João Coelho Capela Guimarães Bello

Bachelor in Chemical and Biochemical Engeneering

Mass Balance to the Production of Polyhydroxyalkanoates from Mixed Microbial cultures with Fruit Pulp

Dissertation submitted in partial fulfillment
of the requirements for the degree of

Master of Science in
Chemical and Biochemical Engeneering

Adviser: Professor Doutor Mário Fernando José Eusébio, Faculdade de Ciências e Tecnologias, Departamento de Química, Universidade Nova de Lisboa

Co-adviser: Fernando Silva, Estudante, Doutoramento, Universidade Nova de Lisboa

Examination Committee

Chair: Dra. Isabel Maria Rôla Coelhoso
Rapporteurs: Dr. Rui Manuel Freitas Oliveira
Dr. Mário Fernando José Eusébio

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

November, 2019

Mass Balance to the Production of Polyhydroxyalkanoates from Mixed Microbial cultures with Fruit Pulp

Copyright © João Coelho Capela Guimarães Bello, Faculty of Sciences and Technology, NOVA University Lisbon.

The Faculty of Sciences and Technology and the NOVA University Lisbon have the right, perpetual and without geographical boundaries, to file and publish this dissertation through printed copies reproduced on paper or on digital form, or by any other means known or that may be invented, and to disseminate through scientific repositories and admit its copying and distribution for non-commercial, educational or research purposes, as long as credit is given to the author and editor.

This document was created using the (pdf)LaTeX processor, based on the "novathesis" template[1], developed at the Dep. Informática of FCT-NOVA [2].
[1] https://github.com/joaomlourenco/novathesis [2] http://www.di.fct.unl.pt

Acknowledgements

I would like thank to Dr. Maria Ascensão, for the fantastic opportunity to work on this project, and be a part of the PHA revolution.

I am also grateful to Dr. Mario Eusébio, Doc-student Fernando Silva for all the scientific, motivation and huge support given during this dissertation. I would also like to the members of the pilot lab, and analysis lab for all the patient and guidance through all the steps.

I would like to thank to all my family, specially my Parents, my brother, my sister, my cousin and last but not least my girlfriend Leonor. I'm very thankful for all the patient, love and infinite support overtime, without it it would not be possible to accomplish this work.

I am also gratefull to my friends, for being my chosen family.

Abstract

Bioplastics, specially polyhydroxyalkanoates(PHA), represent one of the best alternatives to plastics from non-renewable sources. The production of PHA, from mixed microbial cultures with low-cost feedstocks, as in this dissertation fruit pulp requires a process of three stages: treatment of the residues through anaerobic digestion which is used to feed both the selection and accumulation reactor. The biomass PHA-accumulating selection reactor is carried out on a feast and famine regime with uncoupled feeding from nitrogen an carbon, leading to increased selective pressure, where a ratio of COD:N:P(100:6.5:1:) is applied. The accumulation reactor is carried out on a fed-batch, and is inoculated from the sequential batch reactor and therefore is capable of accumulating PHA.

All of the processes were followed along two months of operation, from the Up-flow Anaerobic Sludge Blanket Reactor to the accumulation stage. In the first and second reactors, no alterations were made, and they were just monitored to study its evolution. In the accumulation reactor, 9 batches were made with and without nutrients, to optimize the process productivity. The following ratios were applied COD:N:P (100:6.5:1; 100:3,25:0.5;100:0:), to induce just little growth they were applied only in the first pulse.

To understand how much fruit pulp is needed to produce 1 kg of PHA, mass balance study's were made, to describe the general productivity of the process. The mass balance was made, while the acidogenic reactor had a high OLR, 16.09± $gCODL^{-1}d^{-1}$, and a conversion rate of 77%, it was considered to be the normal value of the operation. To the accumulation reactor, an average of every two batches where the same conditions were applied was used. Concluding that to produce 1kg of biopolymer from fruit pulp, the following quantities are needed: ACC-3/4 = 8011.6 gCOD; ACC(Nutri)-6/7 = 6906.8 gCOD, ACC(Nutri)-8/9 = 6142.6 gCOD.

Keywords: Volatile Fatty Acids, Mixed Microbial Cultures, Low Cost Feedstock, Polyhydroxyalkanoates, Fruit pulp, Ratio COD:N:P, Productivity.

Resumo

Os bioplásticos nomeadamente os polihidroxialcanoatos, representam uma das melhores alternativas aos plásticos produzidos por fontes não renováveis. A produção de polihidroxialcanoatos (PHA), a partir de culturas microbianas mistas com feedstocks de baixos custo, como no caso desta tese a polpa de fruta, requer um processo com várias fazes que envolve desde: o tratamento dos resíduos de comida através da digestão anaeróbia, que serve para alimentar ambos tanto o reactor de seleção como o de acumulação. O reactor de seleção é onde a biomassa acumuladora de PHAs é selecionada, através de um regime de fome e fartura e com a alimentação de azoto desfasada do carbono de forma a aumentar a pressão selectiva. O reactor de acumulação funciona em modo fed-batch, onde a sua biomassa deriva do reactor de seleção e por isso produtora de PHAs.
Todo o processo foi seguido ao longo dois meses, desde o reactor UASB (Reactor anaeróbio com fluxo ascendente e manta de lodo) passando pelo reactor de seleção até ao de acumulação. No primeiro e segundo reactor, não foram feitas alteraçãos ao processo apenas monotorizações de forma a acompanhar a sua evolução. No reactor de acumulação foram feitos ensaios, com e sem a adição de nutrientes de forma a optimizar a productividade do processo. Os seguintes rácios foram testados COD:N:P (100:6.5:1; 100:3,25:0.5;100:0:0), foram alimentados apenas no primeiro pulso.

De forma a compreender, qual a quantidade de polpa de fruta utilizada para produzir 1 kg de bioplástico foram realizados ensaios de balanços de massa de forma a descrever o rendimento geral do processo. O balanço de massa foi feito enquanto o reactor acidogénico estava com a sua maior carga orgânica, cerca de 16.09± $gCODL^{-1}d^{-1}$, e uma taxa de conversão de 77%. Este valor de conversão foi utilizado pois foi considerado o valor normal de operação. Para o reactor de acumulação a foi feita a média entre cada duas experiências realizadas para cada teste. Concluindo que para produzir 1 kg de biopolimero serão necessárias as seguintes quantidade de polpa de fruta: ACC-3/4 = 8011.6 gCOD; ACC(Nutri)-6/7 = 6906.8 gCOD, ACC(Nutri)-8/9 = 6142.6 gCOD.

Palavras-chave: Ácidos gordos voláteis, culturas microbianas mistas, feedstocks de baixo custo, polihidroxialcanoatos, polpa de fruta, rácio COD:N:P, productividade.

Contents

List of Figures

List of Tables

Acronyms

ACC	Accumulation
AD	Anaerobic Digestion
ADD	Aerobic Dynamic Discharge
ADF	Aerobic Dynamic Feeding
ATP	Adenosine triphosphate
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred-Tank Reactor
DO	Dissolved oxygen (mgO_2L^{-1})
EDTA	Ethylenediaminetetraacetic Acid
EPBR	Enhanced Biological Phosphorus Removal
EU	European Union
F/F	Feast and Famine ratio
F/M	Food to Microorganisms ratio
FF	Feast and Famine
Fp	Fermentation products
FSCW	Food Supply Chain Waste
FW	Food Waste
GC	Gas Chromatography
Gp	Global Productivity
HB	3-Hydroxybutyrate
HHx	3-Hydroxyhexanoate
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time

HV	3-Hydroxyvalerate
IC	Inorganic Carbon
KHP	Potassium Hydrogen Phthalate
MCL	Medium-Chain-Lenght
MMC	Mixed Microbial Cultures
NACE	Nomenclature of Economic Activities
OLR	Organic Load Rate (gCOD L^{-1} d^{-1})
PC	Sodium Carbonate
pH	Power of Hydrogen
PHA	Polyhydroxyalkanoates
PHB	Poly(3-Hydroxybutyrate)
PHBV	Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate)
PHHx	Poly(3-Hydroxyhexanoate)
PHP	Potassium Hydrogen Phthalate
PHV	Poly(3-Hydroxyvalerate)
PLA	Polylactic Acid
R1	Reactor 1 - Upflow Anaerobic Sludge Blanket
R2	Reactor 2 - Sequencing Batch Reactor
R3	Reactor 3 - Accumulation Reactor (Fed - Batch)
SBR	Sequential Batch Reactor
SCL	Short-Chain-Lenght
SHC	Sodium Hydrogen Carbonate
SRT	Solids Retention Time
TC	Total Carbon
TOC	Total Organic Carbon
TSS	Total Suspended Solids
UASB	Up flow Anaerobic Sludge Blanket

VFA	Volatile Fatty Acids	*
VSS	Volatile Suspended Solids	

Chapter 1

Introduction

1.1 PHA: Production, Characteristics and Applications

Waste production, a major problem in our society. Europe is responsible for the production of over 17 million tones of plastic waste and 88 million tones of Food Waste (FW). Solutions must be found to reduce or make use of the waste produced[1, 2]. From 1950, Europe had a constantly increasing demand for plastics reaching 51.2 million tonnes in 2017. As shown in Figure 1.1 plastic packaging demand leads with 39.7% and an estimated value of 100 billion euros annually, 95% of which will be only used once[1, 3]. Plastic packaging demand is followed by building and construction, automotive, electrical and electronics, household, agriculture and others (eg. Medical equipment, plastic furniture, technical parts, among others). Most of these types of plastic usually have a lifespan of more than 15 years and require a more specialized recycling process. Even though from 2006 to 2016, waste treatment of packaging plastic improved, recycling (corresponding to 6% of the plastics demand) and energy recovery increased by 74% and 71% respectively, while landfill decreased by 53%, higher efforts to improve waste recovery have to be implemented as only 12.55 million tones were recovered out of the 16.75 million tonnes of waste produced [4, 5].

Conventional plastics derivate from fossil fuels, a non-renewable source known for is a contribution to global warming. The low degradability of these types of plastic leads to the accumulation on the environment. It is estimated that between 150 to 500 thousand tonnes of plastic waste end up in the oceans every year just in Europe, contributing to the destruction of entire ecosystems, and also to the recently discovered plastic aggregation in the Mediterranean sea, severely affecting the environment and local economic activities [6].Individually packaged food and drink is expected to reach 900 billion items sold in

2020, therefore a different approach to the problem must be found, and biopolymers could be the answer[3]. Bio-Based polyesters such as, Polyhydroxyalkanoates (PHA), Polylactic Acid (PLA) could replace plastics with the advantage of being produced from renewable source and wastes (eg. FW, corn, potato, wheat, sugar, starch, paper mill, olive oil mill wastewaters, etc). Biopolymers biodegradability capabilities could prevent the high accumulation and consequent levels of pollution caused by the fossil fuel-based plastics. Their biocompatibility allows for its usage in medical appliances (eg. implants, meshes, scaffolds for tissue engineering) [7–9].

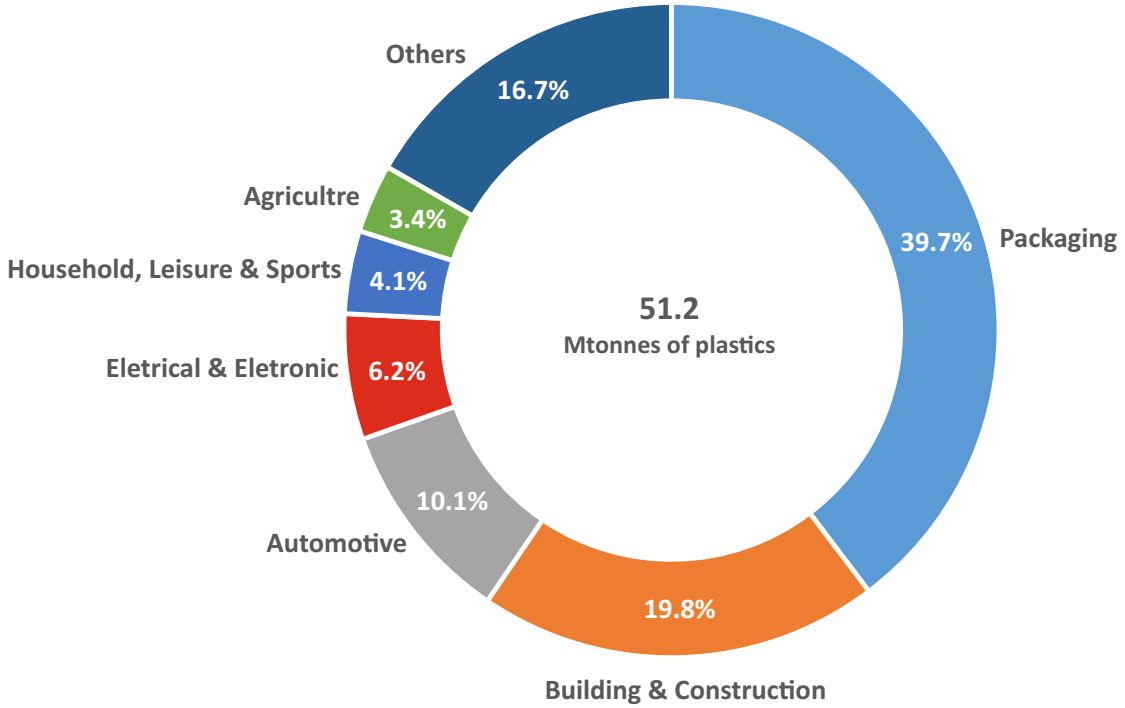

Figure 1.1: Total European plastics demand, adapted from Plastics - The facts 2018 [5]

The ammount of FW produced by European Union (EU) shows the inefficiency of our food supply that annually produces large amounts of accumulative waste, about 87.6 million tonnes, representing a cost of 143 billion euros to the industry [3, 10]. As shown in Figure 1.2, the major sectors that contribute to FW include: production (Agriculture, hunting and forestry, Nomenclature of Economic Activities (NACE) 01-03), processing (manufacture of food products and beverages, NACE 10-11) and food services (accommodation and food and beverage service activities, NACE 55-56). The production and processing sectors are responsible for packaging products to the wholesale & retail (Wholesale and retail trade, NACE 46-47), and food services [2]. Although households represent the majority of FW, its heterogeneous characteristics of it do not allow for an effective valorization, as an opposition to the production and processing sector that have

a more concentrated waste. Increasingly strict directives from EU, regarding the environment waste treatment and carbon emissions, have created the opportunity to a valorization of residues such as FW [11, 12]. While waste treatment through landfill(-75€/ton) represents a cost, revenue opportunities derive from the production of bulk chemicals (900€/ton- average), transportation fuel(200-400€/ton), cattle feed(70-200€/ton) or generation of electricity(60-150€/ton) [12].

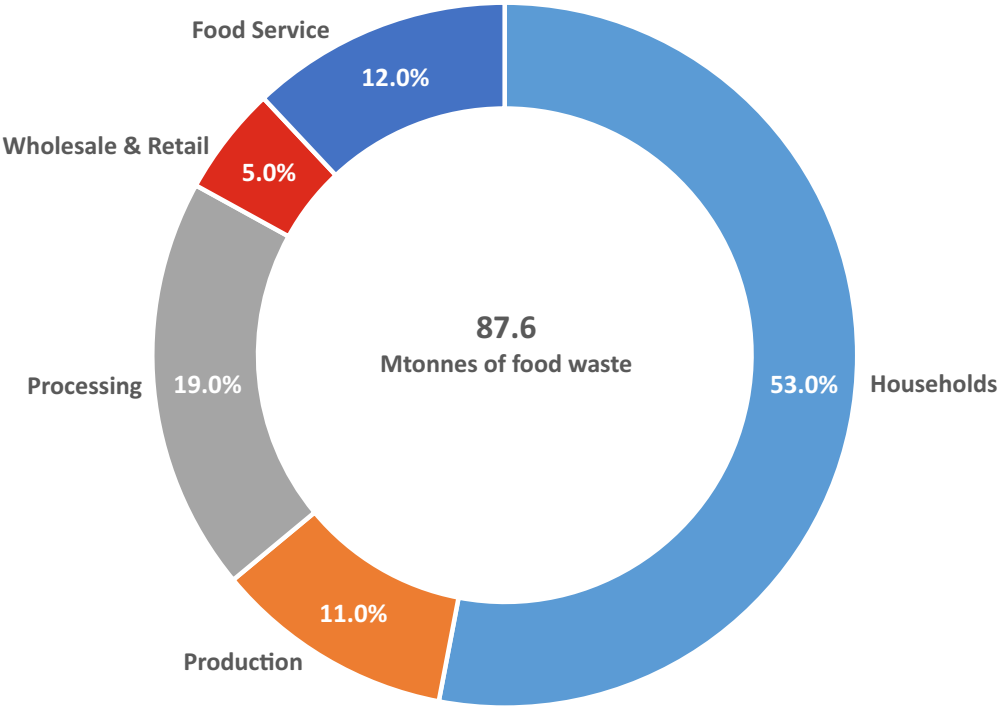

Figure 1.2: Food Waste distribution, adapted from Estimates of European Food Waste levels [2]

PHA production has been widely studied, an high-value commodity (4.5-6€/kg), from FW, which can be a valuable resource since it is considered to be an inexpensive carbon source, due to its high availability [13–15]. FW undergoes a complex procedure, to allow its usage for PHA production. PHA, are one of the most important alternatives to today's most used plastic commodities: they are fully biodegradable, biocompatible, and come from renewable sources[16]. In a perspective of circular economy, the collaboration between two different industries that depend on one another, plastic packaging, and food distribution contributing to a solution that not only, reduces the number of plastics produced from fossil fuels, but also improves the overall value chain [13, 17, 18].

There are more than 150 known hydroxyalkanoic acids which constitute the main component of these biopolymers. Poly(3-Hydroxybutyrate) (PHB), Poly(3-Hydroxyvalerate) (PHV), and Poly(3-Hydroxyhexanoate) (PHHx), are the three main homopolymers. Each one has different physical characteristics in terms of melting temperature, tensile strength,

Young's Modulus, and crystallinity, as shown in the Table 1.1 [8]. The monomer length is directly connected to the thermal and physical properties: with 3 to 5 and 6 to 14 carbon atoms they represent the Short-Chain-Lenght (SCL) and Medium-Chain-Lenght (MCL) respectively. Each chain has different macroscopic and mechanical properties: SCL are brittle, hard and have a high degree of crystallinity whilst MCL are soft, elastic with low crystallinity. During the production stage the blending of these homopolymers allows for a fine tune in their characteristics, in order to get similar, or sometimes even better properties than conventional plastics allowing for their replacement [19–21].

Properties	P(3HB)	P(3HB-co 3HV)	P(3HB-co 3HHx)
Young's Modulus (GPa)	3.5 - 4	0.7 - 2.9	500 (10% HHx)
Tensile Strength (MPa)	50	30 - 38	10 - 23
Elongation at Break (%)	3 - 8	up to 100	400 - 850
Melting Temperature (° C)	173 - 180	137 - 179	115 - 127
Glass Transition Temperature (° C)	5 - 9	-10 - 0	-1 - -4

Table 1.1: Summary of, P(3HB), P(HBHV) and P(HBHHX) thermal and mechanical characteristics, adapted from Anjum et al.[22] and Ching et al.[23]

PHB is the most common and well-studied SCL homopolymer, and as mentioned before, it has a brittle and hard behaviour, it has a crystalline nature, which makes processing difficult. A mixture of monomers such as HV and 3-Hydroxybutyrate (HB), results in a copolymer Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) (PHBV) that is more flexible and resistant material, and could be used for disposable items, such as food packaging or plastic bags [18]. The co-polymer PHBV, as shown in Table 1.2, will change its characteristics along with the increasing proportion of HV, as it will become more elastic and have a lower melting temperature.

	%HV Concentration			
Properties	0	9	20	25
Young's Modulus (GPa)	3.5	1.9	1.2	0.7
Tensile Strength (MPa)	40	38	32	30
Melting Temperature	179	162	145	137
Glass Transition Temperature	10	6	-1	-6

Table 1.2: Variation of the monomer HV proportion thermal and mechanical properties Variation of the monomer HV proportion thermal and mechanical properties, adapted from Ching et al.[23]

1.2 Pure Cultures vs Mixed Cultures

Industrial PHA production is mainly made through pure microbial cultures in their wild form, or by genetically modified strains. The process is conducted in sterile conditions

requires high oxygen demand, and high purity substrates (eg. glucose, sugars, starch), which increases the cost of the process. The value of these substrates could represent 45% of the total process cost, although recent efforts are being maid to find solutions using waste feedstocks instead. Waste feedstocks have different compositions (carbon/nutrients ratio), depending on the source. To get optimal performance, specialized bacteria could be used, which could favor a higher production depending on feedstock composition, considering the nutrients availability as mentioned below [18, 24–26]:

- **Carbon and Nutrients** - A growth associated bacteria, *Alcaligenes Latus*
- **Carbon** - A non growth associated bacteria, *Cupriavidus necator*

Despite the higher costs, an increased bacterial density and process stability, favor the constant product specifications needed by the industry, regarding the polymer characteristics. The process of pure PHA accumulating bacteria is divided into two main stages and is carried out on a fed-batch mode. In the first stage the culture is supplied with a growth substrate to achieve the maximum cellular density, in the second stage the medium supplied enhances PHA storage, with limiting nutrients available, where it usually achieves a PHA content of 80% to 90% [27]. Woo Suk Ahn and colleges reached high productivity rates and densities values of up to 4.6 g PHA/L/h and 200 g/L respectively, using an external cross-flow membranes to recirculate cells into the fed-batch reactor [28].

Even though higher productivities and PHA contents are reached in pure cultures, due to significant costs of the process mentioned before, an increased effort for the production of PHA from MMC has been made. MMC present themselves as a lower-cost alternative, for pure cultures. A 3-stage operation, as shown in Figure 1.3 is carried out in series, the first two stages work in a continues mode. The first stage usually consists of an anaerobic reactor (eg. Continuous Stirred-Tank Reactor (CSTR) or UASB) where the waste feedstock is decomposed in smaller molecules, such as VFA (further explained in the Sub-Section 1.3) and it is used to feed both of the reactors in the following stages. The reactor type used in the second stage is usually an Sequential Batch Reactor (SBR) where the PHA accumulating bacteria are selected under the dynamic operation conditions imposed (further explained in the Sub-Section 1.4); finally the third stage is the production stage, which is carried out on a fed-batch mode.

The lower productivity, and cell density could be compensated by the continuous mode characteristics of the process, which could lead to a much higher specific productivity. This system works in a non-sterile environment and predominantly uses waste feedstocks for feeding, allowing for a decreased cost of the process. A consistent polymer composition is harder to achieve, considering the inherent characteristics of a selected biomass, and the seasonality of the feedstock applied to the anaerobic reactor [8, 9, 29].

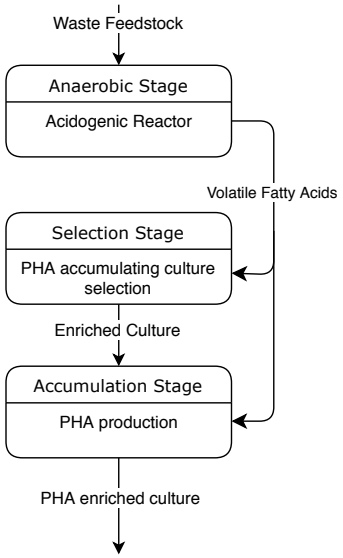

Figure 1.3: Typical 3 stage process to PHA production using MMC

1.3 Acidogenic Fermentation - First Stage

Anaerobic Digestion (AD) is widely used for the treatment of several organic wastes [30]. When comparing it with aerobic systems, the main advantages are the low operation costs since it does not need a oxygen supply, intensive mixing is not required, it has a lower production of sludge, and higher efficiency at treating complex organic molecules such as, proteins, fats and carbohydrates than [31, 32]. AD is a complex biochemical sequential procedure in which, carbon compounds, are hydrolyzed, fermented into intermediate products that are reduced into methane and oxidized to carbon dioxide[17]. This sequence of events are known as hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

As shown in Figure 1.4, the first and very important step is the hydrolysis as whenever the substrate is composed of complex molecules as mentioned before, that need to be broken down into smaller molecules such as amino acids, fatty acids, and basic sugars, as otherwise the uptake can't achieved for such large molecules[33]. It is important to mention that there also other by-products coming from the hydrolysis such as hydrogen and acetate. The second step is acidogenesis, this is where microorganisms digest the sub-products of hydrolysis, and convert them into shorter volatile fatty acids (eg. Acetic Acid, Propionic Acid, Butyric Acid, Valeric Acid), medium volatile fatty acids (eg. Caproic Acid), hydrogen, carbon dioxide, ammonia, and hydrogen sulphide. During the process an acid environment is created, that needs to be controlled to maintain the conversion rates. The acetogenic phase occurs with the formation of volatile fatty acids, so a concrete distinction between them is not clear. Methanogenic phase, is the last one, and is a critical step to the production of bio-gas. Characterized for being the slowest, the

methane-producing bacteria are strictly anaerobes, and are vulnerable even to the lowest concentration of oxygen. The methanogenic bacteria could be divided in two groups, the acetoclastic and hydrogentrophic bacteria. The acetoclastic group produce methane from acetate, while the hydrogentrophic accept hydrogen as a donor and carbon dioxide as electron acceptor to produce methane [34].

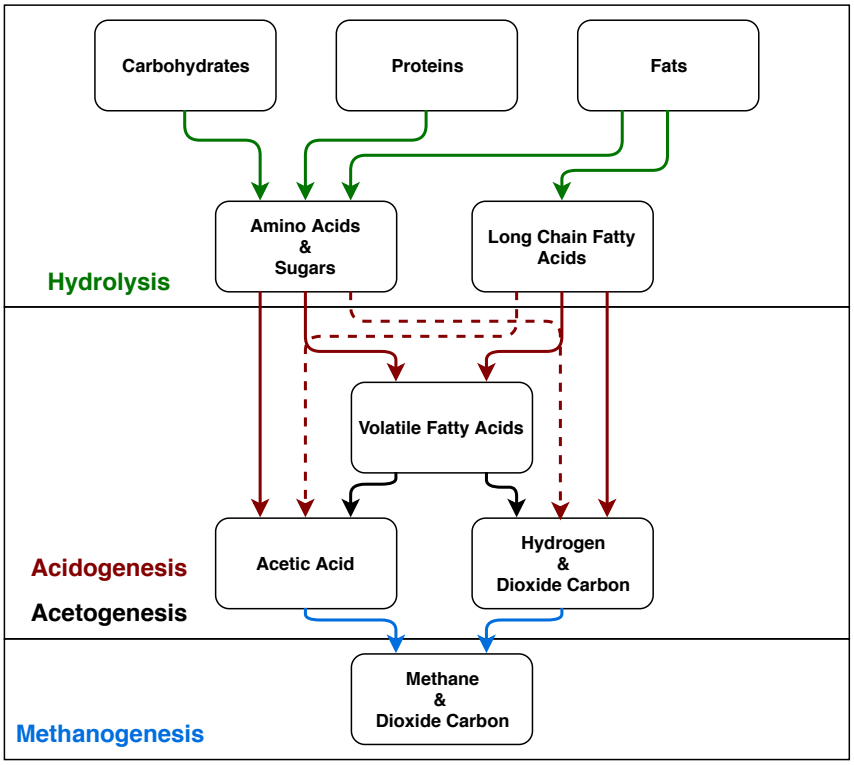

Figure 1.4: Digestion Diagram

Most of the studies made, were about a single-stage system, where the sequence of events mentioned before, occur in the same reactor. A two-stage system, as shown in Figure 1.5 consists in two separated reactors with different settings, Power of Hydrogen (pH), Hydraulic Retention Time (HRT), Organic Load Rate (gCOD L^{-1} d^{-1}) (OLR) and temperature, leading to the selective environment of the desired bacteria's in each phase [35]. Studies have bean made, to identify which conditions both types of microorganisms would prefer. For an UASB reactor, typical operation values for the acidogenic stage are a lower pH, preferably below 6.5 and HRT 1 as on the contrary methanogenic requires higher pH and longer HRT, such as 7 and 5 respectively [35, 36].

These analysis will manly focus on the acidogenic fermentation, as the product of interest is VFA, regarding its importance as they work as precursors to PHA synthesis. The composition of VFAs can also take an important role in the polymer composition, and it can be controlled by altering the pH conditions of the acidogenic reactor[37, 38].

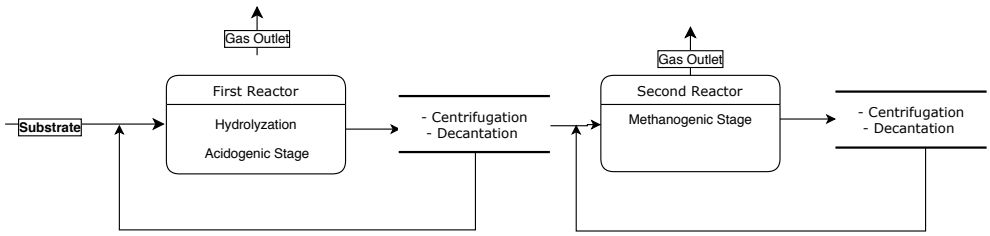

Figure 1.5: Typical Two Stage Biogas plant

Associations to the composition of the polymer have already been made, as it was demonstrated that a higher concentration of even carboxylic chain, for example, acetic acid or butyric acid, is usually associated to the formation of HB, as opposed to when a higher concentration of odd carboxylic chains result in a higher HV content [38, 39].

1.4 PHA Accumulating Culture Selection - Second Stage

MMC is composed of a consortium of microorganisms, who are mainly used for the treatment of wastewater (e.g, wastewater treatment plants, composting facilities)[40]. In Enhanced Biological Phosphorus Removal (EPBR), an anaerobic/aerobic cycle is imposed and polymer storage was generally accepted as a highly important characteristic in the metabolism of Bio-P bacteria, since it stored PHA for cell growth, maintenance and glycogen replenishment where it was first observed[41, 42]. Intracellular PHA's accumulation occurs naturally in more than 300 bacteria types[8], as a source of carbon and energy, usually when not enough nutrients such as nitrogen and phosphorus, or even oxygen is available and consequently growth is limited, and the carbon storage is enhanced[43]. Activated sludge commonly comes from wastewater treatment plants, and as mentioned before it is composed by a variety of microorganisms, but not all of them are able to store PHA's, so a selection process made through Aerobic Dynamic Feeding (ADF) or Feast and Famine (FF) regime is applied and consists in short periods of feast ant long periods of famine, typicality made with an SBR. These steps are vital to enhance the global capacity of the biomass to store biopolymers and improve the substrate consumption rate and growth rate [44].

To manage a sustainable culture, the Feast and Famine ratio (F/F) should remain between the range specified on the table 1.3, where the highest ratio implies a growth response and the lowest storage, and its calculated by dividing the length of the feast phase by the lenght of the famine fase [45], to which a ratio of up to is considered preferable. For the sake of the argument the same cell density, feedstock concentration and volume are considered, manipulation of F/F ratio could be achieved trough the rate in which the feedstock is fed. Considering that it should be maintained under the Food to Microorganisms ratio (F/M) ratio to avoid substrate inhibition, with a higher supply rate the feast phase will be shorter as opposite to a a lower supply rate that will lead to a longer feast phase [46]. In pilot scale operations the pH control will increase the costs of the process and complicate the operation, regarding that the advantage in the selective pressure is not clear, since an higher PHA% could be achieved at the end of the feast, leading to a possible higher substrate uptake ratio and consequent lower F/F [16, 47, 48].

	Operation Parameter	Range
SBR Reactor	SRT (d)	1 - 20
	HRT (d)	1 - 3
	Lenght of SBR cycle (h)	2 - 12h
	pH	7 - 9.5
	Temperature (° C)	20 - 30
Feedstock	OLR (gCOD $L^{-1}d^{-1}$)	1.8 - 31.25
	Substrate Concentration ($gCODL^{-1}$)	0.9 - 31.25
	C/N (gCg^{-1})	9-120
Feedstock and Reactor	F/F ratio	0.1-1.15

Table 1.3: Summary of the most common operation parameters applied in MMC selection to PHA storing microorganisms. Adapted from (Reis et al. 2011[27])

Developments made in the selection process applied to PHA accumulators have been proposed by several authors, an F/F regime was applied as it is considered an essential step in the selection stage [8, 27].

Johnson et al[49] highlighted the selective pressure through manipulation of the operating parameters by gradually increasing the temperature and decreasing the SRT, from 20° to 30° and 4 days to 1 day respectively. A concentration of substrate of 54 VFA/L and a high nutrients ratio (C:N:P - 100:13:5)(Cmmol:Nmmol:Pmmol) were used, in order to sustain an high growth rate needed for 1 day SRT. Up to 89% PHA content with a 1.2 gPHA/ g Xh was achieved during an accumulation set up with a duration of 7.5 hours. The author suggested that possibly the reported high kinetic parameters were due to the higher temperature, long cycles (12h) and low SRT (1d), that result in significantly F/F

ratio of 0.1.

Silva et al.[50] had a different approach for to the problem, taking in account that many types of feedstock used for the PHA production are nitrogen-deficient (e.g: paper mill, cheese whey permeate, sugar-cane molasses or olive oil mill wastewaters) and therefore they are unable to create the conditions for microorganism to grow. Considering that if feeding of nitrogen is coupled with the carbon source, allows for all the non PHA-accumulation bacteria to grow as well, leading to the selection being made during the famine phase. The uncoupled feeding of carbon (Feast Fase) and nitrogen (Famine Fase) lead to a stronger selection, as only the bacteria able to store intracellular PHA, could grow. Oliveira et al. [51] suggest that regarding the imposed selective pressure in this procedure the extent of the famine phase could be lower, as the storage performance remained stable despite the variations on the F/F ratio, and as a result it could lead to an improvement in the general process productivity.

Chen et al. [45], suggested an alternative to ADF, Aerobic Dynamic Discharge (ADD), the main difference between the two methods is that after depletion of carbon a settling phase for a short time (10-15 min), and consequent withdrawal. Throughout the feast phase, the bacteria that had the highest substrate uptake and highest storage response, stored more intracellular PHA. As a consequence got more dense and therefore heavier enhancing the settling capabilities, and making the desired physical selection. This method offered some advantages, regarding that industrial substrates have residual Chemical Oxygen Demand (COD), and in this way a better selection could be made since the conditions imposed were stronger, because as it prevents growth of the undesired non-PHA accumulating microorganisms. The method proposed by both the authors, also has an uncoupled depletion of carbon and nitrogen [45, 52].

1.5 PHA Accumulation - Third Stage

The third step of the process consists in the PHA accumulation reactor, using the preselected microorganisms consortium of the selection stage. To reduce the process cost and improve productivity, several control techniques could be applied such as temperature, pH value, Dissolved oxygen (mgO_2L^{-1}) (DO) quantity and substrate source (Fed-batch or continuous feeding). The nutrients availability, is usually considered secondary in consideration that in this stage, regarding that PHA storage is preferred over growth. However, nutrients availability, in low concentrations, could lead to an increase in productivity, since growth response is lower than PHA storage rate due to active biomass growth [53]. Despite the fact that most studies utilize fed-batch feeding strategies, recent ones show that a continuous feeding could have several advantages regarding, polymer composition

	Operation Parameter	A	B	C
SBR Reactor	SRT (d)	1	4	10
	HRT (d)	1	1	1
	Lenght of SBR cycle (h)	12	6	6
	pH	7	7.6	8-9
	Temperature (∘ C)	30	25	20
Feedstock	OLR (gCOD $L^{-1}d^{-1}$)	1.8 - 31.25	8.5	2
	Substrate ($gCODL^{-1}$)	0.9 - 31.25	10.1	1,008
	C/N (cmol/Nmol)	9-120	17.9	16.6
Feedstock and Reactor	F/F ratio	0.1	0.14	
Results	PHA content (%)	88	ND	74.16

Table 1.4: Summary of the conditons in the SBR Reactor and Results. A - Johnson et al[49] B- Silva et al.[50] C - Chen et al.[45] ND - No Data

and general productivity [18, 20]. Both of the methods mentioned bellow, the substrate uptake rate have changed not only because of the bacterial culture saturation, but also the substrate concentration on the reactor. Michaelis-Menten Equation 1.1, specifies that the concentration of substrate in the reactor (S), will influence the volumetric consumption rate of substrate (r_s), K_m represents the affinity constant, specifically the substrate concentration in which the volumetric consumption rate of substrate is halved [54].

$$r_s = \frac{v_{max} \times S}{K_m + S} \tag{1.1}$$

Pulse wise feeding is DO controlled, the availability of VFAs could be measured according to the substantial difference in DO level, regarding the carbon source availability. Throughout the process when the external carbon source is fed a sudden decrease in DO is measured, in relation with the substrate uptake. Once depleted, an abrupt raise on DO is reached, and new pulse is fed. During the accumulation process, when the selected culture starts to get inhibited by product (Saturated) regarding its high intracellular polymer concentration, a decrease in substrate uptake rate and the variability of DO level is verified, therefore the process is finished. [8, 9].

An alternative to pulse wise feeding, consists of feeding the substrate continuously. The process usually pH and DO controlled. Along the accumulation process a continuous pulse, of reduced flow and higher concentration is fed reducing the probability of inhibition by substrate. In the beginning of the process the DO and pH levels will be at their maximum, when the external carbon source is fed, a sudden decrease in DO level is observed. Along the accumulation DO will increase, in opposition to the pH that will decrease, as a consequence of the substrate uptake rate reduction and the accumulation of none consumed VFA, which have an acidic pH. Higher volumetric productivity and

yield % of PHA were obtained by several authors, considering the advantage of an higher metabolic activity maintained along the process. A pulse wise feeding had variations as result of its characteristics. A better manipulation of the variations in monomers concentration, considering the same given fermented could be achieved, due to the constant availability of all VFA despite its concentration, by virtue of being precursors of PHA synthesis [20, 40].

Chapter 2

Objectives

As mentioned before, solutions to the problems inherent of our consumerist society must be found with the objective of reducing the waste that we produce. Plastics from non-renewable sources are a great source of pollutants, in all the life cycle stage. Their production releases greenhouse gases to the atmosphere and contributes to the global warming, and since they are non-biodegradable the high million of tones of plastics that aren't recovered tend to accumulate in the environment for many years and as a consequence directly affect vital ecosystems. Bio-plastics from PHA accumulating bacteria are one of the most promising alternatives. Nowadays, PHA production is still an expensive process and therefore their price is still higher then the common plastics.

The purpose of this dissertation is to make a contribution, by doing a mass balance of the system in order to comprehend how much fruit pulp is needed to produce 1kg of PHA. An increase of the volumetric production of PHA while maintaining the a high PHA %, was also tested. MMC are seen as the most economical way to produce the desired bio-plastics, as it does not require sterile conditions and low-cost waste feedstocks can be used. Various sources of waste feedstocks (eg. fruit pulp, food waste, olive oil mill wastewaters, paper mill) can be used, and when not enough nutrients are present (Carbon:Nitrogen:Phophorus ratio) to allow bacterial growth, they have to be supplemented. Several studies have applied different ratios, to both SBR reactor and PHA accumulation reactor to optimize the production to a given feedstock.

In the accumulation reactor, PHA accumulation tend to be preferred over growth. High quantities of carbon will be fed, and if enough nitrogen is provided other non-accumulating PHA bacteria have the right conditions to grow and therefore reduce the

concentration of the desired PHA. In the SBR reactor, carbon and nitrogen feed was uncoupled leading to a strong bacterial selection, regarding the high substrate uptake and high PHA accumulating capabilities, and therefore provided good conditions to provide nutrients in the accumulation reactor.

In this context, the goal of this study were to investigate:

- **H**ow will the mass balance of the system vary, considering the most important biomass components, carbon, nitrogen and phosphorus. The studied process, had several stages, from the inoculation of the SBR reactor to what is considered a stable stage.
- **D**ifferent ratios of Carbon:Nitrogen:Phosphorus (100:6.5:1, 100:3.5:0.5, 100:0:0) were applied in the accumulation reactor, to improve PHA volumetric production.

To achieve these objectives, 3 pilot scale (UASB Reactor - SBR Reactor - Accumulation Reactor) reactors representing the different stages of the process were used. The UASB reactor provided the carbon source for both the SBR and Accumulation reactor. Overtime there were variations in the composition of the VFA and therefore, supplementing was needed to maintain the desired OLR on the SBR and polymer composition at the production stage.

Chapter 3

Materials and Methods

3.1 Experimental Set-Up

The setup is consists of three pre-industrial reactors, and each one represents a different stage of the process.

3.1.1 1^{st} Stage - UASB

The acidogenic fermentation was carried out on an UASB, with a nominal volume of 100 L and a working volume of approximately 60 L liquid, and 40 L gaseous phase. The activated sludge utilized to inoculate the reactor, came from a full-scale anaerobic digestor, in the form of granular sludge. The operation was taken at anaerobic conditions, a pressure of 1.1 bar, a controlled temperature at 30° C through a thermostatic jacket, and pH varying from 4.7 and 5.1, being the optimal value 4.9. The reactor, as shown in Figure 3.1 is made of a double wall in acrylic, with 8 apertures. They were used with the following purposes: Monitoring, (pH, liquid Level, temperature, pressure), add substrate, withdraw effluent, gaseous outlet, and re-circulation. The re-circulation flux (average of 2.15 l/min), diffused with inert glass marbles, is enough to allow the fluidization of the reactor. To guarantee a continuous anaerobic operation, two one-way valve's were used, as shown in Figure 3.1. A pressure higher than the atmospheric was achieved, with the strangulation of the gaseous outlet, the pH was kept constant through the addition of Sodium carbonate.

The reactor was controlled manually. The UASB, works in continuous, and it's supplied with 60L/Day of a medium composed by, fruit pulp, Ammonia chloride (NH_4Cl), Mono-potassium phosphate (KH_2PO_4), with a composition of (100:0.5:0.15) (Cmmol: Nmmol: Pmmol), and other micro-nutrients such as Magnesium sulfate ($MgSO_4$), Calcium chloride ($CaCl_2$) and Iron chloride ($FeCl_3$). The amount used depend directly from

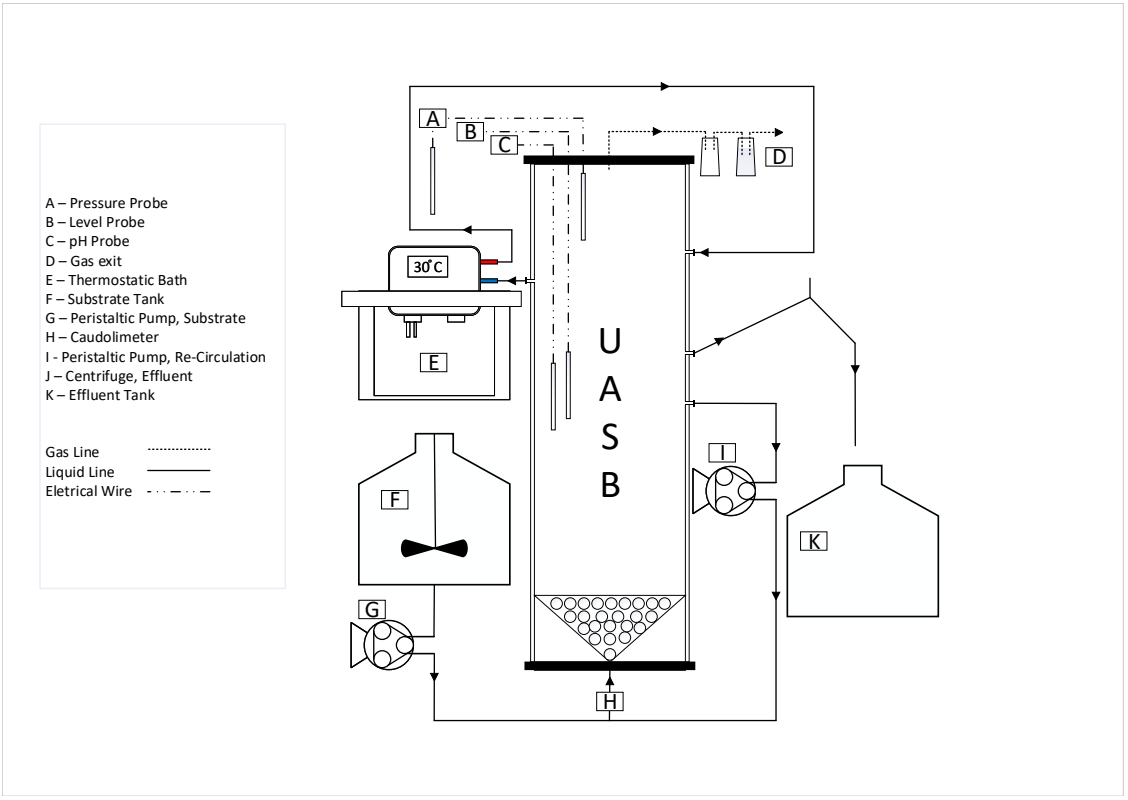

Figure 3.1: UASB Layout

the fruit pulp concentration. For pH buffering purposes sodium bicarbonate is added, and and HRT of one day was applied.

The UASB operation was monitored with daily measurements of pH, pressure, temperature. Three times a week the performance of the system was monitored through the gaseous outlet(D), regarding the concentration of, Methane(CH_4), hydrogen(H_2) and carbon dioxide(CO_2), and VFA concentration of the outlet (K). The effluent (K), every time an SBR cycle or an accumulation was monitored, measurements of Total Suspended Solids (TSS), and sampling of Total Carbon (TC), COD, VFA, ammonia and phophorus concentration were made.

3.1.2 2^{nd} Stage - SBR

The selection of a PHA-storing MMC was taken on a fully aerobic pilot-scale SBR, with a nominal volume of 120 L and a working capacity of approximately 90 L liquid. The activated sludge in which the reactor was inoculated came from Etar de Mutela, a municipal water treatment plant. The operation was taken at fully aerobic conditions, a controlled temperature at (23° C) using a thermostatic bath and pH (8) with the addition of sodium bicarbonate into the carbon source, that came from the UASB reactor. Schematics of the assembling represented in Figure 3.2. The aeration of the reactor was made by an air compressor, with an average output of 0,8 vvm, through a ring sparge

diffuser, and stirred by a double mechanical impeller, to guarantee a non-limiting oxygen concentration (>2mg/L) at all times. The reactor is made of Stainless Steel, and it's operating fully automatically 24 hours a day, in 12 hours phases of the cycle. The SBR in the study followed a strategy of uncoupled carbon and nitrogen/phosphorus feed, where its depletion occurred in the feast and famine phase respectively. The beginning of the cycle, feast phase, the carbon and mineral source are fed, following a reaction time of 120 minutes, where all the carbon source was consumed and mostly stored in the form of PHA's. The feeding of nitrogen and phosphorus, during the famine phase, succeed by a reaction time of 495minutes when the biomass was able to grow. Effluent from UASB was used as carbon source. During a part of this thesis, the SBR had to be re-inoculated, and consequently, the reaction times of the cycle, have changed, feast 180 minutes, famine 435 minutes.

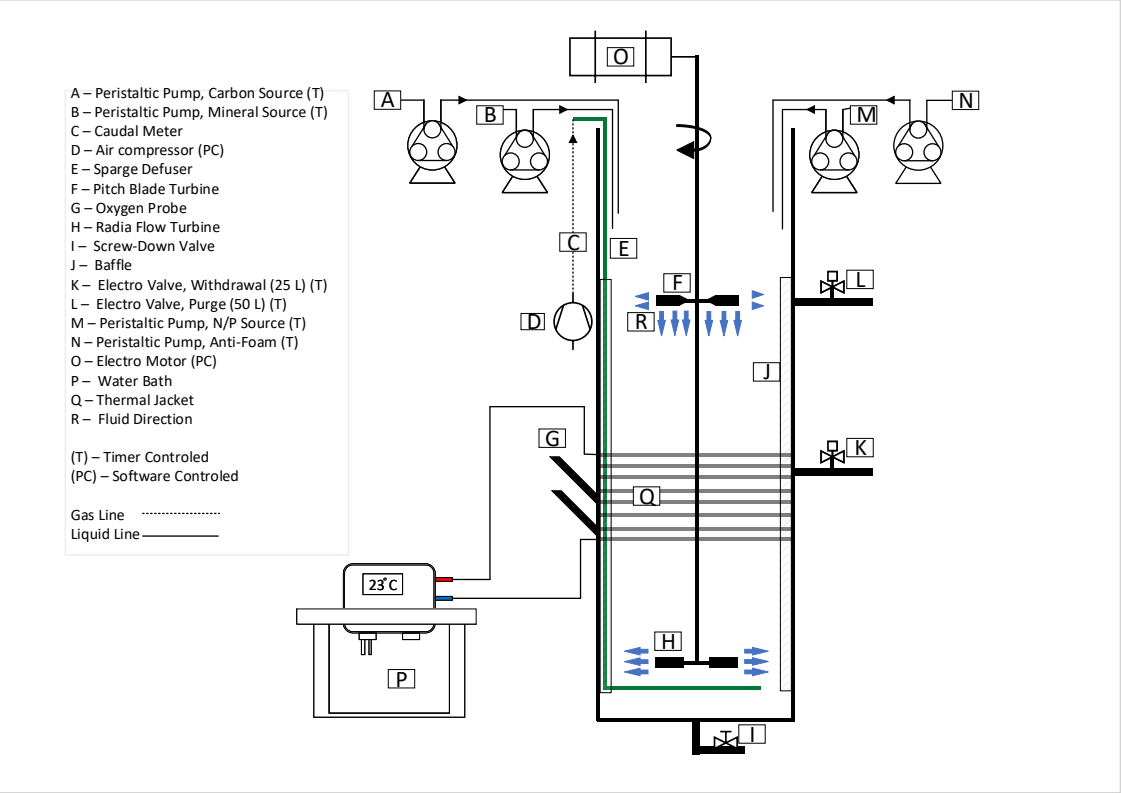

Figure 3.2: SBR Layout

An auxiliary computer software, BioReactor - Lab View, controlled and managed the operation through all of the phases, with a total length of 12 hours, at all time during the study. The ratio of carbon, nitrogen and phosphorus was, (C:N:P) of (100:6.5:1) (C-mmol/N-mmol/P-mmol). HRT is equal to 1d and the SRT 4 d. The mineral medium had a trace elements solution composition of, iron (III) chloride hexahydrate ($FeCl_3.6H_20$), Boric Acid (H_3BO_3), Cobalt Chloride ($CoCl_2.6H_2O$), Manganese Chloride ($MnCl_2.4H_2O$), Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$), sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$), copper sulfate penta hydrate ($CuSO_4.5H_2O$), micronutrients Ethylenediaminetetraacetic

Acid (EDTA), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), calcium chloride dihydrate ($CaCl_2.2H_2O$) . The monitoring of the SBR system, followed the scheme presented in the Table 3.1, 0 represent the beginning of the cycle, 1-6 the uptake of the carbon source (Feast phase), 7-10 the uptake of ammonia and phosphorus (Famine phase), 10 end of the monitoring 5 hours after.

	SBR Cycle						
	VFA	PHA	TSS	COD	TC	PO_4	NH_3
Carbon Feed 0	•	•	•	•	•	•	•
1	•	•					
2	•	•					
3	•	•	•	•	•		
4	•	•					
5	•	•					
6	•	•	•	•	•		
7		•	•			•	•
7A						•	•
7B						•	•
8		•	•	•	•	•	•
9						•	•
10	•	•	•	•	•	•	•
W	•		•	•	•	•	•
P	•	•	•	•	•	•	•

Table 3.1: SBR Sampling (W- Withdrawal, P - Purge)

3.1.3 3^{rd} Stage - Accumulation

Stage 3 of the process, the PHA production. It was taken on a pilot scale with a volume of 65 L and a working capacity of approximately 55 L. The reactor was inoculated with biomass from Section 3.1.2 collected at the end of the famine phase. The accumulation process started one hour after the purge came out, approximately the same time as the sedimentation phase applied on the SBR system. The operation was fully aerobic, an air compressor with an average output of $1vvm$, provided air trough a ring sparge diffuser, stirred by a double mechanical propeller guaranteeing a non-limiting concentration of oxygen (>2mg/L). The effluent from UASB was used as carbon source and it was given through pulses controlled by the level of DO, whenever it raised above a certain level, another carbon pulse was given. The accumulation monitoring, followed the scheme presented in the Table 3.2, 0 represents the initial state of the biomass, 1-5 the carbon source uptake, PHA conversion on the first pulse, after each pulse and consequent carbon depletion (Pre-pulse).

There were two approaches of Accumulation (ACC), with nitrogen and phosphorus feeding on the first pulse, and without. The concentrations applied, are specified on

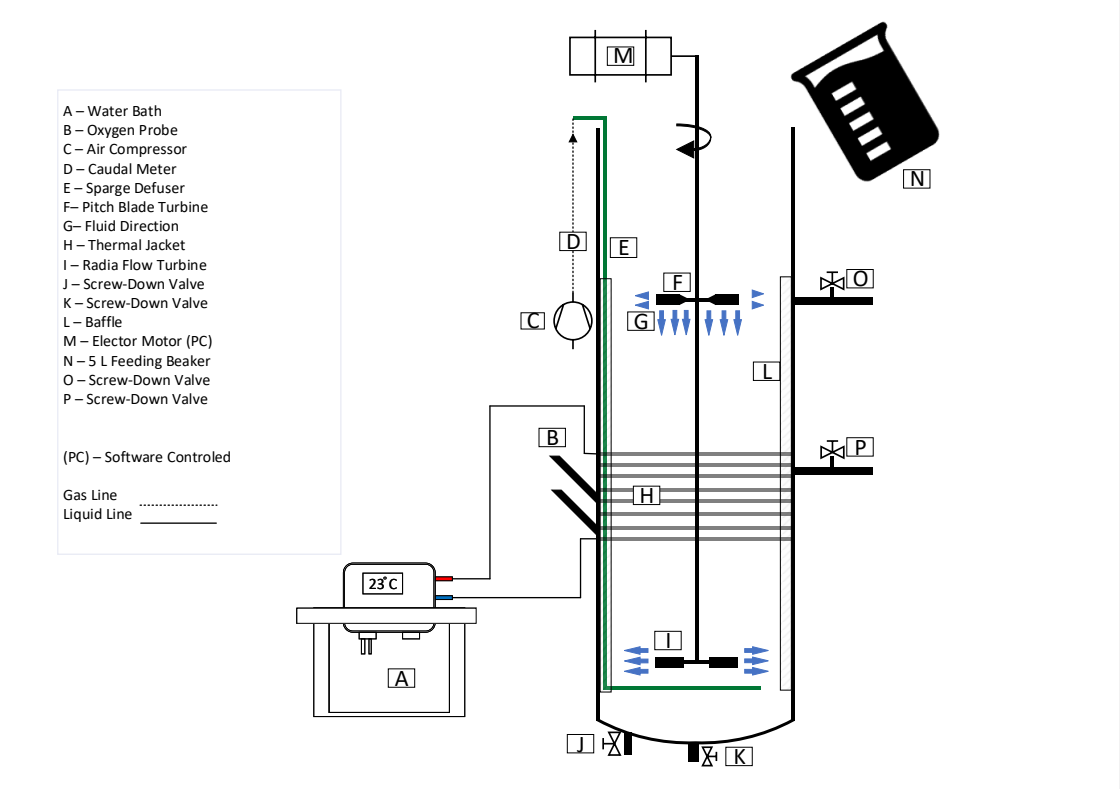

Figure 3.3: Accumulation Fed-Batch Layout

	Accumulation						
	VFA	PHA	TSS	COD	TC	PO_4	NH_3
0	•	•	•	•	•	•	•
1	•	•				$\bullet^a$	$\bullet^a$
2	•	•				$\bullet^a$	$\bullet^a$
3	•	•				$\bullet^a$	$\bullet^a$
4	•	•				$\bullet^a$	$\bullet^a$
5	•	•	•	•	•	$\bullet^a$	$\bullet^a$
Pre-pulse	•	•	•	•	•	$\bullet^a$	$\bullet^a$
End of ACC	•	•	•	•	•	•	•

Table 3.2: Accumulation Sampling (X^a - Only made, when ammonia and phosphorus were added on 0)

the Table 3.3. For each test, at leats two accumulations were done for reproducibility purposes. The biomass initial biological state as referred by Valentino et Al [53], was compared with the SBR monitoring taken on the day before since there were only once cycle between each task, and how long took the first pulse to be consumed in comparison with the SBR feast, at the same day.

Concentration	Carbon (C-mmol)	Nitrogen (N-mmol)	Phosphorus (P-mmol)	$Nr°$ Experiments Uni
1^{st} Test	100.00	6.50	1.00	2
2^{nd} Test	100.00	3.25	0.50	2
3^{rd} Test	100.00	0.00	0.00	7

Table 3.3: Carbon:Nitrogen:Phosphorus Ratio

3.2 Analytical Procedures

3.2.1 Total Suspended Solids and Volatile Suspended Solids

The concentration of the biomass, as well as the PHA's, will have variations along with the monitoring of the selection stage and the accumulation stage. To better understand it, the TSS and Volatile Suspended Solids (VSS) were calculated, the procedure followed the standard methods [55].

A glass microfiber filter (Whatman 1,2 μm) was weighted in a scale (P1) , afterwards two samples of the reactor, were filtered through a vacuum filter(5ml except withdrawal 10 ml). Samples were dried in a kiln, operating at 105° C, for at least twelve hours. After cooling in a desiccator, samples were weighted (P2) again for the TSS calculation. To calculate the VSS the samples were dried in a muffle, operating at 550° C, for two hours, cooled in a desiccator and weighed(P3) again.

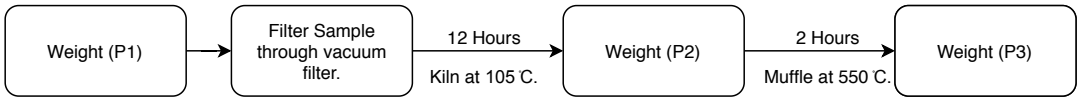

Figure 3.4: Flow Chart of TSS and VSS process

The following equations were used to calculate the parameters :

$$TSS = \frac{P_1 - P_2}{Samples_{Volume}} \quad VSS = \frac{P_2 - P_3}{Samples_{Volume}} \tag{3.1}$$

3.2.2 Chemical Oxygen Demand

The Chemical Oxygen Demand (COD) is usually used as a measure of pollutants in natural or residual waters. It is defined as the amount of a specific oxidant needed, to completely oxidize the biomass present in the sample under controlled conditions. The analysis is made in an acid environment, using silver as a catalyzer and dichromate as a reducer, therefore the dichromate ion oxidizes COD material in the sample during digestion time. Hence, the result of the oxidation is the change of chromium, from hexavalent (VI) to the trivalent (III) state, both are colored and absorb in the spectrum

visible region. The dichromate ($Cr_2O_7^{2-}$), hexavalent chromium, absorbs strongly in the $400nm$ and almost zero at $600nm$, while the chromic ion (Cr^{3+}), trivalent chromium, absorbs strongly at $600nm$, and much less at $400nm$, comparing the same regions for each ion. The method used for this analysis is the SMEWW, 5220 B. Closed Reflux, Colorimetric Method.

3.2.2.1 Solutions preparation

Standard solution - The pattern solution, equivalent to $2g/lO_2$, is made with Potassium Hydrogen Phthalate (KHP), pre-dried at 110° C until the weight does not change. weight, $0.425g$ and dilute it on $250ml$ of distilled water, this solution is stable for a week when refrigerated. The preparation of the different concentration standards was made through the dilution of KHP solution, with distilled water.

Sulfuric Acid Reagent - Weight $5.5g$ of Ag_2SO_4 to one liter of concentrated H_2SO_4 (>97%), let it dissolve for a day or two.

Standard Digestion Solution - To $500ml$ of water add, $10.216g$ of potassium dichromate ($K_2Cr_2O_7$), previously dried for two hours at 150° C, $167ml$ of concentrated sulfuric acid H_2SO_4 (>97%), and $33.3g$ of Silver Sulfate (Ag_2SO_4). Dissolve to 1 liter with distilled water, and let it cool at room temperature.

3.2.2.2 Samples and Standards preparation

Standard Values - The concentration of the samples is measured trough a calibration curve, with its characteristics specified on the Table 3.4. Preferably the value of the coefficient of determination (R_2) should be higher than 0.97.

Standard Concentration	
Std $Nr°$	mg/L O_2
1	0
2	100
3	200
4	400
5	600
6	800
7	1000

Table 3.4: Standards concentrations variation

Sample and Standard Preparation - To the digestion tube, first add $2.5ml$ of the sample/standard, second add $1.5ml$ of Standard Digestion Solution, with the lid agitate a little, third with the digestion tube on the support, add $3.5ml$ of the Sulfuric Acid Reagent, close the lid and agitate.

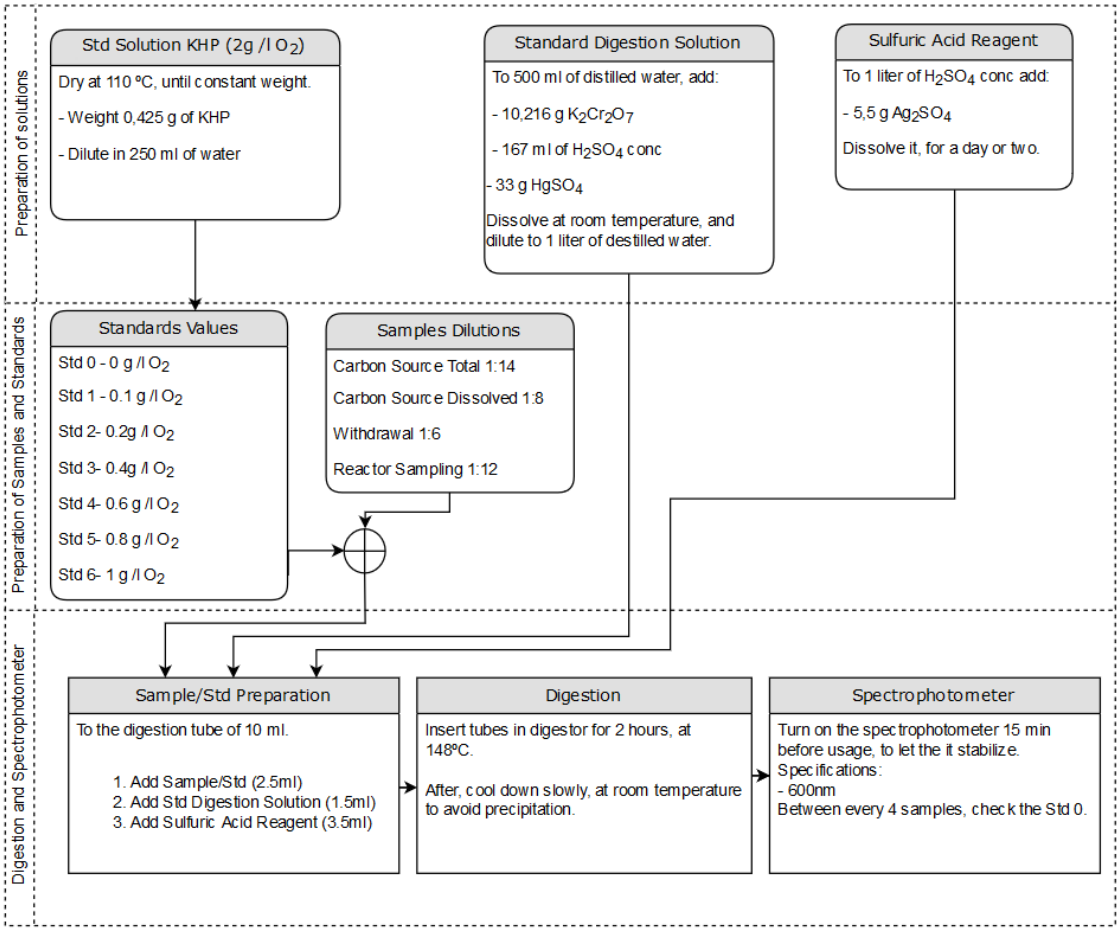

Figure 3.5: Flow Chart of COD: Preparation of solutions, Preparation of Samples and Standards, Digestion and Spectrophotometer

To prepare and guarantee the best results after storage, each sample was acidified (<pH 2) with a solution of sulfuric acid (H_2SO_4) (0.1M). To achieve more trustful results, two samples were prepared at the critical points, the begging and the end, of the selection and accumulation process.

For the sample's preparation, a dilution had to be made to ensure that the value would be in the optimal interval of the analysis, bellow $25mgO_2/L$ tend to be more qualitative rather than quantitative.

Digestion and Spectrophotometer - Insert Samples and Standards on an incubator for 2 hours at 148° C, after letting it cool slowly at room temperature to avoid precipitation. Turn on the spectrophotometer, it should be stabilizing for 15 minutes before using, each sample was read twice and between every four samples the Std 0 was used to verify the machine calibration.

To better understand the composition of the biomass, and its behaviour throughout the studied cycles, some coefficients have been calculated based on the equation of

	Componente ID	Equation	Coefficient
VFA	Acetic	$C_2H_4O_2 + 2O_2 \rightarrow 2H_2O + 2CO_2$	1.07
	Ethanol	$C_2H_6O + 3O_2 \rightarrow 3H_2O + 2CO_2$	2.08
	Propionic	$C_3H_6O_2 + 3.5O_2 \rightarrow 3H_2O + 3CO_2$	1.51
	Lactic	$C_3H_6O_3 + 3O_2 \rightarrow 3H_2O + 3CO_2$	1.07
	Butyric	$C_4H_8O_2 + 5O_2 \rightarrow 4H_2O + 4CO_2$	1.82
	Valeric	$C_5H_{10}O_2 + 6.5O_2 \rightarrow 5H_2O + 5CO_2$	2.04
	Caproic	$C_6H_{12}O_2 + 8O_2 \rightarrow 6H_2O + 6CO_2$	2.20
PHA	HB	$C_4H_6O_2 + 4.5O_2 \rightarrow 3H_2O + 4CO_2$	1.67
	HV	$C_5H_8O_2 + 6O_2 \rightarrow 4H_2O + 5CO_2$	1.92
	HHx	$C_6H_{10}O_2 + 7.5O_2 \rightarrow 5H_2O + 6CO_2$	2.10
Biomass	X_A	$C_5H_7NO_2 + 5O_2 \rightarrow 2H_2O + 5CO_2 + NH_3$	2.10
Fibers	Cellulose	$C_6H_{10}O_5 + 6O_2 \rightarrow 5H_2O + 6CO_2$	1.18
	Pectin	$C_6H_{10}O_7 + 5O_2 \rightarrow 5H_2O + 6CO_2$	0.82

Table 3.5: COD Coefficients

complete carbon oxidation of each compound, which are equal to the mass of oxygen required to full oxidization, as the equation specifies. Where the parameter C, in the equation refers to the stoichiometric coefficient.

$$\frac{gCQO}{gVFA} = \frac{qO_2 * M(O_2)}{qVFA * M(VFA)} \tag{3.2}$$

3.2.3 VFA Analysis

The determination of VFA's concentration helped to understand the variances in the biomass performance along with this study. The equipment used was a High Performance Liquid Chromatography (HPLC) (VWR Hitachi Chromaster), equipped with both a diode array and an IR detector, an Aminex HPX-87H column, operating at a temperature of 60° C.As eluent a solution with 0.01 N sulfuric acid and an elution rate of $0.6ml/min$.

The VFA's concentrations were determined by the calibration curves of each compound, with a range of $(15 - 1000mg/L)$

After sampling from the reactor, the samples were centrifuged for 3 minutes at $10000rpm$ and filtered through a filter with a porosity of $0.2mu$. Because of his high concentration, the carbon source was diluted with the eluent solution, 0.01 M H_2SO_4.

3.2.4 PHA analysis

There were used two pieces of equipment for Gas Chromatography.

GC 430-GC, Bruker equipped with an FID detector and a Restek column ($60m, 0.53mm$ internal diameter, $1\mu m$ film thickness). Helium was used as a carrier gas, at constant pressure (14.5psi). From 0 to 3 min, the temperature started to increase at a rate of 20° C

until 100° C, from 3 to 21 min an increased rate of 3° C until 155° C, from 21 to 32 min, an increased rate of 20° C until 220° C.

GC-FID Varian CP-3800, equipped with a FID detector and a ZB-WAX plus column ($60m, 0.53mm$ internal diameter, $1\mu m$ film thickness), coupled with a guard-column (0.32mm internal diameter). Helium was used as a carrier gas, at constant pressure (14.5psi).

The PHA quantification was achieved according to Braunegg et al [56], Comeau et al [57], which are both later referred at Lahman et al [58], with minor modifications. The method quantity's concentration of each monomer individually, HB, HV and 3-Hydroxyhexanoate (HHx).

All samples were centrifuged at 10 000 rpm for 3 minutes, the pellet frizzed with liquid nitrogen, and lyophilized overnight. An amount between, 3-4.5 g of lyophilized biomass was weighed into a digester tube, where $1mL$ of acidic methanol (20% sulfuric acid v/v) and $1mL$ of chloroform, that contained the internal standard, HD ($1mg/mL$ Heptadecane) were added. After the samples were put in an incubator for 3.5 h at 100° C, subsequently were cooled in an ice bath for 20 minutes, $1mL$ of distilled water was added, and then all the samples were mixed using a vortex for 40 seconds. As a consequence, the solution was separated into two phases, the lower phase was extracted into a GC vial, and sieves were added to remove any residues of water, and analyzed.

3.2.5 Total Carbon analysis

The concentration of Inorganic Carbon (IC), Total Organic Carbon (TOC) is determined by a Shimadzu TOC automatic analyser. Potassium Hydrogen Phthalate (PHP) standards were used (20-500 mgC/L). For the calibration curves a Sodium Hydrogen Carbonate (SHC) and Sodium Carbonate (PC) standard standards solutions were used for TC and IC respectively, both with equal concentrations (1-25 and 20-500mgC/L).

All samples were filtered with a $0.45\mu m$ filter, to the specific vials. All dilutions were made with ultrapure water (Mili-Q).

3.2.6 Nitrogen and Phosphorus analysis

The concentration of ammonia and phosphate was analyzed with a Skalar San++, with a range of 0.2 to $20mg/L$. The procedure for the determination of each component is different, the ammonia is based on the modified Berthelot reaction, where the ammonia is buffered, dialyzed and is chlorinated to monochloramine which reacts with salicylate to 5-aminosalicylate, after oxidation an oxidative coupling green colored complex is formed, and the absorption measured at $660nm$. The phosphate follows the reaction of ammonium heptamolybdate and potassium antimony (III) oxide tartrate, reacts in an acid medium with diluted solutions of phosphate tor form an antimony-phosphomolybdate complex,

which is reduced to an intensely blue colored complex, by L(+) ascorbic acid, then is measured at 880nm.

All samples were centrifuged at 10 000 rpm for 3 minutes, being filtered with a 0.45μm filter, to the Skallar vials. The samples 7A, 7B and 8, as explained in Table 3.1, from the SBR, were diluted with ultrapure (Mili-Q) water.

3.2.7 Data analysis

The evolution and behaviour of all the three reactors UASB, SBR and ACC, were evaluated through the calculation of some kinetic parameters.

In the UASB reactor, the conversion rates (%) of fruit pulp in to Fp, was calculated through the quotient between the Fp gCOD L^{-1} d^{-1} and OLR gCOD L^{-1} d^{-1}.

$$Conversion_{Rate} = \frac{Fp}{OLR} \tag{3.3}$$

In both SBR and ACC reactor the storage rate (r_{PHA}, gCOD_{PHA} h^{-1}) was calculated based on slope created by the increase in the concentration of PHA along time.

$$r_{PHA} = \frac{\Delta PHA}{\Delta h} \tag{3.4}$$

In both SBR and ACC reactor the substrate consumption rate (r_{PHA}, gCOD_{PHA} h^{-1}) was calculated based on slope created by the decrease in the concentration of PHA along time.

$$r_{VFA} = \frac{\Delta VFA}{\Delta h} \tag{3.5}$$

To calculate the storage yield, in the SBR and in the ACC reactor, two methods were used considering that in the SBR there is no difference in the volume whilst on the ACC reactor everytime a pulse is given, the volume increases. For the SBR the storage yield ($Storage_{yield}$, gCOD_{PHA} gCOD_{VFA}[S], L)

$$Storage_{yield} = \frac{\Delta PHA}{\Delta S} \tag{3.6}$$

SBR equation

$$Storage_{yield} = \frac{PHA_{End} * L_{End} - PHA_{Beg} * L_{Beg}}{VFA_{Tot,feed} * L_{Tot,feed}} \tag{3.7}$$

ACC equation

Chapter 4

Results and Discussion

This chapter presents the final results, where the main aim is to increase the global productivity of the process and to do a mass balance of this system, considering the conversion of the waste used as feedstock to produce PHA. As shown in Figure 4.1 the substrate used to feed the UASB reactor was fruit pulp which was then converted into VFAs. While in the SBR, the fermented fruit waste was used to select a PHA accumulating culture from waste activated sludge, the accumulation reactor was inoculated with the culture from the SBR and fed with fermented fruit waste to produce PHA. The laboratory work in the scope of this dissertation was done during an undergoing side project that required high production levels, so whenever needed the VFA substrate was supplemented to keep the desired production levels. No alterations were made to the UASB reactor or the SBR reactor because they were considered to be in optimal operating conditions due to the previously applied research done by Mariana Matos, Fernando Silva, and colleagues.

The SBR reactor has been inoculated and the transient state of the reactor was studied, although since it is not the main purpose of this work only the main parameters were taken into consideration. Five runs were monitored along the process, but only one of these is thoroughly discussed to avoid redundancy and simplify the analysis. The five cycles have the name of SBR-1. SBR-2, SBR-3, SBR-4, and SBR-5. The chosen run to analyze was the SBR-5, considering that was the closest one to the accumulation runs that had the objective to increase the productivity of the process (ACC(Nutrients) -5. ACC(Nutrients)-6, ACC(Nutrients)-7, ACC(Nutrients)-8, ACC(Nutrients)-9).

To increase productivity on the accumulation stage, nine batches were made with and without adding nutrients. The ratios applied to the final stage of the process had the objective of inducing a little growth of the biomass, aiming at increasing the volumetric

productivity without decreasing PHA content in the biomass. Taking into account that is considered to be preferable a higher PHA content, due to the cost of the extraction process increases when a lower PHA content regarding the higher need of solvents to extract it [59, 60]. Along with the transient state of the SBR reactor, two accumulations were made with the name of ACC(Transient)-1 and ACC(Transient)-2, afterward, the bacteria selection process was considered to be completed. Two runs were made with the name of ACC-3, ACC-4 for comparing purposes only with the results obtained from the final five accumulations in which nitrogen and phosphorus were added. The final accumulations had the name of ACC(Nutrients) -5. ACC(Nutrients)-6, ACC(Nutrients)-7, ACC(Nutrients)-8, ACC(Nutrients)-9. As a consequence of the similarity of results between each accumulation test, only ACC-4 and ACC(Nutrients)-9 are thoroughly discussed, for sake of simplicity and redundancy avoidance. ACC-4 and ACC-(Nutrients)-9 were chosen because, from all the batches made with and without the addiction of nutrients were considered to be the optimal candidate due to the similarities in the specific parameters chosen for the analysis.

The global mass balance applied to this process has the purpose to better understand how much fruit pulp is needed for PHA production. In order to optimize PHA production, nutrients were added to the ACC reactor. The studied outlets are represented in Figure 4.1, which were considered to be of most importance regarding the process optimization.

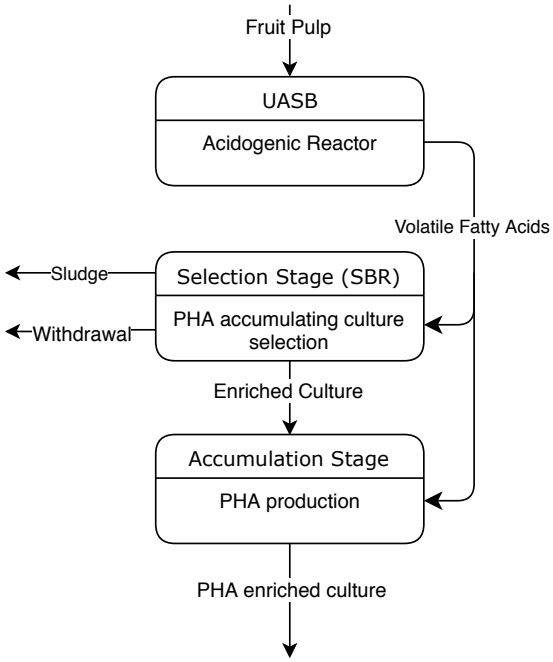

Figure 4.1: Process scheme for the production of PHA using mixed microbial cultures

4.1 UASB Performance

Along this study the conversion of fruit pulp in to VFA was carried out on UASB reactor. It was operated with a one day HRT and a nutrients ratio of (100:0.5:0.15) (Cmmol: Nmmol: Pmmol). Despite other options in the literature, over the usage of an CSTR reactor in the anaerobic stage, the scope of the lab in which this dissertation was made is to get as similar as possible to the conditions applied on an industrial scale, and due to the lower costs of the operation from the UASB is considered to be the best candidate. All calculations regarding the total production of Fp (Including VFA) and conversion rates, were made accordingly to the outlet concentration measured every three days of operation (with duplicates). The concentration of the days in which (regularly two days after) the analysis was not made, it was assumed to be the same as the first day in which the analyses was made, as shown in Figure 4.2.

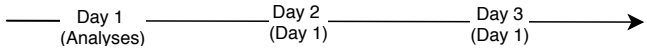

Figure 4.2: Fp analysis, and calculus explanation

This reactor have been inoculated at the beginning of this work, where the evolution of the fermentation products and fruit pulp concentration are specified in Figure 4.4. The activated sludge utilized to inoculate the reactor, came from a full-scale anaerobic digester, in the form of granular sludge to produce methane gas, therefore was mainly composed by methanogenic bacteria. A microbial selection process occurs, by lowering the pH of the reactor to 4.5 considering the sensibility of the methanogenic bacteria towards an acidic environment. This acidic environment improves the conditions for the acidogenic bacteria to grow as stated before in Section 1.3. After thirty days of operation, with an average OLR of 8.24± 1.29 gCOD$L^{-1}d^{-1}$ the first try to increase the OLR was made, but the reactor had become unstable with a consequent reduction on the VFA conversion rate.

To regain the stability needed for the process, another eighteen days passed until the OLR was gradually increased to an average of 12.16 ± 0.76 gCOD$L^{-1}d^{-1}$ and finally to the average 16.09 ± 1.73 gCOD$L^{-1}d^{-1}$. The OLR maximum level was achieved at one hundred and six days of operation. Butyrate, acetate was the most relevant VFAs produced during the operation as shown in Table 4.1. Together Butyrate and acetate represent about 59.91 % of the total production. Valerate concentration has decreased along the time as opposed to the Caproate that seemed to be increasing. A tendency to a higher concentration of Caproate was noticed after each OLR increase and consequent system stabilization. Specifically was noticed between the day 45 - 62, 73 - 85 and 118 - 133. A tendency to the increase of Lactate every time the OLR was increased until the system has stabilized a consequently the concentration decreased. A relation between the increase of concentration of lactate and caproate was measured, whenever

Table 4.1: Total estimated Fp Concentration (After day 104)

	$gCOD_{Fp}L^{-1}$
Lactate	2.35 ± 1.4
Acetate	3.82 ± 1.2
Propionate	1.03 ± 0.56
Ethanol	0.27 ± 0.06
Butyrate	3.50 ± 1.11
Isovalerate	0.00 ± 0.01
Valerate	0.57 ± 0.29
Caproate	0.76 ± 0.53

lactate concentration increased, caproate decreased and the opposite was also verified. Specifically between the day 64-66 and 80-99, this relation could be seen which led to the assumption that could be a result of the instability of the reactor due to the increase of the OLR and consequent biomass adaptation. This dissertation has started on the ninety-nine day of operation.

The average conversion results from the mentioned OLR values and mass balance are presented in Figure 4.3. With the increase in the OLR, the conversion rate has also raised. Due to the composition of the apple, besides sugars (Glucose, Sucrose, and Fructose), it also has other carbohydrates such as up to 20% of fiber (soluble and not soluble) [61]. Fibers are hardly decomposed in this UASB reactor considering the HRT of the only day, whilst for the production of VFAs from sources that contain fiber's, such as cellulose higher HRT (4 days) is needed, therefore since is not digested could influence the efficiency of the reactor [62]. To partially avoid, inert and other non-digestible compounds in both SBR and Accumulation reactor, the result substrate was filtered with a flannel filter that retains part of the non-digestible and inert compounds. It is important to mention that cell maintenance and growing will consume part of the carbon source, regarding the addition of nutrients to the pulp fruit with a constant COD:N:P ratio (100:0.5:0.15).

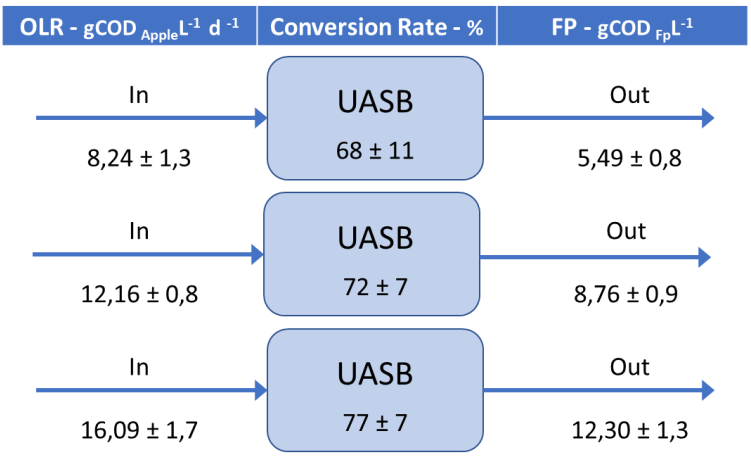

Figure 4.3: Acidogenic Reactor - Mass Balance

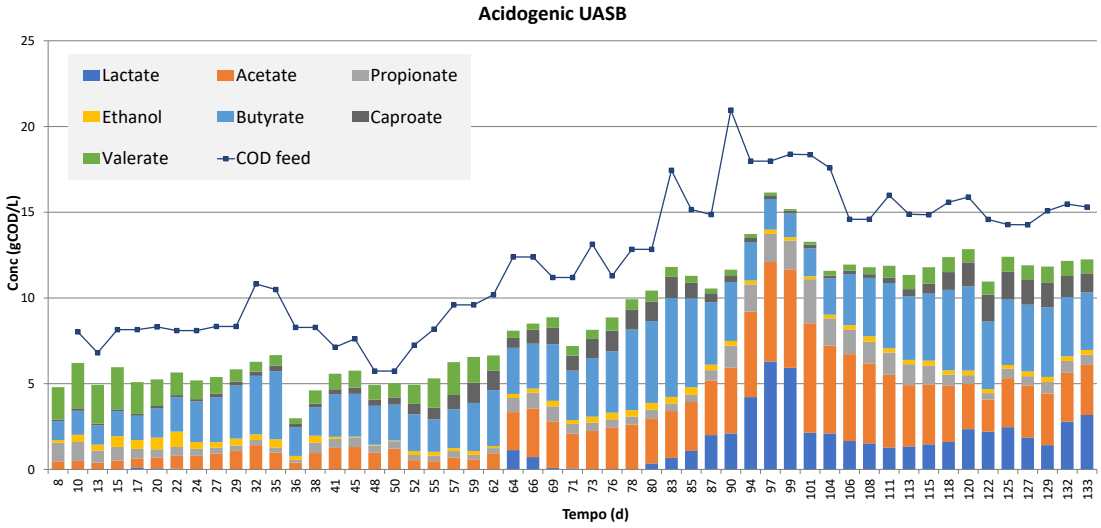

Figure 4.4: Acidogenic Reactor - VFA and Feedstock concentration (Apple Pulp))

4.2 SBR Transient State until Stable Operation

4.2.1 SBR Performance - Overall performance

As stated before, the SBR reactor was fed with the VFA produced by the UASB reactor. The reactor was fed only at the beginning of the cycle, to achieve a FF regime and consequent selection of PHA-accumulating culture. The reactor was operated under a FF regime, at a ratio of (100:6.5:1) (Cmmol: Nmmol: Pmmol) with a 12-hour cycle.

The resume of the results obtained along this study about the SBR performance are shown in Table 4.2. The transient phase, is considered to be the phase after the reactor inoculation until the selection process is completed through a FF regime. The applied ADFFF regime will lead to a competition between the consortium of microorganisms that are able to store intracellular PHA, and the ones that do not have that ability. An uncoupled carbon and nitrogen feeding strategy is used to increase the selective pressure. The VFA composed feedstock from the UASB reactor is fed only at the beginning of the cycle, and since this a sequential reactor the beginning of the cycle corresponds to the end of the previous one. Nitrogen and phosphorus are only fed after the carbon source depletion.

The main objective of the uncoupled strategy is to induce bacterial growth only during the famine phase and therefore increase the selective pressure applied to the system, regarding that only the PHA-Accumulating bacteria have both carbon and nitrogen available for growth purposes. During the transient state, not all of the VFA were consumed as shown in table 4.2, specifically Lactate and Acetate were not depleted until the end of the monitoring. Therefore some of the microorganisms that are not able to store PHA could survive and grow, regarding the available carbon source.

With the several consecutive cycles and a constant selective pressure applied, the bacteria with the highest VFA uptake rate proliferate, and consequently the carbon source is rapidly depleted. Although the feast time has declined with the increase of VFA uptake rate, variations along the process were obtained. It could be explained due to the variability on the substrate provided from the UASB, which leads to the production of less preferred Fp, such as lactate and because of it increase feast duration. Another possible explanation is due to the inherent characteristics of biological systems that are naturally susceptible to some variations over time.

The conversion rate (r_{PHA}) of VFA to PHA, and the yield $Y_{P/S}$ of feedstock conversion to PHA have also increased considering the higher concentration of PHA-Accumulating bacteria. To achieve the desired production, the OLR was gradually increased, taking in to account the F/M ratio to avoid substrate inhibition and undesired accumulation of the carbon source inside the reactor, with the consequent decrease in selective pressure. A considered stable state was reached when no considerable biomass growing between monitoring processes was accounted for.

Over time and with the increase in the OLR, the concentrations of both TSS and VSS have increased as reported on Table 4.3. The concentration of biomass in the reactor is represented by the VSS. The variations in both TSS and VSS during the feast and famine phase could be explained due to the higher PHA concentration after the feast phase when carbon is depleted and converted into PHA. At the beginning of the famine phase in which nitrogen is fed and rapidly depleted by the biomass, growth is induced and the

Table 4.2: Variation in the operation results along the study (a - acetate and lactate Feed - (gCODL^{-1}); OLR - (gCOD$L^{-1}d^{-1}$); Feast (h); r_S ($gCOD_{VFA}$ $L^{-1}h^{-1}$); gCOD$_{PHA}$ $L^{-1}h^{-1}$; gCOD$_{PHA}gCOD_{VFA}^{-1}$; VFAs ($gCOD_{VFA}L^{-1}$)

	Feed	OLR	Feast	FF ratio	r_S	r_{PHA}	$Y_{P/S}$	VFA's
SBR-1	290,00	3,22	2,53	0.21	0.70	1.5	0.622	0.015[a]
SBR-2	618,27	6,87	0,58	0.05	5.18	2.74	0.326	0
SBR-3	585,53	6,51	0,23	0.02	6.57	-	-	0
SBR-4	830,35	9,23	0,57	0.05	5.8	4.11	0.63	0
SBR-5	830,05	9,22	0,43	0.04	9.3	7.78	0.65	0

PHA is used as a carbon source.

Table 4.3: TSS and VSS variation, end of Feast and Famine phase (TSS and VSS g/L)

	Feast		Famine	
	TSS	VSS	TSS	VSS
SBR-1	9.42	8.11	9.90	8.31
SBR-2	10.67	9.47	10.52	9.20
SBR-3	11.08	10.33	11.47	9.99
SBR-4	11.71	10.26	13.54	11.73
SBR-5	13.43	10.91	13.21	10.64

4.2.2 SBR Performance - SBR 5

A typical SBR cycle, represented by the monitoring process of SBR-5 is shown in Figure 4.5 and corresponded to the 125 days of operation in the UASB reactor. The beginning of the cycle in the selection reactor starts with feeding of the fruit pulp based feedstock (Feast Phase). The sudden decrease in the DO level indicates that the aerobic microorganism are consuming the Fps. After the feedstock depletion, the DO level increase again. With the conversion of VFAs to PHA it is possible to observe, an increase in the stored polymer, where TSS and VSS follow that trend. At 0.5h a sudden decrease of PHA content was measured, it was considered to be an error in the analysis and not an actual consumption of bio-polymer because as shown there are still VFAs available. It is assumed that the consumption of an external carbon source is preferred over an internal, regarding the characteristics of the selected biomass, that store bio-polymer to survive when no carbon source is available. Taking also into consideration the increase of the TSS and VSS until the depletion of the carbon source, which could be mean an increase in polymer content, since no nutrients were given and growth is not expected.

Nutrients feeding starts two hours after the beginning of the cycle, where the selected biomass starts to grow using the stored PHA from the previous phase as a carbon source. The gap of two hours increases the selecting pressure, considering that allows for the non-accumulating bacteria to eventually perish. Ammonia is rapidly depleted as shown

in Figure 4.5, whilst phosphorus is not completely consumed until the fifth hour of the cycle. This could be related to the variations in the concentration of phosphorus in the feedstock, with an average of 0.42 ± 0.07 P-mmol. Residual phosphorus was measured at the beginning of cycles: SBR-1 (0.49 P-mmol), SBR-3 (0.52 P-mmol), SBR-4 (0.4 P-mmol), SBR-5 (0.72 P-mmol), but since without ammonia phosphorus does not induce growth, it did not decrease the selection pressure applied.

As mentioned before, the feedstock has to be supplemented to achieve the desired production as shown in Table 4.4. The polymer composition after the feast phase on the SBR-5 cycle was 85% HB and 15% HV, gCOD based, and it was achieved due to the high concentration of Butyric Acid has it works as a precursor for PHB which represents 80% of the feedstock.

All the parameters to evaluate the reactor performance are specified in Table 4.5. The FF ratio of 0.04, was achieved over time and with a high selective pressure imposed on the reactor. During the Feast phase, in which storage yield and observed yielded were calculated, the biomass only stored PHA, growth was not possible since no nutrients were available.

Table 4.4: Supplementing, percentile variation

	gCOD	Lactate	Acetate	Propionate	Ethanol	Butyrate	Valerate	Caproate
FEED	415	0.01%	6.49%	1.52%	n.d	82.61%	9.37%	n.d
UASB	145	19.39%	20.35%	3.95%	2%	34.96&	6.69%	12.67%

Table 4.5: Performance evaluation SBR-5

Operation Parameters	Value	Units
OLR	9.22	$\text{gCOD} L^{-1} d^{-1}$
F/F ratio	0.04	hh^{-1}
Supplied Feed	830.04	$\text{gCOD} d^{-1}$
r_S	9.3	$\text{gCOD}_{VFA}\ L^{-1} h^{-1}$
r_{PHA}	7.78	$\text{gCOD}_{PHA}\ L^{-1} h^{-1}$
Storage Yield	0.65	$\text{gCOD}_{PHA}\ \text{gCOD}_{VFA}^{-1}$
Observed Yield	0.15	$gCOD_X\ \text{gCOD}_{VFA}^{-1}$
Specific productivity	1.1	$\text{gCOD}_{PHA}\ \text{gCOD}_{Xa}^{-1} h^{-1}$

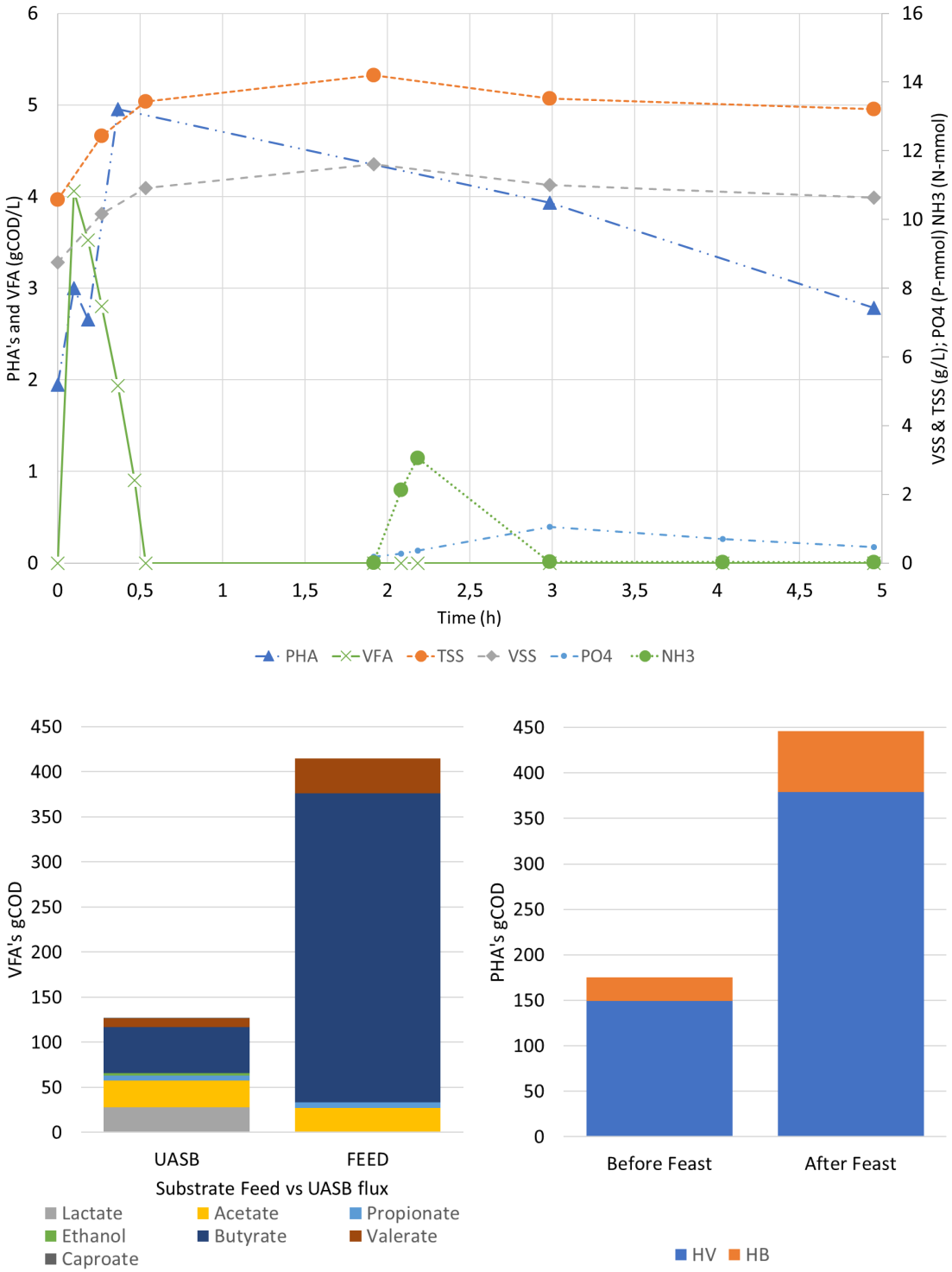

Figure 4.5: SBR-5 Cycle and Mass Balance

4.3 Accumulation Analysis - Overview

The accumulation stage, as stated before is carried out on a fed-batch mode, with pulse-wise feeding controlled by DO. The selected PHA-accumulating culture used to inoculate the reactor came from the SBR reactor at the end of the famine phase. As stated before in Sub-Section 4.2.2 while justified in Figure 4.1, the selected bacteria could have changed during the course of this study regarding the different compositions achieved in the feedstock production on the UASB reactor. Due to the limited number of analyses that could be made, only the first pulse was characterized, regarding substrate uptake, and PHA accumulation. To evaluate the process, Global Productivity (Gp), conversion yield ($Y_{P/S}$) and PHA content were the chosen parameters considering the importance of both in the evaluation of a good accumulation.

Overall with the increased OLR on the selection reactor and consequent increase of the concentration of PHA-Accumulating bacteria, the initial biomass concentration for the accumulation stage has also increased, and it was measured trough VSS. As shown in Figure 4.6 and Figure 4.7, both VSS and TSS concentration have raised along the accumulating as expected, considering that the polymer concentration was getting higher in every giver pulse of VFA.

ACC(Transient)-1, the difference in both VSS and TSS was negligible, as the feedstock has not been supplemented and therefore a higher volume was given increasing the dilution rate of the reactor, to a factor of 1:2 as shown in Table 4.6. A similar dilution rate was achieved in ACC(Nutrients)-5, although the nutrients ratio applied in this accumulation was by excess, as they were fed with the carbon source leading to an exaggerated growth that overcomes PHA production, decreasing the effect of the dilution rate.

Different experiments have been made to improve the global productivity of the accumulation (ACC(Nutrients)-1 - ACC(Nutrients)-9), regarding the applied nutrient ratios as shown in Figure 4.6. The initial ratio applied to the accumulation was already applied in the selection stage reactor and considering the SRT of four days. This meant that considering a stable state theoretically, the biomass would grow 25% a day, considering the two daily cycles applied. The main purpose of adding nutrients to the process was to induce little growth, without overcoming the main objective of high PHA content. A problem in the analysis system, hasn't allowed for precise quantification of the consumption of the given nitrogen and phosphorus(ACC(Nutrients)-5, ACC(Nutrients)-6, ACC(Nutrients)-7, ACC(Nutrients)-8, ACC(Nutrients)-9.

In a direct comparison with the SBR system, were ammonia is depleted under one hour, and phosphorus tends to accumulate in the reactor, it was plausible to assume the same behavior in the accumulation stage. To this assumption, it was also considered the

propensity to residual values of phosphorus that the feedstock has.

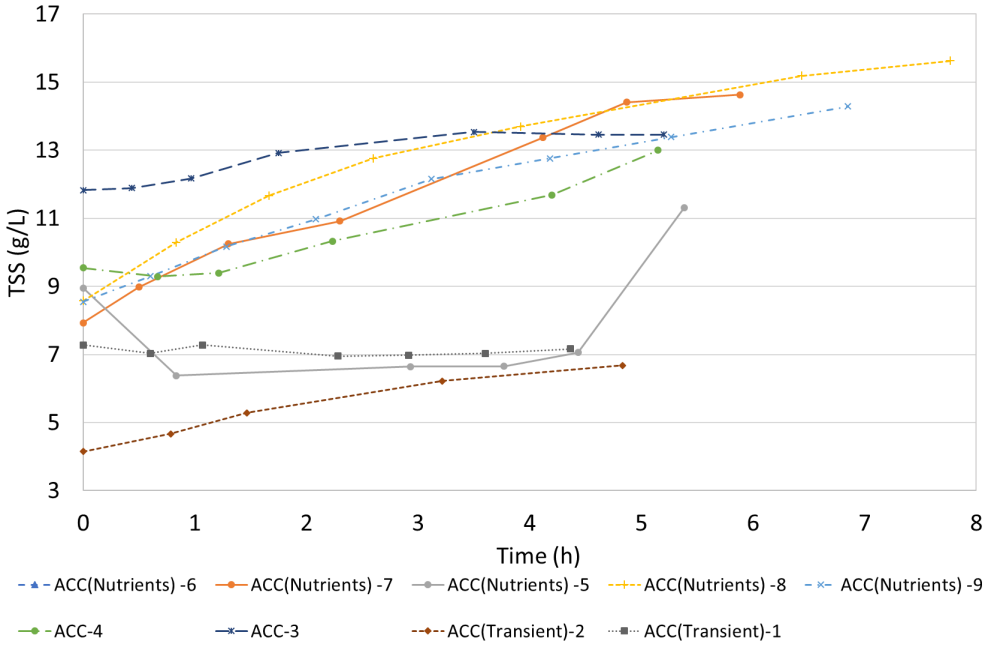

Figure 4.6: Variation of TSS during the accumulations

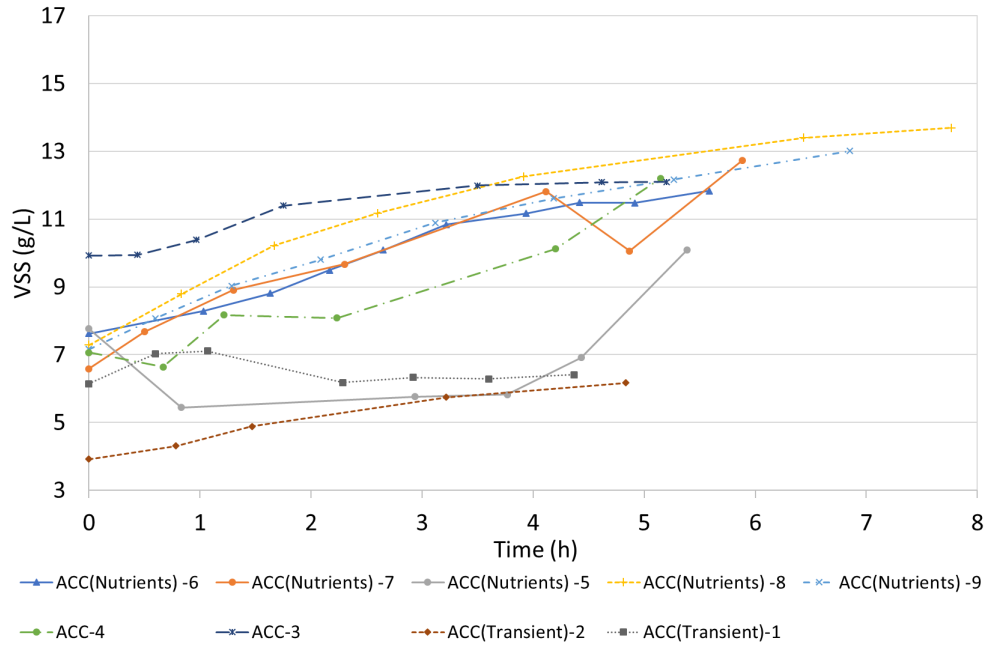

Figure 4.7: Variation of VSS during the accumulations

On the Table 4.6, all the ratios (C-mmol:N-mmol:P-mmol) used in the experiences as displayed. As stated before, ACC(Transient)-1, ACC(Transient)-2, ACC-3, ACC-4, no nutrients were added for comparing purposes, to the ones in which they were. ACC(Nutrients)-5 nutrients were added, every time a carbon pulse was fed. In ACC(Nutrients)-6, ACC(Nutrients)-7, ACC(Nutrients)-8, ACC(Nutrients)-9, nutrients were only added on the first pulse, and therefore the biomass growth was limited to the available nitrogen. Nutrients were added on the first pulse, considering that when growth is induced the number of avaliable cells multiply, and the PHA content tend to divide between daughter cells. This happens due to the cellular regulation during mitosis of both granule localization and granules number per cell, resulting in a dilution of the polymer throughout the biomass [53].

Table 4.6: Accumulations nutrients ratio and dilution ratio; a- nutrients given in every pulse (C-mmol:N-mmol:P-mmol)

	Dilution factor	Nutrients Ratio
ACC(Transient)-1	1:2	100:0:0
ACC(Transient)-2	4:5	100:0:0
ACC-3	2:3	100:0:0
ACC-4	4:7	100:0:0
ACC(Nutrients)-5	2:5	$100 : 6.5 : 1^{a}$
ACC(Nutrients)-6	5:8	100:6.5:1
ACC(Nutrients)-7	3:5	100:6.5:1
ACC(Nutrients)-8	4:7	100:3.5:0.5
ACC(Nutrients)-9	4:7	100:3.5:0.5

As was already mentioned, pulp fruit is composed of several carbohydrates that are not fermented by the UASB, such as fibers or sugars. To search for other carbon sources besides, Fp that can be quantified by HPLC analysis, TOC have been used with this purpose. For simplicity of the analysis, and because the sources have not been identified, only the tendencies for the accumulation or consumption of the external carbon sources were displayed in Table 4.7. It was calculated based on five different moments of the accumulation process, always after feedstock depletion, being the first and last moments always considered. As shown, the overall tendency is to the accumulation of those none consumed carbohydrates sources. The dilution rate, in this case, will lead to a higher accumulation of inert carbohydrates, considering that the total volume of feedstock it's higher (Higher volume of fibers, and sugars added).

As shown in the Table 4.8.ACC (transient)- 2 have reached high values of PHA content, although considering the low biomass concentration, the final production was also low. ACC(Transient) - 1 PHA content, was over 100% due to an error in the analysis. Storage yield, VFA, and PHA uptake rate have increased over time, with the applied cycles on SBR that insured a high selective pressure was being applied. ACC-(Nutrients)-9, storage

Table 4.7: External Sources of Carbon (- failed analysis)

	TOC	TIC
ACC(Transient)-1	-	-
ACC(Transient)-2	2.59	0.01
ACC-3	-	-
ACC-4	1.69	-0.03
ACC(Nutrients)-5	-4.77	0.00
ACC(Nutrients)-6	-1.56	0.03
ACC(Nutrients)-7	0.01	0.03
ACC(Nutrients)-8	1.96	0.01
ACC(Nutrients)-9	0.05	0.03

yield of 0.97, it was probably due to the initial low value of PHA content, since the last analysis were repeated for three times, where the average value was considered. 1^{st} pulse refers to the amount of time in hours, to which the carbon source was depleted regarding the first given pulse. Gp on average, have increased with the addition of nutrients, which could mean that accumulation with nutrients limitation could be preferred over nutrient starvation. The little growth induced, did not seem to affect the biomass capacity and mindset to accumulate PHA.

4.3.1 Accumulation Analysis - ACC - 4; ACC(Nutrients) - 9

Both ACC-4 and ACC(Nutrients)-9, were done under a stable stage of the reactor and considered to have the best results regarding the PHA content and Gp. The feeding strategy was the same in both batches, pulse-wise. The main difference between both the accumulations was the addiction of nutrients in the first pulse. In ACC-4 no nutrients were added, in ACC(Nutrients)-9 nutrients were fed with a ratio of (COD:3.5:0.5) in the first pulse. As mentioned before, the main objective was to induce little growth of the biomass and increase the volumetric productivity without losing PHA content.

As shown on Table 4.8 both the experiments had high polymer content.PHA content was very similar, but the ratio of HB:HV as shown in Table 4.9, was higher in the ACC(Nutrients)-9 then in ACC-4. In the Table 4.10, the feed composition is shown and the concentration of butyric acid is higher in ACC(Nutrients)-9. Silva et Al [50] suggested, the addiction of nutrients could influence the composition of the polymer, favoring the HB concentration. Global productivity and storage yield, have generally increased with all the experiences made with nutrients addiction has it was already show in Table 4.9.

Both accumulations had high feedstock uptakes, with little advantage to ACC(Nutrients)-9. The organic acid lactate was present in a higher concentration in ACC-4 as shown on Table 4.10, and since it is a less preferred carbon source it could take longer to be consumed and could contribute to the lower feedstock uptake. The PHA uptake was higher

in ACC(Nutrients)-9. A lower initial PHA content of ACC(Nutrients)-9 of just 15% when comparing to ACC-4 that had 30%, could explain this difference since the ACC-4 reached it's saturation point, sooner and therefore decreased PHA uptake. Gp could also be influenced by a smaller difference achieved in the PHA content.

Table 4.9: General Accumulation Characteristics (Storage Yield - ($gCOD_{PHA}\ gCOD_{VFA}^{-1}$); Gp - Global productivity ($\Delta\ gCOD_{PHA}\ gCOD_{Xa}^{-1}t^{-1}$, $Total_{Feed}$ gCOD

	Storage Yield	Gp	$Total_{Feed}$	Δ PHA	PHB	PHV
ACC-4	0.71	0.041	573	481.08	74%	26%
ACC(Nutrients)-9	0.97	0.056	685	696.98	76%	24%

Table 4.10: Percentile variation in both Feedstocks, n.d- not detected

	Lactate	Acetate	Propionate	Ethanol	Butyrate	Valerate	Caproate
ACC-4	0.34%	11.58%	3.89%	n.d	73.03%	11.16%	n.d
ACC(Nutri)-9	0.08%	5.54%	1.78%	n.d	78.39%	14.21&	n.d

Both the accumulations were analyzed for external carbon sources in the beginning of each batch as shown of Table 4.11. Any remain of VFA's from the previous SBR cycle would increase the productivity of the process, other carbon sources wer also measured through TOC that weren't accounted for on the HPLC analysis. Nutrients were also analyzed both in the reactor and fruit pulp feedstock, at the same time in which the previously mentioned analyses were made.

Both the accumulations started with similar unidentified, dissolved carbon sources that, that as stated before could be dissolved fibers or sugars as they are part of the apple composition, and as shown in Table 4.11 can accumulate in the system. They are considered to be inert in terms of PHA production, since only the VFA serve as precursors for PHA production, therefore were not considered in the mass balance calculation. Lactate has accumulated at the end of the accumulation in ACC-4.

Table 4.11: Characterization of Feedstock and ACC reactor, carbon and nutrients availability; nd - not detected

	ACC-4			ACC(Nutrients)-9			
Compound	In	Out	Feed	In	Out	Feed	Units
TC	58.57	62.8	751.33	47.42	63.15	1079.56	(C-mmol/L)
TIC	0.6	0.5	0.73	0.44	0.66	0.68	(C-mmol/L)
VFA	nd	0.21	736.45	nd	nd	823.59	(C-mmol/L)
Phosphate	0.245	nd	0.1	nd	nd	nd	(P-mmol/L)
Ammonia	nd	nd	0.08	nd	nd	nd	(N-mmol/L)

Both the accumulations increase in both VSS and TSS along time, as shown in Figure 4.8 and Figure 4.9. The main difference was in the concave shape pattern followed by ACC-4 as opposite to the ACC(Nutrients)-9 that had a more convex shape. Although when compared to other similar accumulations, the results could not be associated with growth.

On ACC(Nutrients)-9, all the pulses had the same quantity of VFAs, it was noticed that along the accumulation the time between each pulse has increased, probably due to the consortium of bacteria got inhibited by product. On the ACC-4, not all the pulses had the same quantity of VFA. Therefore the difference observed in the picture does not correspond to the inhibition by product, but to difference of VFA quantity in each given pulse.

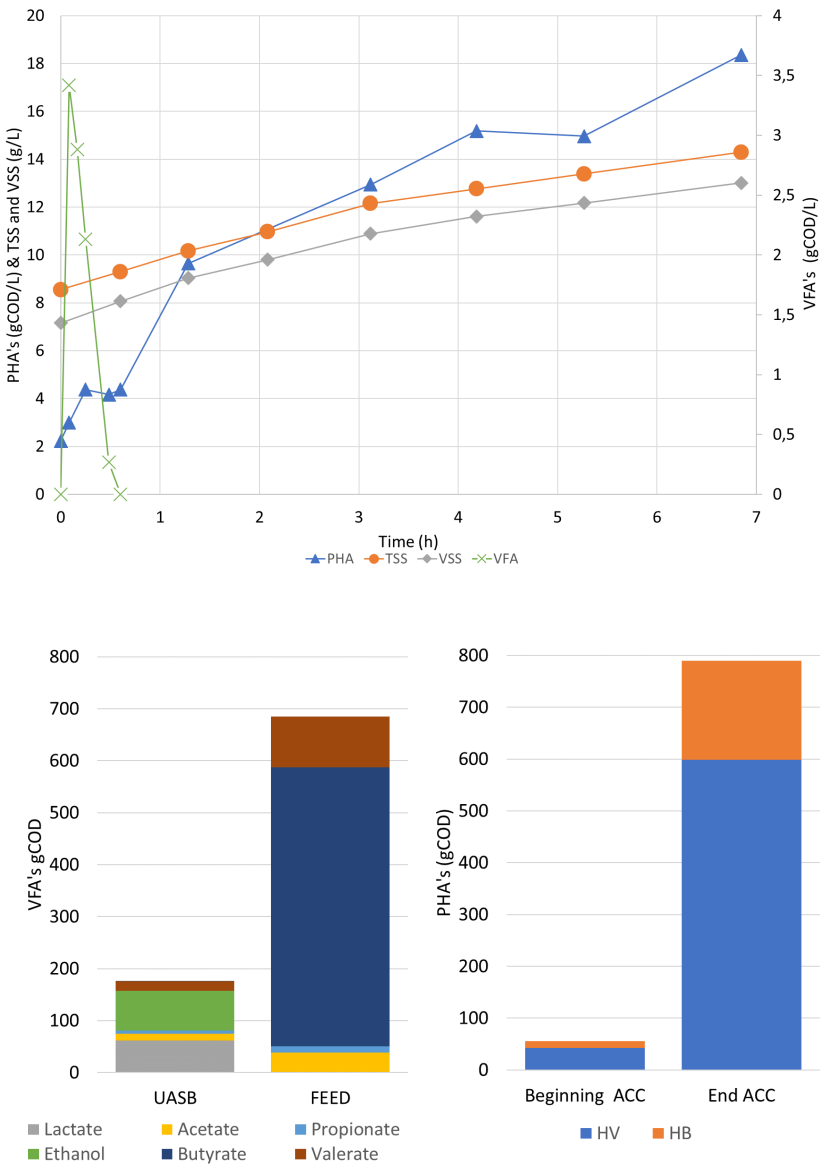

Figure 4.8: Mass Balance - ACC(Nutrients)-9

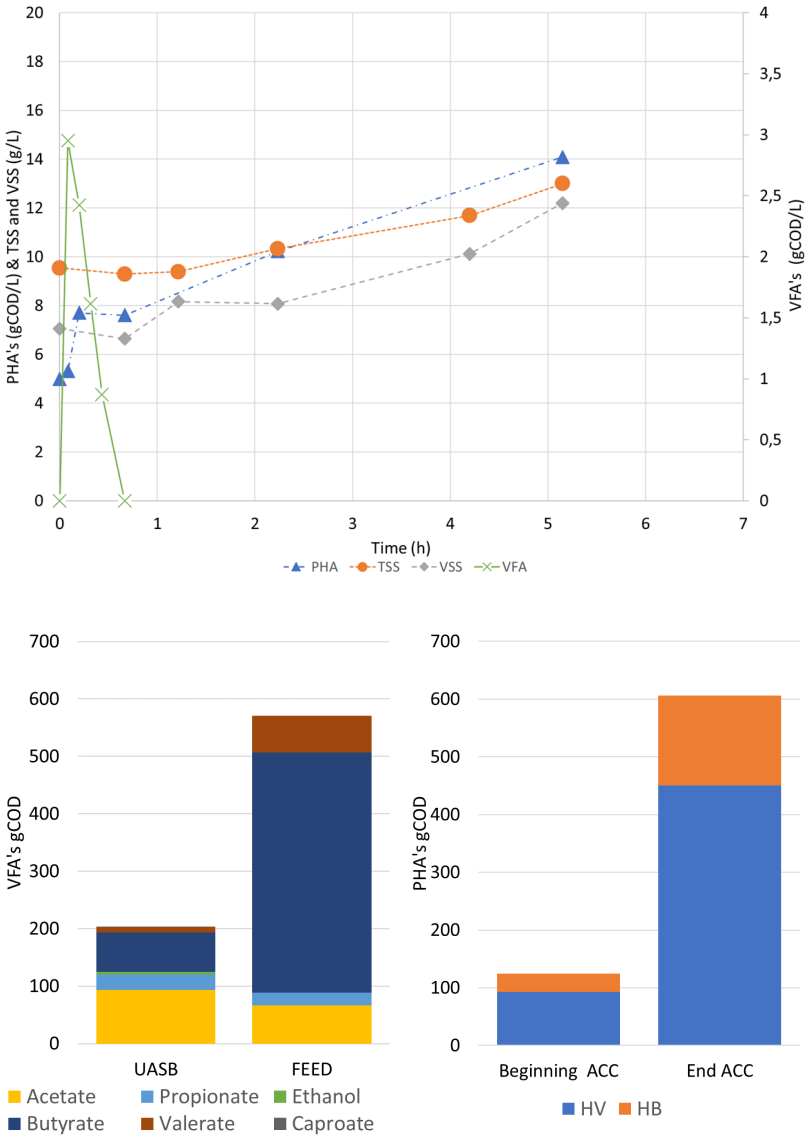

Figure 4.9: Mass Balance - ACC-4

4.3.2 Mass Balance

To make this mass balance, it was not taken into consideration the polymer composition. When supplemented, the concentration of butyrate acid leads to an increase in the production of PHB since it works as a precursor of this monomer. Along with this dissertation, COD units have been used, for comparing purposes between the different processes. The phase considered for the UASB reactor, between 83-159 days of operation, corresponded to what is considered to be closest to standard yield in this reactor. With an average of 16.07 ± 1,78 gCOD/L of pulp fruit used as feedstock, which was converted to Fp with an average conversion rate of 77% and an average value of 12,38 ± 1.30 gCOD/L.

On the production stage due to the inherent differences also achieved during this stage, regarding storage yield, the average value between experiences was used. An average value was considered to be the more representative regarding the fluctuations achieved in storage yield. It was also considered, a 70% HB and 30% glsHV concentration, to produce one kg of PHA (1746.22gCOD equivalent) from fruit pulp and the results are represented in Figure 4.10.

In Figure 4.10, the system applied to the production of PHA is shown. The system starts with the conversion of fruit pulp into fermentation products, an 77% conversion rate is applied. This mass balance scheme considered only the necessary amount of fruit pulp to produce 3 kg of PHA. Besides the production of PHA on the ACC reactor, the production of biomass on the SBR was also taken into account. To produce the desired biomass quantity, and according to the data achieved throughout this thesis at least 3 SBR cycles were needed. Specifically for: ACC 3/4 - 4.5; ACC (Nutri) 6/7 - 3.9; ACC (Nutri) 8/9 - 3.4. The average yield on every group of accumulation is also shown on the previously mentioned Figure in the specific box, for each ACC.

On current A, the amount of fruit pulp needed for each 1 kg of PHA, is represented for each experience, all stages are considered. The current B represents the amount of VFA that the SBR reactor needs for the production of the biomass needed on the accumulation reactor. Therefore it varies with the yield on the ACC reactor. Current I represents, the biomass used on the ACC reactor, that came from the SBR reactor. Current C, D, and E show how much VFA the ACC reactor needs to produce 1 kg of PHA. The current F, G, and H refer to the result of the production.

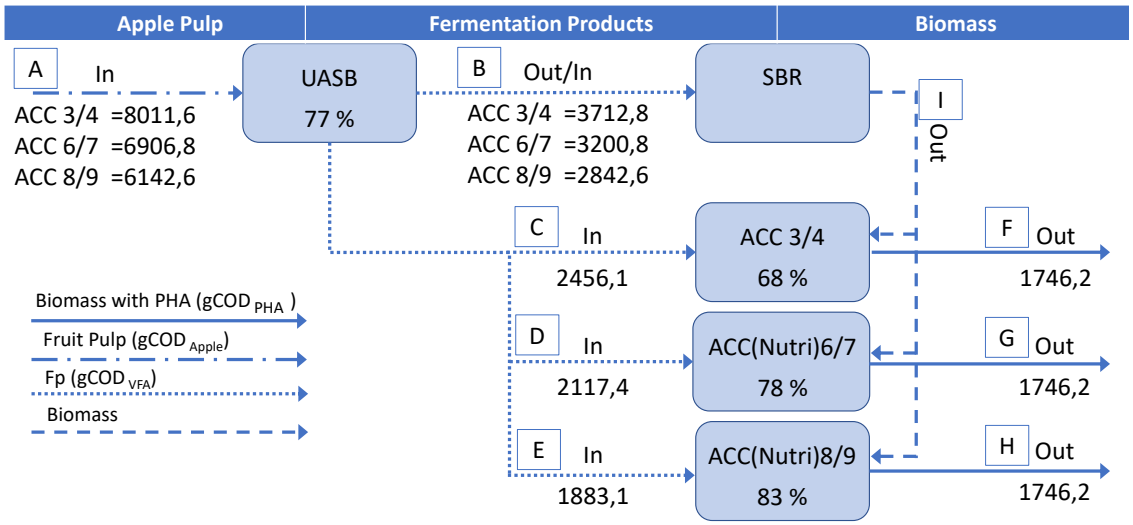

Figure 4.10: Mass Balance - System

Table 4.8: Operational Results (Feed - (gCOD$L^{-1}d^{-1}$); Feast (h); r_S (gCOD$_{VFA}$ $L^{-1}h^{-1}$); ; VFAs (gCOD$L^{-1}d^{-1}$); Storage Yield - (gCOD$_{PHA}$ gCOD$^{-1}_{VFA}$); TSS (g/L); PHB & PHV - (%); PHA - (%); r_{PHA} - (gCOD$_{PHA}$ $L^{-1}h^{-1}$); Gp - (ΔgCOD$_{PHA}L^{-1}h^{-1}$

	1^{St} pulse	r_S	r_{PHA}	Storage$_{Yield}$	Gp	VSS$_{beg}$	VSS$_{end}$	PHB_{Final}	PHV_{Final}	%PHA
ACC(Transient)-1	0.57	1.35	0.79	1.35	0.026	6.14	6.41	67.31	32.69	77.26
ACC(Transient)-2	0.72	5.18	2.74	0.93	0.019	3.92	6.17	73.26	26.74	76.14
ACC-3	0.3	5.55	0.87	0.58	0.017	9.93	12.10	72.05	27.95	47.31
ACC-4	0.58	5.23	1.62	0.84	0.041	7.06	12.20	74.30	25.70	78.86
ACC(Nutrients)-5	0.28	1.25	1.30	0.68	0.012	7.76	10.09	73.30	26.70	53.37
ACC(Nutrients)-6	0.27	7.49	2.42	0.82	0.048	7.62	11.83	79.79	20.21	65.94
ACC(Nutrients)-7	0.32	5.12	1.92	0.83	0.051	6.59	12.73	75.99	24.01	54.65
ACC(Nutrients)-8	0.68	7.06	0.93	0.87	0.034	7.29	13.69	76.58	23.42	54.06
ACC(Nutrients)-9	0.52	7.98	2.45	0.97	0.056	7.16	13.01	75.77	24.23	74.44

Chapter 5

Conclusions and Future Work

In this dissertation, the mass balance of PHA production from MMC with fruit pulp as feedstock was made, regarding the optimization of the process. All of the three reactors were monitored over time since they were inoculated during the course of this work.

The UASB reactor evolution along time with the step-by-step OLR increase, from 8.24± 1.29 gCOD$L^{-1}d^{-1}$ to 16,09 ± 1.73 gCOD$L^{-1}d^{-1}$, achieving 77% of fruit pulp converted to Fermentation products that include, Butyrate, Caproate, Lactate, Acetate, Propionate, Isovalerate, Valerate.

The SBR reactor, was also inoculated and the transient state, was monitored regarding the main parameters needed for a strong selection of PHA-Accumulating bacteria. An uncoupled feed of carbon an nutrients strategy was used, to increase selective pressure. The following parameters were achieved in the first (SBR-1) and and last SBR (SBR-5) monitoring: SBR-1 FF-0.21, r_S = 0.7, r_{PHA} = 1.5 and $Y_{P/S}$ = 0.62; SBR-5 FF= 0.04, r_S = 8.65, r_{PHA} = 7.78 and $Y_{P/S}$ = 0.65.

To optimize the productivity of this process, nutrients were added to the accumulating stage to induce little growth without overcoming the PHA content. In general, global productivity has increased since without nutrients the best results obtained were 0.041 (ACC-4), whilst with nutrients addition 0.056 (ACC(Nutrients)-9) was achieved. Regarding the PHA content, ACC-4 achieved 78,86% and ACC(Nutrients)-9 74.44%.

The main objective of this work was to do a mass balance of the process, from the conversion of fruit pulp in the first stage to the biopolymer production on the last stage. An average of the results in the experiences made was used to get the most significant results due to the variability of the biomass. To produce 1kg of biopolymer: ACC - 3/4 8011.6 gCOD, ACC(Nutri) 6/7 6906.8 gCOD and for ACC(Nutrie) 8/9 6142.6 gCOD.

In a prospect of future work, different ratios should be tested regarding the different

behavior obtained, in the batches that were made. To get a more reliable mass balance, more accumulations batches should be done in order to get more data, due to the variability of the process, considering the variability of the feedstock used for the UASB reactor and also, the PHA accumulating culture that is composed by a consortium of bacteria which could change along with the variations of the UASB reactor.

Bibliography

[1] EuroStat. *Generation of waste by waste category, hazardousness and NACE Rev. 2 activity*. 2016. URL: https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=env_wasgenlang=en.

[2] A. Stenmarck, C. Jensen, T. Quested, G. Moates, M. Buksti, B. Cseh, S. Juul, A. Parry, A. Politano, B. Redlingshofer, S. Scherhaufer, K. Silvennoinen, J. Soethoudt, C. Zubert, and K. Stergren. "Estimates of European food waste levels." English. In: (Mar. 2016).

[3] E. Comission. *How Throwaway plastic is failing to solver Europe's Food Waste Problem*. 2018. URL: https://ieep.eu/uploads/articles/attachments/c3cd1e91-a67e-417b-8bde-d97edcf10bdd/Over%20packaging%20fact%20sheet%20-%20Unwrapped%20Packaging%20and%20Food%20Waste%20IEEP%202018.pdf?v=63690511118.

[4] EuroStat. *Packaging waste by waste management operations and waste flow*. 2016. URL: https://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do.

[5] PlasticsEurope. *Plastics – the Facts 2018*. 2018. URL: https://www.plasticseurope.org/application/files/6315/4510/9658/Plastics_the_facts_2018_AF_web.pdf.

[6] E. Comission. *A European Strategy for Plastics in a Circular Economy*. 2018. URL: https://ec.europa.eu/environment/circular-economy/pdf/plastics-strategy.pdf.

[7] M. Koller. "Biodegradable and Biocompatible Polyhydroxy-alkanoates (PHA): Auspicious Microbial Macromolecules for Pharmaceutical and Therapeutic Applications." In: *Molecules* 23 (Feb. 2018). ISSN: 1420-3049. DOI: 10.3390/molecules23020362.

[8] J. Dias, P. Lemos, L. Serafim, C. Oliveira, M. Eiroa, M. Albuquerque, A. Ramos, R. Oliveira, and M. Reis. "Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product." In: *Macromolecular bioscience* 6 (Nov. 2006), pp. 885–906. DOI: 10.1002/mabi.200600112.

[9] "Response of a three-stage process for PHA production by mixed microbial cultures to feedstock shift: impact on polymer composition." In: *New Biotechnology* 31.4 (2014). Polyhydroxyalkanoate (PHA) by Mixed Microbial Cultures: Fermentation,

Control and Downstream Processing, pp. 276 –288. ISSN: 1871-6784. DOI: https://doi.org/10.1016/j.nbt.2013.10.010.

[10] L. Pfaltzgraff, M. De bruyn, E. C. Cooper, V. Budarin, and J. Clark. "Food waste biomass: A resource for high-value chemicals." In: *Green Chem.* 15 (Jan. 2013), pp. 307–314. DOI: 10.1039/C2GC36978H.

[11] C. Lin, L. Pfaltzgraff, L. Herrero Davila, E. Mubofu, A. Solhy, J. Clark, A. Koutinas, N. Kopsahelis, K. Stamatelatou, F. Dickson, S. Thankappan, M. Zahouily, j Bocklesby, and c Luquek. "Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective." In: *Energy & Environmental Science* 6 (Jan. 2013), pp. 426–464. DOI: 10.1039/C2EE23440H.

[12] C. Tuck, E. Perez Velilla, I. Horváth, R. Sheldon, and M. Poliakoff. "Valorization of Biomass: Deriving More Value from Waste." In: *Science (New York, N.Y.)* 337 (Aug. 2012), pp. 695–9. DOI: 10.1126/science.1218930.

[13] C. Nielsen, A. Rahman, A. Rehman, M. Walsh, and C. Miller. "Food waste conversion to microbial polyhydroxyalkanoates." In: *Microbial Biotechnology* 10 (July 2017). DOI: 10.1111/1751-7915.12776.

[14] 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." In: *Journal of Bioscience and Bioengineering* 126.3 (2018), pp. 282 –292. ISSN: 1389-1723. DOI: https://doi.org/10.1016/j.jbiosc.2018.03.016. URL: http://www.sciencedirect.com/science/article/pii/S1389172317311982.

[15] D Rhu, W Lee, J. Kim, and E Choi. "Polyhydroxyalkanoate (PHA) production from waste." In: *Water science and technology : a journal of the International Association on Water Pollution Research* 482 (Feb. 2003), pp. 221–8. DOI: 10.2166/wst.2003.0472.

[16] L. Serafim, P. Lemos, R. Oliveira, and M. Reis. "Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions." In: *Biotechnology and bioengineering* 87 (Aug. 2004), pp. 145–60. DOI: 10.1002/bit.20085.

[17] F. Silva, L. Serafim, M. Nadais, L. Arroja, and I. Capela. "Acidogenic Fermentation Towards Valorisation of Organic Waste Streams into Volatile Fatty Acids." In: *Chemical and Biochemical Engineering Quarterly* 27 (2013), pp. 467–476.

[18] C. Kourmentza, J. Placido, N. Venetsaneas, A. Burniol-Figols, C. Varrone, H. Gavala, and M. Reis. "Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production." In: *Bioengineering, MDPI* 4 (June 2017), p. 55. DOI: 10.3390/bioengineering4020055.

[19] R. Scaffaro, N. Dintcheva, R. Marino, and F. P. La Mantia. "Processing and Properties of Biopolymer/Polyhydroxyalkanoates Blends." In: *Journal of Polymers and the Environment* 20 (June 2011), p. 2. DOI: 10.1007/s10924-011-0385-2.

[20] M. Albuquerque, V Martino, E. Pollet, L. Avérous, and M. Reis. "Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties." In: *Journal of biotechnology* 151 (Oct. 2010), pp. 66–76. DOI: 10.1016/j.jbiotec.2010.10.070.

[21] E. Bioplastics. *BIOPLASTICS facts and figures*. 2018. URL: https://docs.european-bioplastics.org/publications/EUBP_Facts_and_figures.pdf.

[22] A. Anjum, M. Zuber, K. M. Zia, A. Noreen, M. N. Anjum, and S. Tabasum. "Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements." In: *International Journal of Biological Macromolecules* 89 (2016), pp. 161–174. ISSN: 0141-8130. DOI: https://doi.org/10.1016/j.ijbiomac.2016.04.069.

[23] C. Y. Loo and K. Sudesh. "Polyhydroxyalkanoates: Bio-based microbial plastics and their properties." In: *Malaysian Polymer Journal (MPJ)* 2 (Jan. 2007), pp. 31–57.

[24] S. Khanna and A. K. Srivastava. "Statistical media optimization studies for growth and PHB production by Ralstonia eutropha." In: *Process Biochemistry* 40.6 (2005), pp. 2173 –2182. ISSN: 1359-5113. DOI: https://doi.org/10.1016/j.procbio.2004.08.011.

[25] C. Kourmentza, I. Ntaikou, G. Lyberatos, and M. Kornaros. "Polyhydroxyalkanoates from Pseudomonas sp. using synthetic and olive mill wastewater under limiting conditions." In: *International Journal of Biological Macromolecules* 74 (2015), pp. 202 –210. ISSN: 0141-8130. DOI: https://doi.org/10.1016/j.ijbiomac.2014.12.032.

[26] C. Kourmentza, I. Ntaikou, G. Lyberatos, and M. Kornaros. "Polyhydroxyalkanoates from Pseudomonas sp. using synthetic and olive mill wastewater under limiting conditions." In: *International Journal of Biological Macromolecules* 74 (2015), pp. 202 –210. ISSN: 0141-8130. DOI: https://doi.org/10.1016/j.ijbiomac.2014.12.032.

[27] M Reis, M. Albuquerque, M Villano, and M Majone. "Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus/Wastes as Feedstocks." In: *Comprehensive Biotechnology* 6 (Dec. 2011), pp. 669–683. DOI: 10.1016/B978-0-08-088504-9.00464-5.

[28] S. Y. L. Woo Suk Ahn Si Jae Park. "Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Escherichia coli." In: *Biotechnology Letters* 23 (2001), pp. 235–240. DOI: 10.1023/A:1005633418161.

[29] L. Serafim, P. Lemos, M. Albuquerque, and M. Reis. "Strategies for PHA production by mixed cultures and renewable waste materials." In: *Applied microbiology and biotechnology* 81 (Dec. 2008), pp. 615–28. DOI: 10.1007/s00253-008-1757-y.

[30] M. C. Gould. "Bioenergy and Anaerobic Digestion." In: *Bioenergy*. Ed. by A. Dahiya. Boston: Academic Press, 2015. Chap. 18, pp. 297 –317. ISBN: 978-0-12-407909-0. DOI: https://doi.org/10.1016/B978-0-12-407909-0.00018-3.

[31] C. Bowie, D. Sneddon, and A. Montgomery. "Considerations in Design and Operation of a Biogas Plant." In: *Energy for Rural and Island Communities*. Ed. by J. TWIDELL, F. RIDDOCH, and B. GRAINGER. Pergamon, 1984. Chap. 7, pp. 371 –377. ISBN: 978-0-08-030580-6. DOI: https://doi.org/10.1016/B978-0-08-030580-6.50049-8.

[32] G. Buitrón, G. Kumar, A. Martinez-Arce, and G. Moreno. "Hydrogen and methane production via a two-stage processes (H2-SBR + CH4-UASB) using tequila vinasses." In: *International Journal of Hydrogen Energy* 39.33 (2014), pp. 19249 –19255. ISSN: 0360-3199. DOI: https://doi.org/10.1016/j.ijhydene.2014.04.139. URL: http://www.sciencedirect.com/science/article/pii/S0360319914011975.

[33] O. Sarkar, S. K. Butti, and S. V. Mohan. "Chapter 6 - Acidogenic Biorefinery: Food Waste Valorization to Biogas and Platform Chemicals." In: *Waste Biorefinery*. Ed. by T. Bhaskar, A. Pandey, S. V. Mohan, D.-J. Lee, and S. K. Khanal. Elsevier, 2018, pp. 203 –218. ISBN: 978-0-444-63992-9. DOI: https://doi.org/10.1016/B978-0-444-63992-9.00006-9.

[34] P. Bajpai. "Basics of Anaerobic Digestion Process." In: *Anaerobic Technology in Pulp and Paper Industry*. Singapore: Springer Singapore, 2017, pp. 7–12. ISBN: 978-981-10-4130-3. DOI: 10.1007/978-981-10-4130-3_2.

[35] M. Carvalheira, J. Cassidy, J. M. Ribeiro, B. A. Oliveira, E. B. Freitas, C. Roca, G. Carvalho, A. Oehmen, and M. A. Reis. "Performance of a two-stage anaerobic digestion system treating fruit pulp waste: The impact of substrate shift and operational conditions." In: *Waste Management* 78 (2018), pp. 434 –445. ISSN: 0956-053X. DOI: https://doi.org/10.1016/j.wasman.2018.06.013.

[36] F. G. Pohland and S. Ghosh. "Developments in Anaerobic Stabilization of Organic Wastes - The Two-Phase Concept." In: *Environmental Letters* 1.4 (1971), pp. 255–266. DOI: https://doi.org/10.1080/00139307109434990.

[37] S. K. Khanal, W.-H. Chen, L. Li, and S. Sung. "Biological hydrogen production: effects of pH and intermediate products." In: *International Journal of Hydrogen Energy* 29.11 (2004), pp. 1123 –1131. ISSN: 0360-3199. DOI: https://doi.org/10.1016/j.ijhydene.2003.11.002.

[38] 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." In: *Journal of Biotechnology* 130.4 (2007), pp. 411 –421. ISSN: 0168-1656. DOI: https://doi.org/10.1016/j.jbiotec.2007.05.011.

[39] P. C. Lemos, L. S. Serafim, and M. A. Reis. "Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding." In: *Journal of Biotechnology* 122.2 (2006), pp. 226 –238. ISSN: 0168-1656. DOI: https://doi.org/10.1016/j.jbiotec.2005.09.006.

[40] K. Johnson, Y. Jiang, R. Kleerebezem, G. Muyzer, and M. van Loosdrecht. "Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity." In: *Biomacromolecules* 10 (Jan. 2009), pp. 670–676. DOI: https://doi.org/10.1021/bm8013796.

[41] M. van Loosdrecht, M. Pot, and J. Heijnen. "Importance of bacterial storage polymers in bioprocesses." In: *Water Science and Technology* 35.1 (1997). Sequencing Batch Reactor Technology: Batch Application of Periodic Unsteady-state Processes, pp. 41 –47. ISSN: 0273-1223. DOI: https://doi.org/10.1016/S0273-1223(96)00877-3.

[42] M Reis, M. Albuquerque, M Villano, and M Majone. "Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus/Wastes as Feedstocks." In: *Comprehensive Biotechnology* 6 (Dec. 2011), pp. 669–683. DOI: 10.1016/B978-0-08-088504-9.00464-5.

[43] R. Verlinden, D. Hill, M. Kenward, C. Williams, and I. Radecka. "Bacterial synthesis of biodegradable polyhydroxyalkanoates." In: *Journal of Applied Microbiology* 102.6 (2007), pp. 1437–1449. DOI: 10.1111/j.1365-2672.2007.03335.x.

[44] M Majone, M. Beccari, S. Di Gregorio, D. Dionisi, and G. Vallini. "Enrichment of activated sludge in a Sequencing Batch Reactor for polyhydroxyalkanoates production." In: *Water science and technology : a journal of the International Association on Water Pollution Research* 54 (Feb. 2006), pp. 119–28. DOI: 10.2166/wst.2006.379.

[45] Z. Chen, Z. Guo, Q. Wen, L. Huang, R. Bakke, and M. Du. "A new method for polyhydroxyalkanoate (PHA) accumulating bacteria selection under physical selective pressure." In: *International Journal of Biological Macromolecules* 72 (2015), pp. 1329 –1334. ISSN: 0141-8130. DOI: https://doi.org/10.1016/j.ijbiomac.2014.10.027.

[46] M. Albuquerque, C. Torres, and M. Reis. "Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: Effect of the influent substrate concentration on culture selection." In: *Water Research* 44.11 (2010), pp. 3419 –3433. ISSN: 0043-1354. DOI: https://doi.org/10.1016/j.watres.2010.03.021.

[47] G. Montiel-Jarillo, J. Carrera, and M. E. Suárez-Ojeda. "Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: Effect of pH and N and P concentrations." In: *Science of The Total Environment* 583 (2017), pp. 300 –307. ISSN: 0048-9697. DOI: https://doi.org/10.1016/j.scitotenv.2017.01.069.

[48] C. Kourmentza and M. Kornaros. "Biotransformation of volatile fatty acids to polyhydroxyalkanoates by employing mixed microbial consortia: The effect of pH and carbon source." In: *Bioresource Technology* 222 (2016), pp. 388 –398. ISSN: 0960-8524. DOI: https://doi.org/10.1016/j.biortech.2016.10.014. URL: http://www.sciencedirect.com/science/article/pii/S0960852416314201.

[49] K. Johnson, Y. Jiang, R. Kleerebezem, G. Muyzer, and M. van Loosdrecht. "Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity." In: *Biomacromolecules* 10 (Jan. 2009), pp. 670–676. DOI: https://doi.org/10.1021/bm8013796.

[50] 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." In: *New Biotechnology* 37 (2017). Biopolymers Eu Symposium, pp. 90 –98. ISSN: 1871-6784. DOI: https://doi.org/10.1016/j.nbt.2016.07.013.

[51] 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." In: *New Biotechnology* 37 (2017). Biopolymers Eu Symposium, pp. 69 –79. ISSN: 1871-6784. DOI: https://doi.org/10.1016/j.nbt.2016.10.008.

[52] E. Korkakaki, M. C. van Loosdrecht, and R. Kleerebezem. "Survival of the fastest: Selective removal of the side population for enhanced PHA production in a mixed substrate enrichment." In: *Bioresource Technology* 216 (2016), pp. 1022 –1029. ISSN: 0960-8524. DOI: https://doi.org/10.1016/j.biortech.2016.05.125.

[53] F. Valentino, L. Karabegovic, M. Majone, F. Morgan-Sagastume, and A. Werker. "Polyhydroxyalkanoate storage within a mixed culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels." In: *Water Research* 77 (Mar. 2015), pp. 49 –63. DOI: https://doi.org/10.1016/j.watres.2015.03.016.

[54] K. A. Johnson and R. S. Goody. "The Original Michaelis Constant: Translation of the 1913 Michaelis–Menten Paper." In: *Biochemistry* 50.39 (2011), pp. 8264–8269. DOI: 10.1021/bi201284u.

[55] U. E. P. A. O. of Water. *Total, Fixed, and Volatile Solids in Water, Solids, and Biosolids*. URL: https://www.epa.gov/sites/production/files/2015-10/documents/method_1684_draft_2001.pdf.

[56] L. R. Braunegg G Sonnleitner B. "Gas chromatographic determination of poly- β -hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis." In: *Journal of Chromatography A* 445 (1988), pp. 285 –289. ISSN: 0021-9673. DOI: https://doi.org/10.1016/S0021-9673(01)84535-0.

[57] Y. Comeau, K. J. Hall, and W. K. Oldham. "Determination of Poly- β-Hydroxybutyrate and Poly- β -Hydroxyvalerate in Activated Sludge by Gas-Liquid Chromatography." In: *Applied and Environmental Microbiology* 54.9 (1988), pp. 2325–2327. ISSN: 0099-2240. eprint: https://aem.asm.org/content/54/9/2325.full.pdf.

[58] A. B. Lanham, A. R. Ricardo, M. G. Albuquerque, F. Pardelha, M. Carvalheira, M. Coma, J. Fradinho, G. Carvalho, A. Oehmen, and M. A. Reis. "Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems." In: *Process Biochemistry* 48.11 (2013), pp. 1626 –1634. ISSN: 1359-5113. DOI: https://doi.org/10.1016/j.procbio.2013.07.023.

[59] S. Y. Lee and J. il Choi. "Effect of fermentation performance on the economics of poly(3-hydroxybutyrate) production byAlcaligenes latus." In: *Polymer Degradation and Stability* 59.1 (1998). Biodegradable Polymers and Macromolecules, pp. 387 –393. ISSN: 0141-3910. DOI: https://doi.org/10.1016/S0141-3910(97)00176-6.

[60] M. Reis, M. Albuquerque, M. Villano, and M. Majone. "Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus/Wastes as Feedstocks." In: *Comprehensive Biotechnology* 6 (Dec. 2011), pp. 669–683. DOI: 10.1016/B978-0-08-088504-9.00464-5.

[61] U. D. of Agriculture. *FoodData Central Search Results*. 2017. URL: https://fdc.nal.usda.gov/fdc-app.html#/food-details/577849/nutrients.

[62] B. Ahring, N. Murali, and K. Srinivas. "Fermentation of Cellulose with a Mixed Microbial Rumen Culture with and without Methanogenesis." In: *Fermentation Technology* 07 (Jan. 2018). DOI: 10.4172/2167-7972.1000152.