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Effect of reactor operating conditions on carboxylate production and chain elongation from co-fermented sludge and food waste

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ABSTRACT

Nowadays, fermentation of organic wastes for the production of carboxylic acids as precursors of higher-value products has attracted significant attention. In this paper, sewage sludge and food waste were co-fermented to produce carboxylic acids and study the subsequent chain elongation process. The Copenhagen waste stream scenario was taken as a case study. Firstly, design of experiments was used to investigate the overall carboxylic acids and hexanoic acid production in batch, as a function of the co-fermentation ratio, substrate to co-culture ratio and initial pH. Statistical optimization increased the overall carboxylic acid titer by 41%, while co-fermentation allowed to increase hexanoate annual production up to 77%. Optimal operating conditions for hexanoic acid were obtained with SS/FW 6.61, S/Xo 6.73 and initial pH 6.83. Furthermore, a continuous fermentation experiment was performed to study the effect of reactor operating conditions. The overall carboxylates titer was 2 times higher, which also favored chain elongation compared to batch mode. An increasing loading rate did not affect the overall carboxylate titer, however the hexanoic acid titer increased by 44%. A maximum titer of 4.9 g/l of hexanoic acid was produced, achieving a productivity of 2.46 $g \cdot l^{-1} \cdot d^{-1}$ of hexanoic acid with a retention time of 2 days and no external electron donor

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addition. This would correspond to 610 tons/y of hexanoic acid and 350 tons/y of other carboxylic acids that could be produced, based on the waste availability in Copenhagen.

KEYWORDS

Co-fermentation

Sewage sludge

Food waste

Carboxylic acids

Hexanoic acid

Chain elongation

ABBREVIATIONS

AD	Anaerobic digestion
ANOVA	Analysis of variance
BBD	Box-Behnken design
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
HRT	Hydraulic retention time
MCCA	Medium chain carboxylic acids
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
SS	Sewage sludge
SRT	Solids retention time
TS	Total solids
VFA	Volatile fatty acids

VS	Volatile solids
WAS	Waste activated sludge

1. Introduction

In the last years, resource recovery from organic wastes has become an important opportunity in order to move towards a more circular economy. One of the strategies to achieve this goal is the (co)fermentation of different organic wastes to produce carboxylic acids, also known as the carboxylate platform (Agler et al., 2011). Due to the immense variety of available organic wastes (in terms of biochemical composition, C/N ratio, recalcitrance/bioavailability, etc), any bioprocess should be adapted to the specific type of waste, in order to become economically profitable. Sewage sludge (SS) is one of the most abundant organic wastes and a potential raw material for resource recovery (Healy et al., 2015). A total production of 464,000 tons dry matter was generated only in Scandinavia in 2018 (Dansk Vand- og Spildevandsforening (DANVA), 2019; Statistics Sweden (SCB), 2020; Statistisk sentralbyrå Norway (SSB), 2020), and 13 million tons are estimated in Europe in 2020 (European Commission, 2008). A common strategy to manage sewage sludge is anaerobic digestion (AD) (Zhou et al., 2020), classic example of resource recovery from wastes. Carboxylic acids, intermediate products during AD, have higher market value than biogas (Kleerebezem et al., 2015). Their production can be optimized through different approaches, such as pretreatment of feedstock, optimization of inoculum (i.e. through adaptation, enrichment and/or bioaugmentation) (Varrone et al, 2018) or the mixture of different organic wastes to balance some of the nutrients (to obtain i.e. a more favorable C/N ratio), reduce possible inhibitory compounds and exploit the synergistic effect during co-fermentation (Marone et al., 2015; Mata-Alvarez et al., 2011; Zhou et al., 2014).

Previous studies have investigated carboxylic acids production from sewage sludge, using primary and/or waste activated sludge (WAS) (Luo et al, 2019). These studies were based on the effect of different parameters such as pH (Ma et al., 2016; Zhao et al., 2018), the addition of biosurfactants (Huang et al., 2015) or pretreatment of the substrate material (Chen et al, 2020; Liu et al., 2016; Liu et al., 2020; Zhang et al., 2019). Even though numerous studies focused on developing efficient pretreatment methods, it is worth noting that sewage sludge is mainly composed of water; therefore, pretreatment of the material by mechanical methods or addition of chemical compounds implies an additional expense to valorize this waste.

The addition of another organic waste to sewage sludge has been investigated to produce i.e. methane or volatile fatty acids (VFA), using different sources, such as the organic fraction of municipal solid waste (OFMSW, also known as food waste) (Feng et al., 2011; Gottardo et al., 2015; Koch et al., 2016), conditioning with agricultural or industrial residues (Zhang et al., 2020) or microalgae (Olsson et al., 2014). Moreover, the co-fermentation of sewage sludge and food waste for VFA production has been statistically optimized, based on different parameters such as pH or co-fermentation ratio (Chen et al., 2013; Hong and Haiyun, 2010; Khan et al., 2016; Wu et al., 2016).

Despite the higher value of VFA compared to biogas, their recovery is still challenging as it consists of a mixture of products with very similar properties (Cabrera-Rodríguez et al., 2017). Therefore, some alternative strategies to further use the produced VFA have been investigated, including for instance PHA production (Kourmentza et al., 2017), use as carbon source for biological nutrient removal during wastewater treatment (F. Liu et al., 2016) or chain elongation to medium chain carboxylic acids (MCCA) (Angenent et al., 2016). During chain elongation, short chain carboxylic acids are elongated to MCCA using ethanol (or eventually lactate) as electron donor. Following the reverse β oxidation pathway, after ethanol or lactate oxidation, acetate is elongated to butyrate, which is further elongated to hexanoate

(Contreras-Davila et al., 2020; Spirito et al., 2014). MCCA, such as hexanoic, heptanoic or octanoic acids (also known as caproic, enanthic and caprylic acids, respectively), have higher market value than VFA (Zacharof and Lovitt, 2013) and might thus represent a valuable alternative. Different complex organic wastes and the influence of some key parameters, as for example pH or temperature (Candry et al, 2020; Cavalcante et al., 2017; De Groof et al., 2019), have been studied during the chain elongation process. Furthermore, hexanoic acid production has been investigated using different organic wastes such as food waste (Nzeteu et al., 2018), diluted yeast fermentation beer (Ge et al., 2015) or switchgrass-derived stillage (Scarborough et al., 2018). However, hexanoic acid production varies depending on the operating conditions (e.g. biomass immobilization, use of a two-stage system, etc.) and substrates used. Other key parameters that can influence the chain elongation process are reactor configuration, organic overloading, the type of inoculum and the electron donor. Additionally, in situ extraction has been studied in order to avoid product toxicity, which results in low concentration of MCCA in the fermentation broth (De Groof et al., 2019). During continuous fermentation, productivity values (without additional electron donor) ranged from 0.04 $g \cdot l^{-1} \cdot d^{-1}$ using sewage sludge to 1.42 $g \cdot l^{-1} \cdot d^{-1}$ with switchgrass-derived stillage (Jankowska et al., 2018; Scarborough et al., 2018). To date, one of the highest hexanoic acid production rates was obtained using a two-stage system and food waste as substrate. Ethanol was added to enhance the process and up to 55.8 $g \cdot l^{-1} \cdot d^{-1}$ of hexanoic acid were produced (Grootscholten et al., 2014).

The focus of previous studies has been typically set on the improvement of hexanoic acid production by addition of electron donor or the optimization of reactor configuration. However, the use of sewage sludge as substrate and the co-fermentation of two (waste) substrates are not well studied (Wu et al., 2021). Therefore, there is a lack of knowledge regarding the effect of substrates co-fermentation on chain elongation. The current paper

focuses on carboxylate production followed by chain elongation to hexanoic acid from co-fermentation of two organic wastes, namely sewage sludge and food waste. The addition of food waste to largely abundant sewage sludge, which was the base organic waste, expected to enhance its valorization. The study was based on the real waste scenario of the Copenhagen area; for that reason the experiments were applied to the availability and waste streams collected in the region. In Copenhagen, collection of food waste is followed by pretreatment and storage on industrial scale, which may enhance the fermentation experiments. Firstly, design of experiments was used in order to study the effect of 3 key parameters on the overall batch production of carboxylic acids (2 to 6 carbon atoms) and hexanoic acid. Then, continuous fermentation of the two organic wastes was performed to evaluate the impact of hydraulic retention time (HRT) and organic loading rate (OLR) on the overall production and distribution of the different carboxylic acids. Particular emphasis was set on the chain elongation process and hexanoic acid production.

2. Materials and Methods

2.1. Organic wastes and co-culture

Two different waste streams were used as substrate, namely sewage sludge and food waste. In addition, digested sludge was used as co-culture in order to provide the reactors with a microbial consortium adapted to anaerobic fermentation of sewage sludge. Municipal sewage sludge was obtained from BIOFOS, the largest wastewater treatment plant in Denmark. The sewage sludge consisted of a mixture of primary and secondary sludge, also known as waste activated sludge. Samples of food waste were collected from the waste receiving plant of HCS A/S Transport & Spedition in Glostrup, Denmark. This organic waste consists of a mixture of organic waste from households (80%), leftovers from restaurants and expired food from supermarkets (20%). The food waste was pretreated at HCS with an industrial hammer mill and mixed with rain water to generate a pulp. Copenhagen area was estimated to generate

148 around 600,000 tons of sewage sludge (wet weight) per year, while 57,000 tons of food waste
149 were calculated to be produced every year (BIOFOS, 2017; Ebdrup and Mortensen, 2019).
150 Samples of digested sludge from the AD digester, adapted to sewage sludge and used as co-
151 culture, were also provided by BIOFOS. The main characteristics of these samples are
152 presented in Table 1.
153 *2.2. Experimental design and statistical analysis for batch experiments*
154 A Box-Behnken design (BBD) for three variables, each at three levels, was applied to design
155 the experiments and create the model. The experimental design was applied to minimize the
156 number of experiments needed to optimize the desired response. The software Design Expert
157 (version 11.1.2.0, Stat-Ease Inc., US) was used to design the experiment, which required 42
158 runs: 12 different conditions with triplicates and six replicates at the center point. Preliminary
159 experiments with the three variables were used to define the range of each variable (Figure
160 S1). The three variables were: SS/FW (ratio of sewage sludge to food waste, based on volatile
161 solids (VS) content), S/Xo (substrate to co-culture ratio, based on VS) and initial pH. A fixed
162 working volume of co-culture was selected. As a consequence, the organic load of each bottle
163 varied according to the different SS/FW and S/Xo ratios: firstly, the volatile solids load of the
164 substrate was calculated according to the S/Xo (substrate to co-culture ratio); then, the organic
165 load of food waste and sewage sludge were calculated according to the SS/FW ratio. Finally,
166 the set working volume was reached by addition of distilled water.
167 The overall net titer of carboxylic acids ranging from C2 to C6, expressed in g/l, was set as
168 response. The net titer was calculated by subtracting the initial amount of the carboxylates in
169 the bottle (at time zero) to the final amount, as well as the amount from the control
170 experiments. The design space was defined as presented in Table 2. The experimental design
171 with the design parameters are presented in Table S1.

The duration of the experiments (days of incubation) was chosen based on observations from preliminary batch tests. The regression analysis of the experimental data was performed using analysis of variance (ANOVA). In addition, the fitting of the polynomial model was expressed by the coefficient of determination R^2. The fitted polynomial equation was expressed as three-dimensional surface plots to visualize the effects of the different variables on the design range. The "Point prediction" and "Confirmation" tools in Design Expert were used to validate the model. For this purpose, an additional batch test was set up in triplicate as described in section 2.3.

2.3. Batch experiments

42 identical bottles with a volume of 1125 ml were operated in batch mode according to the experimental design presented in Table S1. In addition, three different control conditions were set in triplicate (one for each level of the variable "initial pH") resulting in a total of 51 bottles. After addition of the correct amount of substrate and co-culture, the final volume was adjusted to 400 ml with distilled water. The batch bottles were adjusted to the corresponding initial pH by addition of 3M HCl or 3M NaOH, flushed with N_2 gas and sealed with a rubber stopper and metal cap. Batch tests were run for 9 days at 37 °C and samples for overall carboxylic acids (C2 to C6 compounds), CH_4 and H_2 were taken daily.

2.4. Continuous experiments

Based on the knowledge gained from the statistical analysis, a continuous experiment was performed in order to evaluate the effect of the operating conditions on the carboxylic acids and hexanoic acid production, as well as to study the long-term stability of the process. For the continuous experiment, performed in duplicate, a continuous stirred tank reactor (CSTR) was used with a volume of 3 liters. The CSTR was first run with 4 days HRT until reaching steady state (based on preliminary experiments, Figure S2), from day 0 to 25, and then decreased to 2 days HRT, from day 25 to 45. The objective was to evaluate different HRT and

197 simple pretreatment of the material to homogenize the feed and reduce the risk of tube
198 clogging. For that purpose, the feed was pretreated by homogenization with a kitchen blender
199 for 20 seconds.
200 The SS/FW ratio of the substrate was kept constant during the whole experiment, the initial
201 pH was 9, and the temperature was kept constant at 37 °C, using a heating jacket connected to
202 a water bath. Total solids (TS), VS and chemical oxygen demand (COD) in the feed were
203 analyzed regularly. A liquid sample was taken directly from the reactor every second day and
204 analyzed for carboxylic acids content (i.e. acetic, propionic, butyric, iso-butyric, valeric, iso-
205 valeric, hexanoic and heptanoic acids). For that purpose, the liquid sample was centrifuged at
206 10,000 rpm and 4 ºC for 20 min. The supernatant was then filtered using a 0.45µm filter. The
207 overall net titer was calculated by subtracting the concentration in the feed to the
208 concentration in the reactor.
209 *2.5. Analytical methods*
210 All the samples were chemically characterized in terms of pH, TS, VS, carboxylic acids
211 content and COD. TS and VS were analyzed according to standard methods (APHA, 2005).
212 COD was determined by spectrophotometric analysis using the kits LCK 514 and LCK 914
213 (Hach-Lange). The concentration of total carboxylic acids and ethanol was determined using a
214 gas chromatograph (Shimadzu GC-2010 Plus) equipped with an Agilent 19095F-123 capillary
215 column (30 m length and 0.53 mm i.d.) followed by a flame ionization detector (FID). The
216 carrier gas was nitrogen at 30 ml/min. The temperatures of the injector and the detector were
217 240 and 240 °C, respectively. Lactic acid concentration was analyzed by HPLC on a Dionex
218 Ultimate 3000-LC system with an Aminex HPX-87H column coupled to a refractive index
219 detector (RID). As mobile phase H_2SO_4 (4 mmol/l) was used. Methane and hydrogen
220 production was analyzed using a gas chromatograph (SRI GC model 310) equipped with a

Porapak Q column (182.88 cm length and 2.1 mm i.d.) followed by thermal conductivity detector (TCD). Nitrogen was used as carrier gas.

3. Results

3.1. Optimization of batch experiments through Box-Behnken design

3.1.1. Overall carboxylic acids

Multiple regression analyses were applied to the experimental data, and a quadratic model with a second order polynomial equation was fitted. In addition, the experimental data was treated by square root transformation with the constant k = 0.003, as suggested by Design Expert and presented in Eq. 1. The influence of the different variables on the response is also presented in Eq. 1: the linear effect of all variables A (SS/FW), B (S/Xo) and C (Initial pH) was positive, while the quadratic effect was negative for all of them. In addition, variable B had the highest linear and quadratic effect compared to the other variables, suggesting a higher effect of the S/Xo on the response.

$$sqrt(Y + 0.003) = -0.11738 + 0.01142A + 0.19563B + 0.13980C - 0.00010AB - 0.00098AC + 0.02097BC - 0.00009A^2 - 0.02134B^2 - 0.011725C^2 \quad \text{(Eq. 1)}$$

The experimental results were further analyzed by ANOVA (Table S2). The quadratic model was significant (with an F-value of 369.75 and low probability value $p<0.0001$), while the lack of fit was not significant ($p = 0.5917$). The determination coefficient R^2 was 0.9905, which indicated that 99% of the variability could be explained by the model (Table S3). The predicted and actual values of this response are presented in Table S4. Figure 1 presents the response surface plots for the combination of the different factors used during the experiment. More specifically, Figure 1A presents the effects of variables A and B when the variable C is in the optimal level. The overall titer increased when SS/FW decreased (i.e. higher food waste load) and S/Xo increased. Figure 1B confirms the influence of S/Xo and pH on the overall

245 titer, as presented in Eq. 1. Likewise, the response was maximized with increasing pH and
246 decreasing SS/FW (Figure 1C). According to the model, the highest predicted response of
247 3.55 g/l was achieved with a SS/FW value of 16.8, S/Xo of 7.68 and initial pH 8.92, which
248 corresponds to a 41% increase compared to the ratio of real waste availability (SS/FW =
249 30/70). However, when SS/FW ratio had a value around 50/50 or higher, the overall titer
250 decreased (the sub-optimal area ranged between SS/FW 0/100 and 50/50). Therefore, the titer
251 decreased as the sewage sludge load increased (Figures 1A and C). In order to facilitate
252 comparison with other studies in literature, the yield results for the different conditions are
253 presented in Table S7. In this study, the highest experimental yield in batch (250 mg/gVS)
254 was achieved with a SS/FW value of 0, S/Xo of 3.25 and initial pH 9.

255 *3.1.2. Hexanoic acid*

256 The experimental data were also used to study the hexanoic acid production, in order to
257 determine the influence of the three variables on chain elongation. As previously described
258 for the overall titer, a quadratic model and second order polynomial equation were also fitted
259 to the hexanoic acid titers. Moreover, the experimental data was treated by square root
260 transformation with the constant k = 0.001, as suggested by Design Expert and presented in
261 Eq. 2.

262

$$sqrt(Y + 0.001) = +0.3279 - 0.0798A + 0.1072B + 0.0349C - 0.0878AB + 0.0564AC + 0.0118BC - 0.0824A^2 - 0.1419B^2 - 0.0111C^2 \quad \text{(Eq. 2)}$$

263

264 The linear effect of variable A (SS/FW) was negative on the titer, while variables B (S/Xo)
265 and C (initial pH) had a positive impact. On the other hand, all variables had a negative
266 quadratic effect. Variable B had the highest effect on the hexanoic acid titer, as previously
267 observed for the overall titer. The ANOVA analysis of the experimental results is presented in
268 Table S5. The quadratic model was significant (with an F-value of 23.72 and low probability

value $p<0.0001$), and the lack of fit was not significant ($p = 0.1576$). The determination coefficient R^2 was 0.89, which indicated that 89% of the variability could be explained by the model (Table S6). The predicted and actual values of this response are presented in Table S4. Response surface plots for the influence of the different factors are presented in Figure 2. The hexanoic acid titer increased as the SS/FW ratio decreased and S/Xo increased (Figure 2A), as occurred for the overall titer in section 3.1.1. However, when S/Xo was higher than 7.3, the hexanoic acid titer started decreasing, in contrast with the model of the overall titer. In addition, a neutral pH value favored the response when variable A was in the optimal level (Figure 2B). The combination of variables SS/FW and pH also indicated a higher titer as SS/FW decreased (Figure 2C). According to Figures 2A and 2B, a higher organic load (S/Xo) also increased the hexanoic acid production (as presented in Eq. 2), with a minimum load of 4 (g VS substrate per g VS co-culture). In the current study, the highest predicted response of 0.155 g/l was achieved with a SS/FW value of 6.61, S/Xo of 6.73 and initial pH 6.83. This corresponds to a 34% increase compared to the ratio of real waste availability. In the optimized conditions, hexanoic acid represented 6.2% of the overall carboxylic acids.

3.2. Continuous co-fermentation

For continuous operation, the combination of the variables was chosen in order to maximize the overall carboxylic acids. The CSTR was run with a SS/FW ratio of 20/80, high start-up organic load (S/Xo) and initial pH 9 (based on the optimized co-fermentation ratio from batch experiments). Preliminary experiments showed that with a HRT of 6 days, the methanogenic bacteria were enriched and the content of carboxylic acids was reduced, favoring methane production (Figure S2). For this reason, HRT > 4 days was excluded from further testing. The final OLR differed for each HRT, resulting in about 15 gVS/l/day for 4 days HRT and 30 gVS/l/day for 2 days HRT. The initial pH of 9 decreased quickly (and remained constant)

293 oscillating between 5.5 and 5 after the first 4 days, without any chemical addition. The main
294 results of the continuous experiment are shown below (Figure 3).

295 *3.2.1. Effect of HRT and OLR*

296 Figure 3 presents the individual and overall carboxylic acid net titer for compounds ranging
297 from 2 to 7 carbon atoms. During the first 8 days, the overall net titer reached a maximum of
298 11 g/l and then decreased to about 7.7 g/l once stabilized (until day 25). However, the titer
299 predicted by the batch model in comparable conditions (in terms of initial pH, SS/FW and
300 S/Xo) was lower, 3.5 g/l. After 25 days of operation, the HRT was decreased from 4 to 2
301 days, and then run during 20 days. Since the substrate was the same during the whole
302 experiment, the decrease in HRT implied an increase in OLR, from 15 gVS/l/day to 30
303 gVS/l/day. The titer of carboxylic acids remained stable around 7.7 g/l both with 4 and 2 days
304 HRT, indicating no change despite the OLR increase. Additionally, the overall yield also
305 remained stable around 130 mg/gVS (Figure S3). The increase in OLR had higher influence
306 on the hexanoic acid production; from day 9 to 25, the average hexanoic acid titer was 3.4 g/l,
307 while after decreasing the HRT it reached 4.9 g/l, thus leading to an increase of 44% and a
308 maximum productivity of 2.46 $g \cdot l^{-1} \cdot d^{-1}$. Ethanol was spiked in day 39 (as indicated by the
309 arrow in Figure 3) to investigate its effect on the hexanoic acid production, but no significant
310 effect was observed on the long term. Nevertheless, from day 39 to 41, butyric and hexanoic
311 acid titers increased by 14 and 12%, respectively.

312 **4. Discussion**

313 *4.1. Influence of selected variables during batch fermentation*

314 *4.1.1. Overall carboxylic acids*

315 The model created with the experimental data for carboxylic acids production showed that the
316 response (overall titer in g/l) increased as variable A (SS/FW ratio) decreased (i.e. higher food
317 waste load) and variables B (S/Xo) and C (initial pH) increased. A high S/Xo value implied

318 that the organic load was high, which could inhibit methanogenic activity due to carboxylic
319 acids accumulation (González-Fernández and García-Encina, 2009; Shahbaz et al., 2019). In
320 addition, the decrease in sewage sludge load in co-fermentation increased the titer, as
321 expected, due to the recalcitrant nature of this substrate and its low VS content (Koch et al.,
322 2016). A high organic load, in combination with high initial pH, increases the overall titer
323 since the carboxylic acids are accumulated in the dissociated form (due to the alkaline pH),
324 thus reducing the inhibition to the microorganisms. Previous studies analyzed the influence of
325 different variables in the VFA yield, utilizing similar substrates, but only taking into account
326 compounds from 2 to 5 carbon atoms and working in semi continuous mode instead of batch
327 mode (Chen et al., 2013; Hong and Haiyun, 2010; Shahbaz et al., 2019).
328 On the other hand, the influence of pH on VFA production has been extensively studied
329 (Jankowska et al., 2017; Jiang et al., 2013; Ma et al., 2016; Zhang et al., 2009). In addition,
330 co-fermentation of WAS with food waste was also studied in batch mode and different pH,
331 showing positive effect of alkaline pH compared to neutral pH (Feng et al., 2011).
332 *4.1.2. Hexanoic acid*
333 As presented in section 3.1.2, increasing food waste load (i.e. decreasing SS/FW), together
334 with increasing S/Xo ratio and initial pH, increased the hexanoic acid titer. However, the titer
335 decreased when S/Xo was higher than 7.3. Previous studies suggested that a S/Xo ratio of 5
336 favors hexanoic acid production, while increasing it to 10 inhibits it (Coma et al., 2017). The
337 increasing food waste load (implying a decrease in the SS/FW ratio) may have favored chain
338 elongation to hexanoic acid due to the ethanol and lactic acid content in food waste (Table 1),
339 which can be used as electron donors during chain elongation. In addition, controlling the pH
340 for hexanoic acid production implies a compromise between primary fermentation and chain
341 elongation, since the optimal conditions differ for each process. While alkaline pH favors
342 VFA fermentation (Feng et al., 2011; Ma et al., 2016), chain elongation is less

thermodynamically favorable under these conditions and higher productions were reported at neutral pH (Liang and Wan, 2015). The highest predicted titer for batch tests in this study was 0.155 g/l, in agreement with previous studies under similar conditions (i.e. complex substrate and no additional electron donor), which reported hexanoic acid titers ranging from 0.02 to 0.12 g/l (in batch mode) within 3 to 5 fermentation days (Jankowska et al., 2015; Weimer et al., 2015). It is worth noting that the influence of these variables and different operating conditions on hexanoic acid production are not well studied in literature, especially for continuous operation and co-fermentation of sewage sludge with food waste. Therefore, a continuous experiment was performed (see section 3.2).

4.1.3. Co-fermentation as waste management strategy

Design of experiments for the statistical optimization of key process parameters can be used as a decision-making tool in order to study different waste management scenarios (Marone et al., 2015). Considering that food waste was the limiting substrate (compared to the huge volumes of sludge available), the highest predicted titer of 3.55 g carboxylates/l would allow to obtain 396 tons of carboxylic acids per year, by valorizing all available food waste together with 9% of the available sludge (which corresponds to 17% of the total available substrates). Additionally, 772 tons of carboxylic acids could be produced by valorizing the remaining sludge alone under the best conditions, which would correspond in total to 1168 tons of carboxylic acids (Scenario A, Table S8). On the other hand, if these waste streams were valorized separately, 203 tons of carboxylic acids could be produced every year only from food waste and 849 tons from sludge, achieving in total 1052 tons of carboxylates (Scenario B, Table S8). Therefore, co-fermentation of substrates in the optimized condition integrated with the mono fermentation of the remaining sludge would increase the carboxylate production by 11%. Furthermore, the SS/FW ratio corresponding to the real waste availability in Copenhagen area (SS/FW 70/30) would produce a total of 1650 tons of per year, according

to the model. This apparent contradiction can be easily explained when considering the overall valorization / fermentation strategy of multiple wastes, when the availability of a feedstock stream represents the limiting factor (as further elaborated below).

In the case of chain elongation, the optimized co-fermentation ratio would produce 12 tons/y of hexanoic acid, but valorizing only 3% of the available sludge (and only 12% of the total available waste). The remaining sludge could produce 33 additional tons/y of hexanoic acid, thus achieving 45 tons/y in total (Scenario A, Table S8). On the other hand, mono fermentation of these waste streams could produce up to 43 tons of hexanoic acid per year. In other words, the optimal co-fermentation ratio would only produce 5% more hexanoic acid than mono fermentation, because only 3% of the sludge would be co-fermented, while the remaining 97% would be fermented separately. It is worth noting that the real waste availability (SS/FW 70/30) would produce 76 tons of hexanoic acid per year.

Clearly, in a real life scenario it would be necessary to design a process that valorizes all the available waste, instead of focusing only on optimizing titers (neglecting the real substrate availability), as often done in lab-scale studies. When designing a feasible waste management system, availability of waste is of particular importance. In fact, assuming that there is no limiting substrate, the optimal co-fermentation ratio would clearly produce more carboxylic acids than the real co-fermentation ratio. However, the Copenhagen real case scenario would produce 41% more carboxylic acids and 69% more hexanoic acid per year than the optimized co-fermentation (Scenario A, Table S8). This is due to the higher amount of material treated in co-fermentation; in fact, while the real ratio would co-ferment 100% of the available material, the optimal carboxylate and hexanoate conditions would only co-ferment 17% and 12% of the available material, respectively. On the other hand, downstream processing costs (strictly related to titers) cannot be neglected either, so an integrated assessment would be necessary.

393 *4.2. Continuous carboxylate production and chain elongation*

394 During continuous co-fermentation, the shorter HRT was used as a strategy to reduce the
395 methanogenic activity and to maximize the acidogenesis. Thus, preliminary inactivation of
396 methanogens was unnecessary and the addition of chemicals to regulate pH could be avoided.
397 The higher OLR, compared to an anaerobic digester focused on biogas production, was
398 another strategy used to decrease methane production. In this way, carboxylic acids are
399 expected to accumulate in the reactor, since methanogenic microorganisms cannot consume
400 them as quickly as they are produced (Yuan and Zhu, 2016).

401 According to (Cheah et al., 2019), the continuous fermentation of food waste under alkaline
402 conditions (pH 10) did not show a significant increase in the VFA yield compared to acidic
403 pH (6). In addition, previous studies found the overall VFA production by co-fermentation of
404 these wastes optimal with a self-regulating pH range between 5.2 and 6.4 (Wu et al., 2016). In
405 the current study, the addition of chemicals to maintain a constant pH during continuous
406 fermentation was discarded in order to avoid additional expenses. This is of particular
407 importance when developing a robust process that could treat up to 657,000 tons/year of
408 waste streams, only considering the Copenhagen region (BIOFOS, 2017; Ebdrup and
409 Mortensen, 2019).

410 *4.2.1. Organic overloading favors chain elongation*

411 As described in section 2.4, the substrate of the continuous experiment was homogenized in
412 order to facilitate reactor operations such as pumping of waste materials. Notably, the overall
413 titer did not change when decreasing from 4 to 2 days HRT, despite the OLR increase,
414 indicating no microbial inhibition due to organic overloading. Nevertheless, the increase in
415 OLR had higher influence on the hexanoic acid production, in good agreement literature (De
416 Groof et al., 2019). The enhancement of chain elongation after increasing the OLR could be
417 due to an adaptation of the microbial population.

During chain elongation, ethanol is typically used as electron donor enabling the carbon incorporation. In fact, previous studies have focused on the addition of ethanol or the use of different substrates to enhance the hexanoic acid production (Duber et al., 2018; Grootscholten et al., 2013). In the current study, the food waste was partially pre-fermented before collection (due to the treatment at the company), thus containing some ethanol and lactic acid. This pre-fermentation could in principle explain the increase in hexanoic acid production as the food waste load increased. However, the ethanol concentration in the reactor was very similar to the concentration in the feed (Figure 3), suggesting that another chain elongation pathway could have been dominant in our reactor.

In order to verify this hypothesis, ethanol was spiked (after reaching steady state) in day 39, but no significant effect on hexanoic acid was observed on the long term. In previous studies, ethanol addition enhanced chain elongation of shorter carboxylic acids to hexanoic acid by 39% (Grootscholten et al., 2013; Roghair et al., 2018). However, in these studies, the biomass was immobilized in the reactor in order to optimize the process, while in the current study there was no such immobilization system. On the other hand, the study by Cavalcante and colleagues suggested that lactic acid can also be used as electron donor for chain elongation (Cavalcante et al., 2017). Interestingly, in the current study, lactate concentration from the feed (resulting from the pretreatment of the material in the company) showed a high consumption in the reactor (Figure 3) and was positively correlated to the hexanoic acid production (Spearman correlation = 0.8), potentially indicating its involvement in the process. This could in principle explain why the ethanol spiking did not show significant effects. Furthermore, previous studies investigated lactic acid production coupled to chain elongation, using food waste as substrate (Contreras-Dávila et al, 2020). The successful consecutive lactate formation and chain elongation avoided the addition of external electron donor for hexanoic acid production, decreasing the chemicals input. Additionally, the self-regulated pH

around 5.5 may have enhanced the chain elongation to hexanoic acid, using lactic acid as electron donor as suggested in previous studies. Candry and colleagues (2020), for instance, studied the influence of pH on the competition for lactic acid between chain elongating and propionic acid producing microorganisms. As a result, the microbial populations shifted depending on the pH, favoring chain elongation to hexanoic acid at mildly acidic pH. Thus, further experiments are needed to clarify the underlying mechanism of chain elongation in the current study, for instance by spiking with lactate and performing a parallel composition analysis of the microbial composition.

4.2.2. Chain elongation as strategy for waste management

Organic overloading has been previously studied as a strategy to enhance the chain elongation process, both at the start-up phase of the reactor (substrate to co-culture ratio, S/Xo) or as increasing OLR during reactor operation (De Groof et al., 2019). This is coherent with our findings, in which increasing OLR (and decreased HRT) led to an enhanced chain elongation. Nevertheless, a higher OLR when using a complex feedstock may not always correspond to a higher production of MCCA, since the feedstock may not be easily biodegraded (especially without any pretreatment). Moreover, the type of reactor, pumping system and the retention time used during fermentation depend on the type of substrate. In the current paper, the focus was set on the valorization of sewage sludge and food waste, two abundant organic wastes. The mixture of these two substrates allowed the use of a CSTR, a simple type of reactor, in which the HRT and solid retention time (SRT) have the same length. In order to compare hexanoic acid production with similar studies, the titer (g/l), productivity ($g \cdot l^{-1} \cdot d^{-1}$) and yield (mg/gVS) are presented in Table 3, both in grams and in COD equivalents. All the studies presented in Table 3 were performed without addition of external electron donor, and depended solely on the composition of the complex waste for the production of MCCA. The HRT in Table 3 varied from 1 to 6 days, and in all cases a CSTR was used for the experiment.

468 The different composition of the substrates in literature (from grass silage to sewage sludge),
469 together with the varying OLR, resulted in productivities ranging from 0.01 up to 2.46 $g \cdot l^{-1} \cdot d^{-1}$
470 . In the current study, a maximum productivity of 2.46 $g \cdot l^{-1} \cdot d^{-1}$ was achieved with a HRT of 2
471 days, among the highest obtained from co-fermentation of these types of wastes, without
472 additional electron donor, to the authors' knowledge. The suitability of food waste as
473 substrate for MCCA production could be explained by its pretreatment in the collection plant,
474 where its size was reduced and it was mixed with rain water to produce a pulp. This
475 pretreatment probably enhanced the hydrolysis step, which accelerated the fermentation and
476 allowed the chain elongation with a short HRT of 2 days. As a result, up to 610 tons of
477 hexanoic acid could be produced every year with this reactor configuration (2 days HRT),
478 valorizing 124,500 tons of waste in Copenhagen area (19% of the yearly total). Additionally,
479 350 tons of other carboxylic acids (acetate, butyrate, valerate and heptanoate) would also be
480 produced at the same time.
481 As previously mentioned, most of the prior studies focused on optimizing the chain
482 elongation process by addition of electron donor or specific reactor configurations. However,
483 the effect of the different reactor operating conditions has been barely compared, and even
484 less the co-fermentation of different complex waste streams. Clearly, the HRT is another key
485 parameter since it is directly related to the OLR and affects the size of the reactor, and thereby
486 the economic feasibility of the waste management process.
487 The current study showed the potential use of food waste and sludge as substrates for stable
488 hexanoic acid (and overall carboxylic acids) production, with a robust process and simple
489 reactor configuration. The pH was not regulated and no external addition of electron donor
490 was necessary (therefore no additional expenses were generated), while reaching among the
491 highest productivities reported in literature within comparable process conditions. On the
492 other hand, more advanced systems using i.e. immobilized biomass and addition of external

carbon and electron source reached significant improvements. Clearly, different reactor configurations could be studied to further optimize the chain elongation process. Although the current study demonstrated the better performance of CSTR compared to a batch system, other semi-continuous operating modes could also be investigated. However, these type of wastes are generated in enormous amounts so a cheap and robust bioprocess should be developed in order to exploit the waste valorization. It would also be necessary to take into consideration the potential downstream technologies that could be applied to the produced carboxylates, both short and medium chain compounds. In fact, previous studies suggested higher affinity for medium chain carboxylates (Fernando-Foncillas et al., 2021; Rebecchi et al., 2016). Additionally, a techno-economic assessment should be performed in order to evaluate the feasibility of the process.

5. Conclusions

In this study, the effect of sewage sludge and food waste co-fermentation ratio (together with the reactor type and operating conditions) on carboxylic acid production and chain elongation were evaluated. During the batch experiment, the overall carboxylic acid titer was highly dependent on the initial pH and co-fermentation ratio, increasing with alkaline pH and decreasing sludge ratio. Chain elongation was rather limited, compared to CSTR operations. However, co-fermentation proved to be a valid waste management strategy, increasing hexanoate annual production up to 77%, compared to mono-fermentation. During continuous experiments, the increase in OLR improved the hexanoic acid titer by 44%. A maximum hexanoic acid titer of 4.9 g/l and productivity of 2.46 $g \cdot l^{-1} \cdot d^{-1}$ were achieved with 2 days HRT and no external electron donor addition, one of the highest with this reactor configuration and types of wastes. This would correspond to 610 tons of hexanoic acid and 350 tons of other carboxylic acids that could be produced, based on the waste availability in Copenhagen.

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760 FIGURE AND TABLE CAPTIONS

761 Table 1. Main characteristics of sewage sludge, food waste and co-culture.

762

763 Table 2. Levels and experimental range of the variables in the design experiments.

764

765 Table 3. Summary of hexanoic acid production from complex wastes without additional
766 electron donor.

767

768 Figure 1. 3D response surface for overall titer. (A) Effect of S/Xo and SS/FW; (B) Effect of
769 pH and SS/FW; (C) Effect of pH and S/Xo.

770

771 Figure 2. 3D response surface for hexanoic acid titer. (A) Effect of S/Xo and SS/FW; (B)
772 Effect of pH and SS/FW; (C) Effect of pH and S/Xo.

773

774 Figure 3. Individual and overall carboxylic acid, ethanol and lactate titers during continuous
775 operation.

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777

778 TABLES AND FIGURES

779 Table 1

Parameter	Sewage sludge	Food waste	Co-culture
pH	6.2 ± 0.4	4.6 ± 0.4	6.9 ± 0.3
Total solids (TS) (g/l)	33.05 ± 1.11	138.39 ± 1.79	16.37 ± 0.06
Volatile solids (VS) (g/l)	26.55 ± 0.92	125.77 ± 1.11	10.38 ± 0.08
Total chemical oxygen demand (tCOD) (g/l)	53.20 ± 3.68	248.50 ± 0.71	16.45 ± 0.78
Total VFA (g/l)	1.53 ± 0.01	2.76 ± 0.22	0.14 ± 0.05
Ethanol (g/l)	0.00 ± 0.00	6.77 ± 0.11	0.00 ± 0.00
Lactic acid (g/l)	0.00 ± 0.00	12.61 ± 0.15	0.00 ± 0.00

780

781 Table 2

Variable	Name	Units	Coded levels		
			-1	0	1
A	SS/FW	% VS	0.00	50.00	100.00
B	S/Xo	gVS/gVS	0.50	4.00	8.00
C	Initial pH		4.50	6.75	9.00

782

783

784 Table 3

Substrate	HRT (days)	OLR ($g \cdot l^{-1} \cdot d^{-1}$)		Titer (g/l)		Productivity ($g \cdot l^{-1} \cdot d^{-1}$)		Yield (mg/g)		Inhibition methanogens	Reference
		VS	COD	VS	COD	VS	COD	VS	COD		
Grass silage	6	10.0	-	3.90	8.58	0.65	1.43	65.0	-	High OLR	(Pakarinen et al., 2011)
Switchgrass-derived stillage	6	-	22.6	8.50	18.70	1.42	3.12	-	137.9	-	(Scarborough et al., 2018)
Ensiled sorghum + cheese way + cow manure	5	11.6	19.27	1.50*	3.30	0.30	0.66	26.0	34.3	Short HRT	(Dareioti and Kornaros, 2015)
Cheese whey	4	12.8	18.0	0.10	0.23	0.03	0.06	2.0	3.2	Initial pH 5.2	(Jankowska et al., 2018)
Sewage sludge mixture	4	8.2	20.0	0.05	0.10	0.01	0.02	1.4	1.3	Initial pH 5.2	(Jankowska et al., 2018)
Food waste + sewage sludge	4	15.0	27.0	3.91	8.60	0.98	2.15	64.0	79.6	High OLR	This study
Ensiled sorghum + cheese way + cow manure	3	17.2	28.6	1.00*	2.20	0.33	0.73	19.4	25.6	Short HRT	(Dareioti and Kornaros, 2015)
Food waste + sewage sludge	2	30.0	54.0	4.91	10.80	2.46	5.40	81.8	100.0	High OLR	This study
Cheese whey	1	51.0	71.9	0.04	0.08	0.04	0.08	0.7	1.1	Initial pH 5.2	(Jankowska et al., 2018)
Sewage sludge mixture	1	32.9	79.9	0.04	0.08	0.04	0.08	1.1	1	Initial pH 5.2	(Jankowska et al., 2018)

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* Estimated values from Figures.

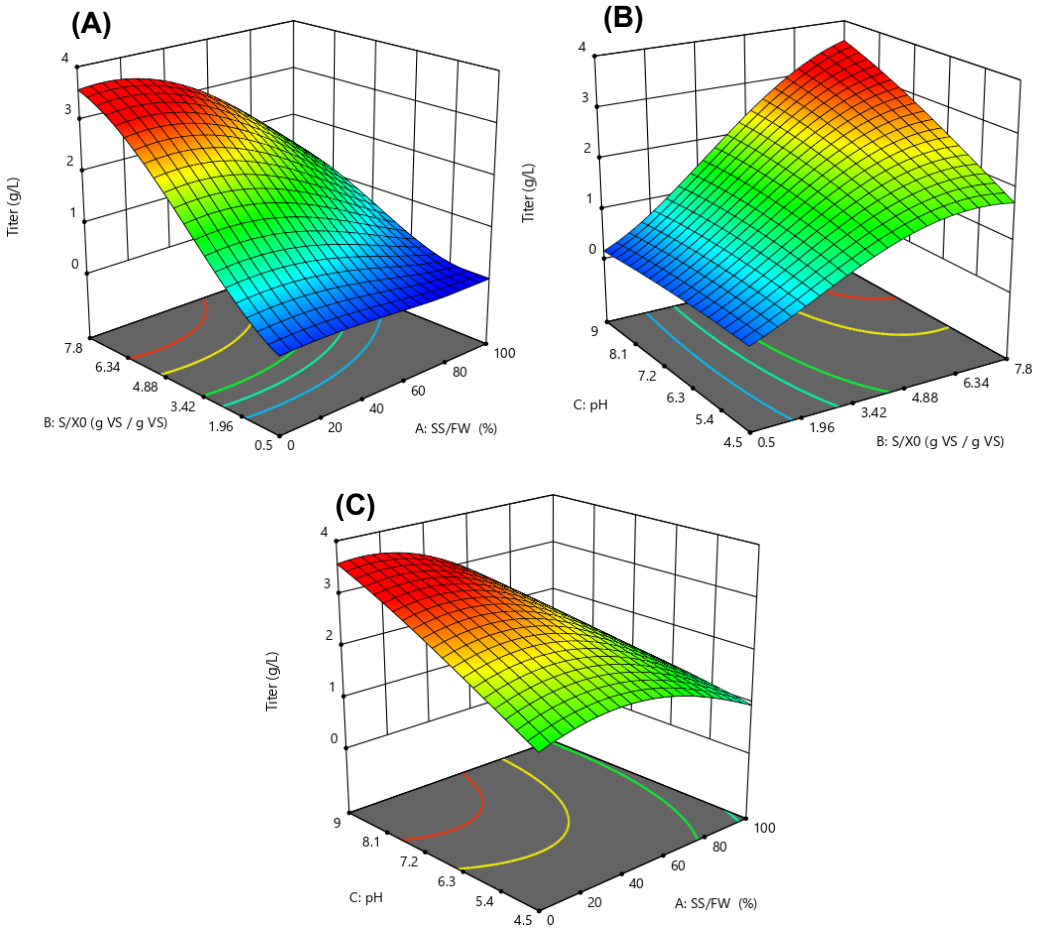
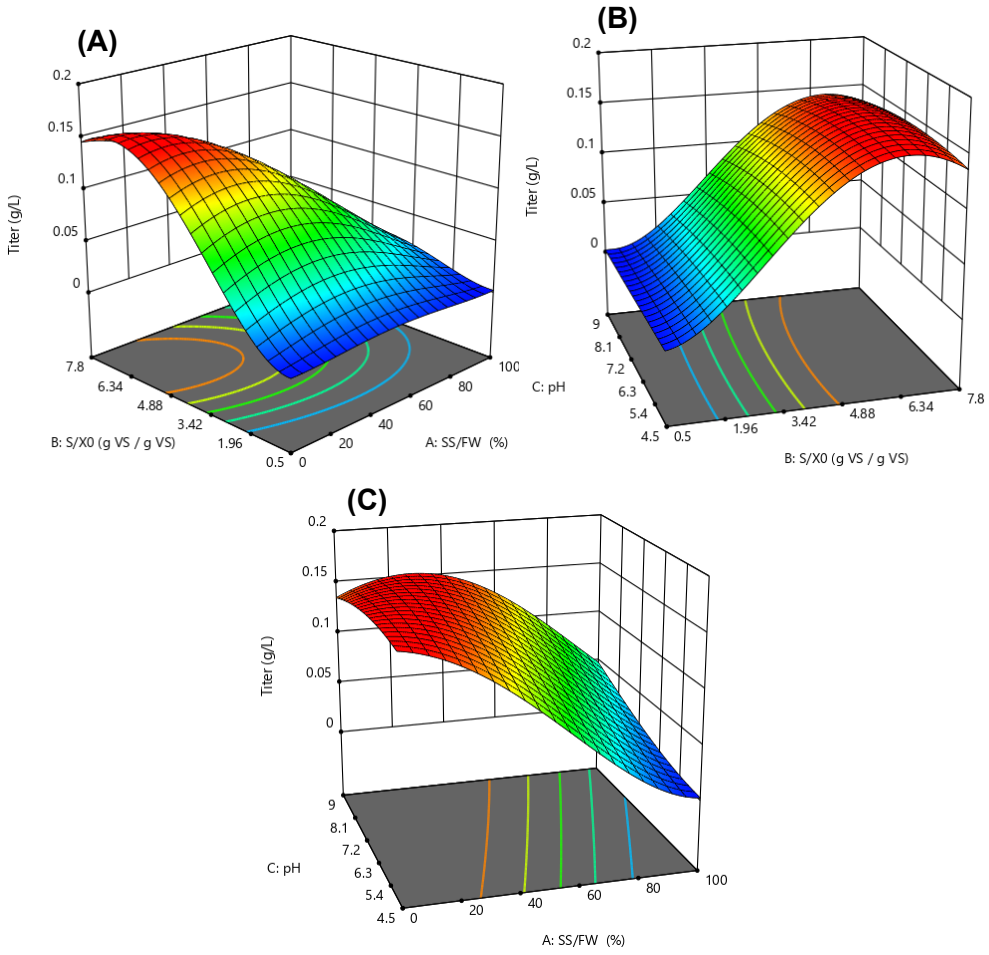

(A)
Titer (g/L)
4
3
2
1
0
7.8
6.34
4.88
3.42
1.96
0.5
B: S/X0 (g VS / g VS)
0
20
40
60
80
100
A: SS/FW (%)
(B)
Titer (g/L)
4
3
2
1
0
9
8.1
7.2
6.3
5.4
4.5
C: pH
0.5
1.96
3.42
4.88
6.34
7.8
B: S/X0 (g VS / g VS)
(C)
Titer (g/L)
4
3
2
1
0
9
8.1
7.2
6.3
5.4
4.5
C: pH
0
20
40
60
80
100
A: SS/FW (%)

Figure 1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Titer (g/L)
0.2
0.15
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7.8
6.34
4.88
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B: S/X0 (g VS / g VS)
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0.2
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B: S/X0 (g VS / g VS)
(C)
Titer (g/L)
0.2
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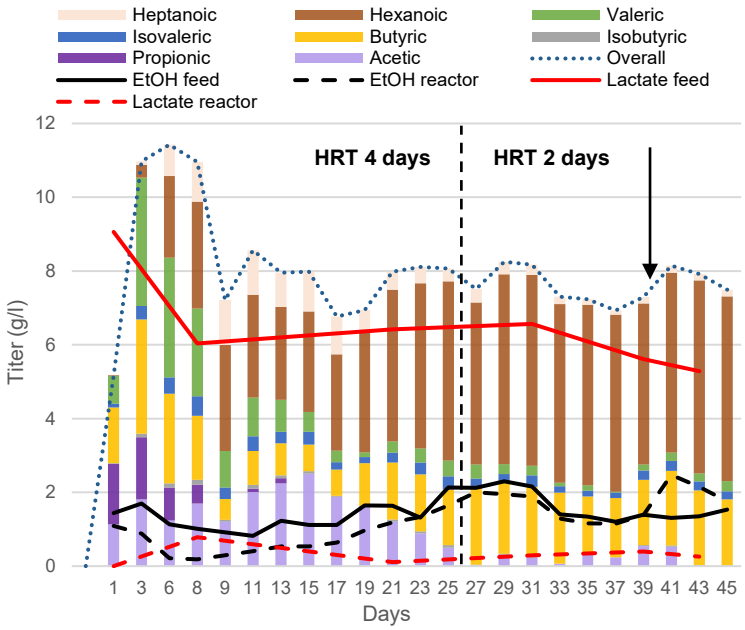
9
8.1
7.2
6.3
5.4
4.5
C: pH
0
20
40
60
80
100
A: SS/FW (%)

Figure 2



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798 Figure 3

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