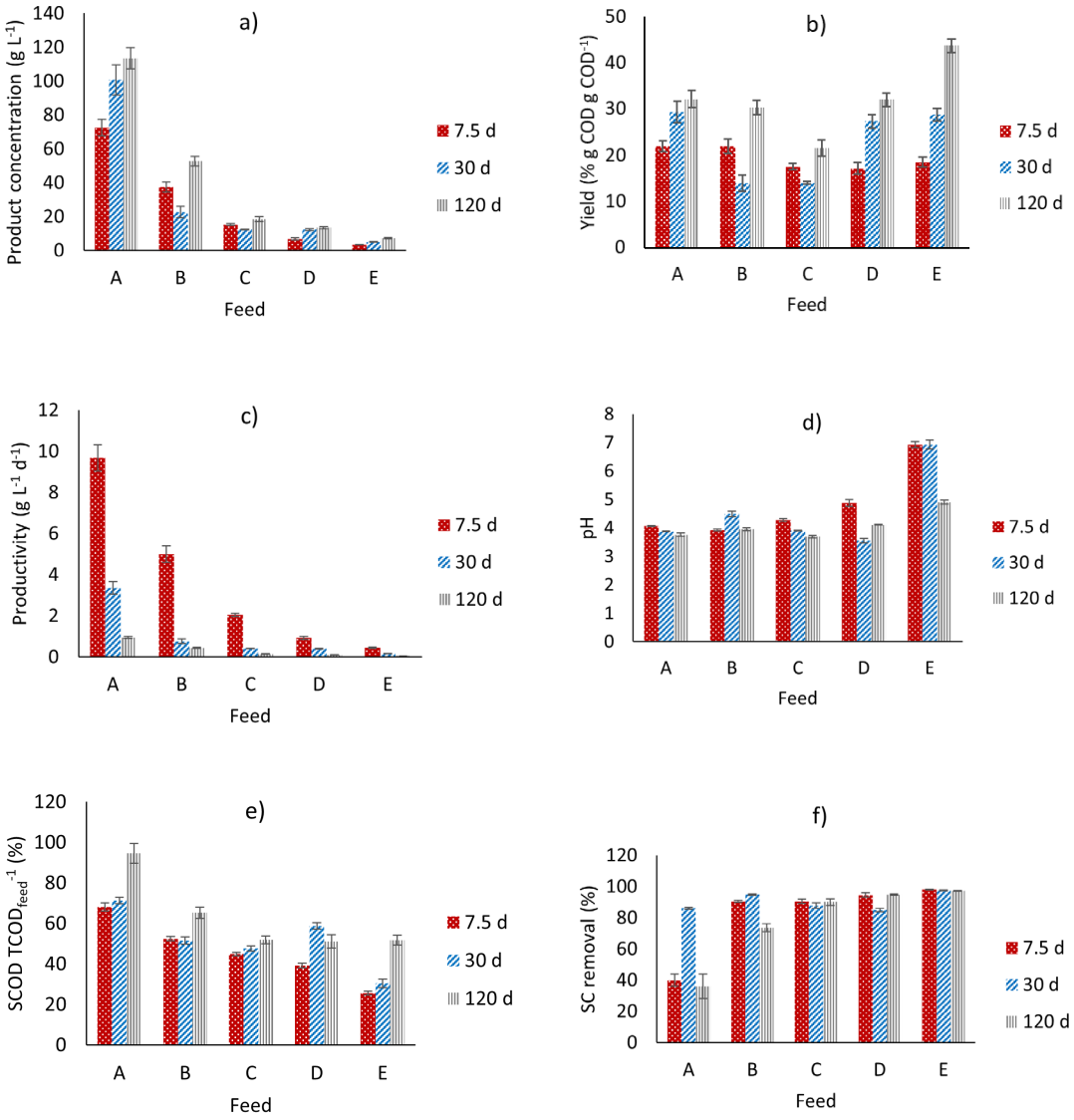

Figure 1. Product concentration (a), yield (b), productivity (c), pH (d), SCOD $TCOD_{feed}^{-1}$ (e) and SC removal (f) of CSTRs (average values with standard errors)

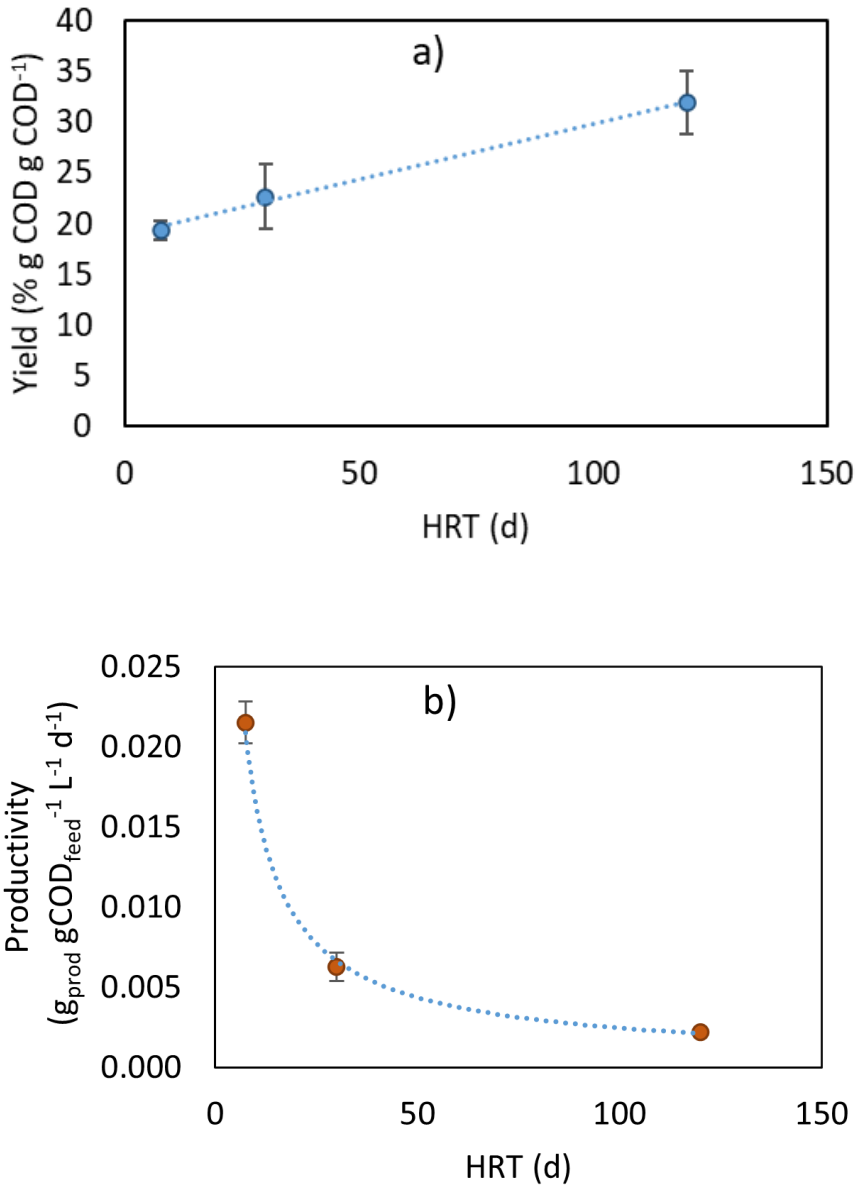

Figure 2. Yield (a) and productivity per unit feed concentration (b) vs HRT of CSTRs (average values with standard errors), independently of the feed concentration

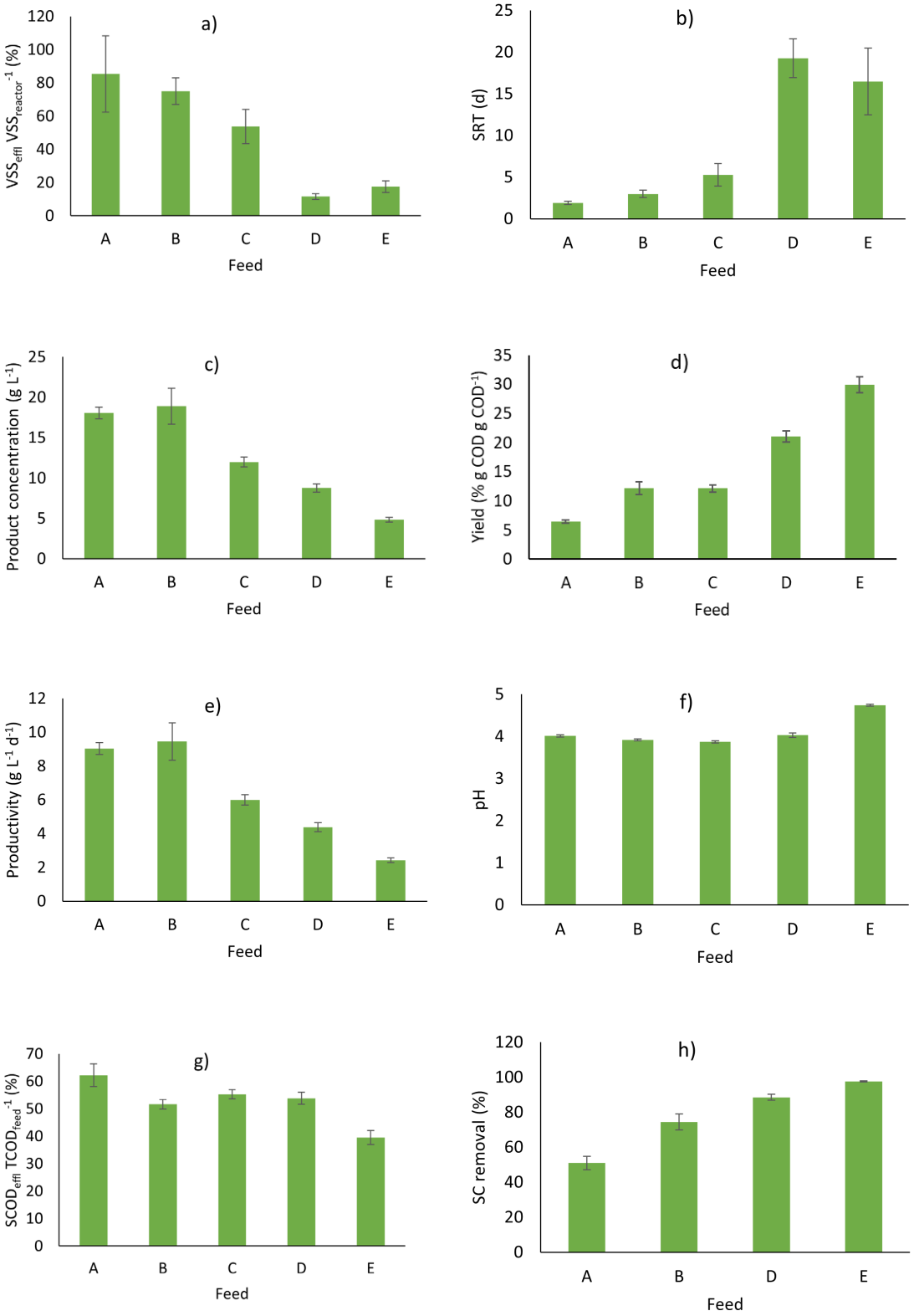

Figure 3. $VSS_{effluent}$ $VSS_{reactor}^{-1}$ (a), SRT (b), product concentration (c), yield (d), productivity (e), pH (f), $SCOD_{effluent}$ $TCOD_{feed}^{-1}$ (g), and SC removal (h) in SBRs (average values with standard errors). SC for SBR C were not measured

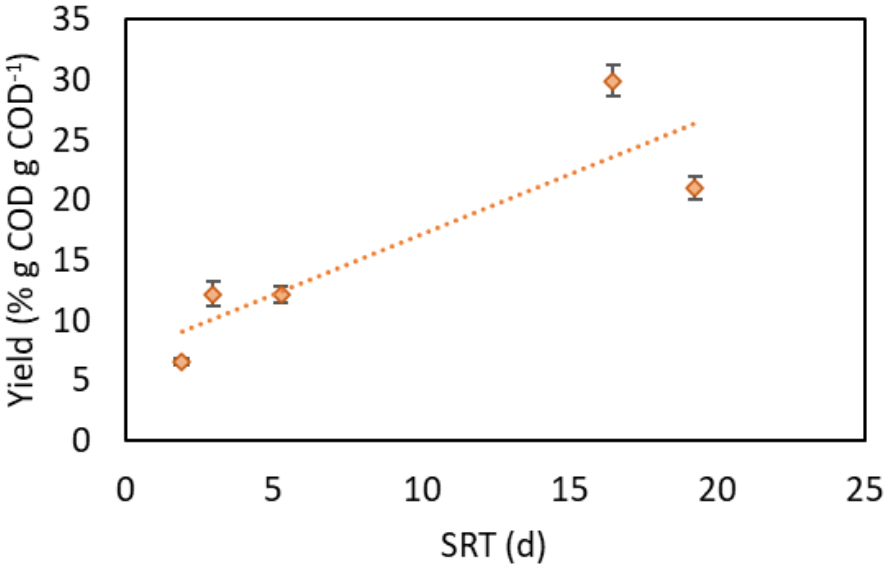

Figure 4. Yield vs SRT of SBRs (average values with standard errors)

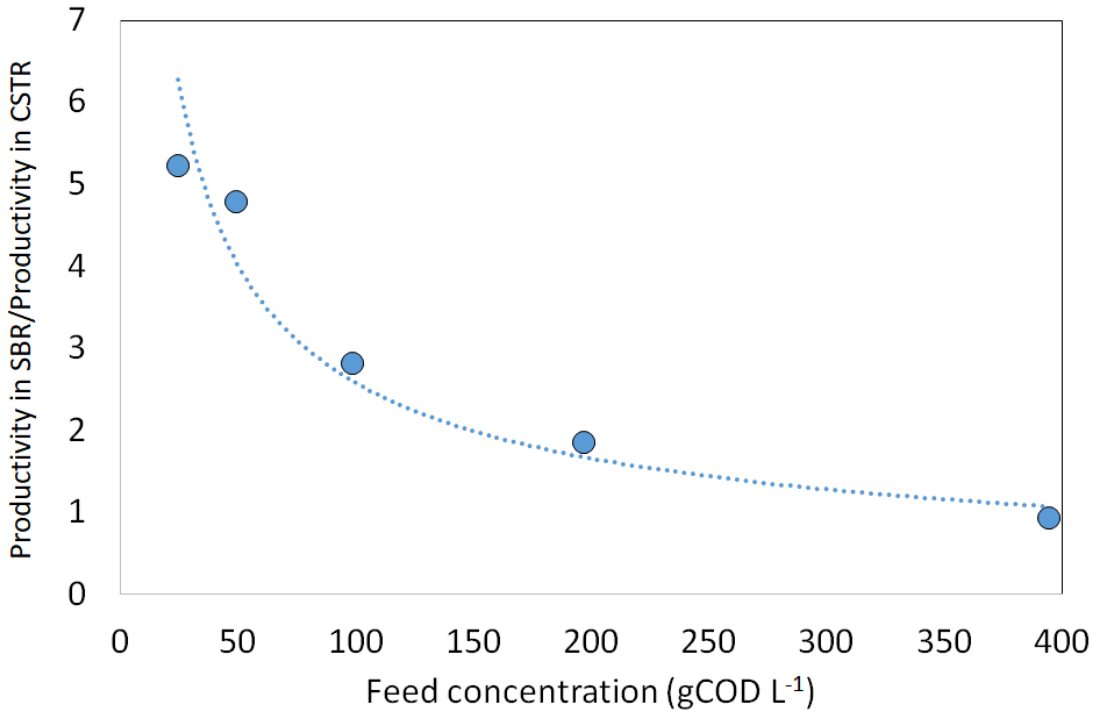

Figure 5. Ratio of the productivity in SBR vs in CSTR for the same feed concentration (data from figures 3e and 1c). For the CSTRs, for each feed concentration the productivity used was the highest obtained, i.e. the productivity at HRT 7.5 d.

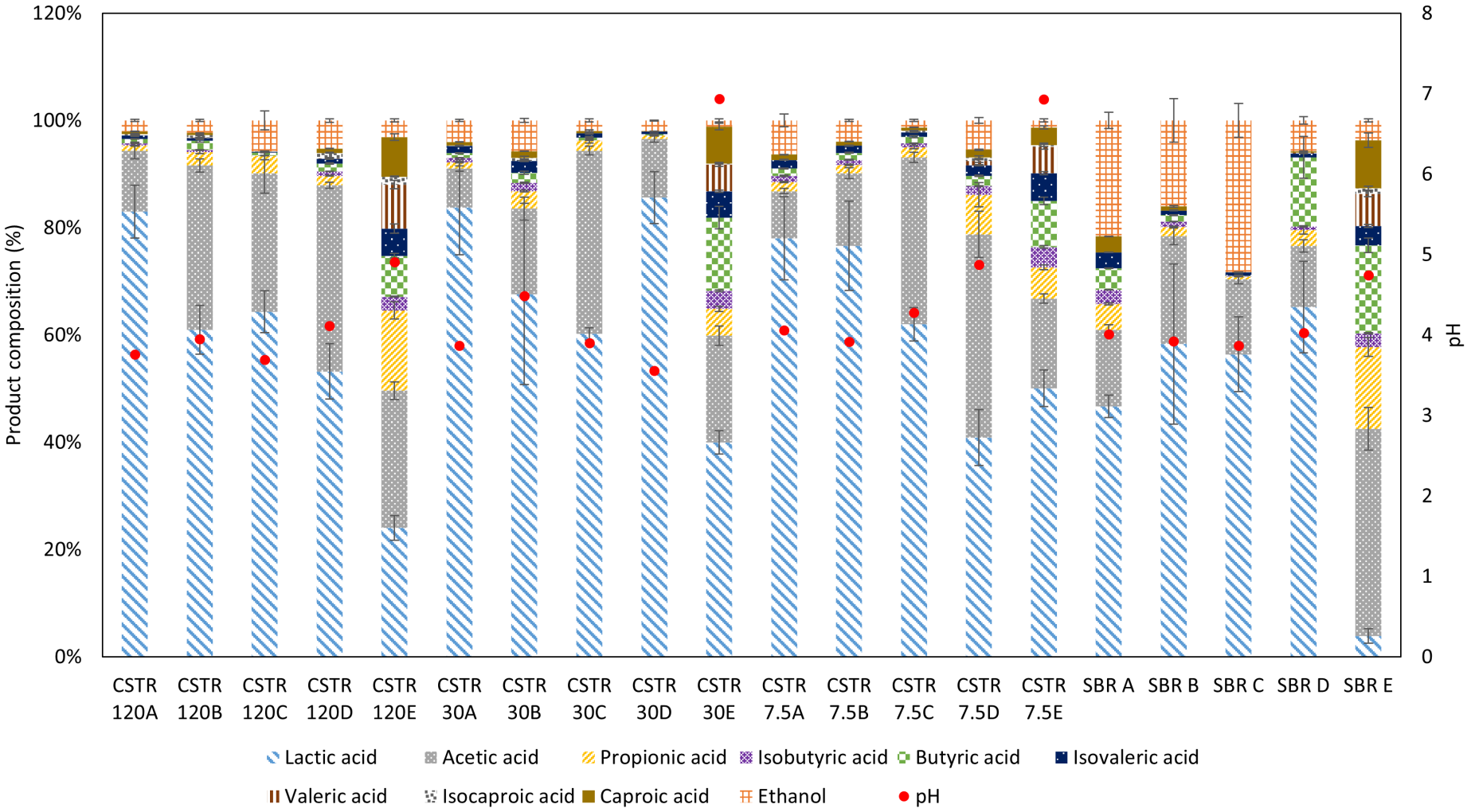

Figure 6. Product composition (% w/w) of CSTRs and SBRs (average values with standard errors)

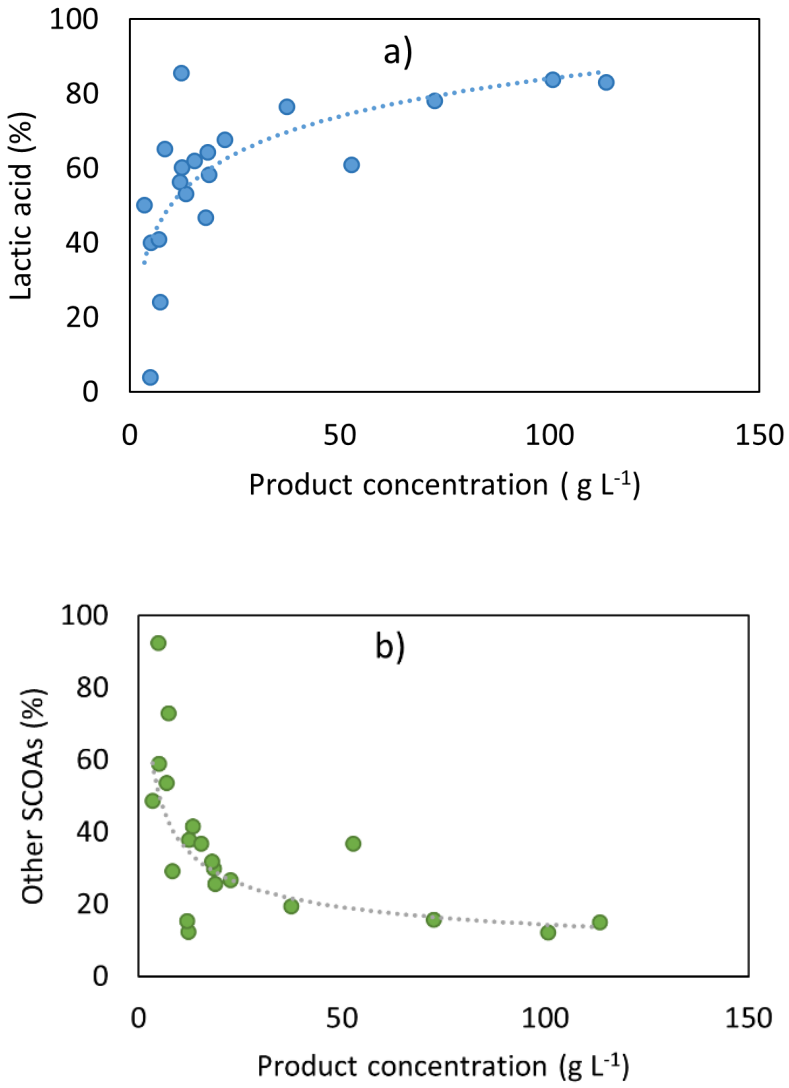

Figure 7. Lactic acid (a) and other SCOAs different from lactic acid (b) % vs product concentration of CSTRs and SBR (average values)

Figure 8. Evolution of the microbial community in CSTR runs 30 A, 30 B, 7.5 A, 7.5 B. Abundance of top 16 taxa at phylum level and top 20 taxa at genus level at each timepoint (days 7 – 91) and in each CSTR