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The influence of essential growth nutrients on PHA production from waste

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Proefschrift

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus, Prof.dr.ir. T.H.J.J. van der Hagen,
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door

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Table of contents

0

Samenvatting

Vluchtige vetzuren (VVZ) kunnen als bouwblokken dienen voor de productie van een type bioplastic genaamd polyhydroxyalkanoaten (PHA). Zowel VVZ als PHA kunnen worden gemaakt uit afvalstromen. Ideale afvalstromen voor PHA productie bevatten een hoge chemisch zuurstof verbruik (CZV) naar nutriënt ratio. Typische ideale afvalstromen zijn het (afval)water van een papierfabriek of van een snoeprepen producent zoals Mars Inc.. Gangbaar wordt industrieel afvalwater anaeroob behandeld met als eindproduct methaan houdend biogas. Dit geproduceerde methaan kan worden gebruikt om bijvoorbeeld elektriciteit te produceren. Mogelijk kan er meer waarde aan de afvalstromen gegeven worden door de productie van VVZ en/of PHA in plaats van biogas.

PHA kan worden geproduceerd door gebruik van microbiële verrijkingsculturen. Enkele voordelen van open cultuur technologie zijn dat er geen sterilisatie nodig is en minder dure apparatuur vergeleken met pure cultuur technologie. Open cultuur technologie kan toegepast worden indien het juiste selectiecriteria voor een gewenste eigenschap is gevonden. Het micro-organisme dat het best kan overleven in de opgelegde selectiecriteria zal de competitie winnen. Met andere woorden: de sterkste zal overleven.

Het onderzoeksdoel van dit proefschrift is om meer inzichten te verkrijgen over het twee-stap PHA-productie proces uit organische afvalstromen. De eerste stap betreft de maximalisatie van de VVZ-concentratie in de voeding voor de PHA producerende bacteriën. De optimalisatie van de VVZ-productie was onderzocht met korrel slib. Door gebruik te maken van korrel slib kan de volumetrische VVZ-productiviteit worden gemaximaliseerd en worden de onopgeloste delen geminimaliseerd. In de tweede stap zijn twee proces variabelen onderzocht die betrekking hebben op het PHA-productie proces. De eerste proces variabele die is onderzocht, was het nauwkeuriger in kaart brengen van de invloed van groei van nutriënten op het PHA-productie proces. De tweede proces variabele heeft betrekking op de PHA-productie waarbij gebruik wordt gemaakt van percolaat water verkregen uit groente, fruit en tuin afval (GFT) op pilot schaal.

In **hoofdstuk 1** wordt een algemene introductie gegeven. Deze introductie geeft context en relevantie aan het onderwerp van dit proefschrift

In **hoofdstuk 2** is het effect van de verblijftijd van de vaste stof op de VVZ-productie onderzocht, gebruikmakend van korrel slib onder zuurstofloze omstandigheden. Drie verrijkingsculturen waren onderzocht bij een verblijftijd van 1-2 d, 10-20 d en 40-50 d. Een gemaximaliseerde VVZ-opbrengst van 0.79 $gCZV_{VVZ}{\cdot}gCZV_{glucose}^{-1}$ was verkregen bij een verblijftijd van 40-50 d. Het product spectrum bestond uit propionaat en acetaat in een verhouding van 2.05:1 (mol:mol). De verkregen cultuur bij een verblijftijd van 40-50 d bestond uit dichte korrels met een slib volume index (SVI) van 11 $mL{\cdot}gTSS^{-1}$.

Voor de productie van PHA uit afvalwater, bestaat de voeding ideaal uit een nutriënt gelimiteerde stroom, zodat er geen microbiële groei kan plaatsvinden in de PHA-accumulatie stap. In **hoofdstuk 3** is een verrijkingscultuur op suiker fermenteerde bacteriën verkregen onder ammonium arme condities. Ammonium arme condities waren gerealiseerd door stapsgewijs de toevoer van ammonium te verlagen naar 0 gNH_4-N·ronde^{-1}. De cultuur was hierdoor aangewezen op het fixeren van de aanwezige N_2 die continue werd toegevoegd aan de reactor. Een succesvolle verrijkingscultuur was verkregen in de afwezigheid van ammonium in de voeding. De N-inhoud van de cultuur verlaagde van 0.10 gNH_4-N·$gVSS^{-1}$ naar 0.04 gNH_4-N·$gVSS^{-1}$. In de afwezigheid van ammonium was de specifieke substraat opname snelheid met 50% afgenomen naar 0.35 $gCZV·gVSS^{-1}·h^{-1}$ vergeleken met de controle cultuur. De VVZ-opbrengst was tussen de 0.61-0.64 $gCZV_{VFA}·gCZV_{glucose}^{-1}$, en het product spectrum was butyraat en acetaat gedomineerd. Het werk beschreven in dit hoofdstuk laat zien dat zuurstofloos korrel slib verkregen kan worden in afwezigheid van ammonium in de voedingsstroom.

Veelal zijn afvalwaterstromen niet strikt nutriënt gelimiteerd, maar door de aanwezigheid van VVZ en koolhydraten worden deze interessant voor PHA-productie. In **hoofdstuk 4** is de simultane groei en productie van PHA onderzocht onder variabele C:N en C:P verhoudingen. Dit onderzoek was parallel uitgevoerd in 8 fed-batch reactoren en gebruikmakend van een voor-verrijkte cultuur die gedomineerd was door *Plasticicumulans acidivorans*, een bekende PHA-productiespecialist. Dit onderzoek heeft aangetoond dat wanneer er gebruik wordt gemaakt van een cultuur van hoge kwaliteit, hoge PHA-percentages behaald kunnen worden wanneer er gelimiteerde hoeveelheid nutriënten aanwezig zijn. Wanneer de voedingsstroom een verhouding heeft van 26 gCZV:gNH_4-N of 511 gCZV:gPO_4-P voor tenminste 12 uur, kan er een hoge PHA percentage bereikt worden (>75 wt% PHA)

In **hoofdstuk 5** is de haalbaarheid voor de productie van PHA op de organische fractie van vast stedelijk afval onderzocht. pH controle was niet haalbaar door de hoge alkaliniteit en de pH varieerde tussen de 7-9. Verder was er een hoge hoeveelheid van total ammoniumstikstof (TAN) aanwezig in de verrijkingsreactor en accumulatie (>500 $mgN·L^{-1}$). Ook waren er een significante hoeveelheid onopgeloste stoffen aanwezig in de voeding (>2$g·L^{-1}$). Ondanks de ruwe microbiële omstandigheden was er een PHA-content van 0.77 ± 0.18 $gPHA·gVSS^{-1}$ (n=3) behaald aan het einde van een PHA-accumulatie. In deze studie was een niet geclassificeerde *Rhodocyclaea* de dominante micro-organisme. Dit toont aan dat superieure PHA-opslagcapaciteit wijdverspreid aanwezig is in de microbiële wereld. Verder toont deze studie aan dat PHA-productie mogelijk is uit complexe stromen.

In het laatste hoofdstuk, **hoofdstuk 6**, worden vooruitzichten gegeven gebaseerd op behaalde resultaten. Onderwerpen voor vervolgonderzoeksvragen worden geopperd, zoals: wat waren de cruciale factoren die leiden tot de behaalde microbiële gemeenschap met een hoog product opbrengt en verschillend productspectrum (zoals in hoofdstuk 2 bepaald) Tevens worden twee grote uitdagingen voor het PHA productie proces besproken, namelijk: de afwezigheid van pH controle en het belang van het behalen van goed bezinkbare bacteriën.

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Summary

Volatile fatty acids (VFA) may serve as building blocks for Polyhydroxyalkanoates (PHA) production and can be derived from waste streams. Ideal streams for PHA production contain a high Chemical Oxygen Demand (COD) to nutrient ratio, such as (waste)water from a paper-mill factory or candy-bar factory. The (waste)water generated by these companies are usually treated anaerobically with the final product being methane containing biogas. Usually, the methane is burned to produce either heat or electricity. Potentially, more value can be added to these streams by producing VFA and/or PHA.

PHA can be produced using microbial enrichment cultures that can be established by cultivation in a selective environment that favours the growth of PHA producing microorganisms. Some advantages of using open cultures are that no sterilization and expensive equipment is required compared to pure culture biotechnology. Open culture biotechnology can be effectively applied when the right selection pressure for a specific microbial trait is identified. The microorganism that is most effective in the given conditions will win the competition, i.e. the strongest will survive. A selection criteria for PHA productis is consuming substrate very fast by first making a storage polymer (in this case PHA) from the supplied substrate. The PHA producers prefer VFA as substrate, hence it is important to maximize the VFA content in the substrate stream. For the production of VFA in the product chain towards PHA it is important to minimize the solid content in the feedstock for PHA production.

The objective of the research described in this thesis was to gain more insight in the two-step upstream process for PHA production from agricultural waste streams. The first step concerns the maximization of the VFA concentration in the feedstock. Optimization of VFA production was investigated using the granular sludge process in order to maximize the volumetric VFA production capacity and to minimize the solids concentration in the effluent. Two process variables were investigated regarding the PHA production process. Firstly, the influence of the presence of nutrients on PHA production was investigated using PHA producing enrichment cultures. Secondly, the production of PHA was investigated using the leachate of the organic fraction of municipal solid waste (OFMSW) at pilot scale.

In **chapter 1** a general introduction is given. In the introduction the background of the work described in this thesis is presented together with the relevance of the work.

In **chapter 2** the effect the solid retention time (SRT) has on the VFA production was investigated using the anaerobic granular sludge process. Three enrichments were performed at 1-2 d, 10-20 d and 40-50 d SRT. A maximized VFA yield of 0.79 $gCOD_{VFA} \cdot gCOD_{glucose}^{-1}$ was obtained at 40-50 d SRT. The product spectrum in the enrichment conducted at 40-50 d SRT consisted solely

out of propionate and acetate in a ratio of 2.05:1 (mol:mol). Furthermore, very dense granules were obtained in the 40-50 d SRT enrichment as a sludge volume index 60 (SVI) of 11 ml·gTSS^{-1} was determined.

For the production of PHA from wastewater ideally a nutrient limited stream is preferred as that restricts microbial growth in the PHA accumulation step. In **chapter3** the enrichment of glucose fermenting anaerobic granular sludge was investigated in conditions of nitrogen limitation. Nitrogen limitation was established by step-wise lowering the ammonium supply to 0 gNH_4-N·cycle^{-1}, limiting the available nitrogen source to N_2 gas that was supplied to the reactor continuously. In this study a viable enrichment was obtained in the absence of NH_4-N in the feed. The N-content of the culture decreased to from 0.10 gN·gVSS^{-1} to 0.05 gN·gVSS^{-1}. In absence of ammonium in the feed, the biomass specific substrate uptake rate was reduced by 50% to 0.35 gCOD·gVSS^{-1}·h^{-1} compared to the control enrichment. The VFA yields were 0.61-0.64 $gCOD_{VFA}$·$gCOD_{glucose}^{-1}$, and the organic acid product spectrum was butyrate and acetate dominated. The work demonstrated that anaerobic granular sludge was enriched successfully without the requirement of ammonium.

Many wastewaters are not nutrient limited, but due to the presence of VFA and/or carbohydrates the wastewater could be attractive for the production of PHA. In **chapter 4** the simultaneous growth and accumulation of PHA was investigated at variable C:N and C:P ratio's. This study was conducted using 8 fed-batch bioreactors using a pre-enriched culture dominated by *Plasticicumulans acidivorans*, a known PHA producer. This study showed that when the enrichment is of high quality, high PHA percentages can be achieved even when growth nutrients are supplied. This study showed that when a limited amount of nutrients are present in the substrate stream (C:N ratio of 26 gCOD:gNH_4-N and a C:P ratio of 511 gCOD:gPO_4-P) for at least 12 hours a high PHA wt% (>75 wt% PHA) can be maintained.

In **chapter 5** the feasibility of producing PHA from the leachate of OFMSW was investigated. Due to the high alkalinity pH control was rendered not feasible, resulting in a pH varying from 7-9. Furthermore, the leachate contained a high amount of total ammonium nitrogen (TAN) in the enrichment/accumulation (>500 mgN·L^{-1}) and a significant amount of solids were present in the feedstock (>2 g·L^{-1}). Despite these harsh conditions a PHA content of 0.77 ± 0.18 gPHA·gVSS^{-1} (n=3) was obtained on average at the end of several PHA accumulations. In this study an unclassified *Rhodocyclaea* was the dominant microorganism, showing that superior PHA storing capacities are widespread in the microbial world. This study showed that PHA can be produced from complex streams.

Chapter 6 gives an outlook based on the results obtained. Topics for follow-up research questions are suggested such as identification of the factors that determined the changes in the microbial community structure described in chapter 2 resulting in the high product yield and distinct product spectrum. Two big hurdles for the upstream production of PHA are also discussed namely, the absence of pH control and the importance of obtaining well-settling biomass.

1

General introduction

Wastewater is daily being generated by amongst others households and industries. In the Netherlands domestic wastewater is collected in the sewer system and treated in sewage treatment plants before being discharged on surface waters. Besides domestic wastewater, industry generates enormous quantities of wastewater as well. Many industries have on-site (partial) cleaning of the wastewater, minimizing the amount that is discharged to the sewer and thus minimizing the expenditures. Different types of industry generate different types of wastewater. Typical examples of industries all around the world that generate large quantities of wastewater are paper-mill industries, agro-industries such as sugar production and potato processing industries as well as many derivatives thereof. Industrial wastewaters that are rich in concentration of organic pollutants are mainly treated using the anaerobic digestion (AD) process (Batstone *et al.*, 2002). In this process complex (solid) waste is converted to methane containing biogas. The AD process consists of 4 phases; (i) *hydrolysis* of complex organic matter to soluble organic monomers, (ii) *acidogenesis*, where all soluble organic monomers are fermented yielding volatile fatty acids (VFA), (iii) *acetogenesis* is a process in which VFA are consumed and acetic acid, H_2 and CO_2 are produced, and (iv) finally, during *methanogenesis* the aforementioned compounds are converted to a mixture of methane and carbon dioxide, so called methane containing biogas. Acidogenesis, acetogenesis, and methanogenesis using wastewater are carried out by microorganisms operating in a non-axenic environment. Currently, the AD process is an established resource recovery technology for treating industrial wastewaters (Puyol *et al.*, 2017). Some advantages of AD are that it is well studied, and AD is widely applicable on all kind of complex different waste streams. Additionally, the sludge production (considered as waste) is minimal. It is an anaerobic process meaning no external input of air thus there are no high costs connected for aeration. Finally, as the sludge production is low in anaerobic processes the requirement for nutrients and the related costs are minimal. Overall, AD is an established technology for the recovery of chemical oxygen demand (COD) through conversion to energy rich methane containing biogas.

One disadvantages of the AD process is that the value of biogas is relatively low. From the organic pollutants in the water higher-value compounds can be produced. In the last decade economically interesting other compounds with a higher-value potential than methane have been investigated on lab/pilot scale. Alternatives could be the production of biohydrogen or biopolymers from wastewater. One specific alternative could be the production of extra polymeric substances (EPS) produced by bacteria in a granule. Granules are a mixture consisting out of bacteria and EPS in which EPS serves as a matrix in which the bacteria are embedded. EPS is also found in non-granular wastewater treatments processes where it serves as flocculant to improve aggregation (Nouha *et al.*, 2018). Initially EPS, just as biomass, was considered as waste in the biological treatment of wastewater. Nowadays, more attention is being given to the purification of EPS from for example granules. Granules can be obtained in non-axenic conditions –open cultures–, if a strong selective pressure can be applied. As granules are an agglomerate of many bacteria together, they sink fast compared to single bacteria. This feature of fast settling can be used in an enrichment-step to obtain granular sludge. Granules comes in different shapes, for example some are larger or smoother around the edges compared to other granules. An example of granules is shown in figure 1.1.

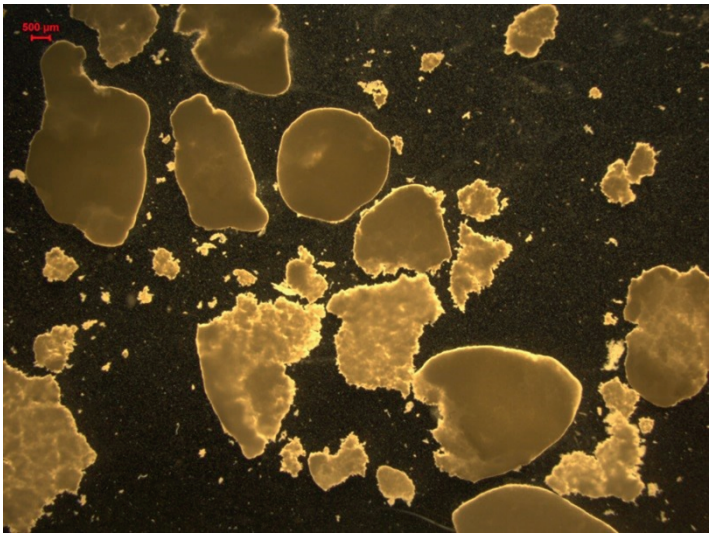

Figure 1.1 – Example of granules of different shapes and sizes.

Bioplastic production from wastewater

Another bio-product that can be produced from agro-industrial wastewater is the bioplastic polyhydroxyalkanoates (PHA). PHA was annotated as bioplastics

as it contains thermoplastic properties similar to that of fossil-fuel derived plastics (Steinbüchel and Füchtenbusch, 1998). The production of PHA from industrial waste water is usually conducted in a two-step process. Figure 1.4 depicts an overall PHA production process from wastewater using mixed microbial cultures. In the first process the amount of VFA is maximized using a fermentation process, the first topic of this thesis. In a fermentation process a myriad of different compounds can be converted to a mixture of VFA done by microorganisms, similar to the *acidogenesis* step in the AD process. After VFA maximization different microorganisms convert the produced VFA to PHA in an aerobic respiration process, which is the focus of the second part of this thesis. There are different selective pressures for obtaining PHA producing specialized bacteria (Kourmentza *et al.*, 2017). An effective selective pressure is pulse-wise feeding the bacterial culture. This creates times of a surplus presence of food (feast) and times when no food is present (famine). Microorganisms that are capable of producing microbial fat are able to growth and stay vital in the famine periods where no food is present. The PHA granules inside a cell can also be seen under a microscope as shown in figure 1.2.

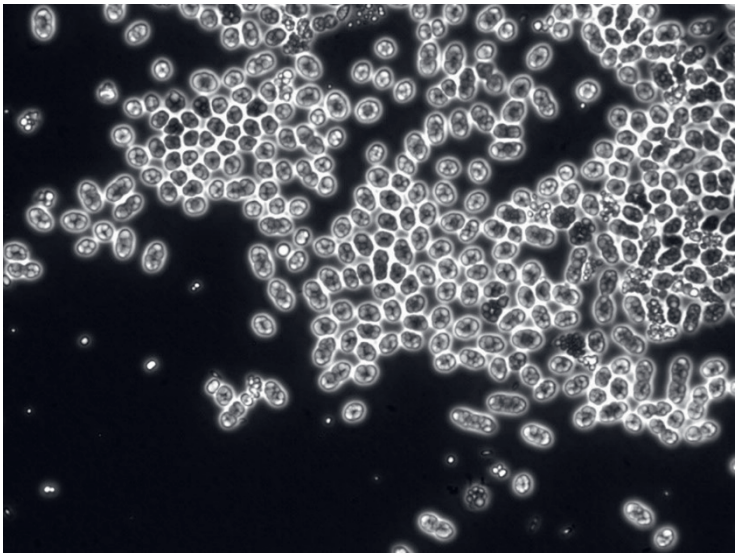

Figure 1.2 – Example of PHA producing bacteria seen under the light-microscope.

The exact economic value of both PHA and EPS are unknown because there is no established market for both compounds. A rough comparison based upon the amount of electrons that can be harvested (chemical oxygen demand COD) from the initial substrate is shown in figure 1.3.

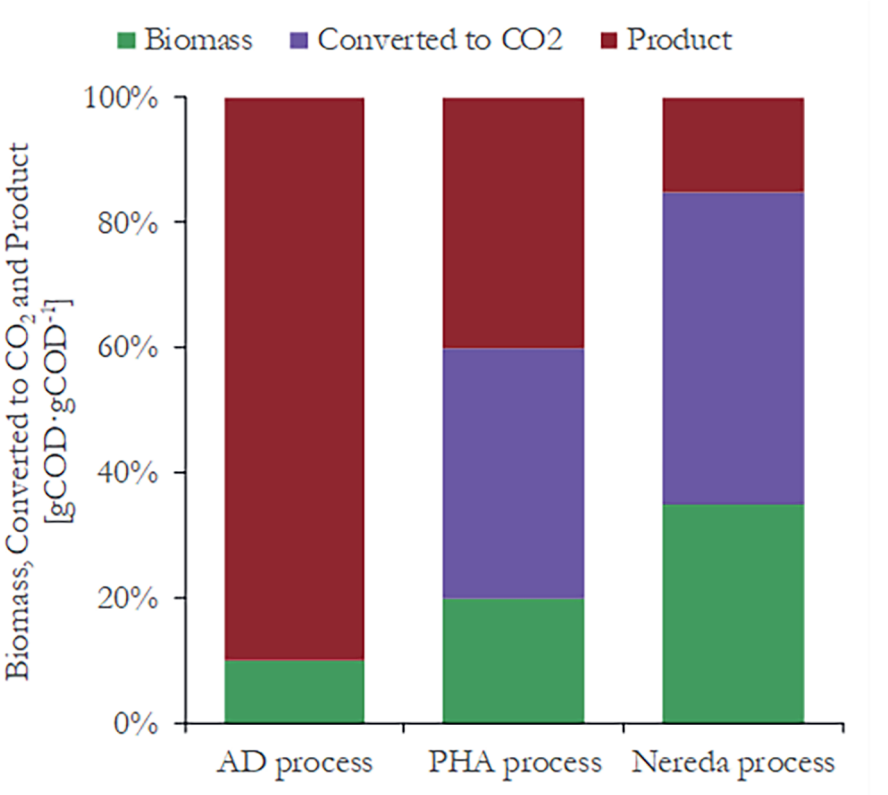

Figure 1.3 – Distribution of COD of the 3 processes competing for producing resources from wastewater.

It is clear that on COD basis the AD process is the big winner as the largest fraction of COD can be recovered as product in this case methane. Also, can be seen that the other 2 competing processes produces more waste in the form of biomass and are consuming more miscellaneous in both cases this is oxygen. It should be kept in mind that this is a COD based analysis and it is not per se a 1 on 1 translation to an economic analysis as methane is a low-value product. Overall, the AD process is an efficient process, though by making higher-value products other than methane from the same wastewater other processes such as production of PHA can compete with this traditional powerhouse.

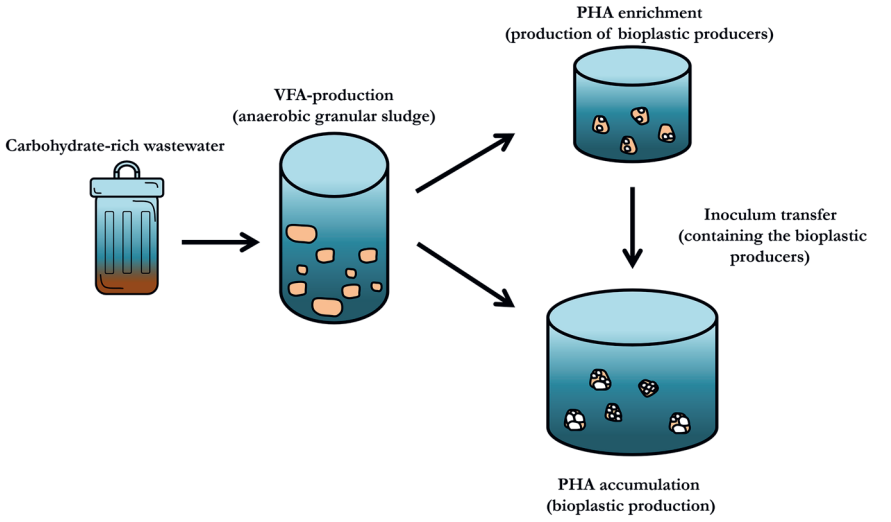

Figure 1.4 – Visualisation of the production chain of bioplastic production from wastewater

VFA production using anaerobic granular sludge

The first step in the two-step process of valorising wastewater to bioplastic is a fermentation step. In this process carbohydrates and amino acids are converted through anaerobic fermentation to a mixture of volatile fatty acids and ammonium. The maximization of VFA is required as consumption of non-VFA COD will not yield PHA (Marang *et al.*, 2014; Korkakaki, van Loosdrecht and Kleerebezem, 2016). One of the open questions in mixed culture fermentation is how operational variables affect the composition of the VFA-mixture produced. Some parameters investigated in a continuous stirred tank reactor (CSTR) are the pH, pH_2, and different carbon sources such as glucose or xylose (Temudo, Kleerebezem and van Loosdrecht, 2007; Temudo *et al.*, 2009; De Kok *et al.*, 2013; Rombouts *et al.*, 2019a). To some extent control over the product spectrum exist, though preferably this knowledge should be more elaborate.

Fermentation processes can be carried out using mixed microbial cultures in different reactor configurations. Fermentation research is often done in CSTR configuration however also a configuration as a granular sludge reactor could be chosen. Advantages of granular sludge compared to CSTR are that the hydraulic retention time (HRT) can be separated from the solid retention time (SRT). Separating the HRT from SRT in combination with granules enables high biomass concentrations resulting in high volumetric rates which can be an order of magnitude larger compared to a CSTR operation(Tamis *et al.*, 2015). This feature enables a low solid content in the supernatant as concentrated amount

biomass can be removed from the sludge bed. This is an advantage in the PHA production process as the product (PHA) is a solid. As the supernatant decanted from the fermentation step contains minimal amount of impurities the PHA purity will be minimally affected. However, using granular sludge entangles more operational difficulties as more factors are playing a crucial role such as creating a substrate gradient over time and/or space. Currently, some challenges in these granular systems are the granulation process, prevention of methanogens, a reduced VFA yield due to H_2 production and a lack of control over the produced organic acid composition.

A partnership program between Paques b.v. and NWO-TTW initiated the so-called 'VFA-platform'. The platform was made to better understand the production of VFA from biomass and waste streams. Secondly, the project aimed to gain more knowledge about VFA derived products. The research described in this thesis was part of this project, and adding knowledge to the waste-based production of VFA and PHA.

Outline of this thesis

The scope of this thesis was to valorise industrial wastewater by means of production of bioplastic from industrial wastewater. This thesis is divided over multiple chapters which will be elaborated shortly in this section. Chapter **two** and **three** focusses on the VFA production from glucose in a granular sludge process as pre-treatment process for PHA production. Chapter **two** entitled: *Volatile fatty acid (VFA) product spectrum as a function of the Solids Retention Time (SRT) in an anaerobic granular sludge process*. In this study an anaerobic granular sludge-based sequencing batch process was operated at a pH of 5.5. The effect of the SRT on the VFA production yield was investigated. By increasing the SRT the overall growth rate will be lowered, and the maintenance will be increased, potentially resulting in a higher VFA-yield in the process. Furthermore, secondary conversions that are characterised by a low growth rate – e.g. homoactogenesis - can still be established if the SRT is increased, adding to the VFA-yield of the process as well. Chapter **three** entitled: *Volatile fatty acid production yield maximization using anaerobic granular sludge by ammonium limitation*. The effect ammonium availability has on the product spectrum from a fermentative granular sludge operated at a pH of 5.5 was investigated. Nitrogen is an important building block for microbial life. In this study ammonium was the sole nitrogen source readily available for the enrichment, besides atmospheric elemental nitrogen. Here the response of a culture was monitored over time when the ammonium supply was cut. Upon cutting the ammonium supply the culture had to either adapt and use N_2 or at least one other microorganism would be enriched capable of using N_2. However, the fixation of bioavailable nitrogen

from N_2 costs additional energy and not all microbes contains the machinery to perform this task.

The second part of this thesis focusses on adding knowledge to the waste-based production of PHA. Chapter **four**, *Simultaneous growth and polyhydroxybutyrate (PHB) accumulation in a Plasticicumulans acidivorans dominated enrichment culture*, focusses on mapping the PHA accumulation to investigate to what extent nutrient limitation is required. Wastewaters are not always strictly nutrient limited. The presence of nutrients cannot exclude growth in the accumulation resulting in lower PHA purities as non-PHA solids (bacteria) are produced. Hence, it is important to investigate to what extent nutrient limitation can be mitigated and/or can be used as an advantage instead of a disadvantage. In chapter **five**, *Pilot-scale polyhydroxyalkanoate (PHA) production from organic waste: process characteristics at high pH and high ammonium concentration*, we investigate the possibility to produce PHA from the leachate produced using the organic fraction of municipal solid waste (OFMSW). The feasibility of producing PHA has already been explored on amongst others wastewater from a candy bar factory (Mars,Veghel) and a paper mill factory (ESKA, Hoogezand)(Tamis, Lužkov, Jiang, M. C. M. va. Loosdrecht, *et al.*, 2014; Tamis *et al.*, 2018). These waters are favourable for PHA production as the COD to nutrient ratio is high indicating growth is restricted during the accumulation process. The leachate from the OFMSW is produced from organic waste and growth nutrients just as carbon compounds get leached out of this waste. The result is that growth cannot be excluded during the accumulation which can be disadvantageous for the overall PHA production process. In chapter **six** an outlook is presented discussing, follow-up research which adds to the valorisation of (industrial) wastewater.

2

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 the production of chemicals and polymers. A technology enabling high-rate VFA production from carbohydrate-rich wastewater is the anaerobic granular sludge process. In this study the characteristics of an anaerobic granular sludge process fermenting glucose was evaluated at different solid retention times (SRT). A lab-scale anaerobic sequencing batch reactor fed with 6 $g \cdot L^{-1}$ glucose was operated at a pH of 5.5 and at SRT-values of 1-2, 10-20, and 40-50 d and operated in total for 215 d. A low sludge volume index (SVI) of 11-44 $mL \cdot gTSS^{-1}$ allowed for the high SRT and high volatile suspended solid (VSS) concentration that reached 59 $gVSS \cdot L^{-1}$. This high VSS concentration enabled a glucose consumption rate of 1100 $gCOD \cdot L^{-1} \cdot d^{-1}$ at an SRT of 40-50 d. Two product spectra were obtained: (i) a propionate:acetate mixture with a ratio of 2.05:1 ($mol_{propionate}$:$mol_{acetate}$) 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 $mol_{acetate} \cdot mol_{VFA}^{-1}$). 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 technologically can be achieved. This works contributes to the implementation of waste-based production of VFA using anaerobic granular sludge.

2.1 – Introduction

Anaerobic digestion (AD) is a process in which complex organic substrate is degraded to yield methane containing biogas (Kleerebezem *et al.*, 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 *et al.*, 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 (Holtzapple and Granda, 2009; Agler *et al.*, 2011). VFA serves in this platform as a building block for the production of potentially higher value chemicals and polymers such as the production of polyhydroxyalkanoates (PHA) (Marang *et al.*, 2013; 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 working at low solid retention times (SRT) and/or working at a low pH and 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 (Temudo, Muyzer, Kleerebezem and Van Loosdrecht, 2008; Rombouts *et al.*, 2019a). 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 (Tamis *et al.*, 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, de Kreuk, *et al.*, 2015). Using granular sludge technology the SRT can be uncoupled from the hydraulic retention time (HRT), resulting in high-rate systems (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 carbohydrate-rich wastewaters.

Even though VFA production from organic waste using anaerobic granular sludge has been demonstrated, many research questions remain unanswered (Tamis *et al.*, 2015). 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 of acetate and ethanol, whereas at a lower pH (5-6) an acetate and butyrate mixture will be obtained (Temudo, Kleerebezem and van Loosdrecht, 2007; De Kok *et al.*, 2013). At lower pH-values, carbohydrate fermentations that produce organic acids often generate H_2-gas as a by-product. Even though in some studies H_2 is considered the main product of the fermentation process, H_2 production lowers the overall VFA product yield (Das and Veziroglu, 2008; Hallenbeck and Ghosh, 2009). The produced H_2 contains electrons originating from the substrate and the H_2 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 *et al.*, 2008; Bolaji and 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 of the SRT on the product yield and product spectrum of anaerobic granular sludge fermenting glucose was investigated. Glucose was taken as model substrate for carbohydrate-rich wastewater such as papermill wastewater, food processing wastewater, or sugar cane molasses (Albuquerque *et al.*, 2007; Tamis, Lužkov, Jiang, M. C. M. van Loosdrecht, *et al.*, 2014; Tamis *et al.*, 2018). A sequencing 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 H_2.

2.2 – 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 ºC 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 (Tamis *et al.*, 2015) using a glucose concentration of 10 $g \cdot L^{-1}$ 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 CO_2 and H_2 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 Tamis et al. (2015) was used and the glucose concentration in the influent was 6 $g \cdot L^{-1}$ (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 besides the biomass washout via the effluent sludge, biomass 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 series. The second water-lock was connected to the outside environment and sparged with N_2 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 CO_2, H_2 and CH_4 in-line with a mass spectrometry system (Omnistar, Pfeifer vacuum). Offline measurements were done for SVI, soluble chemical oxygen demand (COD_{Sol}), 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 COD_{Sol} glucose, and organic acid analysis were filtered directly after sampling using a 0.45 μM pore size filter (PVDF membrane, Millipore, Ireland). The COD_{Sol} 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 H_3PO_4 in Milli-Q water was used with a flow rate of 0.6 $mL \cdot min^{-1}$ 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 °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 ($gTSS \cdot d^{-1}$) 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 was biomass.

A mathematical model was made for obtaining the conversions and qS^{max} 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:

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

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

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

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

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

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

- The biomass conversion factor used was 1.42 $gCOD \cdot gVSS^{-1}$ (Rittmann, Crawford and 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 H_2 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°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.

2.3 – 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 CO_2 and H_2 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 2.1 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 2.2. The start-up period served only as inoculum production for the first enrichment and thus day 0 was chosen to be the first day of the SRT research.

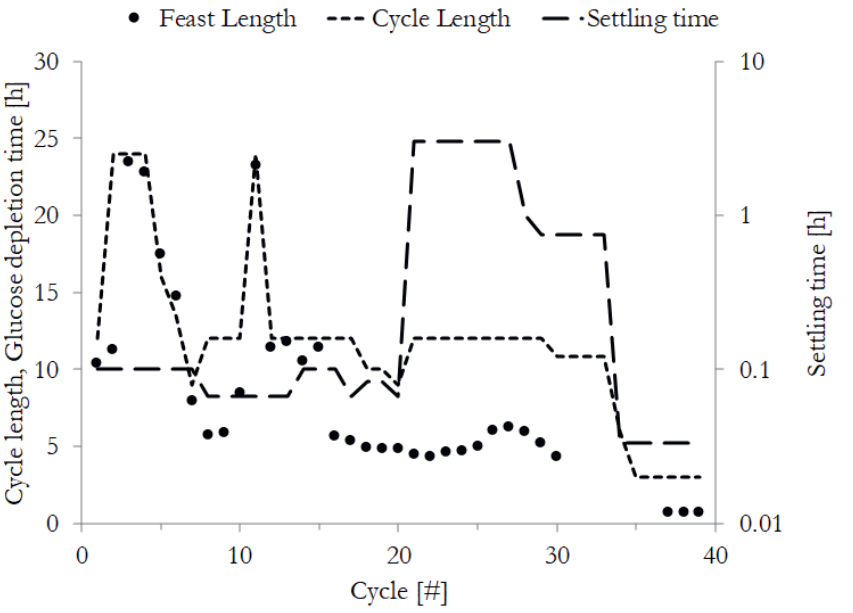

Figure 2.1 – Operational settings chosen, with the measured time needed for glucose depletion during the start-up period for obtaining granules.

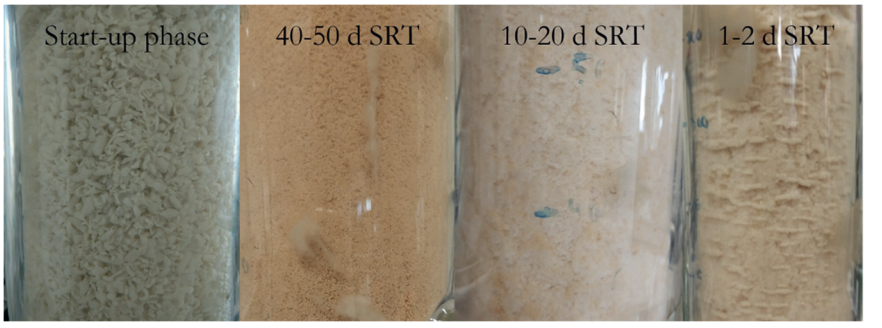

Figure 2.2 – Visual impression of the sludge bed during the different operational periods in this study. From left to right, Start-up phase, 40-50 d SRT, 10-20 d SRT, and 1-2 d SRT.

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 the effluent point of the reactor which was half way the reactor volume 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 H_2 and CO_2 were produced and measured in the biogas. After the start-up period no H_2 was measured in the biogas, the biogas consisted solely of CO_2 (20-40% m/m) and N_2 (60-80% m/m) and no (<0.5%) H_2 or CH_4 were detected. An overview of the culture performance for the 3 SRT is shown in figure 2.3.

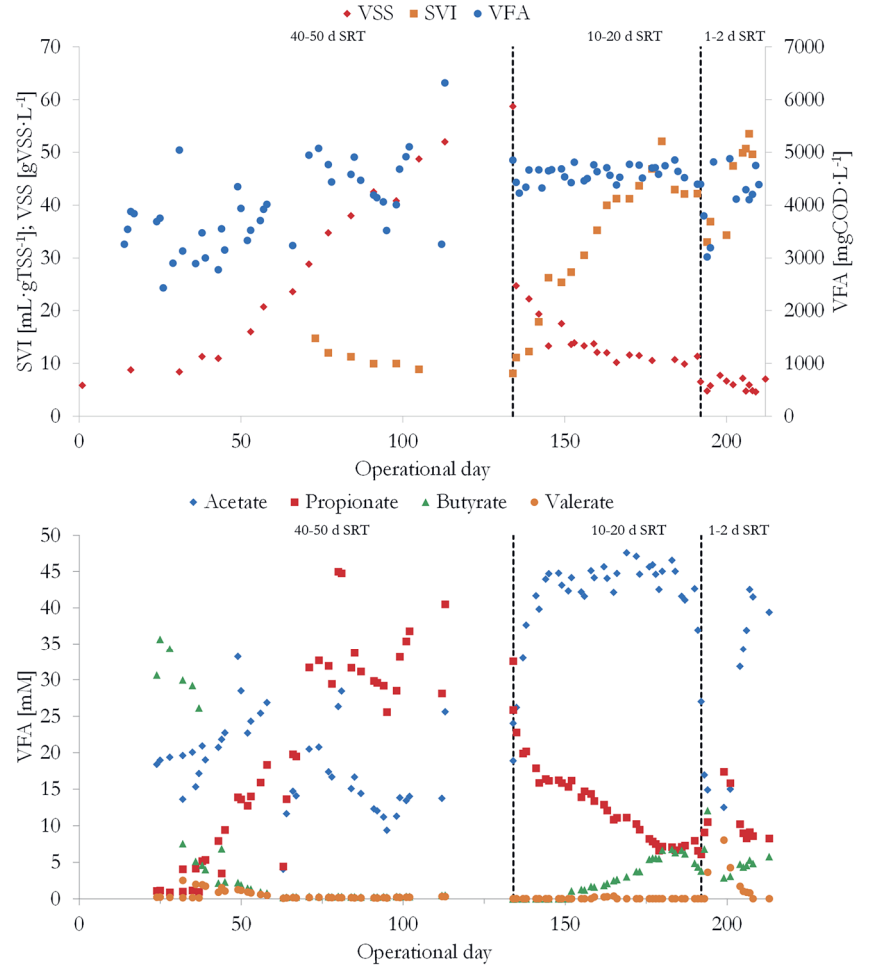

Figure 2.3 – Upper picture is an overview of the reactor performance in terms of VFA, VSS production and the compactness of the culture expressed as SVI60. The bottom picture is the evolution of the product spectrum obtained during each enrichment. Day 0-134 is the 40-50 d SRT, day 135-191 is the 10-20 d SRT and finally day 192-215 is the 1-2 d SRT system.

With increasing the SRT the concentration of biomass in the reactor increased from 6 $gVSS \cdot L^{-1}$ at 1-2 d SRT to 59 $gVSS \cdot L^{-1}$ at 40-50 d SRT. A high SRT could be achieved due to amongst others a very compact sludge bed with an SVI of 11 ± 2 $ml \cdot gTSS^{-1}$ (average ± standard deviation; n=8) and a biomass yield of 0.11 $gCOD_X \cdot gCOD^{-1}$ (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 2.1.

Table 2.1 – Overview system characteristics obtained at different SRT. The $Y_{X/S}$ 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)	$mL \cdot gTSS^{-1}$
$VSS_{effluent}$	0.12 ± 0.04 (n=10)	0.17 ± 0.22 (n=23)	0.23 ± 0.18 (n=67)	$gVSS \cdot L^{-1}$
$Y_{X/S}$	0.17± 0.01 (n=4)	0.14± 0.09 (n=4)	0.11 ± 0.09 (n=4)	$gCOD \cdot gCOD^{-1}$
$Y_{VFA/S}$	0.77 ± 0.06 (n=7)	0.79 ± 0.03 (n= 23)	0.79 ± 0.12 (n=18)	$gCOD \cdot gCOD^{-1}$

In all experimental periods the VSS present in the effluent was low, 0.21 ± 0.18 (n=100) $gVSS \cdot L^{-1}$, compared to the VSS concentrations present in the reactor. The manually removed sludge was 3.1 $gVSS \cdot d^{-1}$, 1.9 $gVSS \cdot d^{-1}$ and 0 $gVSS \cdot d^{-1}$ 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 $gCOD \cdot gCOD^{-1}$. 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 2.3. 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 71-134 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 ($mol_{propionate}$:$mol_{acetate}$). 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) $gCOD_{VFA} \cdot gCOD_{Sol}^{-1}$) (sol = solube). The increasing gap observed in the COD_{Sol} balance during glucose depletion, and subsequent increase of the COD_{Sol} 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 CO_2 partial pressure of 20-40% (m/m) at all SRT. H_2 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 2.4.

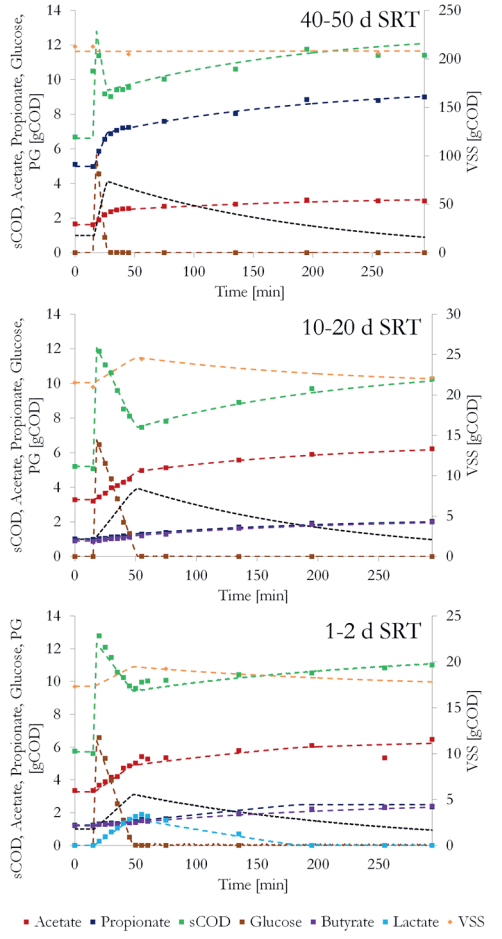

Figure 2.4 – All system characterisations performed in this study. From top to bottom the 40-50 d SRT, 10-20 d SRT and finally the 1-2 d SRT is depicted. Markers reflect measurements and lines are modelled. Soluble COD concentrations (sCOD) were calculated as the sum of the individual substrate and product concentrations. Cycle measurements were performed at the end of each enrichment thus on days: 134, 191 and 212 for respectively 40-50 d, 10-20 d and 1-2 d SRT.

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 2.2.

Table 2.2 – An overview of the obtained of qS^{max}-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	$q_{Glucose}^{max}$ $gCOD \cdot (gVSS \cdot h)^{-1}$	$R_{glucose}$ $gCOD \cdot (L \cdot d)^{-1}$	Y_{glu}^{ace} $gCOD \cdot gCOD^{-1}$	Y_{glu}^{pro} $gCOD \cdot gCOD^{-1}$	Y_{glu}^{but} $gCOD \cdot gCOD^{-1}$	$Y_{glu}^{storage}$ $gCOD \cdot gCOD^{-1}$
1-2	0.7	168	0.43	0.18	0.16	0.31
10-20	0.4	97	0.40	0.14	0.15	0.43
40-50	0.2	1104	0.20	0.57	0	0.47
Tamis et al., (2015) (1-2 d SRT)	1.6	300	0.17	0.03	0.20	0.13-0.19

The amount of stored polymer produced was estimated using the model. The quantity varied per enrichment but was always significant. At an SRT of 40-50 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 $gCOD \cdot COD^{-1}$ respectively at an SRT of 10-20 d and 1-2 d. The maximum specific substrate uptake rate (q_S^{max}) decreased from 0.7 $gCOD \cdot gVSS^{-1} \cdot h^{-1}$ at 1-2 d SRT to 0.2 $gCOD \cdot gVSS^{-1} \cdot h^{-1}$ at an SRT of 40-50 d.

Microbial community structure

During the intial granulation process 16S rRNA showed that the mixed microbial community was dominated by *Clostridium pasteurianum*. An overview of the relative abundance at the start of the SRT research and the end of each SRT period is given in figure 2.5.

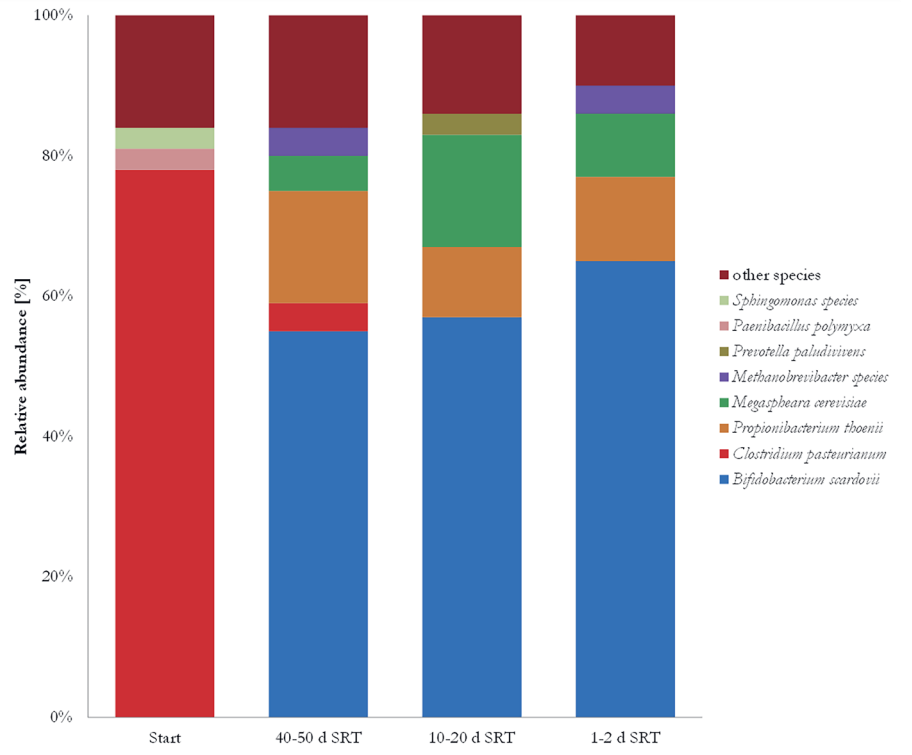

Figure 2.5 – Relative abundance of species detected at the end of each enrichment. Species that were relatively 3% or higher present were taken into account the other species were clustered as 'other species'. Start is the beginning of the SRT-research, day 134, 191 and 212 are respectively the last days of the 40-50 d, 10-20 d and 1-2 d SRT periods.

When the SRT was prolonged to 40-50 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 was obtained, 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) and the *Saccharibacteria* was no longer significantly detected.

2.4 – 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®' process and the upflow anaerobic sludge blanket (UASB) reactor (Lettinga *et al.*, 1980; Pronk, de Kreuk, *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 and van Loosdrecht, 2004). In this work the substrate was added pulse-wise 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 $mL \cdot gTSS^{-1}$ could be achieved, amounting to a very dense sludge bed. At shorter SRT the SVI was higher and was around 34-44 $mL \cdot gTSS^{-1}$. The SVI for previously operated granular systems varied though were in the same order of magnitude; 17-29 $mL \cdot gVSS^{-1}$ for granular acidogenic systems (Tamis *et al.*, 2015) and 12-45 $mL \cdot gTSS^{-1}$ have been reported for aerobic granular sludge in lab and full-scale situation (De Kreuk, Pronk and Van Loosdrecht, 2005; Pronk, de Kreuk, *et al.*, 2015). In UASB reactors the SVI can go as low as 7.8 $mL \cdot gVSS^{-1}$ (Grotenhuis *et al.*, 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 SBR fed pulse-wise with glucose and operated at a pH of 8.0 and a SRT of 8 h (Rombouts *et al.*, 2019a). 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 qS^{max}. 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 microorganisms over the depth of the biofilm are not doing all processes simultaneously. These reasons would result in the underestimation of qS^{max} in a granular system compared to a suspended cell system. The qS^{max} 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}(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 lower q_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 40-50 d a product spectrum consisting of propionate and acetate reached an

overall VFA yield of 77% ($gCOD \cdot gCOD_{glucose}^{-1}$) 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 (Tamis *et al.*, 2015). The difference between the studies are the ratio in which the main fermentation products were obtained and possible H_2 production as shown in table 2.2. In this study more acetate was produced compared to butyrate which was the main fermentation product in (Tamis *et al.*, 2015). The COD-balances as measured in the cycle measurements were 0.94, 0.92, 0.88 $gCOD \cdot gCOD_{glucose}^{-1}$ for respectiveley 1-2 d, 10-20 d and 40-50 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 $gCOD_{Pro} \cdot gCOD_{glucose}$ and 0.14-0.18 $gCOD_{Pro} \cdot gCOD_{glucose}$ 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 ($gCOD \cdot gCOD^{-1}$) 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 $gCOD \cdot gCOD^{-1}$. 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 of yet associated with *Bifidobacterium* (Pokusaeva, Fitzgerald and Van Sinderen, 2011; Falsen *et al.*, 2015; Ventura *et al.*, 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 and 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 (Paikt and Glatz, 1997; Lanjekar *et al.*, 2014). 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 ($mol_{propionate}$:$mol_{acetate}$) 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 ($mol_{propionate}$:$mol_{acetate}$) 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 by 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 N_2. *C. pasteurianum* produced a product spectrum dominated by acetate, butyrate and hydrogen as several studies have reported before for the *Clostridium* genus (Crabbendam *et al.*, 1985; Dabrock, Bahl and Gottschalk, 1992; Lin *et al.*, 2007; 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 PHA that can be separated from the water phase (Tamis, Lužkov, Jiang, M. C. M. van Loosdrecht, *et al.*, 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.

2.6 – 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 and operated for 215 d. Two distinct product spectra were obtained (i) at 40-50 d SRT a propionate:acetate mixture of 2.05:1 ($mol_{propionate}$:$mol_{acetate}$) was obtained; with a VFA production yield of 0.79 ± 0.12 (n=18) $gCOD \cdot gCOD^{-1}$. (ii) At 1-2 d and 10-20 d SRT an acetate dominated, 0.71-0.75 $mol_{acetate} \cdot mol_{VFA}^{-1}$, product spectrum was obtained. Overall, high VFA yields of 0.77-0.79 $gCOD \cdot gCOD^{-1}$ from glucose fermentations were obtained. Furthermore, compact sludge beds were obtained as SVI60 were ranging within 11-44 $mL \cdot gTSS^{-1}$, and *Bifidobacterium scardovii* was the prevailent microorganism in all systems. Substrate specific uptake rates varied from 0.2-0.7 $gCOD \cdot gVSS^{-1} \cdot h^{-1}$. Despite the relatively low qS^{max}, the glucose consumption rate of the systems varied from 100 $gCOD \cdot L^{-1} \cdot d^{-1}$ to a maximum rate of 1100 $gCOD \cdot L^{-1} \cdot d^{-1}$. 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.

2.7 – Appendix

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 (Tamis *et al.*, 2015) and shown in table 2.3. The reaction stoichiometry for reaction (i) and (ii) are given in table 2.3.

Table 2.3 – The reaction stoichiometries used to calibrate the model to the measured data

	rGlu	rSP	rLac
Glucose	-1		
Acetate	$Y_{Ac/Glu}$	$Y_{Ac/SP}$	0.32
Propionate	$Y_{Pro/Glu}$	$Y_{Pro/SP}$	0.63
Butyrate	$Y_{But/Glu}$	$Y_{But/SP}$	0
Lactate	$Y_{Lac/Glu}$	$Y_{Lac/SP}$	-1
SP	$Y_{SP/Glu}$	-1	0
Biomass	$Y_{X/Glu}$	$Y_{X/SP}$	0.06

Kinetics

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

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

- C_j = concentration of compound J [$gCOD \cdot L^{-1}$]
- q_j = the subsrate specific uptake rate for compound j [$gCOD_j \cdot gVSS^{-1} \cdot h^{-1}$]
- X_{lumped} = 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 2.4. 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 2.4 – The kinetic equations used in the study to calibrate the measured data. $K_{s,Glu}$ is the half-saturation constant for glucose, $K_{s,lac}$ is the half-saturation constant for Lactate

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

Model calibration

The difference of each measured sampling point (t_i)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.

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

- j= glucose, aceate, propionate, butyrate, lactate, SP, biomass
- i= time corresponding to each measured data point
- $n_j^{measured}$ = the amount of compound j measured [gCOD]
- n_j^{model} = the modelled amount of compound j [gCOD]

Subsequently the SSE of each component was summed 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):

$$Total\ error = \sum SSE_j \quad (A-3)$$

The total error was minimized adjusting characteristic parameters e.g., qS^{max}, K_{SP}, K_{Lac}, K_{Glu} and the yields shown in table 2.3, 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 initial yields used were 0.5 $gCOD \cdot gCOD^{-1}$, K_{SP} was initially 0.01 min^{-1} and an initial qS^{max} of 1 $gCOD \cdot gVSS^{-1} \cdot h^{-1}$.

3

Volatile fatty acid production yield maximization using anaerobic granular sludge by ammonium limitation

Abstract

Volatile fatty acids (VFA) are building blocks for a variety of (bio)based products such as bioplastics and medium chain length fatty acids. Additionally, VFA are key intermediates in the anaerobic digestion (AD) process. These intermediates can be produced using the principles of the AD process in combination with prevention of the methanogenesis process responsible for the consumption of VFA. In this study the effect of ammonium deprivation was investigated on an anaerobic granular sludge enrichment producing VFA from carbohydrates. A lab-scale reactor was operated for a period 95 d resulting in compact granules with a sludge volume index (SVI) of 33-43 mL·gTSS^{-1} after 2 minutes of settling. The reactor was fed with 10 g·L^{-1} glucose and glucose consumption rates of 125-165 gCOD·L^{-1}·d^{-1} were observed. The main fermentation products for all enrichments were butyrate and acetate (1.3-1.5 $mol_{Butyrate}$:$mol_{Acetate}$). Lowering the ammonium supply resulted in a decrease in the nitrogen content of the volatile suspended solids (from 0.10 to 0.05 gN·gVSS^{-1} upon ammonium deprivation). The VFA product yield remained relatively constant (0.61 to 0.64 $gCOD_{VFA}$·$gCOD_{glucose}^{-1}$) regardless of the amount of ammonium supplied to the enrichment. This work demonstrates that despite ammonium limitation a solid-free effluent rich in VFA can be produced efficiently using the anaerobic granular sludge process.

3.1 – Introduction

Organic waste streams generated by industries are valorised using the anaerobic digestion process, a technology which has been researched extensively (Batstone *et al.*, 2002). During this process organic compounds are hydrolysed and fermented yielding primarily volatile fatty acids (VFA). These VFA are subsequentally converted to methane containing biogas by syntrophic communities of acetogens and methanogens. Herewith VFA are central intermediates in the anaerobic digestion process. These VFA can serve as building blocks for a wide variety of products in the carboxylate platform with a potential higher value than methane containing biogas (Agler *et al.*, 2011). In order to produce VFA from an e.g. carbohydrate-rich wastewater, methanogenesis should be halted, otherwise the produced VFA will be converted to methane containing biogas. The methanogenesis process can be halted by operating at lower pH with a high VFA concentration and/or operating at short solid retention times (SRT) (Kleerebezem *et al.*, 2015).

For the production of VFA from carbohydrate-rich wastewaters open mixed cultures can be utilized. Frequently, in the lab a continuous stirred tank reactor (CSTR) operation is used to study fermentation processes (Temudo, Kleerebezem and van Loosdrecht, 2007; De Kok *et al.*, 2013; Rombouts *et al.*, 2019b). A possible alternative could be the usage of anaerobic granular sludge

technology. A typical characteristic of this technology is that the hydraulic retention time (HRT) is uncoupled from the solid retention time (SRT) enabling higher solid concentrations. Some advantanges of using anaerobic granular sludge are the higher volumetric productivities that can be achieved due to the higher biomass concentrations, and low effluent solid concentrations due to discharge of concentrated biomass from the sludge bed (Tamis *et al.*, 2015). However, the potential of using anaerobic granular sludge for VFA production is just touched upon and currently there is limited control over these type of systems in terms of the product spectrum that can be obtained, the product yield that can be established, and the conditions required to achieve adequate granulation. Furthermore, no clear operational procedure exists linking a specific product spectrum to reactor operation, though parameters such as the pH, or carbon source were shown to play a key role in this process (Temudo, Kleerebezem and van Loosdrecht, 2007; Temudo, Muyzer, Kleerebezem and van Loosdrecht, 2008). Overall, the application of anaerobic granular sludge shows promising properties for high-rate VFA production, but more understanding of the process is desired.

During carbohydrate fermentation to VFA influent COD gets partitioned over (i) biomass production (ii) VFA production and (iii) H_2/CO_2 production. This work aims for maximization of the VFA production yield by minimizing the biomass production. To achieve a decrease in biomass yield on substrate, in this work the amount of ammonium dosed to the system is step-wise decreased to the point that no ammonium is dosed. In the case of ammonium deprivation, the enrichment can only maintain viability by dinitrogen fixation.

3.2 – Materials and methods

Fed batch set-up

Three enrichments were performed using one bioreactor. The enrichments were classified as 100% NH_4-N, 50% NH_4-N and 0% NH_4-N based upon the amount of ammonium dosed. In the 100% NH_4-N enrichment ammonium was present in excess, in the 50% NH_4-N enrichment the ammonium required for growth was halved based on the ammonium requirements in the 100% NH_4-N system. Finally, in the 0% NH_4-N enrichment no ammonium was supplied. The enrichments were carried out in a bioreactor with a working volume of 2.5 L and a headspace of 2.4 L. The inoculum was similar as in (Mulders *et al.*, 2020). The reactor was maintained at 30 ºC using a water jacket around the reactor 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 at a pase 4 times slower than the base addition. The SRT of the reactor for the 100% NH_4-N reactor was maintained at 1-2 d SRT by manual removal of solids from the mixed reactor broth. The SRT was uncontrolled for the 50% and 0% NH_4-N

enrichments, the additional biomass produced left the reactor via the effluent. The HRT of the reactor was 6 hours with a cycle length of 3 hours. For each following enrichment the previous enrichment served as inoculum. Granulation was achieved as described in (Mulders *et al.*, 2020). There it was described that it was critical to retain initially all biomass by using a long settling time of 3 h. The glucose concentration in the feed was 10 $g \cdot L^{-1}$ and the nutrient medium was identical as described in (Tamis *et al.*, 2015). Anaerobic conditions were ensured by continuous input of N_2 gas at a flow rate of 400 $mL \cdot min^{-1}$, mixing in the reactor was performed using gas recirculation at a rate of 2 $L \cdot min^{-1}$. A cycle consisted out of the following phases: Settling phase (2 min), effluent phase (10 min), nutrient feeding phase (5 min), acclimization phase (10 min), glucose feeding phase (3 min) and finally a reaction phase (150 min).

During the characterisation of the enrichment in which ammonium was in excess, a ratio of ammonium consumed per glucose fed of 6.5 (mol glucose: mol NH_4-N) was obtained. This ratio was used in the subsequent reactor operations: for the 50% NH_4-N system the amount of glucose dosed remained constant and the ammonium supplied was lowered obtaining a glucose:ammonium ratio of 13 (mol glucose: mol NH_4-N). Finally, in the 0% NH_4-N system no external ammonium was added to the system while the glucose dosage remained similar to the previous enrichments.

Sampling and analytical methods

The pH and base addition of the bioreactor was monitored online. The biogas produced was measured and quantified for N_2, CO_2, H_2, CH_4 and O_2 using a mass spectrometer (MS) (PRIMA BT Benchtop, Thermo Scientific, UK). The following offline biomass characterisation measurements were conducted: sludge volume index (SVI), total suspended solids concentration ($gTSS \cdot L^{-1}$), volatile suspended solids concentration ($gVSS \cdot L^{-1}$), N-content of VSS ($gN \cdot gVSS^{-1}$), and 16S rRNA. The 16S rRNA quantification was done as described in (Mulders *et al.*, 2020). Samples for TSS were taken from a mixed reactor. Directly after sampling the samples were centrifuged at 2500 g for 20 min and after decanting the supernatant the samples were oven dried at 105 °C. The VSS was obtained according to (Clesceri, Greenberg and Eaton, 1999). Samples taken for organic acids and glucose concentrations were filtered directly after sampling using a 0.45 μm pore size filter (PVDF membrane, Millipore, Ireland). The composition and quantity of the organic acids and glucose concentrations were determined using high-performance liquid chromatography (HPLC) with a BioRadAminex HPX-87H column and an UV/RI detector (Waters 2489). As a mobile phase 1.5 mM H_3PO_4 in Milli-Q water was used with a flow rate of 0.6 $mL \cdot min^{-1}$ and a temperature of 60°C. The SVI was determined by taking the apparent SV in the reactor at the end of the settling

phase and dividing this number by the TSS concentration. The N-content of the TSS was determined as follows: A mixed reactor sample was sonicated approximately for 5 minutes until the sample appeared homogeneous. Next, the N-content of the sample was determined using a commercially available test-kit from Hach-Lange. Next to this sample an extra sample was taken for TSS determination.

Once steady-state was reached in an enrichment, the enrichment was characterised performing a sample campaign. Steady-state was reached when the system was operated for at least 5 SRT under constant reactor operation and a relatively constant product spectrum was obtained.

Data analysis

A chemical oxygen demand (COD) balance over the system was made using all liquid products produced and consumed. Furthermore, the produced VSS was converted to COD equivalents assuming 1.42 $gCOD \cdot gVSS^{-1}$ (Rittmann, Crawford and Tuck, 1986). Lastly, the produced H_2 was quantified and included in the COD balance. The SRT of the system was quantified by the amount of solids removed from the system compared to the total amount of solids present in the reactor. This included both solids removed via the effluent at the end of a cycle and the solids removed manually from the reactor (only in the 100% NH_4-N enrichment solids were removed manually).

A mathematical model was calibrated to the obtained data from a sample campaign. This model was based upon two/three reactions, as described in (Mulders *et al.*, 2020) and shown below with the addition of H_2 production.

1. $\text{Glucose} \rightarrow \text{a Acetate} + \text{b Lactate} + \text{c Propionate} + \text{d Butyrate} + \text{e Biomass} + \text{f Storage polymer} + \text{g } H_2\ [gCOD \cdot gCOD]^{-1}$
2. $\text{Storage polymer} \rightarrow \text{h Acetate} + \text{i Lactate} + \text{j Propionate} + \text{k Butyrate} + \text{l Biomass} + \text{m } H_2\ [gCOD \cdot gCOD]^{-1}$
3. $\text{Lactate} \rightarrow 0.21\ \text{acetate} + 0.73\ \text{propionate} + 0.06\ \text{VSS}\ [gCOD \cdot gCOD]^{-1}$ (Tamis *et al.*, 2015)

Similar assumptions were made listed below:

- All VSS was assumed to be catalytic biomass corresponding to 1.42 $gCOD \cdot gVSS^{-1}$ (Rittmann, Crawford and Tuck, 1986)
- The amount of storage polymer was set at 1 gCOD at the start of the cycle and was designed to be 1 ± 0.05 gCOD at the end of the cycle
- No restriction was made on the COD-balanced to be closed. This was done for the identification of missing products

3.3 – Results

Reactor performance

The same inoculum as described in (Mulders *et al.*, 2020) was used for start-up of the process. The inoculum was a *Clostridium pasteurianum* dominated granular sludge culture. During the start-up and first enrichment ammonium was present in excess. The granules in all periods were creamy-white cloud-like shaped. The granules were stably maintained in the period with ammonium excess and the VSS concentration was successfully maintained around 10 $gVSS \cdot L^{-1}$. When a steady performance was observed an operational cycle was characterised as shown in the product and kinetics section. After the cycle characterisation the ammonium supply was lowered to 50% of what previously was assimilated on average per cycle as described in the material and methods section.

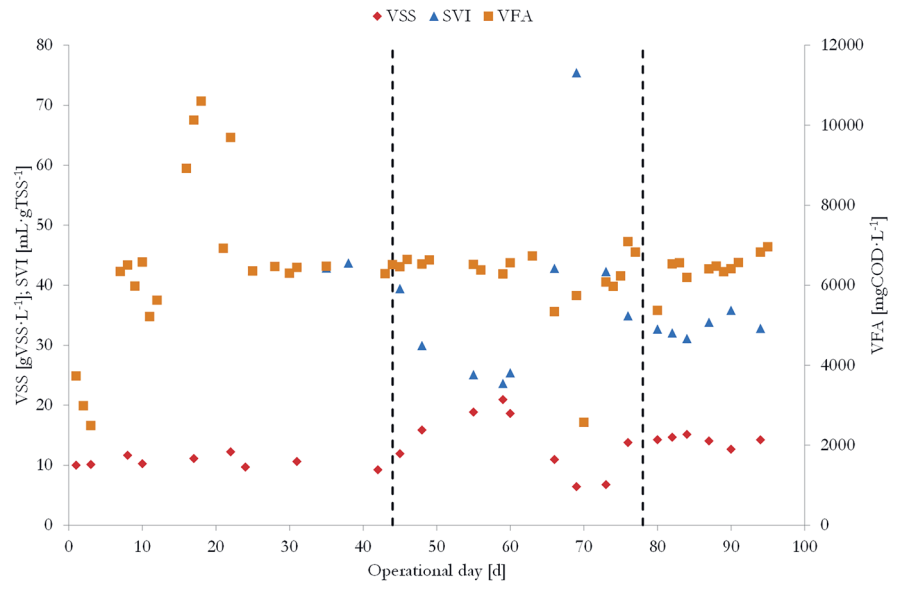

Figure 3.1 – Overview of the 3 enrichments, the 100% NH_4-N enrichment ran from day 0-44, the 50% NH_4-N enrichment ran from day 45-77 and finally the 0% NH_4-N enrichment ran from day 78-95.

When the ammonium dosage was lowered, the extracellular ammonium was usually depleted within the first 30 minutes after substrate supply. In the 50% NH_4-N period and onwards no biomass was removed from the sludge bed to achieve maximum productivity. Quickly after starting the 50% NH_4-N enrichment the integrity of the granules was lost. The settling time and cycle length were temporary increased to subsequently 30 minutes and 4 hours from day 48 to 60. The biomass concentration increased and SVI decreased as a consequence shown in figure 3.1. After 12 d of adaptation the settling time and cycle length were set at the original values (2 min settling time and cycle length of 3 h) in order to wash-out suspended biomass and select specifically for rapidly settling granules. In figure 3.1 around day 60 it can be seen that lowering the settling time induced biomass wash-out. After reaching a minimum biomass concentration of approximately 5 $gVSS \cdot L^{-1}$, granules formation was observed and the biomass concentration increased to values around 10 $gVSS \cdot L^{-1}$. From day 70 onwards granules were observed once again. The compactness of the cultures remained similar with lowering the bioavailable ammonium as the SVI2 went from 43 ± 1 $mL \cdot gTSS^{-1}$ (average ± standard deviation; n = 2) to 39 ± 5 $mL \cdot gTSS^{-1}$ (n=2).

In the last enrichment (day 78-95) no ammonium was supplied to the reactor. During this transition period no loss of granular integrity was observed and the SVI was 33 ± 1.6 $mL \cdot gTSS^{-1}$ (n=6). Overall, a stable system was observed though the VFA yield increased some at the end of the enrichment to 0.69 $gCOD \cdot gCOD^{-1}$. Finally, the overall VFA yield was comparable over all 3 enrichments and was in the range of 0.60-0.65 $gCOD \cdot gCOD^{-1}$. A summary of the most important key system characteristics of all enrichments is shown in table 3.1.

Table 3.1 – Key characteristics off the three enrichments performed. The biomass yield (Y_X) was obtained using all VSS manual removed together with the averaged VSS present in the effluent.

System	N-content	SVI	VSS effluent	Yx	Y_{VFA}
	$gN \cdot gVSS^{-1}$	$mL \cdot gTSS^{-1}$	$gVSS \cdot L^{-1}$	$gCOD \cdot gCOD^{-1}$	$gCOD \cdot gCOD^{-1}$
100% NH_4-N	0.10 (Tamis *et al.*, 2015)	43 ± 0.6 (n=2)	0.73 ± 0.2 (n=24)	0.12	0.63 ± 0.04 (n= 14)
50% NH_4-N	0.051 ± 0.06 (n=11)	38 ± 16 (n=9)	0.67 ± 0.49 (n=15)	0.12	0.61 ± 0.10 (n= 15)
0% NH_4-N	0.048 (n=1)	33 ± 2 (n=6)	1.07 ± 0.33 (n=8)	0.13	0.64 ± 0.03 (n=10)

Product spectrum and kinetics

The VFA composition varied for a short period gradually upon entering the 50% NH_4-N period, before reaching steady state. At the start of the 50% NH_4-N enrichment the product spectrum contained significant amounts of propionate (0.18-0.26 $mol_{pro} \cdot mol_{VFA}^{-1}$). When the system matured this fraction of propionate lowered and an acetate/butyrate dominated system was obtained.

The kinetics and conversions occuring in a cycle were characterised for each enrichment. The cycles were characterised at the end of each enrichment. The obtained data served as basis for a mathematical model describing the conversions and rates as described in the material and methods section. An overview of all the cycle characterisations is shown in figure 3.2.

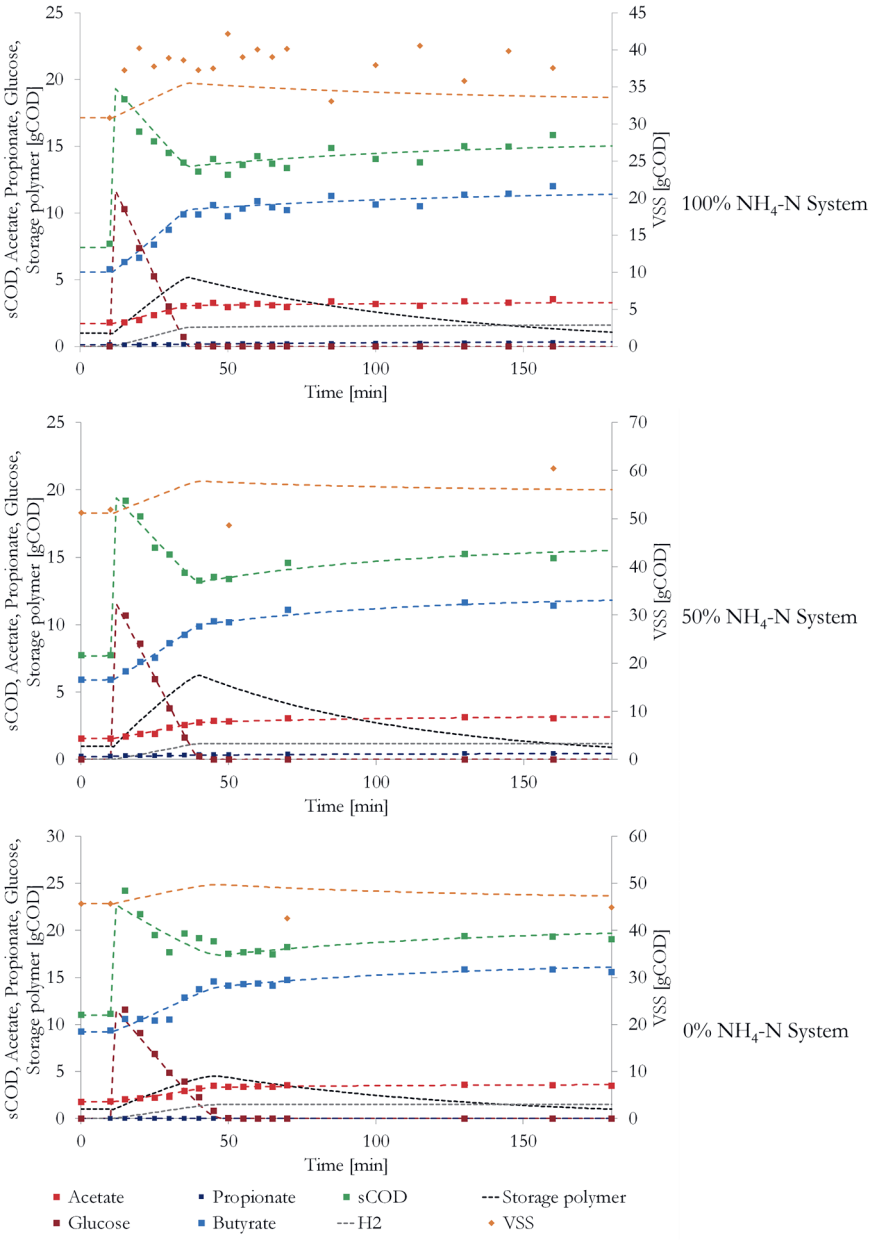

Figure 3.2 – 100% NH_4-N day 43, 50% NH_4-N day 77 and 0% NH_4-N day 95. All COD-balances closed within an accuracy of 1 ± 0.03 gCOD·gCOD^{-1}.

All systems obtained a similar product spectrum during the cycle measurements. In general acetate, butyrate, hydrogen and VSS were the main fermentation

products found. In the 100% and 50% NH_4-N systems a small fraction 0.02 $gCOD \cdot gCOD^{-1}$ was propionate, and in the 0% NH_4-N system no propionate was detected. In contrast to propionate, the fraction butyrate increased from 0.49 to 0.59 $gCOD \cdot gCOD^{-1}$ when lowering the ammonium supply. The main soluble products were identified as the soluble COD consisted mainly out of VFA (0.95 ± 0.06 (n=22) $gCOD_{VFA} \cdot gCOD_{Sol}^{-1}$). Finally, the amount of H_2 produced per glucose consumed was relatively constant 0.10-0.14 $gCOD \cdot gCOD^{-1}$ regardless of the presence of ammonium. A summary of the product spectrum obtained during the cycle characterisations is shown in table 3.2. From the models the lumped biomass maximum specific uptake rates (qS^{max}) could be identified and expressed per gVSS. On average the volumetric loading rate was around 40 $gCOD \cdot L^{-1} \cdot d^{-1}$. However, a cycle included periods in which glucose was not present, excluding these periods an actual glucose consumption rate in the range of 125-165 $gCOD \cdot L^{-1} \cdot d^{-1}$ was obtained.

Table 3.2 – The substrate uptake rates and product spectra obtained using a mathetical model for each different enrichment. The storage polymer was the estimated amount of storage polymer transiently produced.

System	qS^{max} Glucose $gCOD \cdot gVSS^{-1} \cdot h^{-1}$	Acetate $gCOD \cdot gCOD^{-1}$	Propionate $gCOD \cdot gCOD^{-1}$	Butyrate $gCOD \cdot gCOD^{-1}$	H_2 $gCOD \cdot gCOD^{-1}$	Storage polymer $gCOD \cdot gCOD^{-1}$
100% NH_4-N	0.66	0.13	0.02	0.49	0.13	0.42
50% NH_4-N	0.35	0.14	0.02	0.52	0.10	0.57
0% NH_4-N	0.35	0.16	0	0.58	0.13	0.40

After all glucose was consumed there was a gap in the COD balance, which over time decreased indicating the production of a transient storage polymer (SP). The amount of storage polymer was estimated from the fermentation products made in the secondary fermentation. The model showed that in all systems around 0.40-0.57 $gCOD_{SP} \cdot gCOD_{Glu}^{-1}$ was produced. The cycle characterisations elucidated that the lion share of the fermentation products were formed during the presence of glucose and it seemed that the biomass was mainly produced in the famine period. The model prediction for the amount of VSS present over time does not correspond to the measured VSS values. Measuring the amount of VSS during a cycle rendered difficult as a cycle was only 3 h and the SRT was around 2-4 d, thus the production of catalytic VSS was low per cycle.

Microbial community structure

In all 3 enrichments the microbial community structure was analysed using 16S rRNA sequencing. In all 3 enrichments the relative abundance, operational taxonomic unit (OTU), of *Clostridium pasteurianum* was 54%, 79% and 68% respectively for the 100%, 50% and 0% NH_4-N. During the development of the enrichment several other species were detected such as *Bifidobacterium scardovii*, *Ethanoligens species* at lower abundances (below 15% during steady operation). An impression of the granular sludge of the ammonium deprived culture under the light-microscope is shown in figure 3.4.

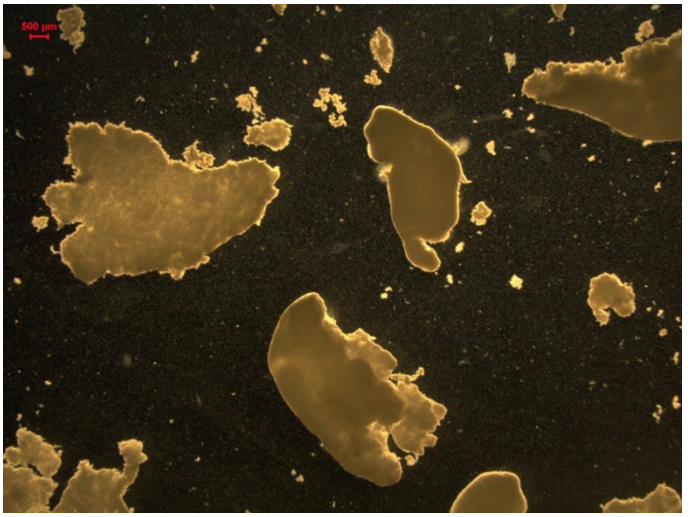

Figure 3.4 – An impression of the granules obtained during the period in which no ammonium was supplied to the reactor (0% NH_4-N enrichment).

3.4 – Discussion

Biomass production under ammonium limited conditions

The objective of limiting the ammonium supply was to stimulate the production of VFA through minimizing the biomass yield on substrate. In ammonium limiting conditions one may speculate that the production of storage polymers provides a competitive advantage using feast-famine conditions. Since organic carbon uptake can be uncoupled from nitrogen fixation and growth which can limit the kinetics of the process. The enrichment had a biomass yield observed in the 100% NH_4-N system of 0.12 $gCOD_X \cdot gCOD_{glucose}^{-1}$ (X= biomass). In this study the VFA yield on glucose was relatively constant, thus a similar amount of catabolic energy can be spent per cycle in each enrichment. Some of this energy expectedly had to be invested in biomass production in order to remain a viable enrichment. Under ammonium deprived conditions another part of this

catabolic energy has to be spent in the fixation of nitrogen. Likely, the SRT of the system will increase as not the equivalent amount of energy can be dedicated to biomass production per the same amount of time. Nonetheless, the biomass production yield in this study measured as VSS was relatively constant 0.12-0.13 $gCOD_X \cdot gCOD_{glucose}^{-1}$. Part of this VSS is not necessarily catalytic biomass, but likely part is polymer. This similar yield throughout all enrichments was not expected as extra energy was required for the fixation of nitrogen. A rough estimate of the expected biomass production is given in figure 3.5, assuming a similar amount of catabolic energy is produced per cycle.

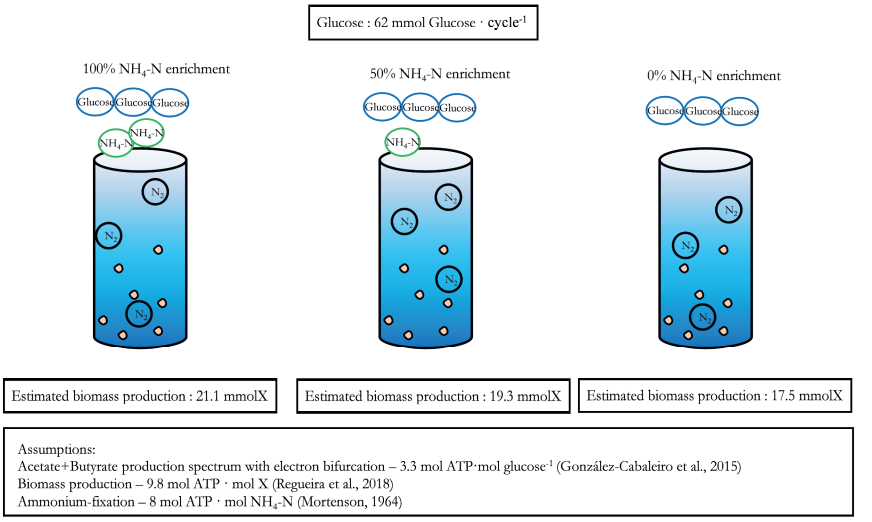

Figure 3.5 – Estimated amount of biomass produced using fixed amount of energy (ATP) requirements for production of biomass and consumption of glucose (Mortenson, 1964; González-Cabaleiro, Lema and Rodríguez, 2015; Regueira *et al.*, 2018).

In the ammonium limited systems the $gN \cdot gVSS^{-1}$ was 0.05 this is lower than the 'conventional' 0.10 $gN \cdot gVSS^{-1}$ present in $C_1H_{1.8}O_{0.5}N_{0.2}$ (Rittmann, Crawford and Tuck, 1986). In a different study comparable to the current study in which glucose was fermented using granular sludge 0.10 $gN \cdot gVSS^{-1}$ was found at ammonium excess conditions (Tamis *et al.*, 2015). In the current study a lower N-content in the VSS was present, thus potentially the catalytic VSS (active biomass) production was lowered. Additionally, it could be that nitrogen-poor EPS (polysaccharides) was overproduced under ammonium limited conditions, which again lowers the N-content expressed as $gN \cdot gVSS^{-1}$. Thus, with the same VSS production yield and a lower N-content likely the catalytic VSS production yield was indeed lower when ammonium was no longer in excess. This is supported by the roughly 50% lower qS^{max} found for the 50% and 0% NH_4-N systems 0.7 vs 0.35-0.4 $gCOD \cdot gVSS^{-1} \cdot h^{-1}$. Overall, the following 3 observations

indicated that the actual yield of catalytic VSS on substrate was decreased upon lowering the ammonium supply: (i) the same microorganism was dominant in all three enrichments, (ii) the $gN \cdot gVSS^{-1}$ was lowered and (iii) the qS^{max} was lowered proportionally to the N-content in the VS at lowering ammonium supply. These results suggest that enriching granular sludge nutrient limited/deprived could be an interesting approach for (over)production of EPS.

Product spectrum and VFA yield

In the presented work the product spectrum in all 3 enrichments was dominated by butyrate, acetate and H_2. In all 3 systems the COD-balances were almost closed as 0.90-0.93 of the COD could be identified on average. Overall, by removal of ammonium from the system the observed yields for VFA and VSS production did not change significantly.

In a previous study investigating the effect of pH (4.5-5.5) using anaerobic granular sludge fermenting glucose a small portion of propionate (±0.05 $gCOD \cdot gCOD^{-1}$) was detected (Tamis *et al.*, 2015). In this work a propionate fraction of 0.02 $gCOD \cdot gCOD^{-1}$ was detected at excess ammonium and at pH 5.5. The production of propionate remained comparable upon reducing the supply of ammonium to the enrichment. Finally, upon complete removal of external ammonium no more propionate was detected. Perhaps microorganisms responsible for propionate production were washed-out because they were not able to fixate nitrogen.

The effect of nitrogen limitation on the fermentation characteristics has previously been studied in continuous stirred tank reactors (CSTR) (Smith and Holtzapple, 2011; Rughoonundun, Mohee and Holtzapple, 2012). Fermentations operated under varying C/N ratios yielded different results compared to the results obtained in this study. Smith and Holtzapple, (2011) found that C/N ratios (g non acid g_{NA}/gN) below 20 favored acetic acid production. Contrary, in the current study no relationship of the C/N ratio and product spectrum was found. The acetate/butyrate ratio was around 0.65-0.78 $mol_{ace} \cdot mol_{but}^{-1}$ obtained from the cycle measurements. Tamis et al., (2015) found an acetate/butyrate ratio of 0.7-0.8 $mol_{ace} \cdot mol_{but}^{-1}$ these ratios were similar to the ratios obtained in this study. Pure cultures chemostat studies using *Clostridium pasteurianum* LMG 3285 obtained $mol_{ace} \cdot mol_{but}^{-1}$ ratios between 0.56-0.80 $mol_{ace} \cdot mol_{but}^{-1}$. The chemostats were operated near pH 5.5, glucose served as carbon source and the chemostat was either nitrogen or phosphorus limited. In general, the obtained product spectrums in this study were comparable to previous found product spectra for *C. pasteurianum* operated around pH 5.5. Additionally, the product spectrum seemed independent of ammonium availability.

Another important fermentation characteristic is the VFA production yield. Previous studies have found a general optimal C/N ratio of 20-40 gC/gN for obtaining the highest VFA yield in batch processes (Liu *et al.*, 2008; Smith and Holtzapple, 2011). In this study the VFA product yield seemed to be independent of the C/N ratio as in all three enrichments a relatively constant VFA yield was observed. Though at the of 0% NH_4-N enrichment the yield seemed higher and around 0.7 $gCOD \cdot gCOD^{-1}$. In this study it seemed that the biomass to EPS ratio changed as the ammonium availability varied. A different mode of operation potentially explains the difference. In this study a carbon/ammonium limited feast-famine regime was used to produce VFA while in the other studies a batch operation was applied. One difference between these systems is the selection criteria. In a feast-famine operated system the selection depends on the substrate uptake rate versus the growth rate in a batch process. Perhaps by circumventing the immediate necessity of growth by producing first storage polymers the VFA production yield was not affected by nitrogen deprivation. Generally, the mode of operation is an important designer choice to investigate the effect of parameters as well the interpretation of their outcomes.

Industrial application

This study investigated the effect of ammonium deprivation on the performance of anaerobic granular sludge for the production of carboxylic acids. The reactor was operated without any addition of ammonium resulting in an ammonium free effluent. This can be beneficial for further processing and (bio)conversion of VFA such as bioplastic production. For an application like bioplastic it is desired to obtain a nutrient limited stream to restrict microbial growth so that all organic carbon can be funnelled to bioplastic production (Johnson, Kleerebezem and Mark C M van Loosdrecht, 2010). Overall, a more stable process can be guaranteed using the fermentation process described in this study as traces of ammonium can be removed during the fermentation process.

Furthermore, two aspects of granular sludge are of important value regarding industrial application, (i) the solid content in the effluent and (ii) the productivity of the system. In this study the VSS in the effluent was between 0.7-1.1 $gVSS \cdot L^{-1}$, this value was obtained without optimization. No manual SRT control was done, thus this number is not informative regarding industrial application and should be optimized. This optimization was done previously by Tamis et al., (2015), after optimization a solid content of 0.05 $gVSS \cdot L^{-1}$ was obtained. The second aspect mentioned was the productivity, we obtained a glucose consumption rate in the range of 125-165 $gCOD \cdot L^{-1} \cdot d^{-1}$. These rates are in the same order of magnitude as found previously namely 150-300 $gCOD \cdot L^{-1} \cdot d^{-1}$ using anaerobic granular sludge fermenting glucose (Tamis *et al.*, 2015). The volumetric loading rate depended for a large part on the amount of biomass

present in the reactor. The obtained qS^{max} in this study was one order of magnitude lower as substrate uptake rates reported previously was 4.3 $gCOD \cdot gVSS^{-1} \cdot h^{-1}$ in a pulse-fed glucose SBR (Rombouts *et al.*, 2019a). The differences in qS^{max} can be potentially explained by a combination of the following reasons. (i) In a granular system significant amount of the VSS is considered as non-catalytic VSS (EPS and/or dead biomass incorporated in the VSS) thus lowering the overall qS^{max} value. (ii) The pH in both systems were different, in the current study the operational pH was 5.5 versus 8.0 in Rombouts., et al (2019b). Thus, in the current study it is more likely to be product inhibited compared to working at a pH of 8. (iii) Lastly, there is the possibility that not all microorganisms are active over the depth of the biofilm. However, in this study more VVS was present in the reactor resulting in volumetric productivities which were at least one order of magnitude larger than conventional chemostat operation. Overall, the application of anaerobic granular sludge for the production of VFA from sugar-rich water contains significant advantages compared to conventional CSTR operations.

3.5 – Conclusion

In this study 3 successful enrichments were achieved using anaerobic granular sludge operated under ammonium access, ammonium-limited and ammonium-deprived conditions. Stable granules where observed regardless of the presence of ammonium. Furthermore, compact structures were obtained as the SVI ranged from 0.33-0.43 $mL \cdot gTSS^{-1}$, and according to 16S rRNA analysis *Clostridium pasteurianum* was the dominant species in all enrichments. However, granular sludge still contains challenges regarding granulation as switching from 100% NH_4-N to 50% NH_4-N resulted initially in unstable granules. Overall, the cultures consumed glucose with a rate of 125-165 $gCOD \cdot L \cdot d^{-1}$ producing VFA, H_2 and VSS. In all 3 enrichments the VFA product spectrum was dominated by butyrate and acetate. The VFA production yield showed an increase upon lowering the ammonium supply as the yield increased from 0.64 to 0.74 $gCOD \cdot gCOD^{-1}$ obtained from the mathematical model. The amount of nitrogen incorporated in the VSS lowered to 0.05 $gN \cdot gVSS^{-1}$ compared to the conventional biomass content of 0.10 $gN \cdot gVSS^{-1}$. Interestingly the VSS production yield remained constant around 0.12-0.13 $gCOD \cdot gCOD^{-1}$. Concluding, ammonium deprivation was an effective way for reaching high VFA yields and simultaneously creating a nutrient limited stream desired for further (bio)processing.

3.6 – Appendix

The mathematical model used in this study was similar to one described earlier by (Mulders *et al.*, 2020). Similar to that study in the current study mainly 3 reactions were assumed to take place namely: (i) the direct conversion of glucose

to organic acids, biomass, gaseous products and storage polymer, (ii) the consumption of storage polymer to organic acids, gaseous products and biomass and (iii) the consumption of lactate to organic acids and biomass. Additionally, to the product stoichiometry used in (Mulders *et al.*, 2020) was extended as shown in table 3.3.

Table 3.3 – The reaction stoichiometries used to calibrate the model to the measured data

	rGlu	rSP	rLac
Glucose	-1		
Acetate	$Y_{Ac/Glu}$	$Y_{Ac/SP}$	0.32
Propionate	$Y_{Pro/Glu}$	$Y_{Pro/SP}$	0.63
Butyrate	$Y_{But/Glu}$	$Y_{But/SP}$	0
Lactate	$Y_{Lac/Glu}$	$Y_{Lac/SP}$	-1
SP	$Y_{SP/Glu}$	-1	0
Biomass	$Y_{X/Glu}$	$Y_{X/SP}$	0.06
H_2	$Y_{H2/Glu}$	$Y_{H2/SP}$	0

The following assumptions were made to implement the H_2-production in the model. The molair volume of gas used was 24.5 $L \cdot mol^{-1}$. Additionally, a constant flow rate of 100% N_2 in the system was constant and equal to 0.264 $L \cdot min^{-1}$.

4

Simultaneous growth and poly(3-hydroxybutyrate) (PHB) accumulation in a Plasticicumulans acidivorans dominated enrichment culture

Submitted as:

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Abstract

The wide variety of organic carbon to nitrogen and phosphorous ratios that are encountered in different wastewaters has a major impact on the poly(3-hydroxybutyrate) (PHB) accumulation potential of microbial communities. In this study we investigated the influence of the substrate composition in terms of the carbon to nitrogen (C/N) or phosphorus (C/P) ratio on the PHB accumulation performance. A multi-reactor set-up was used, enabling parallel experiments using identical inoculum of an enrichment culture dominated by *Plasticicumulans acidivorans*. In all experiments simultaneous PHB production and growth was observed. Generally, when trace amounts of growth nutrients were present the PHB production yield on substrate remained high for at least 12 h. Interestingly, from the carbon to nutrient ratio in the substrate, the PHB wt% could be accurately predicted in the accumulations. This study demonstrates that strict uncoupling of microbial growth and PHA accumulation is not required for achieving high cellular PHA-contents. Herewith the range of wastewaters that enable a cellular PHA content of 80% or higher for at least 12 hours is expanded to C:N and C:P-ratios exceeding COD:N of 26 gCOD:gNH_4-N and COD:P of 511 gCOD:gPO_4-P respectively.

4.1 Introduction

Many products used on a daily basis are fossil-fuel derived. These resources are finite, making alternative raw materials desired. A renewable source that contains large amounts of organic matter is (industrial) wastewater. In the last decade(s) the recovery of various products from wastewater has gotten significant attention. Products vary from biogas -an established technology- to innovative products such as extracellular polymeric substances (EPS) from granular sludge and bioplastics production (Kleerebezem *et al.*, 2015; Van Der Hoek, De Fooij and Struker, 2016).

One way of producing bioplastics from (industrial) wastewater is using enrichment cultures. Poly(3-hydroxybutyrate) (PHB) is a type of bioplastic produced by microorganisms in which it can serve as microbial storage polymer. One effective enrichment technique for obtaining PHB producing microorganisms is pulse feeding the culture, creating periods where there is a surplus of organic substrate and times of starvation. Microorganisms that consume the substrate the fastest store this as PHB and thus can proliferate during the starvation periods in which substrate is absent. This so-called feast-

famine regime creates a situation in which the bioplastic producing microbes will dominate the enrichment. The PHB content of the enriched microbes is maximized in an accumulation, a (fed)-batch process in which preferably no essential growth nutrient such as ammonium or phosphate are present. Using this technology, cultures have been enriched which can store up to 9 times their weight as bioplastic (0.90 gPHA·gVSS^{-1}) (Yang Jiang *et al.*, 2011; Marang *et al.*, 2013).

Lab and pilot studies have demonstrated that the feasibility of waste-based bioplastic production strongly depends on the composition of the waste stream in terms of carbon source and presence of nutrients (Johnson, Kleerebezem and Mark C M van Loosdrecht, 2010; Johnson, Kleerebezem and Mark C.M. van Loosdrecht, 2010; Korkakaki *et al.*, 2016; Korkakaki, van Loosdrecht and Kleerebezem, 2017; Valentino *et al.*, 2018). In general lower polyhydroxyalkanoates (PHA) contents are achieved at pilot scale compared to lab scale studies. Solids present in the influent lower the final PHA purity. Furthermore, waste streams can contain varying amounts of growth nutrients such as ammonium and phosphate. These growth nutrients could be disadvantageous for the PHB production process as microbial growth cannot be excluded to occur in the accumulation phase. Previous studies have shown the disadvantageous effect of growth nutrients present in the PHA accumulation process as generally lower overall PHA purities were obtained (Johnson, Kleerebezem and Mark C M van Loosdrecht, 2010; Morgan-Sagastume *et al.*, 2015). There are techniques present for pre-removal of ammonium and phosphorus. Phosphorus and nitrogen can be removed through biological or physical chemical treatment to reach growth-limited streams (Huang *et al.*, 2018; Ndam *et al.*, 2018). However, the cost-effective removal of growth nutrients remains in many cases a challenge.

The growth nutrients present in a waste stream can also be used as an advantage rather than being considered a disadvantage. The growth nutrients can potentially be used to have growth and PHA production simultaneously. The aim of this study was to investigate the overall PHB producing potential at different C/N or C/P ratios using a pre-enriched PHA producing specialist (*Plasticicumulans acidivorans*) as inoculum (Y. Jiang *et al.*, 2011). Accumulation experiments were conducted in parallel using an inoculum obtained from the same steady-state enrichment reactor. Each accumulation reactor was fed with a designed synthetic medium that contained excess carbon and either ammonium or phosphate as limiting growth nutrient. This study aimed to elucidate in more detail the requirement of nutrient limitation for the production of PHB. Additionally, this study may provide insight in the question if cultivation and accumulation need to be uncoupled, or that some degree of growth during accumulation can benefit the overall PHA process.

4.2 Materials and methods

Inoculum cultivation

The inoculum for each fed-batch experiment was the effluent from a PHB producing enrichment dominated for at least 90% by *Plasticicumulans acidivorans*. This enrichment was maintained in a bioreactor, operated at 30 ± 1 ºC using a warm water jacket on the outside of the reactor. The bioreactor had a working volume of 1.4 L. The pH of the reactor broth was measured and controlled at 7.0 ± 0.1 using 1 M of HCl or 1 M NaOH. To ensure no oxygen and mixing limitation the liquid broth was aerated at a rate of 200 mL·min^{-1} using a fine bubble dispenser and was mixed by a stirrer at 750 rpm. The reactor was operated as a sequencing batch reactor (SBR) according to settings from a previous study (Johnson *et al.*, 2009). A hydraulic retention time (HRT) of 24 h was applied with a cycle length of 12 h. The only difference with Johnson et al., (2009) was an additional amount of trace elements dosed to the bioreactor, we used a final concentration 3.75 mL·L^{-1} of Vishniac trace element solution (Vishniac and Santer, 1957). The presence of *P. acidivorans* in the enrichment was verified and estimated by Fluorescent In Situ Hybridization (FISH) microscopy. In the enrichment reactor the amount of phosphorus or nitrogen supplied was lowered in the cycle prior to the accumulation, minimizing residual phosphorus or nitrogen concentrations

Fed-batch set-up

The fed-batch experiments were performed in 4 separate identical bioreactors, identical to the enrichment reactor. An experiment lasted for 24 hours, from the moment that the reactors were seeded with effluent of a PHB producing enrichment. Each reactor was filled with 700 mL of a mixture of medium and inoculum, the content of the mixture is elaborated later in this section. The reactors were aerated using a fine bubble dispenser at a flow rate of 200 mL·min^{-1}, and stirred at 750 rpm to ensure mixing and no oxygen limitation. The reactors were kept at 30 ± 1 °C by a warm water jacket on the outside of the reactor. There was chosen to use a feed-on-demand feeding regime to mimic a feeding regime that can be applied in a full-scale situation. In the beginning of the experiment alkalinity was generated by addition of a dose of sodium acetate. The pH of the reactor broth was monitored and adjusted to 7.0 ± 0.5 by feeding of substrate (elaborated below) which contained amongst others acetic acid. Acetate consumption elevated the pH and thus activating pH control creating a feed-on-demand feeding regime. When the culture is for example saturated with PHA no more acetic acid will be consumed and thus no more elevation of the pH. This created a self-regulating system and would automatically stop feeding substrate when the culture was saturated with PHA.

For the nitrogen limited (N-limited) experiments, the substrate mixture contained 200 mL mixed effluent of the inoculum reactor, 1.0 mM KH_2PO_4, 0.78 mM $MgSO_4$, 1.0 mM KCl, and 5.3 mL of a trace element solution (Vishniac and Santer, 1957). For the phosphorus limited (P-limited) systems the substrate mixture contained 200 mL mixed effluent of the inoculum reactor, 27 mM NH_4Cl, 0.78 mM $MgSO_4$, 1.0 mM KCl, and 5.3 mL of a trace element solution (Vishniac and Santer, 1957).

Subsequently, each bioreactor got a designed substrate, according to the specifications summarized in table 4.1. The substrates were designed to produce 2 $gX \cdot L^{-1}$ of biomass with a PHB wt% according to the amount of excess carbon. One design substrate bottle will be elaborated in more detail, the rest of the substrate compositions were designed using a similar procedure and shown in table 4.1.

The substrate composition for a system designed to reach N-limited conditions and simultaneously 0.80 $gPHB \cdot gVSS^{-1}$ will be elaborated. The first design parameter set was the amount of biomass to be produced, this was set to 2 $gX \cdot L^{-1}$ (X = biomass). This concentration was chosen so no oxygen limitation should occur. Next a molecular formula of $C_1H_{1.8}O_{0.5}N_{0.2}$ with a molar mass of 25.1 $g \cdot mol^{-1}$ was assumed, so the 2 $gX \cdot L^{-1}$ equals 79.6 $mmolX \cdot L^{-1}$ (Beun *et al.*, 2002; Metcalf & Eddy, 2003). There is 0.2 mol nitrogen per carbon mole of biomass, thus 15.9 $mmolN \cdot L^{-1}$ is required to produce 2 $gX \cdot L^{-1}$. The amount of acetate required for growth was calculated as follows (equation 1):

$$\text{Ac (growth)} = \frac{X}{MW_X} \cdot Y^{\frac{PHB}{X}} \cdot Y^{\frac{Ac}{PHB}}\text{(accumulation)} \quad (1)$$

- Ac (growth) ($mol \cdot L^{-1}$) is the amount of acetate required to produce biomass in the accumulation experiment
- X ($gX \cdot L^{-1}$) is the designed biomass concentration in the accumulation experiment (2 $gX \cdot L^{-1}$)
- MW_x ($gX \cdot mol^{-1}$) is the molecular weight of biomass (25.1 $gX \cdot mol^{-1}$ (Beun *et al.*, 2002))
- $Y^{PHB/X}$ ($Cmol \cdot Cmol^{-1}$) is the amount of PHB required to produce 1 Cmol of biomass (1.5 $CmolPHB \cdot CmolX^{-1}$ (Marang *et al.*, 2013))
- $Y^{Ac/PHB}$ (accumulation) ($Cmol \cdot Cmol^{-1}$) is the amount of acetate required to produce 1 Cmol of PHB (1.2 $Cmol_{acetate} \cdot Cmol_{PHB}^{-1}$ slightly lower than the reported value of 1.5 $Cmol_{acetate} \cdot Cmol_{PHB}^{-1}$ was assumed (Y. Jiang *et al.*, 2011; Marang *et al.*, 2013)). The yield was chosen slightly lower as the yield was reported to decrease over time in an accumulation (Tamis *et al.*, 2018).

With the biomass concentration set at 2 $gX \cdot L^{-1}$, Ac(growth) will be 99.1 $mmol_{Acetate} \cdot L^{-1}$. All other growth-nutrients were supplied in the same acetate to nutrient ratio as in the enrichment reactor (Marang *et al.*, 2013). Ac (growth) was taken to determine the amount of nutrients required for growth. Another design parameter was that the culture should reach and maintain a PHB percentage of 80 wt% PHB. The acetate required for PHB production was obtained as follows (equation 2):

$$\text{Ac (PHB)} = \frac{X \cdot \text{PHB wt\%}}{100 - \text{PHB wt\%}} \cdot MW_{PHB} \cdot Y_{PHB}^{Ac}(\text{accumulation}) \quad (2)$$

- Ac (PHB) ($mol \cdot L^{-1}$) is the amount of acetate required to produce the target PHB wt%
- PHB wt% is the designed target PHB wt%
- MW_{PHB} ($g \cdot Cmol^{-1}$) is the molecular weight of PHB (21.5 $g \cdot Cmol^{-1}$ (Beun *et al.*, 2002))

A target of 80 wt% PHB results in that Ac (PHB) is 310 $mmol \cdot L^{-1}$. In total 409.1 $mmol \cdot L^{-1}$ is required to produce 2 $gX \cdot L^{-1}$ with a PHB content of 80 wt%. A similar approach was used to obtain the other designed substrates.

Table 4.1 – Overview of the substrate compositions used in this study.

Name	Target PHB wt%	Ammonium mmolN	Phosphate mmolP	Acetate mmol
CODN-50	90	16	8.4	797
CODN-26	80	16	8.4	409
CODN-13	60	16	8.4	215
CODN-9	40	16	8.4	151
CODP-996	90	32	0.8	797
CODP-511	80	32	0.8	409
CODP-269	60	32	0.8	215
CODP-189	40	32	0.8	151

Sampling and analytical methods

During all fed-batches a sample was withdrawn every 3 hours. Samples were analysed for PHB content, total suspended solids (TSS), volatile suspended solids (VSS), acetate, NH_4^+-N and PO_4—P concentrations. Samples withdrawn from the reactor that were analysed for (NH_4^+, PO_4^-, acetate) were directly after

sampling filtered using a 0.45 μM PVDF filter (0.45 μm pore size, PVDF membrane, Millipore, Ireland). Soluble samples were analyzed using a Gallery™ Plus Discrete Analyzer (ThermoFischer Scientific), commercially available. TSS and VSS samples were measured according to standard methods (Clesceri, Greenberg and Eaton, 1999), and PHB measurements were performed on dried samples according to a method supplied in an earlier study (Johnson *et al.*, 2009).

Microbial community analysis

Fluorescent In Situ Hybridization (FISH) was used to analyse the microbial community composition. The probes used were a mixture of the probes UCB823 and EUB338I-III. A more detailed version of performing this FISH technique can be found in previous work (Johnson *et al.*, 2009). Commercially synthetized probes with either 5' sulfoindocyanine dyes Cy5 and Cy3 (Thermo Hybrid interactive, Ulm, Germany) were used, summarized in table 4.2.

Table 4.2 – Oligonucleotides probes used for FISH analysis in this study.

Code	Sequence (5' -3')	Specificity	Reference
EUB338 I	gctgcctcccgtaggagt	Bacteria	(R. I. Amann *et al.*, 1990)
EUB338 II	gcagccacccgtaggtgt	Bacteria	(Daims *et al.*, 1999)
EUB338 III	gctgccacccgtaggtgt	Bacteria	(Daims *et al.*, 1999)
UCB823	cctccccaccgtccagtt	*P. acidivorans*	(Johnson *et al.*, 2009)

4.3 Results

In this study two sets of fed-batch experiments were conducted using for each set an enrichment culture from the same steady-state reactor. Operational procedures of the enrichment reactor are described in the materials and methods section. The enrichment was dominated by *Plasticicumulans acidivorans* as determined using FISH microscopy after running a test-accumulation. Following figure 4.1 an estimated dominance of *P. acividorans* of 90% or higher was observed.

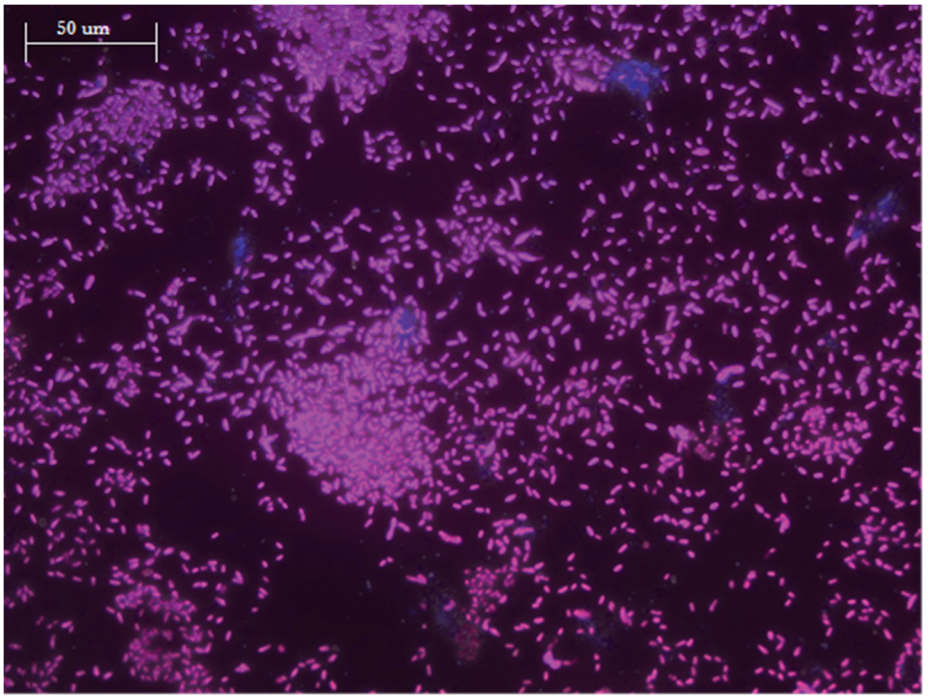

Figure 4.1 – Fluorescent In Situ Hybridization (FISH) picture of polyhydroxyalkanoate (PHA) enriched culture during a test-accumulation. Stained with Cy3-labeled UCB823 Red (*Plasticimulans acidivorans*) and Cy5-labeled EUB338 (Eubacteria).

Four separate bioreactors were used for PHA accumulation experiments at different degrees of nitrogen limitation and four separate bioreactors for phosphorus limitation experiments. The experiments were operated for 24 h. A complete overview of the collected data can be found in the supplementary materials (Appendix I). Two representative datasets for N and P limitation were selected to be elaborated in more detail: 1) the experiment designed to be P-limited and reaching 80 wt% PHB (CODP-511) and 2) the experiment designed to be N-limited and reaching 80 wt% PHB (CODN-26). Nutrients supply with the inoculum was minimized by lowering the ammonium or phosphate dosage in the enrichment reactor the cycle before the experiment was conducted (see the materials and methods section).

Phosphorus limited PHB accumulation targeting 80 wt% PHB (CODP-511)

An experiment was started by supplying a dose of acetate to the reactor to achieve a concentration of 27 mM. After this a feed-on-demand regime (fed-batch) was created as the pH controlling agent was the feed. A feed-on-demand

pattern was chosen to have carbon substrate in access at all time. Within 3 h the culture reached the target 80 wt% PHB, and maintained the target PHB wt% of 80% (within a margin of 5%-point) for 20 h before dropping to 60 wt% PHB. The overall PHA production yield varied over time but remained around 0.54 ± 0.15 $gCOD_{PHA} \cdot gCOD_{Acetate}^{-1}$ (n=7; average ± standard deviation), before the yield lowered to 0.12 $gCOD_{PHA} \cdot gCOD_{Acetate}^{-1}$ from h 20 to 24.

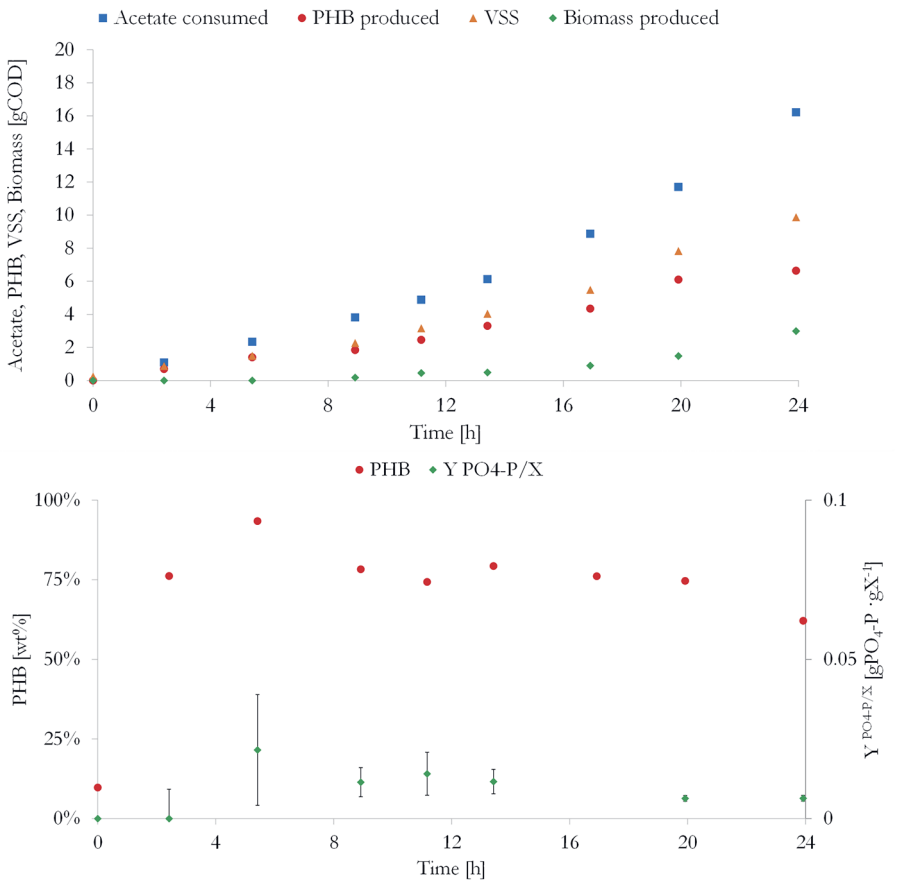

Figure 4.2 – Phosphorus limited accumulation targeting 80 wt% poly(3-hydroxybutyrate) (PHB) (CODP-511). The top graph shows the acetate consumed, PHB produced, total amount of volatile suspended solids (VSS) present, biomass produced, all in Chemical Oxygen Demand (COD) equivalents. The bottom graph shows the PHB wt% and the yield of PO_4-P per X produced with a 95% confidence interval.

In this accumulation experiment the measured phosphate concentration was continuously below the detection limit of 0.01 $mgP \cdot L^{-1}$. The substrate specific uptake rate expressed as $gCOD_{Acetate} \cdot gX^{-1} \cdot h^{-1}$ (X= biomass measured as VSS

minus PHB) was high for the first 5 h of the experiment after which it stabilized around 1-2 $gCOD_{Acetate} \cdot gX^{-1} \cdot h^{-1}$. After the initial 5 h of the experiment biomass growth was detected and the biomass production yield increased shown in figure 4.2. Until 20 h the measured acetate concentration was above 400 $mgCOD \cdot L^{-1}$, the concentration of acetate lowered to 120 $mgCOD \cdot L^{-1}$ at 24 h.

Nitrogen limited PHB accumulation with a target of 80 wt% (CODN-26)

For the second set of accumulation experiments a similar approach was used to initiate the feed-on-demand pattern as discussed before, after initial addition of acetate to the system. The culture reached within 9 h the target PHB wt% of 80% ± 5%-point and maintained this PHB wt% for an additional 9 h before dropping to 70 wt% (figure 4.3). The observed PHB yield until 18 h was 0.64 ± 0.15 $gCOD_{PHA} \cdot gCOD_{Acetate}^{-1}$ (n=6), afterwards the PHB wt% started to decline.

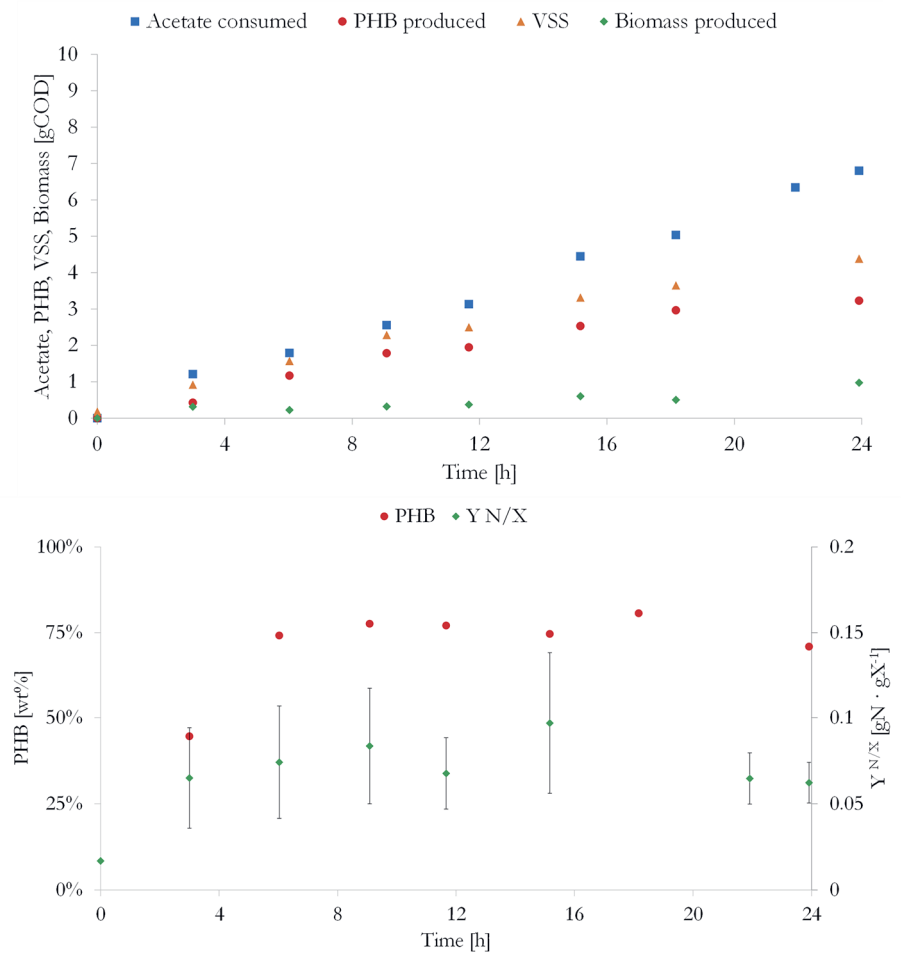

Figure 4.3 – Nitrogen limited accumulation targeting 80 wt% poly(3-hydroxybutyrate) (PHB) (CODN-26). The top graph shows the acetate consumed, PHB produced, total amount of volatile suspended solids (VSS) present, biomass produced, all in Chemical Oxygen Demand (COD) equivalents. The bottom graph shows PHB wt% and the yield of NH_4-N per X produced with a 95% confidence interval.

During the entire experiment the extracellular NH_4^+ concentration was below the detection limit of 0.005 mgN·L^{-1}. In this experiment the substrate specific uptake rate rapidly decreased in the first 4 h. At the start of the fed-batch the $q_{Acetate}$ was around 4.5 $gCOD_{Acetate}$·gX^{-1}·h^{-1}. This $q_{Acetate}$ decreased after the first 4 h and was for the rest of the experiment 0.80 ± 0.31 (n=7) $gCOD_{Acetate}$·gX^{-1}·h^{-1}. The measured acetate concentration was for the duration of the experiment 50 mgCOD·L^{-1} or higher.

Overview of the results

In all experiments the designed nutrient limitation occurred within 6 h and was limited for the remainder of each experiment. In all experiments the acetate concentration was at all times 50 mgCOD·L^{-1} or higher and the highest measured acetate concentration was around 1700 mgCOD·L^{-1}. The system performance was analysed using the PHB wt% of the culture to evaluate whether the target PHB wt% was reached and maintained.

Generally, the PHB target wt% (± 5%-point) was reached within 6 hours for all systems and the target was maintained for at least an additional 6 h. In all the N-limited systems no PHB wt% decline larger than 10%-point compared to the target was observed. In all P-limited systems, a larger than anticipated decline in PHB wt% was observed after 13 h for CODP-189 and CODP-269 and after 20 h for the CODP-511 system.

Despite the similar initial amount of biomass (X_0) in the N-limited systems, the volumetric PHB productivity (gPHB·L^{-1}·h^{-1}) was in general higher in the P-limited systems compared to the N-limited systems. The higher productivity in the P-limited system concurred with the higher amount of active biomass that was produced in these systems during the experiment. This is also reflected in the $Y_{\frac{NH_4-N}{X}}$ (figure 4.3) which remained relatively constant over time in contrast to $Y_{\frac{PO_4-P}{X}}$ (figure 4.2) which slightly lowered over time. This indicated that in the P-limited systems over time more X could be produced per PO_4-P consumed.

In order to assess the stability of the system a variable named $t_{optimal}$ was defined. $t_{optimal}$ was the time point in an experiment when the PHB content deviated more than 5%-point below the target PHB wt% for at least two measurements (table 4.3). All experiments maintained their target PHB wt% at least until 12 h before reaching $t_{optimal}$. As example in the CODP-996 system the PHB wt% was 0.87 gPHB·$gVSS^{-1}$ at 17 h, the following two measurement both were below 0.85 gPHB·$gVSS^{-1}$ making $t_{optimal}$ 17 h.

Table 4.3 – System characteristics obtained for each system at $t_{optimal}$. $t_{optimal}$ was defined as the time in an experiment when the PHB wt% of the culture fell below the target PHB wt% for at least 2 measurements in a row. X stands for biomass.

System	$t_{optimal}$	PHB	$Y^{PHA/Acetate}$	$Y^{X/Acetate}$
	h	gPHB·gVSS^{-1}	gCOD·gCOD^{-1}	gCOD·gCOD^{-1}
CODP-996	17	0.87	0.68 ± 0.27 (n=6)	0.07 ± 0.10 (n=4)
CODP-511	20	0.75	0.54 ± 0.15 (n=7)	0.12 ± 0.11 (n=4)
CODP-269	13	0.61	0.55 ± 0.17 (n=3)	0.21 ± 0.17 (n=3)
CODP-189	13	0.35	0.36 ± 0.55 (n=5)	0.47 ± 0.34 (n=4)
CODN-50	12	0.85	0.66 ± 0.20 (n=2)	0.11 ± 0.23 (n=3)
CODN-26	18	0.81	0.64 ± 0.37 (n=6)	0.05 ± 0.18 (n=6)
CODN-13	22	0.59	0.52 ± 0.33 (n=7)	0.24 ± 0.15 (n=7)
CODN-9	24	0.42	0.20 ± 0.31 (n=7)	0.33 ± 0.28 (n=7)

4.4 Discussion

Wastewaters from all kind of industries are often not strictly growth nutrient limited and this can affect the overall bioplastic production possibilities. Hence, in this study we investigated the production of PHB at different degrees of P- and N-limitation. The initial hypothesis was that the PHB wt% of a culture could be predicted based on the C/N ratio or C/P ratio in the substrate. To test to which extent this hypothesis holds true and for how long the predicted PHB content can be maintained, 8 fed-batch experiments were performed. In the systems targeting lower PHB wt%, e.g. 40% and 60%, initially after the start-up of the experiment PHB contents exceeded the predicted PHB wt% for 6-12 h. After this period the PHB wt% decreased to the designed target wt%. The minimum PHB wt% could be accurately predicted based upon on the C/N and C/P ratio for at least 12 h .

Nitrogen limited PHB accumulation and growth

In the N-limited systems the predicted PHB percentages were achieved for at least 12 h before reaching $t_{optimal}$, the timestamp in an experiment from which the culture started to negatively deviate more than 5%-point from the target PHB wt%. Comparable results were obtained in previous work using a *P. acidivorans* culture in a single N-limited accumulation (Johnson, Kleerebezem and Mark C M van Loosdrecht, 2010). Johnson et al., (2010a) conducted an accumulation experiment at a C/N ratio targeting 60-80 wt% PHB and obtained a PHB content of 77 wt% after 9.6 h. In the current work and in Johnson et al.,

(2010a) a designed medium resulted in different targeted PHB wt% combined with biomass growth. The PHB wt% were predictable from the composition of the substrate, i.e. the C/N ratio. Remarkably, $t_{optimal}$ seemed to be related to the degree of limitation. At lower C/N ratios $t_{optimal}$ was reached later, as $t_{optimal}$ was 24 h for CODN-9 versus 12 h for CODN-50. The production of PHA is a widespread trait throughout the microbial world, however reaching high PHA wt% can only be achieved by specialists (Kourmentza *et al.*, 2017). Reaching lower wt% PHB (<60 wt%) can be done by microorganisms that are present in the activated sludge process from a municipal wastewater treatment plant (Werker *et al.*, 2018). In the CODN-9 and CODN-13 the amount of PHB that the side-population needs to produce can be lower than the target as the culture likely exist of a mixture of *Plasticicumulans* able to reach higher than 60 wt% PHA. The combination of having a PHB specialist with a non-specialist both capable of producing PHB can explain why $t_{optimal}$ was reached later for lower PHB wt% targets. Additionally, the biomass concentration increased significantly in the CODN-9 systems as 12 times the initial the biomass amount was present at 24 h. No significant increase in the substrate uptake rates were observed despite the biomass growth (data shown in supplementary materials). This implied that exponential growth did not occur in the N-limited systems, otherwise an increased substrate uptake rate would have been observed.

Phosphorus limited PHB accumulation and growth

The overall performance of the phosphorus limited systems after $t_{optimal}$ was different from the nitrogen limited systems i.e. after $t_{optimal}$ the PHB content dropped faster. Furthermore, the P-limited experiments could roughly be divided in 2 groups namely, the first group CODP-996 and CODP-511 which reached $t_{optimal}$ relatively late (17-20 h) and CODP-269 and CODP-189 reaching $t_{optimal}$ relatively early (13 h). The lower cellular PHB content after $t_{optimal}$ may indicate two phenomena: (i) the P-content of the biomass was lowered under P-limited conditions, or (ii) a side-population specialized in phosphate uptake starts proliferating in the system at the expense of a decreasing contribution of *P. acidivorans*. If the P-content per unit of biomass was lowered, more active biomass (as VSS) can be produced per phosphate consumed as proposed by Korkakaki et al. (2017). The decrease in the P-content of active biomass therewith results in an increase in the biomass yield on substrate and consequently a decrease in PHA yield and a lower PHB wt%. Finally, a combination of the abovementioned phenomena may have occurred, resulting in a lower PHB content per cell. The phenomenon of adaptation of the phosphorus content of *P. acidivorans* was previously investigated and it was shown that the cellular P-content of *P. acidivorans* was reduced under P-limiting conditions (Korkakaki, van Loosdrecht and Kleerebezem, 2017). The dilution of the phosphorus content per cell did not affect the PHB accumulating capacity

as a high PHB potential was obtained, when accumulating under growth restricted conditions. In this study the effects of the possible P-dilution occurred late as the earliest observed $t_{optimal}$ for the P-limited experiments was 13 h.

Overall system performance

The PHB accumulating potential was evaluated by looking at the PHB production yield at $t_{optimal}$. In general, a higher PHB production yield was obtained at higher PHB wt%. Higher PHB production yields were obtained at higher target PHB wt% as more carbon was allocated to PHB production per biomass-unit produced.

Overall in our study, the final PHB content could adequately be predicted based on the C:N or C:P ratios of the feed. To achieve a high PHB cellular content (>75 PHB wt%) the carbon to nutrient ratio should be high. This observation showed the sensitivity of the accumulation process, and the requirement of using a nutrient-limited (waste)stream to reach a high PHB cellular content regardless of the inoculum.

Outlook

The production of PHA from wastewater using mixed microbial cultures has been studied for over 10 years. Conditions have been identified that enable the enrichment of a superior PHA producing microbial community using a feast-famine regime operated at a short SRT of 1 d and a cycle length of 12 h (Johnson *et al.*, 2009). Later this PHA specialist was isolated and annotated as *Plasticiumulans acidivorans*, and is now a thoroughly studied microorganism (Y. Jiang *et al.*, 2011). The feast-famine setting resulting in the enrichment of a PHA specialist also have been successfully applied on real wastewater streams such as the candy bar factory or the paper mill factory (Tamis, Lužkov, Jiang, M. C. M. va. Loosdrecht, *et al.*, 2014; Tamis *et al.*, 2018). Tamis et al., (2014) found that enriching on fermented candy bar wastewater (containing VFA and other COD) resulted in PHA producing microorganisms and a side-population. The effect of this non-VFA COD on the enrichment step was studied in the lab (Marang *et al.*, 2014; Korkakaki, van Loosdrecht and Kleerebezem, 2016). These demonstrated that enrichments supplied with VFA will result in the PHA producing specialist *P. acidivorans* and non-VFA (methanol) will not result in this PHA producing specialist.

One of the next challenges encountered was that PHA production from the leachate of the organic fraction of municipal solid waste (OFMSW) seemed challenging due to enrichment difficulties (Korkakaki *et al.*, 2016). One approach to circumvent enrichment difficulties applied by Korkakaki et al., (2016a) was to supply the leachate of the OFMSW to a *P. acidivorans* culture that had been

established on synthetic medium. That approach is comparable to the work described in this paper since in both studies the *P. acidivorans* enrichment was fed with a VFA-rich stream containing growth-nutrients.

The results in this study can help facilitate the waste-based production of PHA. It was observed that if the substrate contained a ratio of CODP>511 and/or a CODN>26 a high PHA wt% (PHA wt% > 75%) can be maintained for at least 12 h. This indicates that operating the accumulation can be less stringently as an operational window of at least 12 h exist before the PHA wt% will drop. On the contrary, if the substrate contained more nutrients for example in experiment CODN-13 the highest observed PHA wt% was 0.71 gPHA·gVSS at t = 9 h (shown in the supplementary material). The consequence of this observed PHA_{max} is that likely operating the accumulation is more challenging as the window of reaching the highest possible PHA purities is narrow.

Additional research to elucidate to an even larger extent the effect of nutrients on the accumulation are: (i) performing the same experiments as done in this work though using a higher degree of enriched culture (>99% presence) of *P. acidivorans* to see whether the same effects will be observed. (ii) Using different feeding patterns, in this study the feed was a continuous stream containing both carbon source and nutrient source. It could be interesting to find out whether a culture can maintain longer its PHA producing capability by altering the feeding pattern. For example, when the carbon feed is separated from the nutrient feed, the carbon feed can still be continuous and the nutrient feed can be dosed pulse-wise. Potentially, in this way it can be circumvented that a specialist for nitrogen or phosphorus uptake will take over the reactor at e.g. CODN-13. Overall, the production of PHA is nowadays possible from a wide variety of (waste)streams using a range of different strategies.

4.5 Conclusion

Not all waste streams are strictly growth nutrient limited. The effect of these nutrients on the overall PHA production process was in more detail investigated in this work. In this study we in investigated the effect of N- and P-limitation on the PHA accumulation potential using a pre-enriched *P. acidivorans* dominated culture. 8 accumulation experiments in a multi-reactor set-up were performed with different ratios of excess carbon and 1 growth nutrient either nitrogen or phosphorus designed to be limited. Experimental results demonstrated that for at least 12 hours the PHB wt% (higher or 5%-point lower) could be predicted based upon the substrate composition, i.e. the carbon to nutrient ratio. This study showed that the presence of small amounts of nutrients did not hamper the PHA production process as high PHA purities were maintained for an extended period of time.

Simultaneous growth and poly(3-hydroxybutyrate) (PHB) accumulation in a Plasticicumulans acidivorans dominated enrichment culture

4.6 Appendix

An overview of all experiments is given below. Figure 4.4 shows the nitrogen limited experiments and figure 4.5 show the phosphate limited experiments

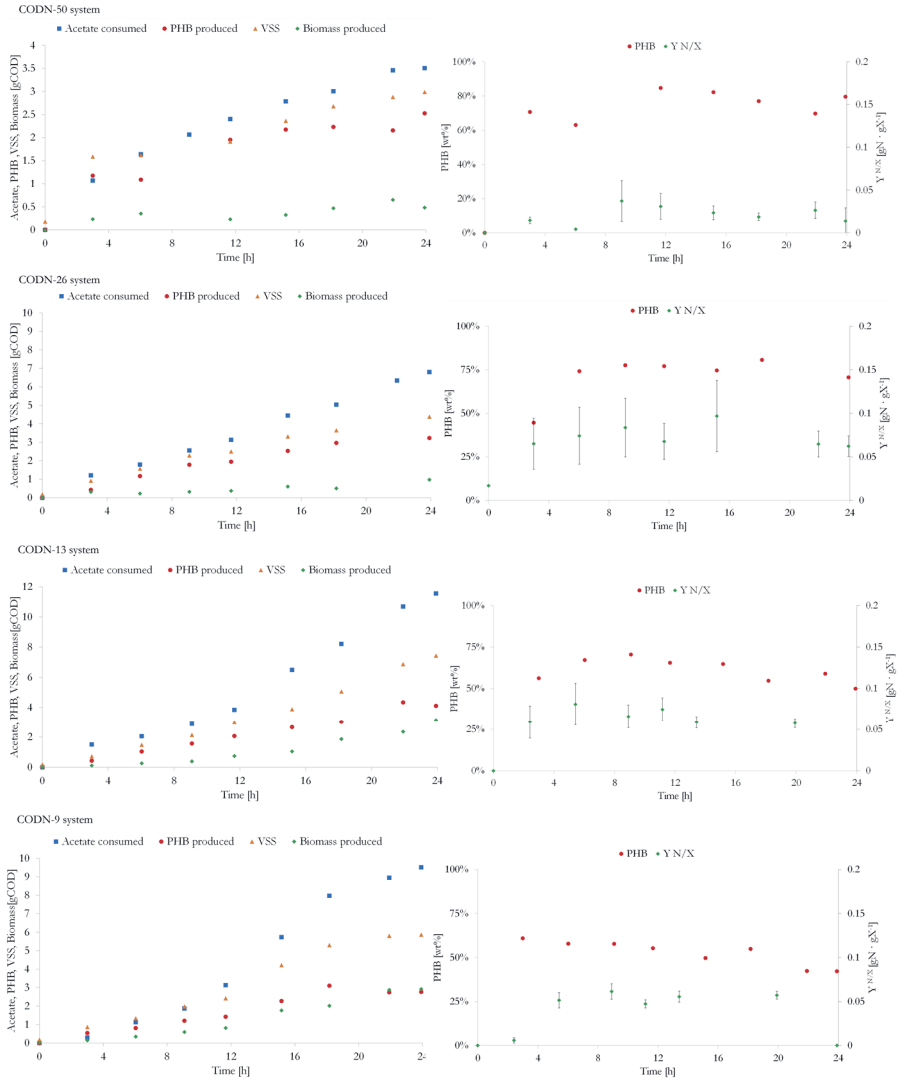

Figure 4.4 – An overview of all nitrogen limited poly(3-hydroxybutyrate) (PHB) accumulations performed with a 95% confidence interval applied on the yields.

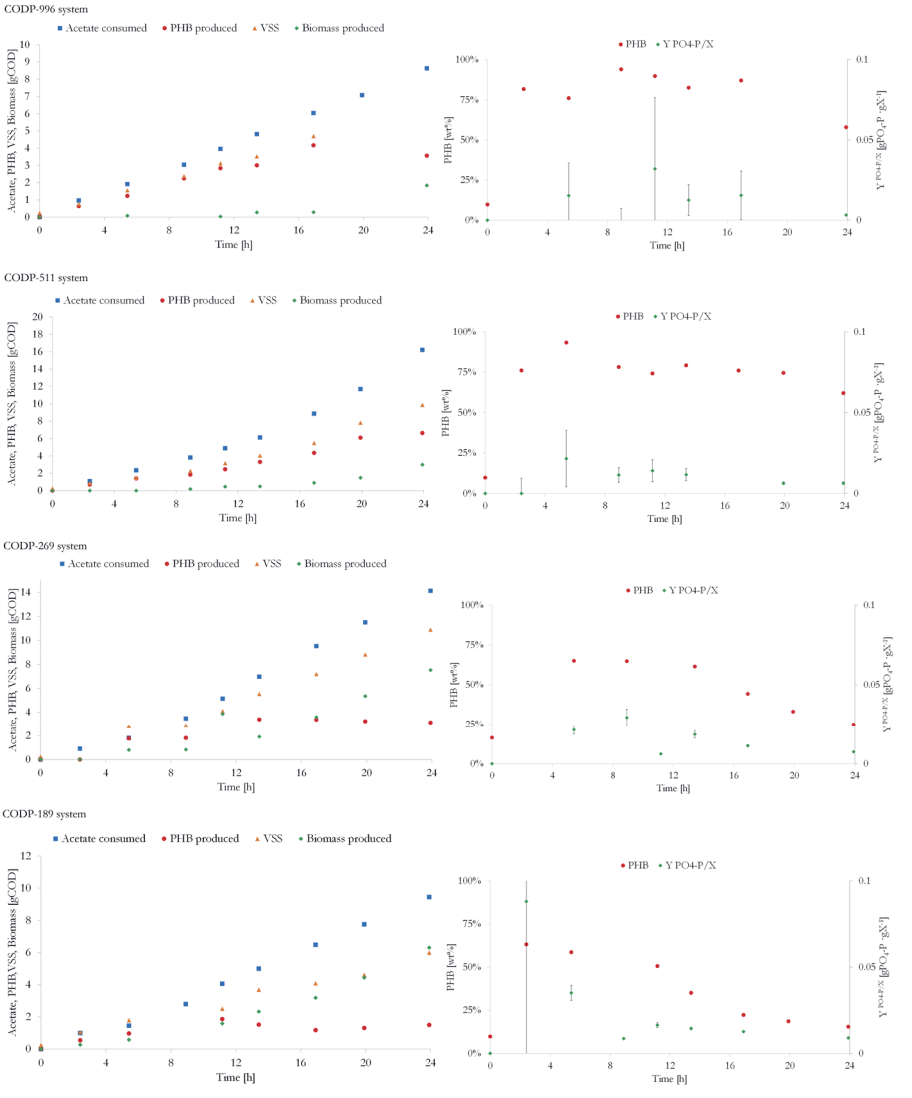

Figure 4.5 – An overview of all phosphorus limited poly(3-hydroxybutyrate) (PHB) accumulations performed with a 95% confidence interval applied on the yield.

5

Pilot-Scale Polyhydroxyalkanoate Production from Organic Waste: Process Characteristics at High pH and High Ammonium Concentration

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Abstract

A polyhydroxyalkanoate (PHA) accumulating microbial enrichment was established on a volatile fatty acids (VFA) containing leachate derived from the organic fraction of municipal solid waste (OFMSW). The enrichment was based on a 12 h feast-famine batch cycle and an exchange ratio of 50% in which VFA were completely consumed in less than 50 minutes during stable periods of operation. No pH control was applied in the system and the pH went as high as 9 due to the presence of amongst others ammonia (500 $mg \cdot L^{-1}$ total ammonia nitrogen (TAN) on average). The degree of enrichment was evaluated with fluorescence in situ hybridization (FISH) and a yet unknown genus of large (3-5 μm diameter) beta-proteobacteria appeared dominant in the culture. A method for estimating the fraction of PHA accumulating active biomass in the total VSS was established: results indicated an increase of this fraction from 25 to 56% after implementing two modifications in the operational protocol: (i) a pretreatment of the substrate removing virtually all settleable solids and (ii) a settling phase in the enrichment reactor after the feast phase, selectively removing non-settleable solids and slowly degradable substrates. The PHA accumulation potential of the culture was 77 ± 18 wt% PHA (n=3) after 3 h in batch accumulation experiments. The results suggest the potential feasibility of PHA production under conditions that were previously considered economically favourable but technically difficult.

5.1 Introduction

In the Netherlands around 1.4 Mton of the organic fraction of municipal solid waste (OFMSW) was generated in 2017 (CBS, 2018). Roughly $^2/_3$ of this waste was directly being processed aerobically to compost and used as soil fertilizer. The remaining $^1/_3$ of OFMSW was anaerobically digested to methane before being processed into compost. In the anaerobic digestion (AD) process, OFMSW is first hydrolysed to yield monomeric compounds of proteins, carbohydrates and lipids. Secondly, volatile fatty acids (VFA) are produced from the monomeric compounds via fermentation. In the last 2 phases, the produced VFA are further degraded via acetic acid, H_2 and CO_2 to methane containing biogas. An interesting alternative to compete with biogas production while treating OFMSW could be the production of PHA bioplastic (Kleerebezem and van Loosdrecht, 2007).

The production of PHA from wastewater with open microbial communities has been widely studied (Johnson *et al.*, 2009; Korkakaki *et al.*, 2016; Colombo *et al.*, 2017), both in the lab and in various pilot studies (Bengtsson, Werker and Welander, 2008; Tamis, Lužkov, Jiang, M. C. M. va. Loosdrecht, *et al.*, 2014; Tamis *et al.*, 2018; Valentino *et al.*, 2018). A common strategy to enrich for PHA producing organisms is the application of a feast-famine regime. Lab studies have reported biomass with PHA contents of more than 85 wt% (gPHA·gVSS^{-1}·100%) on synthetic wastewater (Johnson *et al.*, 2009; Marang *et al.*, 2013). More variable and, in general, lower PHA contents (29-75 wt%) have been reported when using real wastewater in lab and pilot studies (Korkakaki *et al.*, 2016; Tamis *et al.*, 2018). Commonly, these numbers are lower because of non-PHA solids in the wastewater and/or the presence of organic substrates not suitable for PHA production.

One interesting stream for the production of PHA is solid organic waste; but information about the suitability of this substrate is scarce until now, and it is worthwhile to investigate in more detail (Colombo *et al.*, 2017; Valentino *et al.*, 2018). The use of a liquid stream (leachate) derived from organic waste could pose difficulties for microbial PHA production due to its variation over seasons and its complex unknown structure. In this study, a leachate was investigated that was derived from organic waste for the production of methane containing biogas according to the Biocel process (Ten Brummeler, 2000). Characteristics for this type of leachate are a high NH_3 content, high alkalinity, presence of undefined soluble compounds and undefined particulate solids. Previous PHA production pilot studies were conducted on wastewater from a candy bar factory and papermill wastewater (Tamis, Lužkov, Jiang, M. C. M. va. Loosdrecht, *et al.*, 2014; Tamis *et al.*, 2018). These wastewaters typically have a high chemical oxygen demand (COD) to nitrogen ratio and low amounts of undefined (particulate) organic compounds. Furthermore, these pilot studies were operated in a reactor system with pH control and an operating pH of 7. In this study, the leachate had a high alkalinity, rendering pH control expensive. The relatively harsh conditions encountered when using organic waste leachate are reflected in the results from a lab study conducted on similar leachate (Korkakaki *et al.*, 2016), which yielded a poor PHA accumulating potential (29 wt%). Only when the microbial enrichment was established on an artificial VFA mixture, high PHA contents (78 wt%) could be achieved when fed with leachate during accumulation.

Figure 5.1 – Impression of the pilot plant. The open container contained the selector and accumulator bioreactor (red bracket). In the front, there is an IBC sedimentation tank (orange bracket). Behind the sedimentation tank, the influent buffer vessel was located (yellow bracket). (Image by Michel Mulders.)

In this study, a PHA producing feast-famine enrichment without pH control was investigated on pilot-scale, an impression of the pilot is shown in figure 5.1. The substrate was leachate derived from the OFMSW collected on site at 'Orgaworld Vergisting Biocel' in Lelystad, the Netherlands. In this work we do not aim for maximization of the PHA production per unit of OFMSW treated, because the main fraction of VFA will be converted to methane containing biogas. The main research question of this study was whether a good overall PHA production process could be established with a complex feedstock like the leachate of OFMSW. The main criteria used to identify the process performance were the overall PHA production yield ($gPHA \cdot gCOD^{-1}$), the PHA content of the product ($gPHA \cdot gVSS^{-1}$) and the process stability.

5.2 Materials and methods

A pilot plant situated at the 'Orgaworld Vergisting Biocel' located in Lelystad, the Netherlands, was operated for a period of 757 days. A period of 188 days will be elaborated in which the pilot plant was operated under a modified version of the standard feast-famine operation as described by (Johnson *et al.*, 2009). The modified version included a settling phase of 60 minutes after depletion of the rapidly degradable chemical oxygen demand.

Substrate production

The leachate used in this study was produced at the 'Orgaworld Vergisting Biocel' located in Lelystad. At this site, the source separated OFMSW is treated in anaerobic tunnel digesters. Fresh OFMSW is batch-wise mixed with digested OFMSW from the previous batch, which serves as seeding material. The OFMSW is anaerobically digested to methane containing biogas (Ten Brummeler, 2000). Leachate from the tunnels is collected centrally and recirculated over the OFMSW tunnels continuously to enhance transport of substrates between zones in the bed with more or less methanogenic activity. Leachate for feeding the PHA pilot was withdrawn from the central point where leachate was collected.

The collected leachate for the PHA pilot plant was first stored in a 1 m^3 intermediate bulk container (IBC). The leachate was stored for at least 24 hours to allow settling of particulate material; after this period the supernatant (ca. 500 L) was pumped in a buffer vessel of the pilot plant. Finally, the leachate was diluted 2-3 times towards a final concentration of soluble COD (COD_{Sol}) of 6 $gCOD_{Sol} \cdot L^{-1}$. The buffer vessel was 1500 L and kept at 35 ± 5 °C, the substrate was not mixed promoting further settling of solids, thus minimizing solids entering the bioreactors. Diluted leachate was in the buffer vessel for roughly 3 days, after which the buffer vessel was completely emptied and cleaned before being filled again with new substrate, minimizing methanogenic activity in the buffer vessel.

Enrichment reactor

Enrichment of a PHA producing microbial community was conducted in an aerobic reactor. This reactor had a working volume of 180 L and was inoculated with a mixture of a lab enrichment, dominated by *P. acidivorans*, and activated sludge from a domestic wastewater treatment plant (Dokhaven, Rotterdam, The Netherlands). Air was supplied via a fine bubble diffuser at a rate of 100 $L \cdot min^{-1}$ to prevent oxygen limitation and ensure mixing of the reactor broth. The reactor was kept at 30 ± 3 °C through a warm water jacket on the outside of the reactor. The pH of the system was only monitored and not controlled.

A selective environment that favors PHA production was created using a feast-famine regime. The sequential batch reactor (SBR) was operated using a cycle length of 12 hours and a solid retention time (SRT) of 24 hours and a hydraulic retention time (HRT) of 17 hours. A cycle consisted of the following phases:

1. *Feed phase (0-16 min)*

During the feed phase 125 L of substrate was supplied from the buffer vessel to the reactor for 16 min resulting in a working volume of 177 L. In the leachate, the COD_{Sol}:P ratio was 275 ($gCOD_{Sol}$:gPO_4-P) and phosphorus was assumed to

be limiting. Even though all nutrients (except phosphate) were present in excess in the wastewater, additional growth nutrients were supplied in order to make sure that no nutrient limitation would occur. Nutrients were dosed to the reactor, 80-100 mL $cycle^{-1}$, throughout all phases, except for the settling phase. The nutrient mix consisted out of the following components: 3 M urea-N, 0.3 M phosphate, 0.3 M $MgSO_4$, 0.2 M K_2SO_4, 64 mM $FeCl_3$, 3 mM $ZnSO_4$, 2.7 mM H_3BO_3, 2.1 mM $NiCl_2$, 1.5 mM $CoSO_4$, 0.6 mM $CuSO_4$, 0.8 mM Na_2MoO_4.

2. *Reaction phase (16-50 min)*

The reaction phase followed the feed phase. In this phase the microorganisms had the opportunity to consume all readily biodegradable substrate aerobically.

3. *Settling phase (50-110 min)*

50 min after the cycle initialization, a settling phase started. During this phase aeration was turned off for 60 min. After 60 min settling, the top half of the liquid (86 L) was removed from the middle of the reactor.

4. *Growth reaction phase (110-705 min)*

After effluenting the top half of the liquid, the aeration was turned back on for the remainder of the cycle. No substrate was added to the reactor.

5. *Effluent phase (705-720 min)*

At the end of the cycle, around half of the remaining reactor broth was withdrawn (40 L). This biomass containing effluent was used optionally in the subsequent accumulation step.

Accumulation reactor

A separate reactor was used to maximize the PHA content. This reactor had a maximum working volume of 180 L. The reactor was inoculated with 10 L mixed broth from the enrichment reactor. Subsequently, the reactor was filled with 170 L of substrate from the influent buffer tank. Aeration was similar to the enrichment reactor: 100 L·min-1, the temperature was kept at 30 ± 2 °C. After 4 hours, the PHA-rich biomass was harvested using a pilot-scale centrifuge, processing a flow of 200 L·h-1 at 3000 g.

Sampling and analytical methods

Table 5.1 – Overview of the samples withdrawn, from each vessel/reactor and its frequency.

Measurement	Sample point	Sample Time	Frequency	Method
TSS/VSS[a]	Buffer vessel	SoC[g]	Daily	Wet- / Dry- / Ash- weight
	Enrichment reactor	EoC[h], EoF[i]		
SVI30[b]	Enrichment reactor	EoC, EoF	Daily	Imhoff cone
COD[c]	Buffer vessel	SoC	Daily	Spectrophotometric (Hach-Lange)
	Enrichment reactor	EoC, EoF		
Alcohol/VFA[d]	Buffer vessel	SoC	Daily	GC
	Enrichment reactor	EoC, EoF		
PHA[e]	Enrichment reactor	EoC, EoF	Daily	GC
NH_4^+	Buffer vessel	SoC	Every other day	Spectrophotometric (Hach-Lange)
	Enrichment reactor	EoC, EoF		
Conductivity	Buffer vessel	SoC	Daily	Handmeter
	Enrichment reactor	EoC, EoF		
pH	Buffer vessel	Continuous	Online	Ag/AgCl electrode
	Enrichment reactor	Continuous		
DO[f]	Enrichment reactor	Continuous	Online	LDO Sensor

[a] TSS/VSS stands for total suspended solids and volatile suspended solids
[b] SVI30 stands for sludge volume index 30
[c] COD stands for chemical oxygen demand
[d] VFA stands for volatile fatty acids
[e] PHA stands for polyhydroxyalkanoates
[f] DO stands for dissolved oxygen
[g] SoC stands for start of cycle
[h] EoC stands for end of cycle
[i] EoF stands for end of feast

Data was collected over a period of 188 days according to the sampling and measurement scheme shown in table 5.1. Furthermore, to gain more insight in the process, multiple detailed sampling campaigns were executed. During these campaigns the number of samples withdrawn from the reactor was increased (every 10 minutes during the feast period, every hour for 2-3 hours in the famine period and accumulation reactor).

Samples withdrawn from the reactor for volatile fatty acid (VFA), COD_{Sol} and TAN content were filtered before measurement (0.45 μm pore size, PVDF membrane, Millipore, Ireland). TAN and COD_{Sol} were measured using a commercially available spectrophotometric test cuvette kit provided by Hach-Lange. LCK302 was used to quantify the TAN and LCK014 was used for COD_{sol}. The VFA content of the samples were measured using gas chromatography (GC). The GC was equipped with a ZB-WAXplus column (20 m length × 0.18 mm internal diameter, 0.18 μm film) and a flame ionization detector (FID), as described in (Cabrera-Rodríguez *et al.*, 2017), though in this study iso-hexanoic acid was used as internal standard instead of anisole. Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to standard methods (Clescerci et al. 1999). The SVI30 was determined as follows: the SV30 was obtained in an imhoff cone according to standard methods (Clescerci et al. 1999), the SV30 was then divided by the corresponding TSS of the sample minus the TSS of the influent. The PHA content of the dried matter was analyzed according to the method described by (Johnson *et al.*, 2009). The PHA content was extracted and esterified using a mixture of propanol:HCL (4:1) (1.5 mL) and dichloroethane as solvent (1.5 mL) for 3 hours at 100 ºC. After separation of the solvent phase from the water phase the PHA content (in the solvent phase) was quantified using a GC (model 6890N, Agilent, U.S.A.) equipped with a FID on a HP Innowax column.

Data analysis

The VFA fraction of the total soluble COD (COD_{VFA}) concentration was determined as the sum of the individual COD concentrations of acetate, propionate, iso-butyrate, butyrate, iso-valerate and valerate. The amount of catalytic biomass was estimated by subtracting the amount of PHA and inert VSS from the total amount of VSS measured in the bioreactor. The VSS present in the pretreated leachate was assumed to be inert VSS. For practical purposes it was assumed that $Y_{VFA}^{X_{PHA}}$ equals $Y_{BOD_{other}}^{X_{other}}$.

Therewith, the fraction of PHA producing biomass in the total catalytic biomass and the amount of catalytic biomass could be approximated as follows:

$$f_{XPHA} = \frac{\Delta COD_{VFA}}{\Delta COD_{sol}} \quad (1)$$

$$VSS_{catalytic} = \left(VSS_{EoC} - PHA_{EoC} - VSS_{inf}\right) \cdot V_{EoC} \quad (2)$$

$$BOD_{other} = \Delta COD_{sol} - \Delta COD_{VFA} \quad (3)$$

The amount of PHA producers was estimated by using the fraction of COD being consumed by the PHA producing biomass (f_{xPHA}) multiplied with the approximated amount of catalytic biomass.

The expression calculating the amount of PHA producers X_{PHA} is formulated below. Furthermore, it was assumed no growth occurred using particulate COD.

$$X_{PHA} = f_{XPHA} \cdot VSS_{catalytic} \quad (4)$$

The VFA specific substrate uptake rate q_{VFA} ($gCOD_{VFA} \cdot gX_{PHA}^{-1} \cdot h^{-1}$) for the PHA producing biomass was estimated as follows:

$$q_{VFA} = \frac{\Delta COD_{VFA}}{X_{PHA} \cdot feastphase} \quad (5)$$

The VFA specific substrate uptake of all the VSS q^*_{VFA}($gCOD_{VFA} \cdot gVSS^{-1} \cdot h^{-1}$) was estimated as follows:

$$q^*_{VFA} = \frac{\Delta COD_{VFA}}{VSS_{EoC} \cdot V_{EoC} \cdot feastphase} \quad (6)$$

The overall substrate uptake rate of all the VSS q^*_{sCOD} ($gCOD_{Sol} \cdot gVSS^{-1} \cdot h^{-1}$) was estimated as follows:

$$q^*_{sCOD} = \frac{\Delta COD_{sol}}{VSS_{EoC} \cdot V_{EoC} \cdot feastphase} \quad (7)$$

For the accumulation experiments, the amount of VSS was measured at the beginning of the accumulation experiment to obtain the specific substrate uptake rates. Furthermore, instead of the length of the feast phase, the duration of the experiment was used.

The overall biomass production yield ($gVSS \cdot gCOD_{Sol}^{-1}$) was estimated as follows:

$$Y^{X}_{\overline{sCOD}} = \frac{\left(VSS_{EoC} - PHA_{EoC} - VSS_{inf}\right) \cdot Volume_{EoC}}{\Delta COD_{sol}} \quad (8)$$

The overall PHA production yield was calculated using the method proposed in (Bengtsson, Werker and Welander, 2008). First the amount of PHA biomass (X^*_{PHA}) that could be produced from 1 $kgCOD_{Sol}$ was calculated. Next, the amount of PHA (PHA*) required to reach the target wt% PHA could be

calculated using (X^*_{PHA}). Finally, the amount of PHA* was used to calculate the amount of COD_{Sol} required for its production, which was added to the 1 $kgCOD_{Sol}$ used for biomass production, resulting in an overall PHA production yield.

$$X^*_{PHA} = 1\ \mathrm{kgCOD_{sol}} \cdot Y^{\frac{X}{sCOD}} \quad (9)$$

$$PHA^* = \frac{X^*_{PHA}}{1 - PHA\ (wt\%)} - X^*_{PHA} \quad (10)$$

$$Y_{Overall\ PHA\ production} = \frac{PHA^*}{PHA^* \cdot \frac{1}{Y^{\frac{PHA}{\Delta COD_{VFA}}}} + 1\ \mathrm{kgCOD_{Sol}}} \quad (11)$$

Microbial community analysis: gDNA extraction, Cloning and sequencing of full 16S-rRNA genes

Full 16S-rRNA genes (~1500bp), for the purpose of the development of specific FISH probes, were generated by performing a full gene amplification on a sample from the PHA enrichment reactor (May 2017). The first step was extraction of the genomic DNA by using the UltraClean Microbial DNA Isolation Kit (Mobio Inc. USA) according to manufacturer's protocol, except a combination of heating (65 °C) and beat beating was used (minibeater-16, Biospec, USA). Following extraction, a PCR amplification was done by using the primers GM3 and GM4 (Muyzer *et al.*, 1995). The amplified fragments were checked and quantified using an agarose gel, purified using QIAquick PCR purification kit (QIAGEN, Germany) and used for TOPO TA Cloning (Thermo Fisher Scientific, USA). In total 48 clones were picked from plate and used for direct PCR on the plasmid using the primers MF, MR. The final product was checked on an agarose gel and all clones yielded near 1600bp fragments. All these clones were sent for (bi-directional Sanger) sequencing to Baseclear, Leiden, the Netherlands. The sequences were aligned and quality trimmed using CodonCode aligner v.4.2.7. software (Codoncode Corp. USA) resulting in complete 16S-rRNA genes with the primers sites removed. The final consensus sequences were exported and imported into ARB (v5.2) software (Ludwig *et al.*, 2004) and redundant, identical, sequences were removed. The resulting 12 sequences, which were near identical (>99%), were used for probe development (implemented in the ARB software package). Several probes were developed with different properties on various positions. In the end probe JT01 was most specific (matching only the imported sequences) after optimization. The unambiguous clone JT01 which represented all twelve clones was submitted to genbank (NCBI) under the accession number MK575517

The microbial community composition was analysed microscopically using Fluorescent In Situ Hybridization (FISH) with a mixture of the probes JT01, UCB823 and EUB338I-III. A more detailed version of performing this FISH technique can be found in (Johnson *et al.*, 2009). Commercially synthetized probes with either 5' FLUOS or the sulfoindocyanine dyes Cy5 and Cy3 (Thermo Hybrid interactive, Ulm, Germany) were used, summarized in table 5.2.

Table 5.2 – Oligonucleotides probes used for FISH analysis used in this study.

Code	Sequence (5' -3')	specificity	Reference
EUB338 I	gctgcctcccgtaggagt	Bacteria	(R I Amann *et al.*, 1990)
EUB338 II	gcagccacccgtaggtgt	Bacteria	(Daims *et al.*, 1999)
EUB338 III	gctgccacccgtaggtgt	Bacteria	(Daims *et al.*, 1999)
UCB823	cctccccaccgtccagtt	*P. acidivorans*	(Johnson *et al.*, 2009)
JT01	tccacacacgctattcacgca	Clone JT01	This study

5.3 Results

Characterization of the substrate

Pretreatment of the substrate aimed at minimizing suspended solids concentrations in the influent. To this end, the OFMSW-leachate used in this study was stored at ambient temperatures for at least 24 hours in a 1 m^3 IBC vessel prior to usage. After storage, the upper fraction was stored in the substrate buffer vessel. The leachate had a COD_{Sol} concentration of 16.6 $gCOD_{Sol} \cdot L^{-1} \pm$ 5.6 $gCOD_{Sol} \cdot L^{-1}$ (average ± standard deviation; n = 75) and this was diluted with tap water to 6 $gCOD_{Sol} \cdot L^{-1}$ before being used in the PHA production pilot. A summary of the substrate properties as measured in the influent buffer vessel (after dilution) is shown in table 5.3.

Table 5.3 – Summary of the pilot influent characteristics.

	Influent buffer tank (after dilution)	Unit
TSS	1.49 ± 0.77 (n=122)	$gTSS \cdot L^{-1}$
VSS	0.89 ± 0.52 (n=122)	$gVSS \cdot L^{-1}$
COD_{Sol}	5.78 ± 1.13 (n=127)	$gCOD_{Sol} \cdot L^{-1}$
BOD_{other}	0.70 ± 0.07 (n=117)	$gCOD_{other} \cdot gCOD_{Sol}^{-1}$
VFA	0.50 ± 0.13 (n=114)	$gCOD_{VFA} \cdot gCOD_{Sol}^{-1}$
Even VFA	0.56 ± 0.12 (n=114)	$gCOD_{VFA} \cdot gCOD_{VFA}^{-1}$
Odd VFA	0.44 ± 0.12 (n=114)	$gCOD_{VFA}$ $gCOD_{VFA}^{-1}$
TAN	622 ± 159 (n=77)	$mgN \cdot L^{-1}$
Phosphate	20.9 ± 10.8 (n=77)	$mgPO_4\text{-}P \cdot L^{-1}$
pH	7.53 ± 0.42 (Online data August 2017)	
Alkalinity	70 ±10	$meq \cdot L^{-1}$

Here TAN represent the total ammonia nitrogen, which was ammonia (NH_3) + ammonium (NH_4^+). The alkalinity of the substrate was estimated to be around 70 $meq \cdot L^{-1}$ using the measured concentrations of TAN and VFA and estimating a pCO_2 of 9%. (**see** Appendix-I)

Selector characterization

Reactor operation was started in January 2016 and data collection started on 29th of March 2016. The enrichment was monitored for a period of 667 d. Within 13 d after start-up of the reactor, typical feast-famine dynamics were observed (figure 5.3). The length of the feast phase was identified using the dissolved oxygen (DO) profile during the cycle. Multiple stable periods were observed during the operation of the PHA producing pilot plant. A stable period comprised all cycles with a feast length below 50 min with the condition that the standard deviation of the feast length of the period including the surrounding 3 days (7 days in total) was below 15 min. Furthermore, specific days were included that would not pass the qualifications due to for example technical difficulties. An overview of the stable periods is given in figure 5.2.

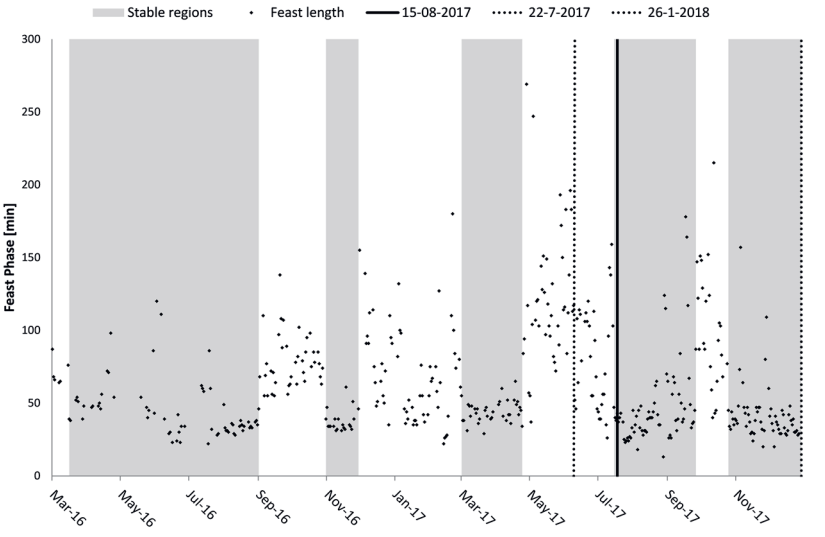

Figure 5.2 – Overview of the performance of the enrichment reactor in terms of the length of the feast phase.

In this study, we describe in detail the period from the 22nd of July 2017 until 26th of January 2018 (188 operational days). During this period, the operational settings of the systems were kept constant, providing a representative overview of the system performance.

During this period a DO pattern typical for the enrichment of PHA producing microorganisms using a feast-famine regime was observed. This typical pattern consisted out of two phases; the first phase, called the feast phase, in which a high oxygen respiration rate was observed; the second phase, called the famine phase, in which the oxygen respiration rate was lower (figure 5.3, operational day: 29-08-2017).

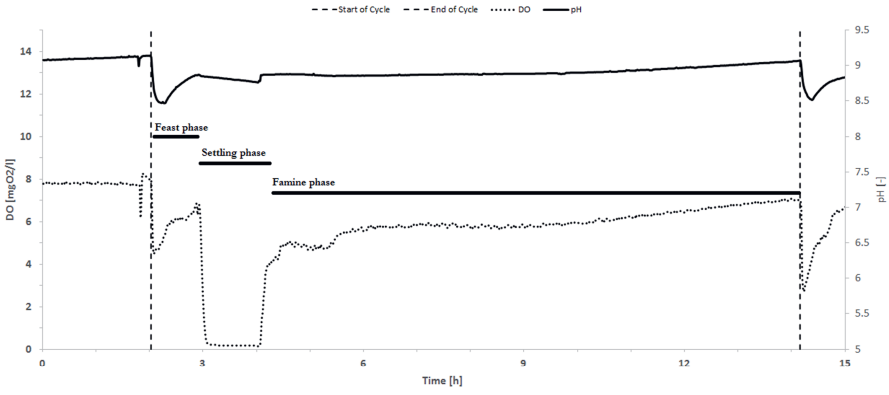

Figure 5.3 – Dissolved oxygen profile and pH profile in the enrichment reactor in a stable period, from operational day 29-07-2017.

The pH dropped during the feeding phase (duration: 16 min); once the feeding was completed the pH gradually increased due to VFA uptake. System characteristics of the enrichment reactor are summarized in table 5.4.

Table 5.4 – Characteristics of the enrichment reactor averaged over time.

	Enrichment reactor	Unit
PHA (end of cycle)	0.04 ± 0.03 (n=27)	gPHA·gTSS^{-1}
TSS (end of cycle)	4.56 ± 1.57 (n=119)	gTSS·L^{-1}
VSS (end of cycle)	3.45 ± 1.16 (n=118)	gVSS·L^{-1}
SVI30 (end of feast)	66 ± 38 (n=120)	mL·gTSS^{-1}
PHA (end of feast)	0.26 ± 0.09 (n=109)	gPHA·gTSS^{-1}
TSS (end of feast)	3.48 ± 1.12 (n=121)	gTSS·L^{-1}
VSS (end of feast)	2.64 ± 0.84 (n=120)	gVSS·L^{-1}
q_{VFA}	4.2 ± 1.9 (n=108)	gCOD$_{VFA}$·gX$_{PHA}^{-1}$·h^{-1}
q^*_{VFA}	2.4 ± 1.2 (n=108)	gCOD$_{VFA}$·gVSS^{-1}·h^{-1}
Y^{PHA}_{VFA}	0.44 ± 0.14	gPHA·gCOD$_{VFA}^{-1}$
Y^{X}_{sCOD}	0.25 ± 0.08	gVSS·gCOD$_{Sol}^{-1}$

During the settling phase the DO dropped to zero; and all settleable biomass settled to the bottom part of the reactor. After the settling phase and removal of the supernatant, the aeration was turned on again entering the famine phase. The DO was slowly increasing in this phase, indicating the depletion of the intracellular PHA; non-PHA VSS increased concurrently, indicating growth. The

pH in this phase was also slowly increasing from 8.5 to 9, likely due to the stripping of CO_2.

Microbial community analysis

FISH microscopy was used to investigate microscopically the diversity of the enriched culture. Initial 16S rRNA (NGS) analysis indicated that an unknown species was present in the enrichment. A specific probe targeting the *uncultured Rhodocyclaceae bacterium clone JT01* was designed as described in the material and methods section. Three different probes for FISH analysis used were, EUB338I-III (Cy5) binding to all prokaryotic bacteria, UCB823 (Fluos) this probe bind to *P. acidivorans*, and JT01 (Cy3) targeting clone JT01. Over the period described in this study multiple FISH slides were prepared and in all of them clone JT01 seemed to be the dominating microorganism in the enrichment. The bacteria appeared to be a large betaproteobacteria, sizes varied and bacteria as large as 3-5 µm were observed. The bacteria species belonged to a yet unkown species and genus, the closest related species was the *Thauera* genus as shown in figure 5.4.

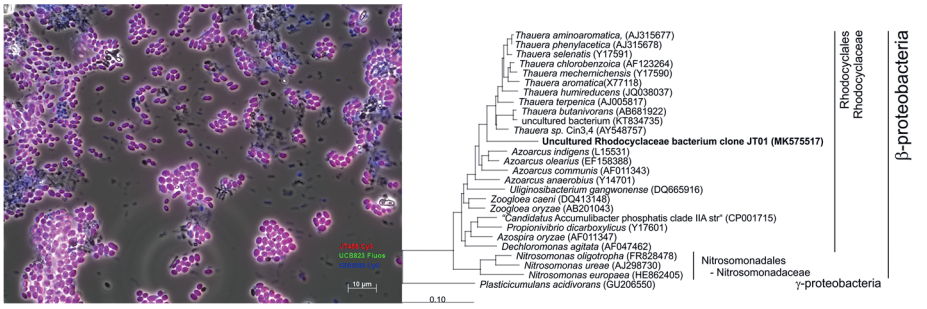

Figure 5.4 FISH microscopy picture of the enriched culture on diluted leachate at pilot scale, using the following probes: Cy3 JT01, Fluos UCB823, Cy5 EUB 338. This phylogenetic tree was constructed off near full length 16S-rRNA genes from clone library and reference database (SSURef_NR99_128_SILVA_07_09_16_opt) using NeighborHood Joining algorithm implemented in ARBv5.2. In total 1206 positions were taken for calculation. A position filter, SSU-Ref: bacteria was applied to select for most common positions in the bacterial 16S-rRNA alignment. The scale bar shows 10% base-pair difference. Two species of the Thermotoga genus were used as outgroup but later pruned from the tree.

Accumulation

To assess the PHA accumulation potential of the enrichment, batch experiments were performed. The experiments were started with a small inoculum containing 10 L of mixed effluent from the enrichment reactor (containing 33 gVSS), and 170 L of influent containing on average 440 $gCOD_{VFA}$. The average COD_{VFA}:P:N ratio was 1:4:108 ($gCOD_{VFA}$:mgP:mgN) suggesting that the accumulations were performed in the presence of nutrients. Data obtained during an accumulation experiment conducted on the 15th of August 2017 is

shown in figure 5.5 as an example. In this experiment, virtually all VFA were consumed in less than three hours, and a PHA content of 0.61 gPHA·gVSS^{-1} was reached. The polyhydroxybutyrate (PHB) content was 0.43 gPHB·gVSS^{-1} and the polyhydroxyvalerate (PHV) content was 0.18 gPHV·gVSS^{-1}.

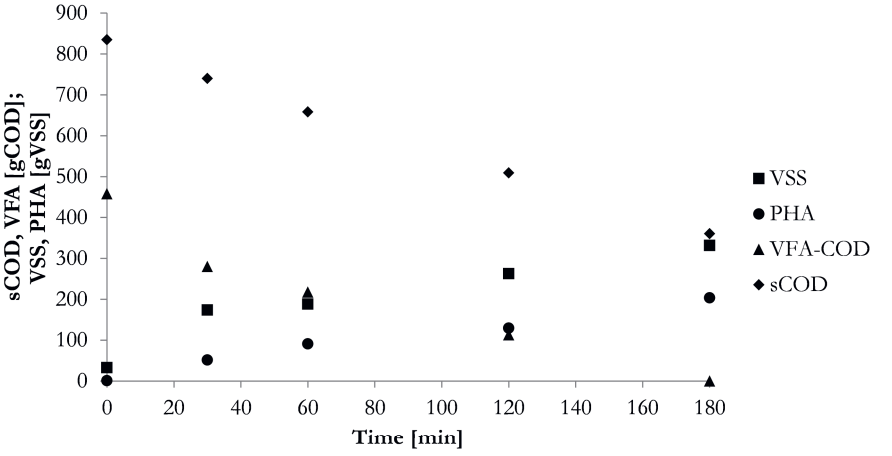

Figure 5.5 – Detailed characterization of a PHA accumulation potential experiment (15-08-2017). Illustrating the evolution of COD_{Sol}, COD_{VFA}, PHA and VSS over time in absolute amounts.

There were two mechanisms assumed for non-PHA VSS contribution in the process: (i) inert particulate solids in the influent, and (ii) biomass growth in the accumulation bioreactor. The initial increase in VSS represents the inert VSS part of the influent added during filling up the reactor and simultaneous production of PHA. After the feeding was finished a constant PHA production rate was observed, resulting in an increase of VSS. Determining the X_{PHA} present in the system as described in the material and methods section, a cellular PHA content of 0.86 gPHA·X_{PHA}^{-1} was estimated. Furthermore, the COD consumption during the accumulation period was for 95% due to the consumption of VFA thus almost no BOD_{other} was consumed. Process characteristics were obtained from the measured data to interpret the performance of the system, these are summarized in table 5.5. The specific uptake rates for the PHA producer (q_{VFA}) obtained in the accumulation were higher than those obtained during the cycle characterization.

Table 5.5 – Characteristics from the PHA producing enrichment exposed to excess substrate in a batch process.

Accumulation reactor		Unit
Y_{VFA}^{PHA}	0.44	$gPHA \cdot gCOD_{VFA}^{-1}$
q_{VFA}	7.5	$gCOD_{VFA} \cdot gX_{PHA}^{-1} \cdot h^{-1}$
q_{VFA}^{*}	4.7	$gCOD_{VFA} \cdot gVSS^{-1} \cdot h^{-1}$
q_{sCOD}^{*}	4.9	$gCOD_{Sol} \cdot gVSS^{-1} \cdot h^{-1}$

Over the period described in this study multiple accumulations were performed. The enrichment was exposed to substrate for at least 3 hours and subsequently settled for 30 minutes prior to sampling. A maximum amount of 0.88 $gPHA \cdot gVSS^{-1}$ was measured. On average a PHA content of 0.61 $gPHA \cdot gTSS^{-1}$ ± 0.14 (n=7) expressed per TSS and 0.77 ± 0.18 $PHA \cdot gVSS^{-1}$ (n=3) expressed per VSS was obtained. The PHB content was on average 0.38 ± 0.13 $gPHB \cdot gVSS^{-1}$ (n=3) and the PHV content was on average 0.38 ± 0.09 $gPHV \cdot gVSS^{-1}$ (n=3).

5.4 Discussion

Enrichment performance

The microbial community established in this work was able to consume the supplied VFA with an estimated PHA specific biomass substrate uptake rate of 4.2 ± 1.9 $gCOD_{VFA} \cdot gX_{PHA}^{-1} \cdot h^{-1}$ (n=108). This q_{VFA} was in a range comparable to lab studies (Johnson *et al.*, 2009; Albuquerque *et al.*, 2011; Yang Jiang *et al.*, 2011; Marang *et al.*, 2013). In lab studies, biomass maximum specific uptake rates of 5.6 gCOD $gX_{PHA}^{-1} \cdot h^{-1}$ were reported for cultures enriched using acetate and 10.6 gCOD $gX_{PHA}^{-1} \cdot h^{-1}$ for cultures enriched on butyrate (assuming $C_1H_{1.8}O_{0.5}N_{0.2}$ correspondig to 25 $gX \cdot cmolX^{-1}$) (Beun *et al.*, 2002; Yang Jiang *et al.*, 2011; Marang *et al.*, 2013). In this study a mixture of different linear VFA was present, of which acetate was the most abundant 0.44 ± 0.11 $gCOD_{Ace} \cdot gCOD_{VFA}^{-1}$.

This study also showed the importance of estimating the PHA producing culture as fraction of the VSS in order to estimate a qS value. The q_{VFA}^{*} was 2.4 ± 1.2 $gCOD_{Sol} \cdot gVSS^{-1} \cdot h^{-1}$ (n=108) which was almost 50% lower than the q_{VFA}. The data clearly suggest that influent solids and non-PHA producing biomass lowered the fraction of PHA producers significantly. The concentration of free ammonia (FA) was around 240 $mgNH_3\text{-}N \cdot L^{-1}$ using the equilibrium constant of NH_3/NH_4^+. This FA concentration is above the inhibition range compared to conventional wastewater treatment inhibition concentrations of FA (Liu *et al.*, 2019). Despite these high FA concentrations q_{VFA} values obtained in this work

were comparable to those observed previously, which demonstrates the capacity of the process to adapt to unfavourable environmental conditions.

An overall VSS yield on COD_{Sol} of $Y_{\frac{X}{sCOD}}$= 0.25 $gVSS \cdot gCOD_{Sol}^{-1}$ was observed and this was lower than values reported in literature for comparable systems: $Y_{\frac{X}{sCOD}}$ = 0.30 $gVSS \cdot gCOD^{-1}$ (Tamis *et al.*, 2018). The data suggest that the high pH and/or TAN concentration did not significantly affect the biomass specific uptake rate, but reduced the efficiency of biomass production. Furthermore, the presence of unstable periods indicated that the process was susceptible for unknown compounds present in the substrate. The leachate of organic waste is such a complex mixture making it virtually impossible to identify the cause of the unstable periods.

Despite the potentially adverse conditions, the obtained microbial culture appeared highly effective with respect to PHA production. According to the FISH picture as shown in figure 5.4 the microbial community was dominated by one species. The dominant bacterium was a large betaproteobacteria most closely related to *Thauera Cin3,4*. The species found in the bioreactor had comparably high substrate uptake rates and oxygen respiration rates which are known for the *Plasticicumulans* genus, even though, both genera are phylogenetically not related. The *uncultured Rhodocyclaceae bacterium clone JT01* described in this work belongs to the betaproteobacteria, family of *Rhodocyclaceae*, whereas *Plasticicumulans* is a gammaproteobacteria. Both bacteria have functional and morphologically similar properties: the bacteria are large, rapid VFA consumers and the ability to store PHA up to high wt%. The results demonstrate that high respiration rates and high PHA storing capabilities are distributed over different sections of the phylogenetic tree.

PHA production from leachate derived from OFMSW required multiple levels of understanding of the production process. The COD_{Sol} present in the stream contained VFA that would result in PHA producers, but the stream also contained COD_{Sol} resulting in non-PHA producers. Furthermore, the leachate contained non-biodegradable (inert) solids, that were divided in settleable solids and non-settleable solids. The settleable solids were removed by a pretreatment method in which 60% of the solids were removed. The non-settleable solids entered the reactor with the influent and were observed in comparable concentrations in the reactor effluent suggesting that they were not incorporated into the settling biomass in significant amounts. The fraction of COD_{Sol} consumed by the PHA producer in the bioreactor was on average 82% based upon the VFA consumed compared to ΔCOD_{Sol}. The impact of BOD_{other} resulting in side population was minizimed by introduction of a settling phase shortly after all VFA were depleted as proposed in (Korkakaki, van Loosdrecht and Kleerebezem, 2016).

Solid partitioning

In an operational cycle, multiple phases could be identified, which influenced the final composition of the VSS. We assumed that three main components contribute to the final VSS composition: (i) the COD_{VFA} which resulted via the production and conversion of PHA in PHA producing biomass, (ii) BOD_{other} consumption resulting in a non-PHA producing side population and (iii) inert VSS present in the substrate. Four time points during a cycle were evaluated (figure 5.6).

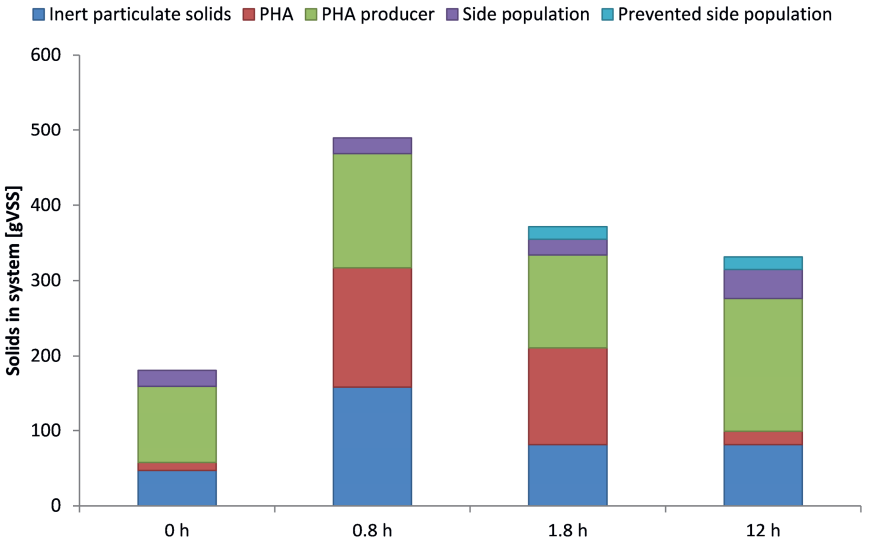

Figure 5.6 – The solid partition over the cycle. Here 0h represented the beginning of the cycle, 0.8 h represented the time point in the cycle when all VFA were consumed, 1.8 h was the solid composition in the reactor after 1 h settling and removal of the supernatant. Finally, 12 h represents the end of the cycle

The starting point of each cycle (t = 0 h) is after biomass removal at the end of the previous cycle.
After t = 0, the reactor was filled with substrate adding COD_{Sol}, nutrients and inert particulate solids in the system. Next, the culture consumed the VFA, resulting in VSS production as PHA and PHA producing biomass. On average 0.82 $gCOD_{VFA} \cdot gCOD_{consumed}^{-1}$ was consumed in the entire cycle, this value was used as $f_{X,PHA}$ (as explained in the method section). After all VFA had been depleted (0.8 h) a settling phase was scheduled lasting from 0.8 h – 1.8 h. In this phase, solids could settle to the bottom of the bioreactor for 60 minutes after which the supernatant (86 L) was removed from the system. With the supernatant at least 2 compounds were removed from the system: (i) inert solids that were not incorporated in biomass and (ii) half of the slowly degradable

BOD_{other} that was not degraded in the feast phase. In the famine phase lasting from 1.8 h – 12 h the PHA was consumed and X_{PHA} was produced. Furthermore, during the famine phase BOD_{other} was consumed which resulted in the production of side population. The side population ($f_{X,other}$) was calculated using the amount BOD_{other} consumed. Finally, the BOD_{other} that was present in the supernatant could be converted to biomass equivalents using the VSS production yield. This potential biomass removed after settling was classified as prevented side population. In the set-up used in this study, the fraction of PHA producer present in the VSS was 0.56 $gX_{PHA} \cdot gVSS^{-1}$. The portion of PHA producers would have been 0.25 $gX_{PHA} \cdot gVSS^{-1}$ without a settling phase and no pretreatment of the substrate. The higher fraction of PHA producing biomass in the set-up with settling, implicates that higher PHA content may be reached: 0.77 $gPHA \cdot gVSS^{-1}$ with the settling vs. 0.61 $gPHA \cdot gVSS^{-1}$ without settling.

Accumulation potential

The PHA accumulation potential of the enrichment was assessed by doing multiple batch experiments to determine the maximum PHA content that could be achieved. One factor that was important enabling the relatively high PHA content was the pretreatment of the influent removing undesired settleable solids. After pretreatment by settling, the substrate still contained 1.5 ± 0.8 $gTSS \cdot L^{-1}$ (n=122) of non-settling solids. In the accumulation experiment shown in figure 5.5 this contributed for 30% of the TSS concentration at the end of the accumulation. In several accumulations it was shown that high apparent PHA wt% such as PHA percentages between 70% and 80% could be established after settling and removal of the supernatant at the end of the accumulation. These experiments showed an additional advantage of the settling technique besides selective removal of side population. Having a settleable culture made it possible to achieve high apparent PHA wt% in a complex environment containing non-settling solids.

Generally, achieving more than 0.60 $gPHA \cdot gTSS^{-1}$ and usage of mixed cultures requires defined environments. The PHA content achieved in this study was higher than the PHA content reached on similar leachate in the lab (0.29 $gPHA \cdot gVSS^{-1}$), (Korkakaki *et al.*, 2016). In Korkakaki et al. (2016b) it was suggested that an unsuccessful enrichment resulted in poor PHA storing capabilities. Remarkably, in the Korkakaki et al. (2016b) study, a culture enriched on synthetic medium was capable of accumulating up to 0.78 $gPHA \cdot VSS^{-1}$ in batch experiments using a similar type of leachate as substrate. That study, performed at pH 7, indicated that in the short term the complex medium (in the leachate) was not significantly inhibiting PHA production, though long-term cultivation on leachate resulted in enrichment of biomass with poor PHA

accumulating abilities. This phenomena of obtaining a bad enrichment at prolonged exposure to this complex substrate seemed absent in this study, because an enrichment with good PHA accumulating capability was obtained. Possibly, better control of nitrogen and phosphorus availability (by measuring these concentrations on a daily basis, and making sure that they are not limiting) could explain the difference. This would make sense since the limitation of N or P during enrichment will give a competitive advantage to microorganisms with high N or P uptake rate instead of high PHA production capability (Johnson, Kleerebezem and Mark C.M. van Loosdrecht, 2010).

To reach a high PHA content a nutrient limited stream is prefered, because of the growth limiting condition created which favors the PHA purity at the end of the accumulation (Johnson, Kleerebezem and Mark C M van Loosdrecht, 2010; Korkakaki, van Loosdrecht and Kleerebezem, 2017). PHA storing potentials using streams containing nutrients were generally lower than those obtained with nutrient limitation. A culture enriched on fermented wastewater treatment plant (WWTP) sludge as substrate for PHA accumulation which was not nutrient limited reached 0.39 gPHA·gVSS^{-1} (Morgan-Sagastume *et al.*, 2015). Leachate as used in this study comprised not only conditions where growth was possible, but also other adverse conditions such as a high pH after aeration and containing a high total ammonia nitrogen content. Despite these conditions 0.61 gPHA·gTSS^{-1} ± 0.14 (n=7) and expressed per organic content 0.77 ± 0.18 gPHA·gVSS^{-1} (n=3) was obtained, highlighting the importance of having a good inoculum (enrichment).

Industrial implemepntation of PHA production

Table 5.6 – Overview of achieved PHA percentages and PHA production yields on different types of waste streams.

PHA percentage	Yield	Substrate	Scale	Reference
$gPHA \cdot gVSS^{-1}$	$kgPHA \cdot kgCOD^{-1}$			
0.29	0.07	Leachate of OFMSW	lab	(Korkakaki *et al.*, 2016)
0.39		Fermented WWTP Sludge	pilot	(Morgan-Sagastume *et al.*, 2015)
0.46		OFMSW-SS mixture	Pilot	(Valentino *et al.*, 2019)
0.48		Leachate of OFMSW	lab	(Colombo *et al.*, 2017)
0.49	0.11	Fermented organic residues	pilot	(Bengtsson *et al.*, 2017)
0.55		Leachate of OFMSW	pilot	(Valentino *et al.*, 2018)
0.76	0.18	Fermented candy bar factory water	pilot	(Tamis, Lužkov, Jiang, M. C. M. va. Loosdrecht, *et al.*, 2014)
0.77	**0.14**	**Leachate of OFMSW**	**pilot**	**This study**
0.80	0.20	Fermented papermill wastewater	pilot	(Tamis *et al.*, 2018)
0.89		Synthetic medium	lab	(Johnson *et al.*, 2009)

The production of PHA on different types of wastewater has been investigated on pilot scale in several studies as shown in table 5.6. The high PHA contents reported, suggest that PHA producing microorganisms have been effectively enriched on different wastestreams. One way of evaluating PHA production from OFMSW was done via the overall PHA production yield. Using an adapted method for obtaining an overall process yield as defined by (Bengtsson, Werker and Welander, 2008), the overall PHA production yield on COD_{Sol} in the wastewater was determined. At the Biocel, a VFA-rich stream was present, thus no pretreatment and COD losses in this step were taken into account for the determination of the overall PHA yield. An overall PHA production yield of 0.14 $kgPHA \cdot kgCOD_{Sol}^{-1}$ was obtained using the following parameters and visualized in figure 5.7:

- A biomass production yield of 0.25 $kg\ VSS \cdot kgCOD_{VFA}^{-1}$
- A PHA production yield of 0.44 $kgPHA \cdot kgCOD_{VFA}^{-1}$
- A PHA purity at the end of the accumulation of 0.77 $kgPHA \cdot kgVSS^{-1}$ (table 5.6)
- A VFA fraction in substrate of 0.50 $kgCOD_{VFA} \cdot kgCOD_{Sol}^{-1}$

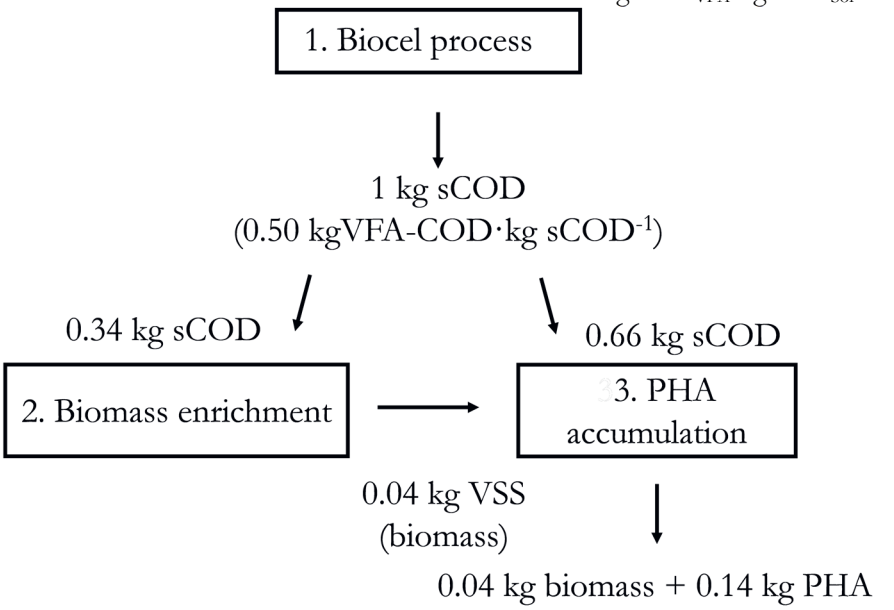

Figure 5.7 – COD partitioning for the enrichment and accumulation reactor, from 1 $kgCOD_{Sol}$ of leaching product obtained from the biocel process.

This yield was lower than the PHA production yield obtained in several other studies shown in table 5.6. The PHA production yield expressed per $kgCOD_{VFA}$

is much higher and equals 0.29 kgPHA·kgCOD_{VFA}^{-1} because the VFA fraction of the COD_{Sol} was 50%. It should be noted that a large part of the remaining COD was inert COD.

The main upstream operational expenses originate from aeration of the reactors and acid/base dosage (Tamis *et al.*, 2018). This study is a case for potentially eliminating one of these cost factors namely, the acid/base dosage. For the enrichment and accumulation in this study no acid or base was used, while both an adequate enrichment of PHA producing biomass, and a high PHA content at the end of an accumulation were achieved. A previous study has shown the economical potential of producing PHA from a stream like leachate instead of biogas (Kleerebezem *et al.*, 2015). One of the key criteria in the economic model is that the operational expensens should be kept low, which can be pursued when pH control is absent. If pH control is not a prerequisite for PHA production from wastewater the overall process becomes economical more feasible.

In this study the substrate used for the pilot plant was diluted 2-3 times with tap water, preventing oxygen limitation in the selector. Besides the COD, other compounds such as ammonium were diluted as well. In a full-scale situation the dilution will be done with effluent recirculation of the PHA process if necessary, to avoid oxygen limitation. The dilution performed with effluent instead of tap water will result in that the ammonia no longer will be diluted and will likely reach 1-2 $gN \cdot L^{-1}$. At operational pH-values of 9 these ammonium concentrations may give rise to inhibition. In a full-scale process, nitrogen removal using the anammox process can be implemented to remove (partly) nitrogen and thus alkalinity from the system after the PHA process. The VFA fraction in $kgCOD_{VFA} \cdot kgCOD_{Sol}^{-1}$ was low in this study due to the origin of the substrate from a methanogenic OFMSW digester, implying the effective removal of VFA by methanogens. Evidently, eventual implementation of the PHA production from OFMSW is anticipated to be based on a hydrolysis pilot that maximizes VFA production and aims for minimization of methane production. This will evidently increase the overall effectiveness of the PHA production process from OFMSW.

5.5 Conclusion

This study shows the successful enrichment of a functional PHA accumulating microbial community grown on OFMSW-leachate without pH control on pilot scale. After accumulation for maximization of the cellular PHA content a maximum PHA content of 0.77 ± 0.18 $gPHA \cdot gVSS^{-1}$ (n=3) was achieved within 3 hours of accumulation. Detailed mass balance analysis demonstrated that the actual PHA content in VFA grown cells was highly comparable to previous studies, and the eventual lower PHA content was the resultant from particulates in the influent and growth of non-PHA producers on substrates other than VFA. This demonstrates the technological feasibility of PHA production in conditions

formerly considered unfavourable for PHA production, such as a pH of 8.5-9 and high ammonium concentrations. Herewith this work contributes to the extension of the operational window of the wastewater based PHA production process and demonstrates that the hoarding strategy based on PHA production is widely distributed in the microbial world.

5.6 Appendix

Leachate alkalinity calculations

The alkalinity of the leachate was estimated using the pCO_2, pH, total TAN and the known VFA concentration. The pCO_2 in the digestion tunnels was unkown, to estimate the pCO_2 the following assumption was made: At the end of a cycle in the enrichment reactor the pCO_2 was estimated to be 0.1% close to atmospheric values as the broth was aerated for 12 hours. At this point in the cycle the pH reached 9.1. This pH and pCO_2 was used as input for the leachate to obtain the alkanity of the sytem. The alkalinity of the system was calculated as follows:

$$Alkalinity = [OH^{-1}] + [VFA^{-1}] + [HCO_3^{-1}] + 2 \cdot [CO_3^{-2}] \quad \text{(A1)}$$

- Alkalinity was the calculated alkalinity expressed in meq·L^{-1} of the leachate
- [OH^{-1}] was the concentration of hydroxide (mM) in the leachate
- [VFA^{-1}] was the averaged concentration of VFA (mM) in the leachate
- [HCO_3^{-1}] was the concentration of bicarbonate (mM) in the leachate
- [CO_3^{-2}] was the concentration of carbonate (mM) in the leachate

The Henry coefficient for CO_2 (H^{CO2}) used was $2.98 \cdot 10^{-2}$ mol·L^{-1}·atm^{-1} for 30 ºC. The pKa values for each compound contributing to the overall alkalinity are summarized in table 5.7.

Table 5.7 – pK_a values used in this study to estimate the alkalinity of the leachate.

pK_a	Chemical reaction
6.38	H_2CO_3 <--> HCO_3^- + H^+
10.33	HCO_3^- <--> CO_3^{2-} + H^+
4.77	VFA <--> VFA^- + H^+
9.30	NH_4^+ <--> NH_3 + H^+

The alkalinity of the leachate was found to be 70 ± 10 meq·L^{-1}. The pH of the substrate was continuously measured when stored in the buffer vessel and was 7.53 ± 0.42. The alkanity obtained at pH of 9.1 and a pCO_2 of 0.1 % should be similar to the alkanity at a pH of 7.5 as measured in the buffer vessel. Using a pH of 7.5 and an alkalinity of 70 meq·L^{-1} a partial pressure for CO_2 of 9% was found.

6

Outlook

The research described in this thesis adds knowledge to the topic of valorisation of industrial wastewater using mixed microbial cultures to produce volatile fatty acids (VFA) and polyhydroxyalkanoates (PHA). In this thesis anaerobic fermentation processes for VFA production were explored using anaerobic granular sludge. We investigated the effect ammonium limitation and prolonging the solid retention time (SRT) on the VFA production yield and product spectrum. In the PHA production process the focus was on exploring to which extent strict uncoupling of growth and accumulation is required. Additionally, we investigated the feasibility of producing PHA from the organic fraction of municipal solid waste (OFMSW) at the Orgaworld treatment facility in Lelystad, The Netherlands. An overview of the processes and on which process the knowledge gained from each chapter could be applied is shown in figure 6.1

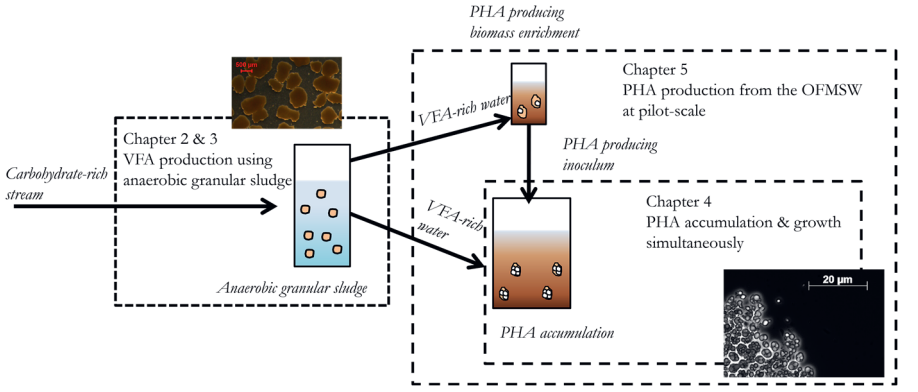

Figure 6.1. Overview of the PHA production upstream production process. First, a fermentation step is producing VFA which in the enrichment and accumulation process are consumed to produce PHA the final product.

Anaerobic VFA producing granular sludge

Biomass granulation

In this work a pragmatic approach was presented for the creation of granular sludge from flocculent sludge for effective start-up of a granular sludge process. This approach is based on visible separation of the sludge from the supernatant regardless of the settling time. There are many different strategies mentioned in literature for creating aerobic granular sludge and anaerobic granular sludge which are often based upon synthrophic relations (Liu *et al.*, 2003; Wilén *et al.*, 2018). A non-syntrophic model is the spaghetti model where filamentous *Methanosaeta* are responsible for forming a three-dimensional network as the basis for a granule (Wiegant, 1988). Another model describing the granulation process is the local dehydration model which states that 'when bacterial surfaces are strongly hydrophobic, irreversible adhesion will occur'. However,

experimental evidence shows that acidogens display hydrophilic properties (Liu *et al.*, 2003). In VFA producing systems such as used in the research for this thesis the microbial consortia consisted mainly out of acidogenic bacteria. Furthermore, in the granules produced in chapter 2 and 3 usually one dominant microorganism was determined according to 16S rRNA analysis, likely showing that not many syntrophic relations were occurring.

Prior to starting this work, the idea of obtaining anaerobic VFA producing granules was that a short settling time immediately should result in granule formation as proposed by Tamis et al., (2015). Granule formation became visible in that study within 2-3 d after inoculation by setting the settling time at only 2 min. In this work (Chapter 2) the start-up strategy proposed by Tamis et al. (2015) could not be reproduced. Instead, biomass granulation was achieved by setting the settling time at 3 h initially based upon previous work where a longer settling time was chosen to acclimatize the slow settling biomass (Pronk, Abbas, *et al.*, 2015). After retaining all biomass the settling time was step-wise lowered to enrich for fast-settling granules as shown in chapter 2. This settling time was based upon that at least 90% of the biomass should be under the effluent point. A next challenge could be to gain a better understanding on how to obtain a stable granular system in continuous fed reactors as the substrate gradient is lower compared to pulse-wise fed systems.

Settling properties

One of the advantages of using granular sludge is the high volumetric productivities that can be achieved. A high biomass concentration enables these high productivities, and the amount of biomass that can be present in the reactor depends on the SVI of the sludge. The process of becoming a dense enrichment (i.e. low SVI <15 mL·gTSS^{-1}) was found to be dependent of the settling time (chapter 2). When the settling time was increased a more compact sludge bed after settling was obtained (defined as a lower SVI). This was shown in chapter 2 where a biomass concentration of 60 gVSS·L^{-1} was obtained in a lab-scale reactor, this corresponded to a biomass concentration in the settled sludge bed of 120 g/L or an SVI of 8. To achieve these high biomass concentration values, biomass removal was only conducted with the effluent, maximizing the biomass concentration. When a long settling time was applied and biomass was actively removed from the sludge bed to achieve a certain SRT (shorter than 40-50 d), the SVI of the culture increased (chapter 2). In chapter 3 also an anaerobic VFA producing granular enrichment was operated. The settling time in that enrichment was set at 2 min and biomass was not actively removed. Despite the maximization of the biomass due to no biomass removal a relatively high SVI was obtained (SVI > 15 mL·gTSS^{-1}). It seemed to achieve a low SVI (<15 mL·gTSS^{-1}) in the lab set-up not only biomass concentration should be maximized but the settling time should be set at least to 60 min as well. An

overview of the different settling settings are visualized in figure 6.2. Thus, there seems to be a cut-off between fast settling and SVI, investigating this trade-off could improve overall system performance. Overall, low SVI cultures can be of interest as a low SVI can be associated with a high biomass concentration.

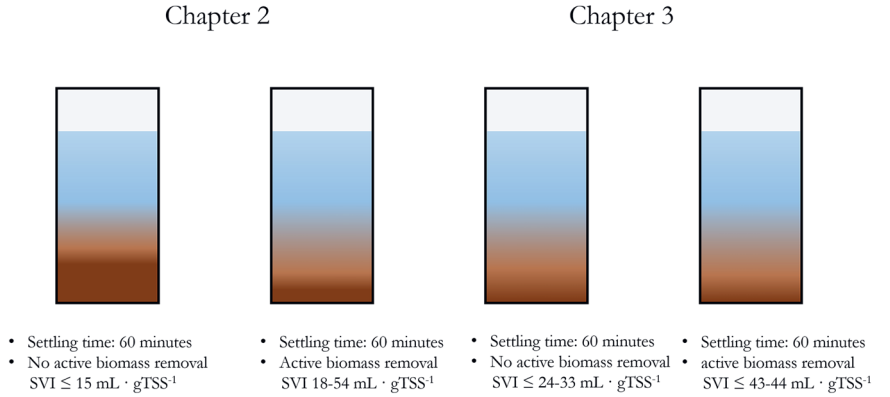

Figure 6.2. Visualisation of some key operational parameters affecting SVI and their outcome.

VFA production using anaerobic granular sludge

The aim of this work (Chapter 2 and 3) was to investigate the effect of different operational parameters on the VFA yield using anaerobic granular sludge. In chapter 2 the following 3 parameters were changed compared to Tamis et al., (2015) with the objective to control the SRT in the process: (i) The settling time was increased to 60 min., (ii) the biogas was recirculated instead of continuous dilution of the off gas by input of N_2, and (iii) the cycle length was increased from 2 h to 6 h.

One of the major changes observed after increasing the SRT in the process was the shift in microbial community structure as identified by 16S rRNA. In the reactor operated according to Tamis et al. (2015) with an SRT of 1-2 d *Clostridium pasteurianum* was the dominant microorganism. *Clostridiae* are often encountered in open culture fermentation under slightly acidic conditions and glucose as carbon source similar to this study (Tamis *et al.*, 2015; Rombouts *et al.*, 2019a). In chapter 2 the reactor with the long settling time and the other 2 changes aforementioned, enriched for *Bifidobacterium scardovii* as dominant species according to 16S rRNA analysis (figure 2.5). After decreasing the SRT to values comparable to those imposed by Tamis et al. (2015), the *Bifidobacterium* remained the dominant species in the subsequent enrichments and the community did not enrich for *Clostridium pasteurianum*. This indicated that either a local optimum was reached in the enrichment or that more than solely the SRT in this case

influenced the microbial community. In a local optimum, a specific functional performance can be stably maintained even though microorganisms active in the global optimum may have more favourable kinetic properties. Still, operation of both systems was not identical and minor differences may have triggered the establishment of a different functional performance and corresponding microbial community structure and it is interesting to gain more knowledge about how and why this change occurred.

Some potential factors that may have contributed to the microbial shift observed will be elaborated in this paragraph. First, the ability to thrive when H_2 and CO_2 are continuous present at elevated concentrations. In the granular work presented so far a *Clostridium* species was obtained when the headspace was continuous flushed with N_2 thus effectively flushing out the produced H_2 and CO_2. As described in chapter 2 gas mixing was changed from continuous input of N_2 to recirculation of biogas. When recirculating the biogas consisted out of significant levels of H_2 and CO_2 (>10% m/m) as a consequence of the *Clostridium* fermentation producing acetate, butyrate, CO_2 and H_2. After this transient time, and changing the SRT towards 40-50 d the headspace basically contained only CO_2 and N_2 and at this point according to 16S rRNA analysis the dominant microorganism was a *Bifidobacterium*. De Kok et al., (2013) showed a lower H_2 production and shift from a butyrate/acetate system to propionate/butyrate/acetate system at lower growth rates (20 h vs 8 h SRT) and high pH_2 (>0.19 atm). This could indicate that pH_2 could play a role at lower growth rate systems such as a granular system. (ii) A second trigger could be the settling time that was prolonged from 2 min to 60 min. The selection is on fast settling when using a short settling period, if a granule/microorganism is not capable in fast settling (<2 min) it will be washed out. When the settling time was increased to 60 min the selection pressure changed slightly. Potentially allowing to select for different microorganisms. Still the microorganism should settle relatively fast. However, more biomass could remain in the reactor if the culture possessed the ability to thicken in, creating denser structures. The obtained *Bifidobacterium* prevails in this regard versus the fast settling ability of *Clostridium* granules as lower SVI were obtained with *Bifidobacterium*. (iii) A third possibility could be the accumulation of dead/inactive biomass in the sludge bed. When the operational settings were such that around 60 $gVSS \cdot L^{-1}$ were present in the reactor, likely part of this biomass was dead/inactive but incorporated in the VSS. This was reflected in the specific substrate uptake rates (q-rate) in Chapter 2, all three enrichments were dominated by the same microorganism though lower q-rates were observed at longer SRT. This old biomass after some time could decay and release proteins/products other cells are not able to produce, this could stimulate some form of auxotrophic behaviour. It is interesting to find out which of these triggers if any induced the shift in microbial community. With the shift of microbial community also a

distinct different product spectrum was obtained. It would be insightful to know exactly what operational conditions enabled the enrichment for the *Bifidobacterium* as a product spectrum without H_2 was obtained resulting in a high VFA yield on glucose. Additionally, it could be interesting to apply this long SRT strategy using other substrates then glucose to investigate if also distinct product spectra can be obtained as a function of the SRT.

PHA production from organic waste(water)

Producing PHA from organic waste streams has been investigated intensively (Johnson *et al.*, 2009; Jiang *et al.*, 2012; Marang *et al.*, 2013; Tamis, Lužkov, Jiang, M. C. M. va. Loosdrecht, *et al.*, 2014). The lion share of the work regarding PHA production from wastewater was focussed on gaining a better understanding of the selection process. In this work the technological feasibility of PHA production from a specific feedstock was investigated: leachate of OFMSW was investigated at pilot-scale (Chapter 5). Furthermore, the impact of the carbon to nitrogen and carbon to phosphorous ratio on the (fed)-batch PHA accumulation process was investigated (Chapter 4).

No active pH control

As mentioned before a lot of research effort has been dedicated to the selection process. In this way a lot of information has been gained about the conditions that enable the effective enrichment of *Plasticicumulans acidivorans*, which was shown to be a superior PHA producing microorganism (Johnson *et al.*, 2009; Marang *et al.*, 2013). Basically, all the lab/pilot work done on *P. acidivorans* was conducted using a controlled environment with amongst others a working pH of 7. In the work done at 'Orgaworld' (Chapter 5) pH control rendered not feasible thus no pH control was done. The resulting pH in the process was 8.5-9 at the end of a cycle, even though, the pH of the leachate in the buffer vessel was around 7. Despite these unfavourable pH-values and the absence of nutrient limitation a PHA amount of 0.77 ± 0.18 (n=3) $gPHA \cdot gVSS^{-1}$ was achieved which was amazing considering the conditions. The dominant microorganism in the culture was identified to be a *Rhodocyclaceae bacterium* through 16S rDNA sequencing and subsequent FISH microscopy. This study showed that nor pH control nor strict nutrient limitation is a prerequisite for obtaining high PHA wt%. Though, the effect of no pH control is not investigated in a more controlled set-up. As shown in Tamis et al., (2018) pH control accounts for a large part the upstream production cost. Eliminating these costs benefits the waste-based production of PHA. Possible scenarios of interesting research could be no pH control under varying alkalinities, or no pH control at different NH_3 concentrations.

Settling capabilities

Another important finding from Chapter 5 was that the elevated solids concentration in the leachate represented a serious limitation in the PHA production process as shown in figure 6.3. In the work described in Chapter 5 the concentration of solids in the influent was minimized by pre-settling of the wastewater for 24 h. Furthermore, to optimize the enrichment of PHA producing biomass, a settling step was incorporated in the operational cycle after the feast phase. This settling step enabled the removal of solids that are not incorporated in the PHA producing biomass. In this way the enrichment of PHA producing biomass was optimized through removal of non-productive solids in the process.

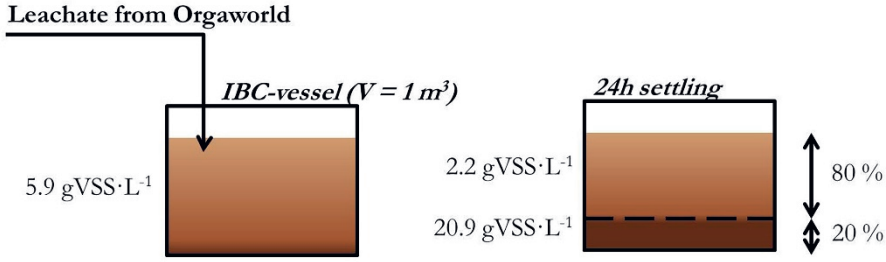

Figure 6.3. The effect of the pre-treatment visualized in which untreated leachate was settled for 1 d in a 1 m³ vessel.

From figure 6.3 can be seen that by pre-treating the leachate a large fraction of the solids was removed that lower the eventual PHA% achieved. In the PHA production process the biomass content gets diluted by non-biodegradable solids in the substrate. Without pre-treatment it would have been more challenging to reach high PHA purities. In this study the settling time was set to 30 min. Potentially, dense granules are not ideal as oxygen limitation can occur inside a granule. Oxygen limitation seems unfavourable as the selective pressure can shift towards an oxygen affinity based system instead of a substrate uptake based selective system. On the contrary compact granules are easy to separate from the liquid, thus it could also benefit the waste-based production of PHA. However, these are all assumptions and should be tested. Gaining more control over the settle-ability of biomass is almost a necessity in order to obtain high purities when working with waters that contain solids.

Industrial application

The research performed in this study was part of the partnership-program initiated by Paques b.v. and NWO-TTW. In this program the aim was to investigate and optimize the waste-based valorisation of VFA. This thesis adds knowledge to this program on two levels. (i) It focusses on VFA production

from carbohydrate-rich wastewater. Done in the lab operating anaerobic granular sludge fed with glucose at a pH of 5.5 and at different operational parameters (chapter 2 and 3). (ii) We looked at valorisation of VFA towards the production of PHA (chapter 4 and 5). Other topics of the program included recovery of VFA recovery by production of ketones and vinyl ester platform chemicals or ester production integrated with acidogenesis which were outside the scope of this thesis.

As shown in this thesis biotechnologically it is possible to produce VFA and PHA from a wide range of different industrial wastewaters. One of the biggest hurdles for adoption of the waste-based production of PHA is the limited amount of product applications. The current status-quo is a chicken or the egg dilemma. Companies would like to test large quantities of PHA, for which in order to produce these quantities a demo-scale or bigger is necessary. To justify the production of a demo-scale or larger preferably a market is guaranteed, hence you get the chicken or the egg dilemma. A potential opportunity for PHA right now is the self-healing concrete application (Jonkers, 2007). In this application concrete is mixed with spores containing food (PHA) and microbes. The result is that widespread throughout the concrete after hardening the spores are present. Cracks occur in the concrete after which water can enter the concrete and corrode the system. The spores present in the concrete are activated by the water and after activation PHA consumption will start. During the consumption of PHA the microbes produces CO_2 which can form with the readily available calcium in the concrete $CaCO_3$ which precipitates. The precipitated $CaCO_3$ fills the crack and the concrete is 'repaired' hence, the term self-healing concrete. As the PHA serves as 'food' the quality can be of low-grade making it a suitable application for a waste-derived product. Other applications could be that PHA serves as a slow-releasing fertilizer. An advantage of using PHA as slow-releasing fertilizer agent would be that PHA is bio-degradable. Additionally, as PHA does not contain nutrients after extraction an ideal mixture can be created to stimulate or inhibit the bio-degradation of the PHA making it a suitable candidate to serve as slow-releasing fertilizer agent. Finally, many high-end products can be derived from PHA making it an important polymer for the biobased economy.

Future research

For the production of VFA using anaerobic granular sludge in this thesis different hydraulic retention times (HRT) were applied over the two different studies. A consequence of this was that different feast to famine ratios were obtained. For follow-up research a uniform reactor setting should be used to research the exact effect of SRT. For this set-up probably a long settling time will be required to reach a low SVI and thus a long SRT, the other factors i.e. (i) HRT (ii) input of fresh N_2 for biogas dilution; and (iii) non-auxotrophic conditions should be investigated separately. These experiments would elucidate

whether the fraction of e.g. propionate in the product spectrum is truly depended on the SRT or some form of combination of the abovementioned arguments. The first explorative experiments have been performed indicating the next set of research objectives. The challenges to elucidate multiple research questions at once will be making a parallel reactor set-up in which all experiments can be conducted uniformly.

In the lab we have shown that applying the granular sludge technique for the production of VFA is feasible. A next step could be to test whether this technique can also be applied on pilot/full-scale and similar results can be obtained. It could be a challenge to operate a stable granular system with a constant product spectrum on wastewater, as wastewater contain various kind of (in)soluble compounds which are not solely glucose as used in this thesis. One of the challenges that can occur on pilot/full-scale due to less process control could be the suppression of methanogens. Finally, to facilitate the waste-based production of VFA other products then PHA should be evaluated. Perhaps, not all streams that can be fermented using granular sludge are suitable for PHA production and/or different products are economically more favourable to produce than PHA.

Another interesting field of research only slightly touched upon in this thesis is the production of EPS from wastewater. EPS is gaining traction at the moment and the first plant dedicated for the production of EPS is being realized using the aerobic granular sludge technology ('Nereda®' technology). With the upswing of EPS, in the future sludge generated at wastewater treatment plants can be considered as being valuable instead of being waste. The research field of producing EPS is wide-open. Only limited knowledge exists linking operational conditions to specific EPS characteristics, for example glucose- or protein-rich EPS. Interesting research topics relating could be to enrich nutrient limited. In chapter 3 was shown that when no ammonium was dosed to the enrichment likely the EPS content increased significantly making this is an attractive strategy for EPS production. Another research field within the EPS research could be the the biodegradability of the EPS. In chapter 2 the enrichment SRT varied from 1 to 50 d. In the 40-50 d enrichment the produced EPS was likely robust as the EPS similar to the microorganisms had to stay for 40-50 on average in the reactor. This in contrast to the 1-2 d SRT enrichment where the EPS had to be less robust as it will leave the reactor on average within 2 d. In chapter 2 the focus was on producing VFA and the EPS was not investigated in great detail, though could have been interesting to still do. There are many other variables that can be researched to investigate the effect of the quality and quantity of EPS production, such as salinity, different substrates and many more possibilities. Generally, EPS is an interesting polymer which can upgrade the value of for example wastewater treatment plants.

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List of publications

J. Tamis, **M. Mulders**, H. Dijkman, R. Rozendal, M.C.M. van Loosdrecht, R. Kleerebezem, "Pilot-Scale Polyhydroxyalkanoate Production from Paper Mill Wastewater: Process Characteristics and Identification of Bottlenecks for Full-Scale Implementation", Journal of Environmental Engineering, 144(10), p. 04018107. doi: 10.1061/(ASCE)EE.1943-7870.0001444 (peer-reviewed article)

E. Korkakaki, **M. Mulders**, A. Veeken, R. Rozendal, M.C.M. van Loosdrecht, R. Kleerebezem, "PHA production from the organic fraction of municipal solid waste (OFMSW): Overcoming the inhibitory matrix" Water Research, 96, pp. 74–83. doi: 10.1016/j.watres.2016.03.033. (peer-reviewed article)

M. Mulders, J. Tamis, B. Abbas, J. Sousa, H. Dijkman, R. Rozendal, R. Kleerebezem, "Pilot-scale polyhydroxyalkanoate (PHA) production from organic waste: Process characteristics at high pH and high ammonium concentration" (peer-reviewed article)

M. Mulders, J. Tamis, G.R. Stouten, R. Kleerebezem, "Simultaneous growth and polyhydroxyalkanoate (PHA) accumulation in one bioreactor using a P. acidivorans enriched culture" (peer-reviewed article)

M. Mulders, Á.E. Alonso, J. Tamis, G.R. Stouten, M. Pronk, R. Kleerebezem, "VFA production using anaerobic granular sludge: product yield maximization by varying the solid retention time" (peer-reviewed article)

Curriculum vitae

Michel Mulders

Michel Mulders was born on 7th of October 1991 in Bergen op Zoom (the Netherlands. After finishing high school, he went on to study Life Science and Technology in Leiden and at Delft University of Technology. He got passionate for the 'technology' of the curriculum after his internship at 'Cell Systems Engineering' at TUDelft. Michel went on to successfully finish his Master of Life Science and Technology and got in touch with open culture technology at the 'Environmental Biotechnology' group.

After a short intermezzo in supporting the bioplastic production research, he started his PhD under the supervision of Robbert Kleerebezem and Mark van Loosdrecht. The research of this PhD was focused on the production of VFA and PHA of organic waste stream, that resulted in this thesis.

He made the switch from academia to consultancy as he is currently working as a water technology specialist at Arcadis.

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De 'papa' van het bioplastic onderzoek bij de TU Delft, want zo mogen we je wel noemen, *Robbert*. Aan het einde van een maaltijd zeiden we altijd: "Papa betaalt wel." Ook al zaten we daar met allerlei mensen: blond haar, bruin haar, groot of klein, noem het maar op. Voor ons was je op dat moment 'papa'. Ik kan niet met woorden beschrijven hoeveel ik van je geleerd heb, daarom ga ik dat ook niet proberen. Jij hebt van mij een betere wetenschapper gemaakt dan ik ooit heb durven dromen. Het belangrijkste voor mij was dat we dit lachend deden. Het was voor mij een groot plezier en een voorrecht om met je te mogen samenwerken. Ik wens je het allerbeste toe.

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Jelmer, maat, wij hebben zoveel gemaakt. We hebben het noordelijk halfrond van Nederland ten behoeve van bioplastic verkend. Ons avontuur begon in Groningen, toen je een geweldige manier had gevonden om bioplastic te drogen. Het waren in ieder geval mooie Breaking-Bad taferelen, achteraf kunnen we er gelukkig om lachen. Ook van jou heb ik zoveel geleerd, zowel op wetenschappelijk vlak, maar ook als mens. Het gaat je goed! Dat we in de toekomst elkaar nog mogen tegenkomen. Succes met een succesverhaal maken van bioplastic.

Gerrie Gerrie (dit is geen 'typfout') de alleskunner en alleskenner. Het daadwerkelijk zaken beter weten dan andere zou een irritante eigenschap zijn, maar gelukkig zit je hart op de juiste plek en ben je een hele toffe peer. Jij had

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Udo, onze patatkoning, mede-Brabander en bovenal YouTube koning. Mijn tijd bij EBT zou niet hetzelfde zijn geweest zonder jou. Wat een gezelligheid breng jij mee.

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