Resource recovery from organic waste streams by microbial enrichment cultures

Proefschrift

ter verkrijging van de graad van doctor

aan de Technische Universiteit Delft,

op gezag van de Rector Magnificus prof. ir. Karel Ch.A.M. Luyben;

voorzitter van het College voor Promoties,

in het openbaar te verdedigen op

woensdag, 6 mei, 2015 om 15:00 uur

door

Jelmer TAMIS

ingenieur, geboren te Alkmaar, Nederland

Dit proefschrift is goedgekeurd door de

promotor: prof. dr. dr. h.c. ir. M.C.M. van Loosdrecht en

copromotor: dr. ir. R. Kleerebezem

Samenstelling promotiecommissie bestaat uit:

Rector Magnificus	voorzitter
Prof. dr. dr. h.c. ir. M.C.M. van Loosdrecht	promotor
Dr. ir. R. Kleerebezem	copromotor

onafhankelijke leden:

Prof. dr. ir. J.B. van Lier	Technische Universiteit Delft
Prof. dr. ir. J.J. Heijnen	Technische Universiteit Delft
Prof. dr. ir. C.J.N. Buisman	Universiteit Wageningen
Prof. dr. ir. L.T. Angenent	Cornell University, United States of America
Prof. dr. E. Paul	University of Toulouse, France
*Prof. dr. ir. J. Pronk	Technische Universiteit Delft, reservelid

This research is supported by the Dutch Technology Foundation STW, which is part of the Netherlands Organisation for Scientific Research (NWO) and partly funded by the Ministry of Economic Affairs (project 11604 and 11605).

Cover illustration: Pascalle Vermeulen

Printing: Sieca Repro, Delft

ISBN: 978-90-9029011-9

Voor Kristina en Augustus

Table of contents

Summary

Polyhydroxyalkanoate (PHA) is a natural product that can potentially replace a part of the chemicals and plastics derived from fossil sources. One of the main barriers for market entry of PHA is its relatively high price compared to conventional (fossil) feedstocks. This high price is related to current industrial production methods which are based on the cultivation of pure microbial cultures of a single species that a.o. has to be protected from contaminations from unwanted microorganisms that invade the systems from the surroundings. These production methods consequently have to rely on expensive substrates and pre-sterilized equipment. It was proposed that the costs of PHA production can be reduced significantly by replacing the existing industrial practices with open cultures that do not require sterile conditions and use organic waste streams as a feedstock. Open culture processes have a free exchange with the surroundings and therefore any organism present in nature can in principle enter these systems. To make an open process for PHA production feasible, a selective environment needs to be applied that enriches for species with high PHA accumulation capacity. PHA is produced by numerous microorganisms in natural ecosystems as a reserve compound to balance metabolic requirements during the absence of external energy and carbon sources. Based on this ecological role of PHA, selective environments can be designed that provide a competitive growth advantage to species with a superior PHA producing capacity. One approach for selective cultivation of PHA producing species is the feast-famine process, in which the substrate is dosed in short pulses followed by relatively long periods (hours) of absence of external substrate. This process is relatively well understood viz. controlled conditions at lab-scale e.g. the enrichment of PHA producing cultures dominated by the specialised genus *Plasticicumulans (*that can accumulate up to 0.9 gPHA $gVSS^{-1}$) was reported for sequencing batch reactors that were operated under feast-famine conditions and at short solid retention time (i.e. 24 h) in a relatively long cycle (12 h) (Johnson et al. 2009; Jiang et al. 2011).

The objective of this thesis was the development of processes for resource recovery from wastewater with microbial enrichment cultures and to evaluate the industrial relevance of waste based PHA production, with a focus on the upstream part of the product chain: the production of PHA rich biomass. To this end, we investigated several topics related to the production of PHA from waste water using a three-step process: (1) pre-treatment to maximize the VFA concentrations, (2) enrichment of a microbial culture with high PHA storing capacity and (3) maximization of the PHA content in a fed-batch accumulation step.

The **first chapter** contains a general introduction of the topic and an explanation of the relevance and scope of the research.

In the **second chapter**, the pre-treatment of organic waste streams was investigated. The goal was to develop a process for efficient production of a VFA, the preferred substrate for PHA production. A granular sludge process for the production of VFA from a model substrate (glucose) at high rate, yield and purity, with minimized operational costs, was developed using an anaerobic sequencing batch reactor (ASBR) at low pH. The inclusion of a short (2 minute) settling phase before effluent discharge enabled effective granulation and very high volumetric conversion rates of 150-300 $kgCOD\ m^{-3}\ d^{-1}$. The product spectrum remained similar at the tested pH range with acetate and butyrate as the main products, and a total VFA yield of 60-70% on chemical oxygen demand (COD) basis. The requirement for base addition for pH regulation could be reduced from 1.1 to 0.6 mol OH^- $(mol\ glucose)^{-1}$ by lowering the pH from 5.5 to 4.5. Moreover, a virtually solid-free VFA stream could be achieved, which is advantageous to achieve high PHA contents in the accumulation step.

Wastewater often contains a fraction of lipids; these are not easily converted to volatile fatty acids in a pre-fermentation step. In the **third chapter** of this thesis, the conversion of lipids in the feast-famine process

was investigated. It was found that lipids do not contribute to PHA production in a standard feast-famine SBR. Instead, lipid-accumulating organisms were enriched. Further optimisation could potentially lead to a process for lipids recovery from wastewater, for instance for the production of biodiesel.

A modelling approach was used to compare the experimental data from the pilot- and lab-scale experiments. There are many models for feast-famine processes found in literature and the differences between the models used by different research groups hinders easy comparison experimental data. To enable better comparison of experimental results, a (concept) generalized model was developed in **chapter four**. Based on experimental data available in literature we have proposed model improvements for (1) modelling mixed substrates uptake, (2) growth in the feast phase, (3) switching between feast and famine phase, (4) PHA degradation and (5) modelling the accumulation phase. Finally, we provide an example of a simple uniform model.

In **chapter five** the industrial relevance of waste-based production is investigated in a pilot experiment at an industrial location. The Mars candy bar factory in Veghel, The Netherlands, was selected because of its favourable waste water properties: high VFA and low nitrogen content. The pilot setup was according the earlier described three-step process: (1) fermentable COD was converted into mainly VFA in an anaerobic pre-treatment step resulting in an average VFA fraction of 0.64 gCOD $gCOD^{-1}$; (2) selective enrichment in a 200 l SBR led to a microbial culture dominated by *P. acidivorans*; (3) the PHA content of the biomass was maximized in a fed-batch reactor resulting in an average PHA content of 0.7 gPHA $gVSS^{-1}$. The dominant presence of *P. acidivorans* indicated that the selective pressure in the pilot experiment was similar to the lab. The difference in the PHA content achieved in pilot and lab (0.9 gPHA $gVSS^{-1}$) could be explained by two main factors: the presence of non-VFA COD and solids in the waste water.

In **chapter six** an outlook for future development is provided. To replace existing chemical and polymer feedstocks with PHA, further optimization of the process is required. Amongst others minimization of acid and base consumption for pH control, production of clean effluent water, and the recycling of effluent water will still significantly contribute to process efficiency. Nevertheless, in the perspective of these results, we believe the optimization of waste-based PHA production in conceptually not limited by the bioprocesses investigated in this thesis. Instead the most important bottleneck for successful market entry is the development of economic down-stream processing and product utilization routes that enable conversion of the PHA-containing sludge into a marketable product.

Samenvatting

Polyhydroxyalkanoaat (PHA) is een natuurlijk product dat een gedeelte van de chemicaliën en plastics die momenteel uit fossiele bronnen (aardolie) gemaakt worden, zou kunnen vervangen. Een van de belangrijkste belemmeringen voor het naar de markt te brengen van PHA is de relatief hoge productiekost in vergelijking met producten uit conventionele (fossiele) bronnen. Deze hoge prijs heeft te maken met de manier waarop PHA momenteel gemaakt wordt; namelijk met pure culturen van micro-organismen die bescherming tegen ongewenste micro-organismen uit de omgeving vereisen. Het zuiver houden van de cultuur vergt het steriliseren van de apparatuur en het werken met schone, pure en dure substraten (bijvoorbeeld suiker).

De productiekosten van PHA kunnen substantieel worden gereduceerd als we deze dure processen met pure culturen vervangen door open culturen, omdat deze geen steriele apparatuur vereisen, en organische afvalstromen als substraat kunnen gebruiken. Open culturen hebben een vrije uitwisseling met micro-organismen uit de omgeving en het organisme dat het best is aangepast aan de omstandigheden in de reactor overleeft. Om het gebruik van open culturen voor PHA productie haalbaar te maken moet er dus een reactor-omgeving verzonnen worden, waarbij PHA producerende organismen een selectief groeivoordeel hebben ten opzichte van andere micro-organismen. In de natuur produceren een groot aantal verschillende micro-organismen PHA als een reserve stof om het metabolisme te faciliteren in perioden waarin geen energie- en/of koolstofbronnen aanwezig zijn in de omgeving. Op basis van deze ecologische rol van PHA, kunnen selectieve omgevingen ontworpen worden die een competitief groeivoordeel bieden aan soorten met een superieure PHA-productiecapaciteit. Een van de meest succesvolle ontwerpen is het zogenaamde *feast-famine* proces, dat bestaat uit het doseren van het substraat in korte pulsen, gevolgd door een relatief lange periode waarin geen substraat aanwezig is. Dit proces is relatief goed in kaart gebracht in het lab, onder gecontroleerde omstandigheden; zoals blijkt uit open culturen waarin het micro-organisme *P. acidivorans* dominant is en die tot wel 0,9 gPHA $gVSS^{-1}$ kunnen ophopen (Johnson et al. 2009; Jiang et al. 2011).

Het doel van de studie beschreven in dit proefschrift was het ontwikkelen van processen voor het terugwinnen van grondstoffen door middel van microbiële open culturen en het beoordelen van de industriële relevantie van PHA-productie uit organische afvalstromen, waarbij de aandacht is gericht op het *upstream* gedeelte van de productieketen: de productie van een PHA-rijke biomassa. Een aantal onderwerpen zijn hiervoor onderzocht: (1) voorbehandeling van organische (afval) stromen om vluchtige verzuur productie (VFA) te maximaliseren; (2) ophoping van microbiële culturen met een superieure PHA-opslag capaciteit; (3) maximalisatie van het PHA gehalte in *fed-batch* reactoren.

Het **eerste hoofdstuk** van dit proefschrift omvat een algemene samenvatting met uitleg over de relevantie en de aandachtspunten van het onderzoek.

In het **tweede hoofdstuk** wordt de voorbehandeling van organische afval stromen onderzocht. Het doel van deze studie was het ontwikkelen van een proces voor efficiënte productie van vluchtige vetzuren (*volatile fatty acids*, VFA), het gewenste substraat voor PHA productie. Een korrelslibproces bij lage pH was ontwikkeld dat met hoge snelheid, opbrengst en zuiverheid VFA produceert met minimale operationele kosten, op basis van een modelsubstraat (glucose). De introductie van een bezinkingsfase van 2 minuten in de cyclus waarbij vervolgens niet goed bezinkende delen worden afgevoerd maakt effectieve korrelvorming en zeer hoge omzettingssnelheden (150-300 $kgCOD\ m^{-3}\ d^{-1}$) mogelijk. Het product spectrum veranderde nauwelijks binnen het geteste pH bereik (4,5-5,5) met een VFA opbrengst van 0,6-0,7 $gCOD\ gCOD^{-1}$. Het baseverbruik van het systeem (benodigd om de pH constant te houden) kon worden verminderd van 1,1 tot 0,6 mol OH^- $(mol\ glucose)^{-1}$ door het verlagen van de operationele pH in de reactor van pH 5,5 naar 4,5. De vaste stof concentraties in het effluent konden vrijwel geheel worden

verwijderd; dit is gunstig voor het verkrijgen van hoge PHA gehaltes later in de vervolgstappen in het proces.

In het **derde hoofdstuk** is de omzetting van lipiden in het *feast-famine* proces onderzocht. Veel soorten afvalwater bevatten lipiden, en deze zijn niet gemakkelijk om te zetten in VFA. Resultaten gaven aan dat lipiden niet worden omgezet in PHA in het *feast-famine* proces. In plaats daarvan werden lipide-ophopende micro-organismen gevonden. Verdere optimalisatie van dit proces zou het economisch terugwinnen van lipiden uit afvalwater mogelijk kunnen maken.

Vergelijking op basis van een model maakt objectieve vergelijking van lab en pilot experimenten mogelijk. Een groot aantal verschillende modellen werd gevonden in literatuur en deze verschillen bemoeilijken onderlinge vergelijking van experimentele resultaten. Om hier een mouw aan te passen is in **hoofdstuk vier** een voorstel gedaan voor een algemeen model voor *feast-famine* processen. Er zijn bovendien voorstellen gedaan voor het aanpakken van een aantal hiaten in bestaande modellen, namelijk (1) het modeleren van substraat mengsels, (2) groei in de *feast* fase, (3) overgang van de *feast* naar de *famine* fase, (4) PHA afbraak en (5) het modeleren van de accumulatie fase. Afsluitend wordt een voorbeeld gegeven van een simpel en uniform model.

In **hoofdstuk vijf** wordt de industriële relevantie van PHA productie uit organische afvalstromen getest in een pilot experiment op een industriële locatie. De chocoladerepenfabriek Mars in Veghel is hiervoor uitgekozen omwille van de gunstige eigenschappen van het afvalwater uit deze fabriek: hoge fracties fermenteerbare organische stoffen en lage stikstof gehaltes. De pilot-opstelling was gebaseerd op het eerder beschreven 3-stappenproces: (1) fermenteerbare COD werd omgezet naar VFA in een voorbehandeling, met gemiddeld 0,64 $gCOD\ gCOD^{-1}$ als resultaat; (2) *feast-famine* verrijking in een 200 l reactor, resulterend in een open cultuur die gedomineerd werd door *P. acidivorans*; (3) het PHA gehalte werd gemaximaliseerd in een *fed-batch*, resulterend in 0,7 $gPHA\ gVSS^{-1}$. De dominante aanwezigheid van *P. acidivorans* wijst erop dat de selectiedruk in de pilot vergelijkbaar was met wat in het lab was bereikt. Het verschil in PHA gehaltes tussen pilot en lab (0,9 $gPHA\ gVSS^{-1}$ experimenten kon grotendeels worden verklaard door de aanwezigheid van niet VFA-COD en vaste stof in het gefermenteerde afvalwater.

In het afsluitende **hoofdstuk zes** worden aanbevelingen gedaan voor verdere ontwikkelingen van het proces. Op basis van de resultaten gepresenteerd in dit proefschrift, lijkt het erop dat de ontwikkeling van een open proces van PHA productie uit organische afvalstromen niet meer wordt belemmerd door de *upstream* bioprocessen die onderzocht zijn in dit proefschrift. In plaats daarvan zijn de belangrijkste beperkende factoren het opzuiveren van het PHA uit de biomassa in combinatie met het ontwikkelen van toepassingen en verkoopbare producten.

1

General introduction

Global energy and resource demand

Planet earth has become densely populated with humans, who have grown not only in number but also in their claim on our planets natural resources (Brown et al. 2014). One of the main drivers[1] enabling this rapid growth has been the ubiquitous availability of fossil energy resources, specifically: oil, coal and natural gas. Human primary resource consumption (excluding food) has increased to 16 TW (mostly for energy and a small part of approx. 5-10% for chemicals) and keeps rising, with a predicted increase of 40% between 2009 and 2035 (IEA World energy outlook 2011). The increase in demand and limited availability of natural resources will lead to an accelerated depletion of fossil reserves. Concurrently, entities that do not have sufficient natural resources (i.e. Europe) become geopolitically dependent on major natural resource producers (i.e. Russia and Saudi Arabia). Finally, human activity is now so gigantic, that it has become a global geochemical driving force (e.g. fossil fuel consumption leading to increased CO_2 levels in the atmosphere and ocean), and is threatening the stability of our planet's environment (Rökstrom et al. 2009). Efficient energy and resource technologies are required to replace the existing fossil sources. Possible solutions relating to renewable energy and material production strategies that have been proposed include nuclear fusion[2], solar and wind energy and bio-based resource technologies.

Historically, humanity has for a long time been a bio-based society[3], using biomass as primary resource until the onset of the industrial revolution in the 19th century AD. Taking into account the much larger human population and per capita resource consumption, a quantitative evaluation is required to assess the potential effects of transition back to a bio-based society. On a global scale, photosynthetic production exceeds (90 TW) the current human energy demand (Hermann et al. 2007). Nevertheless, considering that humans already have appropriated about 1/3 of the total land area on the planet for food production (Wirsenius, 2000) and considering the importance of preserving natural ecosystems, the most optimal employment of biomass as resource should be:

1) Optimization of primary production
    a. minimize environmental impact
    b. maximize areal productivity
    c. employment of alternative areas such as oceans or deserts

2) Use of agro-industrial residues as resource

[1] The correlation between annual GDP and energy consumption has been roughly 0.25 W $\$^{-1}$ (purchase parity and inflation corrected 2000 US international dollar). Currently, the global GDP related to primary energy production is estimated at 10-20%.
[2] Not strictly renewable but practically infinite
[3] Apart from bio-based we have also been a wind-based society for a period of time; e.g. sailing ships and industrial windmills were of prime importance during the renaissance era.

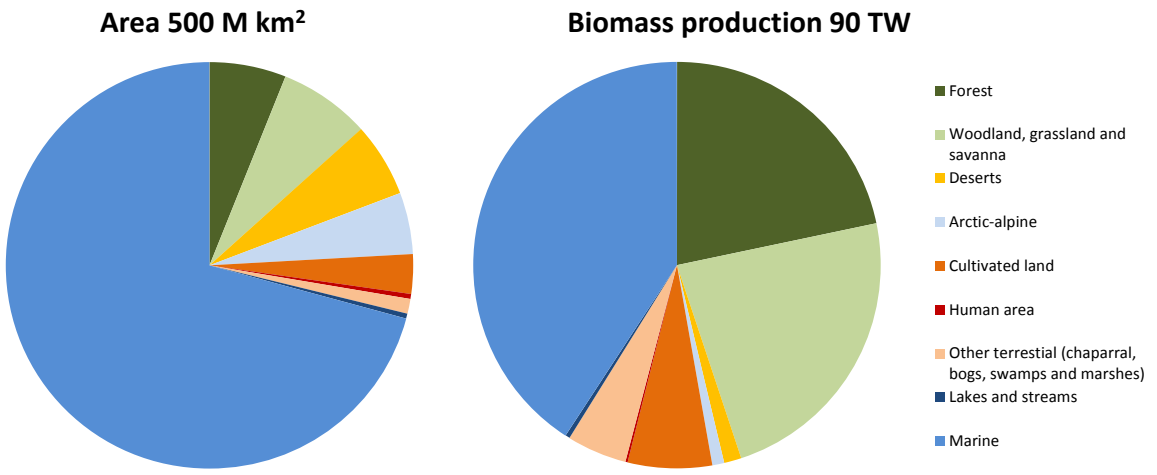


Figure 1.1. Global primary production of biomass for different types of area (data adapted from Vitousek et al. 1986). Cultivated land but also a large part of woodland, grassland and savanna and a part of lakes, streams and marine environment are currently employed for human food production.

The first option, the optimization of primary production, may include increasing the areal productivity, the employment of marginal lands or aquaculture in the oceans, and cultivation methods that have less impact on the environment. The upcoming field of aquaculture, especially the cultivation of algae, is hailed as a great promise for improving primary production (Wijffels and Barbosa, 2010). However, despite the reported high potential, large-scale cultivation of algae for bulk production is hampered by high energy requirements and operational and capital costs. Techniques that enable cultivation of high yield strains in open systems may improve the economic feasibility (Mooij et al. 2014), but also cost-effective product recovery methods are indispensable. Apart from cultivation and harvesting technologies, the real limiting factor for primary production in many locations is the availability of fresh water and nutrients. The price of nutrients is related to complex geopolitical aspects and market variations (Van der Weijden et al. 2014) while water scarcity is often related to the costs of transportation. Detailed analysis of these problems is however outside the scope of this thesis.

The topic of this thesis is related to the second option, the recovery of resources from organic residual streams, such as industrial wastewater, agricultural residues and municipal organic waste. Estimates of the availability of organic residual streams range from 2-7 TW, enough to replace a significant part, but not all, of our total fossil resource consumption (Hoogwijk et al. 2003; Lal, 2005; Heinemo and Junginger, 2009). It should be noted that the actual availability of organic residual streams for energy and bio-based material production is even smaller because a part of these residues is essential to maintain soil quality (Wilhelm et al. 2007). Apart from their limited availability, the recovery of resources from organic residues if often economically difficult due to the nature of these waste streams, which may be diluted, heterogeneous or recalcitrant or a combination thereof. Many organic waste streams are too heterogeneous (or complex) for economic purification of their individual compounds and contain too much water to yield net energy by thermochemical methods i.e. effective combustion, gasification and pyrolysis typically require water content below 0.5 gH_2O (g wet weight)$^{-1}$ (Jenkins et al. 1998; Brammer and Bridgwater, 2002). For these kind of waste streams, biochemical conversion can provide a solution because microorganisms are able to convert the myriad compounds present in the waste stream into a form that enables easier product recovery (figure 1.2). It should be noted that treatment of waste streams with pure cultures is often not economic due to a.o. the requirement for sterilized conditions and open (unsterilized) bioconversion processes are therefore required for efficient valorisation of the waste streams.

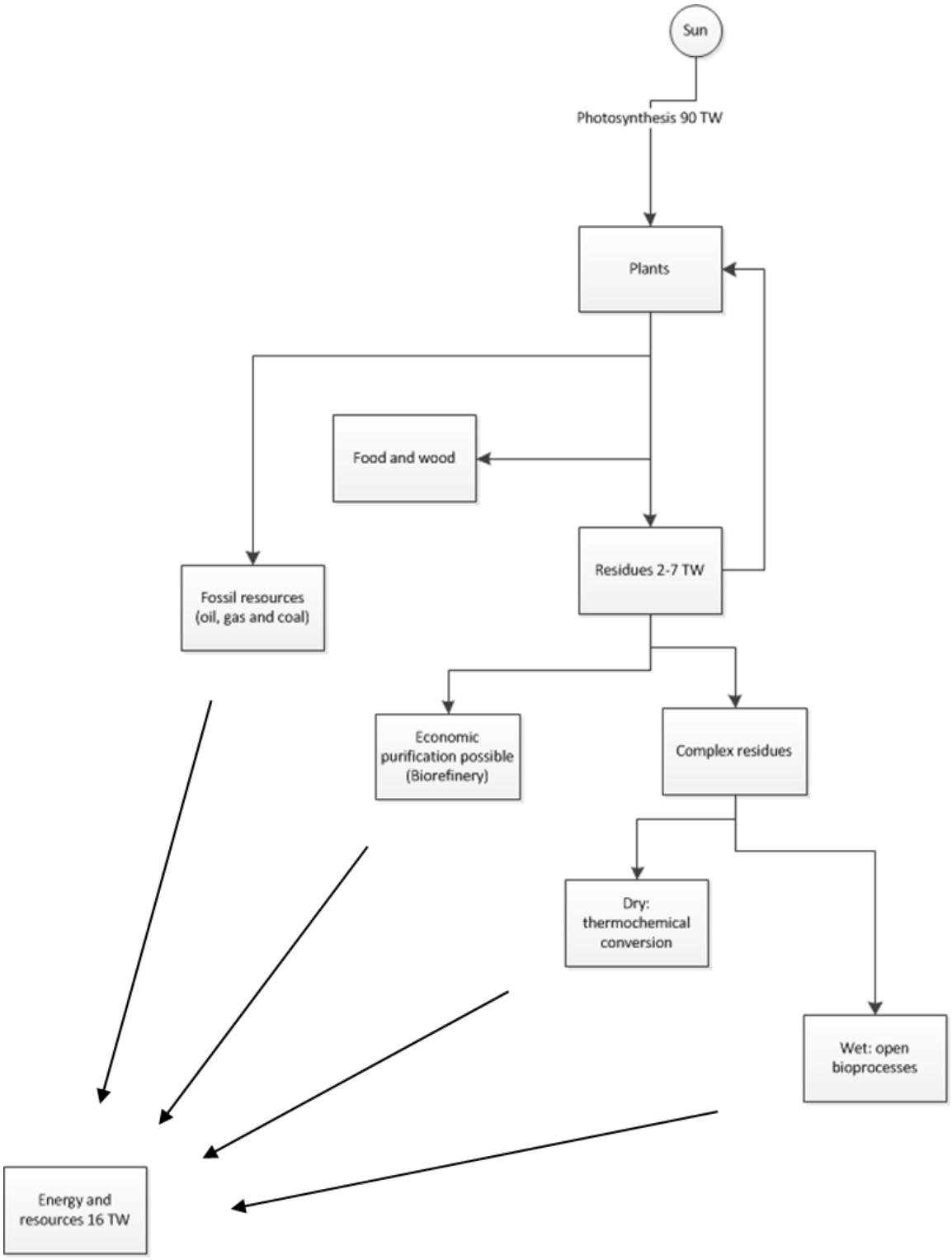


Figure 1.2. Schematic overview of biomass flows with focus on agro-industrial residues.

The traditional open bioconversion process for resource recovery from complex wet organic waste streams is anaerobic fermentation. The large variety of complex organic molecules is hydrolysed, converted into volatile fatty acids (VFA), and finally into methane and CO_2 gas. This biogas can be applied as energy source for the generation of heat and/or power. One of the major drawbacks of this process is that its product has a very limited economic value, even less than natural gas whose 2013 bulk prices ranged from 0.21 (US) to 0.65 (Russia) \$ kg^{-1} (World bank commodity price index database) or

0.05-0.16 $ $kgCOD^{-1}$ (COD: Chemical oxygen demand is used here as a measure of energy content of an organic stream). Consequently, in general, conversion of residual streams for energy production alone is not economically feasible[4].

An alternative option for resource recovery from organic waste streams is conversion to higher added value compounds. Naturally, bio-based bulk materials represent already an important part of global material flows comprising mainly wood, pulp for paper, cotton and rubber (Hoogwijk et al. 2003). Additionally, innovative technologies can provide biobased chemicals, polymers, paints and solvents as alternative for their fossil counterparts. Bio-based bulk chemicals have typically a higher value (e.g. succinic acid 2-3 $ kg^{-1} or 2-3 $ $kgCOD^{-1}$) than energy-carriers such as methane and the production of biomaterials with higher added value is generally regarded as a preferred waste management option (Kemp, 2007). Chemical compounds in general require more energy input than their combustion would produce; therefore they should rather be reused as chemical instead of being burned. The global demand for basic chemicals and polymers is much lower (in the order of 500 - 1000 MT y^{-1}, equivalent to a flow in the order of 1 TW) and thus matches much better with the available organic waste streams (< 2 - 7 TW) than the energy market. Finally, the production of (bulk) higher added value compounds rather than energy is a natural choice because in a fossil free energy society energy can be obtained from many sources whereas chemicals can be almost only be efficiently derived from plant material.

The production of bio-based fine chemicals is usually focussed on processes that use sugar as a substrate and relate to the cultivation of a single microbial species (pure culture) that operates in sterilized environments. This approach can be justified in the perspective of compounds with relatively high value (typically > 5 $ kg^{-1}) but the production of bio-based bulk chemicals and polymers (with a typical value of 1 - 3 $ kg^{-1}) is often economically limited by the substrate costs (e.g. raw sugar was valued at 0.20 - 0.60 $ kg^{-1} between 2004 and 2014) and the capital and operating costs required for pure culture systems. Additionally, sugar-based chemical products compete with food products, increasing food prices. Consequently, it is of economic interest to replace sugar-based processes with waste-based processes that do not require sterilized environments. Various open (non-sterilized) bioconversion technologies for the recovery of resources from organic waste streams are being developed, including the recovery of food protein by insects, the production of alginates from municipal wastewater, the direct production of electrical energy by microbial fuel cells and production of various compounds using VFA as central platform molecules.

[4] An alternative process option is the conversion of the methane into higher value products such as PHA (Herrema et al. 2010). This involves a first step in which all carbon-carbon molecular bonds are broken and a second step in which carbon-carbon bonds are reconstructed, making the process intrinsically inefficient.

The carboxylate platform as a general concept for resource recovery

The carboxylate platform concept is based on the observation that anaerobic fermentation acts as a molecular funnel that converts the multitude of compounds in organic waste streams into only of few types of carboxylate (VFA) molecules (figure 1.3). In traditional anaerobic waste fermentation VFA are subsequently converted into methane but this step can be inhibited by application of the proper conditions (e.g. short residence time, low pH) making VFA available as platform molecule for production of more valuable compounds i.e. medium chain length fatty acids (Spirito et al. 2014) and polymers, i.e. poly-hydroxyalkanoates (PHA) (Reis et al. 2003; Johnson et al. 2009).

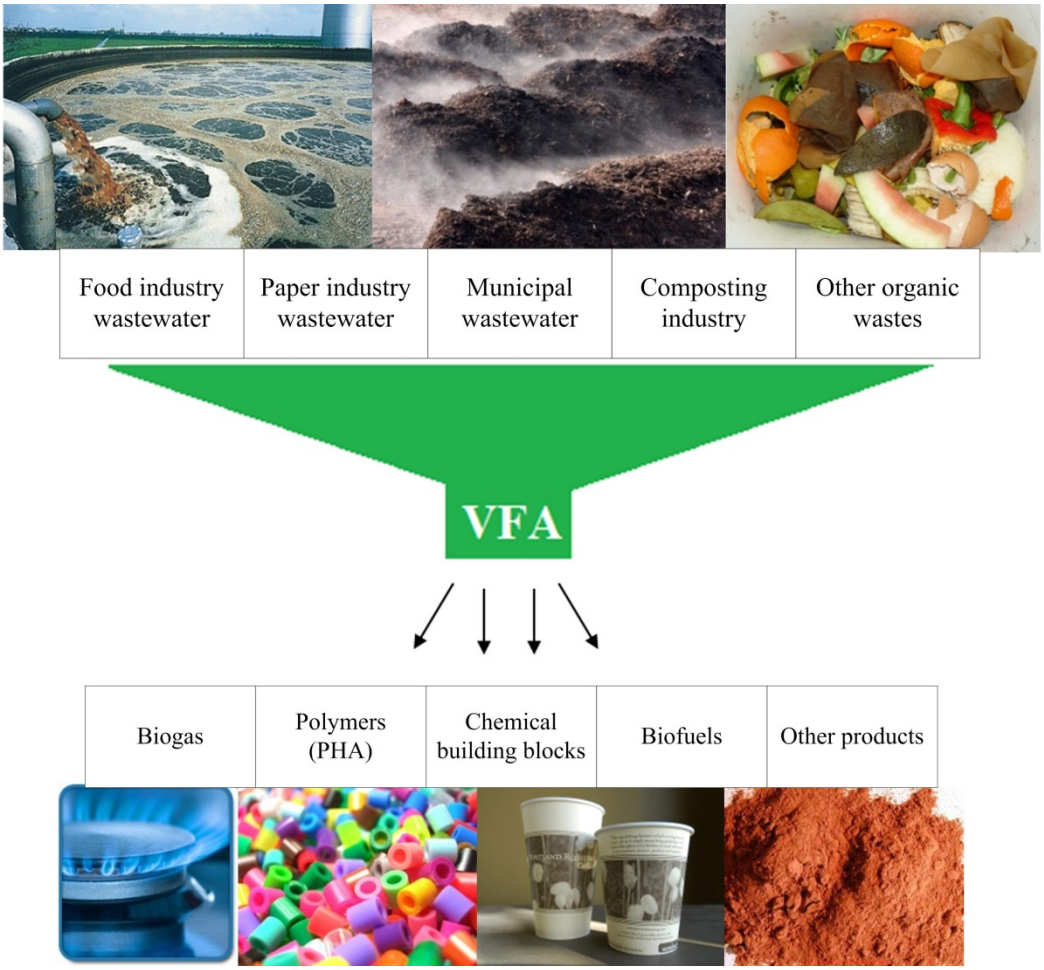


Figure 1.3. The role of carboxylates (VFA) as platform molecule: many different types of compounds from organic waste streams are converted into VFA. A variety of valuable products can be conceived using VFA as feedstock for innovative open bioprocesses.

One route to obtain usable products from VFA is the production of storage polymers (PHA) by open bioconversion processes. This approach has several advantages: 1) it concentrates a product from diluted VFA streams inside a biomass that can be easily separated from a large part of the water (i.e. by settling); and 2) the different VFA molecules present in the wastewater (i.e. acetate, propionate, butyrate and valerate) can in principle be converted into one polymer compounds (i.e. PHBV). Because of a.o. these reasons, waste-based PHA production has been under investigation by many research groups e.g. in Sweden (e.g. Bengtsson et al. 2010), Italy (e.g. Dionisi et al. 2007), The Netherlands (Marang et al. 2014) Portugal (e.g. Oehmen et al. 2007; Serafim et al. 2008), the United States (Liu et al. 2008), Australia (Arcos-Hernandez et al. 2013) and other countries.

PHA was discovered in 1926 (Lemoigne, 1926) as a product that accumulates in bacteria and appears to function as carbon and energy reserve in various microorganisms in a wide range of environments (Steinbuchel and Valentin, 1995). PHA polymers comprise polyesters of hydroxy-fatty-acids (figure 1.4) and have attracted attention as bio-based and biodegradable substitute of petrochemical polymers because of their thermoplastic and mechanical properties (Crank and Patel, 2005).

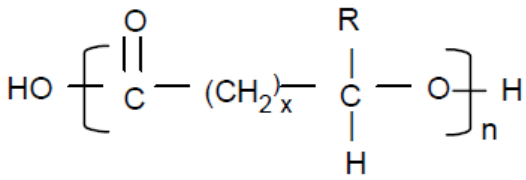


Figure 1.4. Generalized structure of PHA, where R represents a side chain that varies between types of PHA (e.g. for x = 0 and R = CH_3 the result is polylactic acid; for x = 1 and R = CH_3 the result is polyhydroxybutyric acid)

The open microbial process for PHA production was developed over time from the observation that, in nature, some bacteria produce PHA in periods of excess substrate, as survival strategy to buffer carbon and energy supplies in periods of famine. Based on this ecological principle several laboratory studies were performed and two types of metabolism were reported: 1) production of PHA in an anaerobic phase by phosphate- or glycogen accumulating organisms (PAO/GAO), with subsequent conversion to glycogen and new cell material in an aerobic phase (Smolders et al. 1994) and 2) production of PHA by aerobic cultures that are pulse-fed with subsequent growth when substrate is absent (feast-famine) (Beun et al. 2002). By application of the proper environmental conditions these type of organisms could be selectively enriched in bioreactors, both for PAO/GAO (Welles et al. 2014) and for feast-famine cultures (Johnson et al. 2009). It appears that (until now) feast-famine cultures can achieve both a higher PHA content and a higher product yield on substrate than PAO/GAO cultures. Possibly PAO/GAO cultures are limited in PHA storing capacity due to the requirement to also store glycogen (and poly-P). Furthermore, because growth rates are essentially lower in PAO/GAO cultures, a larger fraction of the substrate is required for maintenance processes, resulting in conceptually lower product yields. Since the PHA content has a very strong economic impact (Van Wegen et al. 1998), feast-famine cultures (and not PAO/GAO cultures) were selected in this study as preferred method to reduce the PHA production costs.

Industrial PHA production was for a long time performed only by pure culture techniques because of the high weight fraction of PHA achieved, which is essential for economic feasibility (Van Wegen et al. 1998). Recently, very high PHA content (90%) has been reached by feast-famine open cultures in laboratory experiments (Johnson et al. 2009; Jiang et al. 2011). These PHA contents are comparable to pure cultures and are a first step towards industrial implementation of waste-based PHA production with open microbial cultures.

Outline of this thesis

This thesis is part of the *Waste to Resource* research program (funded by the Dutch technology foundation STW) that aims for paving the road towards industrial implementation of waste-based PHA production from the perspective of a complete product chain, including upstream, downstream and utilization aspects. The objective of this thesis was the development of processes for resource recovery from wastewater with microbial enrichment cultures and to evaluate the industrial relevance of waste based PHA production, with a focus on the upstream part of the product chain (figure 1.5).

The central process setup in this thesis comprises three steps including 1) anaerobic fermentation for the conversion of organics in the wastewater to VFA, the preferred substrate for PHA producing microbial enrichment cultures; 2) enrichment of biomass with superior PHA producing capacity by application of a feast-famine regime, according to the parameters obtained from lab-scale experiments (Johnson et al. 2009); 3) a fed-batch environment, in which the PHA content of the enriched biomass is maximized by over-feeding with VFA.

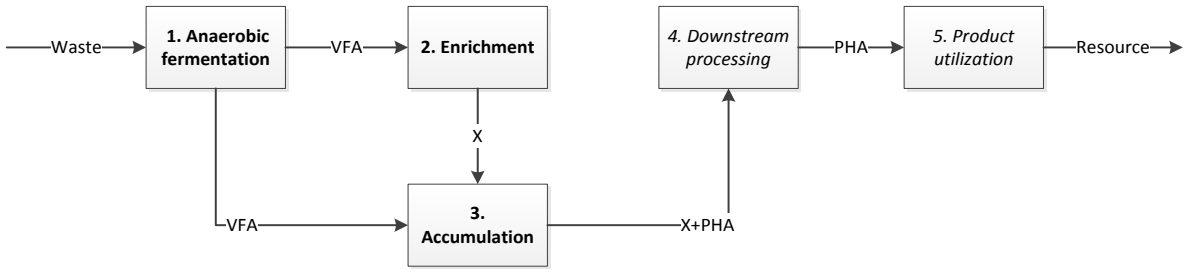


Figure 1.5. Overview of the product chain for waste-based PHA production. The first three unit processes (in bold) are related to the upstream part and the topic of this thesis. The last 2 unit processes (in italics) are important aspect of the product chain but are outside the scope of this thesis.

In this thesis, specific attention was directed to 1) VFA production, 2) the fate of lipids in the feast-famine process, 3) establishment of a generalized model for feast-famine processes and 4) pilot experiments. The efficient production of a VFA stream with favourable properties for PHA production is a major bottleneck for the overall waste-based PHA production chain. To optimize the VFA production, a (lab-scale) granular sludge type process was developed for efficient conversion of a model substrate (glucose) to VFA (**chapter 2**). Secondly, wastewater composition was considered as major factor that influences PHA production and for many compounds the effect on PHA production has been evaluated. However, while lipids are an important constituent in many waste streams, there is little information on their influence in the feast-famine process. This is extra relevant since lipids are generally not converted into VFA in reactors that aim for maximization of VFA concentrations in the effluent. The fate of lipids in the feast-famine process was evaluated with specific attention on potential storage compound production in a lab-scale feast-famine reactor (**chapter 3**). A modelling approach was used to compare the experimental data from the pilot- and lab-scale experiments. There are many models for feast-famine processes found in literature and the differences between the models used by different research groups hinders easy comparison experimental data. To enable better comparison of experimental results, a (concept) generalized model was developed (**chapter 4**). Finally, the industrial relevance, characteristic parameters and potential bottlenecks for up-scaling of PHA production were evaluated in pilot experiments Wastewater from a candy bar factory (Mars, Veghel, The Netherlands) was selected as suitable substrate for the pilot experiments because of its relatively high fermentable sugars content and low toxicity (**chapter 5**). To conclude, an outlook for future development is discussed in **chapter 6**, where the insights acquired in this thesis are evaluated in a perspective of full-scale implementation of waste-based PHA production.

2

High-rate volatile fatty acid (VFA) production by a granular sludge process at low pH

Abstract

Volatile fatty acids (VFA) are proposed platform molecules for the production of basic chemicals and polymers from organic waste streams. In this study we developed a granular sludge process to produce VFA at high rate, yield and purity while minimizing potential operational costs. A lab-scale anaerobic sequencing batch reactor (ASBR) was fed with 10 g l^{-1} glucose as model substrate. Inclusion of a short (2 minute) settling phase before effluent discharge enabled effective granulation and very high volumetric conversion rates of 150-300 gCOD l^{-1} d^{-1} were observed during glucose conversion. The product spectrum remained similar at the tested pH range with acetate and butyrate as the main products, and a total VFA yield of 60-70% on chemical oxygen demand (COD) basis. The requirement for base addition for pH regulation could be reduced from 1.1 to 0.6 mol OH^- $(mol\ glucose)^{-1}$ by lowering the pH from 5.5 to 4.5. Solids concentrations in the effluent were 0.6 ± 0.3 g l^{-1} but could be reduced to 0.02 ± 0.01 g l^{-1} by introduction of an additional settling period of 5 minutes. The efficient production of VFA at low pH with a virtually solid-free effluent increases the economic feasibility of waste-based chemicals and polymer production.

Submitted to Journal of Biotechnology and Bioengineering

Introduction

Increased demand and limited availability of natural resources have directed attention towards the recovery of valuable compounds from wastewater, agro-industrial residues and other organic-rich waste streams (Hoogwijk et al. 2003). The heterogeneous and sometimes diluted nature of these waste streams complicates valorization, but in many cases anaerobic fermentation offers a method to convert the myriad of compounds present in organic waste streams into just a few defined products. Additionally, anaerobic fermentation has the advantage that almost all energy from the substrate remains present in the products. The traditional anaerobic waste fermentation has methane containing biogas as end product, which can be employed as renewable energy source. Furthermore, this process stabilizes organic waste, enabling reuse in agriculture. Anaerobic fermentation consists of three main processes: (1) hydrolysis of complex substrates, (2) fermentation of monomeric compounds to volatile fatty acids, alcohols and hydrogen, and finally (3) methanogenesis (Klass 1984). Due to the slow growth and sensitivity of the methane producing archaea, methanogenesis can easily be inhibited resulting in the accumulation of fermentation product. Methanogenesis is generally minimized in bio-reactors that operate at high dilution rate or at high or low pH. Consequently, the methanogenic waste fermentation can be converted into primarily an organic acids producing process, that besides volatile fatty acids (VFA) may produce ethanol, hydrogen gas and other simple organic fermentation products such as lactate, acetone and succinate.

It has been proposed that VFA may serve as platform molecules in the so-called *carboxylate platform* or *VFA platform* concept (Agler et al. 2011; Holtzapple and Granda 2009), based on product formation routes with a higher added value than methane (figure 1.3). Examples are the production of biopolymers (Kleerebezem and van Loosdrecht 2007; Reis et al. 2003), the production of medium chain length fatty acids (Grootscholten et al. 2014; Spirito et al. 2014) or direct recovery (Angenent et al. 2004). In order to establish efficient product chains in the context of the carboxylate platform, development of specialized bio-reactors is required, that (1) produce a desired VFA mixture in an open process (without sterilization of substrate), (2) minimize solid concentrations in the effluent, (3) require minimal input of chemicals (i.e. for pH control) and (4) operate at high volumetric rates. Control of the product spectrum of anaerobic fermentations is critical because it influences the product quality and process performance in the subsequent steps of the product chain (Albuquerque et al. 2011; Jiang et al. 2011). The complexity and unsterile nature of waste substrates makes the use of pure cultures with defined metabolic pathways unfeasible. Instead, microbial communities have to be selected by adjusting the process conditions so that a competitive advantage is provided for species that produce the desired compounds (Kleerebezem and van Loosdrecht 2007). Several models have been proposed to explain the variation in kinetics and stoichiometry of open anaerobic fermentations as function of process conditions (Hoelzle et al. 2014; Kleerebezem et al. 2008; Rodríguez et al. 2006). Although a comprehensive mechanistic model enabling complete prediction of the product spectrum has not been established yet, the operational pH has been reported as key factor determining the product spectrum and biomass specific conversion rates (Fang and Liu 2002; Horiuchi et al. 2002; Temudo et al. 2007; Zoetemeyer et al. 1982a). Furthermore, the operational pH has special interest from application perspective, since (for waste streams with a relatively low alkalinity) the addition of base chemicals for pH control can be reduced by working at lower operational pH, improving the economic feasibility of large scale implementation. In general, operation at low pH and high organic acid concentrations inhibits microbial growth because more energy is required for maintaining intracellular pH by actively pumping out undissociated fatty acids that diffuse over the cell membrane into the cell (Fukuzaki et al. 1990). Consequently, reactors operated at low pH require a relatively long solids retention time and processes with efficient biomass retention are required to enable the processing of large diluted flows in compact reactors without washing-out the biomass. An additional advantage of biomass retention systems in the context of the carboxylate platform is the minimization of

solid concentrations in the effluent, which may be crucial for further processing of the VFA containing effluent (Tamis et al. 2014a).

An efficient way of uncoupling solid and liquid retention times is the employment of a granular sludge process. Granular sludge based processes offer several advantages compared to other sludge retention systems: (i) granular biomass has a density close to water, enabling efficient mixing with low energy input, (ii) granular sludge has a higher specific surface area compared to biofilms on a carrier, minimizing mass transfer limitations, and (iii) SRT control in granular sludge systems is relatively straightforward compared to biofilm systems, since granular biomass can directly be removed from the reactor compartment.

Granular sludge has already proven its virtue for a number of applications such as anaerobic wastewater treatment coupled to methane production (Lettinga et al. 1980), Anammox (Third et al. 2005; van der Star et al. 2007) and aerobic wastewater treatment processes (de Kreuk and van Loosdrecht 2006). Only limited information is available on the production of VFA by granular sludge processes. The few studies available are from the 1980s and report the use of acidifying granular sludge in a 2-stage methane containing biogas production process (Beeftink and Van den Heuvel 1987; Zoetemeyer et al. 1982b; Zoutberg et al. 1989). These systems were not optimized in the context of the carboxylate platform, and thus did not focus on aspects that are important for efficient VFA production, such as minimizing solids concentrations in the effluent and the consumption of base chemicals for pH control. Moreover, the stability of acidifying granules was reported to be problematic, leading to decreased conversion capacities in biogas systems (Alphenaar 1994; Angenent and Sung 2001).

In this study, the aim was to develop a stable granular sludge type process for the production of VFA (or other fermentation products) from a model substrate (glucose) and to investigate the influence of operation at low pH on the granulation process, effluent solid concentrations, product spectrum and base chemical requirements. To this end, anaerobic sequencing batch reactor (ASBR) systems operated at pH 5.5, 5.0 and 4.5 were investigated.

Materials and methods

Reactor operation

A lab-scale ASBR with a height of 150 cm and an internal column diameter of 6.5 cm (liquid volume of 2.6 l with a headspace of 1.6 l) was inoculated with 100 ml of sludge (approximately 30 gVSS l^{-1}) from an anaerobic digester treating primary and secondary sewage sludge (WWTP Harnaschpolder, the Netherlands). After a start-up period, the system was operated as a sequencing batch reactor with an operational cycle of 2 hours in total, comprising a feed phase of 17 minutes (1.3 l $cycle^{-1}$), a reaction phase of 95 minutes, settling phase of 2 minutes and finally a 3 minute effluent phase, in which half of the reactor liquid (1.3 l $cycle^{-1}$) was decanted to keep a hydraulic retention time (HRT) of 4 h. The solids retention time (SRT) was 1-2.5 days and was established by manual biomass removal and by solid concentrations in the effluent. Nitrogen gas was sparged (0.4 l min^{-1}) to ensure anaerobic conditions. Liquid mixing was achieved by off-gas recirculation at a flow rate of 2 l min^{-1}. The temperature was maintained at 30 ± 1 °C using a water jacket. The pH was controlled (pH ± 0.1) by automatic titration (I/O Tower, Sartorius controlled by SCADA software, Sartorius BBI Systems MFCS/win 3.0) with 2 M NaOH. To prevent foaming, a 2% Antifoam C solution (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added with a flow rate 4 times lower than the base flow rate. The growth medium contained the following compounds (g l^{-1}): glucose 10; NH_4Cl 0.678; KH_2PO_4 0.127; $MgSO_4.7H_2O$ 0.059; KCl 0.020; EDTA 0.036; $ZnSO_4.7H_2O$ 0.012; $CoCL_2.6H_2O$ 0.0009; $MnCl_2.4H_2O$ 0.0029; $CuSO_4.5H_2O$ 0.0009;

$FeSO_4.7H_2O$ 0.0028; $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.0006; $CaCl_2.H_2O$ 0.0041. The glucose and nutrient solutions were prepared separately and autoclaved for 20 min. at 110 ºC and 121 ºC, respectively.

Sampling and measurement

The process was monitored using online measurements of the pH, base dosage, off-gas H_2 and CO_2 and offline measurements of glucose, ammonium, volatile suspended solids (VSS), total suspended solids (TSS), and fermentation products (organic acids and alcohols) in the reactor effluent. After a steady state was reached (according to the definition provided in the following data analysis section), the conversions during a cycle were analyzed by more detailed sampling and measurement, including the formation of polyglucose (PG) in addition to online and off-line measurements as described above. Reactor samples taken for analysis of ammonium, glucose and fermentation product concentrations were immediately filtered with a 0.45 μm pore size filter (PVDF membrane, Millipore, Ireland). The ammonium concentration in the filtrate was measured spectrophotometrically using a cuvette test kit (Hach-Lange, Germany). The glucose and organic acid concentrations in the filtrate were measured using a high-performance liquid chromatograph with a BioRad Aminex 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 min^{-1} and a temperature of 60 ºC. The alcohols in the filtrate were measured using a gas chromatograph (Thermo Scientific Focus, USA) equipped with a HP-Innowax column (length: 30 m, diameter 0.25 mm) and a flame ionization detector. Helium was used as a carrier gas. The temperature of the injector, column and detector were 200 ºC, 65 ºC and 250 ºC, respectively. The TSS and VSS concentrations were determined according to standard methods (Clesceri et al. 1999) and for determination of the sludge volume index (SVI) an adapted method was used with 4 min of settling in a 50 ml tube. For polyglucose (PG) analysis the method proposed by Smolders et al. (1994) was used with two optimizations: an HCl concentration of 0.9 M as proposed by Lanham et al. (2012) was used instead of 0.6 M HCl and the heating time was prolonged from 3 to 6 h. It was verified that cooking for a longer time or at higher HCl concentrations did not results in increased PG yields. The nitrogen content of the biomass was analyzed using a cuvette test for total nitrogen measurement (Hach-Lange, Germany).

Data analysis

The SRT was determined by the rate (gVSS d^{-1}) of solid discharge compared to the amount of solids (gVSS) in the reactor. The reactor was assumed to be in steady state when during at least 3 SRTs (1) the concentration of VSS in the effluent (2) the product spectrum (3) the quantity and duration of base addition during the cycle were constant (≤ 5% variation). Biomass production was estimated from ammonium uptake and the nitrogen content of the biomass. The chemical oxygen demand (COD) balance over the cycle was evaluated by comparison of the influent COD with the fermentation products in the effluent, the off-gas plus the COD content of newly formed biomass. The gap between the influent and the sum of the identified products was classified as "unidentified fermentation products". The charge balance of the system was evaluated by comparison of the amount of base that was added per cycle with the H^+ production estimated by the concentrations of dissociated acids (including acetate, propionate, butyrate, lactate and bicarbonate), and the ammonium uptake. The concentration of dissociated acids was calculated using the model output (appendix 2A) in combination with equation 1.

$$f = \frac{K_a}{K_a + \left[H^+\right]} \qquad \text{(eq. 1)}$$

With f the fraction of compound in dissociated form and apparent pK_a values: acetate 4.66; propionate 4.77; butyrate 4.72; lactate 3.76, bicarbonate 6.25 (estimated values based on an average ionic strength of the medium of 60 Mm and a temperature of 30 ºC).

A mathematical model was established for characterization of the conversions observed (Appendix 2A). The model was based on three reactions (figure 2.1) and comprised: 1) direct conversion of glucose to fermentation products and intracellular polyglucose according to a variable stoichiometry derived from fitting the data, 2) conversion of lactate to propionate, acetate and CO_2 according to a fixed stoichiometry and 3) conversion of polyglucose to fermentation product according to a stoichiometry derived from the direct glucose conversion.

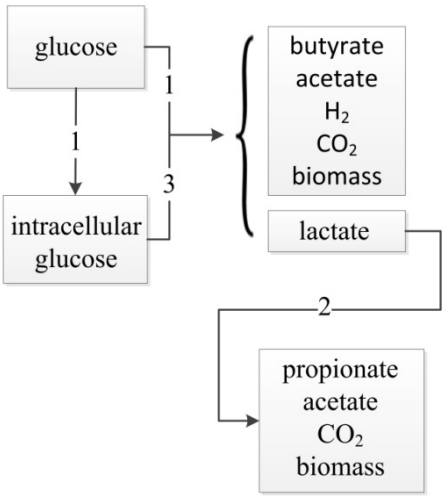


Figure 2.1. Overview of the reactions included in the applied model for anaerobic fermentation of glucose.

Microbial community structure analysis

The microbial diversity in the reactor was analysed by denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) products obtained by using prokaryotic (16S rDNA) primers on DNA extracted from the reactor biomass. A detailed description of the applied method was reported earlier (Johnson et al. 2009). Briefly: genomic DNA was extracted using the UltraClean soil DNA extraction kit (MoBio Laboratories). A 32 cycle PCR program was done using the following primers: Bac341F without a GC clamp and Bac907rM with a GC clamp. The PCR products were separated using an 8% polyacrylamide gel with a 20-70% urea-formamide gradient. The DNA was stained with SYBR gold (Thermo Fisher Scientific) to allow visualization with a blue light safe imager 2.0 (Thermo Fisher Scientific). Individual bands were excised, allowed to diffuse in 40 µl Tris-HCl buffer (10 mM) and re-amplified using the above PCR method but for 25 cycles and without GC clamp. The resulting PCR product was sequenced (Macrogen, The Netherlands) and the obtained 16S rDNA sequences were BLASTed (NCBI BLAST 2.2.29) to identify the corresponding species.

Results

After inoculation with anaerobic digester sludge, the reactor was operated at pH 5.5 for more than 800 cycles (60 days). After reaching steady state, the conversions during the cycle were analyzed. Hereafter the pH was decreased to pH 5.0 for 300 cycles (25 days) and subsequently to pH 4.5 for 300 cycles (25 days). In all the experiments steady state was reached within 20 days. An overview of the most important results are collected in table 2.1, a more detailed description is presented in the subsequent sections.

Table 2.1. Overview of the characteristics of the system (average ± standard deviation over dataset). The yields, SVI and base consumption were determined from steady state measurements. The kinetic parameters (qS^{max} and μ^{max}) were derived by calibration of the model to experimental data from a representative cycle.

pH	4.5	5.0	5.5	
SVI	22 ± 3	29 ± 1	17 ± 2	ml $gVSS^{-1}$
Y_X	0.13 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	gCOD $gCOD^{-1}$
Y_{VFA}	0.66 ± 0.02	0.60 ± 0.03	0.59 ± 0.03	gCOD $gCOD^{-1}$
Y_{H2}	0.12 ± 0.01	0.11 ± 0.00	0.12 ± 0.01	gCOD $gCOD^{-1}$
base	0.66	0.88	1.1	mol OH^- mol glu^{-1}
q_S^{max}	0.7	1.1	1.6	gCOD $gVSS^{-1}$ h^{-1}

Reactor start-up and granulation

The start-up of the reactor system was focused on complete conversion of glucose during the cycle. The base consumption (for pH control) was used as indicator of biological activity related to the conversion of glucose into organic acids. Initially the cycle length was controlled manually: once the consumption of base stopped (indicating glucose depletion), a new cycle was initiated. Granule formation became visible within 2 - 3 days after inoculation (3 - 4 cycles of 16 h). After gradually lowering the cycle length to 2 h in the course of 7 - 8 days (14 - 18 cycles in total) the biomass concentration in the reactor had increased to 6 gVSS l^{-1}. The final working concentration of 10 ± 2 gVSS l^{-1} granular sludge was reached during the consecutive 3-4 days (approximately 40 cycles). The granules were disc-shaped and the size (1-3 mm) and sludge volume index (SVI) varied (17 - 29 ml $gVSS^{-1}$) between the experiments (figure 2.2). At pH 5.5 granules were relatively small (approximately 1 mm in diameter) with an SVI of 17 ± 2 ml $gVSS^{-1}$. Larger granules with a 3 mm diameter and an SVI of 29 ± 1 ml $gVSS^{-1}$ were observed at pH 5.0. At pH 4.5 the granule size was similar to the granules at pH 5.5 with an SVI of about 22 ± 3 ml $gVSS^{-1}$. The nitrogen content of the biomass was 0.10 ± 0.01 gN g^{-1}. With this value the biomass production rate could be estimated on the basis of ammonium uptake, resulting in an estimated growth yield of 0.13 ± 0.01, 0.15 ± 0.01 and 0.16 ± 0.01 gCOD $gCOD^{-1}$ at pH 4.5, 5.0 and 5.5, respectively. The SRT was influenced by the solid concentrations in the effluent and by manual solids removal. VSS concentrations of 0.6 ± 0.2 g l^{-1} were found in the effluent, comprising smaller particles (< 0.5 mm in diameter) of suspended solids, that were likely detached from the granules and were not settling fast enough. Consequently, in all experiments most of the biomass (9 ± 2 g d^{-1}) was removed via the effluent. Additional manual removal of sludge was 1.5 ± 0.7 g d^{-1}, 7 ± 1 g d^{-1} and 9 ± 1 g d^{-1} at pH 4.5, 5.0 and 5.5, respectively. Due to fluctuations in the growth and solid concentrations in the effluent, the SRT of the systems varied between 1-2.5 d during all experiments. It appeared that it was relatively easy to remove the small particles of detached suspended solids present in the effluent by introduction of an additional settling step. For all experiments the VSS concentration could be reduced to 0.02 ± 0.01 g l^{-1} by an additional settling time of 5 minutes (tested in a separate settling column).

Figure 2.2. Photographs of the fermentative sludge granules in the reactor operated at different pH setpoints.

Product spectrum

The product spectra observed in the effluent of the reactors operated at different pH setpoints are shown in figure 2.3. The measured fermentation products and biomass represented 90-95% of the influent COD, indicating the most important products were identified. The results demonstrate that the major fermentation products were not affected in the pH range studied, although the pH decrease caused a small shift in the distribution of fermentation products. A small increase in the VFA yield on glucose from 0.59 (± 0.03) gCOD $gCOD^{-1}$ at pH 5.5 to 0.66 (± 0.02) gCOD $gCOD^{-1}$ at pH 4.5 was observed. In all experiments, analysis of the off-gas showed significant concentrations of hydrogen gas, resulting in a yield of hydrogen on glucose of 0.12 ± 0.01 gCOD $gCOD^{-1}$; methane concentrations in the off-gas were below detection limit, indicating that no significant methane production occurred (< 0.01 gCOD $gCOD^{-1}$). As expected, the base dosage could be reduced by lowering the pH setpoint. A reduction from 1.1 mol OH^- $(mol\ glucose)^{-1}$ at pH 5.5 to 0.66 mol OH^- $(mol\ glucose)^{-1}$ at pH 4.5 was observed, in accordance (< 10% deviation) with the amounts estimated from the charge balance (based on the production of dissociated VFA and consumption of ammonium, as described in the methods section).

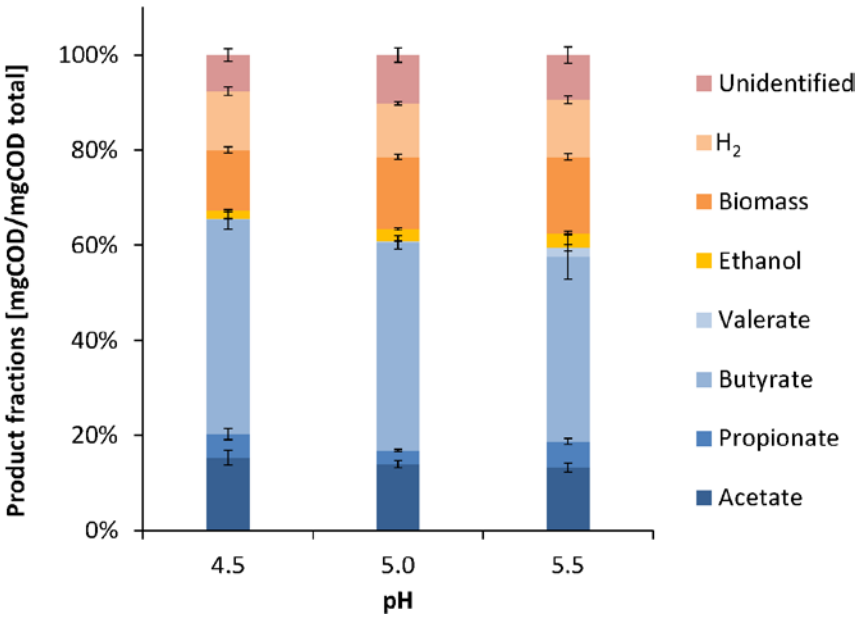


Figure 2.3. Product spectrum in a fermentative granular sludge SBR with glucose as substrate. The products are given as percentage of the influent glucose COD.

Kinetic and stoichiometric characterization

After reaching steady state, conversions during representative cycles were characterized by detailed sampling and measurement (figure 2.4). In all experiments, the majority of product formation occurred during the first part of the cycle when glucose was still present in the reactor. Transient lactate accumulation was observed in the presence of glucose (the first 30-40 minutes of the cycle), with yields of lactate on glucose of 0.13, 0.11 and 0.09 gCOD gCOD^{-1} at pH 5.5, 5.0 and 4.5, respectively. Subsequently, after glucose depletion (around 30-40 minutes after the start of the cycle), all the accumulated lactate was consumed. Furthermore, a significant fraction of the substrate was stored as intracellular carbohydrates (0.13-0.19 gCOD gCOD^{-1}), such as trehalose or polyglucose. The accumulated intracellular carbohydrates (further referred to as polyglucose or PG) were fermented after depletion of the external substrate present in the reactor, resulting in a further increase in fermentation products during the remainder of the cycle.

The data from the cycle experiments was used as basis for a model describing direct conversion of glucose and two secondary reactions related to the transient presence of lactate and intracellular polyglucose (appendix 2A). A good representation of the experimental data could be obtained, indicating that these three reactions sufficed to explain the behavior of the system (figure 2.4). A clear trend could be observed with the model derived values for the lumped maximum biomass specific glucose uptake rate (q_S^{max}), decreasing substantially at lower operational pH: with rates of 1.6, 1.1 and 0.7 gCOD gVSS^{-1} h^{-1} at pH 5.5, 5.0 and 4.5 respectively. The average volumetric loading rate of the system was around 60 gCOD l^{-1} d^{-1} during the entire operational period; however, the operational cycle included a significant period during which glucose was depleted. Consequently, actual volumetric conversion rates during glucose conversion were in the range of 150-300 gCOD l^{-1} d^{-1} with variations due to the fluctuations in the amount of biomass present in the reactor.

Microbial community structure

DGGE results showed the presence of three types of prokaryotes (Appendix 2C). In all experiments *Clostridium pasteurianum* was detected, known for anaerobic fermentation of glucose into a variety of products but typically associated with an acetate and butyrate product spectrum (Rogers 1986; Temudo et al. 2008). Furthermore, in all experiments lactic acid bacteria were detected: *Olsenella* sp. in the experiments operated at pH 5.0 and 5.5 and *Lactobacillus harbinensis* in the experiments operated at pH 4.5. A third type of prokaryote present in all experiments was a *Pectinatus frisingensis*, from a genus with members known to be able to convert lactate into propionate, acetate and CO2 (Membré et al. 1994).

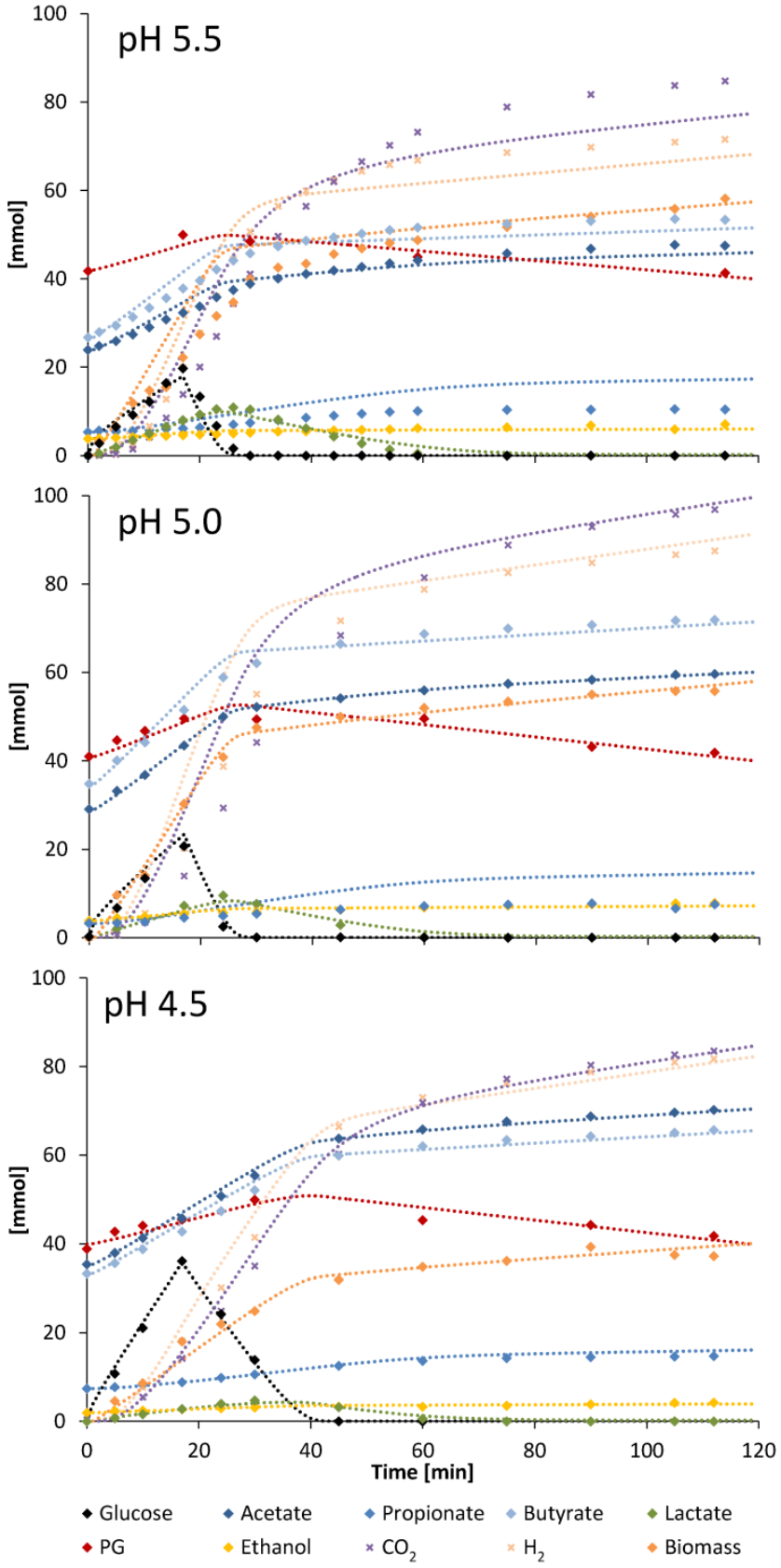


Figure 2.4. Substrate and product profiles during one cycle during steady state operation at different pH setpoints. The amount of biomass in the reactor varied between experiments (pH 4.5: 27 gVSS, pH 5.0: 32 gVSS, pH 5.5: 22 gVSS). The profiles are presented in amounts (mmol) (and not in concentrations) because reactor volume changed during the feed phase. The symbols represent the measured data; the dotted lines represent a simulation of the conversion process.

Discussion

Granule formation and performance

In this study we show the formation of compact fermentative granular sludge (SVI 17-29 ml $gVSS^{-1}$) in an ASBR with a relatively short HRT (4 h) through a cycle that includes a short (2 min) settling phase and subsequent removal of poor settling biomass in the effluent, while keeping the biomass with good settling properties inside the reactor. The granules varied in size (1 mm at pH 4.5 and 5.5 and 3mm at pH 5.0) but we could not explain a direct link between granule size and operational parameters. The enrichment of VFA producing granules in an upflow type system was reported earlier with granules similar in appearance and size (Zoetemeyer et al. 1982b). However, the system of Zoetemeyer was not optimized in the context of the carboxylate platform, and was operated at relatively high pH: 6, with relatively high solid concentrations in the effluent (around 1.5 g l^{-1}). Operation at low pH has generally a problematic effect on the conversion and growth rates of anaerobic fermentations (Temudo et al. 2007; Zoetemeyer et al. 1982a). Here we demonstrate the use of granular sludge for VFA production in a system operated at low pH (4.5-5.5), in which biomass retention enables micro-organisms to survive despite the adverse conditions imposed by the low pH and high concentrations of undissociated fatty acids. A relatively large fraction of the biomass was discharged via the effluent indicating significant detachment of part of the biomass from the granules. This is in line with earlier observations that acidifiers form relatively unstable granules (Alphenaar 1994; Angenent and Sung 2001). The solids concentrations in the effluent of our system were somewhat lower (0.6 g l^{-1}) than in the system of Zoetemeyer et al. (1982b) (1.5 g l^{-1}) at equal HRT (4h) and influent substrate concentration (10 g l^{-1}); but, in this study, we show that virtually all solids (until around 0.02 g l^{-1}) could be removed from the effluent by introduction of an additional settling step of 5 minutes (in a separate vessel).

It appeared that the low pH had a strongly negative influence on the lumped biomass specific conversion rates, with apparent biomass specific glucose uptake rate more than half at pH 4.5 compared to pH 5.5. Although, this apparent decrease in biomass specific uptake rate was reported earlier for systems operated at low pH (Zoetemeyer et al. 1982a), the underlying reason remains unclear. Possible explanations include an intrinsically lower uptake rate of the active biomass at low pH or e.g. a significantly increased fraction of dead biomass in the solids. Nevertheless, due to the feeding regime in our system, with glucose dosed in one short pulse at the beginning of the cycle, species with the highest possible specific uptake rate were enriched. Consequently, even at relatively adverse conditions (pH 4.5), the apparent biomass specific conversion rate was still within the range of values reported for anaerobic fermentations operated at more favorable conditions (pH 6-8) (Temudo et al. 2009; Zoetemeyer et al. 1982b). Although the average volumetric loading rate of the system was around 60 gCOD l^{-1} d^{-1}, actual volumetric rates during the presence of glucose were as high as 150-300 gCOD l^{-1} d^{-1}, an order of magnitude higher than for methane producing granules (McCarty and Smith 1986; Nicolella et al. 2000) and two to three orders of magnitude higher than for CSTR systems operated for VFA production (Temudo et al. 2007). Volumetric conversion rates were limited by the amount of biomass in the reactor (which comprised a sludge bed of only 500-700 ml in a 2.6 l reactor) and may be improved to > 500 gCOD l^{-1} d^{-1} if the amount of biomass in the reactor could be increased.

Product formation and microbial community structure

The product spectra were relatively similar for the tested pH range, with acetate and butyrate as the main products, and a total VFA yield of 60-70% on COD basis. Similar product spectra were reported earlier in a granular system (Zoetemeyer et al. 1982b) and a continuous stirred tank reactor (CSTR) (Temudo et al. 2007). The microbial diversity in our ASBR system was larger than reported for the CSTR system (operated at similar SRT and pH), in which only *Clostridium* sp. was detected (Temudo et al. 2007).

Remarkably, it appeared that there were two types of glucose fermenting organisms coexisting in the reactor i.e. *Clostridium* bacteria and lactic acid bacteria (*Olsenella*/*Lactobacillus*). This coexistence can possibly be explained by the transient presence lactate and polyglucose during the operational cycle. Transient lactic acid production was reported earlier in a similar system (Agler et al. 2012), suggesting an intrinsically higher growth rate of lactic acid bacteria on glucose. A second niche may be created by microorganisms that perform anaerobic polyglucose storage as strategy for rapid glucose uptake. This phenomenon of anaerobic glucose storage was reported earlier as hoarding strategy for microorganisms in environments with dynamic availability of glucose (Shimada et al. 2007). The organisms responsible for the glucose storage in this study were not identified, but presence of intracellular polyglucose in *Clostridium* species has been reported earlier (Hobson and Nasr 1951).

Applications

The carboxylate platform comprises waste valorization processes that rely on the production of VFA or other fermentation products as intermediate product and for this, efficient open fermentation processes are required (Agler et al. 2011; Angenent et al. 2004; Kleerebezem and van Loosdrecht 2007). The granular sludge process in this study enabled VFA production at low pH, thus minimizing consumption of chemicals for pH control, while at the same time, maximizing volumetric productivity. Furthermore, a virtually solid-free effluent could be produced, which may be advantageous for further processing. An example of the importance of a solid-free effluent in further processing was reported earlier: in pilot experiments for PHA production, solids concentrations of around 0.5 g l^{-1} in the substrate caused a decrease in the PHA product purity from roughly 0.9 to 0.8 gPHA gVSS^{-1} (Tamis et al. 2014a). It should be noted that such a decrease in PHA purity has substantial economic impact related to down-stream processing costs (Van Wegen et al. 1998).

Further optimization of anaerobic processes within the carboxylate platform depends, among others, on the desired final product. For example, in the context of PHA production it may be interesting to maximize the VFA yield at the expense of hydrogen formation (de Kok et al. 2013), while for caproic acid production (Grootscholten et al. 2014) it may be relevant to obtain a product spectrum comprising an ethanol:VFA molar ratio of > 2. Additionally, while in this study we used glucose as model substrate, the influence of other industrially relevant model substrates e.g. different sugars, such as xylose and mannose, or alcohols that may be present in (agro)industrial effluents should be evaluated. Possibly, substrates that are more difficult to convert may require operation at longer SRT to prevent biomass washout and it should be evaluated whether methane production can still be inhibited at potentially increased SRT. Nevertheless, the results obtained in this study clearly show the potential of the granular sludge concept for the valorization of organic waste streams.

Conclusions

The valorization of waste streams through VFA intermediates depends on many steps in a long product chain. In this study, we show efficient VFA production at low pH with a virtually solid-free effluent. A compact granular sludge (17-29 ml gVSS^{-1}) was obtained in an ASBR operated at low pH (4.5-5.5) using glucose as substrate. The biomass specific conversion rates were negatively influenced by the low pH but were still relatively fast with 0.7-1.6 gCOD gVSS $^{-1}$ h^{-1} and volumetric rates as high as 150-300 gCOD l^{-1} d^{-1}. Solid concentrations in the effluent were around 0.6 ± 0.3 g l^{-1} but could be reduced to 0.02 ± 0.01 g l^{-1} by introduction of an additional settling period of 5 minutes. Operation at low pH reduced the amount of base chemicals required for pH control by roughly a factor 2. Butyrate and acetate were the major products and the overall VFA yield was 60-70% on COD basis. The efficient production of VFA at low pH with a virtually solid-free effluent improves the economic feasibility of processes that use VFA as feedstock for the production of biopolymers and other valuable biobased compounds.

Appendix 2A – Process model for characterisation of anaerobic fermentations

General

The purpose of the model was to derive characteristic parameters from the experimental data describing the anaerobic conversion of glucose and enabling objective comparison between experiments. The model was based on three reactions: (1) conversion of glucose into fermentation products (including lactate) and polyglucose according to a stoichiometry that was calibrated to the experimental data, (2) conversion of lactate according to a fixed stoichiometry (appendix 2B) and (3) conversion of polyglucose according to a stoichiometry identical to glucose conversion.

Stoichiometry

The model used in this paper consisted of three reactions (table 1). Minor compounds (i.e. concentrations <1 mM) were not considered and therefore omitted from the reaction stoichiometry. The first reaction described glucose conversion to fermentation products. A second reaction was included in the model to be able to describe the transient lactate concentrations that may be observed during acidogenic fermentation (Membré et al. 1994). This fermentation pattern was also observed in preliminary experiments (data not shown here) together with a transient polyglucose accumulation. A third reaction describing fermentation on polyglucose (PG) was included. The PG consuming reaction was assumed to have the same stoichiometry as the glucose consuming reaction except there is no PG produced. Because the glucose and lactate conversion occurred parallel including the same products, it was not possible to calibrate the stoichiometry of both reactions to the experimental data and therefore the stoichiometry of the lactate consuming reaction was fixed (appendix 2B).

Table 1. Stoichiometric matrix used in the metabolic model.

Compound	Glucose consumption	Lactate consumption*	Polyglucose consumption**
Glucose	-1		
Acetate	$Y_{Ac,Glu}$	0.32	$Y_{Ac,PG}$
Propionate	$Y_{Pro,Glu}$	0.63	$Y_{Pro,PG}$
Butyrate	$Y_{Bu,Glu}$		$Y_{Bu,PG}$
Lactate	$Y_{Lac,Glu}$	-1	$Y_{Lac,PG}$
PG	$Y_{PG,Glu}$		-1
Ethanol	$Y_{EtOH,Glu}$		$Y_{EtOH,PG}$
CO_2	$Y_{CO2,Glu}$	0.32	$Y_{CO2,PG}$
H_2	$Y_{H2,Glu}$		$Y_{H2,PG}$
Biomass	$Y_{X,Glu}$	0.15	$Y_{X,PG}$

* Stoichiometric values were derived according to appendix 2B.

** with $Y_{i,PG} = Y_{i,Glu} (1-Y_{PG,Glu})^{-1}$

Kinetics

The substrate uptake rate was established using a differential equation (eq. 1).

$$\frac{dC_i}{dt} = q_i \cdot X_{lumped} \quad \text{(eq. 1)}$$

where X_{lumped} represents the lumped biomass concentration (equal to VSS) and q_i is the apparent biomass specific conversion rate. It was not feasible to establish the actual biomass specific conversion rates for the individual species in the reactor and the specific conversion rate (q) used in this study is therewith an overall apparent rate that only indicates the specific activity of the solids in the reactor. Furthermore, it should be noted that the biomass concentrations did not change significantly during one cycle (since a cycle was 2 hours and the SRT was more than 24 hours) and were therefore assumed to be constant during the cycle. The kinetic equations for biological conversion and mass transfer are presented in table 2.

Table 2. Model kinetics.

Reaction	Rate equation	
Glucose consumption	$q_{Glu}(t) = q_{Glu}^{max} \frac{C_{Glu}(t)}{K_{Glu} + C_{Glu}(t)}$	(eq. 2)
Lactate consumption	$q_{Lac}(t) = q_{Lac}^{max} \frac{C_{Lac}(t)}{K_{Lac} + C_{Lac}(t)}$	(eq. 3)
PG consumption	$q_{PG}(t) = q_{PG}^{max} \frac{C_{PG}(t)}{K_{PG} + C_{PG}(t)} \cdot \frac{K_I}{K_I + C_{Glu}}$	(eq. 4)
H_2 evolution rate	$H_2ER = k_{L,H_2} A\left(C_{H_2(g)} \cdot k_{H,H_2} - C_{H_2(aq)}\right)$	(eq. 5)
CO_2 evolution rate	$CO_2ER = k_{L,CO_2} A\left(C_{CO_2(g)} \cdot k_{H,CO_2} - C_{CO_2(aq)}\right)$	(eq. 6)

The kinetic equations used in the model for biological conversion and mass transfer are presented in table A2. The half-saturation constants for glucose, lactate, PG (K_{glu}, K_{lac}, and K_{PG}, respectively) and the inhibition constant (K_I) were assumed to be 1 mM. Coefficients for H_2 and CO_2 mass transfer were based on measurements for oxygen mass transfer and corrected according to Royce and Thornhill (1991) and using the Stokes-Einstein equation for calculation of the relevant diffusion coefficients.

Model calibration

For each sampling time point (t_i) the modelled data for each compound was compared with the measured data. The errors were calculated, squared, and summed up as shown for the example of acetate:

$$SSE_{Ac} = \sum_{i=1}^{N} \left(n_{Ac}^{measure}(t_i) - n_{Ac}^{model}(t_i)\right)^2 \quad \text{(eq. 7)}$$

The sum of squared errors for the different compounds was summed up to the total error between model and measurements (E_M).

$$E_M = \sum SSE_i \qquad \text{(eq. 8)}$$

with i = Glu, Ac, Pro, Bu, Lac, PG, EtOH, CO_2, H_2, X

The total error was minimized by adjusting characteristic parameters (q_S^{max}, and yields) by the solver tool of Microsoft Excel. The solver was driven by a GRG non-linear algorithm and no additional constraints were used. The initial values for all yields were 0.5 gCOD $gCOD^{-1}$ and for kinetic (q) rates 1 gCOD $gVSS^{-1}$ h^{-1}. It was verified that normalizing the SSE did not change the outcome of the model calibration. Carbon and COD balances were not included as criteria for model calibration and were evaluated separately.

Appendix 2B – Lactate conversion stoichiometry

The lactate fermentation stoichiometry used in this study was based on the thermodynamic principles regarding anaerobic growth on lactate, taking into account catabolic and anabolic reactions (Kleerebezem and Van Loosdrecht, 2010). The catabolic reaction stoichiometry was assumed to be identical to earlier reports concerning lactate fermentation (Seeliger et al. 2002; Tholozan et al. 1996).

$$1\ \text{Lac} \rightarrow {}^{1}/_{3}\ \text{Ac} + {}^{2}/_{3}\ \text{Pro} + {}^{1}/_{3}\ CO_2 \qquad \text{(eq. 9)}$$

$\Delta G_{cat} = 55$ kJ (mol lactate)$^{-1}$

The amount of Gibbs energy required to synthesize 1 Cmol of biomass can be calculated by a correlation proposed by Heijnen et al. (2010). For heterotrophic growth only the nature of the carbon source is important:

$$\frac{1}{Y_{GX}^{max}} = 200 + 18\left(6 - C\right)^{1.8} + e^{\left(\left|3.8 - \frac{\gamma}{C}\right|^{0.32} \times (3.6 + 0.4C)\right)} \qquad \text{(eq. 10)}$$

For lactate ($\gamma_{Lac} = 12$, $C = 3$) the energy required for the synthesis of 1 Cmol biomass ($1/Y_{GX}^{max}$) is equal to 348 kJ.

The amount of substrate that has to be catabolized to generate the energy needed to synthesize 1 Cmol of biomass ($|a_{cat}|$) can be calculated by dividing the amount of energy needed for the synthesis of 1 Cmol biomass by the amount of energy generated by catabolism (ΔG_{cat}).

$$|a_{cat}| = \frac{\frac{1}{Y_{GX}^{max}}}{\Delta G_{cat}} \qquad \text{(eq. 11)}$$

With this parameter the catabolic part of the biomass reaction can be derived:

$$|a_{cat}| \cdot \text{Lac} \rightarrow |a_{cat}| \cdot \frac{1}{3}\text{Ac} + |a_{cat}| \cdot \frac{2}{3}\text{Pro} + |a_{cat}| \cdot \frac{1}{3}CO_2 \qquad \text{(eq. 12)}$$

The anabolic part of the biomass reaction represents the amount of substrate needed to produce 1 Cmol of biomass ($|a_{ana}|$) and can be calculated by dividing the degree of reduction of 1 mol biomass ($\gamma_X = 4.2$) by the degree of reduction of 1 mol lactate ($\gamma_{Lac} = 12$).

$$|a_{ana}| = \frac{\gamma_X}{\gamma_{Lac}} \qquad \text{(eq. 13)}$$

With this parameter the anabolic part of the biomass reaction can be derived (eq. 14). The coefficient $a_{ana,c}$ can be found from the carbon conservation.

$$|a_{ana}|\ \text{Lac} \rightarrow 1\ \text{X} + a_{ana,c}\ CO_2 \qquad \text{(eq. 14)}$$

The total amount of substrate needed to produce 1 Cmol of biomass is thus:

$$|a| = |a_{cat}| + |a_{ana}| \qquad \text{(eq. 15)}$$

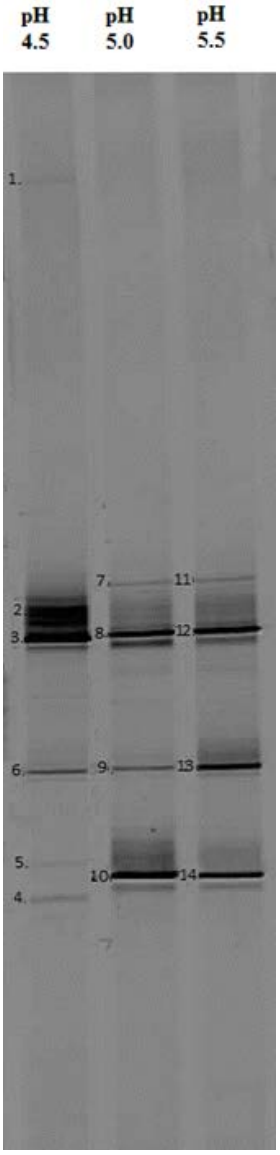
The overall lactate fermentation reaction derived from above equations can therefore be written as:

$$1\ \mathrm{Lac} \rightarrow \frac{|a_{cat}|}{|a|}\frac{1}{3}\mathrm{Ac} + \frac{|a_{cat}|}{|a|}\frac{2}{3}\mathrm{Pro} + \frac{|a_{cat}|+a_{ana,c}}{|a|}\frac{1}{3}\mathrm{CO_2} + \frac{1}{|a|}\mathrm{X} \qquad \text{(eq. 16)}$$

Filling in the values for the parameters derived in equation 11 and 13 and the coefficient $a_{ana,c}$ the overall reaction becomes:

$$1\ \mathrm{Lac} \rightarrow 0.32\ \mathrm{Ac} + 0.63\ \mathrm{Pro} + 0.32\ \mathrm{CO_2} + 0.15\ \mathrm{X} \qquad \text{(eq. 17)}$$

Appendix 2C – DGGE results



Band	Experiment	Closest match	% similarity
1	pH 4.5	*Clostridium pasteurianum*	98-100
2			
3			
4			
5		*Lactobacillus harbinensis*	100
6		*Pectinatus frisingensis*	97
7	pH 5.0	*Clostridium pasteurianum*	96-100
8			
9		*Pectinatus frisingensis*	100
10		*Olsenella* sp.	100
11	pH 5.5	*Clostridium pasteurianum*	96-100
12			
13		*Pectinatus frisingensis*	100
14		*Olsenella* sp.	100

Nomenclature appendix 2

a	substrate requirement for biomass production [mol (Cmol X) $^{-1}$]
a_{ana}	substrate requirement for anabolism [mol (Cmol X) $^{-1}$]
a_{cat}	substrate requirement for catabolism [mol (Cmol X) $^{-1}$]
C	number of carbon atoms
C_i	modeled concentration [mmol l^{-1}]
CO_2ER	CO_2 evolution rate from liquid to gas [mmol min^{-1}]
E_M	total error between model and measurements
H_2ER	hydrogen evolution rate from liquid to gas [mmol min^{-1}]
K_i	half-saturation constant [mmol l^{-1}]
$k_{H,i}$	Henry constant for compound *i*
$k_{L,,i}A$	mass transfer coefficient [l min^{-1}]
$n_i^{measure}$	measured amount [mmol]
n_i^{model}	modeled amount [mmol]
q_i	modeled biomass specific conversion rate [mol (mol X)$^{-1}$ h^{-1}]
q_i^{max}	maximum modeled biomass specific conversion rate [(C)mol ((C)mol X)$^{-1}$ h^{-1}]
r_i	rate of reaction *i*
SSE_i	sum of squared errors between model and measurements for compound *i*
t	model time [min]
Y_i	measured yield on glucose for compound *i*
$Y_{i,j}$	modeled yield of compound *i* on compound *j*
Y_{GX}^{max}	maximum biomass yield on Gibbs energy [(Cmol X) kJ^{-1}]
ΔG_{cat}	Gibbs energy of the catabolic reaction per mol donor [kJ mol^{-1}]
γ_i	degree of reduction for compound *i*

Subscripts

Ac	acetate
ana	anabolic
Bu	butyrate
cat	catabolic
CO_2	carbon dioxide
EtOH	ethanol
Glu	glucose
H_2	hydrogen
Lac	lactate
PG	polyglucose
Pro	propionate
Val	valerate
X	biomass

3

Lipid recovery from a vegetable oil emulsion using microbial enrichment cultures

Abstract

Many waste streams have a relatively high vegetable oil content, which is a potential resource that should be recovered. Microbial storage compound production for the recovery of lipids from lipid-water emulsions with open (unsterilized) microbial cultures was investigated in a sequencing batch reactor using a diluted vegetable oil emulsion as model substrate. After feeding, triacylglycerides (TAG) were accumulated intracellular by the microbial enrichment culture and subsequently used for growth in the remainder of the sequencing batch cycle. Roughly 50% of the added TAG could be recovered as intracellular lipids in this culture. The maximum lipid storage capacity of the enrichment culture was 54% on volatile suspended solids (VSS) mass basis in a separate fed-batch accumulation experiment. The microbial community was dominated by a lipolytic fungus, *Trichosporon gracile*, that was responsible for intracellular lipid accumulation, but also a significant fraction of lipolytic and long-chain-fatty-acid-utilizing-bacteria was present. Herewith, we demonstrate an effective strategy for enrichment of a microbial community that can accumulate significant amounts of lipids from wastewaters without the need for sterilization of substrates or equipment. Further optimization of this process will make recovery of lipids from wastewater possible.

Published as

Tamis, J., Sorokin, D.Y., Jiang, Y., van Loosdrecht M.C.M., Kleerebezem, R. (2015) Lipid recovery from a vegetable oil emulsion using microbial enrichment cultures. Biotechnology for biofuels 8(1):1-11.

Introduction

The use of agro-industrial organic residues for the production of valuable commodities is a logical step towards a bio-based economy. Traditionally, these wastes are used for biogas or compost production. Recently, several alternatives for the production of more valuable compounds have been proposed, for example, the production of biopolymers (Kleerebezem and van Loosdrecht 2007, Reis et al. 2003), volatile fatty acids (Agler et al. 2011), or medium chain length fatty acids (Spirito et al. 2014). One of the critical issues related to resource recovery from wastewater is the efficient up-concentration and purification of the valuable compounds that are present in diluted form in the wastewater. In general, the production of storage compounds with microbial cultures from diluted waste streams improves the efficiency of resource recovery since the product is concentrated inside the biomass and can be readily separated from the water using standard sludge settling (or other separation) methods.

A relatively well studied example of a process based on storage compound production by microbial enrichment cultures is the production of polyhydroxyalkanoate (PHA) and contents up to 90% on VSS mass basis have been obtained by application of selective pressure in the form of feast-famine conditions (Jiang et al. 2011a, Johnson et al. 2009a). This process is currently further evaluated in industrial environments (Anterrieu et al. 2014, Jiang et al. 2012a, Liu et al. 2008). Interestingly, it has been found that different carbon sources in a feast-famine process can result in formation of different storage compounds: microbial cultures enriched on glucose or starch, produced polyglucose as storage compound (Carta et al. 2001, Karahan et al. 2006), a microbial culture enriched on glycerol produced a mixture of polyglucose and PHA (Moralejo-Gárate et al. 2011), microbial communities enriched on different types of volatile fatty acids (VFA) produced different varieties of PHA (Albuquerque et al. 2011, Jiang et al. 2011b), and a microbial culture enriched on methanol did not produce any storage compounds (Marang et al. 2014). While the above examples illustrate the many substrate types that have been investigated, the fate of lipids as substrate in a feast-famine process remains unclear.

Lipids are an important constituent of many types of wastewater, especially triacylglycerides (TAG), present in (among others) effluents from vegetable oil crop processing industries. For example, in the wastewater from the palm oil industry, high concentrations of emulsified oil are found that are hard to recover using physical-chemical methods (Igwe and Onyegbado 2007). Palm oil is a fast growing market with a current production of around 50 Mton y^{-1} (FAO 2014) accompanied by the production of 3 m^3 wastewater per ton oil palm oil produced (Borja and Banks 1994) resulting in an estimated palm oil mill effluent flow of 150 million $m^3\ y^{-1}$.

It was proposed that lipids found in wastewater may be recovered through the use of lipid-accumulating microorganism for the production of biodiesel (Muller et al. 2014). The economic feasibility of lipid extraction for diesel production from sludge will depend on the lipid content accumulated in the sludge, but a cost of around 1 US $ l^{-1} was reported from sludge containing 10% lipids (Mondala et al. 2009). In this approach, the recovery of lipids from wastewater requires a micro-organism with an as high TAG storage capacity as possible. However, not all microorganisms store TAG from wastewater in an appropriate way for biodiesel production.

A variety of metabolic pathways related to the microbial conversion of TAG have been described in literature (figure 3.1). Due to the relatively large molecule size (M_w around 800-1000 g mol^{-1}) and strongly hydrophobic nature of TAG, the uptake mechanism involves an extracellular hydrolysis step yielding long chain fatty acids (LCFA) and glycerol, which are subsequently transported into the cell (Wakelin and Forster 1997). Once the substrate is taken up, different routes are possible for the production of storage compounds: e.g. PHA production by pure cultures of prokaryotic strains was reported (Eggink et al. 1992,

Lageveen et al. 1988, Lee et al. 2000, Ng et al. 2010) as well as the intracellular storage of TAG by pure cultures e.g. by oleaginous yeasts (Liang and Jiang 2013, Zhu et al. 2008) or bacteria (Alvarez and Steinbüchel 2002, Slijkhuis 1984).

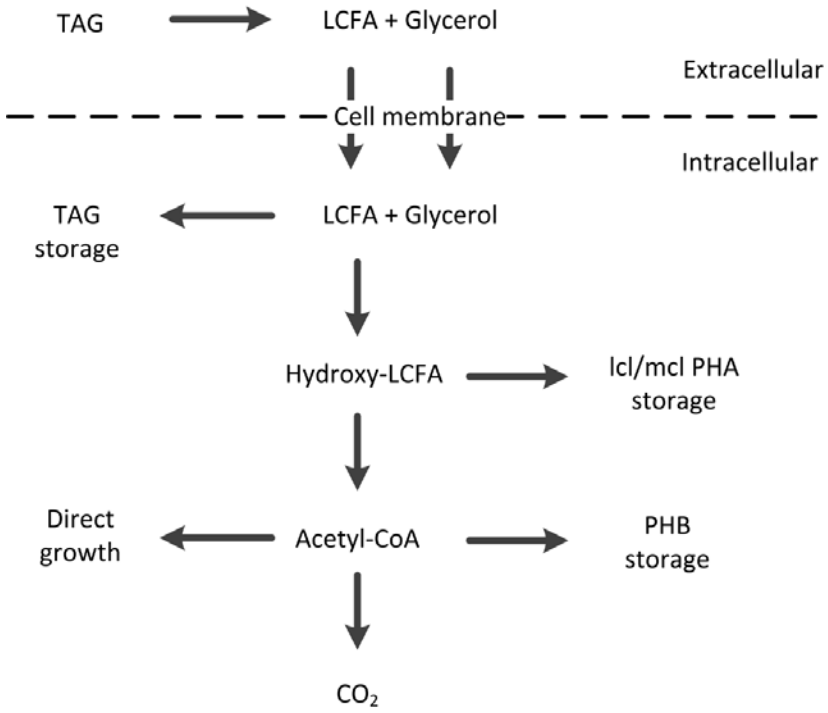


Figure 3.1. Simplified metabolic pathways of storage compound formation and growth on TAG based on literature reports (Wakelin and Forster 1997, Eggink et al. 1992, Lageveen et al. 1988, Lee et al. 2000, Ng et al. 2010, Liang and Jiang 2013, Zhu et al. 2008, Alvarez and Steinbüchel 2002, Slijkhuis 1984, Chipasa and Mędrzycka 2006).

One approach for recovery of lipids from wastewater is the cultivation of pure cultures of strains with very high lipid storage capacity. This approach has been evaluated in laboratory experiments with synthetic substrates and sterilized molasses (Zhu et al. 2008). However, since sterilization of diluted wastewater streams for biodiesel production is likely economically unfeasible, the use of unsterilized wastewater was evaluated (Ling et al. 2013; Peng et al. 2013). In these cases a pre-cultivated pure culture of oleaginous yeast was used to inoculate a batch experiment with acidic wastewater of a distillery as substrate. It was observed that a biomass with a relatively high lipid content (respectively 44% and 14%) could be grown by this method.

A different strategy was proposed by Santamauro et al. (2014) in which an oleaginous yeast could be selectively enriched in a low pH environment. In general oleaginous yeasts thrive relatively well in low pH environments. Nevertheless, many non-oleaginous microorganisms are known to be able to grow under low pH conditions (Magan 2007). The selective pressure imposed by the low pH (or temperature) does not intrinsically provide a competitive advantage for lipid-accumulating microorganisms, which makes the enrichment culture unstable and likely not optimal for lipid accumulation (Mooij et al. 2015).

Since lipid accumulation has been reported as a microbial survival strategy to balance carbon and energy requirements in periods of absence of external substrate (Andreasen and Nielsen 2000), in this study, we propose the application of a feast-famine strategy as a novel approach for the enrichment of lipid-accumulating organisms. This strategy is analogous to the feast-famine process for PHA production (Johnson et al. 2009a) and is based on ecological principles that provide an intrinsic competitive advantage to lipid-accumulating species. This process potentially enables the recovery of lipids from a broader

spectrum of wastewaters, can be operated as sequenced batch or continuously, and alleviates the need for single batch operations with pre-cultivated pure culture inocula. An open (non-sterilized) reactor system was inoculated with wastewater sludge and pulse-fed with a model substrate (soybean oil) to evaluate the potential of the feast-famine principle for the recovery of lipids from diluted lipid-containing emulsions.

Materials and methods

Enrichment reactor setup and operational parameters

A sequencing batch reactor (SBR) with a liquid volume of 2 l was operated aerobically, similar to Johnson et al. (2009). An operational cycle comprised of a feed phase of 30 minutes, during which TAG and nutrients were dosed, followed by a reaction phase of 11 hours. At the end of the cycle, half of the volume of the reactor was discharged, to be replaced with new feed in the next cycle, resulting in a total cycle length of 12 h, a hydraulic retention time (HRT) and solid retention time (SRT) of 24 h. The reactor was completely mixed (1000 rpm) throughout the cycle and biofilms were structurally removed (several times per week) to ensure that the solid retention time (SRT) equaled the hydraulic retention time. The temperature was controlled at 30 °C ± 1 °C using a thermostat, and the pH was maintained at 7.0 ± 0.1 by pH control using 1 M NaOH and 1 M HCl. The gas flow into the reactor was 1.5 l min^{-1} consisting of 0.3 l min^{-1} air and 1.2 l min^{-1} recycled off-gas. The off-gas was cooled to 5 °C by a condenser to minimize water evaporation.

Medium composition

Soybean oil (Markant merk, COOP, The Netherlands) was chosen as representative model substrate for vegetable oil waste. The substrate soybean oil was analyzed by gas chromatography (GC) as described in the 'Sampling and measurement' section and the results can be found in the 'Results' section (table 3.1); the composition was similar to that of soybean oil found in literature (Babayan 1987). In each cycle 1.3 grams (equivalent to 83 Cmmol) soybean oil was dosed into the reactor using a precision syringe pump. Additionally, 200 ml $cycle^{-1}$ nutrient solution containing (g l^{-1}) NH_4Cl 3.6; KH_2PO_4 3.4; $MgSO_4 \cdot 7H_2O$ 1.4; KCl 0.54; EDTA Titriplex III (Sigma-Aldrich, St. Louis, MO, USA) 1.0; $ZnSO_4.7H_2O$ 0.033; $CoCl_2.6H_2O$ 0.024; $CuSO_4.5H_2O$ 0.024; $FeSO_4.7H_2O$ 0.075; $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.017; $CaCl_2.H_2O$ 0.110; according to Vishniac and Santer (Vishniac and Santer 1957), and 0.050 g l^{-1} allylthiourea (to prevent nitrification) was added together with 800 ml $cycle^{-1}$ of water.

Sampling and measurement

The temperature, pH and off-gas composition in terms of CO_2 and O_2 concentrations were monitored online using a standard sensors (Mettler Toledo, Columbus, OH, USA) and an off-gas analyzer (NGA 2000, Rosemount Inc, Shakopee, MN, USA). In addition, samples were taken regularly at the end of the cycle to measure the total suspended solids (TSS) and the volatile suspended solids (VSS) concentrations according to standard methods (Clesceri et al. 1999) and the ammonium concentration using spectrophotometric method (LCK-348, Hach-Lange, Düsseldorf, Germany). For evaluation of the carbon balance, the organic carbon content of the organic solids was measured using a colorimetric method (LCK 381, Hach-Lange, Düsseldorf, Germany).

In order to characterize the dynamics during a cycle, samples were taken to measure the intracellular lipid concentrations, TSS and VSS production, ammonium uptake, CO_2 production and O_2 consumption. Intracellular storage compounds were analyzed as described by Johnson et al. (2009a), except that a modified gas chromatography method was used: analysis of extracted PHA and lipids derivatives was performed using direct injection into a polyethylene glycol column (HP-INNOWax, 60m x 250μm x 0.15μm) installed on a gas chromatograph (model 6890N, Agilent Technologies, Santa Clara, CA, USA)

equipped with a flame ionization detector. The column temperature program comprised an initial oven temperate of 100 ˚C, operated for 2 min, followed by an increase to 170 ˚C at a rate of 20 ˚C min^{-1} and a further increase to 240 ˚C at a rate of 5 ˚C min^{-1} resulting in a total runtime of 30 min per sample. The temperatures of the injector and detector were 230 ˚C and 250 ˚C, respectively. Calibration standards included PHB, PHV, hydroxyhexanoate and hydroxypalmitate for identification of PHA, and oleic-, linoleic-, a-linolenic-, palmitic- and stearic-acid for identification of LCFAs. For each standard, three different concentrations were used to make a calibration curve. For each GC spectrum it was checked whether there were unknown peaks indicating missing compounds.

The lipase activity in the culture was evaluated using a colorimetric method based on the hydrolysis of an artificial lipid, 4-nitrophenyl-palmitate (Vorderwulbecke et al. 1992) both in untreated samples and in samples in which biomass had been removed by centrifugation for 10 min at 2500 rpm. The hydrolysis product, 4-nitrophenyl was measured spectrometrically at 410 nm.

Microbial community structure

The general microbial community composition was investigated using fluorescence *in situ* hybridisation (FISH) analysis as described by Johnson et al. (2009a), using the EUB338 and EUK516 probe mixtures for identification of bacterial and eukaryote species respectively (Alm et al. 1996). Intracellular storage compounds were visualized using Nile blue A staining. The microbial diversity was further analyzed by denaturing gradient gel electrophoresis (DGGE) using eukaryotic and prokaryotic primers (Muyzer 1999). Additionally, traditional cultivation techniques were used to separate the functional groups of microorganisms from the reactor and to isolate pure cultures. For this, serial dilutions in liquid medium containing three different substrates were used: soybean oil, the free LCFA fraction of soybean oil and glycerol. To separate the eukaryotic community from bacteria, a mixture of kanamycine and streptomycine (100 mg l^{-1} each) was applied in a dilution series. The final positive dilutions on each substrate were plated on solid media with corresponding substrates, except that LCFA and soybean oil were first emulsified by sonication with gum arabic to allow detection of the LCFA-utilizing and lipolytic colonies (Sorokin and Jones 2009).

Fed-batch accumulation experiments

The storage capacity of the microbial enrichment culture was assessed using a fed-batch accumulation experiment. Apart from the feeding regime, all other operational parameters were kept identical to the enrichment reactor: pH was maintained at 7.0 ± 0.1, the temperature was maintained at 30 ˚C ± 1 ˚C and the reactor liquid was stirred with 1000 rpm. The biomass from the enrichment reactor was used as inoculum and the oxygen profile was used to monitor the activity in the reactor; when the oxygen consumption decreased, a new pulse of soybean oil substrate was dosed. The biomass was characterized in terms of lipid content over time.

Data analysis and interpretation

It was not possible to take a representative sample for measurement of the substrate concentration from the oil-water emulsion that formed quickly after substrate dosage. Instead the soybean oil uptake rate in the medium was estimated by assuming that all the oil had been taken up after the feast phase. This assumption was verified using microscopy to make sure that all oil had been taken up and was not absorbed to the biomass. The length of the feast phase was estimated using the off-gas data, with the feast phase characterized by relatively high oxygen consumption and CO_2 production rates and the onset of the famine phase by a relatively sharp decline in both these rates (appendix 3A). The active biomass concentration was calculated by subtracting the amount of storage compounds from the amount of VSS in the system. Active biomass concentrations were verified by the evaluation of the ammonium uptake

and nitrogen content of the biomass. Conversion yields and rates were obtained using a modified version of a model used for modelling PHA producing feast-famine cultures (Tamis et al. 2014b). The only modifications in the model were replacement of PHA storage by storage of TAG and inclusion of a fraction of the substrate that was used for direct growth. A description of the model is provided in the supplementary material (appendix 3C).

Results

Enrichment reactor

After inoculation of the sequencing batch reactor (SBR) with activated sludge (WWTP Dokhaven, The Netherlands) a typical feast-famine pattern became apparent in less than 5 days (10 cycles). The length of the feast phase (i.e. time it took to take up the lipid substrate added with the influent) was initially more than 3 h and became more stable after 25 days (50 cycles) with a feast phase length of around 1 - 2 h (figure 3.2). Variations in the length of the feast phase are due to the formation of biofilm in the reactor, which had to be periodically removed.

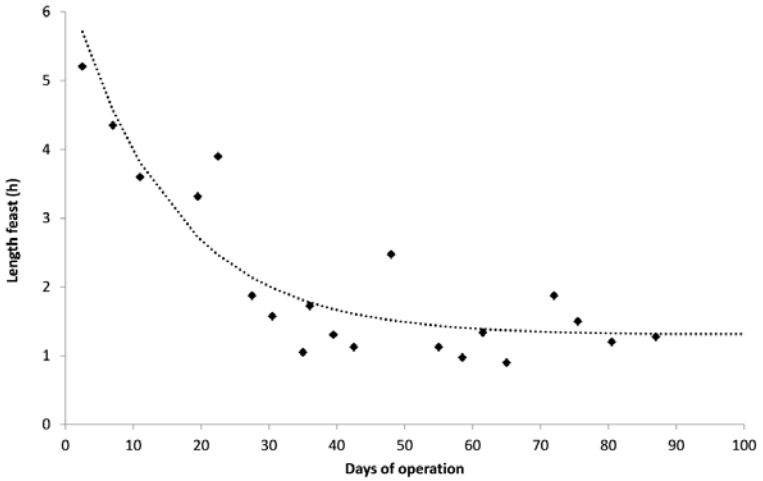


Figure 3.2. Monitoring of the start-up of the sequencing batch reactor by the evolution of the length of the period needed for TAG substrate uptake (feast phase) by the enrichment culture in the reactor.

After the reactor had reached a stable operational performance, the carbon mass balance over the system was evaluated by analysis of the influent and effluent in the period of day 68 until day 76 of operation (16 cycles). The average effluent total suspended solids (TSS) concentration was 1.0 ± 0.16 g l^{-1} (standard deviation over the dataset of 16 cycles) with an ash fraction of 0.14 ± 0.03, resulting in a volatile suspended solids (VSS) concentration of 0.86 ± 0.15 g l^{-1}. Additionally, the amount of biofilm that was formed on the reactor wall was removed and measured, indicating an average biofilm growth of 0.25 gVSS per cycle. TOC measurements indicated that the carbon content of the organic solids in the effluent was 0.59 (±0.03) gC $gVSS^{-1}$ resulting in a total organic solids production equivalent to 59 ± 10 Cmmol per cycle. Off-gas measurements showed a CO_2 production of 17 ± 3 mmol per cycle. Herewith, most of the carbon present in the influent substrate (83 Cmmol per cycle) could be accounted for by carbon in the produced organic solids and CO_2 (76 ± 10 Cmmol per cycle). The 10% deviation in the balance is partly due to the complications arising from the reactor wall growth.

To get further insight into the conversions during the cycle, three experiments were performed. For all three cycles experiments, the biomass concentrations and length of feast phase were within the value range determined during the steady operational period. In figure 3.3 profiles from a typical cycle are

shown. For practical purposes and in order to establish the substrate amount extra accurate, the substrate in these experiments was dosed manually in one short pulse, and the amount of substrate was evaluated by the weight of the dosing syringe before and after addition of the oil.

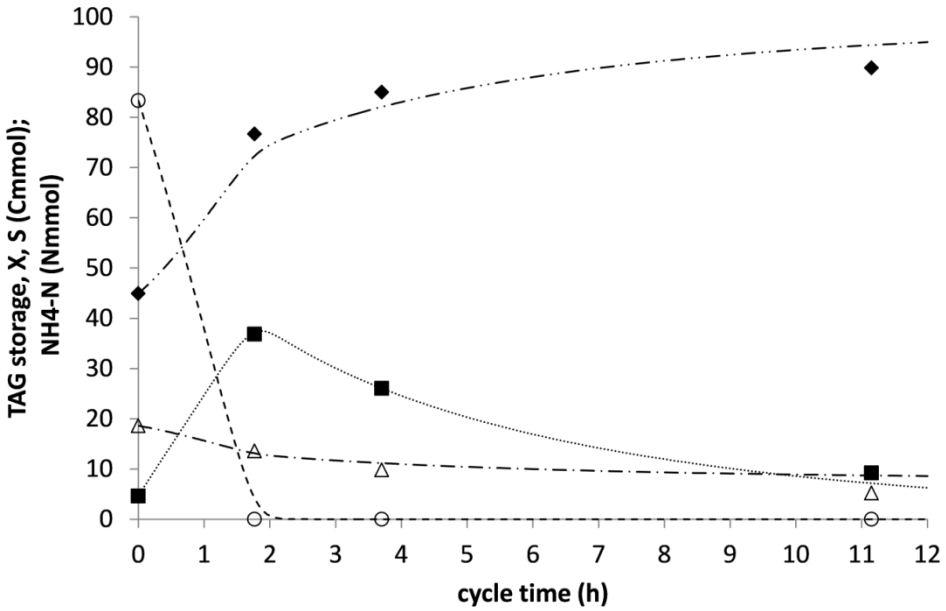


Figure 3.3. Amounts of TAG substrate (○, ----), lipid storage (■, ····), active biomass (♦, -··-··) and ammonium (Δ, -·-·) during a cycle in the TAG-fed sequencing batch reactor. Symbols represent measurement and lines represent model based calculations.

After dosing the vegetable oil, the reactor liquid became opaque, indicating that the mixture of microorganisms and vegetable oils formed an emulsion; this opaque feature disappeared again at the end of the feast phase (after 2 h) when the TAG was depleted from the medium. GC analysis showed that the lipid content of the biomass increased from 6% ± 0.6% on VSS mass basis, at the start of the cycle, to 25% ± 1.5% on VSS mass basis at the end of the feast phase (average ± standard deviation over three cycle experiments). Microscope images of the culture confirmed the indication that significant amount of lipids were stored intracellularly (figure 3.5, right). The composition of the accumulated microbial lipids was almost identical to the substrate oil (table 3.1), indicating direct utilization of the externally hydrolyzed LCFA and glycerol without their *de novo* synthesis or conversion. GC analysis showed only peaks that corresponded with the LCFA standard, and no significant peaks corresponding with the PHA standards, suggesting that TAG was the only relevant storage compound present in the culture at any time during the cycle. The lipid content of the cells decreased during the famine phase indicating growth on the stored lipids. Nitrogen was present throughout the cycle, and it was observed that nitrogen was taken up both during the feast and the famine phase, indicating significant microbial growth during the whole SBR cycle. The increase in VSS concentration in the feast phase was higher than that would be expected from TAG storage only, supporting the indication of growth of microorganisms in both the feast phase and the famine phase. A model was calibrated to the experimental data to identify characteristic process parameters (appendix 3C). It was estimated that about half of the substrate was used for growth while the other half was accumulated as intracellular lipids during the feast phase. An overall biomass specific uptake rate of 1.0 Cmol Cmol^{-1} h^{-1}, a yield of lipid storage on substrate of 0.9 Cmol Cmol^{-1} and biomass on lipids of 0.6 - 0.7 Cmol Cmol^{-1} were estimated from the cycle measurements. A summary of the experimental data obtained during steady operation and cycle measurements in terms of carbon flows, is provided in appendix 3B.

Table 3.1. Comparison of the LCFA constituents from literature, used substrate and intracellular storage compounds. For the composition of the intracellular storage the average and the standard deviation over a dataset of six individual samples are provided.

	Soybean oil (Babayan, 1987)	Soybean oil (this study)	Intracellular storage (this study)
		% of LCFA mass	
Oleic acid	23%	23%	25 ± 5 %
Linoleic	53%	55%	50 ± 6 %
a-Linolenic	8%	6%	7 ± 2 %
Palmitic	11%	12%	11 ± 3 %
Stearic	4%	4%	4 ± 2 %

Accumulation experiments

The maximum lipid storage capacity of the culture was evaluated using fed-batch experiments in which soybean oil was available in excess. The maximum lipid content appeared to be dependent on the availability of nitrogen. In the absence of a nitrogen source, only 24% lipid storage on VSS basis was observed while in another experiment with ammonium present in the reactor liquid (C:N molar ratio was 4/1 Cmol/Nmol), a lipid accumulation of 54% on VSS basis was observed. While the initial lipid accumulation was relatively fast, the lipid content of the culture stabilized after 4 - 5 h of operation, indicating the maximum storage capacity of the culture under these conditions was reached (figure 3.4).

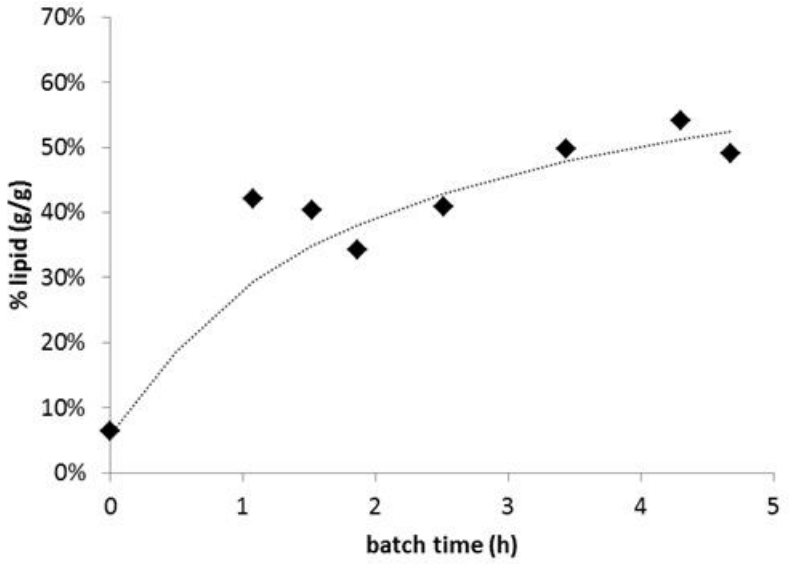


Figure 3.4. The evolution of lipid content of the enriched microbial culture over time in a fed-batch experiment with nutrients present.

Lipase activity assays

The lipase activity was measured using a standard test with 4-nitrophenyl-palmitate (Vorderwulbecke et al. 1992) during both the feast and the famine phases. An activity of 0.80 ± 0.28 mmol l^{-1} min^{-1} was measured during the feast phase (average ± standard deviation over a dataset of six measurements). During the famine phase, an activity of 0.73 ± 0.27 mmol l^{-1} min^{-1} was measured (average ± standard deviation over a dataset of three measurements). This indicated that there was no significant difference in lipase activity between the feast and the famine phases. For all cases, we evaluated the activity of both the reactor broth (with biomass) and the reactor supernatant. The average activity measured in the reactor broth was 0.99 ± 0.18 mmol l^{-1} min^{-1} and in the supernatant 0.57 ± 0.14 mmol l^{-1} min^{-1}. This indicated that a significant part of the enzymatic activity was due to extracellular enzymes but also that part of the lipase activity was cell-

bound. The measured lipase activity was relatively high compared to the lipid uptake rate observed during the feast phase. Using an uptake of 83 Cmmol (approximately 4 mmol ester bonds) in 2 h, and a reactor volume of 2 l, the minimal required lipase activity would be 0.01-0.02 mmol l^{-1} min^{-1}. This indicated that extracellular lipase activity was not limiting the conversion rates.

Microbial community structure

The molecular and microscopic analyses of the reactor biomass showed a presence of a dimorphic fungus and several bacterial morphotypes. The direct microscopy results were confirmed by FISH analysis which indicated the presence of both eukaryotes and prokaryotes (figure 3.5, left). Furthermore, lipid-specific Nile blue A staining indicated that the fungus was involved in lipid storage while the majority of the prokaryotes in the reactor did not contain significant amounts of lipid-like intracellular storage compounds (figure 3.5, right).

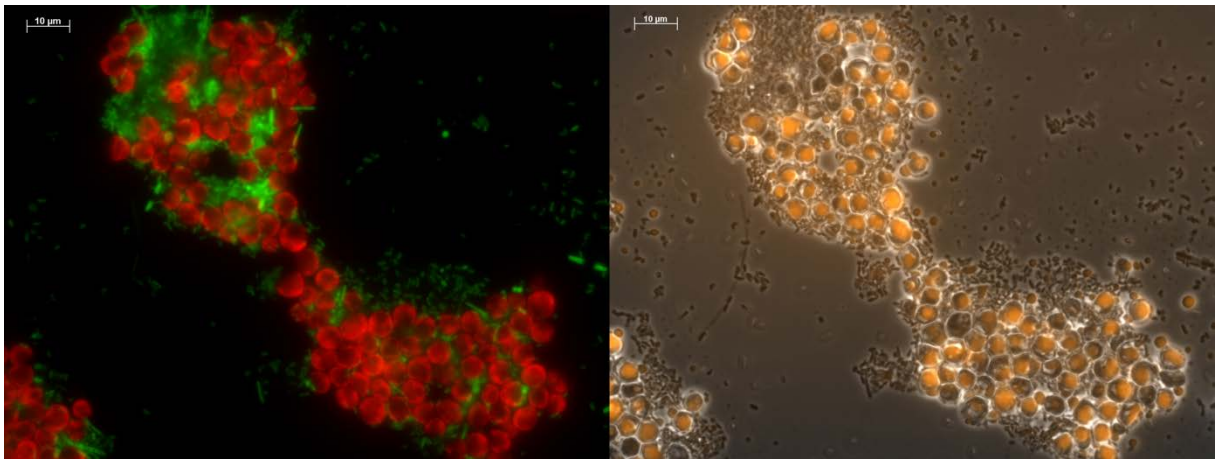
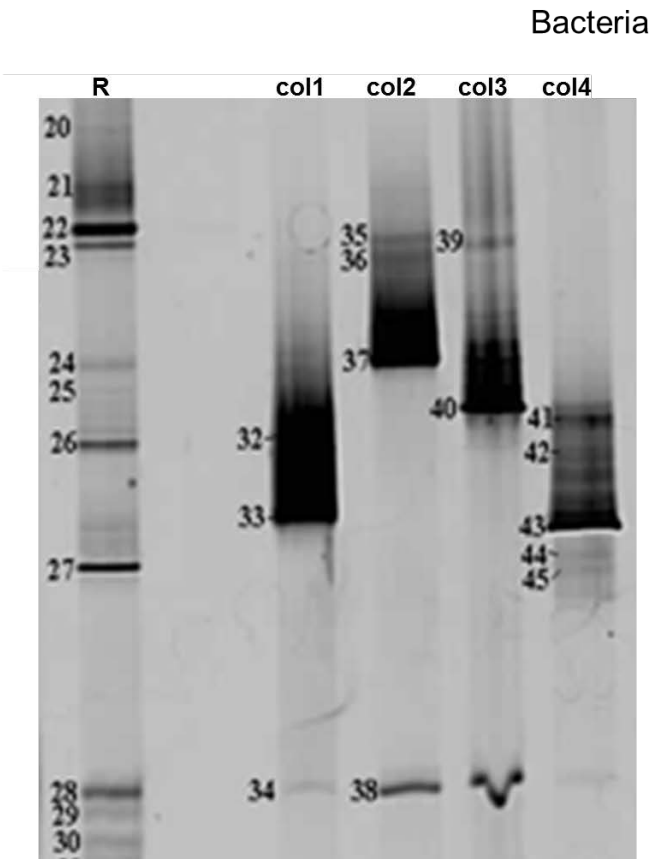


Figure 3.5. Microscope images of a sample from the reactor at the end of the feast phase (GC measurements indicated a lipid content of 25% on VSS mass basis). Left: FISH staining with the EUK516 probe to stain eukaryotes (red) and the EUB338 probe to stain prokaryotes (green). Right: Nile blue A staining to identify lipids (orange).

DGGE was used to characterize the communities. Eukaryotic primer-based PCR products indicated the presence of *Trichosporon gracile* (100% similarity) from a genus known to include several oleaginous species (Zhu et al. 2008, Holdsworth and Ratledge 1988). The bacterial-specific DGGE analysis of the reactor biomass indicated the presence of a few proteobacterial phylotypes closely related to the *Acetinobacter* and *Gordonia* genera (figure 3.6), which are both known to possess metabolic pathways for degradation of hydrophobic substrates (Arenskötter et al. 2004, Juni 1978).



Identification results

Band	Sample	Closest match	% similarity
B20	Reactor	*Acinetobacter seohaensis*	98-99
B21			
B22			
B23			
B24		*Pseudomonas alcaligenes.*	98
B27		*Gordonia* sp	99
B28		*Acinetobacter* sp.	99
B30		*Gordonia* sp.	97
B32	Col 1	*Diaphorobacter* sp.	97
B33			
B34			
B35	Col 2	*Pseudomonas alcaligenes*	99
B36			
B37			
B38			
B39	Col 3	*Shewanella putrefaciens*	99
B40			
B41	Col 4	*Hydrogenophaga intermedia*	99
B43			
B44			

Figure 3.6. Bacterial-specific DGGE analysis of the reactor biomass and isolates. R: Reactor biomass; Col 1: LCFA isolate, long rods; Col 2: lipolytic, short motile rods; Col 3: LCFA isolate, small rods; Col 4: glycerol enrichments.

Cultivation efforts have been made to try to resolve the role of the microbial species present in the reactor biomass in lipid degradation and utilization of the products. Incubations, with and without antibiotics, allowed the isolation of the eukaryote *Trichosporon* and four prokaryote microorganisms. The eukaryotic dimorphic fungus *Trichosporon gracile* was isolated on lipid plates with antibiotics; it formed mycelium-containing colonies surrounded by zones of lipid hydrolysis (figure 7). This organism was most versatile in its metabolism, being able to degrade the TAG, utilize the products of hydrolysis (LCFA and glycerol), and reform the TAG inside the cells. The bacterial enrichments with three different substrates – lipid, LCFA and glycerol, yielded four isolates: (i) a small motile rod-shaped bacterium able to grow with lipid and LCFA with strong lipolytic activity (col2, identified as a *Pseudomonas alcaligenes* from the betaproteobacteria). Isolates (ii) and (iii) are two different types of LCFA utilizers. The first LCFA degrader (col1) is a long rod with the ability to store lipids inside the cells. This microorganism was identified as a representative of the genus *Diaphorobacter* from the betaproteobacterial lineage. Although the genus *Diaphorobacter* is quite common for activated sludge, our isolate is very different in cell morphology from the three species described of this genus. The ability to utilize LCFA has never been tested for this genus. The second LCFA degrader is a small rod with no apparent storage polymers (col3) identified as a representative of the genus *Shewanella* in the gammaproteobacteria. (iv) Glycerol-specific enrichment resulted in a domination of *Hydrogenophaga* sp. (betaproteobacterium) represented by small motile rods. Summarizing, the cultivation results showed two functional parts – a eukaryotic dimorphic fungus, doing a complete job of lipid degradation and lipid accumulation, and a bacterial block, basically doing equivalent work but divided between different species (figure 3.7).

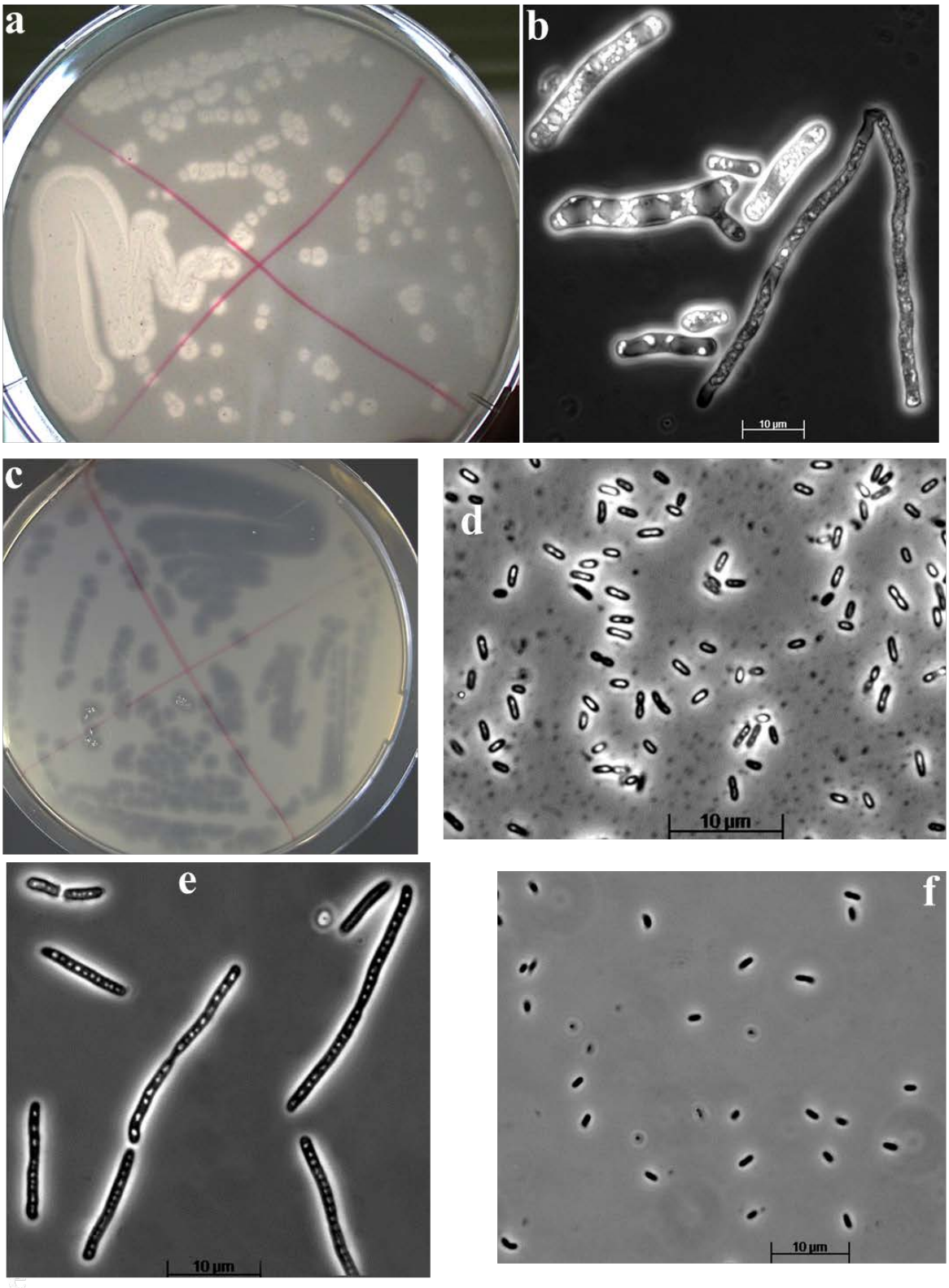


Figure 3.7. Different functionalities from isolates obtained by plating of the reactor culture. Upper panel: Trichosporon gracile (a –lypolytic colonies, b –lipid storing hyphal cells); middle panel: lipolytic Pseudomonas alcaligenes. (col2) (c –lipolytic colonies, d – cells in liquid culture with lipid), bottom panel: LCFA-utilizing bacterial isolates (e –Diaphorobacter sp. (col1) and f –Hydrogenophaga sp. (col3)).

Despite that two of the bacterial species (*Diaphorobacter* and *Pseudomonas*) were clearly recognizable by microscopic inspection of the reactor biomass, only the latter was detected directly by DGGE analysis of the reactor biomass. One of the possible explanations is a DNA extraction bias in the presence of lipids.

Discussion

A microbial culture capable of accumulating lipids from a dilute emulsion was enriched in a sequencing batch reactor with a feast-famine regime. The development of this enrichment from an activated sludge inoculum occurred relatively fast (25 days). A lipid-storing fungus and a variety of proteobacterial species co-existed in the enrichment culture. Microscopic observation of the reactor biomass indicated that the bacterial phenotypes (except a small population of large cells identified as a *Diaphorobacter* sp.) did not store significant amounts of lipids but instead used lipids or its immediate hydrolysis products directly as growth substrates.

Analysis of the microbial community by DGGE indicated the presence of the eukaryotic fungus *Trichopsoron gracile,* which could be readily isolated by microbial plating techniques with antibiotics and appeared to be able to accumulate significant amount of lipids intracellularly. The bacterial population analysis was more complicated since *Gordonia* and *Acinetobacter* species that were detected by DGGE could not be isolated using microbial plating techniques. *Gordonia* and *Acinetobacter* species are important species in the lipid-degrading populations that cause foaming in wastewater treatment plants (Chipasa and Mędrzycka 2006), and especially *Gordonia* species are known to accumulate large amounts of intracellular lipids (Gouda et al. 2008). Furthermore, while *Diaphorobacter* was observed in the biomass (based on its morphology) and could be readily isolated by microbial plating, it could not be detected by DGGE analysis. This shows that microbial analysis tools such as microbial plating and DGGE can easily introduce bias in the population analysis of a reactor. In conclusion, we could identify and culture the dominant microorganism in the culture, a yeast, but the major bacteria could not be exclusively identified by DGGE or microbial plating.

The presence of the bacterial side population in the reactor may be partly explained by the role of bacteria that by themselves were not able to hydrolyse and use TAG as substrate, but could thrive in the reactor by using the LCFA or glycerol that was liberated through the extracellular lipase activity facilitated by e.g. *Trichosporon.* Furthermore, the coexistence of storing and non-storing microbes indicates that lipid uptake and storage by *Trichosporon gracile* was not fast enough to outcompete direct growth by bacterial lipolytic and LCFA-utilizers. The specific uptake rate of the microbial culture was with 1 Cmol Cmol^{-1} h^{-1} relatively low compared to similar feast-famine systems on other substrates, e.g. an uptake rate of around 4 Cmol Cmol^{-1} h^{-1} was reported for a feast-famine culture cultivated using acetate as carbon source (Jiang et al. 2011c).

The measured hydrolytic activity was more than an order of magnitude higher than the observed uptake rate suggesting that hydrolysis was not the rate limiting step. It should be noted that the values obtained from the enzyme assay only serve as rough indication since the lipase activity may vary when TAG is the substrate instead of 4-nitrophenol-palmitate (Vorderwulbecke et al. 1992, Sigurgísladóttir et al. 1993). Factors that potentially limit the lipase activity include transport limitations due to formation of an emulsion during the feast phase (Cirne et al. 2007).

In this study, we show the use of a feast-famine based enrichment strategy for the enrichment of a mixed microbial culture with enhanced lipid accumulation capacity. Remarkably, the maximum accumulation of intracellular lipids of 54% on VSS basis was only achieved when excess nutrients were supplied. This is in contrast with PHA-producing enrichment cultures, which were reported to achieve higher storage

contents during nitrogen-deficient fed-batch experiments (Johnson et al. 2010a). Possibly, the major lipid-storing species could not function under nitrogen-limited conditions. Further research may include investigation of the behavior of pure cultures of *Trichosporon gracile* to evaluate this hypothesis.

The lipid content of the microbial enrichment culture obtained in this study was around 50% on VSS mass basis and was mainly related to the presence of the oleaginous yeast *Trichosporon gracile*. The *Trichosporon* genus is known to be able to accumulate high amount of lipids, e.g. TAG contents higher than 60% were reported in pure culture experiments (Zhu et al. 2008). The high TAG content of these cultures suggest that further improvement may be achieved by development of strategies that provide a competitive advantage for *Trichosporon* over the bacterial side population.

Nevertheless, the achieved lipid content of around 50% is already significantly more than the 10% lipid content in sludge that was used as reference content to evaluate the feasibility biodiesel production (Mondala et al. 2009) or and even more than the oil content of soybeans (FAO 1992) or olives, implicating the potential feasibility of lipid recovery for the production of biodiesel. Herewith, we show that ecological principles can effectively be employed for the enrichment of lipid-accumulating species, enabling potential application on a broad variety of waste streams. The use of enrichment cultures decreases capital and operating costs because (semi-continuous) open cultivation techniques can be used without the need for sterilization or re-inoculation.

Conclusions

A microbial enrichment culture of lipid-storing organisms in co-existence with microorganisms that used TAG for direct formation of biomass was observed in a soybean-oil-fed reactor system. The former was represented by a lipolytic dimorphic fungus, while the latter consisted of a consortium of proteobacteria capable of utilizing either the lipid itself or its hydrolysis products, mainly as a substrate for direct growth. The maximum lipid accumulation capacity of the culture was 54% on VSS mass basis. Results were obtained from an enrichment reactor that was not optimized for maximum storage capacity, suggesting that future optimization may lead to microbial enrichment cultures with even higher lipid storage capacity.

Appendix 3A – Off-gas profiles

Off-gas measurements showed decreasing oxygen and increasing CO_2 concentrations in the first part of the cycle, indicating increased activity defined here as the feast phase, followed by a relatively sharp decline indicating the start of the famine phase (figure 1).

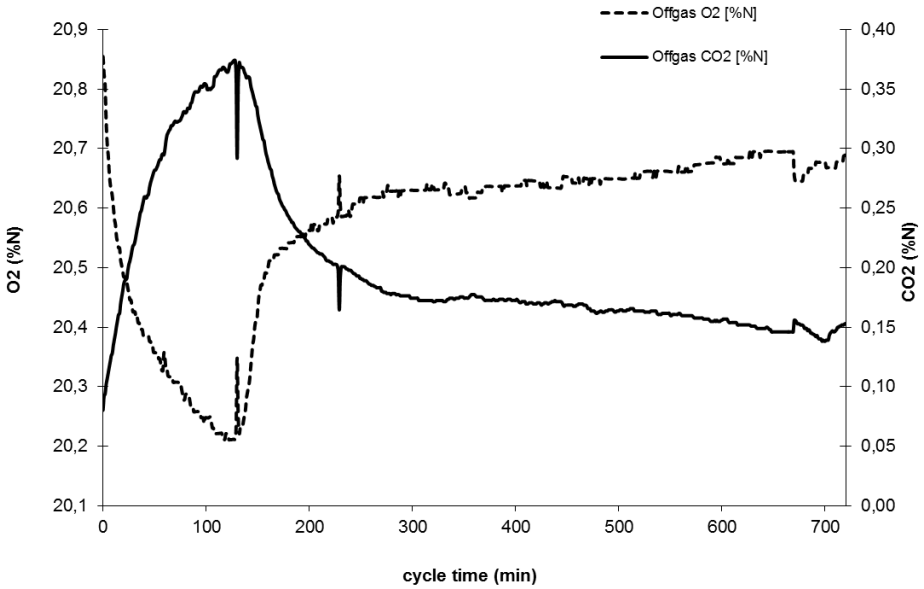


Figure 1. Typical off-gas profile of the reactor, indicating an increased activity after dosing a pulse of vegetable oil at the start of the cycle and a relatively sharp decline of activity after around 120 minutes. The small peaks around 120 and 230 minutes were caused by sampling.

Appendix 3B – Carbon flow sheet

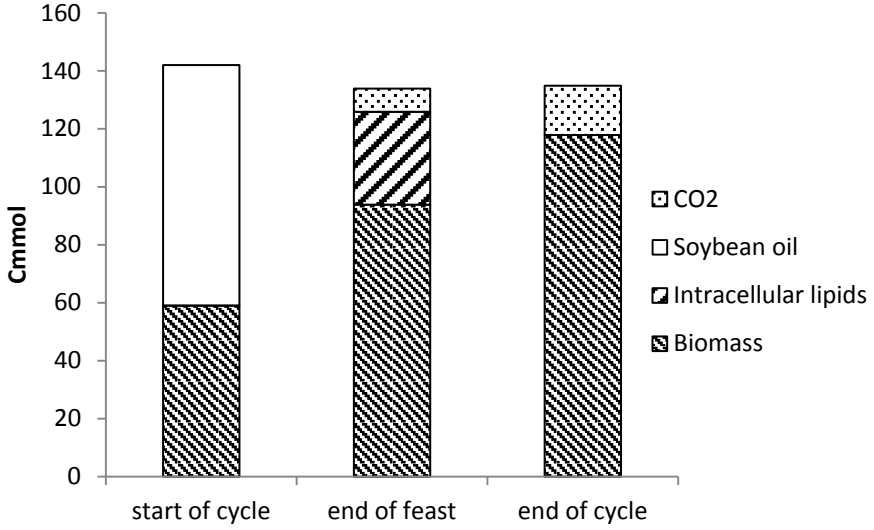


Figure 2. Carbon balances of the enrichment reactor during steady operation.

Appendix 3C – Model

General

The model structure was derived from the metabolic model used to model PHA accumulating microbial enrichment cultures (Tamis et al. 2014b). An explanation for the rationale behind the used kinetic equation can be found in the latter article. Here we describe the most important kinetic equations and the model calibration.

Kinetic equations

Uptake rate kinetics were described by a Monod equation

$$q_S = q_S^{\max} \frac{C_S}{C_S + K_S}$$

The intracellular lipid conversion rate was calculated by subtracting the consumption rate from the production rate.

$$q_{lipid} = Y_{lipid,S} \cdot q_S - k \cdot \left(\frac{C_{X0}}{C_X} \right)^{1/3} f_{lipid}{}^{2/3}$$

The growth rate (μ) was established as the resultant of

1) Direct growth on external substrate
2) Growth intracellular substrate

$$\mu = \left(q_S \cdot (1 - \alpha_{storage}) + k \cdot \left(\frac{C_{X0}}{C_X} \right)^{1/3} f_{lipid}{}^{2/3} \right) \cdot Y_{X,lipid}$$

The nitrogen consumption rate was stoichiometrically coupled to the growth rate using

$$q_N = 0.2\mu$$

Model calibration

For each sampling time point the modelled data for each compound was compared with the measured data. The errors were calculated, squared, and summed up as follows

$$SSE = \sum_{i=1}^{N} \left(n^{measure}(t_i) - n^{model}(t_i) \right)^2$$

The sum of squared errors for the different compounds was summed up to the total error between model and measurements (E_M).

$$E_M = \sum SSE_i$$

with i = S, lipid, X, N

The total error was minimized by adjusting the parameter values for the yields ($Y_{lipid,S}$ and $Y_{X,lipid}$), the fraction of the substrate converted to storage lipids (α) and the biomass specific uptake rate (q_S). The substrate affinity constant was assumed not be relevant for the system and was set to 2 Cmol l^{-1}, effectively minimizing its influence. Minimization was performed by the solver tool of Microsoft Excel. Carbon and COD balances were not included as criteria for model calibration and were evaluated separately.

Model nomenclature

C_S	concentration of substrate [Cmol l^{-1}]
C_X	concentration of biomass [Cmol l^{-1}]
C_{X0}	initial concentration of active biomass [Cmol l^{-1}]
f_{lipid}	ratio of PHA to active biomass [Cmol $Cmol^{-1}$]
k	rate constant for shrinking particle model [Cmol $lipid^{1/3}$ $(Cmol\ X)^{-1/3}$ h^{-1})]
K_S	substrate affinity constant [Cmol l^{-1}]
q_S	biomass specific substrate uptake rate [Cmol $Cmol^{-1} \cdot h^{-1}$]
q_S^{max}	maximum biomass specific substrate uptake rate [Cmol $Cmol^{-1}$ h^{-1}]
$Y_{i,j}$	yield of compound i on compound j [Cmol $Cmol^{-1}$]
α	fraction of the substrate converted to storage [Cmol $Cmol^{-1}$]
μ	biomass specific growth rate [Cmol $Cmol^{-1}$ h^{-1}]

4

Modelling PHA-producing microbial enrichment cultures – towards a generalized model with predictive power

Abstract

Polyhydroxyalkanoate (PHA) production from waste streams using microbial enrichment cultures is a promising option for cost price reduction of this biopolymer. For proper understanding and successful optimization of the process, a consistent mechanistic model for PHA conversion by microbial enrichment cultures is needed. However, there is still a lack of mechanistic expressions describing the dynamics of the feast-famine process. The scope of this article is to provide an overview of the current models, investigate points of improvement, and contribute concepts for creation of a generalized model with more predictive value for the feast-famine process. Based on experimental data available in literature we have proposed model improvements for (i) modelling mixed substrates uptake, (ii) growth in the feast phase, (iii) switching between feast and famine phase, (iv) PHA degradation and (v) modelling the accumulation phase. Finally, we provide an example of a simple uniform model. Herewith we aim to give an impulse to the establishment of a generalized model.

Published as

Tamis, J., Marang, L., Jiang, Y., van Loosdrecht, M.C. and Kleerebezem, R. (2014) Modeling PHA-producing microbial enrichment cultures--towards a generalized model with predictive power. N Biotechnol 31(4), 324-334.

Introduction

Sustainable production of biodegradable polymers has until now been severely hampered by the high cost price of materials with desirable polymer properties. Polyhydroxyalkanoates (PHAs) are a family of molecules exhibiting favorable properties for replacement of part of the petrochemical plastics and for the production of new types of bio-based polymer products. PHAs are currently produced using expensive, pre-sterilized, high-tech equipment and agricultural feedstock, such as glucose, as a substrate (Chen 2009, Crank and Patel 2005). A promising option for cost reduction of PHA is the use of ecological selection principles – as commonly applied in wastewater treatment processes – to obtain microbial enrichment cultures with superior PHA producing capacity (Kleerebezem and van Loosdrecht 2007). Such innovative processes are under development by various research groups (Bengtsson et al. 2010, Dionisi et al. 2007, Marang et al. 2013, Oehmen et al. 2007, Serafim et al. 2008a) as a candidate for integration into the bio-based economy (Chen 2009, Gurieff and Lant 2007). The enrichment procedure utilized is often referred to as the feast-famine process as the microorganisms are cultivated in a selective environment comprising alternating presence and absence of external substrate, preferably short chain organic acids (Reis et al. 2003). Since the uptake of these substrates can be performed intrinsically faster by PHA-accumulating bacteria, a competitive growth advantage over non-storing organisms is established (van Loosdrecht et al. 1997). The proposed production method for PHA formation by microbial enrichment cultures comprises three steps: first, a pretreatment step may be used to convert readily biodegradable COD into substrates suitable for PHA production, such as volatile fatty acids (VFA). Second, the VFA are used to establish the described enrichment culture. Third, this enrichment culture, highly enriched in PHA-producing microorganisms, is fed with the VFA enriched substrate – preferably lacking one or more essential growth nutrients – to obtain biomass with a maximized PHA content.

In order to understand, design, and optimize a feast-famine process, it is useful to have a model that describes the biological and physico-chemical conversions in the reactor. Such a model can be used to fit experimental data in order to identify characteristic kinetic and stoichiometric parameter values which enable objective comparison of process data and allows for prediction of the effect of operational variables.

Currently, several models can be found in literature that – within certain constraints – adequately describe the feast-famine process (Beun et al. 2002, Beun et al. 2000, Ciggin et al. 2013, Dias et al. 2008, Dias et al. 2005, Johnson et al. 2009b, Krishna and van Loosdrecht 1999, Lavallee et al. 2009, Pardelha et al. 2013, Tajparast and Frigon 2013, Third et al. 2003, van Aalst van Leeuwen et al. 1997, van Loosdrecht and Heijnen 2002). However, there is still a lack of mechanistic expressions that describe the dynamics of the feast-famine process, encumbering the establishment of a generalized model with predictive power. The scope of this article is to provide a brief overview of the current models, investigate points of improvement, and contribute concepts for creation of a generalized model with more predictive value for the feast-famine process.

Model comparison

Virtually all current models are based on the metabolic pathways described by van Aalst-van Leeuwen et al. (van Aalst van Leeuwen et al. 1997). Although some differences in kinetic description and stoichiometry exist, a conceptual version of the metabolic model may be described by the reactions in table 4.1. During the feast phase, VFA are taken up and activated at the expense of ATP. A fraction of the VFA is oxidized to carbon dioxide for generation of NADH and subsequently ATP, while the larger part is stored as PHA. Subsequently, during the famine phase PHA is consumed to generate energy and carbon for biomass production.

Table 4.1. Overview of a typical metabolic model for the feast-famine process. Reactions are on carbon mole basis.

1	Substrate uptake	VFA + ATP → VFA-CoA + n_0 CO_2 + n_1 NADH
2	PHA production	VFA-CoA + n_2 NADH → PHA
3	PHA consumption	PHA + ATP → VFA-CoA + n_3 NADH
4	Catabolism	VFA-CoA → n_3 NADH + n_4 CO2
5	Oxid. phosphorylation	NADH + 0.5 O_2 → (P/O) ATP
6	Growth	n_5 VFA-CoA + n_6 NH_3 + $Y_{ATP,X}$·ATP → X + n_7 CO_2 + n_8 NADH
7	Maintenance	ATP → 0

It may be noted that the above model (table 4.1) considers solely the uptake of VFA. Other types of substrates may be present in e.g. waste streams, leading to different dynamics. An extension of the model, that includes the influence of different types of substrates, is discussed in the section "Mixed substrates and mixed cultures".

Some parameters are equal in all models since they originate from conservation principles, i.e. the NADH and CO_2 yield if one type of VFA in converted into another, the NADH consumption for PHA production, the NADH and CO_2 yield of catabolism (respectively n_0, n_1, n_2, n_3 and n_4) just depend on the type of VFA and PHA involved in the reactions. Other parameter values differ between models (i.e. n_5, n_6, n_7, n_8 and $Y_{X/ATP}$) due to e.g. differences in the assumed elemental composition of the active biomass. A comparison of parameters used in the different models is presented in table 4.2. Although differences in the above parameter values may lead to different PHA yields and changes in other stoichiometric parameters, in general, they cause no significant deviation in model behavior.

Table 4.2. Overview of the elemental biomass composition and other parameters used in the compared metabolic models.

P/O ratio mol ATP (mol NADH)$^{-1}$	**m_{ATP}** mol Cmol^{-1} h^{-1}	**Biomass composition**	**Biomass degree of red.**	**$Y_{ATP,X}$** mol (Cmol X)$^{-1}$	**Reference**
1.84	0.102	$CH_{1.73}O_{0.44}N_{0.24}$	4.13	2.16	(van Aalst van Leeuwen et al. 1997)
1.8[a]	0.010	$CH_{2.09}O_{0.54}N_{0.2}P_{0.}$	4.49	1.6	(Murnleitner et al. 1997)
1.6	0.015	$CH_{1.87}O_{0.66}N_{0.17}$	4.04	2.16	(Beun et al. 2000)
2	0.02[b]	$CH_{1.8}O_{0.5}N_{0.2}$	4.2	2.16	(Beun et al. 2002)
3	n/a	$CH_{1.8}O_{0.5}N_{0.2}$	4.2	2.16	(Third et al. 2003)
3.0 ± 0.3	0.042[c]	$CH_{1.4}O_{0.4}N_{0.2}$	4.0	2.16	(Dias et al. 2005)
2.9 ± 0.2	0.02[d]	$CH_{1.4}O_{0.4}N_{0.2}$	4.0	1.7	(Dias et al. 2008)
2.0	Fitted	$CH_{1.8}O_{0.5}N_{0.2}$	4.2	2.16	(Johnson et al. 2009b)

a Taken from Smolders et al. (1994)

b At 20 °C; with increasing temperature m_{ATP} increases according to the Arrhenius equation with an activation energy of 70 kJ mol^{-1}

c Calculated using m_S = 0.084 and method by Van Aalst-van Leeuwen et al. (1997)

d Taken from Beun et al. (2002)

In most models the feast and the famine phase are described by separate sets of kinetic expressions. An overview of kinetic expressions typically used in modelling a feast-famine process is given in table 4.3. The biomass specific substrate uptake rate during the feast phase (q_S) is commonly represented by a Monod equation, and the PHA production rate (q_P) is stoichiometrically coupled to this uptake rate (van Aalst van Leeuwen et al. 1997). At high PHA concentrations – which may occur e.g. in the accumulation process – the PHA production slows down. This phenomenon may be modeled by introduction of an empirical powered inhibition function that is depending on the maximum PHA content of the cell (van Aalst van Leeuwen et al. 1997). Reported power values (α) show high variability between experiments even when

the same species is enriched (Jiang et al. 2011a, Jiang et al. 2011d, Johnson et al. 2010b, Johnson et al. 2010c), making the process poorly predictable. This problem is further discussed in the section "Kinetics at high PHA content".

Table 4.3. Typically used kinetic expressions to describe the feast-famine process (adapted from Johnson et al. 2009b). This table governs only the most important state variables: substrate, PHA and biomass. Other state variables such as CO_2 and O_2 are stoichiometrically coupled.

Reaction	Feast	Famine
Substrate uptake	$q_S = q_S^{\max} \frac{C_S}{C_S + K_S}$	-
Substrate uptake -with inhibition	$q_S = \frac{\mu}{Y_{X,S}} + \cdot \frac{q_{PHA}}{Y_{PHA,S}} + m_S$	-
PHA production	$q_{PHA} = Y_{PHA,S}\left(q_S - \frac{\mu}{Y_{X,S}} - m_S\right)$	-
PHA production -with inhibition	$q_{PHA} = q_{PHA}^{\max} \frac{C_S}{C_S + K_S} \cdot \left(1 - \left(\frac{f_{PHA}}{f_{PHA}^{\max}}\right)^a\right)$	-
PHA consumption	-	$q_{PHA} = k \cdot f_{PHA}{}^{2/3}$
Growth	$\mu = \mu^{\max} \frac{C_S}{C_S + K_S}$	$\mu = Y_{X,PHA}\left(q_{PHA} - m_{PHA}\right)$

The degradation of PHA that occurs during the famine phase is in some models described using a shrinking particle model as a basis (Johnson et al. 2009b, Murnleitner et al. 1997). Others use a Monod expression (Dias et al. 2005, Gujer et al. 1999) or other functions mainly depending on cellular PHA content (Beun et al. 2002, Beun et al. 2000, Dias et al. 2008). The shrinking particle model may be considered an appealing option because it offers a mechanistic explanation for the conversion dynamics, coherent with the observation that PHA granules comprise a PHA polymer core covered with enzymes that facilitate the PHA conversion (Steinbüchel et al. 1995). However, the current shrinking particle model description used for PHA degradation is incomplete and is therefore further evaluated in this paper in the section "PHA degradation kinetics".

The value for the maintenance coefficient (m_{ATP}) is strongly correlated with the P/O ratio, complicating independent identification of these parameter values. Dias et al. (2008) therefore proposed to fix the value of m_{ATP}, whereas the P/O ratio was estimated based on experimental data. The P/O ratio identified by Dias et al. (2008) apparently depended on the selected microbial culture, but also on the carbon substrate fed to the culture: model output resulted in a P/O ratio of around 3 for acetate and around 1 for propionate. Since the actual efficiency of oxidative phosphorylation should be independent of the substrate, the large change in P/O ratio for different substrates suggests that this model structure is not entirely correct. In the model by Johnson et al. (2009b), on the contrary, the P/O ratio was assumed 2 for all experiments and the m_{ATP} was estimated by fitting. Although there is no decisive reason to favor one approach over the other, it would be beneficial for the development of a generalized model to choose to

fix either the m_{ATP} or the P/O value and obtain the other value by calibrating the model to experimental data. There is an extensive body of literature available with estimated maintenance coefficients (m_{ATP}) (Tijhuis et al. 1993). The m_{ATP} tends to vary more strongly than the P/O ratio, for which reasonable estimates are available (Smolders et al. 1994, Jiang et al. 2011d, Lopez-Vazquez et al. 2009, Oehmen et al. 2013). The larger variation in m_{ATP} supports the use of a fixed P/O ratio and subsequent fitting of m_{ATP} as proposed by Johnson et al. (2009b). On the other hand, for fast growing systems (short SRT) the maintenance coefficient has only a relatively small influence on the overall process stoichiometry and the P/O ratio influences the stoichiometry of the process stronger (e.g. the yield of biomass on PHA and the yield of PHA on substrate) making it a more sensitive variable. So, for low SRT processes, fitting the P/O ratio potentially provides insight into the energy efficiency of the microorganism.

All the discussed models are in principle able to generate patterns that resemble the experimental data generated in a typical feast-famine experiment, therewith enabling identification of characteristic kinetic and stoichiometric parameter values. Nevertheless, it would be advantageous to adopt a uniform model structure, so that differences in parameter values due to differences in model structure can be excluded, enabling a direct comparison between results obtained in different studies.

Mixed substrates and mixed cultures

The substrate composition is a key parameter in the feast-famine process, influencing the microbial composition, kinetic properties and polymer content of the culture. It is important to make a distinction between enrichments obtained on a single substrate that are fed substrate mixtures in short-term batch experiments only (Bengtsson et al. 2010, Lemos et al. 2006, Serafim et al. 2008b) and enrichments obtained using mixed substrates. The latter may react very different from cultures enriched on a single substrate (Albuquerque et al. 2007). In this section, only microbial enrichment cultures obtained on mixed substrates will be further discussed (Marang et al. 2013, Pardelha et al. 2013, Jiang et al. 2011d, Albuquerque et al. 2013, Albuquerque et al. 2010a, Bengtsson et al. 2008, Dionisi et al. 2004).

Currently, most models treat the biomass in feast-famine cultures as if it were one species with lumped properties (Beun et al. 2002, Beun et al. 2000, Dias et al. 2008, Dias et al. 2005, Johnson et al. 2009b, van Aalst van Leeuwen et al. 1997). In this section it will be argued that under some conditions biomass should be divided into different fractions with different characteristics when working with enrichment cultures acquired on mixed substrates.

It is well known that some substrates (e.g. VFA and lactate) favor PHA producing species (Reis et al. 2003) while other substrates (e.g. carbohydrates and vegetable oils) give rise to populations that do not store PHA (Carta et al. 2001, Dircks et al. 2001, Jiang et al. 2012b). Non-storing populations were observed by Jiang et al. (2012b) while studying a feast-famine enrichment culture fed with paper mill wastewater. Results from this study indicated that the fraction of non-storing biomass was proportional to the fraction of non-VFA COD in the substrate. The PHA storage capacity of the culture could be explained as the weighted sum of the PHA-storing population (i.e. *P. acidivorans*) and the non-storing population. The overall storage capacity of the enrichment could thus be calculated according to equation 1 (adapted from Jiang et al. (2012b)).

$$f_{PHA,overall} = f_{PHA,1} \cdot \frac{C_{X1}}{C_{X1} + C_{X2}} + f_{PHA,2} \cdot \frac{C_{X2}}{C_{X1} + C_{X2}} \qquad \text{(eq. 1)}$$

In equation 1, f_{PHA} represents the molar ratio of PHA to biomass (Cmol Cmol^{-1}) and C_{Xi} the concentrations of different biomass types.

In many cases, the equation describing the effect of non-PHA storing bacteria on the overall PHA content (equation 1) may be extended to explain other culture characteristics as the weighted sum of distinct types of biomass. For example, Jiang et al. (2011a) found two predominant PHA-producing species in a system fed with an acetate/lactate mixture characteristics that could be described as the weighted sum of an enrichment culture fed with sole acetate and one with sole lactate. In another example, Carta et al. (2001) observed similar behavior for acetate/glucose-fed cultures sustaining PHB and glycogen production as the sum of the individual conversions of acetate and glucose. By treating an enrichment culture on mixed substrates as a sum of enrichment cultures on single substrate, waste streams containing VFA in combination with e.g. glycerol (Moralejo-Garate et al. 2011) or medium chain length fatty acids (Lee et al. 2011) may be included in a generalized model using data from single substrate experiments. It must be noted that such an approach is only valid if different species are involved in the uptake of different substrates.

Besides influencing coexistence of different PHA-storing and non-storing species, different substrates may be converted to different types of PHA. It was observed (Albuquerque et al. 2011, Marang et al. 2013, Pardelha et al. 2013, Jiang et al. 2011d, Albuquerque et al. 2013, Albuquerque et al. 2010a, Bengtsson et al. 2008, Dionisi et al. 2004) that the PHB/PHV ratio was generally proportional to the ratio of VFA with an uneven number of carbon atoms (i.e. propionate and valerate) to VFA with an even number (i.e. acetate and butyrate).

Mixed substrate uptake kinetics

Substrate uptake rates may vary strongly between different types of substrate and microbial enrichment cultures may exhibit preferential uptake for one type of substrate over another. Although some proposals to find a generalized description for substrate uptake kinetics have been published (Pardelha et al. 2013), there is currently no mechanistic model available that predicts how a mixture of VFA is taken up by a feast-famine enrichment: for every new experiment the kinetic parameters have to be obtained by calibrating the model to experimental data.

Understanding the environmental pressures may help elucidating a general mechanism. In an SBR feast-famine process substrate is typically dosed in a short pulse, giving rise to a selective pressure that leads to a competitive advantage for microorganisms that take up carbon the fastest. Preferential butyrate uptake observed in SBR experiments suggests that the maximum biomass specific uptake rate of butyrate is intrinsically higher than the one of acetate (Marang et al. 2013, Albuquerque et al. 2013, Jiang et al. 2012b). Operational setups other than SBR may give rise to different selective pressures. Bengtsson et al. (2008) applied a different operational mode, comprising a 2-stage continuous system in which the substrate was dosed continuously, resulting in substrate limiting conditions in the first reactor. In such an environment the uptake rate of the most competitive species is likely adapted to the feeding rate of the substrate. In this case the influent acetate concentration reported by Bengtsson et al. (2008) was significantly higher than the butyrate concentration, and consequently microorganisms exhibiting a higher acetate uptake rate were favored in this system.

A short overview of functional and microbial data discussed in this section is presented in table 4.4.

Table 4.4. Overview of the functional characteristics and operational parameters of PHA enrichment cultures fed with mixed substrates.

Enrichment conditions		Microbial composition	Storage capacity[a]	Storage composition (PHB/PHV)	Reference
Substrate composition[b]	Operational parameters				
75% VFA + 25% unknown (ac/pro/bu/val = 31/18/29/22)[c]	SBR (12h cycle, 10d SRT)	63% *Azoarcus* sp. + 25% *Thauera* sp. + 12% unidentified	77%	61/39	(Albuquerque et al. 2011)
VFA from fermented molasses (ac/pro/bu/val = 60/10/25/5)	Continuous (10d SRT)	Not reported	61%	85/15[c]	(Albuquerque et al. 2010a)
85% VFA + 15% unknown[d] (ac/pro/bu/val = 63/10/25/2)	SBR (12h cycle, 10d SRT)	*Paracoccus* sp., *Azoarcus* sp., *Thauera* sp.	61%	87/13[c]	(Albuquerque et al. 2013)
Paper mill wastewater[e] 74% VFA on COD basis	Continuous (10d SRT)	Unidentified filamentous bacteria	48%	60/40[c]	(Bengtsson et al. 2008)
Synthetic mixture glucose/acetate	SBR (4 h cycle, 6 d SRT)	Not reported	25%	40/60 (PHB/ polyglucose)	(Carta et al. 2001)
Synthetic mixture Starch/acetate	SBR (4h cycle , 8 d SRT)	Unidentified filamentous bacteria	10%	30/70 (PHB/ polyglucose)	(Ciggin et al. 2012)
Synthetic mixture (ac/lac/pro = 40/40/20)[d]	SBR (2h cycle, 10 d SRT)	Not reported	50%	70/30	(Dionisi et al. 2007)
Organic acids synthetic mixture (ac/lac = 50/50)	SBR (12h cycle, 1d SRT)	*Plasticicumulans. acidivorans* + *Thauera* sp.	84%	100/0	(Jiang et al. 2011a)
VFA synthetic mixture (ac/pro = 50/50)	SBR (12h cycle, 1d SRT)	*P. acidivorans*	80%	50/50	(Jiang et al. 2011d)
Paper mill wastewater[e] 60-64% VFA on COD basis	SBR (12h cycle, 1d SRT)	*Plasticicumulans. acidivorans* + non-storing population	77%	86/14	(Jiang et al. 2012b)
Synthetic mixture Nonanic acid	SBR (24h cycle, 3d SRT)	*Pseudomonas sp.*	49%	mainly mcl-PHA	(Lee et al. 2011)
VFA synthetic mixture (ac/bu = 50/50)	SBR (12h cycle, 1d SRT)	*Plasticicumulans. acidivorans*	88%	100/0	(Marang et al. 2013)
Synthetic mixture Glycerol	SBR (24h cycle, 2 d SRT)	*Defluviicoccus vanus*	80%	92/8 (PHB/ polyglucose)	(Moralejo-Garate et al. 2011)

a The storage capacity reported is the PHA content achieved during batch experiments on the same carbon substrate it was enriched on. The substrate may have been nutrient limited. The PHA content is given as PHA/VSS or PHA/TSS with low (<10%) ash content
b Numbers are on Cmol basis unless indicated otherwise
c Characteristic ratios
d On COD basis

Considering the above-mentioned findings, a generalized model may comprise mapping the substrate into three fractions:

1. Substrates favoring enrichment of PHA-storing species
    a. Acetate/butyrate/lactate → PHB
    b. Propionate/valerate → PHV

2. Other COD, resulting in growth of a non-PHA-producing population

This division may be expanded to include substrates yielding other storage compounds (e.g. medium chain length (mcl) PHAs, polyglucose and triacylglycerides). The use of a simplified division for mapping different substrates onto different types of biomass has clear advantages in explaining or even predicting the overall PHA-producing capacity and other culture dynamics (Pardelha et al. 2013, Tajparast and Frigon 2013, Jiang et al. 2012b).

Growth on external substrate

Traditionally, PHA formation was often regarded as overflow metabolism due to unbalanced growth (Stal 1992). In this scenario bacteria are growing at their maximum rate during the feast phase and PHA production is a consequence of biomass specific substrate uptake rates that exceed the substrate flux that can be used for biomass production.

In proper functioning feast-famine systems the feast phase is relatively short (e.g. under 2 h compared to 10 h famine phase (Jiang et al. 2011a, Beun et al. 2002, Albuquerque et al. 2010a)), complicating measurement of the specific growth rate in this phase. Some researchers reported faster growth during the feast than the famine phase (Beun et al. 2000, Krishna and van Loosdrecht 1999), whereas others reported only slow growth during the feast phase (Marang et al. 2013, Johnson et al. 2009b, Jiang et al. 2011d). To which extent the overflow mechanism can still be considered realistic is unclear. Several alternative hypotheses involving various levels of control have been suggested to explain the mechanism of growth on external substrate. One level of control involves regulation of growth at the proteome/transcriptome level: van Loosdrecht and Heijnen (2002) hypothesized that RNA and proteins are mainly produced in the feast phase and are decaying and being diluted in the famine phase. This dilution is due to cell division which, without an equivalent production of protein, will results in a decrease in protein content per cell. This leads to the amount of RNA setting the ratio for growth to storage fluxes in the feast phase. In this scenario the biomass specific growth rate will increase during the feast phase until reaching its maximum.

Another scenario is based on control of growth and PHA formation at the metabolome level, involving internal metabolites that may become limiting. When uptake of substrate is maximized at the cost of ATP, these ATP levels may become low, thus limiting the ATP dependent growth (Third et al. 2003). Also other levels of control at the metabolome level may be involved like activation and deactivation of enzymes e.g. by allosteric modulation. For establishment of a proper mechanistic kinetic expression for growth in the feast phase, further experiments are needed to elucidate how, and at which level, the metabolism of microbial enrichment cultures is regulated.

The Feast-Famine Switch

Once the external substrate in a sequencing batch reactor (SBR) is depleted, a virtually instant transition from the feast to the famine phase is observed (Johnson et al. 2009b, Krishna and van Loosdrecht 1999). Since research on PHA production by microbial enrichment cultures is commonly performed in SBRs (Jiang et al. 2011c, Johnson et al. 2009b, Lemos et al. 2006, Dionisi et al. 2004, Moralejo-Garate et al.

2011, Beccari et al. 2002), the discussed models all consider the feast and famine phases as separate phases, with their own set of kinetic equations (Beun et al. 2000, Dias et al. 2005, Johnson et al. 2009b, van Aalst van Leeuwen et al. 1997). To switch from feast to famine phase kinetics a conditional statement or Monod-like switch function ($C_i/(C_i + K_i)$) is used. In the case of a conditional statement, the famine phase starts and a different set of kinetic equations is used once the external substrate concentration comes below a certain threshold. The use of a conditional statement has several numerical drawbacks for application in a generalized model and is, moreover, not a mechanistic description. The observed change in the kinetics upon depletion of the external substrate in feast-famine enrichment cultures is not imposed on the culture through a change in environmental conditions, like in biological phosphorus removal systems. In models describing those processes, the strict separation of phases is also applied (Murnleitner et al. 1997) but, there, the alternating anaerobic and aerobic periods imposed on the system justify the use of a conditional statement (although a switch function will also work).

A further disadvantage of using a conditional statement to switch between feast and famine phase kinetics is, that it does not allow to model systems in which the separation between the two phases is less strict, e.g. if instead of the SBR a staged CSTR is used (Bengtsson et al. 2008, Albuquerque et al. 2010b). In such a system, a limited amount of external carbon will be available throughout the famine phase and likely will be taken up by the microorganisms. Substrate uptake during the famine phase is not incorporated in the current models but may be adequately described using a single set of equations, therewith enabling a more generalized description of the feast-famine process.

Because of the rapid nature of the feast-famine switch, it is likely that the feast-famine switch is a result of changes on metabolite level e.g. intracellular levels of ATP, NADH and acetyl-CoA (Beun et al. 2002, Third et al. 2003, Anderson and Dawes 1990). Abundant presence of external substrate, will lead to an increase of the acetyl-CoA/free coenzyme A ratio in the cell. This favors the condensation reaction of two acetyl-CoA to form acetoacetyl-CoA, as catalyzed by 3-ketothiolase in the PHA synthesis pathway (Beun et al. 2002, Anderson et al. 1990). Once the external substrate is depleted, the availability of free coenzyme A and ATP will go up, inducing PHB degradation and biomass growth (Third et al. 2003). Including intracellular metabolites will complicate the model, but allows a more mechanistic description and the development of one universal set of kinetic equations. A major drawback of such an approach is that intracellular metabolites are not readily measurable. In appendix 4B a proposal for a simple generalized model is presented including an alternative approach for the switch between the feast and famine phase.

PHA degradation kinetics

Generally PHA degradation is considered the rate limiting step that determines the growth rate in the famine phase. In many experiments (Beun et al. 2002, Johnson et al. 2009b, van Loosdrecht and Heijnen 2002, Murnleitner et al. 1997), it has been observed that the PHA degradation rate is a function of the ratio of PHA to active biomass (f_{PHA}). Therefore, equation 2 is typically used to describe the PHA degradation.

$$q_{PHA} = k \cdot f_{PHA}^{\ n} \qquad \text{(eq. 2)}$$

This formula represents a shrinking particle model when using n = 2/3 and by assuming that the biomass concentration is constant during the cycle. In that way the PHA degradation rate will be proportional to the available surface of the PHA granules in the cells – thus providing a mechanistic description (Johnson et al. 2009b, Murnleitner et al. 1997). However, although it was expected that, with the same culture, the rate constant (k) would only depend on temperature; Jiang et al. (2011c) found PHA degradation rate constants between 0.13 and 0.56 h^{-1} using experimental data from enrichment cultures dominated by the

same microorganism and operated at the same temperature (30 °C) but with different cycle lengths. The strong variation in parameter value under different operational conditions suggest that the model is not generally applicable and severely limits the possibility to predict PHA degradation dynamics in feast-famine cycles.

In order to investigate this problem, the description for PHA degradation was re-evaluated in this paper. Since the above equation (equation 2) was deduced assuming that the biomass concentration stays constant throughout the cycle, it may not be valid when working with cultures that experience significant biomass growth during a cycle. In order to solve this problem, an adapted expression for the PHA degradation rate was derived (equation 3), representing a shrinking particle model in a system including biomass growth (appendix 4A).

$$q_{PHA} = k \cdot \left(\frac{C_{X0}}{C_X} \right)^{1/3} f_{PHA}^{\ 2/3} \qquad \text{(eq. 3)}$$

In the above equation q_{PHA} is the biomass specific PHA degradation rate (Cmol Cmol^{-1}·h^{-1}) which may be converted to a volumetric conversion rate by multiplying with the actual biomass concentration (C_X).

Using the new equation 3, the PHA degradation rate constants obtained from the experimental data discussed above (Jiang et al. 2011c) remains, however, unexplained: the degradation rate constant (k) still differed for enrichment cultures obtained at the same temperature but at different cycle lengths. In order to provide an explanation for this variation in degradation rate constants, a new, ecology-based approach is proposed. Assuming that microorganisms that have their kinetics adapted to the operational parameters become dominant, it can be inferred that rate constants for degradation kinetics are a function of the PHA content and the length of the famine phase (appendix 4A). Furthermore, since it was observed by Jiang et al. (2011c) that the PHA content at the end of the feast phase in general can be predicted from the cycle length and SRT (assuming the PHA is virtually depleted at the end of the cycle), the rate constant can also be calculated from these key variables (equation 4).

$$k = \frac{3}{t_{fam}} \left(\left(\frac{C_{PHA\_end\_fam}}{C_{X0}} \right)^{1/3} - \left(\frac{C_{PHA\_end\_feast}}{C_{X0}} \right)^{1/3} \right) \qquad \text{(eq. 4)}$$

From the equation it becomes clear that, at a low number of cycles per SRT and consequently a high average PHA content, full PHA degradation can be achieved with a lower rate constant (k). The influence of temperature on the degradation rate constant may also be analyzed using the above approach: at lower temperatures the famine phase becomes shorter and an adapted culture would exhibit a higher rate constant.

The hypothesized relation between the cycle length and the PHA degradation rate constant was evaluated using the abovementioned experimental data (Jiang et al. 2011c) from enrichment reactors operated at different cycle lengths and temperatures. Model-predicted values for the final PHA to biomass ratio (f_{PHA}) and PHA degradation rate constants (k) were compared to values obtained by fitting this experimental data (Jiang et al. 2011c) (figure 1). The general tendency of the values of the rate constants and maximum PHA content was adequately predicted by the hypothesis: the rate constants increased with shorter cycle length and lower temperatures. A further, literature-wide evaluation of the relation between degradation

rate constant and operational parameters is required to make the proposed theory applicable in a generalized model, but this is outside the scope of this article.

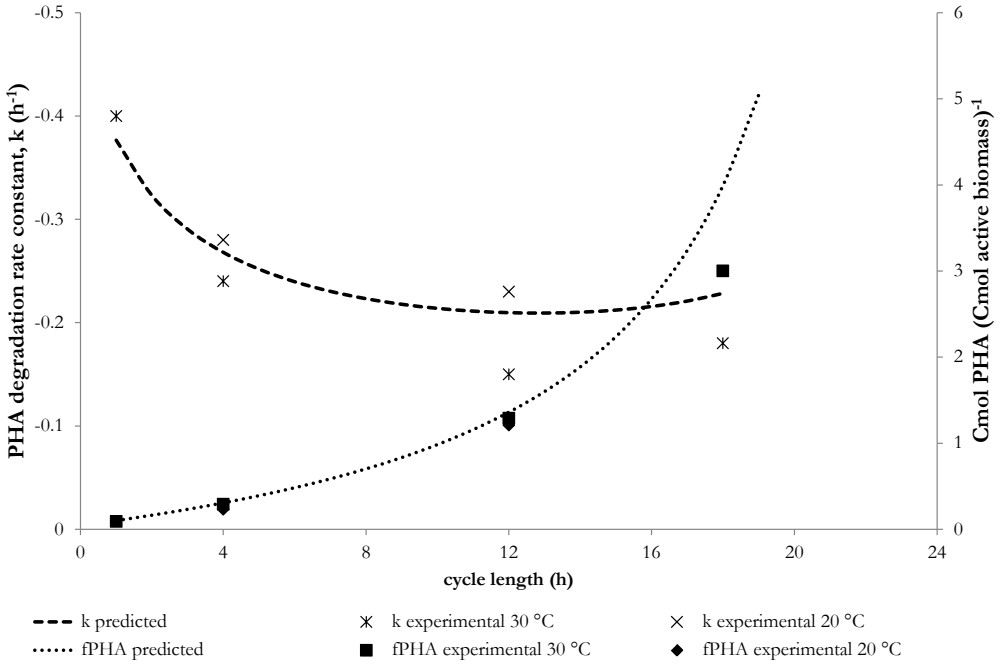


Figure 4.1. Comparison of the predicted and experimentally obtained PHA degradation rate constants (k) and the predicted and experimentally obtained PHA to active biomass Cmolar ratios (f_{PHA}) at the end of the feast phase; f_{PHA} at the end of the cycle was 0.04 in all cases.

It remains a question how the variation in PHA degradation rate constant (k) is regulated within the cell. One possible mechanism is that cells adjust the amount of available reactive surface area by varying the number of granules per cell. Another strategy that cells may employ is to regulate the reactivity of the granule surface. This could be achieved by incorporating less or more depolymerase enzymes into the surface of the granule, for example. Although these options represent realistic scenarios, additional experimental data is needed to support these theories.

Kinetics at high PHA content

The PHA accumulation phase is the last stage in the PHA production process and can be compared with a prolonged feast phase, preferably in absence of an essential growth nutrient. This phase is crucial in the production process since it determines the final PHA productivity and PHA content of the culture. An accurate model for this stage is therewith an important asset, as it can be used to predict and optimize PHA accumulation experiments.

Several existing models consider the kinetics at high PHA content (Dias et al. 2008, Dias et al. 2005, Johnson et al. 2009b, Third et al. 2003, van Aalst van Leeuwen et al. 1997). There are still two important flaws in these models though: the mechanism for termination of PHA synthesis remains unclear, and the formation of side-products is not included. The current models simplify the first issue by adopting a black box model in order to solve numerical problems. This does, however, not help to understand the fundamental PHA synthesis mechanism and has also practical disadvantages. Currently, the end of the PHA accumulation – the moment that the PHA producing bacteria achieve their maximum PHA content – is unpredictable. Many observations indicate that the PHA synthesis rate declines with the increase of the intracellular PHA content. Most of the models therefore describe the PHA synthesis rate during PHA

accumulation according to the equation in table 4.3 on PHA production with inhibition (Dias et al. 2008, Dias et al. 2005, Johnson et al. 2009b, Third et al. 2003, van Aalst van Leeuwen et al. 1997).

In this equation, f_{PHA}^{max} and α indicate the maximum PHA fraction over active biomass and the exponent of the PHA inhibition term, respectively. While f_{PHA}^{max} may be a useful parameter, showing similar values for enrichments cultures in which the same species was enriched, the exponent of the PHA inhibition term (α) showed a high degree of variability for highly similar enrichment cultures. For example a factor ten difference in reported values for α, while the same species, cycle length, SRT and temperature were used (Jiang et al. 2011a, Beun et al. 2000, Johnson et al. 2010b, Johnson et al. 2010c). Such a large variability hinders the construction of a generic model. Studying the fundamental regulatory mechanism of PHA synthesis will help to construct a more accurate model. So far, the termination of PHA synthesis remains unclear. It is simple to attribute termination of PHA synthesis to the depletion of external carbon source, but many observations show that PHA accumulation ends when the external carbon source is still available (Johnson et al. 2010b).

As mentioned previously, a second flaw is that formation of side-products during the accumulation phase is neglected. In current models, the specific substrate uptake rate at high PHA contents is calculated as the resultant of growth, PHA synthesis and maintenance (table 4.3). The observed substrate uptake rate does, however, not decrease to the same extent as the model predicts based on the PHA synthesis rate and growth. It has been reported that cells full of PHA secrete side-products into the medium (Marang et al. 2013). These secreted side-products could be hydroxybutyrate or any other intermediate compound of the pathway for PHA production (Carlson et al. 2005, Durner et al. 2000). The consequences of neglecting the secretion of side-products are a gap in the mass balance and a structural underestimation of the substrate uptake rate by the existing models (table 4.3). Since the yields of biomass and PHA on substrate and maintenance rate are relatively well known, side-product formation may be evaluated by calculating the gap between substrate uptake and substrate use for biomass, PHA and maintenance (equation 5).

$$q_{Pi}(t) = Y_{Pi,S} \cdot \left(q_S(t) - \frac{\mu^{feast}(t)}{Y_{X,S}^{feast}} - \frac{q_{PHA}^{feast}(t)}{Y_{PHA,S}^{feast}} - m_S \right) \qquad \text{(eq. 5)}$$

So far, studies on regulation mechanisms for PHA synthesis termination and side product formation are rare, and no conclusive model is available to predict when PHA synthesis stops. A mechanism explaining the behavior of cells at high PHA content may comprise cell volume limitation as determining factor for maximum cellular PHA content. It has been observed that cells swell with increasing intracellular PHA content (Eberl et al. 1996). After some extent, the cell reaches its limits in maintaining an intact and functional structure. From then on, cells may maintain their capacity for substrate uptake and precursor synthesis, but not for PHA formation. Consequently, consumed substrate may be secreted into the medium in the form of PHA precursor molecules, such as hydroxybutyrate (Carlson et al. 2005, Durner et al. 2000). This theory is supported by several observations. It has been reported that the density of PHA decreases with the increased length of the side chain and that the maximum PHA content (wt%) for short chain length (scl) PHA is higher than for mcl-PHA (Lee et al. 2011, Terada and Marchessault 1999), suggesting that the intracellular space available for PHA is the limiting factor. Moreover, recently isolated wild type PHA producers are relatively large in size (e.g. Jiang et al. (2010) found 2 by 4 μm; Moralejo Garate et al. (2011) found 3 by 3 μm), providing the space to reach a high PHA content. Although in practice, establishing the average cell size may prove difficult, especially in microbial enrichment cultures, it may provide better results in cases where it is desired to predict the maximum PHA content.

Conclusions

For proper understanding and successful optimization of PHA production from waste streams, a consistent mechanistic model for PHA conversion by microbial enrichment cultures is needed. We have proposed a uniform structure based on the experimental data available in literature. The developed model should be confronted with new experiments in order to validate the assumptions and test its predictive power. Development of a generalized model that provides insight into the mechanisms underlying the conversions in the system is a logical next step in the research on the feast-famine process. Herewith we hope to give an impulse to the creation of a model that enables users to objectively compare experimental data with each other, and enables prediction of the behavior of feast-famine enrichment cultures.

Appendix 4A

Establishment of a logical expression for the PHA consumption rate

PHA granules comprise a PHA polymer core inside a phospholipid layer, covered with polymerase and depolymerase enzymes as described by Steinbüchel et al. (1995). This architecture suggests that the volumetric degradation rate (r_{PHA}) is a function of reactive area (A) and the area specific reaction rate (k_A):

$$r_{PHA} = \frac{dC_{PHA}}{dt}\left[\frac{Cmol/l}{h}\right] = k_A A \qquad \text{(eq. A1)}$$

In a simplified system in which all granules are spheres with effectively the same radius, it is possible to relate the reactive surface area to the total mass of PHA in the system, the number (n) and density (ρ) of the granules:

$$r_{PHA} = k_A \cdot n \cdot 4\pi \cdot \left(\sqrt[3]{\frac{3}{4\pi\rho}\frac{C_{PHA}}{n}}\right)^2 = k' \cdot n^{1/3} \cdot {C_{PHA}}^{2/3} \qquad \text{(eq. A2)}$$

Assuming that the number of PHA granules is proportional to the biomass concentration at the start of a cycle (C_{X0}) and the amount of granules does not change during the famine phase, the biomass specific PHA degradation rate (q_{PHA}) can be represented as a function of actual biomass concentration (C_X) and the amount of PHA per amount of biomass (f_{PHA}):

$$q_{PHA} = k \cdot \left(\frac{C_{X0}}{C_X}\right)^{1/3} {f_{PHA}}^{2/3} \qquad \text{(eq. A3)}$$

A new theory for predicting the kinetic properties of enrichment cultures

The variable k-values can be explained by taking into account the adaptive behavior of enrichment cultures in an SBR. In order for a species to maintain itself in the system, a minimal, critical growth rate must be met. In order to be able to match this critical growth rate, species adapt their enzymatic activity so that the amount of PHA produced during the feast phase is matched by the amount degraded during the famine phase. Therewith the k-value in equation 3 may be explicitly evaluated:

$$k = \frac{3}{t_{fam}}\left(\left(\frac{C_{PHA\_end\_fam}}{C_{X0}}\right)^{1/3} - \left(\frac{C_{PHA\_end\_feast}}{C_{X0}}\right)^{1/3}\right) \qquad \text{(eq. A4)}$$

As reported by Jiang et al. (2011c), the amount of PHA accumulated in the feast phase is a function of cycle length (CL) and SRT only.

$$\frac{C_{PHA\_end\_feast}}{C_{X0}} = \frac{1}{Y_{X/STO}\left(\frac{SRT}{CL} - 1\right)} \qquad \text{(eq. A5, adapted from Jiang et al. (2011c))}$$

It may be noted herewith the degradation rate constant becomes mainly a function of cycle length and SRT. By combining equation A4 and A5 and assuming that the famine phase covers the larger part of the cycle – i.e. around 90% of the cycle time (Johnson et al. 2009b) – and that most of the stored PHA is

converted at the end of the cycle, the following expression in equation A6 can be obtained to estimate the k-value. Herein a biomass yield on PHA of 0.67 Cmol Cmol^{-1} was used (Johnson et al. 2009b).

$$k \sim \frac{2.5}{CL \cdot \left(\frac{SRT}{CL} - 1 \right)^{1/3}} \qquad \text{(eq. A6)}$$

Theory evaluation

To evaluate the proposed approach, PHA degradation data from feast-famine cultures enriched under different operational conditions were compared to the model-based degradation rates using the k-value predicted by equation A4 (figure 4.1a). Experimental datasets were obtained from feast-famine cultures enriched in SBRs with a SRT of 24 hours, a cycle length of 1, 4, 12 or 18 hours and temperatures regulated at 20 or 30 °C. All other used cultivation, sampling and measurement methods are described in more detail by Johnson et al. (2009b).

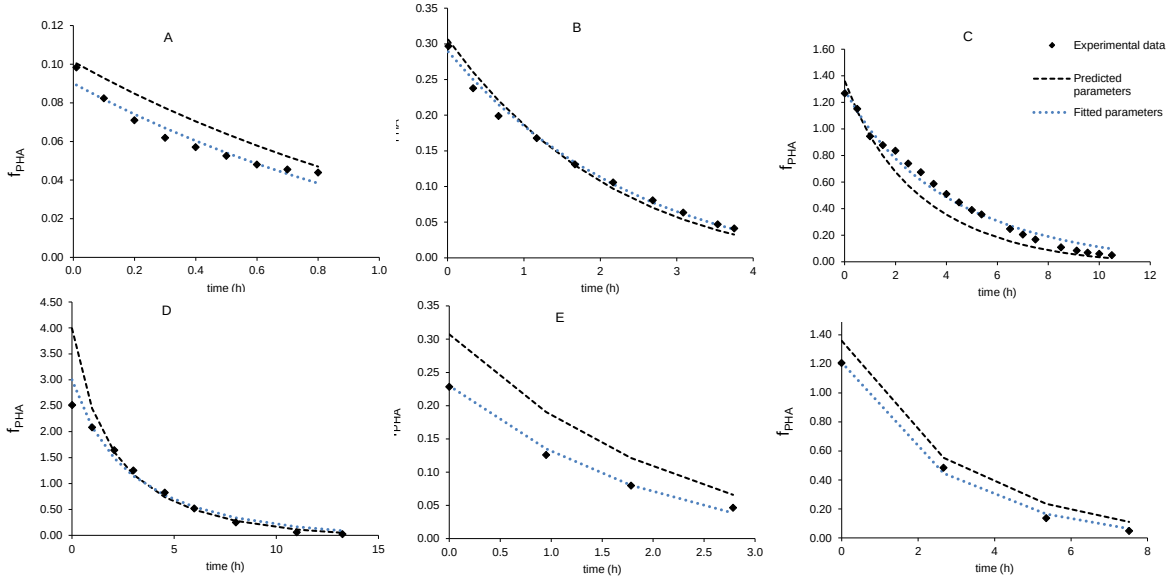


Figure 4.1a. Comparison of model results and experimental data from PHA producing feast–famine cultures enriched in an SBR at different cycle lengths/temperatures: 1 h/ 30 °C (a), 4 h/30 °C (b), 12 h/30 °C (c), 18 h/30 °C (d), 4 h/20 °C (e) and 12 h/20 °C (f). The Cmolar ratio of PHA to active biomass (f_{PHA} in Cmol PHA (Cmol X)$^{-1}$) is represented on the Y-axis, and the time during the famine phase is represented on the X-axis starting at the end of the feast phase.

Appendix 4B

A simple model using a single set of expressions to describe both feast and famine

Until now the feast and famine phase have been described by separate sets of kinetic expressions. For a generalized model it would, however, have the preference to work with a single set of expressions. Herewith the model would not only become more elegant but also universally applicable (e.g. usable in continuous cultivation systems where small amounts of substrate may still be present in the famine phase).

To establish the proper expressions, it is necessary to know the mechanism by which bacteria regulate the metabolic fluxes towards growth or PHA formation during the feast phase. As discussed in the section "Growth on external substrate", this is unfortunately not clear at this moment. Although further experimental evidence is required for elucidation of the actual growth regulation mechanism, here we demonstrate a simple model in which the PHA production during the feast phase and consumption during the famine phase are described with a single set of equations.

To achieve this, uptake rate kinetics were described by a Monod equation, identical as used in most already existing PHA models (table 4.3).

The PHA conversion rate was calculated by subtracting the consumption rate from the production rate (equation B1, adapted from box A).

$$q_{PHA} = Y_{PHA,S} \cdot q_S - k \cdot \left(\frac{C_{X0}}{C_X} \right)^{1/3} f_{PHA}{}^{2/3} \qquad \text{(eq. B1)}$$

The growth rate (μ) was established as the resultant of substrate uptake (q_S) minus PHA production (q_{PHA}, equation B1) and maintenance (m_S) as shown in equation B2.

$$\mu = Y_{X,PHA} \cdot k \cdot \left(\frac{C_{X0}}{C_X} \right)^{1/3} f_{PHA}{}^{2/3} - Y_{X,S} \cdot m_S \qquad \text{(eq. B2)}$$

It may be noted that herewith the growth rate effectively increases during the feast phase, which is in accordance with the hypothesis by Van Loosdrecht and Heijnen (2002) presented in the section "Growth on external substrate". To evaluate model applicability, experimental data previously published by Jiang et al. (2011c) were modeled using the above presented set of equations. The characteristic feast-famine profile could be described properly (figure B1) using parameter values comparable to those reported by Jiang et al. (2011c).

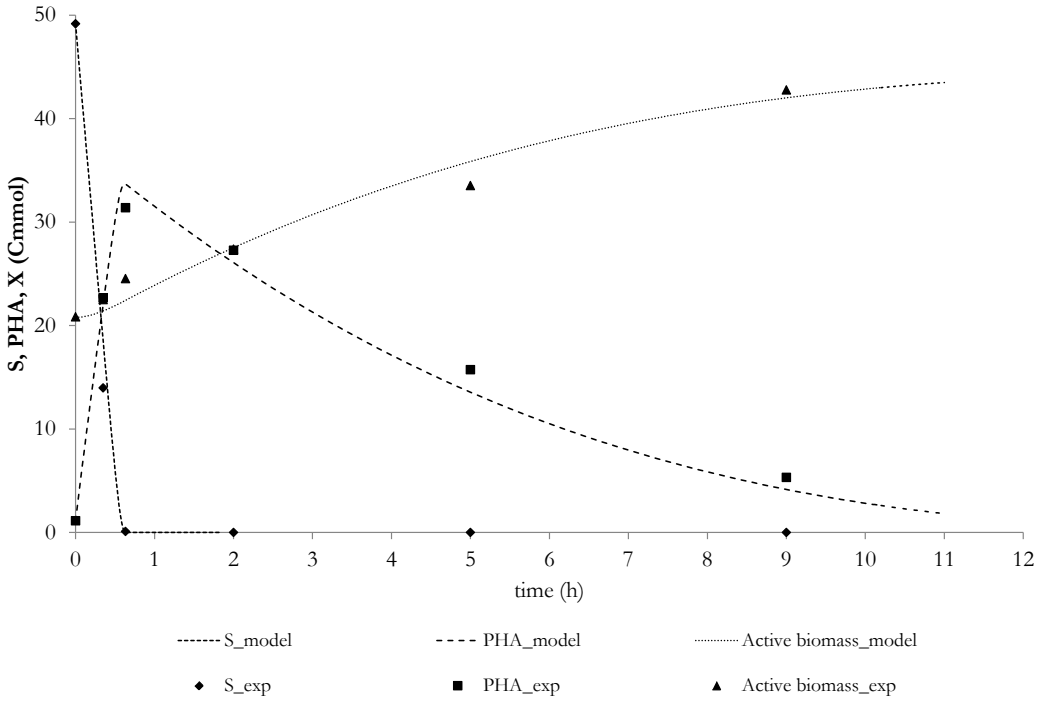


Figure 4.1b. Experimental data obtained by Jiang et al. (2010) calibrated to the presented single set of kinetic equations (Eq B1 and B2). S = substrate, PHA = polyhydroxyalkanoate, X = active biomass. The model was calibrated to the experimental data with Microsoft Excel software by adjusting the parameters so that the differences between the experimental and model values were minimized.

The presented model serves as an example to demonstrate how a feast-famine process can be modeled using a single set of equations. Therewith, it may or may not represent the actual mechanism that regulates the processes in the system. Depending on future insights, the proper mechanisms can be modeled in similar ways. For example, to represent a scenario in which PHA degradation is down-regulated during the feast phase, the equation for PHA conversion may be replaced by the equation B3.

$$q_{PHA} = Y_{PHA,S} \cdot q_S - \left(k \cdot \left(\frac{C_{X0}}{C_X} \right)^{1/3} f_{PHA}^{\,2/3} \right) \left(\frac{1}{1 + C_S / K_i} \right) \qquad \text{(eq. B3)}$$

5

Enrichment of *Plasticicumulans acidivorans* at pilot-scale for PHA production on industrial wastewater

Abstract

A PHA producing microbial culture dominated by *P. acidivorans* was enriched in a pilot plant using fermented wastewater from the Mars candy bar factory. The pilot plant comprised (1) anaerobic fermentation, (2) enrichment of a PHA-producing microbial community and (3) accumulation for maximization of the cellular PHA content. After anaerobic fermentation the wastewater contained mainly VFA (0.64 ± 0.15 gCOD $gCOD^{-1}$) and ethanol (0.22 ± 0.13 gCOD $gCOD^{-1}$). In the enrichment reactor (cycle 12 h, SRT 24 h) a feast-famine pattern was established with a feast phase of around 35 ± 5 min. The culture was able to accumulate 0.70 ± 0.05 gPHA $gVSS^{-1}$. The difference with previous lab-scale results from *P. acidivorans*, in which a PHA content of 0.90 gPHA $gVSS^{-1}$ was achieved, could be attributed to the presence of solids in the influent, the growth of a side population and the accumulation of non-PHA storage compounds that appeared to be related to ethanol consumption.

Published as

Tamis, J., Lužkov, K., Jiang, Y., van Loosdrecht, M.C. and Kleerebezem, R. (2014) Enrichment of Plasticicumulans acidivorans at pilot-scale for PHA production on industrial wastewater. Journal of biotechnology 192, 161-169.

Introduction

The biobased, biodegradable polyhydroxyalkanoate (PHA) polymer can replace petroleum based feedstock in e.g. packaging applications and chemical industry but is until now too expensive for large scale application (Chen, 2009; Crank and Patel, 2005). PHA production from waste streams using microbial enrichment cultures is a promising option for cost reduction of both PHA polymers and treatment of industrial wastewater (Reis et al. 2003; Kleerebezem and van Loosdrecht, 2007; Chen, 2009). Integration of waste-based PHA production into industry would encompass direction of a suitable waste stream towards a PHA production facility, likely in the proximity of the waste source. Here, the organic compounds in the waste stream are converted to a sludge with ideally a high PHA content and at the same time a clean effluent is produced for discharge to surface waters in accordance with local legislation. The PHA containing sludge may be further processed to purified, marketable PHA polymer product or used as feedstock for other added value processes (Chen, 2009).

While many types of wastewater can be used for the production of PHA, high concentrations of fermentable COD, relatively low nitrogen and solid concentrations and low toxicity promote process feasibility. In this perspective, food and paper industry effluents may be considered the most suitable substrates for waste-based PHA production. Other waste streams that may be interesting for PHA production include leachate from the composting industry and municipal wastewater, but it should be noted that these streams pose additional challenges due to e.g. the relatively high nitrogen content and the presence of solids. There are a large number of factors that influence the feasibility of a waste-based PHA production process (Chen, 2009), but in general, the downstream processing represents a major cost factor (Gurieff and Lant, 2007). Apart from optimization of the downstream process itself, the process feasibility can be improved by increasing the PHA content of the biomass, resulting in a reduction of chemicals and energy required for the downstream process, less waste solids and a higher overall process yield (Choi and Lee, 1999; Van Wegen et al. 1998).

The crux of enriching biomass with superior PHA-storing capacity in an open reactor system (an environment in which myriad species constantly invade the system e.g. by being present in the wastewater substrate) is the establishment of a selective environment. The cyclic presence and subsequent absence of volatile fatty acids (VFA) provides a competitive advantage for PHA storing species (Bengtsson et al. 2008; Dionisi et al. 2007; Johnson et al. 2009; Serafim et al. 2008). Based on this principle a three-step process was proposed: (1) anaerobic fermentation to direct the myriad organic compounds in wastewater to VFA (Temudo et al. 2007), (2) enrichment of biomass with superior PHA-producing capacity in a selective environment and (3) maximization of the PHA content of the biomass in an accumulation step by feeding the enriched biomass with VFA in fed-batch mode under growth limiting conditions e.g. in absence of a nitrogen source (Reis et al. 2003).

Although PHA accumulation is a common trait in bacteria, the highest PHA contents reached by microbial enrichment cultures were achieved in reactor systems dominated by a genus identified as *Plasticicumulans*. Lab-scale experiments with synthetic substrates resulted in a biomass with around 0.90 gPHA $gVSS^{-1}$ on acetate (Johnson et al. 2009) or lactate (Jiang et al. 2011b). Lab-scale experiments with wastewater resulted generally in lower PHA content: e.g. 0.75 gPHA $gVSS^{-1}$ on fermented molasses (Albuquerque et al. 2010) and 0.77 gPHA $gVSS^{-1}$ on fermented paper mill wastewater (Jiang et al. 2012). Nevertheless, the above reports show that microbial enrichment cultures can reach similar PHA contents as genetically modified pure cultures that are conventionally used in industrial biotechnology for PHA production. To further assess the industrial relevance of the waste-based PHA process, pilot-scale experiments are required, especially concerning the product quality (separability from the water phase, PHA content of the biomass and PHA quality) under industrial conditions (variable influent, complex

substrate, less strict pH control) and the identification of potential bottlenecks in the process, such as the use of chemicals (e.g. acid, base and nutrients) and the effluent quality of the process.

Only a limited amount of literature was found related to pilot-scale waste-based PHA production. Morgan-Sagastume et al. (2013) used municipal wastewater (with 17-60% of the soluble COD present in the form of VFA) to establish a PHA producing biomass in a pilot reactor with a volume of 500 l. The PHA storing capacity of this biomass was evaluated by feeding it with acetate and this way a maximum PHA content up to 0.34 gPHA $gVSS^{-1}$ was achieved. Chakravarty et al. (2010) tested a concept to harvest a PHA rich biomass and meet effluent discharge regulations at the same time. A pilot system treating 60 - 65 l day^{-1} of dairy industry wastewater produced a sludge with an estimated 0.4 - 0.5 gPHA $gVSS^{-1}$ content. Anterrieu et al. (2014) demonstrated the use of different VFA feedstock for PHA production while treating a food industry wastewater. A biomass that was cultivated on a food industry effluent (Procordia Food, Eslov, Sweden) was fed with effluent from a sugar refinery (Suikerunie, Groningen, The Netherlands). A batch experiment performed on 1000 l scale resulted in a maximum PHA content of 0.60 gPHA $gVSS^{-1}$.

It would be interesting to understand why the reported pilot-scale experiments resulted structurally in a biomass with a lower PHA content than lab-scale experiments. However, the above publications are difficult to compare to earlier published lab-scale experiments because of the use of uncharacterized biomass (Anterrieu et al. 2014), a more difficult waste stream resulting in relatively low PHA contents (Morgan Sagastume et al. 2013), or a conceptually different process setup (Chakravarty et al. 2010).

In this paper, we present results on the enrichment of a PHA producing microbial enrichment on industrial wastewater (Mars, Veghel, the Netherlands) in a pilot installation set-up similar to previous lab-scale studies (Johnson et al. 2009; Temudo et al. 2007; Jiang et al. 2012). In this study we aimed to characterize the enrichment of PHA-producing biomass in a pilot reactor operated under less strictly controlled conditions compared to lab-scale and using variable wastewater characteristics as encountered in industrial practice. In order to compare the performance of the biology in the system, characteristic process parameters were derived using a model based on earlier publications (Tamis et al. 2014b). Additionally, the research was aimed at identification of key limiting factors of PHA production at pilot-scale and explaining the difference in PHA content between lab-scale (Johnson et al. 2009; Jiang et al. 2011b) and pilot-scale experiments in general.

Materials and methods

Experiments were conducted with wastewater from a candy bar factory (Mars, Veghel, the Netherlands). The pilot plant was designed as a three-step process comprising (1) anaerobic fermentation, (2) enrichment and (3) accumulation; a schematic representation of the pilot plant layout is provided in figure 5.1.

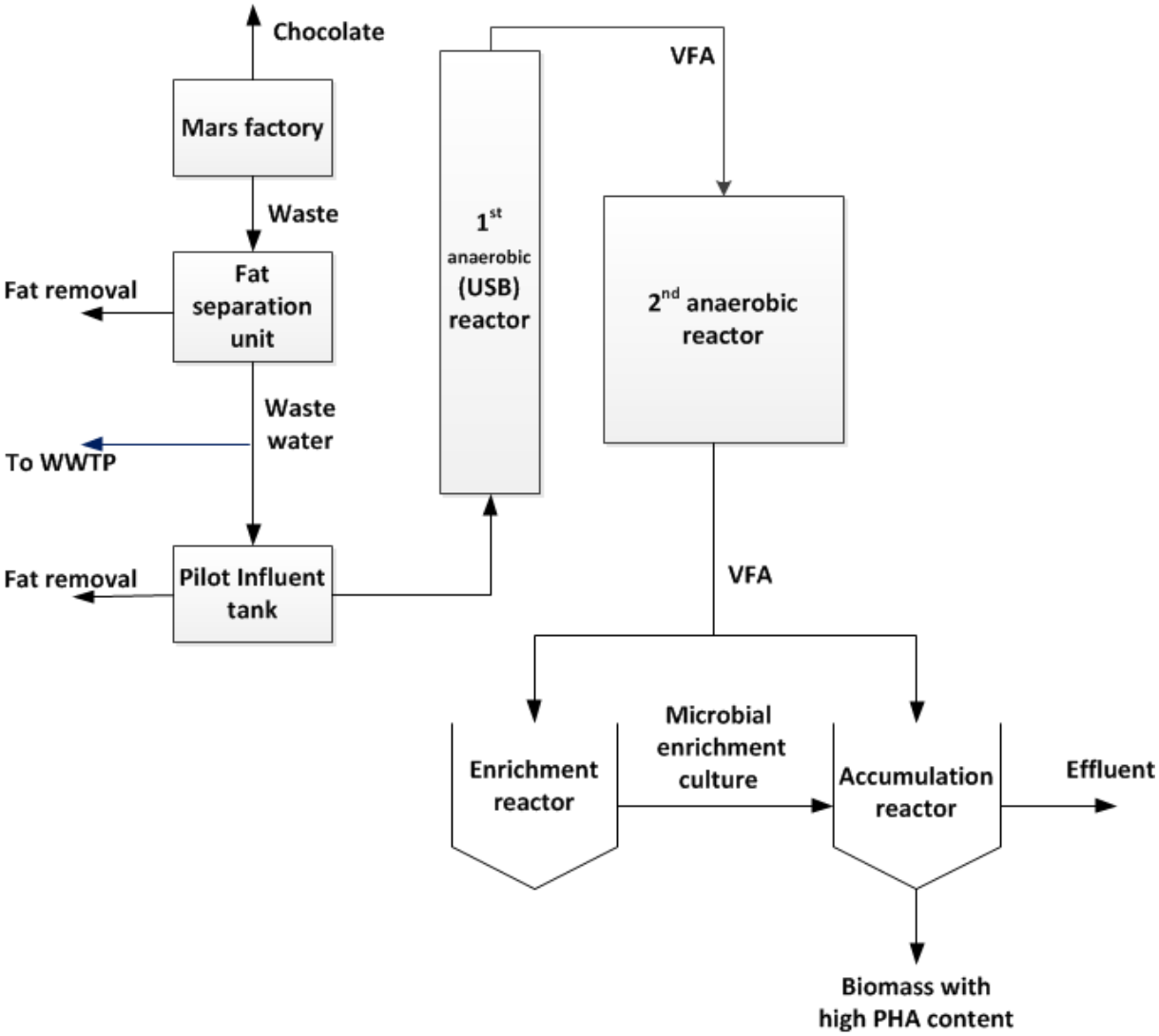


Figure 5.1. Schematic overview of the pilot system for PHA production from wastewater at the Mars candy bar factory, Veghel, The Netherlands.

Wastewater and pilot-scale anaerobic fermentation reactors

The wastewater from the Mars factory was pretreated in a flotation-based fat separation unit before entering the influent tank of the pilot installation. Subsequently, maximization of the VFA concentrations in the wastewater was pursued by application of two anaerobic reactors, operated in series. Firstly, the wastewater was fed to an upflow sludge blanket (USB) type reactor with a working volume of 60 l that was maintained at 30 ± 1 °C and a pH of 4.5 ± 0.1 by addition of controlled amounts of 1 M NaOH. The USB reactor was inoculated with sludge from a lab-scale reactor performing acidification of sugar molasses. The hydraulic retention time (HRT) of the reactor was 4 h and the solid retention time (SRT) was maintained around 4 d by manual sludge removal. To keep the reactor effluent nitrogen depleted (favorable for use in the accumulation reactor later in the process) the target COD:N mass ratio was around 300:1. A nutrient solution containing 3 M nitrogen in the form of urea, 0.3 M phosphate, 0.3 M $MgSO_4$, 0.2 M K_2SO_4, and trace elements (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) was provided to the reactor.

To buffer the volumes of available VFA substrate for the enrichment and accumulation processes, and to secure full conversion of the fermentable COD to VFA, a second anaerobic fermentation reactor was included in the system, comprising an anaerobic tank with a liquid volume of 1500 l with a hydraulic

retention time of 4 d, maintained at a temperature of 40 (±1) °C and a pH of 4.5 (±0.1) by addition of 1 M NaOH. After this second step the fermented wastewater was used as a substrate for the enrichment and accumulation reactors.

Pilot-scale enrichment reactor

The enrichment reactor (working volume 200 l) was inoculated with activated sludge from a wastewater treatment plant (Dokhaven, Rotterdam, The Netherlands) and was operated similar to the lab-scale experiments described by Johnson et al. (2009): the reactor was operated as a sequencing batch reactor (SBR) with a cycle length of 12 h and a solid and liquid retention time of 24 h. The operational cycle consisted of a feed phase, a reaction phase and an effluent phase. During the feed phase 55 l of acidified wastewater (from the second anaerobic fermentation reactor) together with 45 l of clean process water was dosed using a pH controlled pump. The dilution of the substrate with clean process water was to prevent possible oxygen limitation at high COD concentrations. For further upscaling, (treated) process effluent may be used, or the oxygen supply capacity can be increased.

After the substrate was dosed, the reactor was operated for a relatively long period during which no substrate was provided (i.e. around 11 h). At the end of the cycle half of the reactor content was discharged as effluent. The reactor was constantly aerated to achieve mixing throughout the entire cycle and supply oxygen to the system. An air flow of 200 l min^{-1} was supplied through a fine bubble diffuser to ensure no oxygen limitation occurred i.e. dissolved oxygen concentration > 1 mg l^{-1}. The reactor temperature was maintained at 30 ± 2 °C by supplying heat through the reactor walls from a thermostated compartment of warm water that surrounded the reactor. Controlled amounts of 1 M HCl and 1 M NaOH were pumped into the reactor to maintain the pH between 6.5 and 7.5. To ensure that no nutrient limitation occurred during cultivation, a nutrient solution (with composition identical to the nutrient solution used for the fermentation) was dosed. The amount of nutrients dosed was based on daily measurement of the ammonium concentration at the end of the cycle. In this way the concentration of ammonium was maintained between 10 and 30 mgN l^{-1} at the end of the cycle. The resulting COD:N mass ratio in the feed stream was approximately 25:1. It was assumed that ammonium was the limiting growth nutrient with other elements required for microbial growth present in excess. In order to limit the possible influence of nitrification, 10-20 ml of the specific inhibitor allylthiourea (ATU) stock (33 g l^{-1}) was dosed per day during periods in which NO_3^- or NO_2^- concentrations exceeded 5 mgN l^{-1}.

Pilot-scale accumulation reactor

To maximize the PHA content in the cells, a fed-batch reactor (working volume 200 l) was operated. The accumulation experiments were performed as follows: first 100 l biomass from the enrichment reactor was pumped into the accumulation reactor. Subsequently, residual nitrogen was depleted by dosing a small pulse of substrate followed by a regular famine period (providing a last growth advantage to PHA-storing organisms). After this period, the depletion of ammonium was verified manually and the accumulation process was continued by dosing of the acidified wastewater (from the second anaerobic fermentation reactor) using a pH regulated pump. The amount of substrate dosed during an accumulation experiment varied, depending on the amount of PHA that could be accumulated by the culture. The reactor broth was aerated through a fine bubble diffuser with a constant air flow of 200 l min^{-1} and its temperature was maintained at 30 (±2) °C. The accumulation experiment was terminated when the activity (observed as oxygen consumption) was approaching zero. Biomass was harvested using a pilot-scale centrifuge (3000 g, 200 l h^{-1}).

Lab-scale accumulation reactors

To assess the impact of separate substrates on the pilot biomass, two lab-scale accumulation experiments were performed. One accumulation experiment was performed with acetic acid as sole substrate in a fed-batch reactor with a working volume of 2 liter. The experiment was conducted with biomass from the pilot in which ammonium was depleted. The reactor was operated at a temperature of 30 (±1) ˚C and fed with a 1.5 M acetic acid by pH-control. The air flow through the system was 1.5 l min^{-1} and the stirring speed was 750 rpm.

A similar second experiment was performed with ethanol as sole substrate in a fed-batch reactor. This reactor was identical to the acetic acid fed-batch reactor except the following: the substrate was a 1.5 M ethanol solution which was manually dosed. The initial dose was 9 ml of 1.5 M ethanol and additional 3 ml of 1.5 M ethanol was dosed each time the ethanol was depleted. The depletion of ethanol was monitored through the dissolved oxygen (DO) concentration profile, with a sharp increase in the DO concentration indicating substrate depletion. The air flow through this system was 0.6 l min^{-1} and the stirring speed was 500 rpm.

Sampling and analytical methods

The data used in this study were obtained by monitoring the pilot system during a period of 105 days, according to the scheme presented in table 5.1. Apart from the regular sampling, detailed sampling of the operational cycle in the enrichment reactor and the accumulation reactor was performed.

Table 5.1. Sampling and measurement scheme.

Measurement	Sample point	Frequency	Method
COD	Anaerobic fermentors (in, out)	Daily	Spectrophotometric
sCOD	Anaerobic fermentors (in, out)	Daily	Spectrophotometric
VSS/TSS	2nd anaerobic fermentor (out), enrichment reactor	Daily	Dry/ash weight
NH_4^+, NO_2^-, NO_3^-	Enrichment reactor (end of cycle)	Daily	Colorimetric
PHA	Enrichment and accumulation reactor	Daily	GC
alcohol/VFA	Anaerobic fermentors (in, out)	Daily	HPLC and GC
CO_2 and O_2	Enrichment reactor	Online	Off-gas analyzer
pH	All reactors	Online	Ag/AgCl electrode
acid/base dose	All reactors	Online	Calibrated pump time
DO	Enrichment and accumulation reactors	Online	LDO sensor

Filtered (0.45 μm pore size, PVDF membrane, Millipore, Ireland) and unfiltered wastewater samples were collected before and after both anaerobic fermentation reactors to evaluate soluble and total COD, using spectrophotometric analysis (Hach-Lange). It was verified that the filter itself did not release any COD. Additionally, filtered samples were taken for detailed composition analysis on a high performance liquid chromatography (HPLC) and gas chromatography (GC). The VFA concentrations were measured by HPLC (BioRad Aminex HPX-87H column, Waters 2489 UV/RI detector, 1.5 mM H_3PO_4 mobile phase with a flow rate of 0.6 ml min^{-1} and a temperature of 60 ˚C) and the ethanol concentrations were measured by GC (HP-Innowax column with FID detector, helium as carrier gas and injector and detector and column temperatures of 200 ˚C, 250 ˚C and 60 ˚C, respectively). Sludge concentrations (TSS and VSS) and characteristics (SVI30) were analyzed by standard methods (Clesceri et al. 1999). The PHA content of the biomass was evaluated by a method described by Johnson et al. (2009). In brief: weighed biomass samples and standards (pure poly-3-hydroxybutyric acid-co-3-hydroxyvaleric acid with a PHV content of 12 mol%,

(403121, Sigma-Aldrich, USA) as external standard; and benzoic acid in 1-propanol as the internal standard) were heated at 100 °C for 2 hours in a mixture of concentrated HCl, 1-propanol and dichlorethane (volume ratios 1:4:5). The propylesters in the organic phase were analyzed by gas chromatography. Results were expressed as weight percentage of PHB and PHV of the volatile suspended solids. Ammonium, nitrite and nitrate concentrations were analyzed using a colorimetric method (Merckoquant, Merck, Germany). The CO_2 and O_2 concentrations of the gas entering and leaving the reactor were measured by an off-gas analyzer (NGA 2000, Rosemount, USA). The gas was dried with a condenser (4 °C) and filtered through a hydrophobic filter to prevent any water entering the off-gas analyzer.

Microbial community structure analysis

The composition of the microbial community was analyzed by microscopic inspection of fluorescent in situ hybridization (FISH) slides with a UCB823 probe mixture for identification of *P. acidivorans* and an EUB probe mixture that binds in principle with all types of eubacterial species; more detailed information on the used FISH method can be found in an earlier publication by Johnson et al. (2009). In addition, the microbial diversity in the enrichment culture was analyzed using denaturing gradient gel electrophoresis (DGGE) as described by Johnson et al. (2009) with an adapted method for the DNA extraction. The extraction involved an extra treatment step comprising three times repeated freezing of the sample in liquid nitrogen and subsequent bead milling in order to ensure DNA extraction from all microorganisms including species with very tough cell walls.

Data analysis

The total VFA concentration was defined as the sum of the individual COD concentrations of acetate, propionate, butyrate, valerate and caproate. Soluble COD that was not identified as either VFA or ethanol was termed "other soluble COD" or "other sCOD" throughout this paper. The solid COD was calculated by subtracting the COD concentration of a filtered sample from the COD concentration of an unfiltered sample. Active biomass concentrations were calculated by subtracting the amount of storage compounds from the amount of volatile suspended solids. The length of the feast phase was used as indicator for performance of the enrichment and process stability (Jiang et al. 2010a) and was identified by the rapid increase in DO and pH levels after the VFA in the reactor was consumed. Characteristic process parameters were identified by calibrating a process model to the experimental data. The model was based on earlier publications (Tamis et al. 2014b) and is a detailed model description is provided in the appendix 5A.

Results

Wastewater and fermentation

The wastewater originating from the candy bar factory had a temperature of around 30 °C and varied over time in strength and composition. The average soluble COD (sCOD) of the wastewater that was fed to the anaerobic fermentation varied strongly over time (intrinsic to factory operation, e.g. semi-periodic cleaning of equipment) with an average concentration of 7.8 ± 4.1 gCOD l^{-1} (average ± standard deviation over the data set).

The soluble COD of the influent wastewater consisted roughly of 0.2 gCOD $gCOD^{-1}$ of ethanol and 0.1 gCOD $gCOD^{-1}$ of VFA. The remaining soluble COD in the wastewater was not identified on a regular basis but comprised glucose, lactate and COD that could not be readily identified by either the GC or HPLC methods used in this research. In addition to soluble COD, a concentration of 0.8 ± 0.5 gCOD l^{-1}

that could not pass a 0.45 μm pore size filter (PVDF membrane, Millipore, Ireland) was present in the wastewater. The soluble nitrogen concentration in the wastewater was negligible (< 1 mg l^{-1}).

The influent and effluent streams of the two anaerobic fermentation reactors were sampled on a weekly basis (16 samples over a period of 105 days) to evaluate the composition of the wastewater and the conversions in both the anaerobic fermentation reactors (figure 5.2).

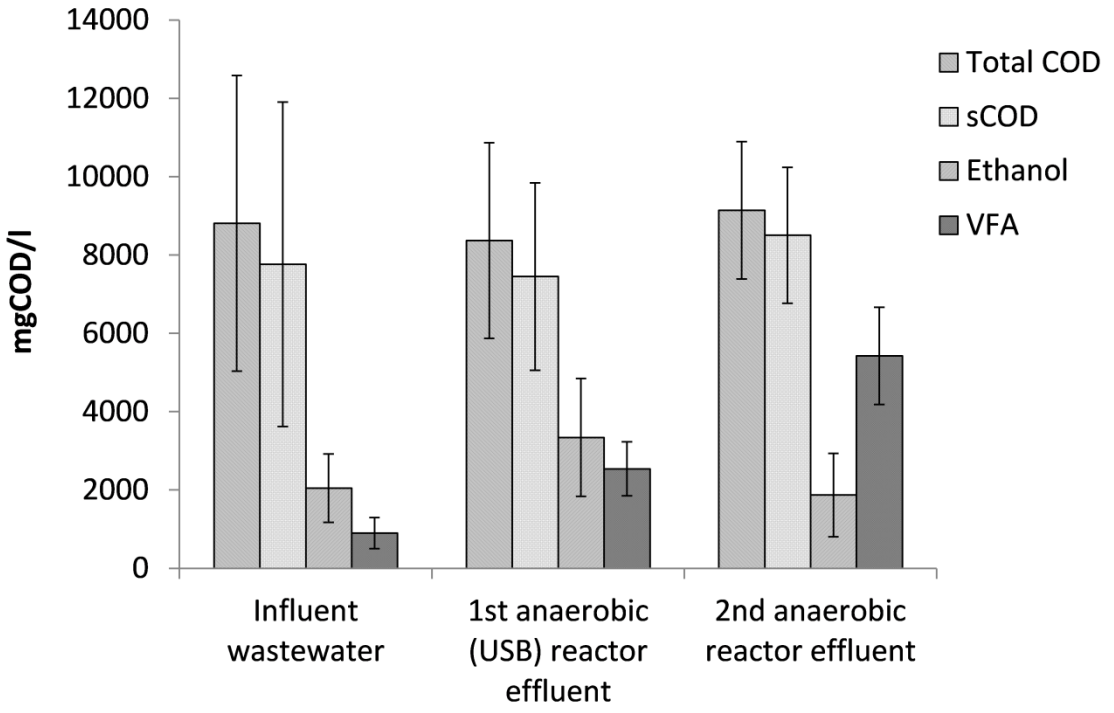


Figure 5.2. Average concentrations of COD in the wastewater before and after fermentation. The COD concentrations of the incoming wastewater varied substantially from day to day and this was represented by the error bars (± standard deviation over the dataset).

A significant net production of ethanol and VFA was observed in the anaerobic upflow sludge bed reactor (the first step of the two-step fermentation). Subsequent fermentation in the second anaerobic fermentation reactor resulted in a net conversion of ethanol to VFA; specifically increased concentrations of butyrate and caproate were found in the effluent of this unit operation, suggesting the occurrence of chain elongation reactions (Agler et al. 2012; Grootscholten et al. 2013). The average fraction of VFA in the soluble COD after the two anaerobic fermentation steps was 0.64 ± 0.15 gCOD $gCOD^{-1}$. The average VFA composition was 32 ± 6 % acetate, 14 ± 3 % propionate, 33 ± 11 % butyrate, 5 ± 3 % valerate and 18 ± 8 % caproate on COD basis. The second most important fraction of soluble COD was ethanol (0.22 ± 0.13 gCOD $gCOD^{-1}$). The remaining soluble COD (0.14 ± 0.09 gCOD $gCOD^{-1}$) in the fermented wastewater could not be readily identified by either the GC or HPLC methods used in this research. The total COD and solid concentrations did not change significantly after conversion in both anaerobic reactors indicating the absence of significant H_2 or CH_4 gas production and also indicating biomass growth was small compared to total COD fluxes. The effluent after the second anaerobic fermentation step was used as substrate for the enrichment and accumulation reactors. It should be noted that the relatively large variation in strength and composition of the incoming wastewater was equalized due to both the conversions in the anaerobic fermentation and the relatively long HRT (4 d) of the second fermentation reactor. An overview of the variations in the raw (entering the anaerobic pre-treatment) and

fermented wastewater (entering the enrichment) is provided in the appendix 5C. The NaOH consumption for maintaining the pH of both reactors at 4.5 was on average 5 mol d^{-1}, equal to around 2 mmol $gCOD^{-1}$.

Enrichment reactor

After startup of the enrichment reactor, a feast-famine pattern was observed within a few cycles, characterized by low dissolved oxygen (DO) concentrations during the feast phase and higher DO concentrations during the famine phase (figure 5.3).

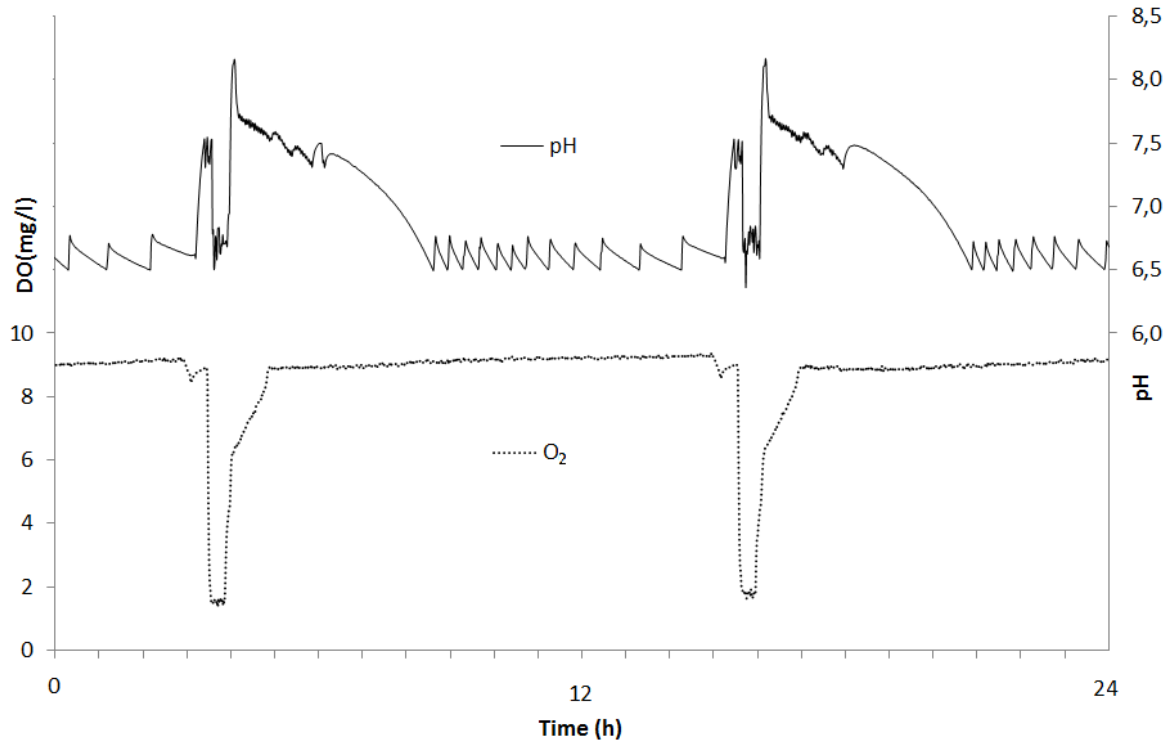


Figure 5.3. Dissolved oxygen (DO) and pH profiles of the operational cycle (12h) in the enrichment reactor during steady state.

Due to its acidic nature, the substrate was dosed intermittently to maintain a minimum pH of 6.5. Shortly after dosing the substrate, a sharp increase in pH was observed concurrently with a sharp increase of the DO concentration, indicating that VFA was depleted. The initial sharp increase of the DO was followed by a period in which the DO was somewhat lower than its value during most of the famine phase. It appeared that other soluble COD besides VFA was consumed during this period. The pH profile during the famine phase was influenced by various factors: shortly after the feast phase the pH had the tendency to increase due to stripping of CO_2 and hydrolysis of the urea in the dosed nutrients, and on average 1.8 mol $cycle^{-1}$ HCl was dosed keep the pH below 7.5. Later in the famine phase, the pH decreased due to ammonium uptake and on average 0.2 mol $cycle^{-1}$ NaOH was dosed to keep the pH above 6.5. The length of the feast phase varied initially between 30 and 90 minutes, but became shorter and more stable (35 ± 5 min) after day 50 of operation (figure 5.4). The average sludge concentration at the end of the cycle was 1.5 ± 0.4 gVSS l^{-1} and the overall yield of biomass on COD was 0.33 ± 0.09 gX $gCOD^{-1}$. The SVI30 of the sludge at the end of the cycle was 283 ± 113 ml g^{-1}.

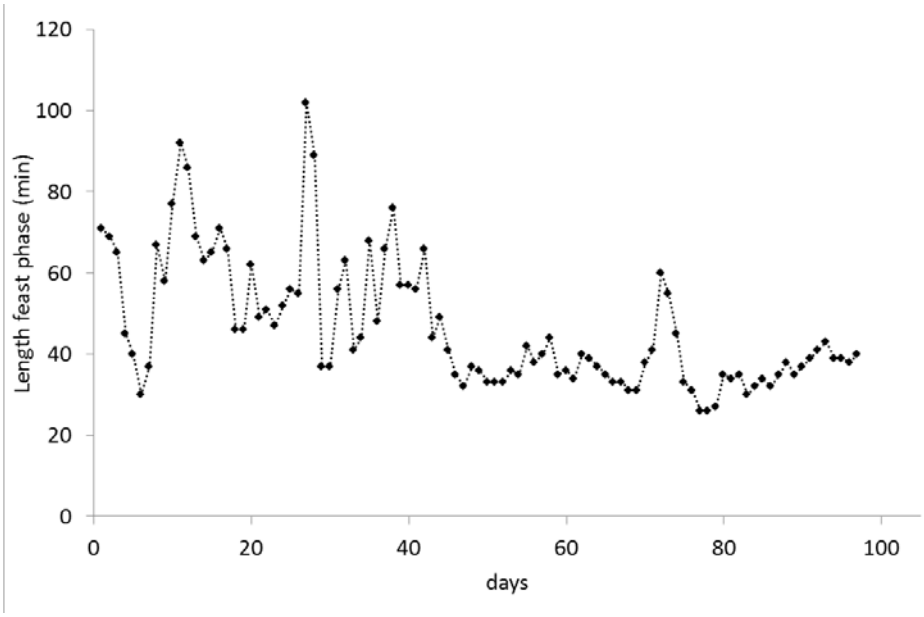


Figure 5.4. The development of the length of the feast phase (based on the DO profile) in the enrichment reactor over the long term.

After a stable operational performance was established, the cycle was characterized by detailed sampling and measurement of the amounts of substrate, PHA and VSS in the reactor. Data from a representative cycle (day 58) is shown in figure 5.5. The PHA content increased during the feast phase when VFA was present. At the end of the feast phase all VFA was consumed and the PHA content of the biomass was 0.40 ± 0.05 gPHA $gVSS^{-1}$. The VSS increased faster than the amount of PHA during the feast phase, indicating that active biomass or another storage compound was produced simultaneously with PHA production. After the VFA was depleted there was still soluble COD present in the reactor, mainly in the form of ethanol, but subsequent uptake of this soluble COD did not increase the amount of PHA in the system. Instead, PHA was degraded while the amount of active biomass (VSS minus PHA) increased throughout the remainder of the cycle, indicating growth on storage. Characteristic process parameters were obtained by calibration of a model (Appendix 5A) to the experimental data from the cycle experiment (table 5.2). The estimated yields were similar to earlier lab-scale experiments (Jiang et al. 2010a) but the biomass specific rates were lower than earlier lab-scale experiments (Jiang et al. 2010a).

Table 5.2. Characteristic parameters derived from the experimental data. Specific rate values represent lumped biomass conversion rates. Calculations were performed using 1.7 gCOD $gPHA^{-1}$ and 1.4 gCOD gX^{-1}.

Reactor	Pilot enrichment	Pilot accumulation	Lab accumulation		
Substrate	Fermented wastewater		Acetate	Ethanol	-
$q_S(VFA)^{max}$	2.8	1.4	2.2	-	gCOD gX^{-1} h^{-1}
$q_S(CODo)^{max}$	0.5	0.2	-	0.4	
$Y_{PHA/S}$	0.37	n.d.	0.50	0.22	g $gCOD^{-1}$
$Y_{X/PHA}$	0.49	-	-	-	
$Y_{X/CODo}$	0.28	-	-	-	
Degradation rate constant (k)	0.21	-	-	-	$gPHA^{1/3}$ $gX^{1/3}$ h^{-1}
Final PHA content	-	0.76	0.80	0.30	gPHA $gVSS^{-1}$

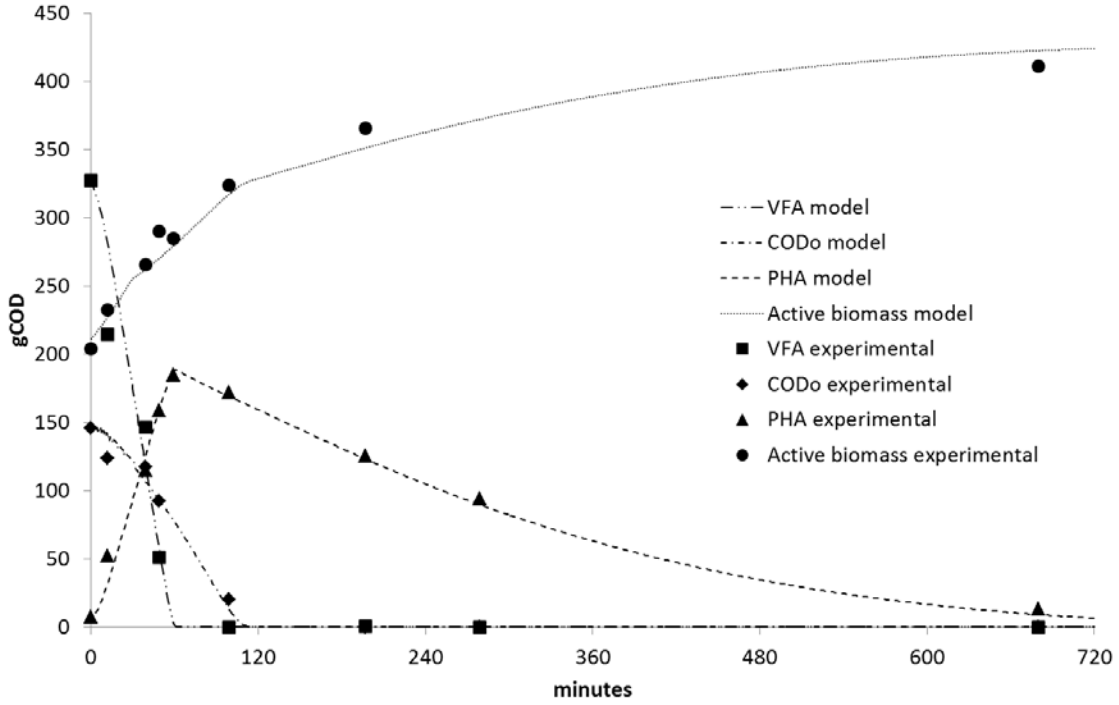


Figure 5.5. Detailed characterization of a representative cycle (day 58) including measurements of the substrate, PHA and active biomass amounts. Due to changing liquid volumes during the reactor feeding, data is represented as amounts and not concentrations.

Microbial community structure

The microbial community structure was evaluated weekly by FISH. In figure 5.6 representative images of the culture in the enrichment reactor are presented. From these images it was clear that from day 50 of operation *P. acidivorans* became the dominant species in the reactor with an estimated abundance of roughly > 70%. In addition, DGGE analysis confirmed the continuous presence of *P. acidivorans* from day 44 of operation. Microscopic inspection showed that a small side-population comprising several species coexisted with *P. acidivorans*, amongst which a tetra-shaped bacterium was prominent, coinciding with DGGE results indicating the presence of *Amaricoccus* sp.

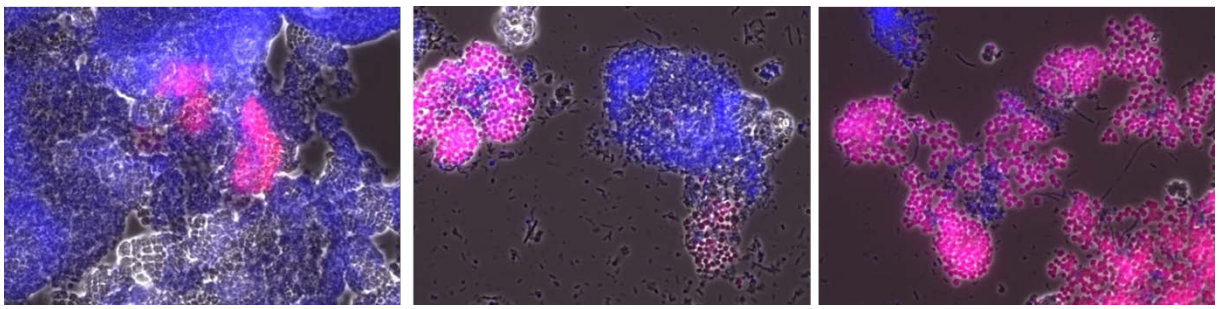


Figure 5.6. FISH microscope images of the biomass in the pilot reactor. *P. acidivorans* in pink (UCB823-probe), other bacteria in blue (EUB-probe), initial culture (left: day 44 of operation) was taken over by *P. acidivorans* (middle: day 51 of operation and right: day 58 of operation).

Pilot-scale accumulation experiments

Six accumulation experiments were conducted with biomass harvested from the enrichment reactor at the end of the operational cycle at active biomass concentrations of typically 1.5 g l^{-1} and initial ammonium, nitrite and nitrate concentrations lower than 5 mgN l^{-1}. The accumulation reactor was fed with fermented wastewater using a pH controlled pump during a period of 4 to 7 hours.

The average PHA content after accumulation was 0.70 ± 0.05 gPHA $gVSS^{-1}$ and a maximum PHA content achieved was 0.76 gPHA $gVSS^{-1}$. The PHB/PHV ratio did not vary strongly (< 10%) and was around 84/16, similar to the fraction of VFA with uneven number of carbon atoms (propionate and valerate) in the substrate, as reported earlier (Albuquerque et al. 2011; Jiang et al. 2011a). The chromatogram for PHA detection had no other significant peaks, indicating PH2MV, PHH and other forms of PHA were not produced in relevant quantities. Detailed experimental results of a PHA accumulation experiment are presented in figure 5.7 (A).

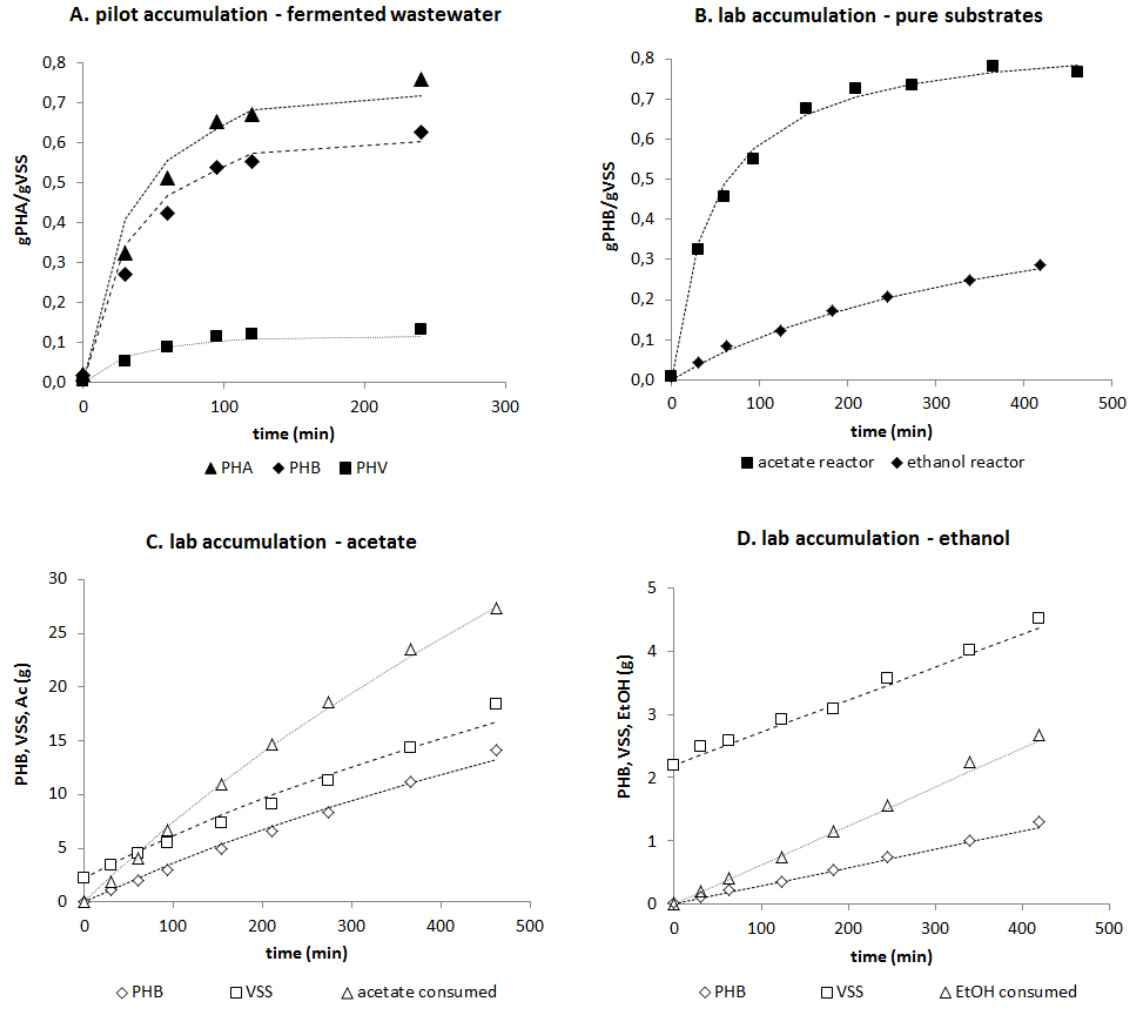


Figure 5.7. Results from accumulation experiments A.) pilot-scale reactor using real wastewater as substrate, B.) lab-scale: comparison of PHB content of the cultures fed with acetate and ethanol C.) lab-scale accumulation experiments fed with acetate and D.) lab-scale accumulation experiments with fed with ethanol.

The biomass from the accumulation experiment was harvested using a centrifuge (3000 g). The TSS concentration of the concentrated biomass was approximately 100 g l^{-1} and the supernatant was free of solids (< 50 mgTSS l^{-1}) and nitrogen (< 1 mgN l^{-1}) but still contained 1-2 g l^{-1} soluble COD. This soluble

COD comprised mainly unidentified COD (0.5 ± 0.1 gCOD $gCOD^{-1}$) and ethanol (0.4 ± 0.1 gCOD $gCOD^{-1}$). It was not possible to identify the PHA yields of the different substrates present in the fermented wastewater independently; instead, the yields obtained from lab-scale experiments were used ($Y_{PHA/VFA}$ = 0.5 gPHA $gCOD^{-1}$; $Y_{PHA/EtOH}$ = 0.22 gPHA $gCOD^{-1}$). The relatively low yield of PHA on ethanol in the fermentation is further contemplated in the section on lab-scale results and in the discussion section.

Lab-scale accumulation experiments

In order to investigate the effects of the separate substrates in the wastewater, two lab-scale accumulation experiments were performed. Two identical reactor vessels with a working volume of 2 l were filled with biomass from the pilot. One reactor was fed with acetate as described by Johnson et al. (2009), another reactor was manually fed with ethanol in pulses as described in the methods section. No ammonium (or other form of soluble nitrogen source) was dosed or present in both experiments. In both systems PHB was produced, although the PHB content in the ethanol reactor was significantly lower than the PHB content in the acetate fed reactor (figure 5.7B). The PHV content was negligible in both experiments.

The yield of PHB on substrate and the biomass specific substrate uptake rate were significantly lower in the ethanol fed-batch reactor. Furthermore, it was found that the VSS concentrations in the ethanol fed reactor increased more than expected on basis of PHB production alone, indicating the production of a substantial amount of non-PHA solids (figure 5.7D). It should be emphasized that no nitrogen source was present in the medium essentially, preventing active biomass formation.

Based on the difference between VSS and PHB production, the estimated yield of these non-PHA solids was 0.37 g solids $gEtOH^{-1}$. This indication for production of an unidentified storage compound was confirmed by evaluation of a carbon balance using reactor off-gas data. A carbon content of the unidentified compound of around 0.5 gC $gVSS^{-1}$ was estimated from solid and carbon measurements, suggesting that it could be a polysaccharide; but this was not explicitly confirmed by independent measurements.

Discussion

Pilot performance and outlook for industrial implementation

The aim of this study was to characterize PHA production from industrial wastewater at pilot-scale using guidelines that were previously established in laboratory research. Enrichment of a PHA producing microbial community able to accumulate 0.70 ± 0.05 gPHA $gVSS^{-1}$ within 4 hours was demonstrated; comparable to previous lab-scale studies with wastewater (Albuquerque et al. 2010; Jiang et al. 2012). The dominant species in the enrichment reactor was *P. acidivorans*, the same organism as enriched in laboratory enrichment cultures under similar regimes (Johnson et al. 2009; Jiang et al. 2010a). This indicates that the selective conditions in the pilot plant indeed matched well with those of laboratory cultures.

The maximum PHA content obtained was substantially lower than for lab-scale microbial enrichments with *P. acidivorans* on synthetic substrates (0.70 vs. 0.90 gPHA $gVSS^{-1}$). In order to explain this difference, three factors were identified: 1) presence of solids in the fermented wastewater, 2) the presence of a non-storing side population and 3) the production of other types of storage compounds from ethanol. A more detailed description of each of these factors can be found in the appendix 5B.

A process yield over the whole process (including anaerobic pre-treatment, enrichment and accumulation steps) of 0.30 ± 0.04 gPHA-COD $gCOD^{-1}$ was established (equal to 0.18 gPHA $gCOD^{-1}$ using 1.7 gCOD $gPHA^{-1}$). Another significant part of the influent COD (0.11 ± 0.02 gX-COD $gCOD^{-1}$ was used for

biomass production in the enrichment step). No significant COD loss was observed in the anaerobic fermentation steps. The COD can be closed by the amount of COD oxidized in the enrichment and accumulation steps (0.55 ± 0.10 gCOD $gCOD^{-1}$). Assuming an energy input for aeration of 2 kWh per kg oxygen supplied, this would result in an electricity consumption of approximately 6 kWh $kgPHA^{-1}$ produced. It should be noted that the overall process yield and related process performance indicators depend on the fraction of VFA in the influent of the enrichment and accumulation steps (which was only around 0.64 in this case) and that the process may be further optimized by directing the anaerobic fermentation to produce more VFA at the expense of ethanol.

A comparison of the volumetric productivity of the pilot with other i.e. pure culture systems is more ambiguous since pure culture systems require initial batch cultivation steps while our pilot system required enrichment and anaerobic pre-treatment steps. A comparison of volumetric productivity based on the accumulation reactor may be more appropriate. Using an initial biomass concentration of 1.5 g l^{-1} and a PHA content of 0.7 gPHA $gVSS^{-1}$ achieved in 4 h, a volumetric productivity of approximately 0.5 g l^{-1} h^{-1} can be estimated. This is in the same order of magnitude as the 0.1 - 4 g l^{-1} h^{-1} range reported for pure culture systems (Huijberts and Eggink, 1996; Chen, 2009). The pilot system was not designed to maximize volumetric productivity but we identified the oxygen mass transfer capacity as principal limiting factor for the productivity.

Another factor that may be further optimized to improve the economic viability is the use chemicals for pH-control. For example, the use of NaOH in the anaerobic fermentation step may be minimized by operating at low pH. In this study the amount of base dosed in the anaerobic fermentation operated at pH 4.5 was around 2 mmol $gCOD^{-1}$, which would translate in about 0.5 kg of NaOH use per kg of PHA produced. This amount could be reduced a factor 2-3 when operating at pH 4.0 instead. Future research should evaluate to what extent it is feasible to efficiently produce VFA at low pH. Furthermore, usage of acid and base for pH control in the enrichment reactor was around 4 mmol $gCOD^{-1}$. These amounts may be further minimized by decreasing the air flow in the famine phase, effectively minimizing CO_2 stripping from the reactor liquid and using it to buffer the pH.

Another critical issue is the use of allylthiourea (ATU) in this pilot study to prevent the conversion of ammonium to nitrate. ATU dosage in full-scale operations would be unacceptable for economic and environmental reasons, but was nevertheless implemented in this pilot study to reduce the amount of unknown factors, as it is currently unknown whether *P. acidivorans* or other species with very high PHA storing capacity can be enriched on nitrate as nitrogen source instead of ammonium. Future research should elucidate this or evaluate alternative strategies for prevention nitrification if necessary (Hunik et al. 1994).

For successful integration of waste-based PHA production into industry, the process must not only produce a sludge with a high PHA content but also facilitate a clean effluent water, in accordance of local legislation. In this study, the effluent of the final process operation (the accumulation step) still contained 1-2 g l^{-1} biodegradable COD. Further development of the process must therefore include minimization of this concentration and a solution for final treatment of the effluent, such as introduction of an aerated post treatment unit operation designed to produce a clean effluent.

Conclusions

Pilot-scale PHA production from industrial wastewater was demonstrated by enrichment of a biomass able to accumulate 0.70 ± 0.05 gPHA $gVSS^{-1}$ within 4 hours - the highest value reported so far on pilot-scale. Removal of suspended solids after the fermentation stage is seen as an important factor for future optimization, as well as repression of ethanol formation and enhancement of VFA production in the fermentation step.

Appendix 5A – Process model

General

The enrichment process was characterized using a modified version of earlier models describing the dynamics of the cycle in the SBR (Tamis et al. 2014b). The model was modified in order to include both VFA and non-VFA COD (termed CODo in the rest of this section) that was present in the substrate. The model comprised three types of biomass: one type of biomass producing PHA from VFA (reaction 1) and subsequently growing on PHA (reaction 2). A second type of biomass exclusively used non-VFA COD for direct growth (reaction 3). A third type of biomass that was considered in the model was inactive biomass (e.g. anaerobic organisms that entered the system via the substrate).

Reactions

1. VFA → $Y_{PHA/VFA}$ PHA + (1- $Y_{PHA/VFA}$) CO_2
2. PHA → $Y_{X/PHA}$ Xp + (1 - $Y_{X/PHA}$) CO_2
3. CODo → $Y_{X/CODo}$ Xo + (1- $Y_{X/CODo}$) CO_2

Differential equations for state variables

The model values for substrate (VFA and non-VFA COD), PHA storage and different types of biomass (X_p: PHA accumulating , X_o: direct growth on non-VFA and X_i: inactive) over time were calculated using the differential equations below. The numerical solutions were established calculated using the Euler method for numerical integration in Microsoft Excel with a time stepsize of 0.5 min.

$$\frac{dVFA}{dt} = \phi \cdot C_{VFA}^{\ in} + q_{VFA} \cdot X_p \quad \text{(eq. A1)}$$

$$\frac{dPHA}{dt} = q_{PHA} \cdot X_p \quad \text{(eq. A2)}$$

$$\frac{dX_p}{dt} = \mu_p \cdot X_p \quad \text{(eq. A3)}$$

$$\frac{dCODo}{dt} = \phi \cdot C_{CODo}^{\ in} + q_{CODo} \cdot X_o \quad \text{(eq. A4)}$$

$$\frac{dX_o}{dt} = \mu_o X_o \quad \text{(eq. A5)}$$

$$\frac{dXi}{dt} = \phi \cdot C_{X\,i}^{\ in} \quad \text{(eq. A6)}$$

Since the experimental dataset included measurements of volatile solids and PHA but no information on the separate types of biomass, the biomass was lumped in a total biomass variable (X)

$$X = X_p + X_o + X_i \quad \text{(eq.A7)}$$

Kinetic equations

The kinetics of the model were adapted from earlier models (Tamis et al. 2014b). The uptake of VFA and non-VFA COD (CODo) were described by Monod kinetics. The PHA production rate was coupled to VFA uptake and the PHA degradation was based on a shrinking particle model (Tamis et al. 2014b). The growth rate of the PHA-producing biomass was stoichiometrically coupled the PHA degradation rate and a maintenance factor. The growth rate of the non-VFA consuming biomass was stoichiometrically coupled to COD uptake rate and a maintenance factor.

$$q_{VFA} = q_{VFA}^{\max} \frac{C_{VFA}}{C_{VFA} + K_{VFA}} \quad \text{(eq. A8)}$$

$$q_{PHA} = Y_{PHA/VFA} \cdot q_{VFA} - k \cdot \left(\frac{C_{Xp}^{0}}{C_{Xp}} \right)^{1/3} \cdot \left(\frac{C_{PHA}}{C_{Xp}} \right)^{2/3} \quad \text{(eq. A9)}$$

$$\mu_p = Y_{X/PHA} \cdot k \cdot \left(\frac{C_{Xp}^{0}}{C_{Xp}} \right)^{1/3} \cdot \left(\frac{C_{PHA}}{C_{Xp}} \right)^{2/3} - Y_{X/VFA} \cdot m_{VFA} \quad \text{(eq. A10)}$$

$$q_{CODo} = q_{CODo}^{\max} \frac{C_{CODo}}{C_{CODo} + K_{CODo}} \quad \text{(eq. A11)}$$

$$\mu_o = Y_{X/CODo} \cdot q_{CODo} - Y_{X/CODo} \cdot m_{CODo} \quad \text{(eq. A12)}$$

Model calibration

The process was characterized in terms of maximum substrate uptake rate for VFA and non-VFA COD (q_{VFA}^{max} and q_{CODo}^{max}), the PHA degradation rate constant (k), the yield of PHA of VFA ($Y_{PHA/VFA}$), the yield of biomass on PHA ($Y_{X/PHA}$) and the yield on biomass on non-VFA COD ($Y_{PHA/CODo}$). The substrate affinity constants (K_{VFA} and K_{CODo}) could not be readily identified from experimental data presented in this paper; instead, it was assumed that they were low compared to the substrate concentrations present in the reactor during the feast phase i.e. 50 mgCOD l^{-1}. The maintenance parameters were assumed to be negligible in a system operated under such a short residence time (SRT = 1 d). The characteristic parameters were obtained using a solver function available in Microsoft Excel (GRG Nonlinear) to minimize the difference between model and experimental values. The initial values for the state variables were chosen assuming steady state conditions for the SBR, i.e. the initial values were half of values at the end of the cycle. The obtained values for the biomass specific rates were related to the individual types of biomass used in the model. However, in some cases it may be more desirable to be able to compare biomass characteristics based on an overall, lumped biomass specific rate, which can be related to the individual biomass specific rates using the fractions of the different types of biomass and equation A13.

$$q_{VFA,lumped} = q_{VFA} \frac{X_p}{X} \quad \text{(eq. A13)}$$

Appendix 5B – Explaining the difference in PHA content between lab and pilot

Several factors were identified to have a significant influence on the PHA content of the biomass. The combined effect of the solids in the substrate that accumulate in the biomass, the presence of a side-population and the production of other storage products are strong enough to account for the difference between the PHA content of 0.90 gPHA $gVSS^{-1}$ obtained with in lab-scale experiments and 0.70 gPHA $gVSS^{-1}$ achieved in this pilot study.

Presence of solids in the fermented wastewater

The substrate that was fed during the pilot experiments (both to the enrichment and accumulation reactor) contained, apart from soluble COD, a fraction of solids. The concentration of solids was around 0.5 gCOD l^{-1}, compared to the total COD concentration of around 8.5 g l^{-1}. With this ratio of solids to soluble COD in the substrate it can be estimated that the PHA content in the biomass would decrease from 0.90 to 0.79 gPHA $gVSS^{-1}$ (see calculations section of this appendix). Clearly it might be advantageous to evaluate the removal of these solids from the influent before the process or after the acidification step.

Side-population

The presence of non-storing microorganisms or microorganism with a lower PHA storage capacity decreases the final PHA content of the biomass after accumulation. However, according to calculations published by Jiang et al. (2012) side-populations up to 50% have only a relatively small influence on the overall PHA content of the biomass. Microscopic analysis of FISH slides indicated that *P. acidivorans* was the dominant species in the population covering at least > 70% of the biomass. It may be noted that the fraction of *P. acidivorans* was roughly proportional to the fraction of VFA in the fermented wastewater confirming earlier observations (Jiang et al. 2012). It is unlikely that the relatively small side-population can be the sole explicatory factor of the lower PHA content in the pilot as compared to lab experiments. In case of a side-population of 30% of the mass of the microbial population, it can be estimated that the side-population may lower the PHA content from 0.90 to 0.86 gPHA $gVSS^{-1}$ (see calculations section of this appendix).

Production of other storage compounds from ethanol

Although it was previously reported that *P. acidivorans* is able to utilize ethanol (Jiang et al. 2010), it appeared that during the lab-scale accumulation experiment with ethanol as substrate (and in absence of a nitrogen source), other storage compounds with a yet unknown identity were produced besides PHA. The yield of VSS on ethanol was 0.84 gVSS $gEtOH^{-1}$ consumed while the yield of PHA was only 0.47 gPHA $gEtOH^{-1}$ suggesting a yield of the other storage compound of 0.37 g $gEtOH^{-1}$.

Since no nitrogen source was present during the accumulation experiments the production of VSS was likely related to storage compounds or extracellular polymeric substances because the synthesis of amino acids, essential for biomass production, is only possible when a nitrogen source is available. Theoretically, the C:N ratio of the biomass can be slightly variable but here it would lead to a unlikely high C:N ratio in the biomass. Moreover as discussed above the resulting side-population is too small to explain a lower biomass PHA content. The influence of the formation of non-PHA solids may be significant: considering the substrate stream fed to the reactor contained on average 0.22 gCOD $gCOD^{-1}$ ethanol, a decrease of the PHA content of the biomass from 0.90 to 0.81 gPHA $gVSS^{-1}$ can be estimated (see the calculations section of this appendix). It should be noted that the production of this unidentified storage compound could not exclusively be linked to *P. acidivorans* in this study, since other microorganisms were still present in the culture.

Calculations

The percentage of PHA was calculated as the mass of PHA per mass of VSS. In an ideal PHA-producing reactor the VSS would only comprise PHA and PHA-producing biomass (X_p). In the pilot plant, the VSS contained additional fractions of solids apart from PHA and PHA producing biomass: a side-population (X_o), other storage compounds derived from ethanol conversion (X_{SC}) and inactive biomass originating from the substrate (X_i):

$$\frac{PHA}{VSS} = \frac{PHA}{PHA + X} = \frac{PHA}{PHA + X_p + X_o + X_i + X_{SC}} \quad \text{(eq. B1)}$$

In this appendix we will provide a description of how each of these fractions can be quantified. The mass of PHA produced can be calculated if the final ratio of PHA to PHA-producing biomass is known.

$$PHA = \left(\frac{C_{PHA}}{C_{Xp}} \right) \cdot X_p \quad \text{(eq. B2)}$$

It was assumed that the PHA producing biomass (i.e. *P. acidivorans*) performed as good in the pilot as in the lab in terms of PHA to biomass ratio i.e. a maximum PHA content of 0.9 gPHA $gVSS^{-1}$. The mass of the side-population is derived using equation B3 (in this paper we used f_o = 30%).

$$X_{side} = X_p \cdot \frac{f_o}{f_{P.A}} \quad \text{(eq. B3)}$$

In order to be able to calculate the mass of other (non-PHA) storage compounds first the total amount of substrate is calculated. If the fractions of ethanol and VFA in the substrate and their PHA yields are known, the total amount of substrate (S) can be calculated using a mass balance based equation (equation B4).

$$PHA = S \cdot \left(f_{VFA} \cdot Y_{PHA/VFA} + f_{EtOH} \cdot Y_{PHA/EtOH} \right) \quad \text{(eq. B4)}$$

Substitution of eq. B2 in eq. B4 and subsequent isolation of *S* results in an explicit equation for the amount of substrate (equation B5).

$$S = \frac{\left(\frac{C_{PHA}}{C_{Xp}} \right) \cdot X_p}{\left(f_{VFA} \cdot Y_{PHA/VFA} + f_{EtOH} \cdot Y_{PHA/EtOH} \right)} \quad \text{(eq. B5)}$$

Subsequently the amount of solids from ethanol can be calculated using equation B6.

$$X_{SC} = Y_{SC/EtOH} \cdot S \cdot f_{EtOH} \quad \text{(eq. B6)}$$

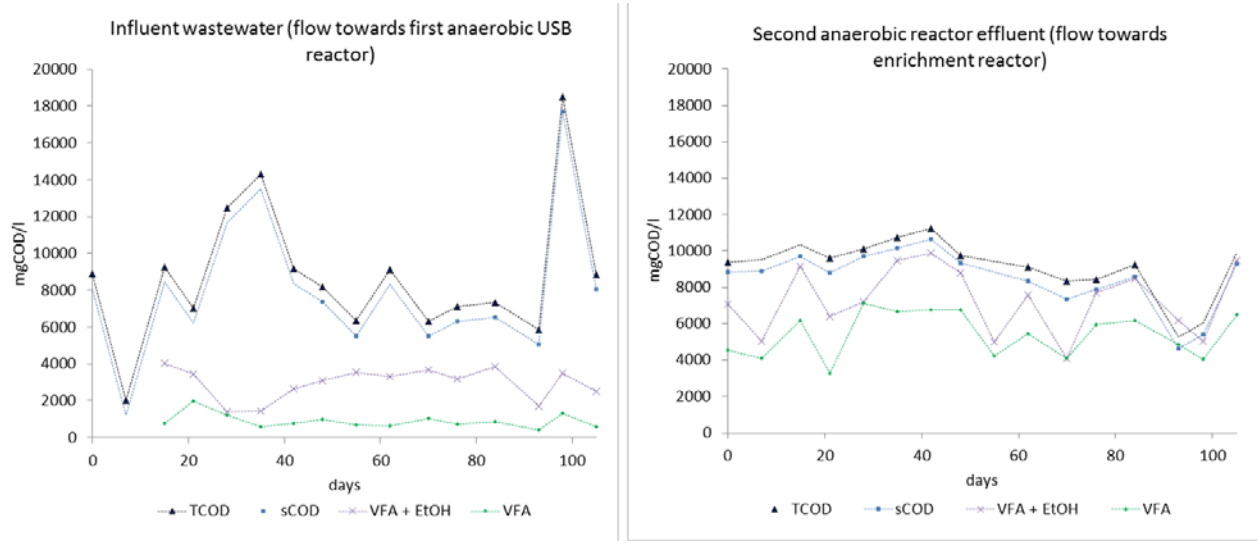
Finally, the amount of solids originating from the influent substrate can be calculated from the amount of solids per amount of substrate (f_{solids}) and the previously calculated amount of substrate (equation B7).

$$X_i = f_{solids} \cdot S \quad \text{(eq. B7)}$$

Nomenclature appendix 5A and B

C_{VFA}	concentration of volatile fatty acids [gCOD l^{-1}]
C_{VFA}^{in}	concentration of volatile fatty acids in the influent [gCOD l^{-1}]
C_{CODo}	concentration of non-VFA COD [gCOD l^{-1}]
C_{PHA}	concentration of PHA [gPHA l^{-1}]
C_{Xp}	concentration of PHA producing biomass [gX l^{-1}]
C_{Xp}^{0}	initial concentration of PHA producing biomass [gX l^{-1}]
C_{Xi}^{in}	concentration of inactive biomass in the influent [gX l^{-1}]
CODo	non-VFA COD [gCOD]
f_o	fraction of side-population of the total microbial population [gX gX^{-1}]
f_{VFA}	fraction of VFA in the substrate [gCOD $gCOD^{-1}$]
f_{EtOH}	fraction of EtOH in the substrate [gCOD $gCOD^{-1}$]
f_{solids}	fraction of solids in the substrate [gCOD $gCOD^{-1}$]
sCOD	soluble chemical oxygen demand [gCOD]
S	substrate [gCOD]
V_R	reactor liquid volume [l]
X_o	biomass growing directly on non-VFA COD [g]
X_p	PHA-producing biomass [g]
X_i	inactive biomass [g]
X_o	side-population biomass [g]
X_{SC}	non-PHA storage compounds produced from ethanol [g]
X	lumped biomass variable [g]
$Y_{PHA/VFA}$	yield of PHA on VFA [gPHA $gCOD^{-1}$]
$Y_{PHA/EtOH}$	yield of PHA on ethanol [gPHA $gCOD^{-1}$]
$Y_{X/PHA}$	yield of biomass on PHA [gX $gPHA^{-1}$]
$Y_{X/CODo}$	yield of biomass on non-VFA COD [gX $gCOD^{-1}$]
$Y_{PHA/S}$	yield of PHA on substrate [gPHA $gCOD^{-1}$]
$Y_{SC/S}$	yield of non-PHA storage compounds on substrate [g $gCOD^{-1}$]
$Y_{CO2/S}$	yield of CO_2 on substrate [mol $gCOD^{-1}$]
μ_p	biomass specific growth rate of PHA producing biomass [d^{-1}]
μ_o	biomass specific growth rate of biomass directly growing on non-VFA COD [d^{-1}]
μ^{max}	maximum growth rate [d^{-1}]
ϕ	volumetric flow [l h^{-1}]
K_{VFA}	substrate affinity constant for VFA [gCOD l^{-1}]
K_{CODo}	substrate affinity constant for non-VFA COD [gCOD l^{-1}]
k	biomass specific PHA degradation rate [$gPHA^{1/3}\,gX^{-1/3}\,h^{-1}$]
m_{VFA}	biomass specific maintenance coefficient for PHA producing biomass [gCOD $gX^{-1}\,d^{-1}$]
m_{CODo}	biomass specific maintenance coefficient for growth on non-VFA COD [gCOD $gX^{-1}\,d^{-1}$]
q_{VFA}	biomass specific uptake rate of volatile fatty acids [gCOD $gX^{-1}\,h^{-1}$]
q_{VFA}^{max}	biomass specific maximum uptake rate of VFA [gCOD $gX^{-1}\,h^{-1}$]
$q_{VFA,lumped}$	lumped biomass specific uptake rate of VFA [gCOD $gX^{-1}\,h^{-1}$]
q_{CODo}	biomass specific uptake rate of non-VFA COD [gCOD $gX^{-1}\,h^{-1}$]
q_{CODo}^{max}	biomass specific maximum uptake rate of non-VFA COD [gCOD $gX^{-1}\,h^{-1}$]
q_{EtOH}	biomass specific uptake rate of ethanol [gCOD $gX^{-1}\,h^{-1}$]
q_{EtOH}^{max}	biomass specific maximum uptake rate of ethanol [gCOD $gX^{-1}\,h^{-1}$]
q_{PHA}	biomass specific PHA production rate [gCOD $gX^{-1}\,h^{-1}$]

Appendix 5C – Influent data



6

Outlook

The objective of this thesis was the development of processes for resource recovery from wastewater with microbial enrichment cultures and to evaluate the industrial relevance of waste based PHA production. Wastewater from the Mars candy bar factory in Veghel, The Netherlands, was selected (because of its relatively high volatile fatty acid (VFA) and low nitrogen content) for pilot experiments for the production of polyhydroxyalkanoates (PHA). In this experiment, a biomass with an average PHA content of 70% was produced. Analysis of the microbial population with FISH showed that the same organism as in the laboratory, *Plasticicumulans acidivorans*, was enriched, indicating that selective conditions similar to the lab could be achieved under industrial conditions and at pilot-scale. The lower PHA content compared to laboratory experiments (70% compared to 90%) was mainly caused by the presence of organic compounds that could not be fermented to VFA in the anaerobic pre-treatment step and the presence of solids in the wastewater.

In this chapter process aspects and bottlenecks of the bioprocesses investigated in this thesis will be discussed (viz. 1: anaerobic fermentation 2: enrichment and 3: accumulation); it should be noted that this chapter is not a complete manual for process operation and design: aspects such as the influence of fluctuations in the wastewater, toxicity, biomass dewatering, product purification and utilization, are, although highly relevant, outside the scope. The two topics that were selected for discussion are the suitability of wastewater for PHA production by open cultures and aspects for process design. The first topic describes the importance of the composition of the wastewater in terms of soluble organic compounds, the ratio of chemical oxygen demand (COD) to nitrogen and concentration of solids. The second topic deals with the pre-treatment of wastewater, oxygen transfer in the feast-famine process, acid and base requirements and effluent quality. To conclude, guidelines for basic process design parameters are summarized and the chapter closes with a brief discussion on barriers and opportunities for market entry of waste-based PHA.

Waste water suitability

Composition of the soluble organics compounds in the waste water

The fraction of VFA (or compounds that can be fermented into VFA) is a prime factor for process performance of the enrichment and accumulation steps. Furthermore, the composition of the VFA mixture determines the PHB/PHV ratio of the product, which is important for the a.o. polymer properties (Albuquerque et al. 2011; Jiang et al. 2011). However, any preference for a specific PHB/PHV composition depends on the requirements of the product utilization route and is outside the scope of this thesis. Although some non-VFA substrates are reported to lead to PHA production (Moralejo-Garate, 2011), substrates that cannot be readily converted into VFA in an acidifying anaerobic fermentation may lead to a reduced final PHA content after the accumulation step. The influence of one substrate that cannot be readily converted into VFA in an acidifying anaerobic fermentation process is investigated in chapter 2, and it was observed that lipids do not contribute to the production of PHA in a feast-famine process. Another non-VFA substrate that was investigated in this thesis is ethanol, which had also a negative effect on the maximum PHA content after accumulation (chapter 5). Moreover, wastewater may contain a significant fraction of COD that cannot be readily identified. Unidentified COD that can be oxidized aerobically in the subsequent enrichment and accumulation steps will likely cause the growth a side population of microorganisms that do not produce PHA. Unidentified COD that is inert under both anaerobic and aerobic conditions will not directly cause growth of side populations, and will therefore not directly reduce the PHA content of the biomass.

COD to nitrogen ratio

Maximization of the PHA content of the biomass in the accumulation process is more effective when biomass growth is prevented, and PHA production is the only assimilative process possible. Consequently, at least one essential growth nutrient should be depleted in the accumulation process. This approach has already been proven effective for nitrogen depletion (Johnson et al. 2010). If the waste stream contains significant amounts of nitrogen compared to COD, depletion of phosphorus should be considered a preferred strategy to prevent biomass growth since, in many cases, phosphorus can easily be precipitated (i.e. by increasing the pH through aeration) while nitrogen removal can be much more problematic. It should be taken into account that small amounts of nitrogen can be removed through biomass growth in the anaerobic fermentation. In this system at least 0.01 gN $gCOD^{-1}$ fermented can be removed if the pH is not too low, i.e. pH > 4.5 (chapter 2). Waste streams with a COD:N mass ratio smaller than 100:1 have likely excess nitrogen and will require phosphate precipitation.

Solids

Solid concentrations in the substrate will decrease the PHA content of the product after accumulation if the solids are entrapped in the biomass. A simplified analysis (see appendix 5B), assuming all soluble COD is VFA and solids in the influent do not hydrolyse during the process, indicates that solids:soluble COD mass ratios above 0.05 g volatile suspended solids (VSS) $gCOD^{-1}$ already have a substantial influence, and should be avoided if possible (figure 6.1).

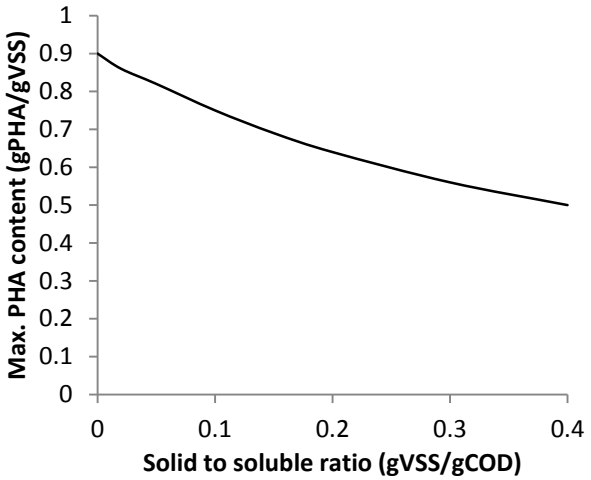


Figure 6.1. The influence of solid:soluble COD mass ratio (gVSS $gCOD^{-1}$) in the substrate on the final PHA content of the biomass after accumulation.

The above mentioned issues provide some guidelines in assessing the suitability of wastewater for PHA production. Even with suitable wastewater the PHA production process should be carefully designed. Some of the most important design aspects are discussed in the next sections.

Design aspects

Anaerobic fermentation by a granular sludge type process as pre-treatment

The goal of the anaerobic pre-treatment step is to maximize the VFA concentrations in the waste stream. The conversions in this process are performed by acidifying bacteria, which have a relatively high growth yield (in the range of 0.10 - 0.15 gCOD $gCOD^{-1}$). The solid production in the anaerobic fermentation is therewith well above the criteria discussed in the section on waste water suitability, and biomass retention systems enable separation of the solids produced in the anaerobic process from the VFA stream. There are several technologies available to achieve solid retention in reactor systems, but the application of a

granular sludge process as described in chapter 2 has two major advantages. Firstly, excess biomass can relatively easy be removed from the system by manual sludge removal, while for other technologies such as trickling filters this may prove more cumbersome. Secondly, in general, anaerobic granular sludge systems can achieve higher maximum volumetric rates since no reactor volume is occupied by carrier material.

Oxygen transfer as principal limiting factor for the productivity of any PHA producing process

One of the prerequisites for establishment of a feast-famine regime is to avoid oxygen depletion in the feast phase (Moralejo-Garate et al. 2013). The oxygen transfer rate was already identified as limiting factor for feast-famine process in earlier studies (Johnson, 2010; Jiang, 2011), and it should be noted that for any PHA producing process (that depends on aeration under standard pressure), whether it is pure culture or open culture, the oxygen transfer rate is the principal limiting factor that determines the maximum volumetric productivity. Therewith, it can be stated that in principle there is no significant difference in maximum volumetric productivity between pure culture and open culture PHA production (assuming comparable yields of product on oxygen).

The physical limit of oxygen transfer in aerated reactors is estimated around 5 g l^{-1} h^{-1}, but it should be noted that this involves expensive industrial fermentation reactors with a very high volumetric power input (Van 't Riet and Tramper, 1991, Basic bioreactor design). Processes based on open microbial cultures, such as the feast-famine process reactors are generally a factor 10-100 cheaper (typically 100 - 300 euro m^{-3} vs. > 10.000 euro m^{-3}) (Sinott, 2005). Although it is possible to apply intensive oxygen input in open culture reactors, it is likely more profitable to work with larger volumes and lower volumetric productivities (Van 't Riet, 1983).

To prevent oxygen depletion, waste water with high concentrations of COD needs to be diluted. This is because the oxygen uptake rate during the feast phase is proportional to the biomass concentration which is in turn proportional to the amount of substrate dosed per cycle (in steady state). The use of process effluent for dilution of the influent should be investigated for these cases to prevent the wastage of large amounts of freshwater. In contrast, for very dilute waste water streams, concentrations of biomass can become limiting, instead of oxygen transfer. In these cases, optimization of the volumetric productivity can be achieved by adequate biomass retention (figure 6.2).

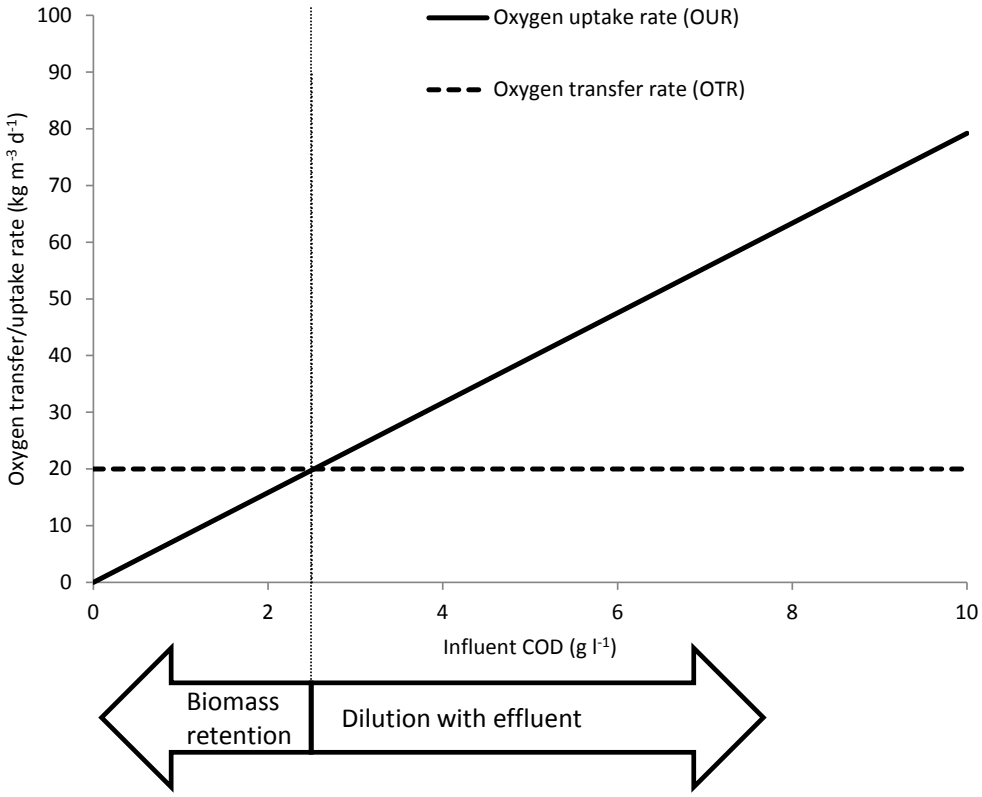


Figure 6.2. Comparison of the oxygen uptake rate (OUR) of the biomass at different influent concentrations with the supply of oxygen to the reactor (oxygen transfer rate). If the OUR is higher than the oxygen transfer rate (OTR), the substrate needs to be diluted in order to maintain aerobic conditions. Conversely, if the OUR is lower than the OTR, the biomass is limiting the volumetric productivity. In this case biomass retention offers a solution to increase the productivity. Calculations were made assuming all COD was in the form of VFA and OTR = 20 kg m^{-3} d^{-1}, $Y_{X/COD}$ = 0.33 gX $gCOD^{-1}$ and q_{O2} = 1 gO_2 gX^{-1} h^{-1}

The basic process setup that was investigated in this thesis comprised a basic setup with a sequencing batch reactor (SBR) in which the substrate was dosed pulse-wise, with consequently a much higher biomass specific oxygen uptake rate in the feast than in the famine phase. Optimization of a.o. the volumetric productivity by establishing a more equal volumetric oxygen consumption throughout the cycle should be investigated e.g. by settling the biomass after the feast phase with subsequent transportation into a separate famine phase reactor.

Acid and base requirements for pH control

The basic process setup comprised three biological processes that require operation within a pH range. For this reason, acid and base dosing may become a significant cost burden for PHA production. The factors that influence the requirement for acid and base in the three unit bioprocesses are discussed in this section.

The first process, anaerobic fermentation, produces organic acids, and in order to prevent extremely adverse conditions imposed by low pH, addition of alkalinity may be required (depending on the alkalinity already present in the influent waste water and the required operational pH in the anaerobic fermentation). In the pilot experiments in chapter 5 the amount of base used was around 2 mmol $gCOD^{-1}$.

The second process is the enrichment of PHA producing bacteria, for which the influence of operational pH on the quality of the enrichment remains unclear, but for which, until now, the best results were reported for laboratory experiments operated at pH 7. While during the feast phase addition of acids

and/or base chemicals may be prevented by dosing the acidic substrate by pH control (see pilot chapter), the dosing of acid and base may be required in the remainder of the cycle. During this period the pH is influenced by a variety of factors: (1) the alkalinity originating from the anaerobic fermentation step may cause a significant increase in pH when the organic acids get depleted. (2) The oxidation of organic substrates will lead to the production of CO_2. Around neutral pH most CO_2 will hydrate to H_2CO_3 and dissociate to HCO_3^- and H^+, leading to a decrease in pH. Subsequently, with a rate depending on the intensity of the aeration, HCO_3^- concentrations decrease again due to CO_2 stripping, accompanied by an increase in pH. (3) Depending on the nutrient source, nutrient uptake will affect the pH as well. For example, in the pilot experiments in this study, urea was dosed as nitrogen source: this caused an initial increase in pH, due to urea hydrolysis to ammonium, and a subsequent decrease in pH, related to ammonium uptake by the micro-organisms. In the pilot experiments in chapter 5, the sum of acid and base usage was around 4 mmol $gCOD^{-1}$. Importantly, in many cases it is required to dilute the fermented wastewater (to prevent oxygen depletion during the feast phase). In these cases the process effluent should be used as dilution water, to minimize the wastage of fresh water (figure 6.2). Alternatively, the process effluent water can already be used to dilute the influent stream of the anaerobic fermentation instead of the enrichment reactor. This way alkalinity may be recycled, which will reduce the amount of base required for pH control in the anaerobic fermentation.

The last biological process studied in this thesis is the accumulation. In this step, substrate may be dosed on the basis of pH control with no additional acid and base dosing required. Depending on the alkalinity of the (pre-treated) waste water and the operational protocol, acid dosing may be required to prevent high concentrations of substrate during the accumulation.

Effluent quality

The waste-based PHA production process is an alternative for conventional wastewater treatment. The resource recovery aspect of this technology sometimes distracts the attention from the main deliverable of the process: clean water. The effluent from the last step of the upstream part of the process can be separated from the biomass by settling. Although most of the COD is oxidized during the process, effluent COD concentrations may still be above the levels required for standard waste water treatment practices. For example, in the pilot study, still 1 - 2 g l^{-1} of COD (but no significant concentrations of nitrogen) was present in the effluent. This system was operated to achieve maximum PHA content in the biomass and not for minimizing COD concentrations in the effluent. Further optimization may reduce COD levels below legally required discharge levels c.q. additional aeration may easily remove residual COD.

Overview of process design aspects

To summarize the design aspects discussed in this thesis, typical values for the most important process parameters are summarized in table 6.1. These values are intended to provide guidelines for basic process design. For design of a full-scale product chain, additional parameters will be required (i.e. operational and capital costs and detailed PHA quality aspects such as M_W and melting temperature). For each unit operation the performance can be evaluated according to the product quality criteria presented in table 6.1. It should be noted that for each unit operation the basic quality criteria are related: i.e. the fraction of VFA in the fermented wastewater determines a.o. the quality of the enrichment, which in turn determines the final PHA content in biomass after accumulation.

Table 6.1. Overview of the process performance criteria and guidelines for basic design parameters (f_{VFA}: fraction of VFA on total COD, f_{PHA}^{max}: maximum PHA content).

	Fermentation	Enrichment	Accumulation	
Product	VFA	Biomass	PHA	
Quality criteria	f_{VFA}	f_{PHA}^{max}	f_{PHA}^{max*}	gCOD gCOD^{-1}
Product yield	0.6-0.9	0.5	0.7	gCOD gCOD^{-1}
Volumetric rate	200	20	20	gCOD l^{-1} d^{-1}
Acid/base dose**	0-2	0-4	0	mmol gCOD^{-1}
Nutrient dose	0-0.003	0.04	0	gN gCOD^{-1}

*for the accumulation the effluent quality is also an important process performance criterion.

** assuming a total of 6 mmol acid and base consumption at a cost of 0.01 euro mol^{-1} and an overall process yield of 0.18 gPHA gCOD^{-1} (chapter 5), the costs of acid and base dosing will be around 0.20 euro kgPHA^{-1} for the basic process setup.

Bottlenecks and market potential for waste-based PHA production

Waste-based PHA can be a resource for the production of bioplastic and platform chemicals. The production of **platform chemicals** from PHA is still in research stage, and will not be considered in this section. The production of **bioplastics** from PHA is already further developed, with (sugar-based) PHA currently entering commercial stage (Shen et al. 2009). Additionally, first estimates of the process costs of waste-based PHA have been reported by Gurieff and Lant (2006). Therefore, this section will mainly focus on barriers and opportunities for waste-based PHA in the plastic market.

Plastics are a growing market with an annual growth of around 4-6% and a volume of roughly 300 MT per year (PlasticsEurope Market Research Group, 2014). The lion's share of the plastic market are polyolefins i.e. polyethylene (PE) and polypropylene (PP) and it has been reported that these can technically (at least for a significant number of applications) be replaced by PHA (Shen et al. 2009). The price at which bioplastics become truly competitive with fossil-based plastics is equal to the price of PP (and PE) and is currently estimated around 1.5 \$ kg^{-1} (2013 - 2014). This price level depends to some extent on the price of crude oil, but additional processing costs add around 1.3 \$ kg^{-1} to the product's value (figure 6.3).

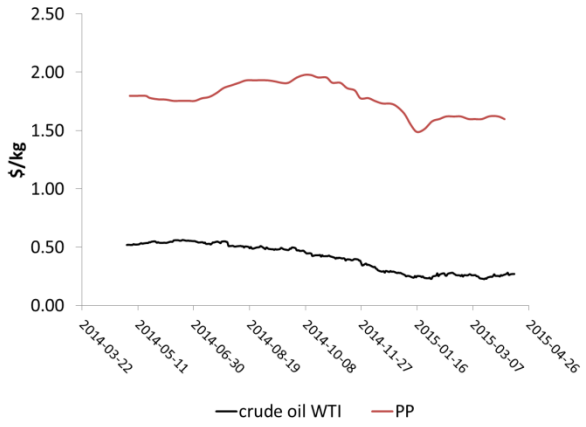


Figure 6.3. Variation of the selling price of crude oil (WTI, US. Energy Information Administration), and PP thermoplastics (injection grade, spot price, FOB Houston, The Plastic Exchange LLC).

Taking into account the relatively low price of fossil-based plastics, a more promising market entry may be achieved in the smaller, but still substantial niche market for biodegradable plastics, where the product can be sold at a premium (a higher price, estimated around 2 - 3 \$ kg^{-1}). Bioplastics in general (i.e. plastics with

biodegradable or bio-based properties or both) are an emerging market that grows even faster than the fast growing plastic market, and current market volumes for biodegradable plastics are estimated at 1 MT per year (Crank and Patel, 2005; Shen et al. 2009). It should be noted however that PHA still has a very small market share within the bioplastic market (a few kT) due to its relatively high price. The reason for this high price is that the types of PHA currently on the market are produced by pure culture processes, that require large capital and operational expenditures. The contribution of sugar-based feedstock to the PHA production costs, is estimated to be substantial with at least 2 kg of sugar (or equivalent) required to produce 1 kg of PHA. Moreover, the costs of sterilization of equipment are added to this burden. In contrast, waste-based PHA production processes do not require either expensive substrate or sterilization. Until recently, waste-based processes were never commercialized because of the lower PHA content of the biomass compared to pure cultures. Van Wegen et al. (1998) estimated that the percentage of PHA in biomass is one of the most important economic factors determining the feasibility of PHA production. Recently, a paradigm shift is occurring, with lab-scale studies showing that waste-based processes can achieve similar PHA contents as in pure culture processes (Johnson et al. 2009; Jiang et al., 2011). It should be noted however, that until now, at pilot-scale the PHA content is still slightly lower, stressing the importance of further optimization of process up-scaling. Nevertheless, assuming that further development will enable the production of biomass with high PHA content at pilot-scale, the waste-based PHA production process acquires a three-fold advantage over current (sugar-based) industrial practices:

1. Benefits because wastewater is cleaned
2. No substrate costs
3. Lower operational costs (no sterilization required, no pre-cultivation required, semi-continuous process)

There are still many aspects in the product chain that require optimization, to enable efficient full-scale implementation of waste-based PHA technology, in particular further development of the downstream processing and product utilization steps. For this, polymer and chemical engineers have to collaborate with down-stream processing engineers to develop a marketable product from the PHA-containing sludge produced in the upstream process. Furthermore, aspects specific to waste-based PHA technology must be considered; for example, it may be interesting to develop methods that can handle a slight variation in polymer properties (like PHB/PHV ratio) that may be caused by variations inherent to waste streams. Moreover, health, legislation and marketing aspects related to this waste derived product should be further investigated.

Nevertheless, in this study the first steps are taken to show the industrial relevance of waste-based PHA technology, with the production of biomass with a high PHA content at pilot-scale, under industrial conditions. In addition, several other aspects related to this process were investigated (VFA production, the fate of lipids in the process and process modelling). Although a detailed techno-economic analysis of the complete value chain is required for evaluation of the feasibility of the process, the results presented in this study suggest that the upstream part of the waste-based PHA production process is reasonably developed. In contrast, the downstream processing (DSP) and product utilization still have an undefined, but probably substantial impact on the overall process economics, and are therewith identified as important bottlenecks for industrial implementation. We expect that the development of innovative DSP-product utilization combinations will enable large-scale market entry of waste-based PHA production in the near future.

References

Agler, M.T., Spirito, C.M., Usack, J.G., Werner, J.J. and Angenent, L.T. (2012a) Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates. Energy & Environmental Science 5(8), 8189-8192.

Agler, M.T., Werner, J.J., Iten, L.B., Dekker, A., Cotta, M.A., Dien, B.S. and Angenent, L.T. (2012b) Shaping reactor microbiomes to produce the fuel precursor n-butyrate from pretreated cellulosic hydrolysates. Environmental Science & Technology 46(18), 10229-10238.

Agler, M.T., Wrenn, B.A., Zinder, S.H. and Angenent, L.T. (2011) Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends in Biotechnology 29(2), 70-78.

Albuquerque, M.G.E., Carvalho, G., Kragelund, C., Silva, A.F., Barreto Crespo, M.T., Reis, M.A.M. and Nielsen, P.H. (2013) Link between microbial composition and carbon substrate-uptake preferences in a PHA-storing community. Isme Journal 7(1), 1-12.

Albuquerque, M.G.E., Concas, S., Bengtsson, S. and Reis, M.A.M. (2010a) Mixed culture polyhydroxyalkanoates production from sugar molasses: The use of a 2-stage CSTR system for culture selection. Bioresource Technology 101(18), 7112-7122.

Albuquerque, M.G.E., Eiroa, M., Torres, C., Nunes, B.R. and Reis, M.A.M. (2007) Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. Journal of Biotechnology 130(4), 411-421.

Albuquerque, M.G.E., Martino, V., Pollet, E., Avérous, L. and Reis, M.A.M. (2011) Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties. Journal of Biotechnology 151(1), 66-76.

Albuquerque, M.G.E., Torres, C.A.V. and Reis, M.A.M. (2010b) Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: effect of the influent substrate concentration on culture selection. Water Research 44(11), 3419-3433.

Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A. and Raskin, L. (1996) The oligonucleotide probe database. Applied and Environmental Microbiology 62(10), 3557.

Alphenaar, P. (1994) Anaerobic granular sludge: Characterization and factors affecting its performance, Ph. D. Thesis, Wageningen Agricultural University, The Netherlands.

Alvarez, H.A. and Steinbüchel, A.S. (2002) Triacylglycerols in prokaryotic microorganisms. Applied Microbiology and Biotechnology 60(4), 367-376.

Anderson, A.J. and Dawes, E.A. (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological Reviews 54(4), 450-472.

Anderson, A.J., Haywood, G.W. and Dawes, E.A. (1990) Biosynthesis and composition of bacterial poly(hydroxyalkanoates). International Journal of Biological Macromolecules 12(2), 102-105.

Andreasen, K. and Nielsen, P.H. (2000) Growth of *Microthrix parvicella* in nutrient removal activated sludge plants: studies of *in situ* physiology. Water Research 34(5), 1559-1569.

Angenent, L.T., Karim, K., Al-Dahhan, M.H., Wrenn, B.A. and Domíguez-Espinosa, R. (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends in Biotechnology 22(9), 477-485.

Anterrieu, S., Quadri, L., Geurkink, B., Dinkla, I., Bengtsson, S., Arcos-Hernandez, M., Alexandersson, T., Morgan-Sagastume, F., Karlsson, A. and Hjort, M. (2014) Integration of biopolymer production with process water treatment at a sugar factory. New Biotechnology 31(4), 308-323.

Arcos-Hernandez, M., Pratt, S., Laycock, B., Johansson, P., Werker, A. and Lant, P. (2013) Waste activated sludge as biomass for production of commercial-grade polyhydroxyalkanoate (PHA). Waste and Biomass Valorization 4(1), 117-127.

Arenskötter, M., Bröker, D. and Steinbüchel, A. (2004) Biology of the metabolically diverse genus *Gordonia*. Applied and Environmental Microbiology 70(6), 3195-3204.

Babayan, V.K. (1987) Medium chain triglycerides and structured lipids. Lipids 22(6), 417-420.

Beccari, M., Dionisi, D., Giuliani, A., Majone, M. and Ramadori, R. (2002) Effect of different carbon sources on aerobic storage by activated sludge. Water Science and Technology 45(6), 157-168.

Beeftink, H. and Van den Heuvel, J. (1987) Novel anaerobic gas-lift reactor (AGLR) with retention of biomass: Start-up routine and establishment of hold up. Biotechnology and Bioengineering 30(2), 233-238.

Bengtsson, S., Pisco, A.R., Reis, M.A.M. and Lemos, P.C. (2010) Production of polyhydroxyalkanoates from fermented sugar cane molasses by a mixed culture enriched in glycogen accumulating organisms. Journal of Biotechnology 145(3), 253-263.

Bengtsson, S., Werker, A., Christensson, M. and Welander, T. (2008) Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. Bioresource Technology 99(3), 509-516.

Beun, J.J., Dircks, K., Van Loosdrecht, M.C.M. and Heijnen, J.J. (2002) Poly-beta-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. Water Research 36(5), 1167-1180.

Beun, J.J., Paletta, F., Van Loosdrecht, M.C.M. and Heijnen, J.J. (2000) Stoichiometry and kinetics of poly-beta-hydroxybutyrate metabolism in aerobic, slow growing, activated sludge cultures. Biotechnology and Bioengineering 67(4), 379-389.

Borja, R. and Banks, C. (1994) Anaerobic digestion of palm oil mill effluent using an up-flow anaerobic sludge blanket reactor. Biomass and Bioenergy 6(5), 381-389.

Brammer, J. and Bridgwater, A. (2002) The influence of feedstock drying on the performance and economics of a biomass gasifier–engine CHP system. Biomass and Bioenergy 22(4), 271-281.

Brown, J.H., Burger, J.R., Burnside, W.R., Chang, M., Davidson, A.D., Fristoe, T.S., Hamilton, M.J., Hammond, S.T., Kodric-Brown, A. and Mercado-Silva, N. (2014) Macroecology meets macroeconomics: Resource scarcity and global sustainability. Ecological engineering 65, 24-32.

Carlson, R., Wlaschin, A. and Srienc, F. (2005) Kinetic studies and biochemical pathway analysis of anaerobic poly-(R)-3-hydroxybutyric acid synthesis in *Escherichia coli*. Applied and Environmental Microbiology 71(2), 713-720.

Carta, F., Beun, J.J., Van Loosdrecht, M.C.M. and Heijnen, J.J. (2001) Simultaneous storage and degradation of PHB and glycogen in activated sludge cultures. Water Research 35(11), 2693-2701.

Chakravarty, P., Mhaisalkar, V. and Chakrabarti, T. (2010) Study on poly-hydroxyalkanoate (PHA) production in pilot scale continuous mode wastewater treatment system. Bioresource Technology 101(8), 2896-2899.

Chen, G.Q. (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chemical Society Reviews 38(8), 2434-2446.

Chipasa, K. and Mędrzycka, K. (2006) Behavior of lipids in biological wastewater treatment processes. Journal of industrial Microbiology and Biotechnology 33(8), 635-645.

Choi, J. and Lee, S.Y. (1999) Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Applied Microbiology and Biotechnology 51(1), 13-21.

Ciggin, A.S., Insel, G., Majone, M. and Orhon, D. (2013) Model evaluation of starch utilization by acclimated biomass with different culture history under pulse and continuous feeding. Bioresource Technology 138(0), 163-171.

Ciggin, A.S., Majone, M. and Orhon, D. (2012) Intracellular storage of acetate/starch mixture by fast growing microbial culture in sequencing batch reactor under continuous feeding. Bioresource Technology 119(0), 331-338.

Cirne, D.G., Paloumet, X., Björnsson, L., Alves, M.M. and Mattiasson, B. (2007) Anaerobic digestion of lipid-rich waste—Effects of lipid concentration. Renewable Energy 32(6), 965-975.

Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. (1999) Standard methods for the examination of water and wastewater, American Public Health Association.

Crank, M. and Patel, M. (2005) Techno-Economic Feasibility of Largescale Production of Bio-Based Polymers in Europe. European Commission.

Dabrock, B., Bahl, H. and Gottschalk, G. (1992) Parameters affecting solvent production by *Clostridium pasteurianum*. Applied and Environmental Microbiology 58(4), 1233-1239.

de Kok, S., Meijer, J., van Loosdrecht, M.M. and Kleerebezem, R. (2013) Impact of dissolved hydrogen partial pressure on mixed culture fermentations. Applied Microbiology and Biotechnology 97(6), 2617-2625.

de Kreuk, M.K. and van Loosdrecht, M.C.M. (2006) Formation of aerobic granules with domestic sewage. Journal of Environmental Engineering-Asce 132(6), 694-697.

Dias, J.M.L., Oehmen, A., Serafim, L.S., Lemos, P.C., Reis, M.A.M. and Oliveira, R. (2008) Metabolic modelling of polyhydroxyalkanoate copolymers production by mixed microbial cultures. Bmc Systems Biology 2.

Dias, J.M.L., Serafim, L.S., Lemos, P.C., Reis, M.A.M. and Oliveira, R. (2005) Mathematical modelling of a mixed culture cultivation process for the production of polyhydroxybutyrate. Biotechnology and Bioengineering 92(2), 209-222.

Dionisi, D., Majone, M., Papa, V. and Beccari, M. (2004) Biodegradable polymers from organic acids by using activated sludge enriched by aerobic periodic feeding. Biotechnology and Bioengineering 85(6), 569-579.

Dionisi, D., Majone, M., Vallini, G., Di Gregorio, S. and Beccari, M. (2007) Effect of the length of the cycle on biodegradable polymer production and microbial community selection in a sequencing batch reactor. Biotechnology Progress 23, 1064-1073.

Dircks, K., Beun, J.J., van Loosdrecht, M., Heijnen, J.J. and Henze, M. (2001) Glycogen metabolism in aerobic mixed cultures. Biotechnology and Bioengineering 73(2), 85-94.

Durner, R., Witholt, B. and Egli, T. (2000) Accumulation of poly[(R)-3-hydroxyalkanoates] in *Pseudomonas oleovorans* during growth with octanoate in continuous culture at different dilution rates. Applied and Environmental Microbiology 66(8), 3408-3414.

Eberl, L., Givskov, M., Sternberg, C., Møller, S., Christiansen, G. and Molin, S. (1996) Physiological responses of *Pseudomonas putida* KT2442 to phosphate starvation. Microbiology 142(1), 155-163.

Eggink, G., van der Wal, H., Huijberts, G.N. and de Waard, P. (1992) Oleic acid as a substrate for poly-3-hydroxyalkanoate formation in *Alcaligenes eutrophus* and *Pseudomonas putida*. Industrial crops and products 1(2), 157-163.

Fang, H.H. and Liu, H. (2002) Effect of pH on hydrogen production from glucose by a mixed culture. Bioresource Technology 82(1), 87-93.

FAO (1992) Technology of production of edible flours and protein products from soybeans. FAO agricultural services bulletin no. 97.

FAO (2014) FAOSTAT, Food & Agriculture Organization of the United Nations, Rome, Italy.

Fukuzaki, S., Nishio, N., Shobayashi, M. and Nagai, S. (1990) Inhibition of the fermentation of propionate to methane by hydrogen, acetate, and propionate. Applied and Environmental Microbiology 56(3), 719-723.

Gouda, M.K., Omar, S.H. and Aouad, L.M. (2008) Single cell oil production by Gordonia sp. DG using agro-industrial wastes. World Journal of Microbiology and Biotechnology 24(9), 1703-1711.

Grootscholten, T., Strik, D., Steinbusch, K., Buisman, C. and Hamelers, H. (2014) Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol. Applied Energy 116, 223-229.

Grootscholten, T.I.M., Kinsky dal Borgo, F., Hamelers, H.V.M. and Buisman, C.J.N. (2013) Promoting chain elongation in mixed culture acidification reactors by addition of ethanol. Biomass and Bioenergy 48(0), 10-16.

Gujer, W., Henze, M., Mino, T. and Loosdrecht, M.v. (1999) Activated sludge model No. 3. Water Science and Technology 39(1), 183-193.

Gurieff, N. and Lant, P. (2007) Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production. Bioresource Technology 98(17), 3393-3403.

Heijnen, J. (2010) Biosystems Engineering II, pp. 139-162, Springer.

Heijnen, J.J., Kleerebezem, R. and Flickinger, M.C. (2009) Encyclopedia of Industrial Biotechnology, John Wiley & Sons, Inc.

Heinimö, J. and Junginger, M. (2009) Production and trading of biomass for energy–an overview of the global status. Biomass and Bioenergy 33(9), 1310-1320.

Hermann, W.A. (2006) Quantifying global exergy resources. Energy 31(12), 1685-1702.

Herrema, M., Kimmel, K. and Creelman, E. (2010) Process for the production of polyhydroxyalkanoates, Google Patents.

Hobson, P. and Nasr, H. (1951) An amylopectin-type polysaccharide synthesised from sucrose by *Clostridium. butyricum*. J. Chem. Soc., 1855-1857.

Hoelzle, R.D., Virdis, B. and Batstone, D.J. (2014) Regulation mechanisms in mixed and pure culture microbial fermentation. Biotechnology and Bioengineering 111(11), 2139-2154.

Holdsworth, J.E. and Ratledge, C. (1988) Lipid turnover in oleaginous yeasts. Journal of general microbiology 134(2), 339-346.

Holtzapple, M.T. and Granda, C.B. (2009) Carboxylate platform: the MixAlco process part 1: comparison of three biomass conversion platforms. Applied biochemistry and biotechnology 156(1-3), 95-106.

Hoogwijk, M., Faaij, A., van den Broek, R., Berndes, G., Gielen, D. and Turkenburg, W. (2003) Exploration of the ranges of the global potential of biomass for energy. Biomass and Bioenergy 25(2), 119-133.

Horiuchi, J.-I., Shimizu, T., Tada, K., Kanno, T. and Kobayashi, M. (2002) Selective production of organic acids in anaerobic acid reactor by pH control. Bioresource Technology 82(3), 209-213.

Huijberts, G. and Eggink, G. (1996) Production of poly (3-hydroxyalkanoates) by Pseudomonas putida KT2442 in continuous cultures. Applied Microbiology and Biotechnology 46(3), 233-239.

Hunik, J.H., Bos, C.G., van den Hoogen, M.P., De Gooijer, C.D. and Tramper, J. (1994) Co-immobilized *Nitrosomonas europaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in κ-carrageenan gel beads. Biotechnology and Bioengineering 43(11), 1153-1163.

IEA. (2011) World energy outlook 2011, IEA.

Igwe, J. and Onyegbado, C. (2007) A review of palm oil mill effluent (POME) water treatment. Global Journal of Environmental Research 1(2), 54-62.

Jenkins, B., Baxter, L. and Miles, T. (1998) Combustion properties of biomass. Fuel processing technology 54(1), 17-46.

Jiang, Y. (2011) Polyhydroxyalkanoates production by bacterial enrichments, TU Delft, Delft University of Technology.

Jiang, Y., Hebly, M., Kleerebezem, R., Muyzer, G. and van Loosdrecht, M.C.M. (2011a) Metabolic modeling of mixed substrate uptake for polyhydroxyalkanoate (PHA) production. Water Research 45(3), 1309-1321.

Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G. and van Loosdrecht, M.C.M. (2011b) Effect of temperature and cycle length on microbial competition in PHB-producing sequencing batch reactor. International Society for Microbial Ecology 5, 896-907.

Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G. and van Loosdrecht, M.C.M. (2011c) Polyhydroxybutyrate production from lactate using a mixed microbial culture. Biotechnology and Bioengineering 108(9), 2022-2035.

Jiang, Y., Marang, L., Tamis, J., van Loosdrecht, M.C.M., Dijkman, H. and Kleerebezem, R. (2012) Waste to resource: Converting paper mill wastewater to bioplastic. Water Research 46(17), 5517-5530.

Jiang, Y., Sorokin, D., Kleerebezem, R., Muyzer, G. and van Loosdrecht, M.C.M. (2010) *Plasticicumulans acidivorans* gen. nov., sp. nov., a polyhydroxyalkanoate-accumulating gammaproteobacterium from a sequencing-batch bioreactor. International Journal of Systematic and Evolutionary Microbiology doi:ijs.0.021410-0.

Johnson, K. (2010) PHA production in aerobic mixed microbial cultures, TU Delft, Delft University of Technology.

Johnson, K., Jiang, Y., Kleerebezem, R., Muyzer, G. and van Loosdrecht, M.C. (2009a) Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromolecules 10(4), 670-676.

Johnson, K., Kleerebezem, R. and van Loosdrecht, M. (2010a) Influence of ammonium on the accumulation of polyhydroxybutyrate (PHB) in aerobic open mixed cultures. Journal of Biotechnology 147(2), 73-79.

Johnson, K., Kleerebezem, R. and van Loosdrecht, M.C.M. (2009b) Model-based data evaluation of polyhydroxybutyrate producing mixed microbial cultures in aerobic sequencing batch and fed-batch reactors. Biotechnology and Bioengineering 104(1), 50-67.

Johnson, K., Kleerebezem, R. and van Loosdrecht, M.C.M. (2010b) Influence of ammonium on the accumulation of polyhydroxybutyrate (PHB) in aerobic open mixed cultures. Journal of Biotechnology 147(2), 73-79.

Johnson, K., van Geest, J., Kleerebezem, R. and van Loosdrecht, M. (2010c) Short-and long-term temperature effects on aerobic polyhydroxybutyrate producing mixed cultures. Water Research 44(6), 1689-1700.

Juni, E. (1978) Genetics and physiology of *Acinetobacter*. Annual Reviews in Microbiology 32(1), 349-371.

Karahan, Ö., van Loosdrecht, M.C.M. and Orhon, D. (2006) Modeling the utilization of starch by activated sludge for simultaneous substrate storage and microbial growth. Biotechnology and Bioengineering 94(1), 43-53.

Kemp, R. (2007) Innovations Towards Sustainability, pp. 87-94, Springer.

Klass, D.L. (1984) Methane from Anaerobic Fermentation. Science 223(4640), 1021-1028.

Kleerebezem, R., Rodriguez, J., Temudo, M.F. and van Loosdrecht, M.C. (2008) Modeling mixed culture fermentations; the role of different electron carriers. Water Sci Technol 57(4), 493-497.

Kleerebezem, R. and Van Loosdrecht, M.C. (2010) A generalized method for thermodynamic state analysis of environmental systems. Critical Reviews in Environmental Science and Technology 40(1), 1-54.

Kleerebezem, R. and van Loosdrecht, M.C.M. (2007) Mixed culture biotechnology for bioenergy production. Current Opinion in Biotechnology 18(3), 207-212.

Krishna, C. and van Loosdrecht, M.C.M. (1999) Effect of temperature on storage polymers and settleability of activated sludge. Water Research 33(10), 2374-2382.

Lageveen, R.G., Huisman, G.W., Preusting, H., Ketelaar, P., Eggink, G. and Witholt, B. (1988) Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Applied and Environmental Microbiology 54(12), 2924-2932.

Lal, R. (2005) World crop residues production and implications of its use as a biofuel. Environment International 31(4), 575-584.

Lanham, A.B., Ricardo, A.R., Coma, M., Fradinho, J., Carvalheira, M., Oehmen, A., Carvalho, G. and Reis, M.a.M. (2012) Optimisation of glycogen quantification in mixed microbial cultures. Bioresource Technology 118, 518-525.

Lavallee, B., Frigon, D., Lessard, P., Vanrolleghem, P.A., Yuan, Z. and van Loosdrecht, M.C.M. (2009) Modelling using rRNA-structured biomass models. Water Science and Technology 59(4), 661-671.

Lee, S.H., Kim, J.H., Mishra, D., Ni, Y.-Y. and Rhee, Y.H. (2011) Production of medium-chain-length polyhydroxyalkanoates by activated sludge enriched under periodic feeding with nonanoic acid. Bioresource Technology 102(10), 6159-6166.

Lee, S.H., Oh, D.H., Ahn, W.S., Lee, Y., Choi, J.i. and Lee, S.Y. (2000) Production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) by high-cell-density cultivation of *Aeromonas hydrophila*. Biotechnology and Bioengineering 67(2), 240-244.

Lemoigne, M. (1926) Products of dehydration and of polymerization of β-hydroxybutyric acid. Bull. Soc. Chem. Biol 8, 770-782.

Lemos, P.C., Serafim, L.S. and Reis, M.A.M. (2006) Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding. Journal of Biotechnology 122(2), 226-238.

Lettinga, G., Vanvelsen, A.F.M., Hobma, S.W., Dezeeuw, W. and Klapwijk, A. (1980) Use of the Upflow Sludge Blanket (Usb) Reactor Concept for Biological Wastewater-Treatment, Especially for Anaerobic Treatment. Biotechnology and Bioengineering 22(4), 699-734.

Liang, M.-H. and Jiang, J.-G. (2013) Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. Progress in Lipid Research 52(4), 395-408.

Ling, J., Nip, S. and Shim, H. (2013) Enhancement of lipid productivity of *Rhodosporidium toruloides* in distillery wastewater by increasing cell density. Bioresource Technology 146, 301-309.

Liu, H.-Y., Hall, P.V., Darby, J.L., Coats, E.R., Green, P.G., Thompson, D.E. and Loge, F.J. (2008) Production of polyhydroxyalkanoate during treatment of tomato cannery wastewater. Water Environment Research 80(4), 367-372.

Lopez-Vazquez, C.M., Oehmen, A., Hooijmans, C.M., Brdjanovic, D., Gijzen, H.J., Yuan, Z.G. and van Loosdrecht, M.C.M. (2009) Modeling the PAO-GAO competition: effects of carbon source, pH and temperature. Water Research 43(2), 450-462.

Magan, N. (2007) 6 Fungi in Extreme Environments. Environmental and Microbial Relationships: Environmental and microbial relationships. IV 4, 85.

Marang, L., Jiang, Y., van Loosdrecht, M. and Kleerebezem, R. (2014) Impact of non-storing biomass on PHA production: An enrichment culture on acetate and methanol. International Journal of Biological Macromolecules.

Marang, L., Jiang, Y., van Loosdrecht, M.C.M. and Kleerebezem, R. (2013) Butyrate as preferred substrate for polyhydroxybutyrate production. Bioresource Technology.

McCarty, P.L. and Smith, D.P. (1986) Anaerobic wastewater treatment. Environmental Science & Technology 20(12), 1200-1206.

Membré, J.M., Tholozan, J.L., Delattre, G., Eulalie, B. and Albagnac, G. (1994) Volatile fatty acid production during beer spoilage by *Pectinatus* sp. Food Quality and Preference 5(1-2), 25-29.

Mondala, A., Liang, K., Toghiani, H., Hernandez, R. and French, T. (2009) Biodiesel production by *in situ* transesterification of municipal primary and secondary sludges. Bioresource Technology 100(3), 1203-1210.

Mooij, P.R., Stouten, G.R., Tamis, J., van Loosdrecht, M.C. and Kleerebezem, R. (2013) Survival of the fattest. Energy Environ. Sci. 6(12), 3404-3406.

Mooij, P.R., Stouten, G.R., van Loosdrecht, M.C.M. and Kleerebezem, R. (2015) Ecology-based selective environments as solution to contamination in microalgal cultivation. Current Opinion in Biotechnology 33(0), 46-51.

Moralejo-Gárate, H., Kleerebezem, R., Mosquera-Corral, A. and van Loosdrecht, M.C.M. (2013) Impact of oxygen limitation on glycerol-based biopolymer production by bacterial enrichments. Water Research 47(3), 1209-1217.

Moralejo-Gárate, H., Mar'atusalihat, E., Kleerebezem, R. and Loosdrecht, M.M. (2011) Microbial community engineering for biopolymer production from glycerol. Applied Microbiology and Biotechnology 92(3), 631-639.

Morgan-Sagastume, F., Valentino, F., Hjort, M., Cirne, D., Karabegovic, L., Gerardin, F., Johansson, P., Karlsson, A., Magnusson, P. and Alexandersson, T. (2013) Polyhydroxyalkanoate (PHA) production from sludge and municipal wastewater treatment. Water Science & Technology Vol 69 (1), 177–184

Muller, E.E., Sheik, A.R. and Wilmes, P. (2014) Lipid-based biofuel production from wastewater. Current Opinion in Biotechnology 30, 9-16.

Murnleitner, E., Kuba, T., vanLoosdrecht, M.C.M. and Heijnen, J.J. (1997) An integrated metabolic model for the aerobic and denitrifying biological phosphorus removal. Biotechnology and Bioengineering 54(5), 434-450.

Muyzer, G. (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Current opinion in microbiology 2(3), 317-322.

Ng, K.-S., Ooi, W.-Y., Goh, L.-K., Shenbagarathai, R. and Sudesh, K. (2010) Evaluation of jatropha oil to produce poly (3-hydroxybutyrate) by *Cupriavidus necator* H16. Polymer Degradation and Stability 95(8), 1365-1369.

Nicolella, C., van Loosdrecht, M. and Heijnen, S.J. (2000) Particle-based biofilm reactor technology. Trends in Biotechnology 18(7), 312-320.

Oehmen, A., Marques, R., Noronha, J.P., Carvalho, G. and Reis, M.A.M. (2013) Propionate addition enhances the biodegradation of the xenobiotic herbicide propanil and its metabolite. Bioresource Technology 127(0), 195-201.

Oehmen, A., Zeng, R.J., Keller, J. and Yuan, Z.G. (2007) Modeling the aerobic metabolism of polyphosphate-accumulating organisms enriched with propionate as a carbon source. Water Environment Research 79(13), 2477-2486.

Pardelha, F., Albuquerque, M.G.E., Reis, M.A.M., Dias, J.M.L. and Oliveira, R. (2012) Flux balance analysis of mixed microbial cultures: Application to the production of polyhydroxyalkanoates from complex mixtures of volatile fatty acids. Journal of Biotechnology 162(2–3), 336-345.

Pardelha, F., Albuquerque, M.G.E., Reis, M.A.M., Oliveira, R. and Dias, J.M.L. (2010) Metabolic modeling of polyhydroxyalkanoates (PHA) production from complex mixtures of competing volatile fatty acids (VFA) by mixed microbial cultures (MMC). Journal of Biotechnology 150, Supplement(0), 565.

Pardelha, F., Albuquerque, M.G.E., Reis, M.A.M., Oliveira, R. and Dias, J.M.L. (2013) Dynamic metabolic modelling of volatile fatty acids conversion to polyhydroxyalkanoates by a mixed microbial culture. New Biotechnology.

Peng, W.-f., Huang, C., Chen, X.-f., Xiong, L., Chen, Y. and Ma, L.-l. (2013) Microbial conversion of wastewater from butanol fermentation to microbial oil by oleaginous yeast *Trichosporon dermatis*. Renewable Energy 55, 31-34.

Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R. and Van Loosdrecht, M.C.M. (2003) Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess and Biosystems Engineering 25(6), 377-385.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F.S., Lambin, E.F., Lenton, T.M., Scheffer, M., Folke, C. and Schellnhuber, H.J. (2009) A safe operating space for humanity. Nature 461(7263), 472-475.

Rodríguez, J., Kleerebezem, R., Lema, J.M. and van Loosdrecht, M. (2006) Modeling product formation in anaerobic mixed culture fermentations. Biotechnology and Bioengineering 93(3), 592-606.

Rogers, P. (1986) Genetics and Biochemistry of *Clostridium* Relevant to Development of Fermentation Processes. Advances in applied microbiology 31, 1-60.

Royce, P.N.C. and Thornhill, N.F. (1991) Estimation of dissolved carbon dioxide concentrations in aerobic fermentations. AIChE Journal 37(11), 1680-1686.

Santamauro, F., Whiffin, F.M., Scott, R.J. and Chuck, C.J. (2014) Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources. Biotechnology for biofuels 7(1), 34.

Seeliger, S., Janssen, P.H. and Schink, B. (2002) Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. FEMS Microbiology Letters 211(1), 65-70.

Serafim, L.S., Lemos, P.C., Albuquerque, M.G.E. and Reis, M.A.M. (2008a) Strategies for PHA production by mixed cultures and renewable waste materials. Applied Microbiology and Biotechnology 81(4), 615-628.

Serafim, L.S., Lemos, P.C., Torres, C., Reis, M.A.M. and Ramos, A.M. (2008b) The Influence of Process Parameters on the Characteristics of Polyhydroxyalkanoates Produced by Mixed Cultures. Macromolecular Bioscience 8(4), 355-366.

Shen, L., Haufe, J. and Patel, M.K. (2009) Product overview and market projection of emerging bio-based plastics PRO-BIP 2009. Report for European Polysaccharide Network of Excellence (EPNOE) and European Bioplastics 243.

Shimada, T., Zilles, J., Raskin, L. and Morgenroth, E. (2007) Carbohydrate storage in anaerobic sequencing batch reactors. Water Research 41(20), 4721-4729.

Sigurgísladóttir, S., Konráòsdóttir, M., Jónsson, Á., Kristjánsson, J. and Matthiasson, E. (1993) Lipase activity of thermophilic bacteria from icelandic hot springs. Biotechnology Letters 15(4), 361-366.

Sinnott, R. (2005) Coulson & Richardson's Chemical Engineering. Volume 6: Chemical Engineering Design, Elsevier, Oxford.

Slijkhuis, H.V.G., Johan W; Kylstra, Dirk J (1984) *Microthrix parvicella*, a filamentous bacterium from activated sludge: growth on Tween 80 as carbon and energy source. Journal of general microbiology 130(8), 2035-2042.

Smolders, G.J.F., Vandermeij, J., Vanloosdrecht, M.C.M. and Heijnen, J.J. (1994) Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnology and Bioengineering 44(7), 837-848.

Sorokin, D.Y. and Jones, B.E. (2009) Improved method for direct screening of true lipase-producing microorganisms with particular emphasis on alkaline conditions. Microbiology 78(1), 125-130.

Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J.M. and Angenent, L.T. (2014) Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Current Opinion in Biotechnology 27(0), 115-122.

Stal, L.J. (1992) Poly(hydroxyalkanoate) in cyanobacteria: an overview. FEMS Microbiology Letters 103(2–4), 169-180.

Steinbüchel, A., Aerts, K., Liebergesell, M., Wieczorek, R., Babel, W., Föllner, C., Madkour, M.H., Mayer, F., Pieper-Fürst, U., Pries, A. and Valentin, H.E. (1995) Considerations on the structure and biochemistry of bacterial polyhydroxyalkanoic acid inclusions. Canadian Journal of Microbiology 41(13), 94-105.

Steinbüchel, A. and Valentin, H.E. (1995) Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiology Letters 128(3), 219-228.

Tajparast, M. and Frigon, D. (2013) Genome-scale metabolic modeling to provide insight into the production of storage compounds during feast-famine cycles of activated sludge. Water science and technology : a journal of the International Association on Water Pollution Research 67(3), 469-476.

Tamis, J., Lužkov, K., Jiang, Y., van Loosdrecht, M.C. and Kleerebezem, R. (2014a) Enrichment of *Plasticicumulans acidivorans* at pilot-scale for PHA production on industrial wastewater. Journal of Biotechnology.

Tamis, J., Marang, L., Jiang, Y., van Loosdrecht, M.C. and Kleerebezem, R. (2014b) Modeling PHA-producing microbial enrichment cultures--towards a generalized model with predictive power. N Biotechnol 31(4), 324-334.

Tamis, J., Sorokin, D.Y., Jiang, Y., van Loosdrecht, M.C. and Kleerebezem, R. (2015) Lipid recovery from a vegetable oil emulsion using microbial enrichment cultures. Biotechnology for biofuels 8(1), 1-11.

Temudo, M., Mato, T., Kleerebezem, R. and van Loosdrecht, M.M. (2009) Xylose anaerobic conversion by open-mixed cultures. Applied Microbiology and Biotechnology 82(2), 231-239.

Temudo, M.F., Kleerebezem, R. and van Loosdrecht, M. (2007) Influence of the pH on (open) mixed culture fermentation of glucose: A chemostat study. Biotechnology and Bioengineering 98(1), 69-79.

Temudo, M.F., Muyzer, G., Kleerebezem, R. and van Loosdrecht, M.C. (2008) Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source. Applied Microbiology and Biotechnology 80(6), 1121-1130.

Terada, M. and Marchessault, R.H. (1999) Determination of solubility parameters for poly(3-hydroxyalkanoates). International Journal of Biological Macromolecules 25(1-3), 207-215.

Third, K.A., Newland, M. and Cord-Ruwisch, R. (2003) The effect of dissolved oxygen on PHB accumulation in activated sludge cultures. Biotechnology and Bioengineering 82(2), 238-250.

Third, K.A., Paxman, J., Schmid, M., Strous, M., Jetten, M.S.M. and Cord-Ruwisch, R. (2005) Enrichment of Anammox from Activated Sludge and Its Application in the CANON Process. Microbial Ecology 49(2), 236-244.

Tholozan, J.L., Membré, J.M. and Kubaczka, M. (1996) Effects of culture conditions on *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis* metabolism: a physiological and statistical approach. Journal of Applied Bacteriology 80(4), 418-424.

Tijhuis, L., Van Loosdrecht, M.C.M. and Heijnen, J.J. (1993) A thermodynamically based correlation for maintenance gibbs energy requirements in aerobic and anaerobic chemotrophic growth. Biotechnology and Bioengineering 42(4), 509-519.

van Aalst van Leeuwen, M.A., Pot, M.A., vanLoosdrecht, M.C.M. and Heijnen, J.J. (1997) Kinetic modeling of poly(beta-hydroxybutyrate) production and consumption by *Paracoccus pantotrophus* under dynamic substrate supply. Biotechnology and Bioengineering 55(5), 773-782.

van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M., Picioreanu, C. and van Loosdrecht, M.C.M. (2007) Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. Water Research 41(18), 4149-4163.

Van der Weijden, W.J., E. Hees, T. Bastein and H.A. Udo de Haes (2014) Geopolitiek rond grondstoffen voor landbouw en voedsel. Deel B: Analyse. Platform Landbouw, Innovatie en Samenleving. Culemborg.

van Loosdrecht, M.C.M. and Heijnen, J.J. (2002) Modelling of activated sludge processes with structured biomass. Water Science and Technology 45(6), 13-23.

van Loosdrecht, M.C.M., Pot, M.A. and Heijnen, J.J. (1997) Importance of bacterial storage polymers in bioprocesses. Water Science and Technology 35(1), 41-47.

Van Wegen, R.J., Ling, Y. and Middelberg, A.P.J. (1998) Industrial Production of Polyhydroxyalkanoates Using *Escherichia Coli*: An Economic Analysis. Chemical Engineering Research and Design 76(3), 417-426.

van't Riet, K. (1983) Mass transfer in fermentation. Trends in Biotechnology 1(4), 113-119.

Vishniac, W. and Santer, M. (1957) Thiobacilli. Bacteriological Reviews 21(3), 195-213.

Vorderwulbecke, T., Kieslich, K. and Erdmann, H. (1992) Comparison of lipases by different assays. Enzyme and Microbial Technology 14(8), 631-639.

Wakelin, N.G. and Forster, C.F. (1997) An investigation into microbial removal of fats, oils and greases. Bioresource Technology 59(1), 37-43.

Welles, L., Lopez-Vazquez, C., Hooijmans, C., van Loosdrecht, M. and Brdjanovic, D. (2014) Impact of salinity on the anaerobic metabolism of phosphate-accumulating organisms (PAO) and glycogen-accumulating organisms (GAO). Applied Microbiology and Biotechnology 98(17), 7609-7622.

Wijffels, R.H. and Barbosa, M.J. (2010) An outlook on microalgal biofuels. Science(Washington) 329(5993), 796-799.

Wilhelm, W.W., Johnson, J.M., Karlen, D.L. and Lightle, D.T. (2007) Corn stover to sustain soil organic carbon further constrains biomass supply. Agronomy journal 99(6), 1665-1667.

Wirsenius, S. (2000) Human use of land and organic materials: modeling the turnover of biomass in the global food system (PhD Dissertation).

Yu, J., Si, Y., Keung, W. and Wong, R. (2002) Kinetics modeling of inhibition and utilization of mixed volatile fatty acids in the formation of polyhydroxyalkanoates by *Ralstonia eutropha*. Process Biochemistry 37(7), 731-738.

Zhu, L., Zong, M. and Wu, H. (2008) Efficient lipid production with *Trichosporon fermentans* and its use for biodiesel preparation. Bioresource Technology 99(16), 7881-7885.

Zoetemeyer, R., Van den Heuvel, J. and Cohen, A. (1982a) pH influence on acidogenic dissimilation of glucose in an anaerobic digestor. Water Research 16(3), 303-311.

Zoetemeyer, R.J., Matthijsen, A., Vandenheuvel, J.C., Cohen, A. and Boelhouwer, C. (1982b) Anaerobic Acidification of Glucose in an Upflow Reactor. Biomass 2(3), 187-199.

Zoutberg, G.R., Willemsberg, R., Smit, G., de Mattos, M.T. and Neijssel, O. (1989) Aggregate-formation by *Clostridium butyricum*. Applied Microbiology and Biotechnology 32(1), 17-21.

Curriculum vitae

Personal data

Name	Jelmer Tamis
Date and place of birth	9 February 1983, Alkmaar, The Netherlands
Address	W.H. van Leeuwenlaan 156 2613 ZH Delft The Netherlands
Nationality	Dutch
Telephone	+31616691609
E-mail	info@jelmertamis.nl

Education

Master of Science: Life Science and Technology
TU Delft, 2008

General secondary education
Fioretti college, Lisse, 2002

Work experience

Jan 2015 – present	**Process specialist** Paques BV *Development of technology for PHA production from organic waste streams*
May 2011 – Dec 2014	**PhD candidate** TU Delft, Environmental Biotechnology *Resource recovery from organic waste streams by microbial enrichment cultures; www.w2r.nl*
Dec 2010 – present	**Treasurer and co-founder** Stichting Katalysator *Supporting sustainable businesses in developing countries (a.o. Mali, Ghana and Colombia); www.stichtingkatalysator.nl*
Apr 2008 - Dec 2010	**Process engineer** SR Technologie BV *Development of a technology for sludge reduction by aquatic worms*

List of publications

Journal articles

Tamis, J., Sorokin, D.Y., Jiang, Y., van Loosdrecht M.C.M., Kleerebezem, R. (2015) Lipid recovery from a vegetable oil emulsion using microbial enrichment cultures. Biotechnology for biofuels 8(1):1-11.

Tamis, J., Marang, L., Jiang, Y., van Loosdrecht, M.C.M. and Kleerebezem, R. (2014) Modeling PHA-producing microbial enrichment cultures—towards a generalized model with predictive power. New biotechnology 31(4), 324-334.

Tamis, J., Lužkov, K., Jiang, Y., van Loosdrecht, M.C.M. and Kleerebezem, R. (2014) Enrichment of *Plasticicumulans acidivorans* at pilot-scale for PHA production on industrial wastewater. Journal of Biotechnology.

Mooij, P.R., Stouten, G.R., **Tamis, J.**, van Loosdrecht, M.C.M. and Kleerebezem, R. (2013) Survival of the fattest. Energy & Environmental Science 6(12), 3404-3406.

Jiang, Y., Marang, L., **Tamis, J.**, van Loosdrecht, M.C.M., Dijkman, H. and Kleerebezem, R. (2012) Waste to resource: Converting paper mill wastewater to bioplastic. Water research 46(17), 5517-5530.

Tamis, J., Van Schouwenburg, G., Kleerebezem, R. and van Loosdrecht, M. (2011) A full scale worm reactor for efficient sludge reduction by predation in a wastewater treatment plant. Water research 45(18), 5916-5924.

Tait, S., **Tamis, J.**, Edgerton, B. and Batstone, D.J. (2009) Anaerobic digestion of spent bedding from deep litter piggery housing. Bioresource technology 100(7), 2210-2218.

Dankwoord

Na ruim honderd pagina's over micro-organismen te hebben geschreven, wil ik op deze laatste pagina mijn dank betuigen aan een aantal inspirerende hogere levensvormen:

Robbert. Ook wel de bezem genoemd, Il Brumo of Roba Scoba. De onbetwiste gebieder van Excel. Robbert, vooral dankzij jouw ongecensureerde uitspraken blijft het hier aangenaam toeven. Opvallend is ook dat jij nog steeds veel eerder door hebt wat er aan de hand is met de reactor. Bedankt alle hulp en voor alle mooie tijden en dat we nog maar veel plastic mogen maken.

Mark. De spin in het web ook wel de zaag genoemd (waar Robbert de bezem is), kameraad konijn, de grote roerganger, de man van de vele namen, de slijper van onze geest. Mark, jij hebt ondanks je drukke leven met ontelbare projecten, altijd tijd, of juist vrijheid, gemaakt als het nodig was. Bedankt voor al je hulp en ook bedankt voor het oprichten van EBT, het is een grote eer en plezier hier te mogen werken. Dat er nog vele vreemde nieuwe (nuttige of fundamenteel interessante) micro-organismen in de EBT reactoren mogen groeien.

Peter en Gerben. Die vaak letterlijk het dichtst bij mij stonden. Een betere bezetting van ons kantoor, de monkey's office, had ik me niet kunnen wensen. Bedankt voor alle hulp en de diamanten muesli.

EBTers. Udo van Dongen aka de lunch professor voor vis, FISH, bbq het betere drone- en duikwerk en vele andere talenten. Dirk Geerts, voor het betere soldeerwerk en het voorkomen van stroomschokken in het algemeen. Ben Abbas en zijn trouwe kameraad Mitchell, de mannen van het DNA. Taalpurist Matthijs Daelman, Mario –Korrel- Pronk, Leo de goud en beton vorser en alle anderen EBTers!

Mijn team bioplastic collega's: de veteranen: Yang Jiang, de absolute labmeester en verhalenverteller, Leonie Marang, dresseuse der *Plasticicumuli* en onze Spaanse schone, Helena Moralejo Garate – Karate and Emma. Mijn favoriete studenten Tianye Zheng, Zhen Zhen Zheng, Noa, Engjell, Pascalle Vermeulen (special thanks voor het maken van de micro-organismen op de kaft), Bart Joosse (die nu op grote hoogte verder gaat als project acid hat), Katlin Lužkov (The Estonian princess of PHA), Casper Borsje, Janis Gedusevs aka mr 25 kV. De marsmannen Hein Mous en Frans, voor het lekkere afvalwater en de kantine waarvan ze in Delft nog wat kunnen leren. Mijn collega's van team bioplastic$^+$ Gizela Mikova, Patricia van Dam, Henk Dijkman, Fokje Muizelaar en Rene Rozendal. Jans Kruit voor het compost project weer op de rails zetten. Maria Albuquerque en Alan Werker voor perspectieven en wijn.

Mijn beste vrienden: Sjoerd Hoogewerf aka Stokvis de surfende sexwax legende, met vele mooie en holle pockets op onze kerfstok en de rest van de strand-gang. Coen van Gennep aka Captain Knut de grote Knutselaar, producer van biogas, jacuzzi's, jacuzzi's op biogas, samen met Alain Simons, de cadeautjesman en admiraal van Stichting Katalysator, van mooie projecten in barre oorden, Hans Buijs, die voor de duivel nog niet bang is. Mijn trouwe ouwe vrienden, Laurens Welles aka de kniftige gnip en neef Steef, voor de betere discussies over het circus der wetenschap, de *G. erectus* en andere wezens om ons heen. Mijn oude vrienden in Lisse, special thanks to Sven, Stefan en Arturo, Arjan, John, Freek, Peter, en Joyce, bedankt voor de mooie tijd.

Mijn familie. In de eerste plaats Sjan en Willem. Jullie hebben altijd voor onze familie klaargestaan, met raad en daad, zonder jullie waren we nergens. Mijn Ouders, Wil en Hetty, mijn grootouders en zusjes, Suzanne en Rozemarijn voor alle liefde en inspiratie.

Kristina. Voor jouw schoonheid en voor jouw gift aan ons, Augustus. En Augustus voor jou.


wastetoresource
PRODUCING BIOPOLYMERS FROM ORGANIC WASTE