TÉCNICO LISBOA

Production of PHA using Mixed Microbial Cultures and a Mixture of Complex Feedstocks

Filipa Carolina dos Santos Souto Pedro

Thesis to obtain the Master of Science Degree in

Biological Engineering

Supervisors:
Professor Maria da Ascensão Carvalho Fernandes Miranda Reis
Doctor Maria Teresa Ferreira Cesário Smolders

Examination Committee

Chairperson: Professor Frederico Castelo Alves Ferreira
Supervisor: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis
Member of the Committee: Professor Helena Maria Rodrigues Vasconcelos Pinheiro

January 2021

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Biochemical Engineering (BIOENG) group, in the Faculty of Sciences and Technology (FCT) of Universidade NOVA de Lisboa (UNL), between June and November, 2020. This thesis was supervised by Dr. Maria da Ascensão Carvalho Fernandes Miranda Reis and MSc. Fernando Ramos Silva and co-supervised at Instituto Superior Técnico by Dr. Maria Teresa Ferreira Cesário Smolders.

Acknowledgements

Nesta pequena página gostaria de agradecer a todas as pessoas que me ajudaram não só esta etapa mas durante todo o período de aprendizagem que me levou até aqui.

Em primeiro lugar, expresso o meu enorme agradecimento à minha orientadora, Professora Doutora Maria Ascensão Reis, pela oportunidade de realizar esta tese em plena pandemia num excelente grupo de investigação. À Doutora Teresa Cesário, agradeço toda a disponibilidade mostrada ao longo destes meses.

Um enorme e sincero agradecimento ao Fernando Silva, o orientador não oficial, que me transmitiu imenso conhecimento, proporcionou bons momentos e me motivou ainda mais para este tema. Ao João Bello, quero agradecer por me ensinar a como pôr as mãos na massa e fazer de mim mais engenheira. Ao Bruno e à Eliana, agradeço do fundo do coração, por nesta fase final estarem sempre disponíveis para me ouvirem, motivarem sempre que achava que não era possível e animarem sempre que necessário. Agradeço ao fantástico grupo de investigação, BionEng, com quem pude partilhar espaço de laboratório, gargalhas e muito trabalho. Um enorme obrigada à Elisabete, à Elsa e à Soraia, as fadas madrinhas dos equipamentos, sem as quais não era possível reportar todos os dados que aqui estão.

A todos aqueles que começaram como colegas de curso e acabaram como amigos um enorme obrigada, em especial ao Manuel, à Margarida, à Beatriz e à Helena, por todo o apoio que me deram ao longo destes anos. Ao NAPE, tenho a agradecer as competências que ganhei no curto tempo que fiz parte da equipa, e aos amigos que levo para a vida e que neste ano nunca me largaram: Bruno, Sara, Madalena, Filipe e Rodrigo, um sincero obrigada. Não é possível esquecer as meninas que sempre fizeram parte da minha vida e que após 10 anos ainda cá continuam, um enorme abraço para a Marta, as Carolinas, a Mariana, a Bárbara e para a Maria Inês.

Por fim, o agradecimento mais sentido e importante faço-o aos meus pais, que me possibilitaram todo o caminho que fiz até este momento, que me sustentaram de todas as formas possíveis, que depositaram toda a confiança em mim e no meu trabalho e, por nesta última fase, me acalmarem quando necessário.

Obrigada a todos!

Abstract

Nowadays, there is a huge demand for biomaterials, such as polyhydroxyalkanoates (PHA), produced from renewable sources. PHA production by mixed microbial cultures in sequential batch reactors with several waste feedstocks was already reported in literature. In this study, PHA production at pilot-scale (60 L) with two waste feedstocks, namely fermented fruit pulp waste (Feed-C) and the fermented organic fraction of municipal solid waste (Feed-N) was studied. The first is rich in carbon but limited in nutrients, while the latter has a low carbon content but a high nutrients titre. Three operational conditions were sequentially tested under the same organic loading rate of 5.8 gCOD L^{-1} d^{-1}(150C-mmol L^{-1} d^{-1}) and an SRT of 4 days. In the first condition, Feed-C was used as carbon source and synthetic ammonia solution as nitrogen source (uncoupled feeding). The PHA content achieved in the accumulation assays was 63.93 $\pm$ 8.70 % (g_{PHA} $g_{VSS}{}^{-1}$) . Further, in condition 2, the synthetic ammonia solution was replaced by Feed-N as a nitrogen source. A PHA content of 31.59 $\pm$ 2.46 % (g_{PHA} $g_{VSS}{}^{-1}$) was achieved. In the third condition, the operation using both feedstocks under a lower HRT (0.66 days) was studied. In this case, PHA content of 53.44 $\pm$ 0.06 % (g_{PHA} $g_{VSS}{}^{-1}$) was obtained. The global productivity achieved in the first condition was 0.62 gPHA L^{-1} h^{-1}, decreasing to 0.42 gPHA L^{-1} $h^{-1}$with the culture selected with both feedstocks (in condition 3). The accumulation stage was also performed with Feed-N. The results showed a significant decrease in PHA content (14.95 $\pm$ 1.02 % (g_{PHA} $g_{VSS}{}^{-1}$) and 26.61 $\pm$ 2.37 % (g_{PHA} $g_{VSS}{}^{-1}$) , for condition 2 and 3, respectively) which was due to a predominant growth response of the culture. For the two feedstocks the selected culture was dominated by *Amaricoccus* and *Paracoccus* genera which are know as PHA storing microorganisms. The present work opens the possibility of using different low cost feedstocks as carbon and nitrogen source for PHA production, lowering the associated costs and contributing to a circular economy.

Keywords: Mixed microbial cultures, polyhydroxyalkanoates, waste feedstocks, culture selection

Resumo

Atualmente, existe uma procura colossal por biomateriais, como os polihidroxialcanoatos (PHAs), produzidos a partir de fontes renováveis. A produção de PHAs por culturas microbianas mistas, em reatores descontínuos sequenciais, com diversos substratos de baixo custo já foi extensamente descrita na literatura. Nesta dissertação, foi avaliada a produção de PHA à escala piloto (60 L) com dois substratos, resíduos de polpa de fruta fermentada (*Feed-C*) e a fração orgânica de resíduos sólidos municipais fermentada (*Feed-N*), o primeiro deficiente em azoto e o segundo em carbono. Foram testadas três condições de operação, sequencialmente, com uma carga orgânica de 5,8 gCOD L^{-1} d^{-1}(150 C-mmol L^{-1} d^{-1}) e um SRT de 4 dias. Na primeira condição, a aclimatação da biomassa ocorreu com *Feed-C* como fonte de carbono e uma solução de amónia como fonte de azoto. Obtendo-se, na fase de acumulação, um conteúdo em PHA de 63,93 $\pm$ 8,70 % (g_{PHA} $g_{VSS}{}^{-1}$) . Seguidamente, na condição 2, a solução sintética de amónia foi substituída pelo *Feed-N*, atingindo-se um conteúdo de PHA de 31.59 $\pm$ 2,46 % (g_{PHA} $g_{VSS}{}^{-1}$) . Na última condição estudou-se a influência de um HRT mais baixo (0,66 dias) no processo com os dois substratos. Foi possível observar uma melhoria no conteúdo em PHA de 53,44 $\pm$ 0,06 % (g_{PHA} $g_{VSS}{}^{-1}$) . Na primeira condição obteve-se uma produtividade global de 0.62 gPHA L^{-1} h^{-1}, diminuindo para 0,42 gPHA L^{-1} h^{-1}na condição 3. Apesar da fase de acumulação ter sido realizada com *Feed-C* também foi avaliada a influência do *Feed-N* na capacidade de acumulação por parte da cultura. Os resultados mostraram uma diminuição no conteúdo de PHA obtido (14,95 $\pm$ 1,02 % (g_{PHA} $g_{VSS}{}^{-1}$) e 26,61 $\pm$ 2,37 % (g_{PHA} $g_{VSS}{}^{-1}$) , na condição 2 e 3, respectivamente) e uma predominante resposta de crescimento. Diferentes organismos acumuladores de PHA, do género *Amaricoccus* e *Paracoccus*, foram identificados na cultura selecionada com ambos os substratos. O presente trabalho abre a possibilidade de utilizar resíduos com composições complementares, diminuindo os custos de produção de PHA associados e contribuindo para uma economia circular.

Palavras-chave: Culturas microbianas mistas; polihidroxialcanoatos; substratos de baixo custo; seleção de cultura

Contents

List of Tables

List of Figures

Acronyms

C/N Carbon to Nitrogen.

COD Chemical Oxygen Demand.

DO Dissolved Oxygen.

EU European Union.

F/F Feast to Famine.

FBR Fed Batch Reactor.

Feed-C Fermented Fruit Pulp Waste.

Feed-N Fermented Food Waste.

FISH Fluorescence in Situ Hybridization.

FP Fermentation Products.

GC Gas Chromatography.

HB Hydroxybutyrate.

HHx Hydroxyhexanoate.

HRT Hydraulic Retention Time.

HV Hydroxyvalerate.

ICI Imperial Chemical Industries.

IUPAC Internacional Union of Pure and Applied Chemistry.

LA Lactic Acid.

LCL Long-chain-length.

LDPE Low-density Polyethylene.

MCL Medium-chain-length.

MMC Mixed Microbial Cultures.

OFMSW Organic Fraction of Municipal Solid Wastes.

OLR Organic Loading Rate.

PA Polyamide.

PBAT Polybutylene adipate-coterephthalate.

PBS Polybutylene Succinate.

PCL Polycaprolactone.

PE Polyethylene.

PEF Polyethylene Furanoate.

PET Polyethylene Terephthalate.

PHA Polyhydroxyalkanoates.

PHB Poly(3-hydroxybutyrate).

PHBV Poly(3-hydroxybutyrate-co-3-hydroxyvalerate).

PHOHH Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate).

PHV Poly(3-hydroxyvalerate).

PLA Polylactic Acid.

PP Polypropylene.

PTT Polytrimethylene Terephthalate.

SBR Sequencing Batch Reactor.

SCL Short-chain-length.

SRT Sludge Retention Time.

TCA Tricarboxylic Acid.

TSS Total Suspended Solids.

UASB Upflow Anaerobic Sludge Blanket Reactor.

VFA Volatile Fatty Acid.

VSS Volatile Suspended Solids.

WWTP Waste Water Treatment Plant.

X Biomass Concentration.

Chapter 1

Introduction

1.1 From Plastic to New Alternatives?

At the beginning of the 20th century, many industrial materials like dyes, solvents, and synthetic fibers were made from trees and agricultural crops. Despite that, in the late 50s, many of these bio-based chemical products were replaced by petroleum - based materials [1]. The use of plastic during World War II was major, and after the end, they became widespread and competitive with more traditional materials, such as metal, glass, wood [2]. A new challenge arrived in 1970s, with the energy crisis, where many countries had to deal with shortages of petroleum, which sparked the interest for processes for the synthesis of fuels and materials from bioresources [1].

The plastic industry continues to growth and a life without plastic seems inconceivable. In 2018, global plastics production reached almost 360 million tonnes, and in Europe alone reached 62 million tonnes. Since 2006, the amount of plastic waste sent to recycling has doubled, however in 2018, in European Union (EU), only 32.5% of collected plastic was recycled, being 42.6% used for energy recovery and 24.9% sent to landfills [3].

Plastics are an extensive family of different materials with specific characteristics, making them ideal for the intended application [3]. Plastic properties are numerous, for the most part, they are lightweight, versatile and can be manufactured inexpensively and mass produced. Due to these characteristics they can be used in a number of applications, such building, construction, packaging, medical applications and many others [1]. Plastic materials can be produced from different sources. Its raw materials can be of fossil origin (crude oil, gas, etc) or renewable (sugar cane, starch, vegetable oils, etc) or even mineral base (salt). On one hand, the biodegradable ones can become compost after proper collection and treatment [3], on the other hand plastics derived from fossil hydrocarbon accumulate, rather than decompose, in landfills or the natural environment. Only by thermal treatment, such as combustion or pyrolysis, plastic from non-renewable sources can be permanently eliminated [4]. The treatment chosen to manage plastic waste depends on the country, and the most common options are reutilization, recycling, incineration, and landfill disposal. At this moment there's no uniform waste management practice in EU, being landfill disposal the first or second option in many countries [2].

In January of 2018, the EU presented the European Strategy for Plastics in a Circular Economy, which is based on the following principles: achieve a better design of plastic products and higher recycling rates, leading to better quality recyclates and greater added value [5]. The report discusses the need to promote investment in new solutions, such as innovative materials and alternative feedstocks for plastic production, more sustainable compared to the non-renewable alternatives. It's necessary to turn today's challenges into opportunities [6]. The Ellen MacArthur Foundation, launched in 2010, promotes a faster transition to circular economy, being the New Plastics Economy one of its several activies. In

this context, in October 2018, ”The New Plastics Economy Global Commitment” was launched, in collaboration UN Environment Programme (UNEP) and more than 450 organisations, such as packging companies (represent 20% of the signatures), NGOs, universities, investors, and others, with the common vision and an ambitious set of targets to address plastic waste and pollution at its source, by 2025. The first annual report, published in 2019, provides an remarkable level of transparency on how these signatories are taking action [7]. In March 2020, Europe joins The Plastics Pact, that is a network of local and regional initiatives that bring together key stakeholders to implement solutions towards a circular economy for plastics. The European Plastics Pact, with 81 members have committed to set several ambitious targets by 2025, such as make all plastic packaging and single-use plastic products reusable where possible, and in all cases recyclable, reduce the need for virgin plastic products and packaging by at least 20% and others [8].

Bio-based polymers have the potential to replace petroleum-based polymers and help solve some of the most urgent problems caused by the overuse of petroleum-based polymers, like water and soil pollution, greenhouse emission and overdependence on petroleum. However, the economic and technical aspects of these bio-based polymers create a barrier to rapid adoption at least in the near future [9].

1.2 Biorelated Polymers

Accordingly to IUPAC, the term bio-based implies that the material is composed or derived totally or in part from the biomass [10]. On the other hand, biodegradable plastics, experience biological degradation through the actions of microorganisms [11] by reducing the molar masses of macromolecules that form the substances [10]. Being bio-based is not necessarily being biodegradable, for instance, 100% bio-based polyethylene (bio-PE) is not biodegradable whereas 100% petrochemical based polybutylene adipate-coterephthalate (PBAT) is biodegradable. In short, the biodegradability of a plastic material it's related to its chemical structure and not to its carbon source [11].

What about bioplastics? According to European Bioplastics, a bioplastic is admited as a bio-based and/or biodegrabdable product, but both conditions don't need to be fulfilled simultaneously [12]. Therefore, the family of bioplastics is divided into three main groups:

- Bio-based and non-biodegradable plastics such as bio-based PE, polypropylene (PP), polyethylene terephthalate (PET) or polyamide (PA);
- Both bio-based and biodegradable, such as polylactic acid (PLA), polybutylene succinate (PBS) and polyhydroxyalkanoates (PHA);
- Based on fossil resources and biodegradable, such as polybutylene adipate terephthalate (PBAT) and polycaprolactone (PCL).

The bioplastics that fall into the second category are considered the green polymers of the future since they are expected to gradually substitute conventional plastics. For these reason, they should mimic the physicochemical, thermal, and mechanical properties of PP and low-density polyethylene (LDPE) [13].

PLA is a aliphatic polyester produced from the monomer of lactic acid (LA) by two well-known processes: the direct polycondensation route and the ring-opening polymerization route. Latic acid is a common industrial chemical with an annual production exceeding 400,000 metric tonnes. In the industry, LA is made using the fermentation process by latic acid bacteria (more then 20 genera in the phylum *Firmicutes* have this capacity), and is mostly performed in batches during 3-6 days. Today PLA is widely use in food packaging, being the attractive glossy appearance well appreciated. This biopolymer is also

used in the medical industry as implants, stents and bone supporting splints.*Nature Works* is nowadays the world's larger producer of PLA [9, 14].

PBS is also an aliphatic polyester, chemically synthesized through polycondensation of 1,4-butanediol and succinic acid. The two monomers that constitute this biopolymer can be produced by using petrochemical feedstocks or through fermentation route. The succinic acid can be produced by some microorganisms such as *Anaerobiospirillum*, *Propionibacterium*, *Escherichia coli* and *Saccharomyces cerevisiae* taking a saccharide such as sugarcane or corn as raw material. This succinic acid can be easily reduced to the other monomer, producing 100% bio-based PBS. This biopolymer is used in the packing industry, in the form of compostable waste bags, film packaging for foods whit short shelf life and others. The main players in the PBS production are the producers of succinic acid, like BioAmber, Myriant, Reverdia [9, 15].

While PLA and PBS are produced upon polymerization of lactic and succinic acid respectively, PHA polymerization is performed naturally by bacteria. In the next subsection, special attention is paid to PHA, referring its structure, properties, metabolism, among others aspects.

1.3 Polyhydroxyalkanoates

Unlike PLA and PBS, each of which refers to one specific type of compound, PHA is a big family of biodegradable polymers, having as basic unit R-hydroxyalkanoic acids. In 1996, Lee defined PHAs as polyesters synthesized by numerous bacteria in the form of granules (in the cytoplasm of cells) for carbon and energy storage [16], under nutrient - limiting conditions. The very first observation of PHA aggregates, was done in 1888 by Beijerinck, and in 1926, poly(3-hydroxybutyrate), namely PHB was reported by Lemoigne in 1926 [17]. In 1974, Wallen and Rohwedder identified poly(3-hydroxyvalerate) (PHV) and 3-hydroxyhexanoate (HHx), as the major and minor compounds in an activated sewage sludge sample [18]. More than 150 different monomers of hydroxyalkanoate have been discovered, that combined can form varied polymers with diverse properties [9]. These intracellular inclusions can be stored by more than 300 species of Gram-positive and Gram-negative as well as a wide range of archae.

1.3.1 Structure and Properties

Several factors can influence the monomeric composition of PHA polymers, such as the producing organism, the carbon source used for growth and how it's metabolized by the cells and the type of PHA synthase used to synthesize the polymer [19]. The molecular weight of the PHAs varies from $2 \times 10^5 - 3 \times 10^6$ Da, and granules diameter ranges from 0.2–0.5 µm [20]. Due to their high refractivity, PHA granules may be visualized clearly with a phase contrast light microscope, and stained with Sudan black B or oxazine dye Nile Blue A, exhibiting a strong orange fluorescence [18].

PHA monomers may be divided into three classes, according to chain-length. SCL, short-chain-length PHAs, which consist of 3–5 carbon units; MCL, medium-chain-length PHAs, with chain lengths between 6 and 14 carbon units; and lastly, LCL, long-chain-length PHAs, which are composed of monomers with carbon chain lengths greater than 14 units, which is uncommon and the least studied [19], [21].

PHAs can be classified as homopolymers or heteropolymers. Homopolymers contain a single repeating monomer unit, such as poly(3-hydroxybutyrate), the most studied, poly(3-hydroxyhexanoate), and poly(3-hydroxyoctanoate) whereas heteropolymers contain more than one type of monomer unit such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly (3-hydroxyhexanoate-co-3-hydroxyoctanoate) [20]. SCL-PHAs, mostly PHB is generally found to be brittle in nature, have a high melting point and

crystallinity, on the other hand MCL have a low melting point, low crystallinity, and a lower value of tensile strength [22]. The use of PHAs is tailored to the application, depending on the specific combinations of different monomers incorporated into the polymer chain [23]. Table 1.1 shows a comparison of the properties of various PHAs with polypropylene and the Figure 1.1 shows the molecular structure of the polymers presented Table 1.1.

Table 1.1: Comparison of the properties of different PHAs with polypropylene. 1 - HV content is 14%; 2 - HV content is 3%.

Properties	PHB	PHBV[1]	PHBV[2]	PHOHH	PP
Melting temperature (Tm ℃)	177	150	170	61	176
Glass Transition Temperature (Tg ℃)	4	4	8	-35	-10
Tensile strength (MPa)	40	37	37	9	1.7
Young's modulus (GPa)	3.5	1.9	2.9	0.0008	38
Elongation to break (%)	6	-	-	380	400
Reference	[22]	[24]	[24]	[22]	[19]

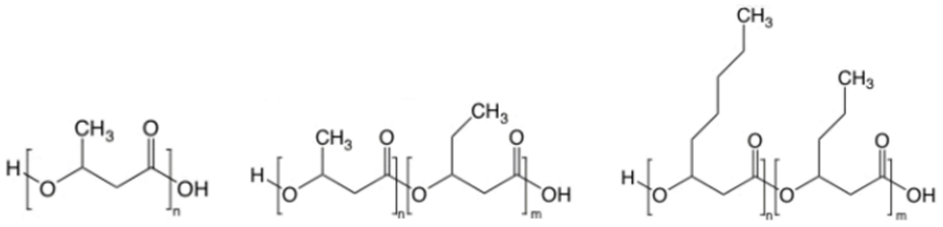


Figure 1.1: Molecular structure of polyhydroxybutyrate (PHB), polyhydroxybutyrate-co-hydroxyvalerate (PHBV) and polyhydroxyhexanoate-co-hydroxyoctanoate (PHOHH).

Comparing PHB with PP it's notable that although the elongation break is lower, which makes PHB more stiffer and brittle, all the other properties are similar [24]. Copolymers like PHBV are less stiff and brittle than PHB, while retaining most of the other mechanical properties of PHB [25].

It's important to highlight some general characteristics of PHA, such as being insoluble in water and relatively resistant to hydrolytic degradation; having good ultra-violet resistance, soluble in chloroform, non- toxic and biocompatible, being suitable for medical applications [26]. Another feature that makes PHAs so attractive it's their ability to degrade naturally in the environment - soil, lake water, marine water and even sewage water - resulting into water and carbon dioxide. This makes PHAs a promising and interesting material [27].

1.3.2 Metabolic Pathways

Biosynthesis of PHA occur under certain conditions, namely, in carbon excess and limitation of essential growth nutrients, such as phosphate and nitrogen. PHAs provide energy (reduction equivalents via the TCA cycle) and building blocks (β-oxidation metabolites such as (R)-3-hydroxyalkanoyl-CoAs) to other cell activities. For this reason, PHA metabolism can't be classified as a unidirectional metabolic process, but a bidirectional one, in which there is a continuous cycle of synthesis and degradation [28, 29].

For the formation of PHA, 14 pathways have been reported [30], being highlighted here 5 of them. Most PHA-producing bacteria used in pure cultures, such as *C.necator* convert sugars through glycolysis, to acetyl-CoA. By β- ketothiolase (PhaA), two molecules of acetyl-CoA form acetoacetyl-CoA, which

is reduced by the NADPH dependent acetoacetyl-CoA reductase (PhaB) into (R)-3-hydroxybutyryl-CoA. After, this molecule is incorporated as 3HB by PHA synthase (PhaC) [31].

In the case of feedstocks mainly constituted by short chain volatile fatty acids, these are transported across the cell membrane and activated to the corresponding acyl-CoA, that serve as a precursor for different monomers. For SCL-PHA, acetic, propionic, butyric and valeric acids are essentially used. From acetate, two molecules of acetyl-CoA give rise to PHB as described above. Propionate is converted in propionyl-CoA, which can then be combined with an acetyl-CoA, forming (R)-3-hydroxyvaleryl-CoA, and finally PHV. Propionyl-CoA can be decarboxylate yielding acetyl-CoA and CO_2 that can give rise to PHB. Butyrate and valerate can be used directly for the production of PHB and PHV or also used through β-oxidation [32, 33]. The above mentioned reactions are summarized in Figure 1.2.

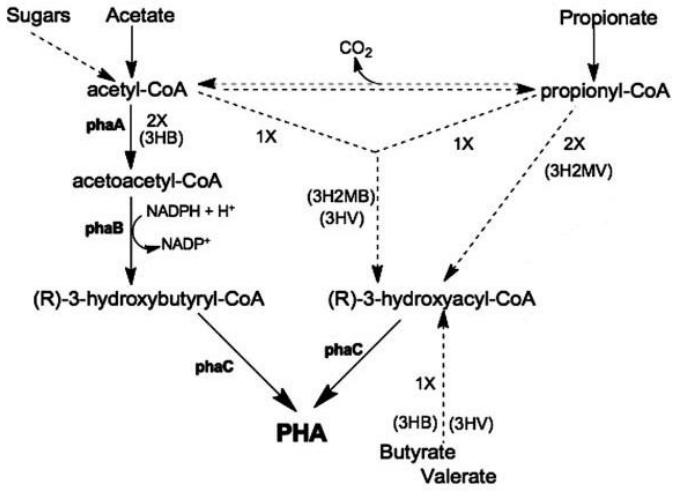


Figure 1.2: Metabolic pathways involved in SCL-PHA synthesis, from sugars and directly from volatile fatty acids. Adpated from [33].

MCL-PHAs synthesis can be associated to three metabolic pathways: *de novo* fatty acid synthesis, fatty acid β-oxidation and chain elongation, commonly used by a variety of *Pseudomonas* spp [31]. These metabolic pathways produce various intermediate precursors of MCL-PHA, such as (R)-3-hydroxyacyl-acyl carrier protein (ACP), 2-trans-enoyl-CoA, (S)-3-hydroxyacyl-CoA, and 3-ketoacyl-CoA. Next figure presents a brief description of the reffered metabolic pathways [34].

In the first pathway, simple carbon sources (glucose, gluconate, ethanol) are oxidized into acetyl-CoA, followed by their conversion into malonyl-CoA, culminating in (R)-3-HA-acyl carrier protein. After, this intermediate is converted to the corresponding (R)-3-hydroxyacyl-CoA monomer, by a specific CoA transferase (PhaG). With β-oxidation pathway, fatty-acids are converted into 2-*trans*-enoyl-CoA, (S)-3-hydroxyacyl-CoA and 3-ketoacyl-CoA. By distinctive enzymes, 3-hydroxyacyl-CoA percursor is formed. The third pathway is a chain elongation reaction, in which acetyl-CoA is extended to 3-hydroxyacyl-CoA, entering in β-oxidation pathway. In the final step of all the metabolic pathways presented, PhaC activates the conversion of (R)-3-hydroxyacyl-CoA molecules into MCL-PHAs [34, 31, 28].

The types of PHA formed depend not only on monomer supply pathways, but also on specificity of PHA synthases [30]. If the criteria relies on constituent subunits, then class I and II synthases are constituted by a single unit, PhaC for activity, while classes III and IV require an additional subunit. On the other hand, if the substrate specificity is taking into account, classes I, III and IV polymerize SCL monomers (C3–C5), while class II PHA synthase utilizes MCL monomers (C6–C14), yet this varies within species [31, 28]. Regulation of PHA metabolism can take place at different levels, such as the activation

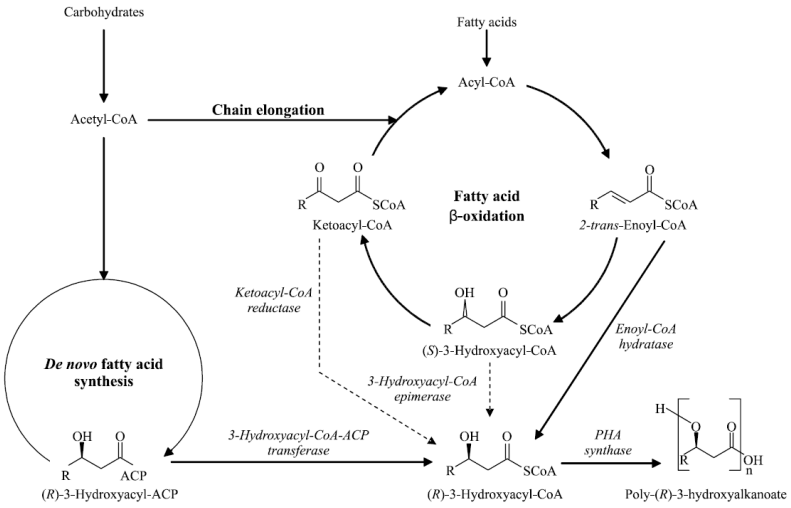


Figure 1.3: Metabolic pathways involved in MCL-PHA synthesis, from sugars and directly from volatile fatty acids. Adpated from [34].

of gene expression due to environmental influences (nutrient starvation) or an inhibition of metabolic enzymes of competing pathways. For example, during normal bacterial growth, PhaA is inhibited by free coenzyme-A coming out of the Krebs cycle, but when nutrients are limited, acetyl-CoA is restricted from entering into the Krebs cycle, being channeled into PHA biosynthesis [35]. It's important to highlight that the knowledge of the metabolic pathways involved in PHA production allows to anticipate polymer composition, and therefore, structure and mechanical properties as well as applications [33].

1.3.3 Organisms for PHA production

PHA-producing microorganisms can originate from almost all conceivable habitats and ecological niches, from marine microbial mats to waste water treatment plants (WWTP) [36].

Pure Cultures

In 1982, Imperial Chemical Industries (ICI) produced, at industrial scale, the first bioplastic under the name of Biopol, using *Cupriavidus necator* as a pure culture [2]. Another well-known species are involved in industrial PHA production, such as *Pseudomonas putida and Alcaligenes latus*, not being necessary, for the last one, a growth limitation in order to store PHA [13].

This production process generally involves two steps, the first one consists in providing the feedstock and when high cellular density is reached, growth limiting conditions are imposed (second step) in order to induce PHA storage [32, 37]. Using pure cultures is possible to achieve maximum PHA contents up to 80-90% of the cell dry weight. After 8h of nitrogen limitation, in *A. latus* batch cultures, a PHB content of 88% was reported, which translates in a productivity of 5 g L^{-1} h^{-1}, approximately [38]. With *Cupriavidus necator*, was observed a cell density over 200 g L^{-1}containing over 80% of PHB after 60h of fermentation [39].

The use of recombinant organisms, such *E.Coli*, can be advantageous since higher growth rates and cell densities can be achieved. The ability to use inexpensive carbon sources and produce PHA without inducing stress is seen as a strength [40, 39]. For example, cell density of 194 g L^{-1}, PHB of 87% and global productivity of 4.6 gPHA L^{-1} $h^{-1}$was achieved when recombinant *E.Coli* harbored PHB operon from *A. Latus* [41].

Although high productivity can be achieved, the use of pure cultures for PHA production is associated with high costs, namely the high price of refined feedstocks, production in batch or fed-batch modes, sterilization requirements and large amounts of solvents and/or labor regarding the downstream stage [32, 2, 37].

Mixed Microbial Cultures

Mixed cultures have been identified as effective systems in many fermentation processes. As already mentioned, in 1974 Wallen and Rohwedder were the firsts to report PHA production by mixed cultures [42]. MMC become an advantage over pure cultures since no sterile conditions are required, less expensive carbon sources can be used, fewer process controls are required and instead of resorting to genetic or metabolic engineering, MMC methods are focused on nature's concepts of selection and competition (evolutionary engineering). Additionally, since the variety of organisms working with complex substrates is higher, more diverse PHAs are formed [37, 36, 43]. It's assumed that mixed cultures metabolism is similar to the one reported for pure cultures, using identical carbon sources [33].

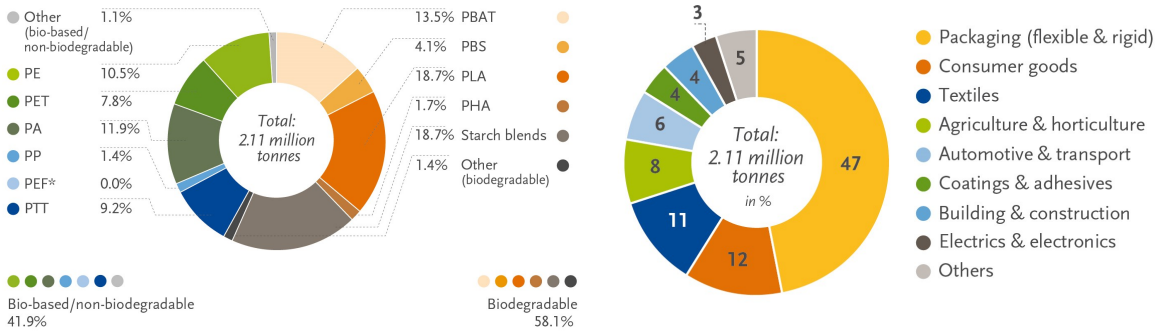
Although numerous descriptions of the microbial structure in PHA-storing MMC was already made, the microorganisms actively storing PHAs are rarely established. PHA-accumulating microorganisms in MMC are mostly from *Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria* classes, being already identified *Azoarcus, Thaurea, Paracoccus* and other genera [44]. Production of PHA from several carbon sources, selection strategies and operation conditions are reported in the next chapters.

1.3.4 Applications and Market

PHA have a wide range of applications for the industry, as molded items, fiber and film. They can be employed in furniture, sports equipment and tools and in consumer electronic devices. PHAs versatility makes it a perfect bioplastic material for caps, bottles, blister packs, and other containers. It may also be used in packaging applications for food-processing items such as bottles, laminated foils, fishnets, flower pots, sanitary products, fast foods, disposable cups, agricultural foils and textile fibers [45]. One of the most attractive characteristic of PHA is their biocompatibility and biodegradability being for this reason well tolerated by the mammalian cells and the human body. A wide range of studies have been performed on the application of the different forms of PHA as drug delivery vehicles. This biopolymers are also potential candidates for the production of scaffolds for tissue engineering [46]. Later this year, a new application for waste-derived PHA was conquered: self-healing concrete. M.Vermeer *et al.,* showed that a PHA-based healing agent can induce crack healing in concrete specimen, therefor reducing water permeability of the cracks[47].

At an industrial level Danimer Scientific (U.S.) is the largest player in the Polyhydroxyalkanoate market, with Nodax®trademark. In november of 2020, Danimer Scientific partnered with Eagle Beverage Produtcs to produce a PHA straw, that will be fully degrade in a matter of months [48]. Several other companies already produce PHA at industrial level, such as Biomer Inc. (Germany), TianAn Biologic (China) and Tianjin GreenBio Material Co.(China) [2].

Since more diversed biopolymers are arising and applications seem endless, the market for bioplastics is continuously growing. According to *Bioplastics market data 2020* from European Bioplastics, bioplastics represented 1% of the 368 million tones of plastic produced annually. However, it's expected in 5 years that bioplastics production capacity increase from 2.11 million tonnes to 2.87 [49].



(a) Global production capacity of biorelated polymers (by material type) in 2020. Adapted from [49].

(b) Biobased plastics by market segment in 2020. Adapted from [49].

Figure 1.4: Global production capacity of biorelated polymers by material type and biobased plastics distribution by market segment, in 2020. PE (polyehtylene), PET (polyethylene terephthalate), PA (polyamide), PP (polypropylene), PEF (polyethylene furanoate), PTT (polytrimethylene terephthalate), PBAT (polybutylene adipate-co-terephthalate), PBS (polybutylene succinate), PLA (polylactic acid), PHA (polyhydroxyalkanoate). *predicted to be available at commercial scale in 2023.

Bio-PA, starch blends and PLA represent the biggest fraction of the 2.11 million tonnes of bio polymers produced in 2020. While PHAs only account for a small proportion of global production capacity (1.7%), they are the polymer family that is expected to grow the most in the next five years. Although bioplastics are used with several applications, packaging remains the largest market segment for bioplastics, as it's possible to see inf Figure 1.4b [49]. For PHA to succeed in the market, the high cost associated to it's production must be tackled. In this context, production with MMCs will now be introduced.

1.4 PHA Production by MMC

1.4.1 Substrates

Unlike pure cultures, MMC prefere volatile fatty acids for PHA production, since they are readily available and quickly converted to PHA. Therefore one of the requirements for any possible feedstock is that it should be easily fermentable in order to fermentation products dominate in total soluble COD (Chemical Oxygen Demand) [43]. Around 40-48% of the total cost of PHA production can be attributed to substrate [50]. So waste feedstocks present themselves as great candidates for PHA production since they are free substrate (making the cost of the biopolymer cheaper) and turn the waste management easier for industries [51]. Depending on their origin, the waste streams have been classified as agro-alimentary, industrial not agro-alimentary, food, urban wastes treatment plant and synthetic substrate [50]. As an example, PHA production from rice bran, pea-shells ([52, 53]), grape pomace ([54]), candy bar factory waste water ([55]), cheese whey ([56]) and several others waste feedstocks have been studied.

Urban Wastes

Accordingly with the last estimation, 88 million tones of food waste are generated in the EU each year with associated costs estimated at 143€ billion. Generally, these wastes are characterized by both high moisture and high biodegradability, which brings inevitable environmental problems regarding its disposable: odours, fires, groundwater contamination, global climate changes, as well as an high associated disposal cost. For a better management, food waste should be firstly treated by biological treatments, anaerobic digestion or composting, and whenever unfeasible a thermal process, as incineration, should

occur, being landfilling seen as the last option [57]. Despite the high biological value of food waste, traditional management solutions do not consider it as a precious resource, adding only a small amount of value to the final product, however added value molecules, such as lactic acid, citric acid, sucinic acid, single cell oils, enzymes and polymers (such as PHA) were reported as an more rewarding production then compost or biomethane [58].

The organic fraction of municipal solid wastes (OFMSW), separatelly collected, is an abundant type of food waste that comes from households, restaurants, small businesses, yards and garden wastes, and it's composition depends on different factors such as season, climate, geographic location and others. However, it's mainly constituted by carbohydrates, proteins and lipids, posing as a substrate with high potential application in biotechnological processes [59, 60]. In fact, some studies described different methods for PHA production from the OFMSW, and related sources such as leachate, percolate, and a mixture of OFMSW with primary sludge [61]. Main characteristics and yields of this studies are presented in Table 1.3. In Table 1.2 the main characteristics of the substrates used in the studies described are presented.

The possibility to integrate PHA production by MMC, in infrastructures typically adopted for biowaste residuals and wastewater treatment is seen as an advantage to turn this technology more economically and environmentally sustainable [61]. Other factors that weigh in the integration of PHA production into existing industrial plants are seasonal availability and substrate variability [22]. OFMSW is available though the year, in more or less quantities, presenting as another advantage for scaling up. Regarding variability, it's known that substrate's composition should present minimal variability and allow stable storage, to ensure that final quality of the polymer is uniform and reproducible [36]. Although OFMSW presents some variations, as mentioned, its matrix can be considered constant. As a negative aspect, such complex waste streams can also contain non-fermentable components and compounds that have inhibiting effects on growth and production kinetics of microbes [62]. Hence, it is crucial to optimize upstream processing of feedstocks in order to provide highly concentrated carbon-rich feeding solutions with lower levels of inhibiting ingredients, being for this reason, the first step of PHA production by MMC an acidogenic fermentation, as explained next.

Table 1.2: Composition of OFMSW feedstocks and derivatives reported in literature for PHA production.

Date	**TSS (gTSS L^{-1})**	**COD_{TOT} (gCOD L^{-1})**	**COD_{SOL} (gCOD L^{-1})**	**COD_{VFA} (gCOD L^{-1})**	**Ammonia (mgN L^{-1})**	**Phosphate (mgP L^{-1})**	**Ref**
2017	—	0.12-0.13	—	—	46-58	3.1-5.6	[63]
2018	—	—	30.78	16.2 $\pm$0.5	27$\pm$3 (C)	—	[64]
2019	56$\pm$3 (A)	62 $\pm$5	20$\pm$1	2.5$\pm$0.5	342 $\pm$28	113 $\pm$7	[61]
2020	1.49 $\pm$ 0.77	—	5.78$\pm$1.13	0.50$\pm$0.13 (B)	622$\pm$159 (D)	20.9 $\pm$10.8	[65]
2020	64$\pm$2 (A)	—	36$\pm$2	31 $\pm$ 2	689$\pm$15	202$\pm$6	[66]

(A): in g/Kg
(B): in $gCOD_{VFA}/gCOD_{SOL}$
(C): in gTKN/gTS
(D): referes to TAN value

Table 1.3: Overview of achieved PHA content, yield, global productivity, specific storage rate and implemented conditions of operations on different types of waste streams.

Date	Substrate and Pre-treatment	Inocolum	SRT [(a)]	HRT [(a)]	Working Volume [(b)]	OLR [(c)]	Cycle Length [(d)]	q_{PHA}	$y_{PHA/s}$	PHA content	Global Produc.	Ref
2016	Lecheate after phosphate removal	Activated sludge from WWTP	1	1	2	—	12	0.86	0.1	0.29	—	[67]
2017	After percolation, centrifugation and ammonia stripping of the supernatant	Activated sludge from WWTP	5	1	0.8	1.3	12	—	0.66-1.13	0.41-0.48	4.3-7	[63]
2018	Lecheate after solid/liquid separation	Activated sludge from WWTP	1	1	100	3.4-2.0	6	0.67	0.33	0.38	1.09	[64]
2019	Squeezed OFMSW mixed with SS produced in the WWTP. After fermentation, centrifugation to reduce solids content	Activated sludge from WWTP	1	1	120	4.0	6	0.28-0.3	0.44-0.50	0.43-0.46	0.26-0.36	[61]
2020	Leachate, after 24h of settling, the supernatant was pumped	P. acidivorans, and activated sludge from WWTP	1	0.71	180		12	—	0.44	0.77	—	[65]
2020	Mixture of 30% v/v WAS and 70% v/v OFMSW, followed by a solid/liquid separation stage	Activated sludge from WWTP	HRT=SRT (1 or 2)		100	4.0	6	0.35	0.62	0.59	—	[66]

(a): in day^{-1}; **(b)**: in L; **(c)**: in gCOD L^{-1} d^{-1}; **(d)**: in h;
Specific PHA storage rate: $\mathbf{q_{PHA}}$, $gCOD_{PHA}$ $gCOD_X{}^{-1}$ h^{-1}; Storage yield: $\mathbf{y_{PHA/s}}$, $gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$;
PHA content, in g_{PHA} $g_{VSS}{}^{-1}$;
Global Productivity, in gPHA L^{-1} h^{-1}

1.4.2 3-stage process

PHA production by MMC usually takes place in three separate stages [13, 40, 51]:

- Acidogenic fermentation: raw complex substrates are fermented in order to obtain readily biodegradable organic matter, the fermentation products;
- Selection: by applying transient conditions, with the substrate obtained previously, is possible to select an enriched-culture of PHA-storing organisms;
- PHA accumulation: the enriched culture is fed with the fermentation products resulted in the first stage, aiming to maximize PHA production.

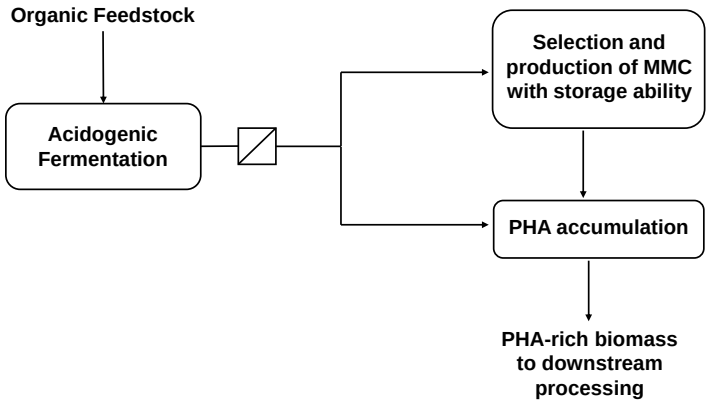


Figure 1.5: Schematic representation of the 3-stage process for PHA production with mixed microbial cultures. Adapted from [43].

Acidogenic Fermentation

Anaerobic Digestion (AD) for power supply dates in the 10th century B.C, allegedly. Four consecutive biochemical stages describe anaerobic digestion. First hydrolysis, where complex organic matter is converted into soluble molecules, as sugars, amino acids and long chain fatty acids. After, acidogenesis allows the conversion of previous molecules in volatile fatty acids, alcohols, carbon dioxide and hydrogen. This stages involve a consortium of obligate and facultative anaerobes. Subsequently, in acetogenesis, acetate, H_2 and CO_2 are formed. In the end, this products are converted in methane and water [68]. Therefore to enhance the production of volatile fatty acids is necessary to suppress the last two stages of anaerobic digestion, favouring acidogenisis. VFA are short-chain fatty acids consisting of six or fewer carbon atoms which can be distilled at atmospheric pressure, being the most common acetic, propionic and butyric acids [69]. However, PHA enrichment and production was already reported with substrates such as methanol in pulp, paper mill foul condensate, hardwood spent sulfite liquor. These are already enriched in readly biodegradable organic matter, not being required this first step of PHA production [43].

Parameters, such as type of substrates, temperature, pH, retention time (HRT/SRT), and organic loading rate influence the type of volatile acids produced [70], which ultimately influences the type of

polymer produced. A recent study, reviewed data from 551 experiments (where almost half of the experiments were performed using food waste, followed by protein-rich substrates and sludge), and concluded that feed concentration and residence time had the most important effect on VFA production, higher product concentration and productivity are related and higher productivities where achieved at an HRT of 2.5 days [71]. Methanogenic bacteria can be prevented by using low SRT, low pH, low temperature or a combination of different approaches [43].

A.R. Gouveia *et al.* with cheese whey as feedstock were able to tailor PHA composition through the manipulation of pH of the acidogenic reactor [72]. Using cheese whey wastewater and paper mill effluent, in batch tests, S. Bengtsson *et al* tested different HRTs and pH, obtaining acetate, propionate and butyrate with different profiles since a higher percecentage of propionate was achieved with higher HRTs.

In summary, if acidogenic fermentation is the first stage of PHA production, controlling process conditions is one way of affecting the properties of the produced PHA polymer. On the other hand, fermentation products resulting from this stage should dominate in total and soluble COD. An high ratio between them enables to achieve high conversion rates and yields of COD to PHA, moreover the presence of non solubilized fermentation products may affect the following aerobic selective pressure [64, 43]

Culture selection

The selection stage aims to obtained a culture with high PHA storage capacity at the highest possible rate [32], since the better the MMC selection the higher the PHA production in the following step [73]. As mentioned previously by engineering the ecosystem, PHA can be produced under dynamic conditions, such as cyclic feast-famine regimes [36]. The most common types of enrichment are anaerobic/aerobic (AN/AE) and aerobic dynamic feeding (ADF). To increase biological phosphate removal and PHA production yield, AN/AE strategy was extensively reported in the literature. Polyphosphate Accumulating Organisms (PAOs) and Glycogen Accumulating Organisms (GAOs) accumulate PHA during the anaerobic phase, where the electron acceptor becomes limiting [13, 74]. Under ADF conditions (or feast-famine FF regime) the limiting factor is substrate availability.

A feast phase is a period characterized by an abundance in carbon source whereas famine by a lack of substrate. Hence, microorganisms have to adapt to survive and growth under the lack of substrate and compete on the occasions where substrate is available. During the feast phase, bacteria that are able to convert carbon into PHA present a competitive advantage over the non-PHA storing organisms since they can use PHA as carbon and energy reserve and external nitrogen to grow in the famine phase. Organisms with no storage capacity are unable to survive and ultimately are eliminated. During starvation periods, the activity of cells is reduced to a minimum, since a considerable amount of RNA and enzymes are necessary and might not be available during this period. This means that growth is inhibited in the next feast period and cell physiological adaptation is needed to reach maximum growth rates. However, PHA synthase remains active and substrate uptake is mainly driven toward polymer storage during these adaptation phase. This internal limitation is the key selective pressure to enrich the reactor and higher PHA contents are obtained when compared to AN/AE enrichment strategy [75, 43, 13, 32].

Usually, selection stage occurs in a selection batch reactor (SBR) and is influenced by several parameters, such as feast to famine ratio (F/F), organic loading rate (OLR), sludge retention times (SRT) and C/N ratio, which will be described in the next paragraphs.

A feast to famine ratio (F/F) up to 0.2-0.3 was proven to allow for a efficient enrichment [75, 73, 43]. However, F/F ratio varies greatly from one study to another, especially because of different substrate type and concentrations used. The length of feast phase must ensure complete substrate depletion and the length of famine phase should allow a significant consumption of the accumulate PHA, promoting

a strong internal limitation and a accumulation over growth response in the next feast phase [51]. Low F/F ratios are obtained by implementing low OLR or long cycle lengths. Regarding OLR, it's increase is in principle an advantage for the process productivity, since biomass concentration also increases. However, it's possible that at higher loading rates there is not enough selective pressure to enrich the -biomass [76]. OLR from 1.8 to 31.25 gCOD L^{-1} d^{-1}were already reported in literature studies [32].

Sludge retention time also plays an important role, since a lower SRT will lead to lower storage yields, since higher fraction of substrate is used for growth. This parameter influences F/F ratio and the weeding out effect and Chen, Z., *et al* stated that these two effects exist simultaneously and compete with each other [77]. In literature, high enriched PHA cultures were selected under a SRT of 1 day and 10 days, so it's inconclusive what's the best strategy to adopted for a better selection [32].

During the selection stage proper growth has to occur, so all nutrients required for that must be available, such as nitrogen (N) and phosphorus (P). Different strategies have been implement regarding nitrogen limitation and availability during feast/famine phase. Generally, studies have found that the polymer storage ability of the biomass can be improved under dynamic conditions with nitrogen deficiency, when compared to a nutrient excess scenario [78, 32]. The objective of nutrient limitation is to shift the metabolic pathways from cell growth towards PHA production, since under N limitation, protein synthesis decreases and the carbon flow is redirected to PHA synthesis [79]. So, in general, low carbon to nitrogen ratio (C/N) ratio is beneficial for cell growth while higher C/N ratios boost PHA accumulation [80]. Regarding feeding strategies, nitrogen can be added when the carbon source (in the feast phase) or uncoupled (in the famine phase). The latter will create a double growth limitation, since it creates a phase with carbon availability and nitrogen absence (feast phase) and a famine phase with intracellular carbon availability and external nitrogen availability. This strategy showed improvements in PHA production [73] and brings the advantage of implementing higher OLRs without compromising selection efficiency [75]. Some waste streams, such as cheese whey, some activated wastewater sludge and OFMSW and derivates contain the necessary nutrient with acceptable C/N/P ratio and supply the medium for cell growth [50], eliminating the associated costs of supplementation. Other authors have proposed the mixture of waste carbon sources to optimize the final substrate, for example Basset *et al.* showed that PHA production yield was increased when OFMSW was added to the wastewater [81].

In summary, after an effective selection stage, characterized by the growth of a robust culture with high PHA storage capability, the next step is maximize PHA production.

Accumulation

The last stage for PHA production from MMC is aimed at achieve the maximum capacity production of the enriched culture, ideally with little to no consumption of PHA. This step reflects not only the optimization of the accumulation step as well as the efficiency of the strategy applied in the previous step [33]. The output of this stage has been related to the final biomass PHA content, which should be as high as possible to improve the economy in the downstream processing [82].

Different feeding strategies are reported in literature, such as pulse fed-batch, continuous feeding and variations of this ones [13]. Regarding nutrient supply, literature is not yet consensual on the best strategy to apply. Nitrogen starvation has generally led to higher saturation levels of PHA content in the biomass [83, 84]. On the other hand, studies have shown that the role of nitrogen during the accumulation stage is secondary, since PHA storage was preferred over growth [85, 86]. The idea of limiting PHA accumulation with nitrogen is to prevent the growth of flanking populations. Based on fed-on-demand, Valentino *et al.* reached an optimal range of combined N and P limitation with N/C from 2 to 15 mg/g and P/C from 0.5 to 3 mg/g that considered to offer improvement of productivity, when compared to nutrient starvation. Due to active biomass growth of the PHA storing biomass, productivity increased without an

observed risk of a growth response overtaking PHA storage activity [87]. These results are very promising for PHA production using waste feedstocks as carbon sources, since, as mentioned, many are far from nutrient-limiting.

1.5 Pilot Scale Productions

Prior to production in large scale, tests in pilot scale need to be done. The next table presents the achievements of some pilot scale facilities (adding to the ones expressed in table 1.3) and a short description of the main characteristics is provide, based on [13]:

- Few studies were developed to the production of PHA with MMC, and only after 2010 gained the desired attention;
- Generally, effluents/feedstocks were always fermented prior to PHA production;
- It was made an effort to integrate this 3-stage process in a existing industry, in order to reduce the production costs;
- Mass transfer limitations, feedstock variability and other factors are responsible for lower PHA yields and cell contents when compared to those reported in lab-scale experiments.

Table 1.4: Examples of pilot scale projects for PHA production with different waste streams. * Yield calculated on a COD basis, in $gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$.

Pilot Plant, Location	Feedstock	Origin of MMC and Enrichment Strategy	$Y_{P/S}{}^{*}$	PHA Content (%)	HB:HV Ratio (%w/w)	Ref
Nagpur, India	Pre-fermented milk and ice cream processing wastewater	Activated Sludge	0.425	39-43	—	[88]
Eslov, Sweden	Beet processed water, 38% in VFA	PHA producing MMC from pre-fermented effluent of Procordia Foods	—	60	85:15	[89]
Brussels, North WWTP, Belgium	Pre-hydrolyzed and fermented WWTP sludge	Sludge under anerobic feast famine	0.25-0.38	27-38	66-74:26-34	[90]
Leerwarden WWTP, Friesland, Netherlands	Fermented residuals from green-house tomato production	Sludge under anoxic feast/ aerobic famine	0.30-0.39	34-42	51-58:42:49	[91]
Mars company, Veghel, Netherlands	Fermented wastewater from a candy bar factory	Activated sludge under aerobic feast famine with inhibitor of nitrification	0.30	70-76	84:16	[55]

Several European projects, at pilot scale demonstrate the feasibility of PHA production by MMCs with waste feedstocks. The PHARIO project, from the Dutch Water Authorities and industrial partner,

achieved a routine kilogram scale PHA production over 10 months. Additionally, life cycle analysis performed indicated that this project has a 70% lower environmental impact compared with pure cultures production [51]. In response to the European Commission: Circular Economy Action Plan and European Plastics Strategy, several Horizon 2020 projects aiming at demonstrate PHA production at pilot scale have been funded. For example, RES-URBIS: REsources from URban BIo-waSte, in its 36-month report notes that production of PHA from organic waste is feasible and implementable in already existing industries [92]. Other H2020 projects are SMART Plant, that aims to recover PHA and struvite from enhanced biological phosphorous removal and the INCOVER, that intents to use the microalgae-bacteria platform and wastewater for also recovering PHA and phosphorous. Finally, YPACK (2019) project aimed at optimising food packaging applications of PHA [51].

1.5.1 Downstream process

Since PHAs are intracellular compounds, downstream processing constitutes a considerable cost factor in the polymer production process, particularly when mixed cultures and waste feedstocks are involved, not only because PHA has to be extracted from a more complex matrix but also because the polymer is considerably diluted as a result of the low cell densities attained during production with MMCs. It's estimated that downstream process can account for half of the production costs [93].

The applicability of a PHA recovery method depends on the several factors, such as the microbial production strain used, the type of PHA produced, PHA content, the required final product purity and others [94]. Generally, PHA downstream is based on the following steps: after fermentation biomass is separate from the broth. Centrifugation, filtration, sedimentation are the most common methods to achieve this. With the goal of increasing the permeability of the bacterial cells, a pre-treatment can be applied, such as heating, freezing, adding salts, grinding in liquid nitrogen and using hot compressed water. Then, pre-treated or not, biomass is subjected to an extraction process in which there is either a solubilization of cellular material surrounding the PHA, or solubilization of the polymer itself. In the end, PHA is subsequently separated from the disrupted cellular matter and purified [93]. For efficient polymer recovery, a high intracellular polymer fraction together with rather large PHA granules is beneficial to optimize the recovery process [95].

Regarding the extraction step, C. Pérez-Rivero, *et al.* review the lasted developments of this stage. The most common technique used is solvent extraction, however it's toxic for human and environmental [93]. The generation of hazardous wastes during extraction is contradictory to the environmentally friendly nature of the PHA production [80], therefore upcoming researches should focus on this aspect. Lastly, the level of purity is related to the intended use of the polymer [87], since, for example, PHA used in medical applications, should privileged high purity over price cost.

Chapter 2

Objectives

Nowadays, the production of new biobased-materials with similar properties and final applications as the based-fossil plastics is crucial. Polyhydroxyalkanoates are one of the most promising alternatives in this field due to its main characteristics: biobased, thermoplastic, biodegradable and biocompatible. Although from the environmental point of view, PHA-production overcomes the plastic accumulation and contributes for our planet sustainability, since they are biodegradable and produced from non-renewable sources, there is still the economical aspect on PHA production that need to be solved before they can be market competitive.

Several waste feedstocks were already implemented in a 3-stage production process of PHA with MMC. Some of these feedstocks are nitrogen deficient, requiring supplementation to promote cellular growth which leads to higher associated costs. On the other hand, there are feedstocks characterized by a high nitrogen content, which can promote a growth response over PHA storage, causing low polymer productivities. In this work, two feedstocks with complementary composition were studied: (1) Fermented fruit pulp waste (Feed-C); (2) Fermented food waste (Feed-N). Both feedstocks are rich in organic matter but different in nitrogen availability: Feed-C has no nitrogen content whereas Feed-N has a high nitrogen content. Taking advantage of the complementarity of the composition between the two wastes, an integrated process for PHA was developed. The main objective was to evaluate the possibility of replacing the synthetic nutrient solution by a low-cost feedstock rich in nitrogen, thus contributing for the reduction of the operational costs and ultimately to a more cost-effective process.

Hence, a MMC 3-stage process was implemented at pilot scale and the specific goals were:

- To characterize the Feed-C and *Feed-N* used as feedstock for PHA production;
- To operate a sequencing batch reactor (SBR) to select a PHA accumulating culture, using Feed-C and a chemically-defined solution of nutrients to enable growth. This study was used as reference;
- To implement a culture selection strategy by uncoupling feeding of Feed-C and Feed-N;
- To evaluate the efficiency and productivity of the culture to produce PHA, under the different operational conditions, through batch accumulation assays;
- To compare and evaluate both operational conditions with respect of PHA production process;

Chapter 3

Materials and Methods

3.1 Experimental Procedures

3.1.1 Experimental Set-Up

The experimental set-up is illustrated in Figure 3.1, consisting in an acidogenic fermentation followed by the selection of a PHA-accumulating culture and accumulation assays. From condition to condition, the experimental set-up suffered changes,in the selection stage, being for that identified with different colours.

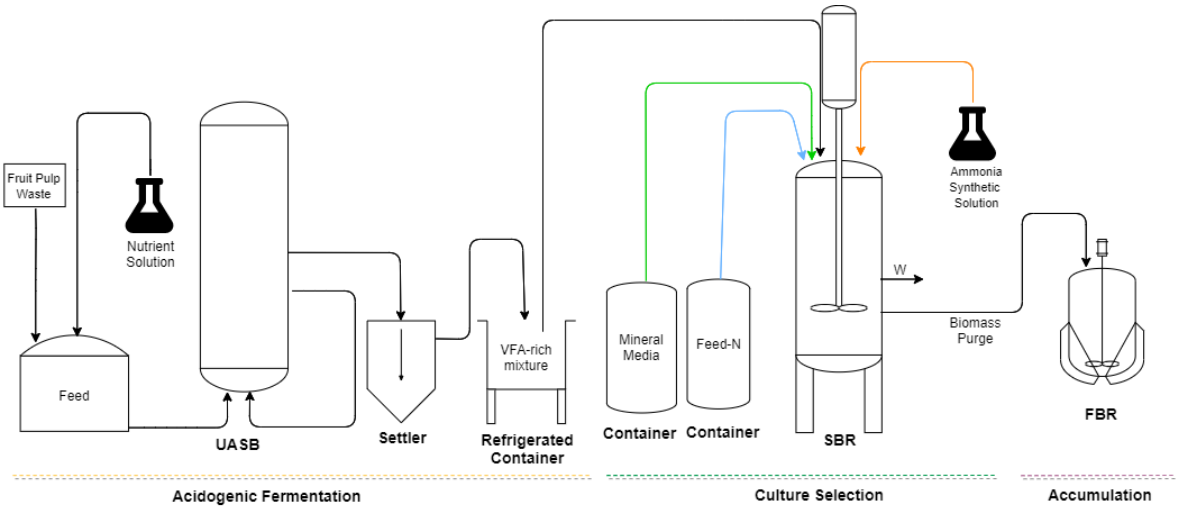


Figure 3.1: Experimental set-up of the 3-stage PHA production process. UASB - Upflow Anaerobic Sludge Blanket Reactor; SBR- Sequencing Batch Reactor; FBR - Fed Batch Reactor; W- Withdrawal. Condition 1 corresponds to green and orange lines. Condition 2 is represented by the blue line. Condition 3 takes into account blue and green lines.

3.1.2 Acidogenic Fermentation

The acidogenic fermentation stage took place in a 100-L acrylic UASB reactor, where fruit pulp waste provided by Sumol+Compal S.A. was used to produce a stream rich in fermentation products.

The feedstock was stored at -20℃ to prevent degradation and the UASB feed solution was prepared twice a week in a 500 L refrigerating tank. The fruit waste was diluted with tap water down to the desired

substrate concentration and the feed solution was supplemented with a nutrient solution, composed of: ammonium chloride, NH_4Cl, and potassium dihydrogen phosphate, KH_2PO_4 , in order to establish a COD:N:P ratio of 100:0.5:0.1. To maintain granules consistency, cellular structure and activity, calcium ($CaCl_2$: 480 mg L^{-1}), magnesium, ($MgSO_4.7H_2O$: 120 mg L^{-1}) and iron ($FeCl_3$: 80 mg L^{-1}) were also added to the medium.

The reactor was operated continuously with a working volume of 60L and a recirculation flow of 1.45 L min^{-1}. A MMC culture from the brewery industry (Super Bock Group, S.A.) of an anaerobic process was used to inoculate the reactor. Feeding and recirculation stream are located in the bottom part of the reactor, keeping the biomass granules in suspension, and the fermentation products outlet stream, in the upper part. During this operation an OLR of 20 gCOD L^{-1} $d^{-1}$was applied. The outlet stream suffered a settling by gravity, for solids removal, and after was kept in a refrigerator container ready to be used in the selection stage.

It's important to highlight that the described reactor was not operated by the author of this thesis, only being described to provide some notion of the process as a whole and to point out the influence of acidogenic fermentation in the selection and accumulation stages.

3.1.3 Culture Selection in SBR

The culture selection stage was conducted in a SBR with a total volume of 60 L. Due to the global pandemic COVID-19 and for safety reasons, was not possible to inoculate the reactor with activated sludge from WWTP. Therefore, a purge from a 100L SBR, operated under similar conditions, within the scope of GLOPACK project was used as inoculum. The GLOPACK reactor was inoculated before the global pandemic so no risk was taking when the purge was removed. The reactor was made of stainless steel, it has 12 holes, allowing the inlet and outlet of liquid, aeration and probes: dissolved oxygen and pH. The aeration was provided by a compressor and regulated by a flow meter (1 vvm) and a mechanical impeller allowed for stirring, ensuring aerobic conditions. DO levels were monitored through an immersion probe (ISM ®, Mettler Toledo) as well as pH. Temperature as control and kept between 18-22 ℃ using a thermostatic jacket. A computer software (BioCTR 59m) was used to acquire data from the probes, which allowed to monitor the different phases of the cycle, kept for 12 hours. Keeping in mind that the SBR was operated with nitrogen supply uncoupled from the main carbon source, the three operation condition imposed will be explained in detail. In all, the organic loading rate was constant and equal to 150 C-mmol L^{-1} d^{-1}, corresponding to 6 gCOD L^{-1} d^{-1}.

Condition 1

In a first approach the reactor was operated with a carbon rich feed, namely pulp fruit waste (Feed-C), and synthetic ammonia solution, within a working volume of 53L. The cycle had the following sequence: a feed phase where carbon (7.5L) and mineral medium (15.2L) were fed, a reaction phase where the carbon was consumed, a famine phase where only nitrogen feed was supplied (approximately 2 hours after carbon entrance, 0.3L), settling of the biomass and withdrawal phase. The reactor operated at two cycles per day, by the end of the night cycle, a biomass purge of 13.25 L was carried out and after a 45 minutes settling, a 9.75 L of supernatant was removed whereas in the morning cycle, the volume discarded was 23 L (supernatant). The total volume removed, in the form of purge and supernatant, was equal to the sum of the volumes added with carbon, mineral medium and nitrogen feed. Overall, this results in an SRT of 4 days and an HRTof 1 day. The reactor was under stirring and agitation during the entire cycle, except during the settling phase and supernatant withdrawn.

The stream from the UASB reactor, was supplemented with synthetic VFA, to adjust HB:HV precursors as well as the desired concentration of the feed, 600 Cmmol/L. pH of the carbon feed was adjusted

to 5, with NaOH (5M). Simultaneously with the carbon source, the mineral medium with the composition showed in Table 3.1 was added to the reactor. In this condition, a typical COD:N:P ratio of 100:6.5:1 (Cmmol:Nmmol:Pmmol) was achieved by adding a synthetic solution of ammonium chloride and potassium phosphate, which translates into a proportion of 75:4.875:0.75 (Cmmol:Nmmol:Pmmol) per cycle. Carbon feed and N/P solution were prepared every three days and mineral medium, twice a week.

Table 3.1: Mineral medium composition.

Reagent	Concentration (mg/L)
$FeCl_3 \cdot 6H_2O$	12
H_3BO_3	1.2
$CoCl_2 \cdot 6H_2O$	1.2
$MnCl_2 \cdot 4H_2O$	1
$ZnSO_4 \cdot 7H_2O$	1
$Na_2MoO_4 \cdot 2H_2O$	0.47
$CuSO_4 \cdot 5H_2O$	0.24
KI	0.24
EDTA	3395
$MgSO_4 \cdot 7H_2O$	2370
$CaCl_2 \cdot 2H_2O$	277

The SBR performance was monitored through weekly sampling: 5/6 samples during the feast phase, 1 sample right before nitrogen feeding and 4 samples during 90 minutes, covering the impact of the ammonia addition. In the remaining part of famine phase, at least 3 samples were taken. All the samples collected were analysed through GC for PHA quantification and composition. For total suspended solids and volatile suspended solids, roughly 3 to 4 samples were taken during feast phase, another 3 during nitrogen uptake and the same amount during the famine phase. The samples from the feast phase were analyzed through HPLC for VFA quantification, and samples after nitrogen feeding were analyzed in Skalar. The SBR was started-up on the 24th of June and this first condition remained until the 21st of July. Besides the monitoring, accumulation assays were performed in each condition, and will be further explained in the next subchapter.

Condition 2

In this condition, the reactor was operated with Feed-C, derived from UASB, and a feedstock provided by *Valorsul*, a nitrogen-rich food waste obtained by canteens and hotels, abbreviated by Feed-N. In the company, the food collected suffers hydrolysis, resulting in a VFA-rich stream, that also contained lipids, proteins and carbohydrates. After collection, the feedstock was stored in 10 L containers, some in a cold chamber (4℃) and others in the freezer (-20℃).

Although the SRT and HRT were kept equal to the previous condition, and the cycle preserved the same structure, the working volume of the reactor was changed to 40L. For this, volumes of withdrawal, purge and feeds were modified being respectively, 10L, 10L and 20L (sum of Feed-C, water and Feed-N). Every other cycle the reactor was purged, whenever it wasn't withdrawal was twice as large. A ratio of 100:7:1 Cmmol:Nmmol:Pmmol was applied and to maintained the desired OLR, the volumes of Feed-N and Feed-C were constantly adjusted. Feed-C was diluted with tap water in a 230L container and suffered the adjustments described in condition 1, prior to enter in the reactor. To meet the desired ratio, in Feed-N supplementation with monopotassium phosphate (KH_2PO_4) was necessary. pH control took place, being the maximum value 8.5, regulated by a pH pump connected to the computer software, adding 1.5M of H_2SO_4 whenever necessary.

SBR performance was monitored and characterized as refereed in condition 1 and Accumulation

assay tests were also performed.

Condition 3

This condition is similar to condition 2, regarding the use of Feed-C and Feed-N. However, feed C, was not diluted and when fed to the reactor, mineral media was fed simultaneously, as in condition 1. Since the SRT was kept constant but HRT was changed to 0.66 day, the frame of the cycle was changed. Two and a half hours after Feed-C was added, Feed-N was feed to the reactor. After two hours, a first settling phase took place (for about 1hour). After withdrawal of the supernatant, 10 L of mineral media were added. Apart from this alteration the rest of the volumes were kept equal to the ones in condition 2. Table 3.2 sums up the changes made in each condition.

Table 3.2: Comparison between SBR conditions and relevant parameters.

Parameters	Condition 1	Condition 2	Condition 3
OLR (C-mmol L^{-1} d^{-1})	150		
SRT (day)	4		
HRT (day)	1		0.66
Working Volume (L)	53	40	
Nitrogen Source	Synthetic Solution	Feed-N	
C:N:P Ratio (Cmmol:Nmmol:Pmmol)	100:6.5:1	100:7:1	
Day of operation	1^{st} - 26^{th}	43^{rd} - 89^{th}	106^{th} - 128^{th}
Mineral medium	Present	Absent	Present

Between conditions, SBR operation continued in unsteady conditions, explained in Chapter 4, therefore the conditions presented were the ones that gave stability to the reactor.

3.1.4 PHA accumulation tests

To assess the PHA accumulation capacity of the culture selected under each condition, in the SBR, batches tests were performed in a separated 1L reactor, BIOSTAT ® plus. Aeration was controlled by a flowmeter at 4 vvm, and a mechanical impeller allowed for stirring, ensuring once again aerobic conditions. This tests occurred when the reactor was considered stable, in each condition. A biomass purge from SBR was harvested and used to inoculate the reactor for each accumulation test (between 0.4 to 0.6L). In every experiment, two 1L reactors were operated in parallel, with the same conditions.

DO levels were acquired through a DO probe (ISM ®, Mettler Toledo) as well as pH. A pulse-wise strategy was implemented, controlled by DO concentration, since the rising of the DO indicated consumption of fermentation products, being a new pulse of carbon source fed to the reactor. This procedure was repeated during 5 to 8 hours. Temperature was controlled with a thermostatic jacket and kept at 20 ℃.

For condition 1, accumulation tests were only performed with one carbon source, Feed-C, on the contrary, for condition 2 and 3, were conducted with Feed-C and Feed-N, separately with each feed, being therefore realized in different days. Based on the organic loading rate applied in the SBR, and the value of volatile suspend solids of the nearest monitoring, a food to microorganism ratio was kept constant in each test.

The performance of the accumulation reactor was monitored through periodic samples in each pulse. For PHA quantification and substrate consumption, 6-7 samples were collected in the first two pulses, and in the rest only 3 (as soon as the pulse is fed, mid-time, and when DO rises). Suspended solids were measured in the beginning of the assay and every time a new pulse was injected.

Feed Composition

The composition in fermentation products of each Feed-C and N referred in the present work, in SBR monitoring and accumulation assays are present in the next table.

Table 3.3: Composition in concentration (gCOD L^{-1}) and in % COD of each feed used in the present work.

Sample I.D	Feed-C gCOD L^{-1}	Composition (% COD) Lact:Ace:Prop:EtoH:But: iVal:Val:Cap:Hep:Oct	Feed-N gCOD L^{-1}	Composition (% COD) Lact:Ace:Prop:EtoH:But: iVal:Val:Cap:Hep:Oct
Monitoring 15 th day	24.6	0:5:1:0:56:0:11:27:0:0	—	—
Monitoring 21 th day	23.5	0:2:0:0:68:0:15:15:0:0	—	—
Monitoring 68 th day	3.1	0:1:0:0:48:0:14:37:0:0	27.6	1:11:1:1:16:2:3:47:7:12
Monitoring 77 th day	17.1	1:8:2:0:55:0:10:24:0:0	32.9	1:10:2:0:16:2:3:44:3:19
Monitoring 120 th day	22.8	0:3:1:0:55:0:11:31:0:0	18.4	0:14:9:0:23:3:11:29:8:2
Monitoring 123 th day	28.3	0:2:0:0:62:0:12:24:0:0	22.1	0:18:1:0:29:3:11:30:8:0
Accumulation assay 1,2	22.4	0:3:0:0:52:0:13:32:0:0	—	—
Accumulation assay A,B	20.6	0:2:0:0:46:0:13:38:0:0	—	—-
Accumulation assay C,D	—	—	33.2	1: 10:1:0:16:2:3:42:6:20
Accumulation assay E,F	16.3	0:4:0:0:49:0:14:34:0:0	—	—
Accumulation assay G,H	—	—	27.5	0:13:9:1:24:3:9:28:11:2

3.2 Analytical Procedures

Whenever analytical procedures here described, require a filtration, with 0.2µm pore size filter, for Feed-N and reactor samples from condition 2 and 3, a prior filtration, with 0.45 µm pore size, was also applied.

3.2.1 Suspended Solids Determination

TSS concentrations were estimated according to standard procedure described in [96]. First, a g lass fibre filter (VWR, Glass fibre filters 47 mm, 1.2 µm) was placed in aluminium dish and weighed. With a vaccumm filtration system, samples are filtered and then placed in an oven at 105 ℃ overnight. The sample volume was fixed at 4 mL for condition 1, and due to higher viscosity, 2 mL for samples of condition 2, 3 and Feed-N. The weight of the dried sample matches TSS value. For VSS, the dried samples are reduced to ash in a muffle (Nabertherm, B 150) for 2 hours at 550 ℃. VSS concentration is calculated with the weight of the sample before and after ignition.

3.2.2 Protein Quantification

Protein content was determined with Lowry cholorometric assay, as described in [97]. Among other substances, the Folin phenol solution (Folin, Panreac), interacts with cuprous irons and the side chains of tyrosine, tryptophan, and cysteine to produce a blue-green color that can be detected at 750 nm in a spectrophotometer (Hach Lange DR 2800). A standard calibration curve of bovine serum albumin (BSA. 98%, Sigma Aldrich) was used to calculate protein concentration.

3.2.3 Total Sugars Content

Total sugars content was measured through the Dubois method [98]. Samples are incubated at room temperature with phenol 5% and concentrated H_2SO_4. Absorbance is measured at 490 nm and

total sugars concentration is estimated based on a standard calibration curve of D-glucose monohydrate (LABCHEM), in the range of 0-100 mg L^{-1}.

3.2.4 Fermentation Products Analysis

High performance liquid chromatography was used to measure the concentration of fermentation products, such as ethanol and organic acids (lactic, acetic, propionic, butyric, valeric, caproic, heptanoic and octanoic and iso forms). A VWR Hitachi Chromaster equipped with a RI detector 5450 and Diode Array Detector 5430, a Biorad Aminex HPX-87H 300x7.8MM column and a Biorad 125-0129 30x4.6mm pre-column was used for the current analysis. Sulphuric acid, H_2SO_4 0.01 N, was applied as eluent at a flow rate of 0.6 mL/min and temperature of 60 ℃. The concentration of the various fermentation products was estimated based on a standard calibration curve ranging between 4-1000 mg/L. For this analysis a filtration (0.2 µm pore size filter) is required.

3.2.5 Chemical Oxygen Demand

For Feed-N characterization, the total amount of organic matter was measured through chemical oxygen demand (COD). Using Hach Lange spectrophotometric kits, with a concentration range of 0-60 mg $L^{-1}O_2$, was possible to estimate total COD (samples wihtout treatment) and soluble COD (centrifuged and filtered samples). After sample preparation accordingly to kit instruction, digestion (in Hach Lange HT200S) at 148 ℃ for 15 min, and cool down to room temperature, COD was measured in a spectrophotometer (Hach Lange DR 2800).

3.2.6 Nitrogen and Phosphorus measurement

Ammonium and phosphate, NH_4^+ and PO_4^{2-} concentration were determined by a colorimetric method in a flow segmented analyzer (Skalar San ++, Skalar Analytical, Netherlands). A calibration curve of 4-20 mg $L^{-1}$of ammonium cloride, NH_4Cl 99%, Sigma, and ortho-phosphoric acid, H_3PO_4 85%, Panreac, allowed the estimation of nutrients concentration. Samples were diluted with mili-Q water and doubled filtered, when necessary.

3.2.7 PHA Quantification

Through gas chromatography (GC) PHA quantification and characterization (monomer identification) of all the collected samples was possible. With some slight changes, protocol was adapted from [99]. After centrifuging (11000 rpm, 3 min) and discard supernatant, samples were frozen with liquid nitrogen and freeze-dried overnight in a Telstar LyoQuest freeze dryer (-51 ℃, 0.09 mbar). Th resulting pellets were weighed (2-5 mg) in 15 mL tubes, where 2 mL of acid methanol (20% H_2SO_4) and 2 mL of chloroform were added. To improve accuracy and reduce error, heptadecanoate (HD), at around 1 g L^{-1}, was added to chloroform solution, acting as a internal standard. Tubes were placed in a digestor, for 4h at 100 ℃, for cell lysis, depolymerization of PHA in monomers and it's transesterification to occur. After cooling down to room temperature, 2 mL of distilled water is added to samples, and by means of a vortex mixer, samples are shaken quickly (30 seconds). A 10 minute waiting time allows the separation of two phases in the tube, having the organic one the desired monomers, being ready for extraction and to put in vials. For this step molecular sieves (0.3 nm, Merck) were used to absorb any residual water.

A gas chromatograph (430-GC, Bruker), equipped with a Restek column (60 m, 0,53 mm internal diameter, 1 µm film thickness, crossbond, Stabilwax) was used for quantification. The carrier gas was helium gas at a flow rate of 1 mL/min and a constant pressure of 14.50 psi. Each run lasted 32 minutes and a volume of 2 µL of sample was injected in the equipment. The temperature started at 40 ℃ and

rose at 20 ℃/min up to 100 ℃ (3 min), followed by a period during which temperature rose up to 155 ℃ at 3 ℃/min (18 min and 20 s), embracing the elution times of the hydroxyalkanoate monomers. Lastly, temperature was raised up to 220 ℃, again at 20 ℃/min (3 min and 15 s), and kept for 7 more minutes for cleaning.

Calibration standards were made using an Aldrich copolymer of P(3HB-co-3HV) containing 12%(HV) and 88% (HB) (%(w/w)) with concentrations between 0 and 6.3 g L^{-1}. Standards were prepared the same way as samples, and since HD was used as internal standard, calibration curves relate the ratio, between monomer and HD, of the peak area with the ratio of the mass.

3.2.8 Cell visualization

PHA-accumulating microorganisms were confirmed through staining with Nile Blue, according to [100]. A drop of Nile blue stain was added to about 1 mL of sample, which was then placed in a thermostatic bath at 55 ℃ for 15 min. After this incubation period, the sample was examined under a Olympus BX51 epifluorescence microscope at 1000X, equipped with an Olympus XM10 camera (Cell-F software) . With this method, intracellular PHA granules could be observed as bright orange fluorescence.

3.2.9 Microbial assessment

For the analysis of the dynamic of the bacterial community and study of the abundance of PHA accumulating microorganisms in the SBR, Fluorescence *in situ* Hybridization (FISH) was performed as previously described by [101]. In every condition, samples were collected from the SBR at the end of the feast phase and fixed with 4% paraformaldehyde (PFA, Gram-negative bacteria), according to [102]. It's important to refer that out of curiosity, a sample from Feed-N was also prepared and analyzed through this technique. This fixation begins with the addition of 1 mL of PFA to 0.5 mL of sample, incubation at 4℃, for 4 hours, centrifugation at 12782 x g rpm for 3 min, supernatant discarding, and wash the remaining pellet with 1 mL of phosphate-buffered saline (PBS). This washing step was repeated once more, and lastly, the cells were resuspende in 0.5 mL of absolute ethanol and stored in -20℃.

After, samples were applied to the wells of specific glass-slides for FISH analysis,identified and placed in an oven at 46 ℃ for 10 min. Next, samples suffered a dehydration in a grading series of ethanol solutions (50%, 80% and 98%) and were dried under compressed air. Hybridization buffer containing 2M NaCl, 10% sodium dodecyl sulphate (SDS), 1M Tris-HCl, formamide and miliQ water, pH 8, was applied to each well (8 µL). It's important to refer that the amount of formamide in the buffer was adjusted accordingly to the probes used and miliQ water was also adjusted to make a final volume of 2 mL. Then 0.7 µL of EUBMIX (see bellow) and 0.7 µL of the specific probe were added to each well. To end this step, each slide was placed in a falcon tube containing a moisturizing tissue with the remaining hybridization buffer and incubated in the oven at 46 ℃ for 1.5-3 hours.

Later, a washing step took place. The washing buffer (2M NaCl, 10% SDS, 1M Tris-HCl, 0.5M EDTA and miliQ water) was prepared in a 50 mL falcon tube and pre-heated to 48 ℃ in a thermostatic bath. The slides were then place in the buffer tube for 10-15 min, washed with mMiliQ water at 4 ℃ and dried with compressed air. In the end, vectahield mounting media was added to the dried slides (enough to cover all the wells). The slides were visualized using an epifluorescent microscope Zeiss Imager D2 at 1000 X.

The EUBMIX probe is a mixture of EUB338 [103], EUB338II, EUB338III [104], and it is used to differentiate biomass from detritus, staining all types of bacteria.This probe was labelled with fluorescein isothiocyanate (FitC), which emits in green light, and specific probes, presented in table 3.4, were labelled with Cyanine 3 (Cy3), which emits in red light when excited. As mentioned, all the probes used are referenced in Table 3.4.

Table 3.4: FISH probes used in this analysis.

Probe Name	Targeted Group	Reference
AMAR839	Amaricoccus	[105]
LGC0355	Firmicutes	[106]
Azo644	Azoarcus	[107]
THAU832	Thauera	[108]
LAMP444	Lampropedia	[109]
CF319a	Flavobacteria	[110]
Zra23a	Zoogloea	[111]
Meg938/1028*	Meganema	[112]
UCB823	Plasticicumulans acidivorans	[113]
PAR651	Paracoccus	[114]
HGC69A	Actinobacteria	[115]
ARC915	Archaea	[116]

3.3 Data Analysis

Feast/famine ratio (F/F, h h^{-1}) was determined as the ratio between the lengths of the feast phase and the famine. In condition 2 and 3, feast phase length is given by the sum of Feed-C and Feed-N feast length. Famine phase length is calculated by subtracting feast time to total cycle time. The OLR is determined by multiplying the concentration of fermentation products, in C-mmol L^{-1} d^{-1}or gCOD L^{-1} d^{-1}, by the flow (Q, Ld^{-1}) divided by unit of volume (V, L).

PHA content was calculated as a percentage of VSS on a mass basis, considering that VSS was constituted by cells (X) and PHA.

$$PHA(\%, g_{\text{PHA}}{g_{\text{VSS}}}^{-1}) = 100 \cdot \frac{PHA}{VSS} \tag{3.1}$$

The generic formula of $C_5H_7NO_2$ was considered [117], and a factor of 1.42 gCOD $X^{-1}$was considered. For PHA the sum of the respective monomers was considered, considering the following conversion factors for HB, HV and HHx, respectively: 1.47 $gCOD_{PHA}$ ${g_{PHA}}^{-1}$, 1.92 $gCOD_{PHA}$ ${g_{PHA}}^{-1}$ and 2.11$gCOD_{PHA}$ ${g_{PHA}}^{-1}$, based on the oxidation stoichiometry. Δ PHA content was determined as the PHA content in the end of an accumulation assay minus the PHA content at the begining of the cycle.

The specific substrate uptake rate, -q_S in $gCOD_{FP}$ ${gCOD_X}^{-1}$ $h^{-1}$was determined from the linear regression of concentrations plotted trough time, dividing by the X value in the beginning of the cycle. The same principle for specific ammonium uptake rate, -q_N in mgN ${gCOD_X}^{-1}$ h^{-1}, but the X value considered was the one in end of feast 1. For accumulations assays only the maximum values of this parameters were presented. For specific PHA storage rate the maximum value obtained was considered, q_{PHA} in $gCOD_{PHA}$ ${gCOD_X}^{-1}$ h^{-1}.

Storage yield, $Y_{PHA/S}$, in $gCOD_{PHA}$ ${gCOD_{FP}}^{-1}$, was calculated by dividing the amount of PHA formed by the amount of fermentation products consumed. Similarly, growth yield, $Y_{X/S}$ in $gCOD_X$ ${gCOD_{FP}}^{-1}$ was estimated with biomass concentration dividing by the fermentation products consumed. Global productivity, gPHA L^{-1} h^{-1}considered the final point of accumulations assays.

Chapter 4

Results and Discussion

The present study aimed at the production of PHA, by mixed microbial cultures, using pulp fruit waste (Feed-C) as nitrogen deficient feedstock and food waste (Feed-N) with high nitrogen content. First, a proper characterization of each feedstock is presented, summarizing its composition and potential functions in the established process. After, the selection of a mixed culture able to produce PHA was evaluated under 3 different operational conditions.

Condition 1 was based on the operation of the SBR using Feed-C and a chemically-defined solution of nutrients to enable growth. In Condition 2, the synthetic solution was replaced by Feed-N. Lastly, Condition 3 appears as an optimization of the previous condition, evaluating the influence of a lower HRT in the selection and accumulation process. In the end, a comparison between each condition is presented.

4.1 Feedstock Characterization

During the 130 days of operation, 3 batches of 150 L of Feed-N were received from Valorsul and although all were analysed by HPLC and skalar, only two of them were fully characterized. Table 4.1 presents a comparison between the two substrates used in the SBR, Feed-N and Feed-C, regarding TSS, protein (Lowry assay), total carbohydrates (Dubois method) and COD.

Table 4.1: Food waste (Feed-N) and pulp fruit waste (Feed-C) characterization.

Parameter	**Feed-N**	**Feed-C**
TSS (gTSS L^{-1})	18.2 ± 1.0	0.5
Protein (gCOD L^{-1})	12.7 ± 0.4	0
Carbohydrates (gCOD L^{-1})	3.3 ± 0.1	—
Fermentation Products (gCOD L^{-1})	29.4 ± 1.1	13.0
COD_{SOL} (gCOD L^{-1})	28.9 ± 0.4	14.2
COD_{FP}/COD_{SOL} (%)	97.9	91.5
COD_{TOT} (gCOD L^{-1})	56.0 ± 1.4	14.8
COD_{FP}/COD_{TOT} (%)	52.6	87.8
Ammonia (gN L^{-1})	2.1 ± 0.8	0
Phosphate (gP L^{-1})	0.3 ± 0.1	0

Comparing the two feedstock a lot of differences stand out. The amount of solids present in Feed-C is almost negligible, which suggests that fruit pulp can be seen as a ”clean” substrate. Another factor that contributes for this classification is the fact that fermentation products are roughly 88% of the total organic matter, indicating that acidogenesis stage was successfully performed. Taking these values into account, Feed-C presents as a good feedstock for PHA production. Due to the fact that no nitrogen nor

phosphate is present, in a reactor, bacterial growth would only occur if a synthetic solution of ammonia or another nitrogen rich feedstock was added.

In contrast, Feed-N presents more solids, and a higher percentage of non-fermented matter, since only 53% of total COD is composed by fermentation products. The concentration in fermentation products and the ratio between it and soluble COD is high, therefore this feed is source of fermentation products. Food waste is denominated as Feed-N since is characterized by a high nitrogen content, as presented in table 4.1. Therefore and in opposition to Feed-C, Feed-N can act as a nitrogen source and allow bacterial growth. It's known that OFMSW is composed of carbohydrate, protein, starch, fat, cellulose, lipids [60] and that their qualitative proportion is a function of origin and seasonality of waste collection. Since the sum, in gCOD L^{-1}, of proteins, carbohydrates and fermentation products doesn't justify total COD, it's possible that other components, such as lipids are present. Apart from the mentioned components, Zhang *et al* study showed the present of mineral salts, such as sulphur, calcium, magnesium and others, in a similar substrate with identical properties [118]. As such, and with the lack of analysis regarding these parameters, the presence of mineral salts is also considered.

4.1.1 Feedstock Storage

Previously to the reactor operation, an initial simple test was conducted to conclude if feedstock storing temperature affected the composition of Feed-N, regarding fermentation products, ammonia and phosphate. A 2L sample from *Valorsul* was collected and stored in the cold chamber (4℃) and at room temperature (22℃). After 3, 7, 10 and 28 days samples were collected from the two storage units and compared with the first one. The results are presented in Figure A.1 in Appendix A. It is possible to conclude, the trends for all the variables in study are quite similar in both temperatures. So, it was assumed that storage temperature didn't affect the composition of Feed-N samples. Therefore, for logistics aspects several containers were frozen (-20℃) and others stored in the cold chamber (4℃).

Before being fed to the reactor (in Condition 2 and 3), 2 containers of 10 L each were analyzed for ammonium, phosphate and fermentation products concentration. Figure 4.1 presents the referred values over the 130 days of operation. It's clear the variability from container to container and within batches.

Figure 4.2 shows the difference between the substrates in relation to fermentation products analysis. Feed-N presents as more diverse, due to the present of octanoic, heptanoic and a higher percentage of acetic and propionic acids in several samples. As illustrated, samples from the same batch (between dashed lines) don't present the same composition, in fermentation products, neither the same concentration of fermentation products (analysis of Figure 4.1). Minimal variability should be promoted, in order to ensure stable polymer quality [36].

The dashed line, in Figure 4.2a, indicates the change in batch for Feed-N. In the first, food waste was mostly composed by caproic, butyric and acetic acids, being the percentage of heptanoic and octanoic acid very low; in the second batch, heptanoic and octanoic acids are present in higher percentage, being caproic the dominant acid in the feed composition; the third batch was the most homogeneous, with a very low percentage of octanoic acid, and a higher percentage of propionic acid when compared with the other batches. Regarding Feed-C, it's possible to say that from sample to sample, the differences in VFA composition are minimal, being the feed mostly composed by butyric, caproic and valeric acids.

When compared with other studies for PHA production with OFMSW and derivatives (Table 1.2), soluble and total COD values are in agreement. Ammonia concentration seems slightly higher when compared to those, however the comparison is between feedstocks that suffer distinct pre-treatments. These pre-treatments were mostly aimed to reduce solid content and ammonia/phosphate removal,

therefore resulting in lower values. Regarding fermentation products distribution, the previously mentioned studies show a higher abundance of butyric acid, acetic acid, propionic acid, valeric acid and caproic acid, Valentino *et al.* also reported heptanoic acid in his study. As previously mentioned, FP distribution is directly related with the conditions implemented on the acidogenesis stage.

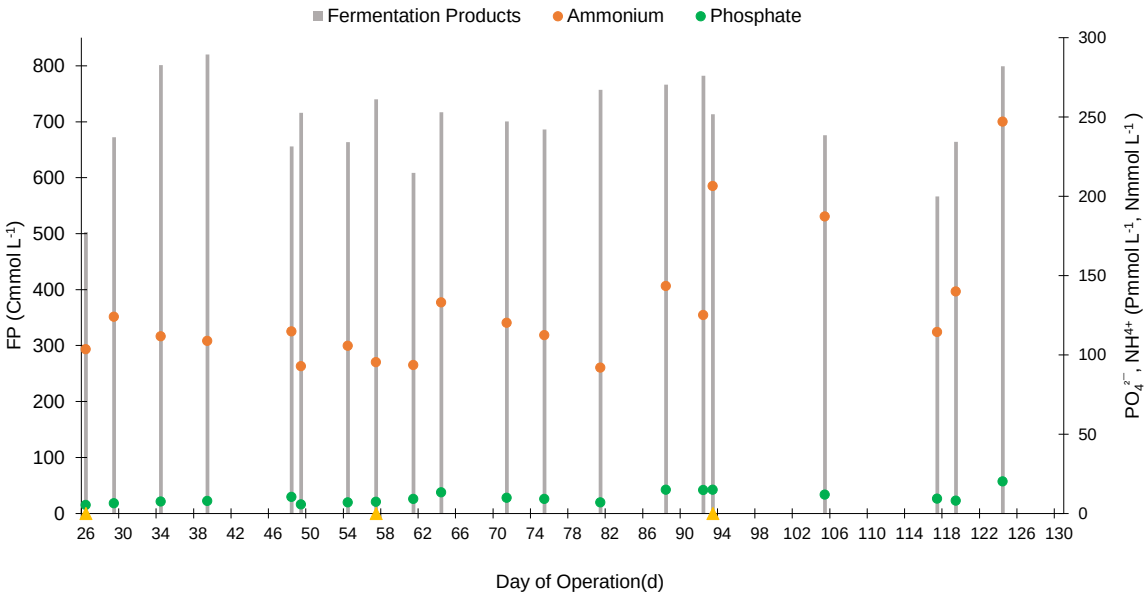
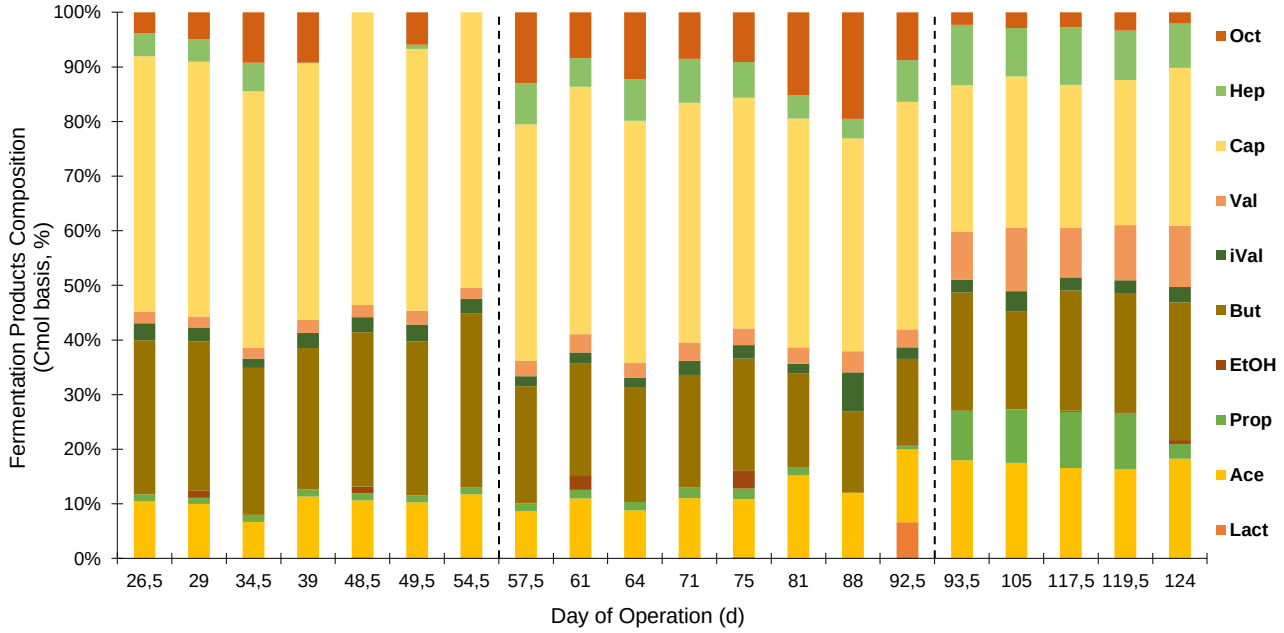
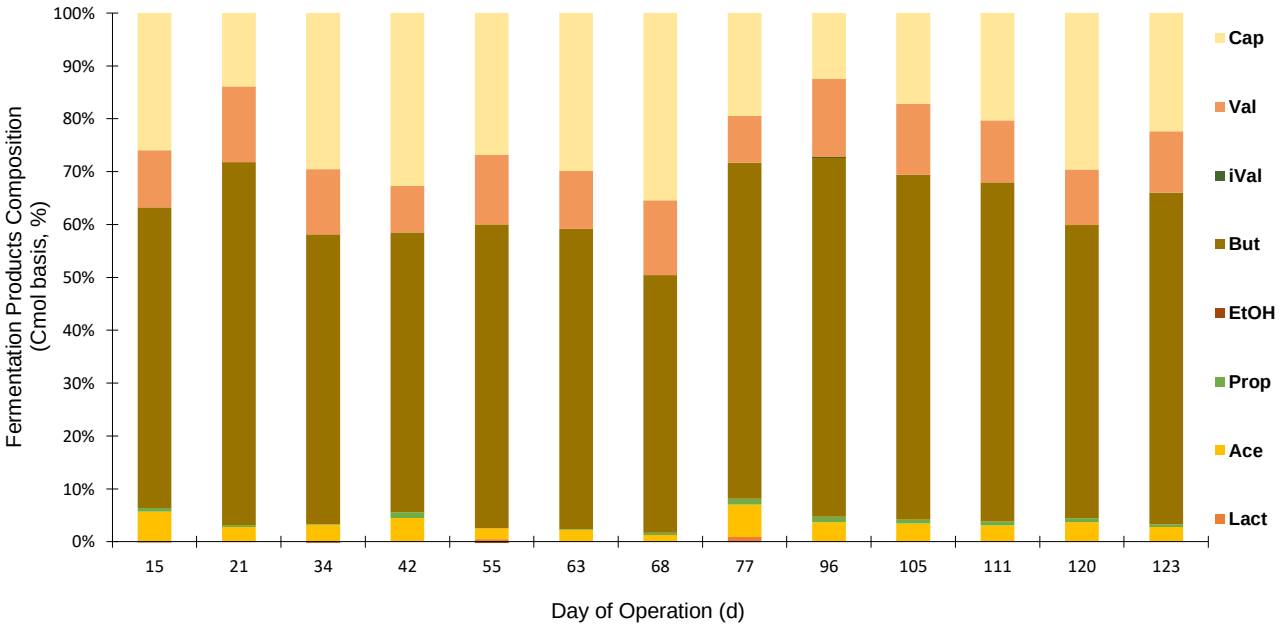


Figure 4.1: Fermentation products (Cmmol L^{-1}), ammonium (Nmmol L^{-1}) and phosphate (Pmmol L^{-1}) concentration of Feed-N prior to feeding. Changes in batch are marked in yellow.



(a) Food waste feedstock: composition on fermentation products (Cmol L^{-1}basis, %) over SBR operation.



(b) Pulp fruit feedstock: composition on fermentation products (Cmol L^{-1}basis, %) over SBR operation.

Figure 4.2: Fermentation products (Cmol basis, %), in terms of octanoic acid (Oct), heptanoic acid (Hep), caproic acid (Cap), valeric acid (Val), isovaleric acid (iVal), butyric acid (But), propionic acid (Prop), acetic (Ace) acid, and ethanol (EtOH) and lactate (Lact), of the two substrates used during SBR operation. The dashed black line marks the change of batch for Feed-N.

4.2 SBR Performance

In order to achieve a proper selection of PHA-accumulating bacteria with two waste feedstocks 3 operational conditions were tested: Condition 1, 2 and 3. A detailed experimental set-up and operational changes can be consulted in chapter 3. As previously mentioned, a SRT of 4 days and an HRT of 1 day were defined for Condition 1 and 2. For Condition 3, HRT was changed to 0.66 days. The OLR of the process was 143.9 $\pm$ 6.19 C-mmol L^{-1} d^{-1}, around 5.8 gCOD L^{-1} d^{-1}.

Adding up to the described parameters, that influence the selective pressure in favor of PHA-storing organisms, feast to famine length ratio (F/F) presents as one of the main factors, since low F/F favours PHA storage. Figure 4.3 shows the feast to famine ratio during the 130 days of SBR operation. Marked in green are the days where conditions were changed, the description of each phase is detailed in the next sections. These values were estimated based on DO profiles, and confirmed with data from the SBR monitoring cycles. With the addition of Feed-N to the process, feast phase length is given by the sum of the two feast phases (feast 1 for Feed-C and feast 2 for Feed-N), which brings an associated error since the DO profile is not as clear as for Feed-C. DO profiles and their analysis will be detailed in each condition.

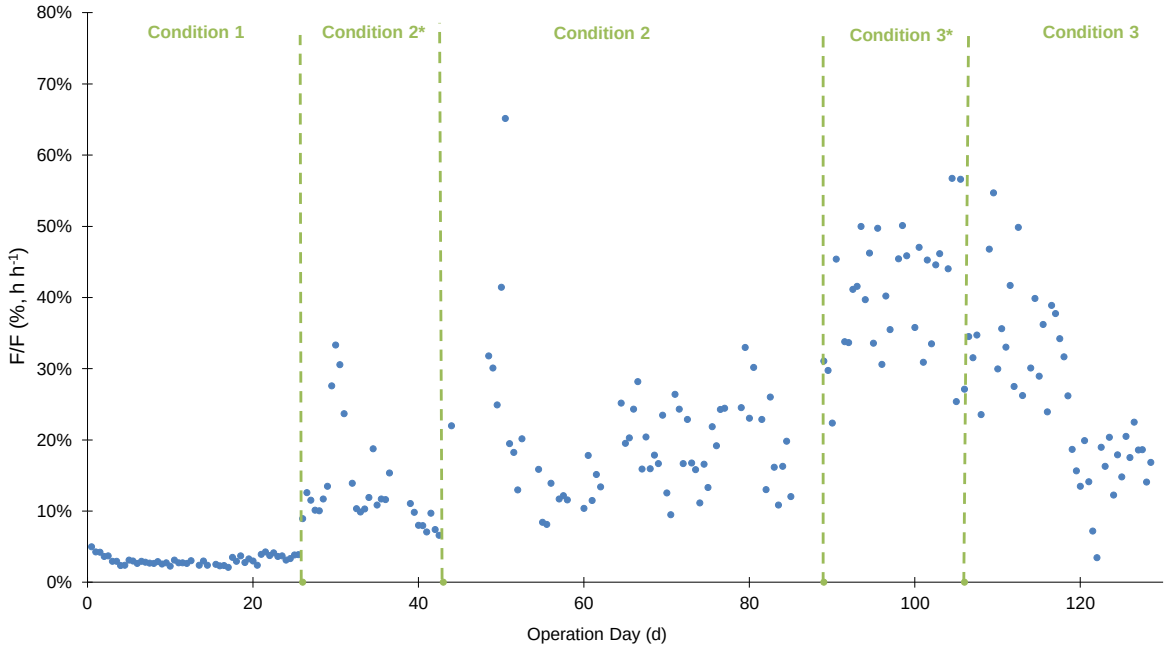


Figure 4.3: Feast to famine ratio (F/F, % h h^{-1}) over the operation days of the SBR, during the 3 studied conditions.

For a good enrichment of a MMC during selection stage, values of F/F lower then 0.2-0.3 (h h^{-1}) [75, 43] are required for inducing PHA storage, since an effective internal limitation is achieved through a long famine phase. In condition 1, these values were reached, SBR is considered stable due to the fact that variation in F/F and feast length is minimal from one day to the other. Condition 2* and 3* are transient states that lead to changes in operation, resulting in Condition 2 and 3, respectively. In the end of Condition 2, from the 67th day of operation forward, F/F presents values between 0.3 and 0.4, however when compared with Condition 1, data reproducibility is not achieved, but F/F ratio is lower then 0.4h h^{-1}. Condition 3 presents a similar behaviour, and after the 120th day of operation the values are within the same range (0.10-0.25 h h^{-1}). Since the biggest difference between condition 1 and the others is the source of nitrogen in the SBR, can be hypothesized that the values of feast N caused the instabilities

in F/F values. In Mulders *et al,.* a similar graphic is presented, with some variation in feast length during a considered ”stable” phase, and since only OFMSW lecheate was used, it can corroborate the obtained results.

In the next sections, the results for each condition are well detailed, taking into account data from cycle's monitoring and accumulation assays. These were collected after, at least, 3 SRT.

4.2.1 Condition 1

In this condition, SBR was operated with Feed-C and a chemically-defined solution of nutrients to enable growth as described in Chapter 3.1.3. Achieving a feast and famine regime it's fundamental to proper select a PHA storing culture, in the selection stage, and the DO profile presented in the Figure 4.4 proves it. When the substrate was fed to the reactor, during 10 minutes, a sudden decrease in the dissolved oxygen concentration, suggesting that fermentation products are being consumed. Once DO concentration sharply increases, it is assumed that carbon source is exhausted and famine phase is starting. The decrease in DO concentration after 2 hours of the new carbon feeding shows the uptake of the nitrogen by the cells. Literature proves that uncoupling the nitrogen from the carbon source allows a faster selection of a more efficient PHA storing culture [75], resulting in higher PHA contents at the end of the feast phase and restricting the growth of non-PHA accumulating bacteria [13]. In the end of the cycle, DO decreases again, during the settling phase and withdrawal removal, starting all over again 12h later, matching the end of a cycle with the beginning of a new one.

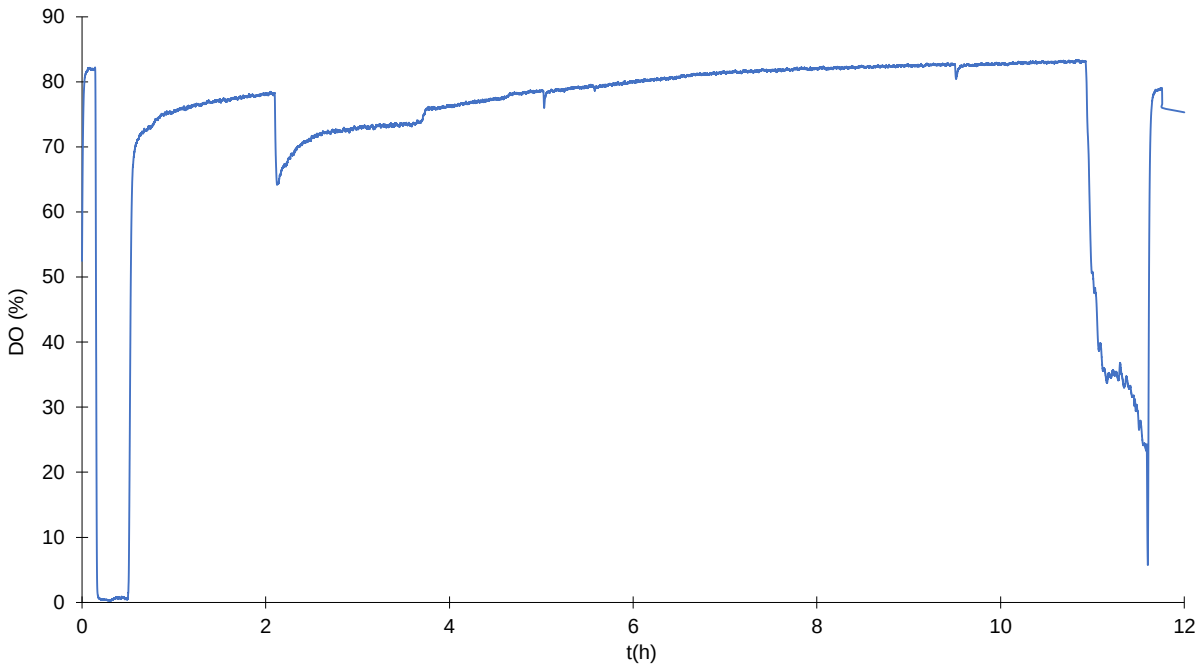


Figure 4.4: Profile of dissolved oxygen concentration (%) during a typical SBR cycle in condition 1.

As mentioned, each condition of operation was characterized by monitoring the cycle and accumulation tests, when the reactor was considered stable, after at least 3 SRT. For this condition the 15th and the 21th day were chosen to be monitored, and the stability of the reactor was achieved in few days due to the fact that the biomass used as an inoculum was already acclimatized with similar conditions. The F/F ratio are very low for both days, 2.4% and 3.3%, respectively, which according to literature it's favorable for PHA storage in feast phase [43, 75]. Figure 4.5 presents TSS/VSS and biomass (X) in g L^{-1}, NH_4^+ concentration in mgN L^{-1}, concentration of fermentation products (FP) in gCOD $L^{-1}$and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)) during the cycle of the 20th day of operation. VSS trend follows the expected: increase during the feast phase, due to the production of PHA, then decrease until the nitrogen is fed into the reactor, when this happens, biomass grows, increasing VSS. After ammonium consumption, during famine phase, VSS start to decrease, since PHA reserves serve as carbon and energy for cells. Butyric acid followed by caproic acid are preferential to the culture selected, since they present the higher -q_S rate, 1.78 $gCOD_{But}$ $gCOD_X{}^{-1}$ $h^{-1}$and 0.87$gCOD_{Cap}$ $gCOD_X{}^{-1}$ h^{-1}, respectively .

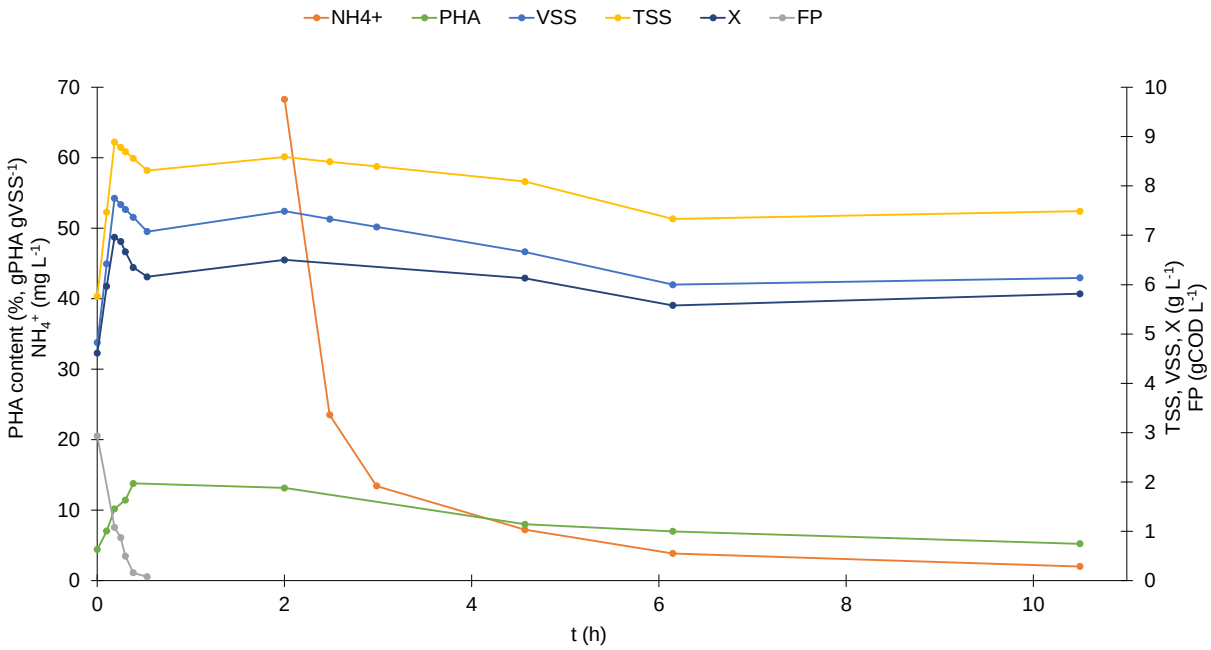


Figure 4.5: Trend of total and volatile suspend solids and biomass (TSS, VSS, X g L^{-1}), fermentation products (FP, gCOD L^{-1}), ammonium concentration (NH_4^+ (mgN L^{-1})) and PHA content (PHA, % (g_{PHA} $g_{VSS}{}^{-1}$)) during a cycle in condition 1.

Biomass concentration is given by subtracting the amount of PHA from VSS, increasing when nitrogen solution is fed to the reactor, since enables bacterial growth. Table 4.2 sums up the variation of several parameters that characterize condition 1. The ammonium uptake rate was estimated in the first 3 hours after the feeding.

Table 4.2: Average values (n=2) of total suspended solids concentration (TSS, g L^{-1}), biomass (X, g L^{-1}), PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)), fermentation products consumption rate ($gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1}), ammonium consumption rate (mgN $gCOD_X{}^{-1}$ h^{-1}), PHA composition, storage yield ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$) and growth yield ($gCOD_X$ $gCOD_{FP}{}^{-1}$) at three different times in SBR the cycle, for condition 1. (a): n=27; (b): maximum PHA content.

Parameter	Beginning of cycle	End of feast	End of cycle
TSS (g L^{-1})	6.80 $\pm$ 1.03	9.05 $\pm$ 0.74	8.31 $\pm$ 0.83
X (g L^{-1})	5.71 $\pm$ 1.10	7.26 $\pm$ 0.92	6.74 $\pm$ 0.93
Feast phase length (h)	—	0.37 $\pm$ 0.11[a]	—
-q_S ($gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1})	—	0.67 $\pm$ 0.14	—
-q_N (mgN $gCOD_X{}^{-1}$ h^{-1})	—	4.69 $\pm$ 1.67	—
PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	4.68	21.03 [b]	6.88
PHA composition (% HB: % HV: % HHx)	56:22:22	62:25:14	58:23:19
$Y_{PHA/S}$($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	—	0.45	—
$Y_{X/S}$($gCOD_X$ $gCOD_{FP}{}^{-1}$)	—	—	0.01

An high storage yield and lower growth yield were obtained, indicating that substrate is being used for PHA storage. It was obtained a polymer that contains HB, HV and HHx in its composition. Butyrate and acetate can be used for the production of PHB whereas propionate and valerate can be used for PHV production. Since Feed-C is composed mainly by the referred acids (see Figure 4.2b) the monomer composition presented is expected. The presence of 3-hydroxyhexanoate it's a reflection of the presence of caproate which is the precursor of this monomer.

Accumulation tests: 1,2

After the selection of PHA-accumulating bacteria in SBR, accumulation tests were performed, on the 16th day of operation. The SBR purge, from the night cycle was collected and used on the two reactors, described in Chapter 3.1.4, with the purpose of maximising PHA production. In every condition, 2 simultaneous accumulations assays were performed, but for simplicity only one will be totally described, however for calculations, both were taking into account. The accumulation test that will be described in detailed, in each condition, was performed always in the same reactor. Generally, the accumulation tests performed lasted for about 5h to 7h, in fed-batch mode being the substrate fed pulse-wise (in this case, 8 pulses were applied). Once the substrate is consumed, DO starts to increase and a new pulse is fed to the reactor.

Taking into consideration the organic loading rate, 6 gCOD L^{-1} d^{-1}, applied in the SBR, and the value of VSS of the end of the cycle of the nearest monitorization, a food to microorganism ratio was calculated (9.6 C-mmol $gVSS^{-1}$) and kept constant during all the assay. Figure 4.6 shows DO and pH variation, during the accumulation test, the global consumption of fermentation products (gCOD L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)).

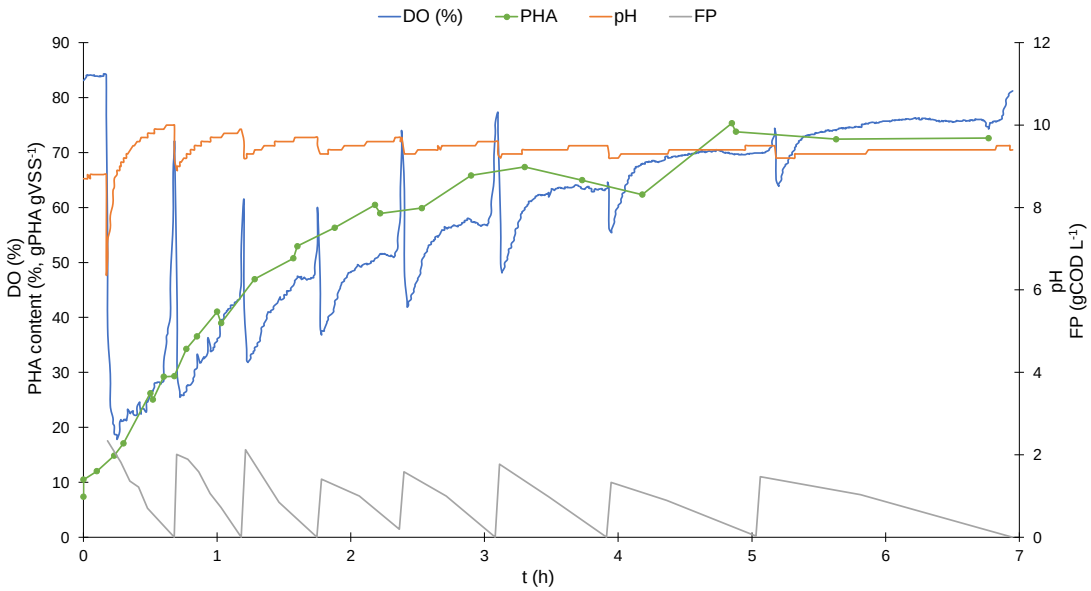


Figure 4.6: Trend of DO (%), pH, global fermentation products (gCOD L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)) throughout accumulation test of condition 1.

As expected, when the reactor is fed, DO drops, which corresponds to the consumption of fermentation products as can be seen in the Figure 4.6. Since the biomass went through a long famine period and consumed part of the PHA stored intracellular, the first and second pulse will present the highest consumption rate of fermentation products. Fermented consumption rates decrease, from -4.7 $gCOD_{FP}$ h^{-1} to -4.4 $gCOD_{FP}$ h^{-1} to -4.0 $gCOD_{FP}$ h^{-1} in the first three pulses, and similar in the next. After feeding the first pulse, at first the cells restore the levels of NADH and ATP, and only after NADH is in excess, consumption of FP is induced, which can explain why the first pulse sometimes is not the one with the higher consumption rate. As PHA saturation is reached, consumption of NADH decreases, resulting in a decrease on FP uptake [119]. In this cases, maximum substrate uptake rate of 1.37 $\pm$ 0.09 $gCOD_{FP}$ $gCOD_X{}^{-1}$ $h^{-1}$was achieved in the first pulse, which indicates that NADH levels were restored fast.

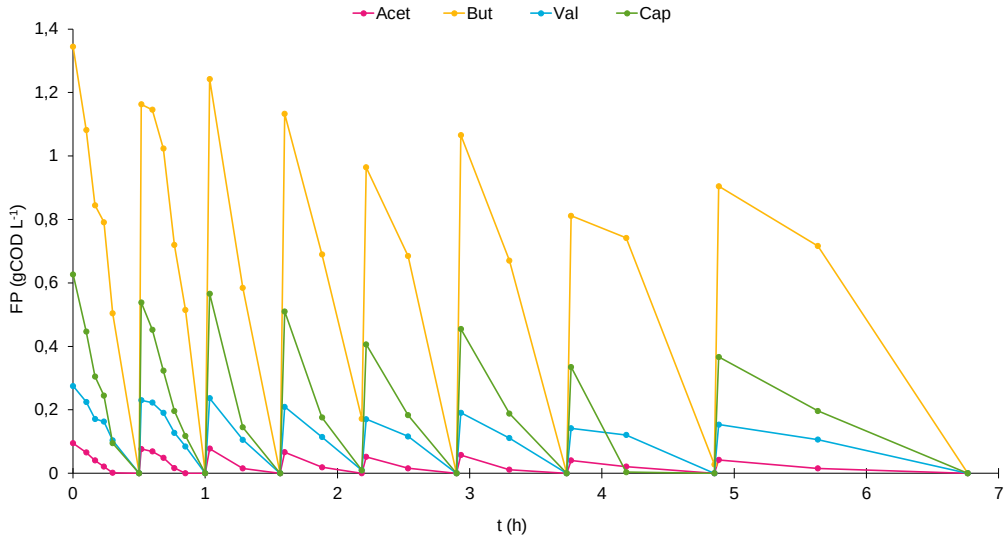


Figure 4.7: Trend of fermentation products, caproic (Cap), valeric (Val), butyric (But) and acetic (Ace) acids (concentration, gCOD L^{-1}) throughout accumulation test of condition 1.

PHA trend presented in figure 4.6, follows the expected: increasing over time. However in the last three pulses there's a sightly decrease, which can be explained by the fact that PHA-accumulating organisms reached the maximum storage capacity, and eventually started to consume the polymer. The highest PHA content of 75.33 (% (g_{PHA} $g_{VSS}{}^{-1}$)) was achieved after 4.85 hours of accumulation. For the assay performed in parallel, the maximum PHA content was obtained after 5.63 hours of accumulation and is roughly 55.92 % (g_{PHA} $g_{VSS}{}^{-1}$) . Figure 4.7 presents the trend of fermentation products during accumulation assays.

Table 4.3 shows an overview of the most important parameters that characterize condition 1. The values present some differences between tests which is expected due to operator error.

Table 4.3: Summary of parameters calculated for assays 1 and 2, for Condition 1: initial VSS, storage and growth yield, global productivity, maximum specific PHA storage rate, final PHA content, Δ PHA content and initial and final PHA composition.

Parameter	Test 1	Test 2	Average (n=2)
Initial VSS (g L^{-1})	7.40	6.47	6.94$\pm$ 0.46
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	0.67	0.50	0.59 $\pm$ 0.05
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}{}^{-1}$)	N.A	N.A	N.A
Global Productivity (gPHA L^{-1} h^{-1})	0.67	0.57	0.62 $\pm$ 0.05
Max q_{PHA} ($gCOD_{PHA}$ $gCOD_X{}^{-1}$ h^{-1})	0.15	0.09	0.12 $\pm$ 0.03
Final PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	72.63	55.23	63.93 $\pm$ 8.70
Δ PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	65.27	50.45	57.86 $\pm$7.41
Initial PHA composition (% HB: % HV: % HHx)	79:13:9		N.A
Final PHA composition (% HB: % HV: % HHx)	75:15:10	76:14:10	N.A

From the values obtained for Δ PHA and PHA content is possible to concluded that the culture was really subjected to the selective pressure of feast and famine regime and a culture enriched in PHA-accumulating organism was obtained. The selected biomass presents good and identical storing capacity, PHA composition is also very alike in both tests, being also present the HHx monomer. PHA production was reported with non-fermented apple fruit waste, at lab scale with pure cultures, from co-culture of *Cupriavidus Necator* and *Pseudomonas Citronellolis* by Rebocho *et al.*. Although studies reported in literature, at pilot scale, see Table 1.4, are mostly with processing wastewater, PHA production with MMC from fermented fruit waste presents storage yields and PHA contents within the expected, when compared with other studies at the same scale.

4.2.2 Condition 2

With the aim of achieving a PHA-accumulating culture, using two "real" feedstocks, after the stabilization with fruit pulp waste and proper characterization, the substrate provided by *Valorsul*, Feed-N was used as the nitrogen source of the process, also contributing to the organic loading rate. As reported in Table 4.1 Feed-N presents a more complex composition than Feed-C, since the amount of solids, proteins and other components are potential obstacles to biomass acclimatization. The volume of N-feed to be added depended on the ammonia concentration (in Nmmol L^{-1}), since the ratio 100:7:1 (Cmmol:Nmmol:Pmmol) was imposed. In this way, every time new Feed-N was fed to the reactor the volume was adjusted as well as the Feed-C volume, taking into account the OLR goal.

Condition 2*

In a first approach, during 17 days, from the 26th to the 42nd day of operation the SBR volume was 60L. On the first day after the replacement of ammonium source, the reactor started foaming a lot, resulting in biomass loss. Although an anti-foam strategy was applied, this was not enough to improve the reactor stability, in this period. The foam in the reactor was severed, which might be related to the complexity of Feed-N, and didn't allow to have an HRT and SRT of 1 and 4 days, respectively.

In order to improve reactor stability (condition 2) the working volume was decreased to 40L. Though the foam was not eliminated, an higher headspace was created an the biomass was not washed out from the reactor. Figure 4.3 shows that biomass acclimatization took several days, but the performance of the reactor was indeed improved comparing with condition 2*.

For Condition 2 characterization, the cycles monitored on the 68th and 77th day of operation were chosen. For simplicity both will be take into account for calculations but only the trends of one will be presented, namely the first one.

Figure 4.8 shows DO variation throughout a cycle, and although the behaviour is similar to the one presented in Figure 4.4, there are some differences. After feeding Feed-C, DO drops, indicating the depletion of fermentation products, then DO increases (until 80%), reaches a plateau and, increases in the end of the feast. This trend suggests that the consumption rate of some fermentation products in the feed it's higher then the others, as reported by [120, 121]. In the last increase, the residual of the fermentation products are consumed. DO decreases again, 2h after the first feeding, with the entrance of Feed-N. Comparing this drop with the one presented on figure 4.4, DO reaches a lower value, due to the fact that fermentation products are also being fed into the reactor. As in the first feast, the formation of tree levels is visible. Specific uptake rates were estimated and it's interesting to see that for the first feast, butyric acid and caproic acid are preferential, in this order, with 0.23 $\pm$ 0.02 $gCOD_{But}$ $gCOD_X{}^{-1}$ $h^{-1}$and 0.16 $\pm$ 0.04 $gCOD_{Cap}$ $gCOD_X{}^{-1}$ h^{-1}, and in the second feast the order changes. Caproic acid presents a specific susbtrate uptake rate of -0.08 $\pm$ 0.01 $gCOD_{Cap}$ $gCOD_X{}^{-1}$ $h^{-1}$and butyric acid one of 0.08 $\pm$ 0.01 $gCOD_{But}$ $gCOD_X{}^{-1}$ h^{-1}. The drop in DO profile, roughly 3h after the beginning of the cycle matches with the addition of anti-foam (the same for the visible drop around 6h). The last rise of DO, correlates with the depletion of all fermentation products. As mentioned above, the concentration of non-fermented organic matter in Feed-N is very high which are likely metabolized during the famine phase since DO doesn't arise to the value in the beginning of the cycle.

Figure 4.9 presents the trends of the most relevant parameters for condition 2. TSS, VSS and biomass (g L^{-1}), ammonium and fermentation products consumption over time (mgN L^{-1}, gCOD L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)). The most considerable difference between condition 1 and this, is the fact that, Feed-N, besides being a major source of nitrogen, also contributes to the organic loading rate of the reactor. Since two feasts occur, it's expected to see a similar tendency between them. In the end of the first feast, VSS, TSS and PHA content follow the trend described in condition 1. Maximum PHA content, roughly 14% occurs in the end of the first feast. Composition of Feed-C and Feed-N for this monitored cycle can be seen in Table 3.3.

As expected, 2h after Feed-C another consumption of fermentation products occurs, together with ammonium consumption (grey and orange line of Figure 4.9). After Feed-N feeding PHA content increases slightly and biomass grows, as indicated by the dark blue line.

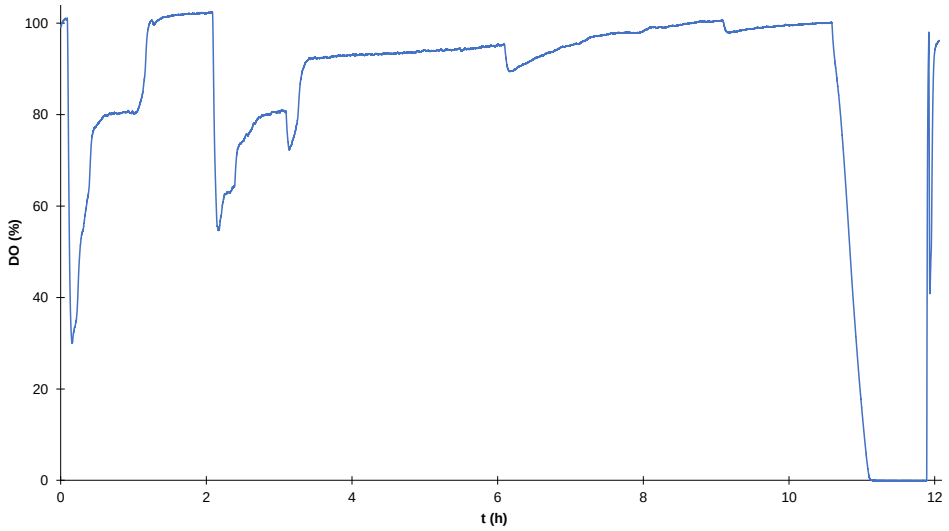


Figure 4.8: Profile of dissolved oxygen concentration (%) during a typical SBR cycle in condition 2.

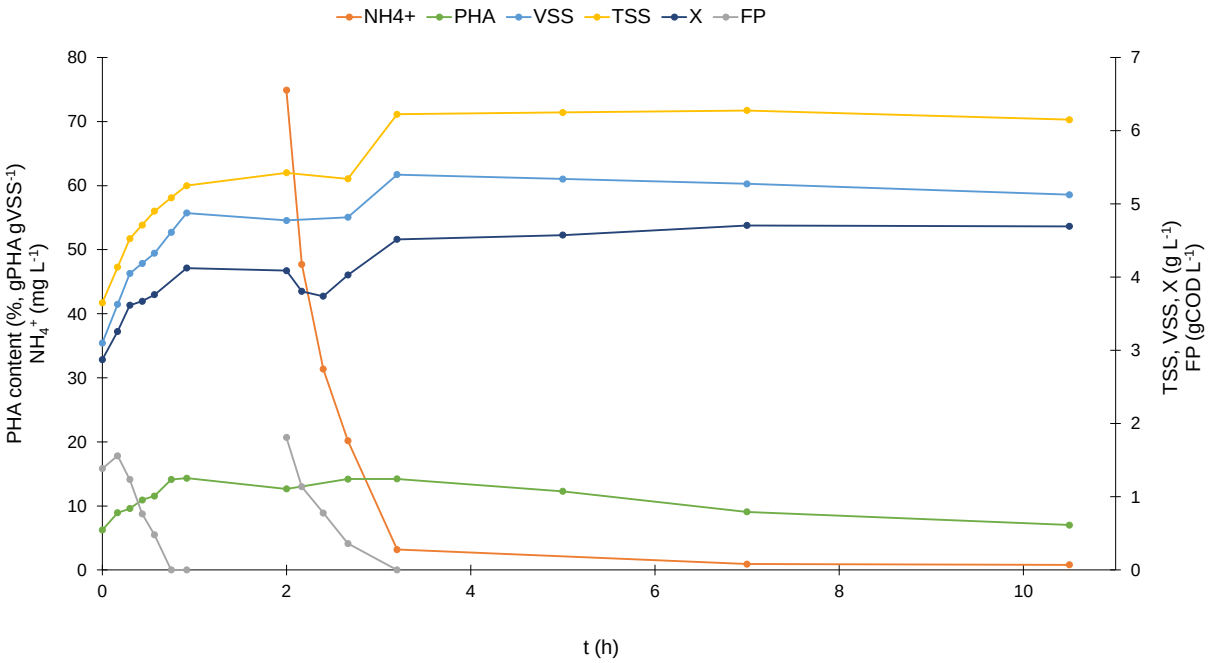


Figure 4.9: Trend of total and volatile suspend solids and biomass (TSS, VSS, X, g L^{-1}), fermentation products (FP, gCOD L^{-1}), ammonia concentration (NH_4^+, mgN L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)) during a cycle in condition 2.

Table 4.4 presents the values of several parameters during the cycle, for condition 2. Regarding TSS and biomass, lower values were achieved, when compared with condition 1. It's important to state that this condition took more time to stabilize, as can be seen in Figure 4.3. Substrate uptake rate for feast 1 is higher than feast 2, but lower than for condition 1. Ammonium uptake rate took in consideration the end of the feast, roughly 3.23 hours, similar to the 3 hours considered for the calculation in condition 1, being the value significantly higher.

Table 4.4: Average values (n=2) of total suspended solids concentration (TSS, g L^{-1}), biomass (X, g L^{-1}), PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)),substrate uptake rate ($gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1}), ammonium uptake rate (mgN $gCOD_X{}^{-1}$ h^{-1}), PHA composition and storage yield ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$) at three different times in SBR the cycle, for condition 2.

Parameter	Beginning of cycle	End of feast 1	End of feast 2	End of cycle
TSS (g L^{-1})	3.04 $\pm$ 0.61	4.53 $\pm$ 0.72	5.55 $\pm$ 0.68	5.47 $\pm$ 0.68
X (g L^{-1})	2.43 $\pm$ 0.44	3.40 $\pm$ 0.73	4.05 $\pm$ 0.47	4.33 $\pm$ 0.37
-q_S ($gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1})	—	0.47 $\pm$ 0.004	0.32 $\pm$ 0.08	—
-q_N (mgN $gCOD_X{}^{-1}$ h^{-1})	—	—	15.70 $\pm$ 1.90	—
Feast phase length (h)	—	1.01 $\pm$ 0.09 0.00	1.23 $\pm$ 0.03	—
PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	5.36 $\pm$ 0.87	16.24 $\pm$ 1.91	14.01 $\pm$ 0.20	6.06 $\pm$ 0.96
PHA composition (% HB: % HV: % HHx)	87:13:0	86:14:0	87:13:0	87:13:0
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	—	0.34 $\pm$ 0.001	0.04 $\pm$ 0.01	—
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}{}^{-1}$)	—	—	—	0.02 $\pm$ 0.01

Regarding storage yield, the values for both conditions and feasts with Feed-C are very similar. In condition 2, it's achieve a lower PHA content, indicating that biomass lost some ability to storage PHA. During the period of Condition 2, from the weekly monitoring, was estimated a loss of 4.28 $\pm$ 2.45 g $L^{-1}$of VSS, in the withdrawal phase. This explains why the values of biomass are so different between the end of the cycle and the beginning of another one. In condition 1, biomass values were much higher, being F/F ratio more constant and the SBR stable.

PHA composition is the biggest change when compared with condition 1, since the monomer HHx is no longer part of the polymer composition. As mentioned in section 1.3.2, substrate composition is key for the type of polymers formed. Therefore, it is hypothesized that the culture selected under these conditions, doesn't present class II PHA synthase, which is responsible for HHx synthesis. Apart from this hypothesis, it can also be speculated that some compound present in Feed-N (other than fermentation products) could influence monomer composition. With a similar substrate, in literature, only polymers composed by HB and HV were reported, which emphasizes the last option. In literature studies, with similar feedstocks, only HB and HV were present in the polymer obtained [65, 61, 63, 64].

For condition 2 characterization, accumulations assays were performed on the 70 th and 75th day of operation, with Feed-C and Feed-N. As in the previous described assays, the SBR purge was collected, and PHA production was maximized using the culture selected through condition 2. The two parallel assays were used for calculations, but only the trend of one will be presented. For simplicity, tests performed with Feed-C will be denominated A and B, and with Feed-N, C and D. The composition of each feed can be found in Table 3.3.

Accumulation tests: A, B

With the same pulse wise strategy, 5 pulses were fed to the reactor, over 8 hours. Considering an organic loading rate of 75 C-mmol L^{-1} $cycle^{-1}$and a value of 5.12 g $L^{-1}$for VSS, a food to microorganism ratio of 14.6 C-mmol $gVSS^{-1}$was kept constant during all the assay. DO variation follows the same trend as the one presented in Figure 4.6. Figure 4.10 presents the trend for PHA production and global fermentation products consumption over the assay.

PHA increases over time, decreasing in the last pulse, which is related to the fact that maximum consumption could already had been achieved. For test A, a maximum PHA content of 30.3% was obtained in the end of accumulation (7.93 h) and for test B the maximum achieved was 30.0% at 7.08

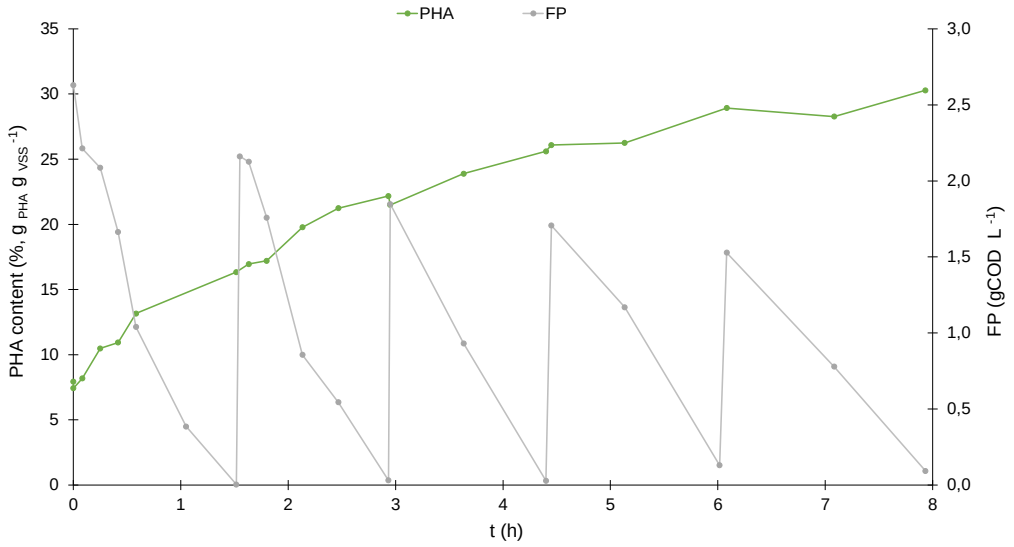


Figure 4.10: Trend of global fermentation products (gCOD L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)) throughout tests A and B for condition 2.

h. Maximum substrate uptake rate was -0.48 $\pm$ 0.05 $gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1}, achieved in the first pulse. This value is much lower when compared with accumulation tests 1 and 2, but is very close to the one in the first feast of the SBR (see Table 4.4). As for condition 1, Table 4.5 sums up the most important parameters for condition 2 with Feed-C as feedstock for accumulations.

Table 4.5: Summary of parameters calculated for assays A and B, for Condition 2: initial VSS, storage and growth yield, global productivity, maximum specific PHA storage rate, final PHA content, Δ PHA content and initial and final PHA composition.

Parameter	Test A	Test B	Average (n=2)
Initial VSS (g L^{-1})	4.79	4.73	4.76 $\pm$ 0.03
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	0.36	0.39	0.38 $\pm$ 0.02
$Y_{X/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$	N.A	N.A	N.A
Global Productivity (gPHA L^{-1} h^{-1})	0.27	0.24	0.25 $\pm$ 0.02
q_{PHA} ($gCOD_{PHA}$ $gCOD_X{}^{-1}$ h^{-1})	0.02	0.02	0.02 $\pm$ 0.002
Final PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	30.30	29.13	31.59 $\pm$ 2.46
Δ PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	24.95	20.04	22.49 $\pm$ 2.46
Initial PHA composition (% HB: % HV: % HHx)	85:15:0		N.A
Final PHA composition (% HB: % HV: % HHx)	88:12:0		N.A

In this condition, a lower initial cell density was used. The storage yield is very similar with condition 1, highlighting that the amount of substrate that is used for polymer storage is almost the same, besides the fact that condition was changed. A lower productivity and maximum specific PHA storage rate were achieved. Final PHA content was also lower when compared with condition 1. In that condition, higher values of VSS, therefore higher values of biomass were achieved, resulting also in higher values for accumulation performance parameters. Since the biggest difference from one condition to the other is the fact that two real feedstocks are used in the selection stage, it can be hypothesized that, in a first approach, Feed-N induces instability due to it's complexity (more variability in fermentation products, more solids, more non-organic matter), lowering specific PHA storage values and global productivity. Furthermore, the lower productivity can also be explained by a less efficiency on the culture selection than in the condition 1.

Accumulation tests: C, D

For a full characterization of condition 2, Feed-N was also used in order to maximise PHA production. The results obtained will be compared with the previous ones. Again, a pulse wide strategy was followed, and 6 pulses were fed to the reactor, over 6 hours. A ratio of 15.0 C-mmol $gVSS^{-1}$for F/M was considered and kept constant during all the test. DO (%) profile for this assay is presented in the Figure 4.11, along with the trend of fermentation products (gCOD L^{-1}). Although the sharp increase in DO was not so visible as in Figure 4.6, during the assay was decided to follow the same feeding strategy. As illustrated in Figure 4.11 global consumption of fermentation products occurred in the first pulse and a tendency for accumulate in the media took place in the rest.

Figure 4.12 shows that longer acids, such as octanoic acid, heptanoic acid, caporoic acid and isovaleric acid were consumed at a lower rate from the first pulse on, leading to some accumulation in the media. If a closer look is done, it's possible to realize butyric e acetic acids are entirely consumed, which can be related to the feed composition. Table B.1, in Appendix B, presents, for each pulse, the consumption rates of each fermentation product. Feed composition is depicted in Table 3.3. Since a total consumption of fermentation products didn't took place is not possible to ensure that PHA production was maximize.

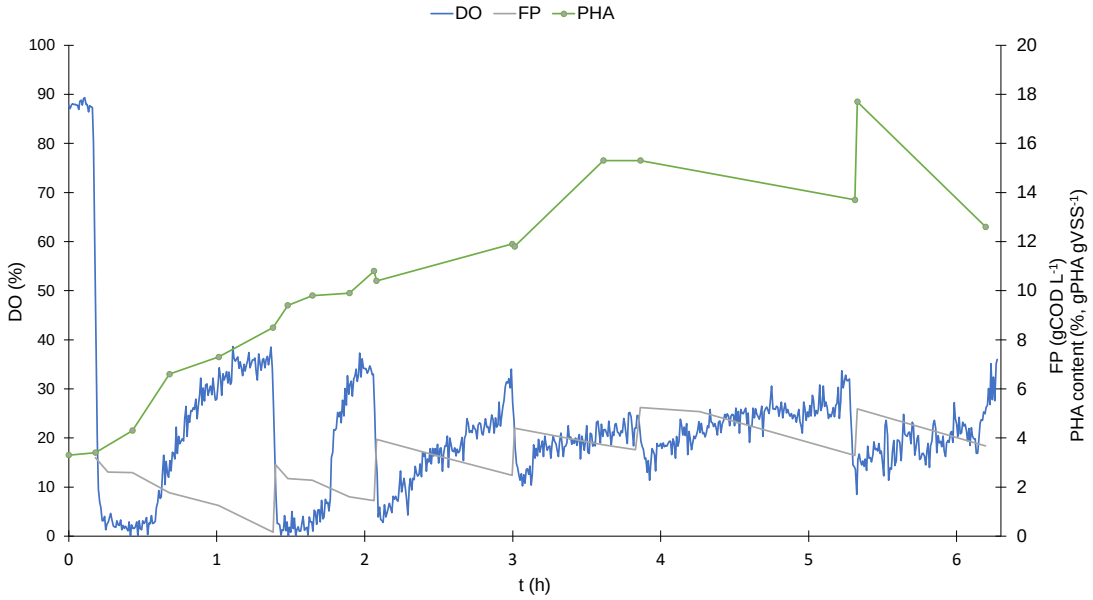


Figure 4.11: Trend of dissolved oxygen (%) global fermentation products (gCOD L^{-1}) and PHA content (% (g_{PHA} g_{VSS}^{-1})) through tests C and D for condition 2.

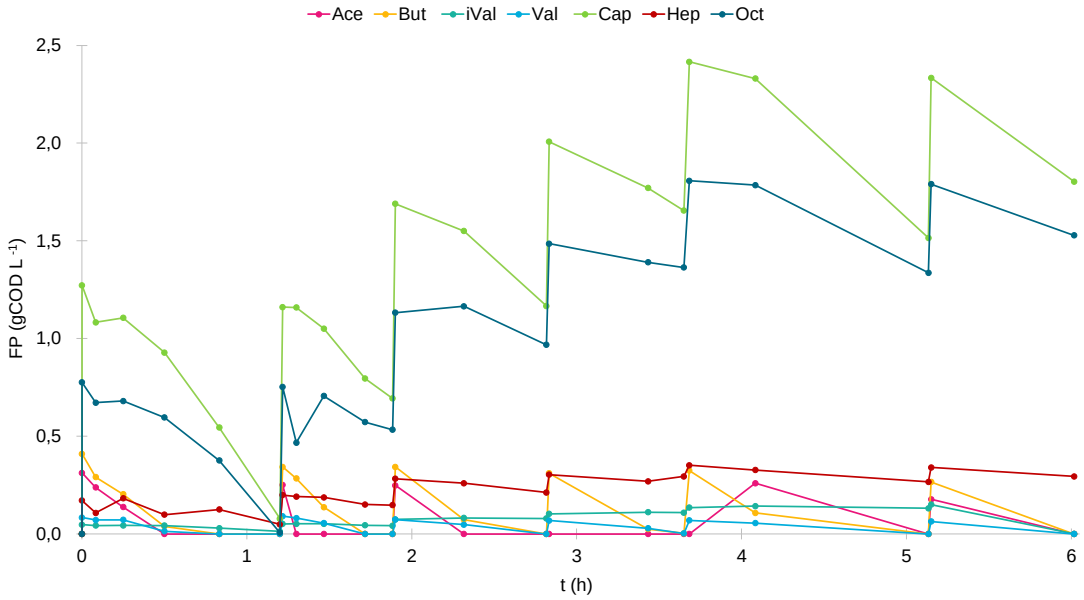


Figure 4.12: Trend of fermentation products (gCOD L^{-1}): acetic acid (Ace), butyric acid (But), iso-valeric acid (i-Val), valeric acid (Val), caproic acid (Cap), heptanoic acid (Hep) and octanoic acid (Oct) in test C, for Condition 2.

As in the previous accumulation assays presented, PHA trend follows the expected: increasing over time. For test C and D, a maximum PHA content was achieved at 5.13 hours, respectively, 15.0 and 16.8 % (g_{PHA} $g_{VSS}{}^{-1}$) . Maximum substrate uptake rate of -0.26$\pm$ 0.03 $gCOD_{FP}$ $gCOD_X{}^{-1}$ $h^{-1}$was achieve in the first pulse, lower than condition 1 and tests A and B, and in agreement with the data obtained for feast 2, in SBR selection (see Table 4.4). This can be related to the fact that an higher variability of fermentation products is present, when compared to the Feed-C used in tests 1 and 2, see Table 3.3. The feed used was mainly composed by caproic, octanoic and butyric acid and a major difference, in % COD, between the preferential acid (butyric) occurs between feeds, from 52% in tests 1,2 to 16% in this assay.

Table 4.6 presents the most relevant parameters for tests C and D. For the first time, a growth yield was estimated, due to the presence of ammonium in Feed-N. An ammonium uptake rate of -6.57 $\pm$ 0.42 mgN $gCOD_X{}^{-1}$ $h^{-1}$was obtained for the first pulse in tests C and D.

Table 4.6: Summary of parameters calculated for assays C and D, for Condition 2: initial VSS, storage and growth yield, global productivity, maximum specific PHA storage rate, final PHA content, Δ PHA content and initial and final PHA composition.

Parameter	Test C	Test D	Average
Initial VSS (g L^{-1})	6.29	6.57	6.43 $\pm$ 0.14
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	0.19	0.18	0.19 $\pm$ 0.005
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}{}^{-1}$)	0.17	0.12	0.14$\pm$ 0.02
Global Productivity (gPHA L^{-1} h^{-1})	0.22	0.24	0.23 $\pm$ 0.01
q_{PHA}($gCOD_{PHA}$ $gCOD_X{}^{-1}$ h^{-1})	0.02	0.03	0.02 $\pm$ 0.01
Final PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	13.93	15.98	14.95 $\pm$ 1.02
Δ PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	10.61	12.87	11.74 $\pm$ 1.13
Initial PHA composition (% HB: % HV: % HHx)	85:15:0		N.A
Final PHA composition (% HB: % HV: % HHx)	93:7:0		N.A

Comparing with test A and B, storage yield is much lower and similar to growth yield, pinpointing a growth response over a storage one. Specific storage rate was lower than in tests A and B, but, global productivity is very similar to the one obtained in previous assays, indicating that due to microbial growth

was possible to achieve comparable values. Several studies [85, 86], although with different substrates, paper mill wastewater and glycerol, have concluded that the role of nitrogen during the accumulation step does not influence PHA yield and content. This limitation is seen as a way to restrict the growth of non-storing bacteria, improving the selection under FF regime. Valentino *et al,.* demonstrated that ratios of N/C from 2 to 15 mg/g could improve productivity [87], since a biomass growth didn't overtake PHA storage capacity. In the accumulations performed a ratio of N/C of 47 mg/g was used and although global productivity didn't suffer much change, PHA content was lower compared to tests A and B.

One of the major differences is the difference between final and initial PHA composition. Feed composition in relation to fermentation products influences polymer composition. When compared with Feed-C composition in test A and B, Feed-N in test C and D presents a higher percentage of HB percursors, lactic, acetic and butyric acid (shown in Table 3.3. Therefore, an increase in HB monomer was expected.

4.2.3 Condition 3

With Condition 2 it was proven that the enrichment of a PHA-accumulating culture is feasible using two complex feedstocks, but it could be enhanced. If higher cell densities were obtained, PHA productivities could be further increase and comparable results to pure cultures could be reached [36], and for that, an improvement in stability of the proposed process is necessary. Condition 3 appears was an optimization of the previous one. As previously mentioned, around 4.28 $\pm$ 2.45 g $L^{-1}$of biomass were being lost in withdrawal phase. Adding to this, an analysis resembling the settling rate test (SV30) was performed several times, being inconsistent, which lead to conclude that settling was not efficient. The stability of the system can depend of several parameters such as the HRT. As an example, in Mulders *et al.,* with an HRT of 0.71 days, inert solids- who were not incorporated in the biomass and the compounds that would be slowly degrade in the famine phase - were removed in the settling phase, specifically in the supernatant. This approach proved that side population could be reduced and the system stability improved [65]. In this study, a decrease of HRT to 0.66 days was applied in order to improve the settling phase, and contribute to the reactor's stability.

Condition 3*

During 17 days, from the 89th to the 106th day of operation, two settling phases were performed during the SBR cycle, one 1h30 hours after feeding Feed-N and other in the end of the cycle, for about 1 hour each. After the first settling phase, 10L of tap water were added to the reactor, after the second a new cycle began and Feed-C was added. Figure 4.3 illustrates that feast phase length increased significantly, as well as F/F ratio, and sometimes was not even possible to assess its end through DO profile, since it coincided with the feeding of Feed-N. For this reason it was decided to feed Feed-N, 2.5 hours after Feed-C, instead of the 2 hours established for the other conditions.

Feast phase increased because biomass concentration decreased in this period. In the beginning of the cycle, VSS was 2.43 $\pm$ 0.19 g L^{-1}, in the end of each feast, 1 for Feed-C and 2 for Feed-N, respectively, 3.59 $\pm$ 0.09 g $L^{-1}$and 4.24 $\pm$ 0.21 g $L^{-1}$and in the end of the cycle 2.90 $\pm$ 0.10 g L^{-1}. When compared to previously values lower cell densities were achieved. It was hypothesized that micronutrients fundamental for cellular activity, were being washed out. Therefore, not being assimilated by the culture, resulting in low cell density values. In order to overcome this limitation, a mineral media, with the composition reported in Table 3.1 was added, replacing tap water.

Similar to Condition 1 and 2, Condition 3 was characterized through 2 cycles monitoring, from the 120th and 123rd days of operation. For calculations both were considered but only the trends of one will

be presented. First, the DO profile for a typical SBR cycle in Condition 3 is presented. Although very similar to Figure 4.8, since a settling phase occurs 1h30 hours after Feed-N feeding, air and agitation are turned off, causing DO to drop, turning on again 1h after settling and withdrawal phase. Figure 4.13 presents the DO profile referred.

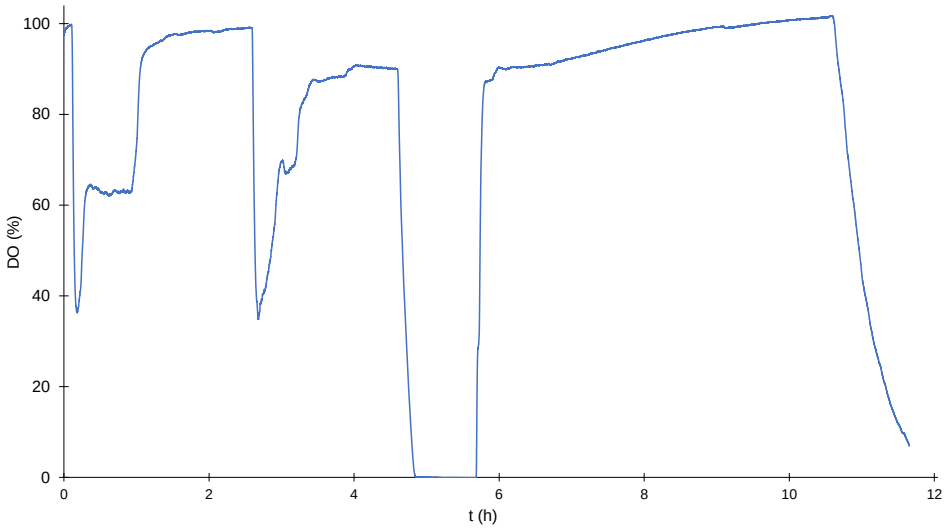


Figure 4.13: Profile of dissolved oxygen concentration (%) during a typical SBR cycle in condition 3.

In Figure 4.14, a typical trend for VSS, TSS, X, PHA, NH_4^+ and FP, in condition 3 is presented. Higher values of TSS, VSS and biomass were achieved, when compared with condition 2. A similar trend occurs in FP and ammonium consumption. Regarding PHA content, the value obtained is higher. A higher ammonium uptake rate was obtained, when compared to condition 2. Since the process is nitrogen limited [99] the competition is based on ammonium uptake rate, hence if in this condition the value is higher, a better selection is occurring. As for the other conditions, a higher production of PHA occurs in the first feast. Only after some consumption of PHA in the 2nd feast, the biomass presents some growth, suggesting that PHA producers are competing with other culture. Non-PHA producers likely uptake carbon from Feed-N, whereas PHA-accumulators consume PHA stored intracellularly in order to consume ammonium to grow, and fermentation products, presenting an advantage over the non-producers. If the slopes of NH_4^+ and FP were compared, in the second feast, it's possible to deduce that consumption speed is higher for ammonium.

Table 4.7 summarizes the most relevant parameters for condition 3 during a typical SBR cycle. PHA trend for condition 3 is similar to previous ones, being mostly produced in the first feast and consumed in the second. The PHA content obtained in the first feast is much higher when compared with the other conditions described.

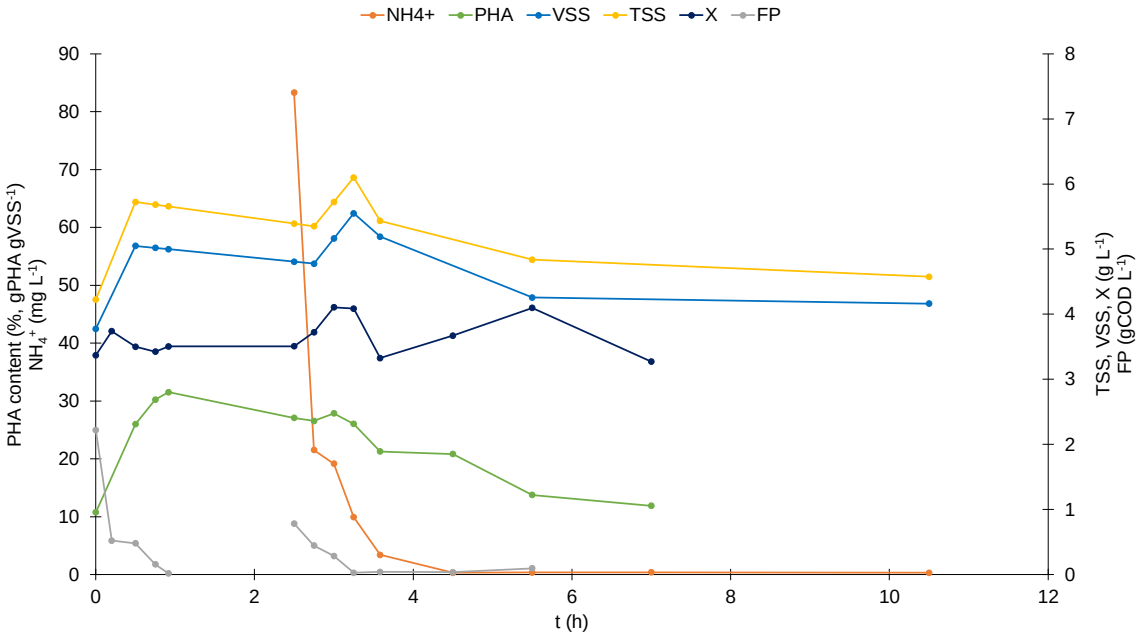


Figure 4.14: Trend of total and volatile suspend solids and active biomass (TSS, VSS, X, g L^{-1}), fermentation products (FP, gCOD L^{-1}), ammonium concentration (NH_4^+, Nmmol L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)) during a cycle in condition 3.

Table 4.7: Average values (n=2) of total suspended solids concentration (TSS, g L^{-1}), biomass (X, g L^{-1}), PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)), substrate uptake rate ($gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1}), ammonium uptake rate (mgN $gCOD_X{}^{-1}$ h^{-1}), PHA composition and storage yield ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$) at three different times in SBR the cycle, for condition 3. a: maximum PHA content.

Parameter	Beginning of cycle	End of feast 1	End of feast 2	End of cycle
TSS (g L^{-1})	3.71 $\pm$ 0.52	6.26 $\pm$ 0.61	4.81 $\pm$ 0.31	5.44 $\pm$ 0.86
X (g L^{-1})	2.90 $\pm$ 0.24	3.19 $\pm$ 0.23	3.79 $\pm$ 0.04	3.38 $\pm$ 0.11
Feast phase length (h)	—	1.02 $\pm$ 0.10	1.17 $\pm$ 0.09	—
-q_S ($gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1})	—	0.50 $\pm$ 0.09	0.143 $\pm$ 0.001	—
-q_N (mgN $gCOD_X{}^{-1}$ h^{-1})	—	—	12.39 $\pm$ 0.57	—
PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	11.66 $\pm$ 0.90	32.14 [a] $\pm$ 0.62	26.63 $\pm$ 5.36	22.24 $\pm$ 1.42
PHA composition (% HB: % HV: % HHx)	78:22:0			
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	—	0.31 $\pm$ 0.13	—	—
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}{}^{-1}$)	—	—	—	0.05 $\pm$ 0.02

Condition 3 accumulations tests were performed on the 125 [th] and 126 [th] day of operation, with Feed-C and Feed-N. In each day, PHA production was maximized using the culture enriched under condition 3 (from SBR purge). Two parallel assays were performed and used for calculations, although only one of the trends will be used. For simplicity, tests performed with Feed-C will be denominated E and F, and with Feed-N, G and H. Once again, feed composition is detailed in Table 3.3.

Accumulation tests: E,F

During 7 hours, 4 pulses of Feed-C were fed to the reactor, keeping a F/M ratio of 17.0 C-mmol $gVSS^{-1}$. DO profile is similar to the previous ones, with a sharp increase seconds before another pulse is added, indicating consumption of fermentation products. Figure 4.15 presents the variation of total fermentation products and PHA content during test E. Maximum PHA content of 53% (g_{PHA} $g_{VSS}{}^{-1}$) was achieved in the end of the assay, 7.22 hours in both tests. Maximum substrate uptake rate, - q_S was 0.25$\pm$ 0.02 $gCOD_{FP}$ $gCOD_X{}^{-1}$ $h^{-1}$and achieved in the first pulse. Similarly, Table 4.8 presents the values for the most important parameters of the accumulation tests performed with Feed-C and a culture selected under Condition 3.

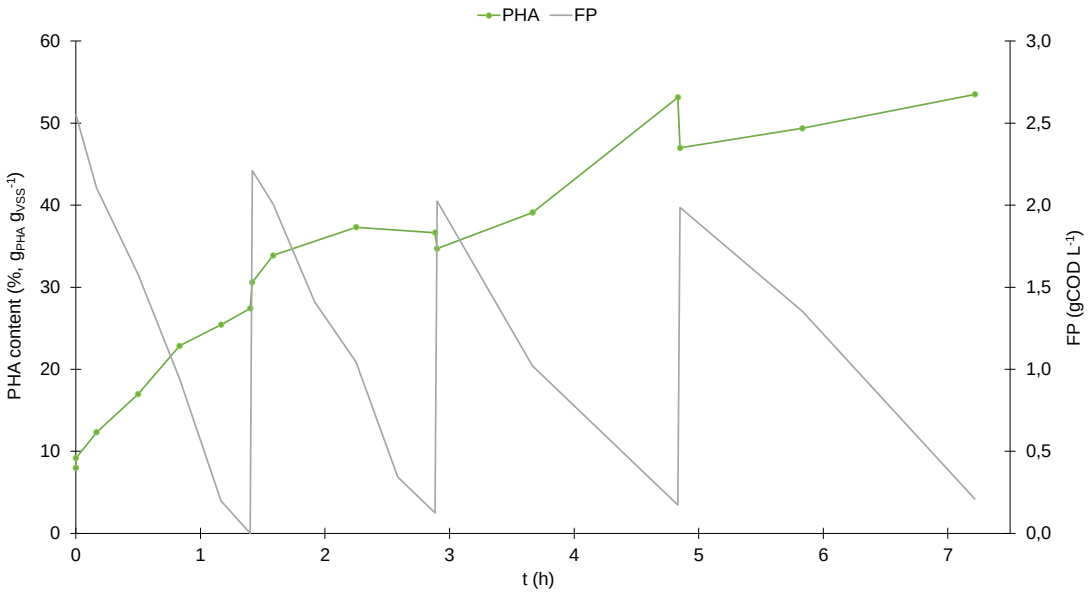


Figure 4.15: Trend of global fermentation products (gCOD L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)) throughout tests E and F for condition 3.

Table 4.8: Summary of parameters calculated for assays E and F, for Condition 3: initial VSS, storage and growth yield, global productivity, maximum specific PHA storage rate, final PHA content, Δ PHA content and initial and final PHA composition.

Parameter	Test E	Test F	Average (n=2)
Initial VSS (g L^{-1})	5.08	5.00	5.04 $\pm$ 0.04
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	0.88	0.72	0.80 $\pm$ 0.08
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}{}^{-1}$)	N.A	N.A	N.A
Global Productivity (gPHA L^{-1} h^{-1})	0.46	0.37	0.41 $\pm$ 0.04
q_{PHA}($gCOD_{PHA}$ $gCOD_X{}^{-1}$ h^{-1})	0.11	0.09	0.10 $\pm$ 0.01
Final PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	53.50	53.38	53.44 $\pm$ 0.06
Δ PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	45.51	45.39	45.45 $\pm$ 0.06
Initial PHA composition (% HB: % HV: % HHx)	83:17:0		N.A
Final PHA composition (% HB: % HV: % HHx)	89:11:0		N.A

Accumulation tests: G,H

In assays G and H, Feed-N was used to maximize PHA production with the culture selected under condition 3. A food to microorganism ratio of 17.0 C-mmol $gVSS^{-1}$was kept constant during 7.48 hours of accumulation, being 4 pulses fed. Figure 4.16 shows the variation of fermentation products and PHA

content during the G assay. For test E, a maximum of 25.1% (g_{PHA} $g_{VSS}{}^{-1}$) was produced at 4.97 hours, and for test F, this value was 29% (g_{PHA} $g_{VSS}{}^{-1}$) at 7.48 hours. Maximum substrate uptake rate, -q_S was 0.25 $\pm$ 0.05 achieved in the first pulse. In these assays, contrary to tests C and D, a better consumption of longer acids is achieved in every pulse, being the accumulation in the media minimal.

Similar to assays C and D, a estimation of produced biomass was accomplished, as well as a growth yield. For the first pulse, an ammonium uptake rate, -q_N, of 9.89$\pm$ 0.51 mgN $gCOD_X{}^{-1}$ $h^{-1}$was achieved. Table 4.9 presents the values for the most important parameters.

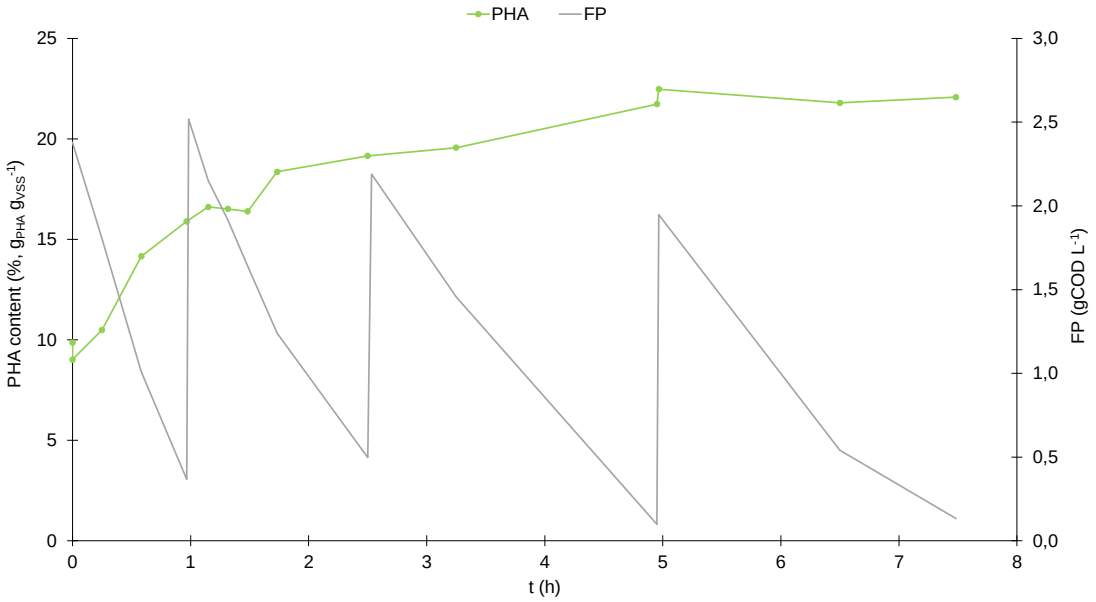


Figure 4.16: Trend of global fermentation products (gCOD L^{-1}) and PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$)) throughout tests G and H for condition 3.

Table 4.9: Summary of parameters calculated for assays G and H, for Condition 3: initial VSS, storage and growth yield, global productivity, maximum specific PHA storage rate, final PHA content, Δ PHA content and initial and final PHA composition.

Parameter	Test G	Test H	Average (n=2)
Initial VSS (g L^{-1})	6.00	5.53	5.76 $\pm$ 0.24
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	0.40	0.46	0.43 $\pm$ 0.03
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}{}^{-1}$)	0.52	0.44	0.48$\pm$ 0.04
Global Productivity (gPHA L^{-1} h^{-1})	0.28	0.31	0.29 $\pm$ 0.01
q_{PHA}($gCOD_{PHA}$ $gCOD_X{}^{-1}$ h^{-1})	0.06	0.05	0.06 $\pm$ 0.01
Final PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	22.01	28.98	25.53 $\pm$ 3.90
Δ PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	13.55	18.29	15.92 $\pm$ 2.37
Initial PHA composition (% HB: % HV: % HHx)	84:16:0		N.A
Final PHA composition (% HB: % HV: % HHx)	84:16:0		N.A

Comparing both assays, good storage yields were obtained, being demonstrated again the influence of nitrogen in accumulation assays, since it decreases PHA content due to microbial growth. From tests 1,2 and E,F an improvement on parameters occur, being related to the fact that lower cell density was achieved, increasing the parameters that rely on VSS. When compared to accumulation assays A and B an overall improvement is achieve, and since biomass concentration didn't suffer a lot of changes, a better selection of the biomass occurred.

4.3 Culture Dynamics

In order to assess for presence of PHA-accumulating microorganisms in the culture and to visually confirm the production of intracellular PHA granules, Nile Blue staining was performed on samples collected during a cycles monitoring and accumulation assays.

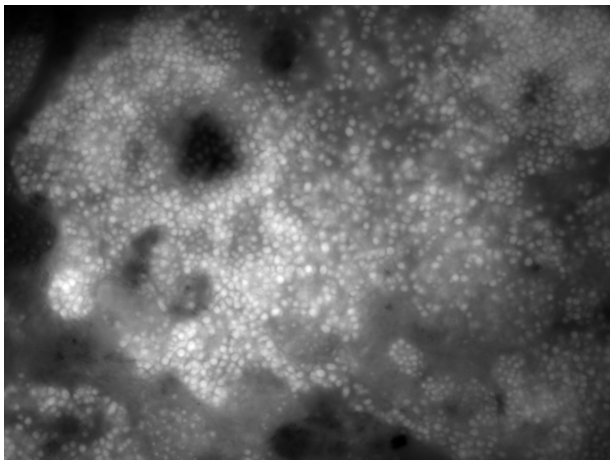


Figure 4.17: Fluorescence image of the biomass sample taken from the accumulation assay G, at 1000X, in the last point. The images show the PHA granules stained with Nile Blue, which are enhanced under fluorescent light.

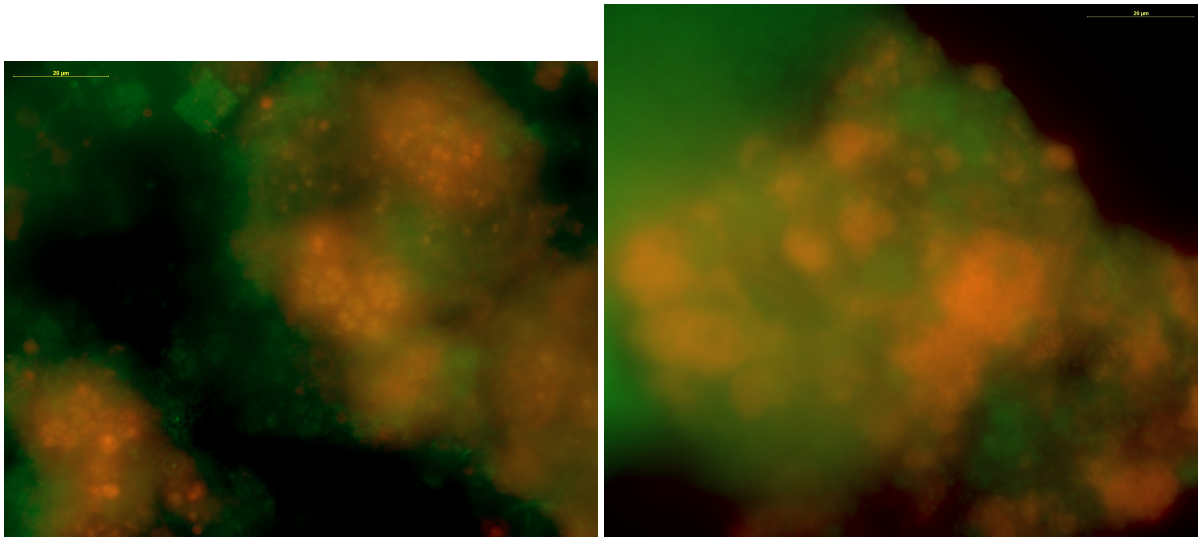
For the analysis of the dynamic of the bacterial community and study of the abundance of PHA accumulating microorganisms, FISH analysis was performed, for inoculum, Condition 1, 2 and 3. For curiosity, Feed-N was also analyzed to see if any dominant culture influenced the SBR culture. For that several probes were selected and the results are presented in Table 4.10. Since microbial cultures can vary in terms of substrate, operation conditions and environmental factors, further information on microbial communities and PHA-accumulating microorganisms in mixed microbial cultures is still required [122].

Table 4.10: FISH results for the evaluation of the MMC microbial population over the SBR operation.

Probe Name	Target Group	Inoculum	Condition 1	Condition 2	Condition 3	Feed N
AMAR839	*Amaricoccus*	-	-	++	++	
Azo644	*Azoarcus*	•	++	+	+	
THA832	*Thauera*	-	-	•	•	
LAMP444	*Lampropedia*	-	-	-	-	
Zra23a	*Zoogloea*	-	-	•	•	
UCB823	*Plasticicumulans acidivorans*	-	•	•	•	
Meg938/1028	*Meganema*	-	-	-	-	
PAR651	*Paracoccus*	++	++	++	++	
LGC355	*Firmicutes*	++	++	•	+	++
CF319a	*Flavobacteria*	-	-	+	•	+
HGC69A	*Actinobacteria*	++	•	•	•	•
ARC915	*Archaea*				+	

(-) Non-present (0%); (•) Almost non-existing (1% - 5%); **(+)** Present (5% - 25%); **(++)** Abundant (25% - 50%); **(+++)** Dominant ($>$ 50%)

For every condition an enriched culture was obtained, although no genera is dominating, the genera *Paracoccus* seems to be present in every condition. From *Alphaproteobacteria*, the genera *Amaricoccus* presented a shift in presence from condition 1 to the others. *Gammaproteobacteria*, identified with probes UCB823 and Meg938/1028 don't have a significant presence in any condition, the same happens for *Lampropedia* genera. From *Betaproteobacteria*, *Thauera* and *Zoogloea* genera were almost non-existent, contrary to *Azoarcus*, that showed a decrease presence from condition 1 to the others. For Feed-N was confirmed the presence of *Archaea* and bacteria associated with acidogenic stages, such as *Firmicutes*. As a result, the FISH analysis demonstrated the presence of several generas of bacterial population known for their ability to accumulate PHA [122], already seen with Nile Blue staining. It's important to highlight that, a shift in the culture occurred from Condition 1 to the others and *Paracoccus* prevailed in every condition. Further studies are needed to conclude if change in culture is directly related to change in polymer composition. It can also be hypothesized that the selection imposed in this study allow the enrichment in a robust culture, that prevailed with the use of two different feedstocks, which can bring several advantages for the use of MMC in industrial scale with real and variable feedstocks.



(a) Cy3-labelled AMAR839 probe.

(b) Cy3-labelled PAR651 probe.

Figure 4.18: FISH images of the biomass samples taken on Condition 3 and Condition 1, of operation of the SBR, at 1000X. The images show bacteria hybridized with FITC EUBmix probe (green) and Cy3-labelled probes (red): AMAR839 (a) and PAR651 (b). The cells where the general and specific probes overlap, result in a yellow color. The scale bars for all panels are 20 µm.

4.4 SBR Overview and Comparison

With the aim of selecting a PHA-accumulating culture using MMC and two complex waste feedstocks, fruit pulp and food waste, a pilot-scale process was set-up and 3 operating conditions were evaluated. A SRT of 4 days and a OLR of 144 C-mmol L^{-1} d^{-1}(medium value) (5.8 gCOD L^{-1} d^{-1}) was kept constant during operation days. A brief description of each condition is presented and summarized in Table 4.11.

- **Condition 1**: Presents as the baseline for the study. A working volume of 60L was considered as well as an HRT of 1 day and a C:N:P ratio of 100:6.5:1 (Cmmol:Nmmol:Pmmol). Ammonium feeding occurs 2h after Feed-C and is provided by a synthetic solution.
- **Condition 2**: Feed-N was implemented as nitrogen source, 2 h after Feed-C as well. As a first attempted, the working volume was unchanged with lead to extreme foaming. In a second and successful approach, working volume was defined in 40L, allowing after several days, SBR stabilization and characterization.
- **Condition 3**: With the aim of improving settling and biomass loss in withdrawal phase, this condition was tested. By setting an HRT of 0.66 day, in a first approach, cell density decreased probably by the washout of several micronutrients. Feed-N feeding was delayed and only added 2.5 h after Feed-C. By adding mineral media instead of tap water was possible to reach a stable phase in the reactor which allowed characterization.

Table 4.11: Average performance parameters of enriched MMC in selection reactor and an overview of PHA-accumulation assays with the selected culture in each condition of operation. a: Considered at the end of the cycle.

	Condition 1	**Condition 2**		**Condition 3**	
Selection Performance Parameters	Feed-C	Feed-C	Feed-N	Feed-C	Feed-N
Initial VSS (g L^{-1})	5.80 ± 0.97	2.60 ± 0.50		3.37± 0.60	
Final X (g L^{-1})	6.74 ± 0.93	4.33 ± 0.37		3.38± 0.11	
Feast Length (h)	0.37 ± 0.11	1.01 ± 0.09	1.20 ± 0.00	1.02± 0.10	1.17± 0.09
-q_S ($gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1})	0.67 ± 0.14	0.47 ± 0.0004	0.32 ± 0.08	0.50± 0.09	0.143± 0.001
-q_N (mgN $gCOD_X{}^{-1}$ h^{-1})	4.67 ± 1.67	—	15.70 ± 1.90	—	12.39± 0.57
PHA Composition [a] (%HB: %HV: %HHx)	58:23:19	76:24:0		78:22:0	
Accumulation Performance Parameters					
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$)	0.59± 0.05	0.38± 0.02	0.17 ±0.007	0.80± 0.08	0.43± 0.03
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}{}^{-1}$)	N.A	N.A	0.14 ± 0.02	N.A	0.48± 0.03
Final PHA content (% (g_{PHA} $g_{VSS}{}^{-1}$))	63.93 ± 8.70	31.59 ± 2.46	14.95 ± 1.02	53.44 ± 0.06	26.61 ± 2.37
q_{PHA}($gCOD_{PHA}$ $gCOD_X{}^{-1}$ h^{-1})	0.12 ± 0.03	0.02± 0.002	0.02 ± 0.01	0.10± 0.01	0.06 ± 0.01
Global Productivity (gPHA L^{-1} h^{-1})	0.62 ± 0.05	0.43± 0.03	0.23± 0.01	0.41 ± 0.04	0.29± 0.01

Condition 1 vs Condition 2

As detailed above, with the aim of producing PHA, a selection of an enriched culture occurred under three distinctive conditions. Since two waste feedstocks were aimed to be used in the developed bioprocess, the first condition served to acclimatize biomass to its main carbon source Feed-C. The main nutrients, nitrogen, phosphorus and others, that enable bacterial growth, were provided through a synthetic solution. From Figure 4.3 stability was reached after few cycles, given that the inoculum came from another reactor operated with the same feedstock and under similar conditions. A low F/F ratio was obtained, which accordingly to literature is essential for an efficient enrichment. A good cell density was achieved for MMC, which is reflected in the low feast phase lengths presented. With MMC, this parameter is usually less than 10 gVSS L^{-1}, lower when compared with pure cultures [13, 39]. Feed-C is composed by a variety of fermentation products, which influences the type of polymer obtained, than in this case is composed by HB, HV and HHx. This highlights one of the advantages of using a complex feedstock, with a variety of fermentation products, for PHA production: the possibility to generate new copolymers with a range of properties. Regarding the accumulation parameters presented, the culture selected under these conditions presents a good storing capacity, a good specific PHA storage rate, and final PHA content values similar to the ones presented in literature with MMC at pilot scale. The use of waste streams as feedstocks for PHA production allows to reduce operation costs but it is associated with low productivies. Further research should be addressed to this, since higher productivies would enhance the use of food wastes in PHA production.

The use of additional streams, such as synthetic solutions, is very common in the described process from waste streams since some don't provide an acceptable C/N/P ratio for PHA production, increasing the cost of the process. By using food waste hydrolysate-fermentate as the nitrogen source, the cost associated with supplementation in the previous condition can be eliminated. Due to the complexity of Feed-N, high solid content, fluctuations in composition, variable values within the same batch and an high presence of non-organic matter, stability of the reactor took several days and forced changes in operation (40 L of working volume). From Figure 4.3 is not possible to assume that a "true stability" was achieved, although F/F ratio is lower than 0.4 h h^{-1}, and this value varies notably from study to study and is influenced by the type of substrate used. This instability caused lower biomass concentrations, hence a higher feast length and higher specific uptake rates. The culture derived from this enrichment was only capable of producing a PHA polymer constituted by HB and HV, losing from condition 1 the HHx monomer. With similar substrates, OFMSW and derivatives, presented in Table 1.3, only polymers with HB and HV were obtained. It's possible to hypothesis that some compound, for example the non-fermented solids of Feed-N can influence polymer composition, being further investigation needed on this topic.

Comparing condition 2 accumulation assays with Feed-C with condition 1, lower storage yield, specific PHA production rate and global productivity were achieved. It suggests that culture lost some accumulation capacity, which reflects the poor stability and selection of the culture.

In the accumulation assays with Feed-N, a higher variability of fermentation products was present, and the preferential one for depletion, butyric acid is only 16% of feed's composition, in opposition with the 52% of tests of condition 1, this lead to an accumulation of fermentation products in the media, not allowing the maximization of PHA production. Adding this reason to the fact that lower biomass concentrations were achieved, lower performance parameters were obtained. As previously mentioned, the role of nitrogen in the accumulation stage is still not consensual, however in this condition is possible to concluded that without nitrogen supply higher PHA contents were obtained. In this accumulation a similar storage response as well as a growth one were seeing, and since PHA accumulations benefit

from a storage response over a growth one, the assay presented is not indicated for PHA accumulations. When compared with the studies reported in Table 1.3, PHA content from accumulation assays with Feed-N is lower than the values presented, as well as specific PHA storage rate reported, indicating that there is room for improvement.

The stability of this condition was not entirely achieved although an enriched culture was obtained, confirmed from FISH analysis. A shift between *Azoarcus* genera to *Amaricoccus* occurred, which is directly related to the shift in feedstock used. The presence of *Paracoccus* genera in the culture seemed permanent and not influenced by condition change. With the establishment of this condition was proved that is possible to produce PHA with a MMC enriched with two food waste feedstocks. However, SBR stability should be improved, in order to achieve higher values of PHA content and productivities, for that a lower OLR could have been considered, hence would promote a lower F/F ratio. In the previous studies with food waste fermentates, upstream to the process at least one or two stages of solid/liquid separation took place, usually centrifugation followed by a membrane separation, with the goal of reducing solid content. Since stability could be improved, condition 3 was tested.

Condition 2 vs Condition 3

Due to biomass loss in condition 2 in withdrawal phase and poor settling, and with the aim of improving the stability of the reactor, the HRT was lowered to 0.66 days. This strategy also allowed the reduction of solid content in the reactor since two settling phases occur. As can be seen in Figure 4.3, condition 3* provoked instability in the reactor, higher F/F ratios were obtained, which can be related to the fact that the withdrawal phase washed out important micronutrients provided by Feed-N. Hence, instead of tap water, mineral media was added, which allowed a better stability in the reactor, when compared with condition 2. The F/F ratio values are not as close together as in condition 1, but close and low enough that stability can be considered, as can be seen in Figure 4.3. From condition 2 to 3, there's a slight change in biomass values and feast length is, as expected, approximately the same. Since higher PHA contents are obtained in the first feast, substrate uptake rate is lower in the second feast, and lower then condition 2. The drop in specific ammonium consumption can be related to the fact that a better selection was performed, since the PHA accumulator community began to consume ammonium quicker to gain a competitive advantage over non-producers. Regarding accumulation parameters, an improvement from condition 2 to 3 is noticeable and can be seen in Table 4.11, this can be justified by the fact that an lower HRT helps to eliminate a part of the non-fermented solids and clear potentially inhibitory compounds of the system, or non-soluble solids, introduced by Feed-N or secreted by bacteria, improving the settling capacity of the culture, resulting in a better selection. Comparing with condition 2, a higher storage yield, PHA content and specific PHA storage rate were obtained, in both accumulations. When compared with condition 1, overall parameters are lower, with exception to storage yield. It shows that although a better assimilation of carbon source to PHA is performed, lower PHA contents are obtained as well as a lower global productivity. Once again, in accumulations assays with Feed-N a lower PHA content was obtained and the growth response was similar to the storage one. Since TSS concentration in Feed-N is very high, as can be seen in 4.1, VSS is also high and for this reason, with substrates that present similar characteristics, an adjustment should be considered, in order to estimate an accurate value of VSS and therefore of biomass. This concern is taken into account, for example, in Mulders *et al.* study. From FISH analysis, the culture selected didn't suffer a significant change, being the presence of *Amaricoccus* and *Paracocus* abundant.

Chapter 5

Conclusions and Future Work

In this work, the PHA production by MMC using two feedstocks was evaluated in three different operational conditions. Many industrial wastes used for PHA production are rich in carbon compounds but poor in nitrogen and phosphorus, making nutrient supplementation necessary during the culture selection. First, a detailed characterization of both feedstocks was performed.

The results showed that both feedstocks are suitable for the bioprocess established presenting a high percentage of fermentation products per soluble COD. Feed-N has a high ammonium content which promotes cellular growth while fermented fruit pulp waste (Feed-C) does not have any ammonia. This shows that Feed-N could be used as the only feedstock of the process instead of Feed-C which requires supplementation of nitrogen source. Although Feed-N has a huge availability, the high content of nitrogen can promote a growth response over a PHA storage one. Adding to this, fluctuations in composition in each batch, related also with seasonal changes, and high solid content influences the downstream process. Thus, the process should be optimized according to these limiting boundaries.

The culture selection was performed using an SBR reactor operated at pilot scale under an OLR of 5.8 gCOD L^{-1} d^{-1}. The selection was achieved through a feast and famine regime, using Feed-C as the main carbon source and an uncoupled nitrogen source (synthetic or Feed-N). The results from the first condition, where synthetic solution was used as nitrogen source, promoted the biomass acclimatization. The stability of the system was achieved under a low F/F ratio (lower than 0.1) which coupled to a high OLR lead to an enriched culture in PHA accumulating microorganisms such as *Paracoccus* and *Azoarcus*. A storage yield of 0.59$\pm$ 0.05gCOD$_{PHA}$ gCOD$_{FP}{}^{-1}$and a PHA content of 63.93 $\pm$ 8.70 % (g_{PHA} $g_{VSS}{}^{-1}$) were obtained, showing a good storage response. These results were in line with the global productivity obtained and specific PHA storage rate and similar to the ones presented in literature at pilot scale. The polymer obtained is composed by HB, HV and HHx monomers.

Then, the synthetic solution used as a nitrogen source was replaced with Feed-N (Condition 2), contributing for cellular growth and OLR. Several problems occurred when Feed-N was introduced into the system and although many changes were made in the operation, the system showed a poor stability, lower cell densities and a higher feast length. The results reveal that biomass acclimatization took longer when compared to condition 1 and although a stable state was not achieved, the F/F ratio was between 0.1-0.4 h h^{-1}indicating a good selection of PHA accumulating microorganisms. In accumulations assays with Feed-C lower storage yield, 0.38 $\pm$ 0.02 gCOD$_{PHA}$ gCOD$_{FP}{}^{-1}$, PHA content, 31.59 $\pm$ 2.46 % (g_{PHA} $g_{VSS}{}^{-1}$) and global productivity, 0.43 $\pm$ 0.03 gPHA L^{-1} h^{-1}were obtained when compared with the previous condition. Polymer composition shift drastically being mainly composed by HB and HV

monomers.

In the last condition (condition 3) it was studied the impact in the system of operating under a lower HRT, 0.66 days. The main goal was to improve SBR stability and reduce the non-fermented solids in the reactor. The selection culture presented higher accumulation performance parameters under similar biomass concentrations. A PHA content of 53.44 $\pm$ 0.06 % (g_{PHA} $g_{VSS}{}^{-1}$) was achieved with similar productivity. The abundance of *Amoricoccus* and *Paracoccus* genera in the culture effectively confirms a good selection with MMC in this condition. When Feed-N was used to perform accumulations assays, in condition 2 and 3, lower yields were obtained, highlighting the influence of nitrogen abundance in the accumulation stage. In the best case, a PHA content of 26.61 $\pm$ 2.37 % (g_{PHA} $g_{VSS}{}^{-1}$) was obtained.

In summary, the main goal of producing PHA with these two feedstocks was achieved in condition 3. This result can lead to the application of a similar bioprocess in other industries, by taking advantage of the complementary between substrates. The results showed that Feed-N should not be used in the accumulation stage as the high nitrogen concentrations promoted a growth response over the PHA storage resulting in low PHA contents. In the future, the system should be operated under a lower OLR in order to achieve a more sustainable PHA production if higher cell densities and consequently higher productivities were reached.

Bibliography

[1] A. J. Ragauskas, C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Frederick, J. P. Hallett, D. J. Leak, and C. L. Liotta, "The path forward for biofuels and biomaterials," *science*, vol. 311, no. 5760, pp. 484–489, 2006.

[2] A. A. Fra-Vazqueza, V. del Rıoa, and A. T. Palmeiro-Sancheza, "Transformation of organic contamination from wastewater into bioplastics (polyhydroxyalkanoate) by microorganisms," *Wastewater Treatment Residues as Resources for Biorefinery Products and Biofuels*, p. 415, 2019.

[3] P. Association of Plastic Manufacturers, "Plastics–the facts 2019 an analysis of european plastics production, demand and waste data," Oct 2019. https://www.plasticseurope.org/application/files/9715/7129/9584/FINAL_web_version_Plastics_the_facts2019_14102019.pdf?fbclid=IwAR30ezU4i2anq6TRikmzdyl-VjAulKQArMldd_6v1F_uVqS9yzVJQ2PTFDc, last accessed on 2020-20-04.

[4] R. Geyer, J. R. Jambeck, and K. L. Law, "Production, use, and fate of all plastics ever made," *Science advances*, vol. 3, no. 7, p. e1700782, 2017.

[5] E. C. Environment, "European comission environment 2020," Mar 2020. https://ec.europa.eu/environment/waste/plastic_waste.htm, last accessed on 2020-20-04.

[6] "A european strategy for plastics in a circular economy." https://ec.europa.eu/environment/circular-economy/pdf/plastics-strategy-brochure.pdf, last accessed on 2020-22-04.

[7] E. M. F. ANBI, "A european strategy for plastics in a circular economy." https://ec.europa.eu/environment/circular-economy/pdf/plastics-strategy-brochure.pdf, last accessed on 2020-24-04.

[8] E. M. F. ANBI, "Europe becomes the first regional initiative to join the foundation's plastics pact network." https://www.ellenmacarthurfoundation.org/news/european-plastics-pact-first-regional-initiative-to-join-the-foundations-plastics-pact-network last accessed on 2020-24-04.

[9] A. Meraldo, "Introduction to bio-based polymers," in *Multilayer Flexible Packaging*, pp. 47–52, Elsevier, 2016.

[10] M. Vert, Y. Doi, K.-H. Hellwich, M. Hess, P. Hodge, P. Kubisa, M. Rinaudo, and F. Schué, "Terminology for biorelated polymers and applications (IUPAC Recommendations 2012)," *Pure and Applied Chemistry*, vol. 84, no. 2, pp. 377–410, 2012.

[11] S. Lambert and M. Wagner, "Environmental performance of bio-based and biodegradable plastics: the road ahead," *Chemical Society Reviews*, vol. 46, no. 22, pp. 6855–6871, 2017.

[12] E. Bioplastics, "Fact sheet." https://docs.european-bioplastics.org/publications/fs/EuBP_FS_What_are_bioplastics.pdf, last accessed on 2020-30-04.

[13] C. Kourmentza, J. Plácido, N. Venetsaneas, A. Burniol-Figols, C. Varrone, H. N. Gavala, and M. A. Reis, "Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production," *Bioengineering*, vol. 4, no. 2, p. 55, 2017.

[14] L. T. Sin and B. S. Tueen, *Polylactic acid: a practical guide for the processing, manufacturing, and applications of PLA*. William Andrew, 2019.

[15] M. Niaounakis, *Biopolymers: processing and products*. William Andrew, 2014.

[16] S. Y. Lee, "Bacterial polyhydroxyalkanoates," *Biotechnology and bioengineering*, vol. 49, no. 1, pp. 1–14, 1996.

[17] G. Rathna, B. S. T. Gadgil, and N. Killi, "Polyhydroxyalkanoates: The Application of Eco-Friendly Materials," *Biodegradable and Biobased Polymers for Environmental and Biomedical Applications*, pp. 25–54, 2016.

[18] K. Sudesh, H. Abe, and Y. Doi, "Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters," *Progress in polymer science*, vol. 25, no. 10, pp. 1503–1555, 2000.

[19] J. Lu, R. C. Tappel, and C. T. Nomura, "Mini-review: biosynthesis of poly (hydroxyalkanoates)," *Journal of Macromolecular Science®, Part C: Polymer Reviews*, vol. 49, no. 3, pp. 226–248, 2009.

[20] P. Basnett, S. Ravi, and I. Roy, "Science and Principles of Biodegradable and Bioresorbable Medical Polymers," Woodhead Publishing, 2016.

[21] B. Kunasundari and K. Sudesh, "Isolation and recovery of microbial polyhydroxyalkanoates," *Express Polymer Letters*, vol. 5, no. 7, 2011.

[22] M. Koller, A. Atlić, M. Dias, A. Reiterer, and G. Braunegg, "Microbial PHA production from waste raw materials," in *Plastics from bacteria*, pp. 85–119, Springer, 2010.

[23] C. Lee, S. Sapuan, R. Ilyas, S. Lee, and A. Khalina, "Development and processing of PLA, PHA, and other biopolymers," in *Advanced Processing, Properties, and Applications of Starch and Other Bio-Based Polymers*, pp. 47–63, Elsevier, 2020.

[24] C. Y. Loo and K. Sudesh, "Polyhydroxyalkanoates: bio-based microbial plastics and their properties," *Malaysian Polymer Journal*, vol. 2, no. 2, pp. 31–57, 2007.

[25] R. A. Verlinden, D. J. Hill, M. Kenward, C. D. Williams, and I. Radecka, "Bacterial synthesis of biodegradable polyhydroxyalkanoates," *Journal of applied microbiology*, vol. 102, no. 6, pp. 1437–1449, 2007.

[26] E. Bugnicourt, P. Cinelli, A. Lazzeri, and V. A. Alvarez, "Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging," 2014.

[27] S. Y. Ong, J. Y. Chee, and K. Sudesh, "Degradation of polyhydroxyalkanoate (PHA): a review," 2017.

[28] J. Możejko-Ciesielska and R. Kiewisz, "Bacterial polyhydroxyalkanoates: still fabulous?," *Microbiological Research*, vol. 192, pp. 271–282, 2016.

[29] A. Prieto, I. F. Escapa, V. Martínez, N. Dinjaski, C. Herencias, F. de la Peña, N. Tarazona, and O. Revelles, "A holistic view of polyhydroxyalkanoate metabolism in Pseudomonas putida," *Environmental microbiology*, vol. 18, no. 2, pp. 341–357, 2016.

[30] Meng, De-Chuan and Shen, Rui and Yao, Hui and Chen, Jin-Chun and Wu, Qiong and Chen, Guo-Qiang, "Engineering the diversity of polyesters," *Current opinion in biotechnology*, vol. 29, pp. 24–33, 2014.

[31] R. Kniewel, O. Revelles, and M. A. Prieto, "Biogenesis of medium-chain-length polyhydroxyalkanoates," 2017.

[32] M. Reis, M. Albuquerque, M. Villano, and M. Majone, "Mixed culture processes for polyhydroxyalkanoate production from agro-industrial surplus/wastes as feedstocks," 2011.

[33] L. S. Serafim, P. C. Lemos, M. G. Albuquerque, and M. A. Reis, "Strategies for PHA production by mixed cultures and renewable waste materials," *Applied microbiology and biotechnology*, vol. 81, no. 4, pp. 615–628, 2008.

[34] H. W. Kim, M. G. Chung, and Y. H. Rhee, "Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates," *The Journal of Microbiology*, vol. 45, no. 2, pp. 87–97, 2007.

[35] B. Laycock, P. Halley, S. Pratt, A. Werker, and P. Lant, "The chemomechanical properties of microbial polyhydroxyalkanoates," *Progress in Polymer Science*, vol. 38, no. 3-4, pp. 536–583, 2013.

[36] M. Koller, L. Maršálek, M. M. de Sousa Dias, and G. Braunegg, "Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner," *New biotechnology*, vol. 37, pp. 24–38, 2017.

[37] H. Pakalapati, C.-K. Chang, P. L. Show, S. K. Arumugasamy, and J. C.-W. Lan, "Development of polyhydroxyalkanoates production from waste feedstocks and applications," *Journal of bioscience and bioengineering*, vol. 126, no. 3, pp. 282–292, 2018.

[38] F. Wang and S. Y. Lee, "Poly (3-Hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-Batch Culture of Alcaligenes latus under Nitrogen Limitation.," *Appl. Environ. Microbiol.*, vol. 63, no. 9, pp. 3703–3706, 1997.

[39] G.-Q. Chen, "A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry," *Chemical Society Reviews*, vol. 38, no. 8, pp. 2434–2446, 2009.

[40] C. Nielsen, A. Rahman, A. U. Rehman, M. K. Walsh, and C. D. Miller, "Food waste conversion to microbial polyhydroxyalkanoates," *Microbial biotechnology*, vol. 10, no. 6, pp. 1338–1352, 2017.

[41] W. S. Ahn, S. J. Park, and S. Y. Lee, "Production of poly (3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Escherichia coli," *Biotechnology Letters*, vol. 23, no. 3, pp. 235–240, 2001.

[42] L. L. Wallen and W. K. Rohwedder, "Poly-. beta.-hydroxyalkanoate from activated sludge," *Environmental science & technology*, vol. 8, no. 6, pp. 576–579, 1974.

[43] F. Valentino, F. Morgan-Sagastume, S. Campanari, M. Villano, A. Werker, and M. Majone, "Carbon recovery from wastewater through bioconversion into biodegradable polymers," *New biotechnology*, vol. 37, pp. 9–23, 2017.

[44] F. Morgan-Sagastume, "Characterisation of open, mixed microbial cultures for polyhydroxyalkanoate (PHA) production," *Reviews in Environmental Science and Bio/Technology*, vol. 15, no. 4, pp. 593–625, 2016.

[45] P. Poltronieri and P. Kumar, "Polyhydroxyalkanoates (PHAs) in industrial applications," *Handbook of Ecomaterials. Cham: Springer International Publishing*, pp. 1–30, 2017.

[46] E. Elmowafy, A. Abdal-Hay, A. Skouras, M. Tiboni, L. Casettari, and V. Guarino, "Polyhydroxyalkanoate (PHA): applications in drug delivery and tissue engineering," *Expert review of medical devices*, vol. 16, no. 6, pp. 467–482, 2019.

[47] C. M. Vermeer, E. Rossi, J. Tamis, H. M. Jonkers, and R. Kleerebezem, "From waste to self-healing concrete: A proof-of-concept of a new application for polyhydroxyalkanoate," *Resources, Conservation and Recycling*, vol. 164, p. 105206.

[48] "Danimer scientific and eagle beverage to produce biodegradable drinking straws," Nov 2020. https://danimerscientific.com/2020/11/17/danimer-scientific-and-eagle-beverage-to-produce-biodegradable-drinking-straws/, last accessed on 2020-09-12.

[49] E. Bioplastics, "Bioplastics market development update 2020." https://docs.european-bioplastics.org/conference/Report_Bioplastics_Market_Data_2020_short_version.pdf.

[50] S. Rodriguez-Perez, A. Serrano, A. A. Pantión, and B. Alonso-Fariñas, "Challenges of scaling-up PHA production from waste streams. A review," *Journal of environmental management*, vol. 205, pp. 215–230, 2018.

[51] G. Mannina, D. Presti, G. Montiel-Jarillo, J. Carrera, and M. E. Suárez-Ojeda, "Recovery of polyhydroxyalkanoates (PHAs) from wastewater: A review," *Bioresource Technology*, vol. 297, p. 122478, 2020.

[52] P. Kumar, S. Ray, and V. C. Kalia, "Production of co-polymers of polyhydroxyalkanoates by regulating the hydrolysis of biowastes," *Bioresource technology*, vol. 200, pp. 413–419, 2016.

[53] S. K. Patel, P. Kumar, M. Singh, J.-K. Lee, and V. C. Kalia, "Integrative approach to produce hydrogen and polyhydroxybutyrate from biowaste using defined bacterial cultures," *Bioresource technology*, vol. 176, pp. 136–141, 2015.

[54] S. Follonier, R. Riesen, and M. Zinn, "Pilot-scale production of functionalized mcl-PHA from grape pomace supplemented with fatty acids," *Chemical and Biochemical Engineering Quarterly*, vol. 29, no. 2, pp. 113–121, 2015.

[55] J. Tamis, K. Lužkov, Y. Jiang, M. C. van Loosdrecht, and R. Kleerebezem, "Enrichment of Plasticicumulans acidivorans at pilot-scale for PHA production on industrial wastewater," *Journal of Biotechnology*, vol. 192, pp. 161–169, 2014.

[56] B. Colombo, T. P. Sciarria, M. Reis, B. Scaglia, and F. Adani, "Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture," *Bioresource technology*, vol. 218, pp. 692–699, 2016.

[57] F. Girotto, L. Alibardi, and R. Cossu, "Food waste generation and industrial uses: a review," *Waste management*, vol. 45, pp. 32–41, 2015.

[58] E. Uçkun Kıran, A. P. Trzcinski, and Y. Liu, "Platform chemical production from food wastes using a biorefinery concept," *Journal of Chemical Technology & Biotechnology*, vol. 90, no. 8, pp. 1364–1379, 2015.

[59] J. P. López-Gómez, M. Latorre-Sánchez, P. Unger, R. Schneider, C. C. Lozano, and J. Venus, "Assessing the organic fraction of municipal solid wastes for the production of lactic acid," *Biochemical Engineering Journal*, vol. 150, p. 107251, 2019.

[60] G. K. Dinesh, R. Chauhan, and S. Chakma, "Influence and strategies for enhanced biohydrogen production from food waste," *Renewable and Sustainable Energy Reviews*, vol. 92, pp. 807–822, 2018.

[61] F. Valentino, G. Moretto, L. Lorini, D. Bolzonella, P. Pavan, and M. Majone, "Pilot-scale polyhydroxyalkanoate production from combined treatment of organic fraction of municipal solid waste and sewage sludge," *Industrial & Engineering Chemistry Research*, vol. 58, no. 27, pp. 12149–12158, 2019.

[62] D. K. Solaiman, R. D. Ashby, T. A. Foglia, and W. N. Marmer, "Conversion of agricultural feedstock and coproducts into poly (hydroxyalkanoates)," *Applied microbiology and biotechnology*, vol. 71, no. 6, pp. 783–789, 2006.

[63] B. Colombo, F. Favini, B. Scaglia, T. P. Sciarria, G. D'Imporzano, M. Pognani, A. Alekseeva, G. Eisele, C. Cosentino, and F. Adani, "Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture," *Biotechnology for biofuels*, vol. 10, no. 1, p. 201, 2017.

[64] F. Valentinoa, L. Lorinia, P. Pavanb, D. Bolzonellac, and M. Majonea, "Organic Fraction of Municipal Solid Waste Conversion into Polyhydroxyalkanoates (PHA) in a Pilot Scale Anaerobic/Aerobic Process," *CHEMICAL ENGINEERING*, vol. 74, 2019.

[65] M. Mulders, J. Tamis, B. Abbas, J. Sousa, H. Dijkman, R. Rozendal, and R. Kleerebezem, "Pilot-Scale Polyhydroxyalkanoate Production from Organic Waste: Process Characteristics at High pH and High Ammonium Concentration," *Journal of Environmental Engineering*, vol. 146, no. 7, p. 04020049, 2020.

[66] G. Moretto, L. Lorini, P. Pavan, S. Crognale, B. Tonanzi, S. Rossetti, M. Majone, and F. Valentino, "Biopolymers from Urban Organic Waste: Influence of the Solid Retention Time to Cycle Length Ratio in the Enrichment of a Mixed Microbial Culture (MMC)," *ACS Sustainable Chemistry & Engineering*, vol. 8, no. 38, pp. 14531–14539, 2020.

[67] E. Korkakaki, M. Mulders, A. Veeken, R. Rozendal, M. C. van Loosdrecht, and R. Kleerebezem, "PHA production from the organic fraction of municipal solid waste (OFMSW): Overcoming the inhibitory matrix," *Water research*, vol. 96, pp. 74–83, 2016.

[68] H. Yesil, A. Tugtas, A. Bayrakdar, and B. Calli, "Anaerobic fermentation of organic solid wastes: volatile fatty acid production and separation," *Water science and technology*, vol. 69, no. 10, pp. 2132–2138, 2014.

[69] W. S. Lee, A. S. M. Chua, H. K. Yeoh, and G. C. Ngoh, "A review of the production and applications of waste-derived volatile fatty acids," *Chemical Engineering Journal*, vol. 235, pp. 83–99, 2014.

[70] Y. Li, D. Su, H. Feng, F. Yan, H. Liu, L. Feng, and G. Liu, "Anaerobic acidogenic fermentation of food waste for mixed-acid production," *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, vol. 39, no. 7, pp. 631–635, 2017.

[71] S. Simonetti, A. Saptoro, C. Fernández Martín, and D. Dionisi, "Product Concentration, Yield and Productivity in Anaerobic Digestion to Produce Short Chain Organic Acids: A Critical Analysis of Literature Data," *Processes*, vol. 8, no. 12, p. 1538, 2020.

[72] A. R. Gouveia, E. B. Freitas, C. F. Galinha, G. Carvalho, A. F. Duque, and M. A. Reis, "Dynamic change of pH in acidogenic fermentation of cheese whey towards polyhydroxyalkanoates production: Impact on performance and microbial population," *New biotechnology*, vol. 37, pp. 108–116, 2017.

[73] F. Silva, S. Campanari, S. Matteo, F. Valentino, M. Majone, and M. Villano, "Impact of nitrogen feeding regulation on polyhydroxyalkanoates production by mixed microbial cultures," *New Biotechnology*, vol. 37, pp. 90–98, 2017.

[74] F. Ahmadi, A. Zinatizadeh, A. Asadi, and H. Younesi, "Influence of Different Culture Selection Methods on Polyhydroxyalkanoate Production at Short-term Biomass Enrichment," *International Journal of Engineering*, vol. 32, no. 2, pp. 184–192, 2019.

[75] C. S. Oliveira, C. E. Silva, G. Carvalho, and M. A. Reis, "Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities," *New biotechnology*, vol. 37, pp. 69–79, 2017.

[76] D. Dionisi, M. Majone, G. Vallini, S. Di Gregorio, and M. Beccari, "Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor," *Biotechnology and bioengineering*, vol. 93, no. 1, pp. 76–88, 2006.

[77] Z. Chen, L. Huang, Q. Wen, H. Zhang, and Z. Guo, "Effects of sludge retention time, carbon and initial biomass concentrations on selection process: From activated sludge to polyhydroxyalkanoate accumulating cultures," *Journal of Environmental Sciences*, vol. 52, pp. 76–84, 2017.

[78] B. Basak, O. Ince, N. Artan, N. Yagci, and B. K. Ince, "Effect of nitrogen limitation on enrichment of activated sludge for PHA production," *Bioprocess and biosystems engineering*, vol. 34, no. 8, pp. 1007–1016, 2011.

[79] L. Cavaillé, E. Grousseau, M. Pocquet, A.-S. Lepeuple, J.-L. Uribelarrea, G. Hernandez-Raquet, and E. Paul, "Polyhydroxybutyrate production by direct use of waste activated sludge in phosphorus-limited fed-batch culture," *Bioresource technology*, vol. 149, pp. 301–309, 2013.

[80] K. Khatami, M. Perez-Zabaleta, I. Owusu-Agyeman, and Z. Cetecioglu, "Waste to bioplastics: How close are we to sustainable polyhydroxyalkanoates production?," *Waste Management*, 2020.

[81] N. Basset, E. Katsou, N. Frison, S. Malamis, J. Dosta, and F. Fatone, "Integrating the selection of PHA storing biomass and nitrogen removal via nitrite in the main wastewater treatment line," *Bioresource technology*, vol. 200, pp. 820–829, 2016.

[82] L. Lorini, F. di Re, M. Majone, and F. Valentino, "High rate selection of PHA accumulating mixed cultures in sequencing batch reactors with uncoupled carbon and nitrogen feeding," *New Biotechnology*, vol. 56, pp. 140–148, 2020.

[83] C. Kourmentza and M. Kornaros, "Biotransformation of volatile fatty acids to polyhydroxyalkanoates by employing mixed microbial consortia: The effect of pH and carbon source," *Bioresource technology*, vol. 222, pp. 388–398, 2016.

[84] M. Albuquerque, M. Eiroa, C. Torres, B. Nunes, and M. Reis, "Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses," *Journal of biotechnology*, vol. 130, no. 4, pp. 411–421, 2007.

[85] H. Moralejo-Gárate, T. Palmeiro-Sánchez, R. Kleerebezem, A. Mosquera-Corral, J. L. Campos, and M. C. van Loosdrecht, "Influence of the cycle length on the production of PHA and polyglucose from glycerol by bacterial enrichments in sequencing batch reactors," *Biotechnology and Bioengineering*, vol. 110, no. 12, pp. 3148–3155, 2013.

[86] Y. Jiang, L. Marang, J. Tamis, M. C. van Loosdrecht, H. Dijkman, and R. Kleerebezem, "Waste to resource: converting paper mill wastewater to bioplastic," *Water research*, vol. 46, no. 17, pp. 5517–5530, 2012.

[87] F. Valentino, L. Karabegovic, M. Majone, F. Morgan-Sagastume, and A. Werker, "Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels," *Water research*, vol. 77, pp. 49–63, 2015.

[88] P. Chakravarty, V. Mhaisalkar, and T. Chakrabarti, "Study on poly-hydroxyalkanoate (PHA) production in pilot scale continuous mode wastewater treatment system," *Bioresource Technology*, vol. 101, no. 8, pp. 2896–2899, 2010.

[89] S. Anterrieu, L. Quadri, B. Geurkink, I. Dinkla, S. Bengtsson, M. Arcos-Hernandez, T. Alexandersson, F. Morgan-Sagastume, A. Karlsson, and M. Hjort, "Integration of biopolymer production with process water treatment at a sugar factory," *New biotechnology*, vol. 31, no. 4, pp. 308–323, 2014.

[90] F. Morgan-Sagastume, M. Hjort, D. Cirne, F. Gérardin, S. Lacroix, G. Gaval, L. Karabegovic, T. Alexandersson, P. Johansson, and A. Karlsson, "Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale," *Bioresource technology*, vol. 181, pp. 78–89, 2015.

[91] S. Bengtsson, A. Karlsson, T. Alexandersson, L. Quadri, M. Hjort, P. Johansson, F. Morgan-Sagastume, S. Anterrieu, M. Arcos-Hernandez, and L. Karabegovic, "A process for polyhydroxyalkanoate (PHA) production from municipal wastewater treatment with biological carbon and nitrogen removal demonstrated at pilot-scale," *New biotechnology*, vol. 35, pp. 42–53, 2017.

[92] M. Majone, "Res urbis - resources from urban biowaste interim financial and technical report." `https://www.resurbis.eu/sites/default/files/Summary_for_publication_month_36.pdf`.

[93] C. Pérez-Rivero, J. P. López-Gómez, and I. Roy, "A sustainable approach for the downstream processing of bacterial polyhydroxyalkanoates: State-of-the-art and latest developments," *Biochemical Engineering Journal*, vol. 150, p. 107283, 2019.

[94] M. Koller, H. Niebelschütz, and G. Braunegg, "Strategies for recovery and purification of poly [(R)-3-hydroxyalkanoates](PHA) biopolyesters from surrounding biomass," *Engineering in life sciences*, vol. 13, no. 6, pp. 549–562, 2013.

[95] A. D. Tripathi, T. Joshi, K. Khosravi-Darani, M. Koller, S. P. Singh, A. Shrivastava, and S. Mishra, "Recovery and characterization of polyhydroxyalkanoates," *Recent Advances in Biotechnology*, vol. 2, pp. 267–303, 2016.

[96] A. P. H. Association, A. W. W. Association, W. P. C. Federation, and W. E. Federation, *Standard methods for the examination of water and wastewater*, vol. 2. American Public Health Association., 1915.

[97] O.-i. Lowry, N. Rosebrough, A. Farr, and R. Randall, "Protein determination by a modified Folin phenol method," *J. biol. Chem*, vol. 193, pp. 265–275, 1951.

[98] M. Dubois, K. A. Gilles, J. K. Hamilton, P. t. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical chemistry*, vol. 28, no. 3, pp. 350–356, 1956.

[99] L. S. Serafim, P. C. Lemos, R. Oliveira, and M. A. Reis, "Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions," *Biotechnology and Bioengineering*, vol. 87, no. 2, pp. 145–160, 2004.

[100] S. Bengtsson, J. Hallquist, A. Werker, and T. Welander, "Acidogenic fermentation of industrial wastewaters: effects of chemostat retention time and pH on volatile fatty acids production," *Biochemical Engineering Journal*, vol. 40, no. 3, pp. 492–499, 2008.

[101] R. I. Amann, "In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes," in *Molecular microbial ecology manual*, pp. 331–345, Springer, 1995.

[102] P. Nielsen and H. Daims, *FISH handbook for biological wastewater treatment*. Iwa publishing, 2009.

[103] R. I. Amann, B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl, "Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations.," *Applied and environmental microbiology*, vol. 56, no. 6, pp. 1919–1925, 1990.

[104] H. Daims, A. Brühl, R. Amann, K.-H. Schleifer, and M. Wagner, "The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set," *Systematic and applied microbiology*, vol. 22, no. 3, pp. 434–444, 1999.

[105] A.-M. Maszenan, R. Seviour, B. Patel, and J. Wanner, "A fluorescently-labelled r-RNA targeted oligonucleotide probe for the in situ detection of G-bacteria of the genus Amaricoccus in activated sludge," *Journal of applied microbiology*, vol. 88, no. 5, pp. 826–835, 2000.

[106] K. B. Hallberg, K. Coupland, S. Kimura, and D. B. Johnson, "Macroscopic streamer growths in acidic, metal-rich mine waters in North Wales consist of novel and remarkably simple bacterial communities," *Applied and environmental microbiology*, vol. 72, no. 3, pp. 2022–2030, 2006.

[107] A. Hess, B. Zarda, D. Hahn, A. Häner, D. Stax, P. Höhener, and J. Zeyer, "In situ analysis of denitrifying toluene-and m-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column.," *Applied and environmental microbiology*, vol. 63, no. 6, pp. 2136–2141, 1997.

[108] A. Loy, C. Schulz, S. Lücker, A. Schöpfer-Wendels, K. Stoecker, C. Baranyi, A. Lehner, and M. Wagner, "16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order "Rhodocyclales"," *Applied and environmental microbiology*, vol. 71, no. 3, pp. 1373–1386, 2005.

[109] N. Lee, C. M. Cellamare, C. Bastianutti, R. Rossello-Mora, P. Kämpfer, W. Ludwig, K. H. Schleifer, and L. Stante, "Emended description of the species Lampropedia hyalina," *International journal of systematic and evolutionary microbiology*, vol. 54, no. 5, pp. 1709–1715, 2004.

[110] W. Manz, R. Amann, W. Ludwig, M. Vancanneyt, and K.-H. Schleifer, "Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment," *Microbiology*, vol. 142, no. 5, pp. 1097–1106, 1996.

[111] R. A. Rosselló-Mora, M. Wagner, R. Amann, and K.-H. Schleifer, "The abundance of Zoogloea ramigera in sewage treatment plants.," *Applied and environmental microbiology*, vol. 61, no. 2, pp. 702–707, 1995.

[112] T. R. Thomsen, L. L. Blackall, M. A. De Muro, J. L. Nielsen, and P. H. Nielsen, "Meganema perideroedes gen. nov., sp. nov., a filamentous alphaproteobacterium from activated sludge," *International journal of systematic and evolutionary microbiology*, vol. 56, no. 8, pp. 1865–1868, 2006.

[113] K. Johnson, Y. Jiang, R. Kleerebezem, G. Muyzer, and M. C. van Loosdrecht, "Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity," *Biomacromolecules*, vol. 10, no. 4, pp. 670–676, 2009.

[114] A. Neef, A. Zaglauer, H. Meier, R. Amann, H. Lemmer, and K.-H. Schleifer, "Population analysis in a denitrifying sand filter: conventional and in situ identification of Paracoccus spp. in methanol-fed biofilms.," *Applied and Environmental Microbiology*, vol. 62, no. 12, pp. 4329–4339, 1996.

[115] C. Roller, M. Wagner, R. Amann, W. Ludwig, and K.-H. Schleifer, "In situ probing of Gram-positive bacteria with high DNA G+ C content using 23S rRNA-targeted oligonucleotides," *Microbiology*, vol. 140, no. 10, pp. 2849–2858, 1994.

[116] D. A. Stahl, B. Flesher, H. R. Mansfield, and L. Montgomery, "Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology.," *Applied and Environmental Microbiology*, vol. 54, no. 5, pp. 1079–1084, 1988.

[117] D. Orhon, F. G. Babuna, and O. Karahan, *Industrial wastewater treatment by activated sludge*. IWA Publishing, 2009.

[118] R. Zhang, H. M. El-Mashad, K. Hartman, F. Wang, G. Liu, C. Choate, and P. Gamble, "Characterization of food waste as feedstock for anaerobic digestion," *Bioresource technology*, vol. 98, no. 4, pp. 929–935, 2007.

[119] F. Pardelha, M. G. Albuquerque, M. A. Reis, J. M. Dias, and R. Oliveira, "Flux balance analysis of mixed microbial cultures: Application to the production of polyhydroxyalkanoates from complex mixtures of volatile fatty acids," *Journal of biotechnology*, vol. 162, no. 2-3, pp. 336–345, 2012.

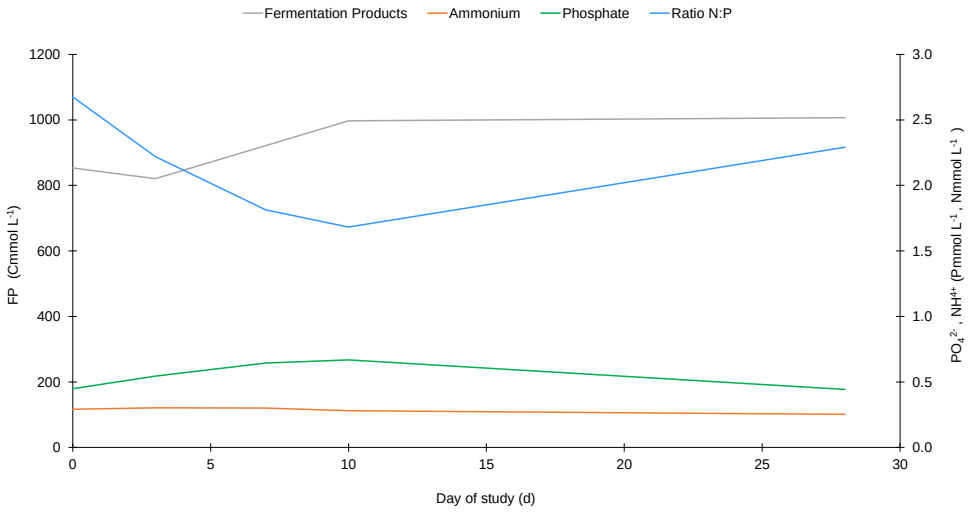
[120] L. Marang, Y. Jiang, M. C. van Loosdrecht, and R. Kleerebezem, "Impact of non-storing biomass on PHA production: An enrichment culture on acetate and methanol," *International journal of biological macromolecules*, vol. 71, pp. 74–80, 2014.

[121] D. Queirós, A. Fonseca, P. C. Lemos, and L. S. Serafim, "Long-term operation of a two-stage polyhydroxyalkanoates production process from hardwood sulphite spent liquor," *Journal of Chemical Technology & Biotechnology*, vol. 91, no. 9, pp. 2480–2487, 2016.

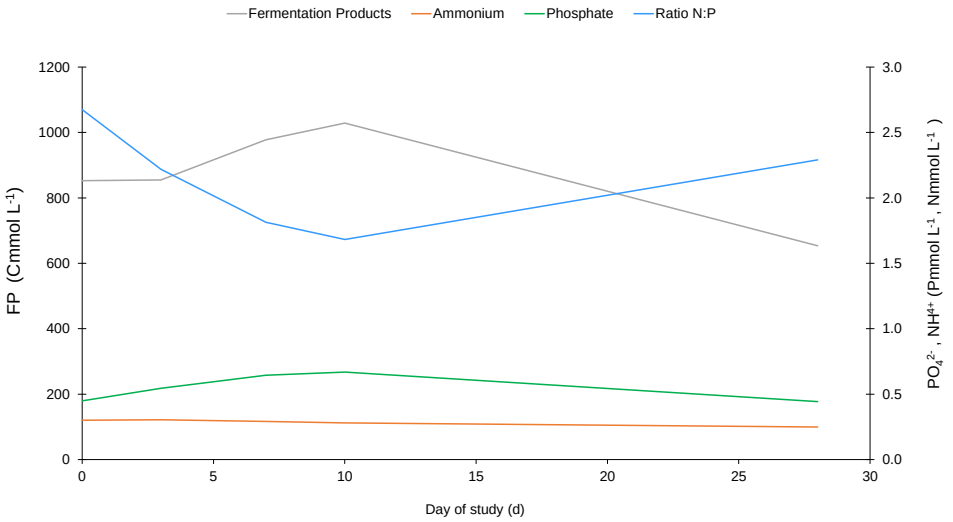
[122] S. Donhatai, T. Parinda, and S. B. Boonchayaanant, "In situ identification of polyhydroxyalkanoate (PHA)-accumulating microorganisms in mixed microbial cultures under feast/famine conditions," *Scientific Reports (Nature Publisher Group)*, vol. 10, no. 1, 2020.

Appendix A

Feedstock Characterization



(a) Fermentation products (Cmmol L^{-1}), ammonium (Nmmol L^{-1}) and phosphate (Pmmol L^{-1}) concentration of feed-N at room temperature, throughout the study.



(b) Fermentation products (Cmmol L^{-1}), ammonium (Nmmol L^{-1}) and phosphate (Pmmol L^{-1}) concentration of feed-N at 4℃, throughout the study.

Figure A.1: Influence of storage temperature of Feed-N over 28 days, in the most relevant parameters: fermentation products, ammonium and phosphate concentration.

Appendix B

Kinetic C

Table B.1: Consumption rate of each fermentation product, for test C, for Condition 2, accordingly with pulse number.

	Pulse Number					
Fermentation Product (gCOD L^{-1} h^{-1})	**1**	**2**	**3**	**4**	**5**	**6**
Acetic	-0.37 $\pm$ 0.01	-0.18 $\pm$ 0.04	-0.11 $\pm$ 0.16	0.02 $\pm$ 0.02	-0.06 $\pm$ 0.05	-0.19 $\pm$ 0.01
Butyric	-0.45 $\pm$ 0.03	-0.29 $\pm$ 0.07	-0.18 $\pm$ 0.02	-0.20 $\pm$ 0.00	-0.10 $\pm$ 0.01	-0.28 $\pm$ 0.02
Iso-Valeric	-0.03 $\pm$ 0.01	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01	0.03 $\pm$ 0.01	0.01 $\pm$ 0.00	-0.09 $\pm$ 0.08
Valeric	-0.10 $\pm$ 0.01	-0.04 $\pm$ 0.05	0.03 $\pm$ 0.00	-0.01 $\pm$ 0.00	-0.02 $\pm$ 0.00	-0.07 $\pm$ 0.01
Caproic	-0.69 $\pm$ 0.09	0.60 $\pm$ 0.26	0.93 $\pm$ 0.02	0.31 $\pm$ 0.02	-0.33 $\pm$ 0.04	-0.58 $\pm$ 0.04
Heptanoic	-0.05 $\pm$ 0.01	0.11 $\pm$ 0.05	0.15 $\pm$ 0.03	0.03 $\pm$ 0.01	-0.03 $\pm$ 0.00	-0.07 $\pm$ 0.01
Octanoic	-0.29 $\pm$ 0.14	0.40 $\pm$ 0.09	0.56 $\pm$ 0.26	0.21 $\pm$ 0.08	-0.12 $\pm$ 0.06	-0.21 $\pm$ 0.10