Volatile fatty acid (VFA) product spectrum as a function of the solids retention time (SRT) in an anaerobic granular sludge process

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Abstract

Volatile fatty acids (VFA) may serve as building blocks for chemicals and polymers. A technology enabling high-rate VFA production from carbohydrate-rich water is the anaerobic granular sludge process. In this study the characteristics of an anaerobic granular sludge enrichment was evaluated at different solid retention times (SRT). A lab-scale anaerobic sequencing batch reactor fed with 6 g·L-1 glucose was operated at a pH of 5.5 and at various SRT of 1-2, 10-20, and 40-50 d. A low sludge volume index (SVI) of 11-44 mL·gTSS-1 allowed a high volatile suspended solid (VSS) concentration that reached 59 gVSS·L-1 . This high VSS concentration enabled a glucose consumption rates in the range of 1100 gCOD·L-1·d-1. Two product spectra were obtained: (i) an propionate:acetate mixture with a ratio of 2.05:1 (molpropionate:molacetate) produced at an SRT of 40-50 d; (ii) an acetate dominated product spectrum was obtained at 1-2 d and 10-20 d SRT (0.71-0.75 molacetate·molVFA-1). Overall a high VFA yield between 0.77-0.79 was obtained throughout all enrichments. This work demonstrates that high rate VFA production combining high yields and low solid concentrations in the effluent can be achieved. This works contributes to the implementation of waste-based production of VFA using anaerobic granular sludge.

Introduction

Anaerobic digestion (AD) is a process in which complex organic substrate is degraded to yield methane containing biogas (Kleerebezem, Joosse, Rozendal, & Van Loosdrecht, 2015). A technology used in the AD process present-day is the high rate granular sludge technology which enables effective production of biogas from wastewater (Lettinga, van Velsen, Hobma, de Zeeuw, & Klapwijk, 1980). Key intermediates in the AD process are volatile fatty acids (VFA). These VFA can serve as a platform molecule, in the so-called carboxylate platform (Agler, Wrenn, Zinder, & Angenent, 2011; Holtzapple & Granda, 2009). VFA serves in this platform as a building block for the production of chemicals and polymers such as the production of polyhydroxyalkanoates (Marang, Jiang, van Loosdrecht, & Kleerebezem, 2013; Jelmer Tamis et al., 2018). Organic waste, of unknown composition and complexity can be degraded to these platform molecules using a fermentation process. In order to acquire VFA from an AD process, methanogenesis should be prevented. Methanogenesis is the conversion of (in)organic C1 and C2 intermediates to methane and carbon dioxide containing biogas. This process can be stopped by inhibiting the methanogens by e.g. working at low solid retention times (SRT), and/or working at a low pH with high VFA concentrations (Kleerebezem et al., 2015). Combining the granular sludge technology and the inhibition of methanogens, the principles of the AD process using granules can be applied to produce VFA from complex substrates.

Fermentation processes to yield VFA are usually conducted in continuous stirred tank reactors (CSTR) fed with glucose (Rombouts, Mos, Weissbrodt, Kleerebezem, & Van Loosdrecht, 2019; Temudo, Muyzer, Kleerebezem, & Van Loosdrecht, 2008). A drawback of using a chemostat type process is that low volumetric productivities are achieved and that all biomass produced is present in the effluent (J. Tamis, Joosse, van Loosdrecht, & Kleerebezem, 2015). Another type of operation would be the usage of granular sludge, an established technology in the AD process through the development of the Upflow Anaerobic Sludge Bed (UASB) reactor or more recently the 'Nereda®' technology applied in municipal wastewater treatment (Lettinga et al., 1980; Pronk et al., 2015). Using granular sludge technology the SRT can be uncoupled from the hydraulic retention time (HRT), resulting in high-rate systems (J. Tamis et al., 2015). Additionally, a lower solid content can be realized in the effluent using granular sludge through biomass removal from the sludge bed. Low solid contents are beneficial in processes converting the VFA produced into higher-value non soluble products like bioplastics. However, the application of granular sludge technology for the production of VFA is just touched upon and granulation and system control need to be persistent. In general, anaerobic granular sludge could serve as a platform to produce on an industrial scale VFA from organic wastewaters.

Even though VFA production from organic waste using anaerobic granular sludge has been demonstrated, many research questions remain (Jelmer Tamis et al., 2018). For example, the factors determining the product spectrum of the fermentation of glucose and other carbohydrates remain unclear. For non-granular processes it has been demonstrated that at higher pH values (6-8) the product spectrum will mainly consist out of acetate and ethanol, whereas at a lower pH (5-6) an acetate and butyrate mixture will be obtained (De Kok, Meijer, Van Loosdrecht, & Kleerebezem, 2013; Temudo, Kleerebezem, & van Loosdrecht, 2007). At lower pH-values, carbohydrate fermentations that produce organic acids often generate H2-gas as a by-product. Even though in some studies H2 is considered the main product of the fermentation process (Das & Veziroglu, 2008; Hallenbeck & Ghosh, 2009), H2 production lowers the overall VFA product yield. The produced H2 contains electrons originating from the substrate and the H2 leaves the bioreactor via the off-gas, resulting in a decrease of the VFA yield on substrate. Lastly, also biomass production can be considered as an unwanted side product of the fermentation process. In anaerobic processes where no (strong) electron acceptor is present the biomass yield is relatively low compared to aerobic processes. Still, in order to maximize VFA production the biomass yield should be minimized. Increasing the SRT (and therewith lowering the biomass specific growth rate) may result in higher VFA yields because the biomass yield is reduced due to higher substrate demands for maintenance purposes. Increasing the SRT can lead to a higher VFA yield as shown in previous studies (Bengtsson, Hallquist, Werker, & Welander, 2008; Bolaji & Dionisi, 2017). Overall, the fermentation of organic waste is a complex process and the factors that determine the product spectrum are largely unknown.

In this study the effect the SRT has on the product yield and product spectrum of the fermentation of glucose to VFA was investigated. A sequenting batch reactor (SBR) operated at pH 5.5 was used in which pulse-wise glucose was fed to anaerobic granular sludge. The main process performance indicators were; the VFA yield, the product spectrum; the sludge volume index (SVI) and the microbial community structure as identified using 16s rRNA sequencing. Three operational SRT were chosen namely, 1-2 d SRT, 10-20 d SRT and uncontrolled SRT. Challenges for this procces will be granulation at different SRT, avoidance of methanogenesis at longer SRT and reduced VFA production yield due to production of H2.

Materials and methods

Inoculum cultivation

A single bioreactor was operated, the bioreactor had a height of 150 cm and an internal diameter of 6.5 cm. The working liquid volume was 2.5 L and the headspace was 2.4 L. The reactor was maintained at 30 ± 1 ^{o}C and the pH was controlled at 5.5 using 2 M NaOH (Biostat Stedim, Sartorius, the Netherlands). A 2% Antifoam C solution was added to the reactor 4 times slower than the base addition. Before intiating the different SRT a start up period was required to obtain granular sludge. The carbon and mineral medium used were identical as in (J. Tamis et al., 2015) using a glucose concentration of 10 g·L-1 in the influent

(1.25 L). The reactor was seeded with anaerobic sludge digesting mixed primary and secondary sludge from the sewage treatment plant Harnaschpolder (Delft, The Netherlands). During the granulation process two general rules were applied determining the cycle length and settling time. The rules were that glucose should be fully consumed and all biomass should be retained in the reactor. Firstly, glucose was considered fully depleted once the base addition flattened out and CO2 and H2 were below 1% in the off-gas. Secondly, the settling time for biomass started at 3 hours and was manually shortened to 2 min, over a period of 20 d.

Enrichment set-up

The same bioreactor, pH, and temperature as used for the inoculum cultivation was used for the subsequent three enrichments. Similar mineral composition as in (J. Tamis et al., 2015) was used and the glucose concentration in the influent was 6 g·L-1 (influent volume: 1.25 L). For all three enrichments the settling time was set to 60 minutes ensuring biomass retention. The cycle length was 6 hours and the HRT was 12 hours. A cycle length consisted out of the following phases: Settling phase (60 min), effluent phase (6 min), nutrient feeding phase (5 min), acclimization phase (10 min), glucose feeding phase (3 min) and finally a reaction phase (276 min). The first SRT investigated was uncontrolled SRT, for this SRT no sludge was manually removed. Hence, the SRT was determined by the biomass washout via the effluent. The other SRT investigated were 1-2 d and 10-20 d SRT. In these systems biomass, besides the biomass washout via the effleuent sludge was manually removed at the end of the reaction time when the reactor was completely mixed. The reactor was mixed by recycling the produced biogas from the culture, and anaerobic conditions were maintained by two water-locks connected to the bioreactor in serie. The second water-lock was connected to the outside environment and sparged with N2 gas keeping the bottle anaerobic.

Sampling and analytical methods

The bioreactor was monitored online using measurements of the pH, and base addition. The biogas was measured qualitatively for CO2, H2 and CH4 in-line with a mass spectrometry system (Omnistar, Pfeifer vacuum). Offline measurements were done for SVI, soluble chemical oxygen demand (CODSol), glucose concentration, organic acids concentration, total suspended solids (TSS) and volatile suspsended solids (VSS). The VSS content of the reactor was determined by taking a VSS sample at the end of the reaction phase, when the reactor was completely mixed. The reactor was sampled in more detail at the end of the enrichment elucidating the conversions during a cycle. To this end the reactor was sampled every 5 min during the presence of glucose and once every 30 min after glucose depletion. Samples taken for CODSol glucose, and organic acid analysis were filtered directly after sampling using a 0.45 μM pore size filter (PVDF membrane, Millipore, Ireland). The CODSol was measured using commercially available spectrophotometrically test-kit from Hach-Lange. The organic acid composition and quantity were measured using a high-performance liquid chromatography with a BioRadAminex HPX-87H column and a UV/RI detector (Waters 2489). As a mobile phase 1.5 mM H3PO4 in Milli-Q water was used with a flow rate of 0.6 mL·min-1 and a temperature of 60°C. The TSS content of a sample was obtained by first centrifuging a sample at 2500 g after decanting the supernatant the sample was oven dried at 105 ^{o}C. The VSS content was obtained according to (Clesceri et al. 1999). The SVI60 was obtained by taking the apparent sludge volume (SV) at the end of the settling phase in the bioreactor and dividing this by the measured TSS from the mixed reactor.

Data analysis

The SRT was determined by the amount of solids (gTSS·d-1) discharged from the system compared to the total amount of solids (gTSS) present in the reactor. The chemical oxygen demand (COD) balance could only be made over the soluble compounds measured in the effluent and averaged VSS production. Furthermore, it was assumed that all VSS consisted for 100% out of biomass.

A mathematical model was made for obtaining the conversions and qSmax of the enrichment. An elaborated description of the model is shown in the supplementary material. The model was based upon two/three reactions.

(i) Direct glucose fermentation towards extracellular products, biomass and storage polymer:

$$Glucose \rightarrow a\ Acetate + b\ Lactate + c\ Propionate + d\ Butyrate + e\ Biomass + f\ Storage\ polymer\ [gCODgCOD]^{-1}$$

(ii) The conversion of storage polymer to extracellular products and biomass

$$Storage\ polymer \rightarrow g\ Acetate + h\ Lactate + i\ Propionate + j\ Butyrate + k\ Biomass\ [gCODgCOD]^{-1}$$

(iii) A fixed stoichiometry for lactate conversion as described in (J. Tamis et al., 2015)

$$Lactate \rightarrow 0.21\ acetate + 0.73\ propionate + 0.06\ VSS\ [gCODgCOD]^{-1}$$

Furthermore, to make the model fitting the following assumptions were made:

- The biomass conversion factor used was 1.42 gCOD·gVSS-1 (Rittmann, Crawford, & Tuck, 1986)
- The storage polymer amount was set to 1 gCOD at the start of a cycle and should be 1 ± 0.05 gCOD at the end of the cycle
- There was no restriction for the COD-balances to be closed, allowing identification of missing fermentation products
- The production/consumption of H2 was not incorporated in the model

Microbial community structure

DNA from mixed bioreactor samples were extracted using the DNeasy UltraClean Microbial Kit (Qiagen, The Netherlands). Approximately 250 mg wet biomass was treated according to the standard protocol except an alternative lysis was implemented. This included a combination of 5 minutes of heat (65^{o}C) followed by 5 minutes of bead-beating for cell disruption on a Mini-Beadbeater-24 (Biospec, U.S.A.). After extraction the DNA was checked for quality by gel electrophorese and quantified using a Qubit 4 (Thermo Fisher Scientific, U.S.A.).

After quality control, samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341-806) on an Illumina paired-end platform. After sequencing, the raw reads were quality filtered, chimeric sequences were removed and OTUs were generated on the base of [?] 97% identity. Subsequently, microbial community analysis was performed by Novogene using Mothur & Qiime software (V1.7.0). For phylogenetical determination a most recent SSURef database from SILVA (http://www.arb-silva.de/) was used.

Results

Granulation and enrichment performance

Initial reactor operations were aimed at complete retention of the biomass. The cycle length varied in the beginning, starting with a long cycle length of 24 h. Both a flat base addition profile and CO2 and H2 concentrations in the off gas below 1 % (m/m) were used as indicators of full conversion of glucose into organic acids. During the reaction time the biomass could grow and consume the glucose dosed. A short settling time (2-10 min) resulted in non-granular biomass at the start. After increasing the settling time to 3 h a visible improvement of biomass retention was achieved. After 6 d the settling time was manually lowered as shown in figure 1 to promote granule formation.

The granules formed during the start-up period were used as starting material for the first enrichment. An impression of the reactor operated at different SRT is depicted in figure 2. The start-up period served only as inoculum production for the first enrichments and thus day 0 was chosen to be the first day of the SRT research.

After the start-up phase the settling time was increased to 60 min and the cycle length to 6 h, to prolong the SRT. In this first period the SRT was uncontrolled, this meant that TSS was not actively removed from the

system. TSS could still leave the system when a maximum sludge volume (SV) was reached (which meant that the sludge bed reached until the effluent point of the reactor which was half way the reactor height) and additional biomass production left the reactor with the discharge effluent. Using this approach an SRT of 40-50 d was achieved. After characterisation of the system, the SRT was manually controlled at 10-20 d SRT and 1-2 d SRT. During the start-up phase H2 and CO2 were produced and measured in the biogas. After the start-up period no H2 was measured in the biogas, the biogas consisted solely out of CO2 (20-40% m/m) and N2 (60-80% m/m) and no (<0.5%) H2 or CH4 were detected. An overview of the culture performance for the 3 SRT is shown in figure 3.

With increasing the SRT the concentration of biomass in the reactor increased from 6 gVSS*L-1 at 1-2 d SRT to 59 gVSS*L-1 at 40-50 d SRT. An high SRT could be achieved due to amongst others a very compact sludge bed with a SVI of 11 +- 2 ml*gTSS-1 (average +- standard deviation; n=8) and a biomass yield of 0.11 gCODX*gCOD-1 (X = biomass). The sludge bed became dense as it showed a strong compressive ability. After starting the settling phase an initial sludge bed was formed within 10 minutes. After the initial settling the sludge bed compressed for the remainder of the settling time (total settling time 60 minutes) resulting in a very low SVI60. An overview of system characteristics is given in table 1. In all experimental periods the VSS present in the effluent was low, 0.21 +- 0.18 (n=100) gVSS*L-1, compared to the VSS concentrations present in the reactor. The manual removed sludge was 3.1 gVSS*d-1, 1.9 gVSS*d-1 and 0 gVSS*d-1 for the 1-2 d SRT, 10-20 d SRT and 40-50 d SRT respectively. The biomass yield was estimated over multiple cycles for each SRT and was in the range of 0.09-0.17 gCOD*gCOD-1. The assumption was made that all VSS could be classified as biomass as described in the material and methods.

Product Spectrum

The development of the product spectrum in time during typical operational cycles is shown in figure 3. In the 40-50 d SRT system the measured biomass and VFA produced covered for 90% the influent COD on average. For the VFA an average was made from day 71-136 as the product spectrum was constant in this period. In this period the product spectrum consisted mainly out of acetate and propionate produced in a ratio of propionate:acetate 2.05:1 (molpropionate:molacetate). At SRT-values of 1-2 d and 10-20 d the measured biomass yields together with the produced VFA covered for 92-94% the influent COD on average for the entire enrichment period. There were no significant amounts of unknown additional fermentation products formed as the measured soluble COD concentration corresponded to the sum of the measured compounds (0.97 +- 0.09 (n=55) gCODVFA*gCODSol-1). The increasing gap observed in the CODSol balance during glucose depletion, and subsequent increase of the CODSol balance due to secondary VFA production, suggests that part of the substrate was converted to a storage polymer. This storage polymer was not quantified, as no distinguishment could be made between storage polymer and extracellular polymeric susbtances (EPS) after extraction. When the SRT was shortened to 1-2 d, next to storage polymers, lactate was produced during the glucose consumption phase. Transiently produced lactate and storage polymers were converted to a mixture of acetate, propionate and butyrate. Inline gas measurements showed a CO2 partial pressure of 20-40% (m/m) at all SRT. H2 partial pressures measured ranged from 0.01% to 0.2 % and corresponded in all cases to less than 1 % of the COD supplied to the system.

Kinetics

The conversions within a cycle were investigated in cycle measurements. The measurements were conducted once the system reached a steady functional performance in terms of product spectrum and substrate uptake rates. An overview of all cycle measurements performed is shown in figure 4.

In all systems the majority of the conversions happened in a short period when glucose was present. A process model was made to investigate the stoichiometric and the kinetic characteristics of the conversions observed as described in the material and methods section. An overview of the obtained kinetic parameters and final product spectrum is given in table 2.

The amount of stored polymer produced was estimated using the model. The quantity varied per enrichment but was always significant. At an SRT of 50 d 0.47 gCOD storage polymer was estimated to be produced

per gCOD glucose consumed. This amount of storage polymer lowered to 0.43 and 0.32 gCOD*COD-1 respectively at an SRT of 10-20 d and 1-2 d. The maximum specific substrate uptake rate (qSmax) decreased from 0.7 gCOD*gVSS-1*h-1 at 1-2 d SRT to 0.2 gCOD*gVSS-1*h-1 at an SRT of 50 d.

Microbial community structure

During the intial granulation process 16S rRNA showed that the mixed microbial community was dominated by *Clostridium pasteurianum* . When the SRT was prolonged to 50 d the microbial community shifted to a community dominated (>50% operational taxonomic unit (OTU) based) by *Bifidobacterium scardovii* . Additionally,*Propionibacterium thoenii* and *Megasphaera cerevisiae* were detected and contributed for 20-30% in total to the community structure based upon 16S rRNA. This community structure remained similar at an SRT of 10-20 d. While decreasing the SRT to 1-2 d a more diverse community structure consisting of 4 species, next to the aforementioned species the 4th observed species was*Saccharibacteria* . Once the enrichment matured, again *B. scardovii* was the dominant microorganism (>65% OTU based).

Discussion

Granulation

There exists a variety of operational conditions, either aerobic or anaerobic, that can result in the formation of dense granular structures. In this study we investigated the characteristics of compact anaerobic granular sludge producing VFA cultivated at varying SRT-values (1-50 d). Other processes creating granules are the 'Nereda(r)' process and the upflow anaerobic sludge blanket (UASB) reactor (Lettinga et al., 1980; Pronk et al., 2015). In both these systems the influent is fed in the bottom of the reactor and passes through a sludge bed which is not (or only partly) mixed. The microorganisms encounter the highest concentration of substrate at the bottom of the reactor, creating a substrate concentration gradient along the height of the sludge bed. Rapidly settling biomass therewith has a competitive advantage through location in the lower section of the sludge bed. Low substrate concentrations as found in CSTR type reactors negatively affect granule formation (de Kreuk & van Loosdrecht, 2004). In this work the substrate was added pulswise in a mixed reactor, creating a substrate concentration gradient only in time and not in space and time as described for the other processes. The substrate gradient is a prerequisite for providing a competitive advantage for microorganisms growing in a biofilm. Effective biomass granulation in this study was achieved using the startup procedure as described in the material and methods section. Enrichment of biomass growing in granules is a complex process, in which the following operational steps were found to be crucial to achieve effective granulation.

- First, to initiate granulation in a mixed reactor system full biomass retention was aimed for, requiring a long settling time. In these experiments an intial settling time of 3 h was implemented, achieving full retention of biomass in the first 12 cycles of operation.
- Second, to achieve adequate selection of biomass growing in granules, the settling time was step-wise reduced.
- Thirdly, the glucose was dosed pulse-wise creating a substrate gradient over time

In this study at an SRT of 40-50 d a SVI60 of 11 +- 2 mL*gTSS-1 could be achieved, amounting to a very dense sludge bed. At shorter SRT the SVI was higher and was around 34-44 mL*gTSS-1. The SVI for previously operated granular systems varied though were in the same order of magnitude; 17-29 mL*gVSS-1 for granular acidogenic systems (J. Tamis et al., 2015) and 12-45 mL*gTSS-1 have been reported for aerobic granular sludge in lab and full-scale situation (De Kreuk, Pronk, & Van Loosdrecht, 2005; Pronk et al., 2015). In upflow anaerobic sludge bed (UASB) reactors the SVI can go as low as 7.8 mL*gVSS-1 (Grotenhuis, Kissel, Plugge, Stams, & Zehnder, 1991). These results demonstrate well-settling granular sludge and dense microbial structures can be obtained under varying process conditions.

Kinetic properties of the enrichment

The $q_S^{\max}$of the culture decreased at increasing SRT. At an SRT of 1-2 d a $q_S^{\max}$ of 0.7 gCOD*gVSS-1*h-1

was achieved, which reduced to a value of 0.2 gCOD*gVSS-1*h-1 at an SRT of 40-50 d. Rombouts et al., (2019) found a q_S^{max} of 4.3 gCOD*gVSS-1*h-1 in a pulse-fed glucose SBR operated at a pH of 8.0 and a SRT of 8 h (Rombouts et al., 2019). This q_S^{max} value is an order of magnitude higher than the values obtained in this study. Possibly the pH, SRT or a combination of both played a crucial role. In Rombouts et al. (2019) the influent contained 4 g*L-1 glucose however, the operating pH was 8.0 and product inhibition was not likely to occur at only 4 g*L-1 glucose, possibly enabling this high qSmax. Another reason for the lower q_S^{max}values found for granular sludge is the production of EPS in granular sludge. When in both cases the VSS concentration gets classified as catalytic biomass the biomass content gets overestimated in a granular system as more parts of the VSS is EPS and not catalytic biomass. Furthermore, part of the biomass could be inactive but retained in the system as VSS incorporated in a granule resulting in lower observed qS values. Additionally, there is the possibility that microorganism over the depth of the biofilm are not doing all processes simultaneously. These reasons would result in the underestimation of qSmax in a granular system compared to a suspended cell system. The qSmax value obtained in this work at an SRT of 1-2 d was lower compared to the values found previously in comparable conditions (0.7 versus 1.6 gCOD*gVSS-1*h-1(J. Tamis et al., 2015). Potentially, the different history of the enrichments obtained in both studies resulted in a different local optimum, characterized by different types of dominant microorganisms and corresponding kinetic properties, i.e. in this study *Bifidobacterium scardovii* was the dominant microorganism versus *Clostridium pasteurianum* in Tamis et al. (2015).

Despite the relatively low observed q_S^{max} a volumetric glucose consumption rate of 1100 gCOD*L-1*d-1 was found in the 40-50 d SRT system. This is orders of magnitude larger than continuous stirred tank reactors (CSTR) operated using similar conditions as in this study. A consumption rate of 12 gCOD*L-1*d-1 can be estimated when the HRT is 12 h and the influent concentration is 6 gCOD*L-1. The overall volumetric capacity in the granular sludge process is orders of magnitude higher compared to a chemostat due to effective biomass retention, and despite the lowerq_s^{max} values achieved.

Product formation (stoichiometry) linked to reactor operation

Operation at different SRT values, long settling times and biogas recirculation resulted in distinct differences in product spectrum in this work. At an SRT of 50 d a product spectrum consisting of propionate and acetate reached an overall VFA yield of 77% (gCOD*gCODglucose-1) obtained during a cycle measurement. At lower SRT values the product spectrum contained less propionate and additionally butyrate was measured in the product spectrum at the end of a cycle. During the 1-2 d SRT there was transient production of lactate observed during glucose consumption. The product spectra obtained at 1-2 d SRT and 10-20 d SRT were comparable to those obtained in a previous study operated at a pH of 5.5 and using granular sludge (J. Tamis et al., 2015). The difference between the studies are the ratio in which the main fermentation products were obtained and possible H2 production as shown in table 2. In this study more acetate was produced compared to butyrate which was the main fermentation product in (J. Tamis et al., 2015) The COD-balances as measured in the cycle measurements were 0.94, 0.92, 0.88 gCOD*gCODglucose-1 for respectiveley 1-2 d, 10-20 d and 50 d SRT. This indicated that the lion-share of the fermentation products were found.

Despite the high SRT, methane production was effectively repressed probably due to the relatively low operational pH. As opposed to previous studies, in this work at an SRT of 40-50 d a relatively higher percentage of the product spectrum consisted out of propionate compared to operating at lower SRT. Namely, 0.57 gCODPro*gCODglucose and 0.14-0.18 gCODPro*gCODglucose respectively for the 40-50 d and 1-2 d & 10-20 d SRT. Tamis et al., (2015) operated an anaerobic sequential batch reactor using granular sludge reactor at a pH of 5.5 and at an SRT of 1-2 d, around 0.05-0.10 (gCOD*gCOD-1) of the product spectrum contained propionate. Chemostat studies have found varying results. (De Kok et al., 2013) demonstrated that by increasing the dilution rate in a glucose-fed chemostat from 0.05 to 0.125 h-1 the COD-based propionate yield decreased from 0.23-0.29 to 0.02-0.03 gCOD*gCOD-1. These results indicate that the SRT plays an important role in the product spectrum and high SRT favour propionate production at the expense of butyrate production. The answer to question why elevated SRT-values seem to favour propionate production in this study remains to be elucidated.

Product formation (stoichiometry) linked to microbial community structure

The microbial community structure at 40-50 d SRT was dominated by *Bifidobacterium scardovii* and supported by *Megasphaera* and *Propionibacterium* based on 16S rRNA analysis. This microbial structure could explain the observed product spectrum of propionate and acetate. *Bifidobacterium* are known for the production of acetic and lactic acid using the phospho-ketolase pathway from glucose using the 'bifid shunt', though no propionate production has been as yet associated with *Bifidobacterium* (Falsen et al., 2015; Pokusaeva, Fitzgerald, & Van Sinderen, 2011; Ventura, Delgado, Milani, O'callaghan, & Van Sinderen, 2016). The microorganism is lacking the key enzymes for the glycolysis and hexose-monophosphate pathway and uses the phospho-ketolase pathway for catabolism (de Vries & Stouthamer, 1967). Possibly, the other observed species *Megasphaera cerevisiae* and *Propionibacterium thoenii* capable of consuming the lactate and producing propionate were responsible for the production of propionate in this study (Lanjekar, Marathe, Ramana, Shouche, & Ranade, 2014; Paikt & Glatz, 1997). Potentially, lactate consumption was faster than lactate production at elevated SRT-values (10-20 d and 40-50 d) since no transient lactate was observed. Looking at the direct fermentation of glucose to propionate and acetate a product spectrum of 2:1 (molpropionate:molacetate) can be expected (Gonzalez-Garcia et al., 2017). In this study a product spectrum at 40-50 d SRT of propionate:acetate of 2.05:1 (molpropionate:molacetate) was found, this reflects nicely the proposed stoichiometry by Gonzalez-Garcia et al., (2017). *Bifidobacterium* was producing lactate and acetate, and subsequently a different microorganism could produce propionate and acetate from lactate.

The microorganism that dominated the process shortly after startup *Clostridium pasteurianum* was also encountered in (J. Tamis et al., 2015). In that work *C. pasteurianum* dominated the granular biomass over a pH range of 4.5-5.5. Two differences between that study and this study for obtaining *C. pasteurianum* were that the settling time was set to 2 min and the biogas was continuous diluted by input of fresh N2. *C. pasteurianum* produced a product spectrum dominated by acetate, butyrate and hydrogen as several studies have reported before for the *Clostridium* genus (Crabbendam, Neijssel, Tempest, & Amsterdam, 1985; Dabrock, Bahl, & Gottschalk, 1992; Lin et al., 2007; J. Tamis et al., 2015). This study shows that there is still a knowledge gap for obtaining specific product spectrums using granular sludge for the production of VFA from a carbohydrate-rich stream.

Application

VFA produced from carbohydrate-rich streams may serve as building blocks for the production of higher-value compounds compared to low-value biogas (Kleerebezem et al., 2015). Open culture fermentation can be used to produce organic acids from a complex substrate such as wastewater. Combining open culture fermentation with granular sludge technology, the production of a VFA rich effluent with a low solid content is possible. This is ideal for processes where the consumption of VFA results in a particulate endproduct like polyhydroxyalkanoates (PHA) that can be separated from the water phase (Jelmer Tamis, Lužkov, Jiang, Loosdrecht, & Kleerebezem, 2014). Tamis et al., (2014) demonstrated that minimizing the influent solid concentrations in the PHA production process enables maximization of the PHA content of biomass.

The granular sludge technology provides besides a low solid effluent, a high volumetric productivity. Uncoupling of the solid and liquid retention enables reduction of the bioreactor volume while maintaining similar output in terms fermentation products formed. This study showed the diversity of the product spectrum that can be obtained through control of the SRT. There was no mechanistic explanation found why in this study one product spectrum prevailed over others at different SRT.

Conclusion

This study showed the successive establishment of anaerobic granular sludge cultures enriched on glucose at 1-2 d, 10-20 d and 40-50 d SRT at a pH of 5.5. Two distinct product spectra were obtained (i) at 40-50 d SRT a propionate:acetate mixture of 2.05:1 (molpropionate:molacetate) was obtained; with a VFA production yield of 0.79 ± 0.12 (n=18) gCOD·gCOD-1. (ii) At 1-2 d and 10-20 d SRT an acetate dominated, 0.71-0.75 molacetate·molVFA-1, product spectrum was obtained. Overall, high VFA yields of 0.77-0.79 gCOD·gCOD-1

from glucose fermentations were obtained. Furthermore, compact sludge beds were obtained as SVI60 were ranging within 11-44 mL·gTSS-1, and *Bifidobacterium scardovii* was the prevailent microorganism in all systems. Substrate specific uptake rates varied from 0.2-0.7 gCOD·gVSS-1·h-1. Despite the relatively low qSmax, the glucose consumption rate of the systems varied from 100 gCOD·L-1·d-1 to a maximum rate of 1100 gCOD·L-1·d-1. Overall, this work showed the benefits of granular sludge technology for fermenting carbohydrate-rich water resulting in a VFA rich effluent with a low concentration solids. The possibilities of applying anaerobic granular sludge are just touched upon and more understanding is desired to control the product spectrum and granulation.

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Tables

Table 1. Overview system characteristics obtained at different SRT. The YX/S was obtained by averaging the VSS values obtained in the effluent together with the biomass growth in the reactor and/or manual removal of biomass from the reactor.

SRT	1-2 d	10-20 d	40-50 d	
SVI60	44 ± 8 (n=8)	34 ± 12 (n=17)	11 ± 2 (n=8)	mL·gTSS-1
VSSeffluent	0.12 ± 0.04 (n=10)	0.17 ± 0.22 (n=23)	0.23 ± 0.18 (n=67)	gVSS·L-1
YX/S	0.17± 0.01 (n=4)	0.14± 0.09 (n=4)	0.11 ± 0.09 (n=4)	gCOD·gCOD-1
YVFA/S	0.77 ± 0.06 (n=7)	0.79 ± 0.03 (n= 23)	0.79 ± 0.12 (n=18)	gCOD·gCOD-1

Table 2. An overview of the obtained of qSmax-values, and overall product stoichiometry for acetate (ac), propionate (pro), butyrate (but) and storage polymer produced. The storage polymer was intermittently produced during the consumption of glucose, and assumed to be fully consumed in the famine phase

SRT	$q^{\max}_{\text{Glucose}}$	R_{glucose}	$Y^{\frac{\text{ace}}{\text{glu}}}$	$Y^{\frac{\text{pro}}{\text{glu}}}$	$Y^{\frac{\text{but}}{\text{glu}}}$
	$gCOD \cdot (gVSS \cdot h)^{-1}$	$gCOD\,(Ld)^{-1}$	$gCODgCOD^{-1}$	$gCODgCOD^{-1}$	$gCODgC$
1-2	0.7	168	0.43	0.18	0.16
10-20	0.4	97	0.40	0.14	0.15
40-50	0.2	1104	0.20	0.57	0
Tamis et al., (2015) (1-2 d SRT)	1.6	300	0.17	0.03	0.20

Figure legends

Figure 1. Operational settings chosen, with the measured feast length during the start-up period for obtaining granules.

Figure 2. Visual impression of the sludge bed during the different operational periods in this study. From left to right, Start-up phase, 40-50 d SRT, 10-20 d SRT, and 1-2 d SRT.

Figure 3. Upper picture is an overview of the reactor performance in terms of VFA, VSS production and the

compactness of the culture expressed as SVI60. The bottom picture is the evolution of the product spectrum obtained during each enrichment. Day 0-136 is the 40-50 d SRT, day 137-192 is the 10-20 d SRT and finally day 193-212 is the 1-2 d SRT system.

Figure 4. All system characterisations performed in this study. From top to bottom the 40-50 d SRT, 10-20 d SRT and finally the 1-2 d SRT is depicted. Markers reflect measurements and lines are modelled. Soluble COD concentrations (sCOD) were calculated as the sum of the individual substrate and product concentrations. All cycle measurements were performed on the last day of the enrichment thus on days: 136, 192 and 212 for respectively 40-50 d, 10-20 d and 1-2 d SRT.

Appendix A

The mathematical model made in this study was aimed to derive characteristic parameters from the experimental data. The model was based upon 3 reactions: (i) the direct conversion of glucose to organic acids, storage polymers, and biomass; (ii) the conversion of storage polymers to organic acids and biomass; (iii) when lactate was one of the organic acids produced a third reaction was used describing the conversion of lactate to acetate, propionate and biomass. Glucose and lacatate conversions occurred in parallel and resulting in (partial) similar products thus these could not be calibrated separately. For this reason the reaction stoichiometry for lactate was fixed and similar to that used by (J. Tamis et al., 2015) and shown in table A.1. The reaction stoichiometry for reaction (i) and (ii) are given in table A.1

Table A.1. The reaction stoichiometries used to calibrate the model to the measured data

	rGlu	rSP	rLac
Glucose	-1		
Acetate	YAc/Glu	YAc/SP	0.32
Propionate	YPro/Glu	YPro/SP	0.63
Butyrate	YBut/Glu	YBut/SP	0
Lactate	YLac/Glu	YLac/SP	-1
SP	YSP/Glu	-1	0
Biomass	YX/Glu	YX/SP	0.06

Kinetics

All substrate uptake rates were established as shown in equation (A-1):

$$\frac{dC_j}{\mathrm{dt}} = q_j X_{\mathrm{lumped}} \quad (A-1)$$

- Cj = concentration of compound J [gCOD·L-1]
- qj = the subsrate specific uptake rate for compound j [gCODj·gVSS-1·h-1]
- Xlumped = the lumped amount of biomass present in the reactor

In this study the biomass was lumped as total VSS minus the storage polymer solids present in the reactor. It was not feasible to obtain fractions of biomass (species differentiation) from the total amount of VSS and thus it was not possible in obtaining specific activities. Therefore, the activities rather represent the activity of the total amount of catalytic VSS present in the reactor. The substrate kinetics equations for glucose, storage polymer (SP) and lactate consumption are shown in table A.2. For the consumption of the storage polymer a shrinken particle model was used. Furthermore, it was assumed that the consumption of the storage polymer started after all extracellular glucose was consumed. For the model the starting amount of SP was set to 1 gCOD and the final value (end of the cycle) was set to be 1 ± 0.05 gCOD.

Table A.2. The kinetic equations used in the study to calibrate the measured data. Ks,Glu is the half-saturation constant for glucose, Ks,lac is the half-saturation constant for Lactate

Glucose consumption	$q_{\mathrm{Glu}}(t) = q_{\mathrm{Glu}}^{\mathrm{Max}} \frac{C_{\mathrm{glu}}}{K_{s,GLu}+C_{\mathrm{Glu}}}$
Storage polymer consumption	$\mathrm{SP}\ (t) = SP\ (t-1)K_{\mathrm{SP}}$
Lactate consumption	$q_{\mathrm{lac}}\ (t) = q_{\mathrm{lac}}^{\mathrm{Max}} \frac{C_{\mathrm{lac}}}{K_{s,\ \ lac}+C_{\mathrm{lac}}}$

Model calibration

The difference of each measured sampling point (ti)with the modelled data was made. This was done for every component and for every measured data point. All the obtained differences were squared and summed to obtained the sum of squared errors (SSE) as shown in equation A-2.

$$\mathrm{SS}E_j = \sum_{i=1}^{N} \left(n_j^{\mathrm{measure}}\ (t_i) - n_j^{\mathrm{model}}\ (t_i)\right)^2 (A-2)$$

- j= glucose, aceate, propionate, butyrate, lactate, SP, biomass
- i= time corresponding to each measured data point
- njmeasured = the amount of compound j measured [gCOD]
- njmodel = the modelled amount of compound j [gCOD]

Subsequently the SSE of each component was summated to each other obtaining the total error of the model. The VSS was not taken into account for the SSE, as the SRT was significantly longer than the HRT the quantification of VSS was assumed not to be accurate within one cycle. The SSE was obtained as follows (eq. A-3):

$Total\ error = \sum \mathrm{SS}E_j\ (A-3)$

The total error was minimized adjusting characteristic parameters e.g., qSmax, KSP, KLac, KGlu and the yields shown in table A.1, done by the solver tool of Microsoft Excel. The solver was used to obtain the minimal total error, and the solver was set at GRG non-linear method. No additional constraints were made for the model then the constraints mentioned before. The intial yields used were 0.5 gCOD·gCOD-1, KSP was initially 0.01 min-1 and an initial qSmax of 1 gCOD·gVSS-1·h-1.

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