Ana Catarina Martins de Oliveira

Bachelor degree in Cellular and Molecular Biology

Valorization of brewery waste through polyhydroxyalkanoates production

Dissertation for Master degree in Biotechnology

Supervisor: Anouk F. Duque, Post-doctoral researcher, FCT/UNL
Co-Supervisor: Mónica Carvalheira, Post-doctoral researcher, FCT/UNL

Jury:
President: Prof. Dr. Susana Filipe Barreiros
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Para a minha família

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Abstract

Polyhydroxyalkanoates (PHA) are a natural polymer synthesized by many microorganisms which have physical-chemical characteristics similar to conventional plastics, and therefore becomes a viable alternative to petrochemical based plastics. PHA production through mixed microbial cultures (MMCs) has been studied as an alternative to pure cultures, since they do not require sterile conditions and can be fed with cheaper substrates (such as agro-industrial waste), decreasing the operational costs.

This study was focused on the valorization of brewer's spent grain (BSG), through PHA production using MMCs which requires a three-step process: an anaerobic acidogenic fermentation step, where BSG is converted into volatile fatty acids (VFA); a aerobic step, performed in a sequencing batch reactor (SBR) where PHA accumulating bacteria are selected, and finally the accumulation step, to produce and achieve the maximum PHA content.

In order to optimize the acidogenic fermentation using BSG hydrolysate different organic loading rates (30 and 50 gCOD/L) and pH (5, 5.5 and 6) were tested. Fermentation products profile changed with different conditions applied, allowing the manipulation the PHA composition with specific characteristics. The yield of fermentation products varies between 0.6 and 0.9 gCOD-FP/gCOD-S. The PHA accumulating bacteria selection and PHA accumulation were studied using a VFA-rich effluent from an anerobic solid waste fermentation reactor. In the SBR different organic loading rates were tested. With the increase in the OLR, PHA storage rate increased, as well as the storage yield, reaching a maximum of 0.33±0.08 CmmolPHA/CmmolX.h and 0.91 ± 0.24 CmolPHA/CmmolFP, respectively. PHA accumulation allowed to reach a maximum PHA content of 41% with a 50% of hydroxybutyrate and 50% of hydroxyvalerate composition.

Keywords: Polyhydroxyalkanoates (PHA); Brewer's spent grain (BSG); Mixed microbial cultures (MMCs); Acidogenic fermentation; Sequencing batch reactor (SBR)

Resumo

Os polihidroxialcanoatos (PHA) são polímeros naturais, sintetizados por muitos microrganismos, que possuem características físico-químicas semelhantes aos plásticos convencionais tornando-se numa alternativa viável a estes. A produção de PHA através de culturas microbianas mistas (CMMs) tem sido estudada como uma alternativa às culturas puras, uma vez que não requerem condições estéreis e podem ser alimentadas com substratos mais baratos (como resíduos agroindustriais), diminuindo os custos operacionais.

Este estudo foi focado na valorização da dreche da cerveja, através da produção de PHA usando CMMs que requer um processo composto por três etapas: uma etapa de fermentação acidogénica anaeróbica, onde a dreche é convertida em ácidos gordos voláteis (AGV); um passo aeróbico, realizado num *sequencing batch reactor* (SBR) onde as bactérias que acumulam PHA são selecionadas e, finalmente, a etapa de acumulação, para se produzir e atingir o teor máximo de PHA.

Para estudar a fermentação acidogénica utilizando o hidrolisado de BSG foram testadas diferentes cargas orgânicas (30 e 50 gCOD/L) e pH (5; 5,5 e 6). O perfil dos produtos de fermentação muda com as diferentes condições aplicadas, o que permite a manipulação da composição do PHA e por sua vez as características específicas do polimero. O rendimento dos produtos de fermentação varia entre 0.6 and 0.9 gCOD-FP/gCOD-S. Em seguida, a seleção de bactérias acumuladoras de PHA e a acumulação de PHA foram estudados usando um efluente rico em AGV de um reator de fermentação anaeróbio de resíduos sólidos. No SBR, cargas orgânicas distintas foram testadas. Com o aumento da carga orgânica, aumenta também a taxa de armazenamento de PHA, assim como o rendimento de armazenamento, atingindo 0,33 ± 0,08 CmmolPHA/CmmolX.h e 0,91 ± 0,24 CmolPHA/CmmolFP, respectivamente. A acumulação de PHA permitiu atingir um teor máximo de PHA de 41% com uma composição de 50% de hidroxibutirato e 50% de hidroxivalerato.

Palavras-chave: Polihidroxialcanoatos (PHA); dreche de cerveja; Culturas microbianas mistas (CMMs); Fermentação acidogénica; *Sequencing batch reactor* (SBR)

List of Contents

List of figures

List of tables

List of abbreviations and variables

BSG	Brewery's spent grains
C/N/P	Carbon to nitrogen to phosphorus ratio, in C-mol/N-mol/P-mol
COD	Chemical oxygen demand
DA	Degree of acidification, in % (gCOD-FPs/gCOD-S)
Eff_1	Effluente from acidogenic fermentation reactor with HRT and SRT of 40.7 days and a OLR of 6.1 gCOD/l.d
Eff_2	Effluente from acidogenic fermentation reactor with HRT and SRT of 16 days and a OLR of 21.2 gCOD/l.d
F/F ratio	Feast and Famine ratio, in h/h
FP	Fermentation products
GC	Gas chromatography
HAc	Acetic acid
HB	Hydroxybutyrate
HBut	Butyric acid
HIsobut	Isobutyric acid
HIsoval	Isovaleric acid
HLac	Lactic acid
HPLC	High Performance Liquid Chromatography
HProp	Propionic acid
HRT	Hydraulic retention time, in days
HV	Hydroxyvalerate
HVal	Valeric acid
LCL	Long chain length
MCL	Medium chain length
MMC	Mixed microbial culture
OLR	Organic loading rate, in C-mmol/L.d
P(3HB)	Poly(3-hydroxybutyrate)
PHA	Polyhydroxyalkanoates
PHA max	Maximum PHA content, in %
qFP	Fermentation products production rate, in gCOD-FP/gVSS.h)
qPHA	PHA production rate, in C-mol-PHA/C-mol-X.h
-qS	Substrate uptake rate, in g-COD S/gVSS h or C-mol-S/C-mol-X.h
qX	Growth rate, in C-mol-X/C-mol-X.h
SBR	Sequencing Batch Reactor
SCL	Short chain length
SRT	Sludge retention time, in days
TSS	Total suspended solids, in gTSS/L;
VFA	Volatile fatty acids

VSS	Volatile suspended solids, in gVSS/L;
X	Active biomass
$Y_{FP/S}$	FPs yield, in gCOD-FP/gCOD-S
$Y_{PHA/FP}$	PHA storage yield, in C-mol-PHA/C-mol-FP
$Y_{X/FP}$	Growth yield, in C-mol-X/C-mol-FP

1. Introduction

1.1. Problem

In recent years there it was observed a population increase, which led to an increase of the industry in order to ensure the population needs, and which consequently caused an increase in the quantity and variety of waste generated. At the same time, there has been a growing reliance on materials derived from crude oil, including plastic. Both aspects contribute not only to a more polluted world but also to the expenditure of non-renewable resources. (Hopewell et al., 2009; Manahan, 2017)

These problems, may have several solutions, but both can be minimized through the production of bioplastics from agro-industrial waste, as demonstrated by Braunegg et al., 1998.

Bioplastic production has emerged as an alternative to conventional plastics having similar characteristics. Bioplastics are biodegradable, biocompatible, non-toxic and can be produced from waste, thus contributing to circular economy (Abid et al., 2018; Reddy et al., 2003; Reis et al., 2011).

The concept of circular economy aims at reducing environmental impacts and resource use by maximizing the service of the material by resource. In this case extend the viability of the waste generated by the industry for the production of bioplastic (Tisserant et al., 2017).

1.2. Plastics

Since the beginning of the plastic industry (1950), it is almost unthinkable to imagine our daily routine without plastics.(Geyer et al., 2017) Indeed this polymeric material is present in our daily routine, due to its strength, lightness, resistance, low cost and durability. Moreover it presents the ability to be molded into a variety of products that can be used in several industries, such as, packaging, building and construction and automotive (figure 1.1) (Hopewell et al., 2009; PlasticsEurope, 2017).

Regarding to the recovery of plastic, in 2016 and for the first time, recycling (31.1%) exceeded the landfill (27.3%) (PlasticsEurope, 2017). However the majority of plastics are nondegradable and even the degradable ones may persist for a considerable time, accumulating in the natural environment and contaminating soils and water resources representing a growing concern (Thompson et al., 2009). Furthermore, plastics also represents a problem to the marine environment, due to their accumulation and consequent effect on marine life (Reddy et al., 2003).

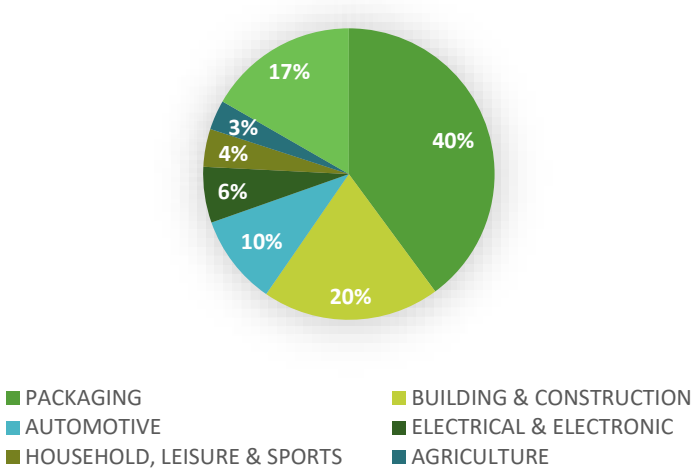


Figure 1.1: Plastics converter demand main market sectors (PlasticsEurope, 2017)

The problem of excess and accumulation of plastic is that most of them are derived from petrochemicals produced from fossil oil and gas, which not only contributes to increased pollution but also the depletion of non-renewable natural resources (Hopewell et al., 2009). Nevertheless has been increasing public concern regarding the environment and climate change. This has pressing governments, compannies and researchers to start developing sustainable alternatives such as production of biodegradable plastic through sustainable feedstocks (Reddy et al., 2003)

1.3. Agro-industrial residues

The population increase led to an increase on food and agriculture industries and consequently to an increase in waste production (Vandamme, 2014). If these agroindustrial wastes are not properly treated they can lead to environmental, animal and human health complications (Sadh et al., 2018).

Most of these residues, such as sugarcane and beet molasses, cheese whey effluents, plant oils, swine waste liquor, vegetable and fruit wastes, effluents of palm oil mill, olive oil mill, paper mill, pull mill, and hydrolysates of starch are waste with high organic fraction making them a viable option for various applications such as production of polyhydroxyalkanoates (PHAs). Some of these residues have carbohydrates that are not used by some mixed cultures, however a pre-treatment of these residues, such as fermentation, allows the transformation of carbohydrates into volatile fatty acids (VFAs) that are precursors of the production of bioplastics (Reis et al., 2011; Sadh et al., 2018).

Since the substrate used for PHA production using mixed culture represents 40% of the production cost (Dias et al., 2006; Salehizadeh et al., 2004) their selection is an important factor in order to optimize the production of the bioplastic by cost reduction and recovery of waste. (Salehizadeh et al., 2004).

Comparing different agroindustrial wastes (table 1.1), the brewery's spent grains (BSG) obtained from beer industry presents a combination of high amounts of hemicellulose sugars and proteins. Beer is the fifth most consumed drink in the world and BSG represents about 85% of

the total waste from beer industry (Roberto, 2006; Simate et al., 2011). So BSG are an excellent residue for several applications, such as energy production, animal feed or PHA production. (Carvalheiro et al., 2004; Lamghari et al., 1998; Mussatto, 2014; Rabelo et al., 2011).

Table 1.1: Composition of diverses agro-industrial wastes

Agricultural by-products	Composition (g/kg dry weight)				Reference
	Cellulose	Hemicellulose	Lignin	Protein	
Rice Straw	434	229	172	NR	(Mussatto, 2014)
Sugar cane molasses	384	232	250	NR	(Rabelo et al., 2011)
Spent Coffee	86	367	NR	136	(Mussatto, 2014)
Fruit pulp waste	75	69	343	513	(Lamghari et al., 1998)
Brewery's spent grains	219	296	217	246	(Carvalheiro et al., 2004)

NR: Not reported

1.3.1. Brewer's spent grain

In the brewing process, the main ingredients used are malt, water, yeast and hops.

Firstly the barley grains are hydrated with water to promote the synthesis and activation of the enzymes that act in the malt hydrolysis process. Then the hydrated grains are dried and designated as malt. Malt is crushed to break the beans that will be heated from 37º to 78º slowly in water to convert the malt into fermentable sugars and the proteins into peptides and amino acids, through the action of the previously activated enzymes. At the end of this stage we get brewery's spent grains (BSG) mixed with the wort (liquid fraction), which are separated by filtration. The wort is subjected to the remaining brewing process, which includes boiling, fermentation and maturation, where other by-products are also obtained. However, , BSG represents about 85% of the total by-products produced (Mussatto 2014; Mussatto 2005; Roberto 2006).

As BSG is derived from the wort formation and is the largest by product from brewing process representing about 85% of the total by-products produced. BSG composition may vary depending on the variety of grains used, when it was harvested, the conditions used for malting and mashing, the type and amount of the adjuncts added in mixture with the barley malt when the wort is formed. (Mussatto 2014; Mussatto 2005; Roberto 2006).

In general, BSG is a lino-cellulosic material, rich in proteins (about 30% of total proteins are composed of essential amino acids and the remaining 70% by non-essential amino acids) and fibers (hemicelluloses, cellulose and lignin), which represent about of 20% and 70% of its

composition, respectively. Hemicellulose and cellulose are essentially composed of sugars, mainly xylose, arabinose and glucose. Lignin contains various polyphenolic components, in particular ferulic, p-coumaric, syringic, vanillic and p-hydroxybenzoic acids. Beside proteins and fibers, BSG can also contain vitamins such as biotin, folic acid, thiamine and riboflavin and some minerals such as silicon, phosphorus, calcium, colbate, copper, iron, magnesium, potassium and sulfur (Gupta et al 2010; Mussatto 2014).

Due to the high moisture content, organic nature and high fermentable sugars content, BSG presents a rapid degradation at room temperature (about 7 to 10 days) and their prolonged exposure to air favors the proliferation of microorganisms, causing loss of nutrients and the formation of toxic products harmful to the environment. It is therefore important that this waste is treated or used for various applications (Gupta et al 2010)

Due to its composition, BSG can have several areas of application such as, feed and food, energy production, chemical processes and biotechnological processes (Table 1.2) (Mussatto,2014).

Table 1.2: Main areas of application of BSG (Mussatto, 2014)

Area of aplication	Aplication	Description
Feed and Food	Animal feed	Animal feeding is currently the main application of BSG. It is excellent for use in feeding cows, since is rich in protein and fiber and when combined with urea as a source of nitrogen, allows complete nutrition to the animals. Promotes the increase of milk production and the decreases of the fat content without affecting the fertility of animals. Its use has also been studied for other animals, such as pigs, fish and birds.
	Human diet	The use of BSG in the human diet is one of the possible applications, and the most studied has been the use of BSG in bakery products. However it is necessary to control the amount used, in order to avoid the browning and unwanted flavors. Due to the nutritional values, BSG also has health benefits, such as lowering cholesterol, diabetes and relieving constipation and diarrhea.
	Food aplications	BSG has phenolic compounds (essentially ferulic and p-coumaric) that have a remarkable application in human health, since it reduces the incidence of diabetes and cancer. Moreover can have antioxidants, anti-inflammatory and anti-allergic effects. The incorporation of BSG into food avoids the use of synthetic compounds.
Energy production	Thermochemical Conversion	BSG can also used to generate energy from thermochemical conversion, using processes such as pyrolysis and combustion. However it is necessary to pay special attention to emission of particles and toxic gases that can occur using these techniques, so whenever possible to avoid or minimize these situations.
	Biogas production	The anaerobic fermentation of BSG allows the production of biogas with a composition rich in methane and carbon dioxide and some traces of hydrogen and water vapor. This process is composed of two stages, a acidogenic stage in which macromolecules are converted to volatile fatty acids and methanogenic stage in which microorganisms convert acetate to methane.

Area of aplication	Aplication	Description
	Ethanol production	In recent years ethanol has been studied as alternative fuel. BSG is considered a promising raw material due to its composition rich in hemicellulose and celulose that are needed to ethanol production.
Chemical processes	Extraction Of valuable compounds	The use of chemical processes such as hydrolysis (with different conditions) and solvent extraction allow the extraction of value-added compounds, such as sugars, lignin and antioxidant phenolic compounds.
	Paper production	Due to the presence of fiber in BSG has been studied to use in the manufacture of paper towels, cards and coasters.
	Adsorbent material	The use of BSG as an adsorbent is another very promising application. BSG can be used to adsorb heavy metals, as well as, dyes from wastewater. It can also be used for the production of activated carbon that has an adsorption capacity like commercialized products, and may have several applications, among which the treatment of waters and gases.
Biotechnological processes	Substrate for microorganism	BSG can be used as a substrate for the growth of microorganisms and in turn enzyme production. However it is necessary to take into account that its water content must be reduced in order to prevent its rapid degradation.
	Extraction Of valuable compounds	Through biotechnological processes such as enzymatic hydrolysis, it is possible extractvaluable compounds from BSG, such as sugars and hydroxycinnamic acids. This method has the advantage of not produce toxic effluents.
	Fermentation processes	BSG's fermentation processes give rise to value-added products, as ethanol or biogas. There is also the possibility of producing VFAs through fermentation, which are precursors of bioplastic production, such as polyhydroxyalkanoates.

1.4. Bioplastics

According to European Bioplastics, bioplastic is a bio-based and/or biodegradable product, which means that it is derived from biomass or renewable resources and whose microorganisms available in the environment are able to convert into natural substances, as water and carbon dioxide (European Bioplastics, 2017).

Bioplastics can be divided in three groups: bio-based; biodegradable and bio-based and biodegradable (European Bioplastics, 2017). The first one are plastics derived from biomass or renewable resources but can not be biodegradable such as polyethylene (PE), polyethylene terephthalate (PET) and Nylon 11 (NY11). The bioplastics biodegradable are based on fossil resources but can be degraded by microorganisms, which is the case of Polycaprolactone (PCL) and polybutylene adipate terephthalate (PBAT). Finally, bio-based and biodegradable are produced from biomass or renewable resources, and are biodegradable such as polylactic acid (PLA), polyhydroxyalkanoates (PHA), polybutylene succinate (PBS) and starch blends (European Bioplastics, 2017; Tokiwa et al., 2009).

The use of bioplastics allows the reduction of the use of fossil oil dependency and, greenhouse gas emissions. Also the increase of resource efficiency through a circular economy is possible since waste is used for its production. In this way plastics do not become waste after use, since they re-enter the economy circular system as valuable technical material or biological nutrients (European Bioplastics, 2017). Only in 2017 were produced 2.05 million tonnes of bioplastic and it is expected that this value will increase in next years, which leads to the creation of many jobs (23 000 jobs in Europe, 2017) (European Bioplastics, 2017).

The polyhydroxyalkanoates are an example of a natural polymer that are synthesized by many microrganisms. PHA are accumulated intracellularly up to high levels and can be used by microorganisms as a source of energy under stress conditions. In addition, PHAs have physico-chemical characteristics similar to conventional plastics, and therefore they become a viable alternative to petrochemical based plastics (Reddy et al., 2003).

1.5. Polyhydoxyalkanoates

Polyhydroxyalkanoates are polyesters composed of several units of hydroxyalkanoates (Figure 1.2) synthesized by many organisms and internally accumulated as carbon and energy reserves (Reddy et al., 2003). PHA accumulation can occurs up to 90% of biomass dry weight in the presence of excess carbon and limitation of other nutrients such as nitrogen, oxygen or phosphorus (Verlinden et al., 2007).

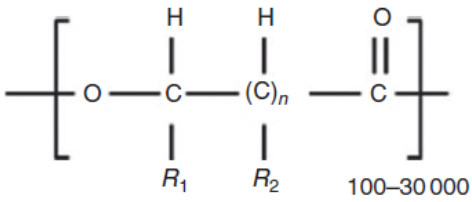


Figure 1.2: General chemical structure for polyhydroxyalkanoate (PHA). n, number of carbon atoms in the linear polyester structure; R1 and R2, variable hydrocarbon side chains.*(Reis et al.,2011)*

There are more than 150 hydroxyalkanoate monomers which generate PHA with different properties and chemical composition (homo-or copolymer). In general PHA are water insoluble, but soluble in chloroform and other chlorinated hydrocarbons. , have good resistance to ultra-violet and to hydrolytic degradation and they are biocompatible, biodegradable and notoxic therefore suitable for medical applications (Junyu et al., 2017). Furthermore sinks in water, furthering anaerobic biodegradation (leads to the production of methane instead of carbon dioxide). (Abid et al., 2018; M. Reis et al., 2011).

In structural terms PHAs can be divided into three groups depending on the number of carbons in their main chain: short-chain-length (SCL) PHAs, , whose monomers contain from 3 to 5 carbon atoms, medium-chain-length (MCL) PHAs, formed by monomers with 6 to 14 carbon atoms and long-chain-length (LCL) PHAs with more than 14 carbons atoms (less known and studied) (Abid et al., 2018)

Poly(3-hydroxybutyrate) (P(3HB)) is the most PHA known and studied. Despite P(H3HB) have a good thermoplastic properties, they also present high crystallinity (55-80%) and originate plastics that are extremely stiff and brittle with poor impact strength (Reis et al., 2011). However these properties can be greatly improved by the introduction of different monomers in order to mimic the characteristics of conventional plastics, such as polypropylene (with greater flexibility, around 400%). Table 1.3 presents the comparison of the physical properties of P(3HB) and copolymers with polypropylene. It is possible to see that the physical properties of PHA depend mostly on the constituent monomers and that copolymers presents physical properties more similar with polypropylene. (Akaraonye et al., 2010)

Table 1.3: Comparison of the physical properties of P(3HB) and copolymers with polypropylene (Akaraonye et al., 2010)

	Meltin Temperature (ºC)	Glass transition temperature (ºC)	Young's modulus (GPa)	Elongation to break (%)	Tensile strength (MPa)
P(3HB)	180	4	3.5	5	40
P(3HB-co-20% 3HV)	145	-1	1.2	50	20
P(3HB-co-16% 4HB)	150	-7	-	444	26
P(3HB-co-10% 3HHx)	127	-1	-	400	21
P(3HB-co-6% 3HD)	130	-8	-	680	17
Polypropylene	176	-10	1.7	400	34.5

1.5.1. Biosynthesis of PHA

The synthesis of PHA occurs under conditions of excess of carbon and limitation of other nutrientes and as all biopolymers they are synthesized by enzymatic processes (Verlinden et al., 2007).

The main enzymes involved in PHA biosynthesis are β-ketothiolase (phaA), acetoacetyl-coA redutase (phaB), PHA synthase (phaC) and 3-hidroxyacyl-acyl carrier protein-coenzime and there are three main pathways for PHA synthesis (Figure 1.3) (Rehm & Steinbu, 1999). Briefly, in the first metabolic pathway (pathway 1), two acetyl-coA generated by the carbon source are converted to acetoacetyl-coA by the enzyme B-ketothiolase. Then acetoacyl-coA redutase convert acetoacetyl-coA to 3-hydroxybutyryl-CoA which is converted into PHB under the catalysis of PHA synyhase. Pathway 2 implies *in situ fatty acid synthesis cycle* which produce R-3-Hydroxyacyl-ACP from acetyl-coA. 3-Hydroxyacyl-acyl carrier protein-coA turn R-3-hydroxyacyl-ACP into R-3-hydroxyacyl-CoA which transformed by phaC to PHA. Finally, pathway 3 involves β-oxidation cycle to obtain R-3-hydroxyacyl-CoA (from fatty acids) which like pathway 2 is converted to PHA through action of phaC (Braunegg et al., 1998; Chen et al., 2015).

Thus depending on the carbon source and/or the microorganisms, different metabolic pathways may be followed and different types of PHA can be produced.

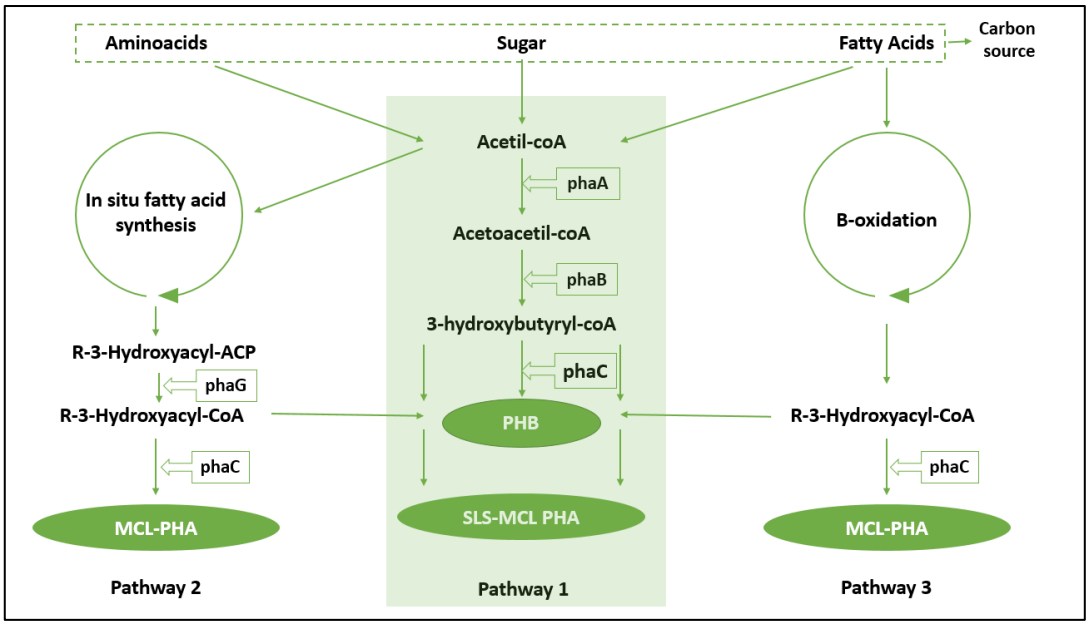


Figure 1.3: Three main pathways related to the biosynthesis of PHA. Adapted from Chen et al, 2015

1.6. Bacterial culture for PHA production

The production costs of bioplastics when comparing with petroleum-based plastics is the main obstacle for its industrialization, since 1kg of PHB has a cost of 9 € while 1kg of petroleum-based plastics has a cost of 1 € (Reis et al., 2003). This difference is due to the high cost of the substrate and high downstream processing costs when using pures cultures (Reis et al., 2003). For that reason the use of mixed cultures allows to decrease the costs since the microorganisms are able to use cheaper substrates (e.g. agroindustrial wastes), and also have the capacity to adapt to the changes of the substrate and avoids the need of sterile (Salehizadeh et al., 2004).

1.6.1. Pure cultures

In the last years the industrial production of PHA is carried out using pure cultures such as *Ralstonia eutropha* (Dennis et al., 1998), *Alcaligenes latus* (Wang et al., 1997), and *Burkholderia sacchar* (Rocha et al., 2008), and is performed in a two stages process. The first stage requires a carbon and nutrients source for the growth of bacteria, the second stage is limiting on an essential nutrient, promoting PHA accumulation. However it is an expensive process due to the high cost of sterile conditions,to the high fermentation costs (mainly due to the price of the substrate) and downstream processing costs (Dias et al., 2006; Laycock et al., 2013; Reis et al., 2011)

Recombinant strains for cost-effective PHA production have been studied in order to develop microorganisms which possess rapid growth and high cell density, and also are capable of using various substrates and whose polymer purification process is more simples (Dias et al., 2006). For exemple, *recombinant Escherichia coli harboring R. eutropha* can accumulate about 80-90% of its dry cell weight (Dias et al., 2006).

Although pure cultures exhibit high PHA yields, this process can not compete directly with the conventional plastics process, since the use of sterile conditions and the requirement of refined substrates (carbohydrates) such as molasses and glucose increase the cost of production(Reis et al., 2011).

1.6.2. Mixed cultures

PHA production through mixed microbial cultures (MMCs) has been studied as an alternative to pure cultures, since they do not require sterile conditions and can be fed with cheaper substractes (such as agro-industrial waste), which allow to decrease the production costs (Reis et al., 2011).

The PHA production requires a three-step process: An anaerobic acidogenic fermentation step, where the organic matter from agroindustrial waste is converted into VFAs; a aerobic step , performed in a sequencing batch reactor (SBR) and where the culture is selected (PHA accumulating bacteria) using the fermented effluent feed ; the third designated accumulation, where the culture (purge from the previous step) is fed with successive concentrated pulses of fermented effluent in order to achieve the maximum of PHA content (Bem et al., 2016; Dionisi et al., 2004; Queirós et al., 2014; Villano et al., 2013).

1.6.2.1. The Acidogenic Fermentation Stage

The traditional anaerobic fermentation is composed of three stages: (1) substrate hydrolysis; (2) acidogenic fermentation (the organic content of the feedstock is converted into VFAs) and (3) methanogenesis. However the VFAs produced in stage 2 can also be used as precursors for the production of PHA by MMCs (Liu et al., 2012; Tamis et al., 2015).

Tipically organics copounds from agro-industrial wastes are converted to lactic acid, propionic, acetic, butyric and other fermentation products (M. Reis et al., 2011). So depending on the feedstock, the operacional conditions, such as temperature, pH and organic loading rate (OLR), different compositions of VFAs are obtained, which consequently varies the amount of HB and HV precursors. (M. Reis et al., 2011; Tamis et al., 2015). It is important to study these variations when it is desired to obtain a polymer having a specific composition of HB and HV.

1.6.2.2. The culture selection and PHA accumulation

The culture selection of PHA accumulating bacteria can be carried out by at least two types of strategies: aerobic and anaerobic cycles and feast and famine cycles. (Laycock et al., 2013; Villano et al., 2013).

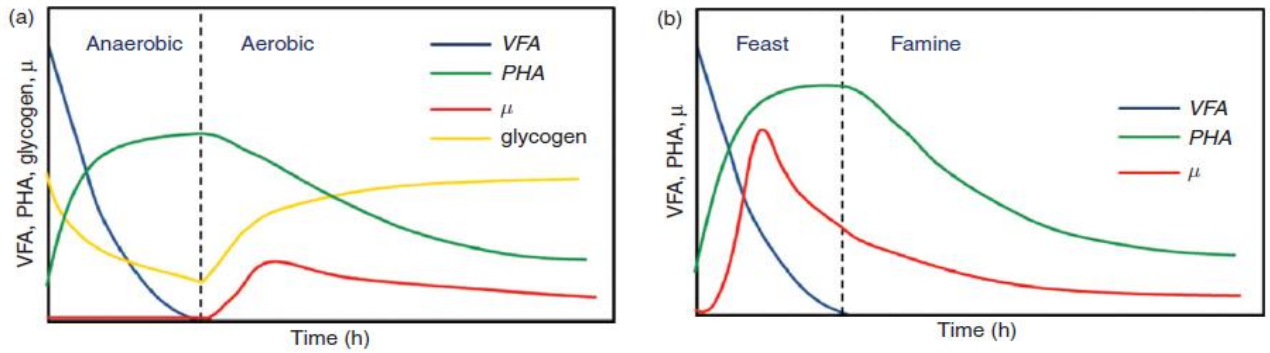
The first time there was observed PHA accumulation using MMC was by polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) through anaerobic and aerobic cycles(Reis et al., 2003; Serafim et al., 2008). In the anaerobic phase carbon is consumed by PAOs that release phosphates as energy source for PHA accumulation.

Meanwhile GAOs gain energy by degrading the internal glycogen to convert the VFAs into PHA. In the aerobic phase, microorganisms use PHA as energy source to replenish glycogen and polyphosphates and also for their growth and maintenance. The change between these phases creates a driving force in bacteria for the storage of the alternative energy source (Dai et al., 2007; Reis et al., 2003; Serafim et al., 2008). Although it is possible to accumulate PHA from these organisms, they present low productivities (Dai et al., 2007).

The other strategy for PHA accumulation, and the most used, is to subject MMCs to two different phases of carbon supply, known as feast and famine cycles, under aerobic conditions. In the feast phase the microorganisms are supplemented with excess external carbon, promoting the storage of the polymer. The famine phase is reached when the culture has already consumed all the substrate supplied and starts to consume the accumulated PHA for its growth and maintenance (Figure 4) (Albuquerque et al., 2007; Dias et al., 2006; Serafim et al., 2004). This selective pressure allow the microorganisms capable of storing internal reserves become dominant in the system, (Majone et al., 1996).

Comparing both regimes, it is verified that the cell growth rate and the PHA productivity is much higher when feast and famine regime is used (Figure 1.4b) (Reis et al., 2011).

Figure 1.4: Mechanisms for PHA storage by mixed microbial cultures under (a) anaerobic/aerobic cycles and (b) fully aerobic feast and famine cycles. PHA,



Polyhydroxyalkanoate; VFA, Volatile fatty acid (Reis et al., 2011)

The type of reactor used for culture selection is usually a sequencing batch reactor that is ideally suited to be operating continuously under the feast and famine regime. The cycles feast and famine are composed of 6 phases: (1) Fill, where it is added fresh medium to the reactor under controlled conditions of agitation and agitation, and corresponds to the beginning of the feast phase; (2) React, where reactions occur, in this case corresponds to the consumption of all carbon and PHA production (feast) followed by consumption of PHA (famine); (3) Biomass withdrawal consists of the disposal of part of the biomass to set the sludge residence time (SRT) of the reactor; (4) Settle, when agitation and aeration are turned off for biomass to settle; (5) Decant, the last step of a continuous cycle, which discards the supernatant to a controlled volume of the reactor (Jafarinejad, 2017; L. K. Wang & Li, 2009)

1.7. PHA aplications

PHA presents interesting properties and a variable composition which allow them to be used in several industries and applications, such as champoo bottles, kitchenware, and various types of packaging (Taylor et al., 2010).

In medical and pharmaceutical area, several applications for PHA have also been investigated, among which therapeutic effects on memory improvement and learning ability (Zou et al., 2009), drug delivery (Michalak et al., 2017). Moreover, due to their biodegradability and biocompatibility, PHAs can also be applied in tissue engineering or as medical implants (Wu et al., 2017).

Other areas of interest include biofuels or fuel additives, an excellent prespective since can be used MMC and is not require high purified PHA, so an inexpensive option (Gao et al., 2011). Further textile industry through the controllable crystallization of PHA fibers (Wang et al., 2014).

2. Motivation and scope of the thesis

Environmental concern related with the high consumption of conventional plastics led to the development of green alternativessuch as bioplastics (e.q. PHA) (Reddy et al., 2003).

PHAs have similar characteristics to conventional plastics, however the cost of production is higher, essentially because pure cultures requiring sterile conditions and specific substrates are used which become more expensive. In this way the hypothesis of PHA production with mixed cultures appeared, that not require sterile conditions and can use agroindustrial residues as substrate (Dias et al., 2006; Reis et al., 2003).

As the population increases, agro-industrial waste becomes increasingly abundant and therefore it is important to reduce them or give them added value. (Ben et al., 2016; M. Reis et al., 2011; Vandamme, 2014). Beer is among the five most consumed beverages worldwide, and about 85% of the waste formed is BSG. BSG is composed of large amounts of fermentable sugars, so it becomes a viable alternative as a substrate for PHA production (Roberto, 2006; Simate et al., 2011).

This research focused on a three-step process for producing PHA. The first step is anaerobic acidogenic fermentation, the second step is areobic performed on an SBR to select the PHA accumulating culture and finally a PHA accumulation step. Acidogenic fermentation was studied in batch assays with BSG hydrolyzate to determine the effect of pH and organic load on this step. The second and third steps were performed based on the most ecofriendly fermentation, derived from a solid acidogenic fermentation of BSG, which aims to confirm the production of PHA from BSG.

In this work the main objectives were (1) to study the effects of different operating conditions (pH and OLR) on acidogenic fermentation of BSG hydrolyzate and (2) to study the PHA production using BSG as substracte.

3. Materials and experimental methods

3.1.Batch assays: Acidogenic Fermentation Stage

Brewer's spent grain: feed preparation

The raw brewer's spent grain used in this batch assays was supplied by UNICER. It was in a wet-form mainly composed by protein, lipids, lignin and carbohydrates.

Firstly BSG was dried at 70ºC during one week in order to reduce the moisture content (Carvalheiro et al., 2004). After the BSG was smashed using a circulating milling system (Dietz-motoren, Mot. Nr. 2893989, 2840 min^{-1} , Emilio de Azevedo Campos) in order to reduce the particle size, which allowed to increase the speed of the chemical reaction (hydrolysis). The feedstock material was then stored in plastic bags with zippers simulating a vacuum storage until required for processing.

Acid Hydrolysis

In order to have a higher sugar extraction were two hydrolyses for the same lot of BSG. Hydrolysis were carried out in schotts of 500 mL where 50g of pretreated BSG was added to 388ml of distilled water and 12ml of sulfuric acid (3% concentration). The schotts were agitated before going to the autoclave where the occurred the hydrolysis at 121 ° C for 20 min.

After hydrolysis, the mixture was centrifuged for 15 min at 10 000 g promoting the separation of the pellet from the supernatant. The pellet was dissolved in distilled water (maintaining the initial volume) and stored in the fridge until the second hydrolysis was carried out. The hydrolyzate was filtered using coffee filters, and then stored at the fridge. The filtrate was analysed in terms of chemical oxygen demand (COD).

The second hydrolysis had a procedure similar to the first hydrolysis. To the the pellet dissolved in water was added the 12mL of sulfuric acid and proceeded with the protocol of the first hydrolysis. Both hydrolysates were mixed and analysed in terms of COD.

Prior to fermentation, pH was adjusted to 7 by addition of CaOH. The precipitate was removed by centrifugation (10 000g, 15min) and the COD content analysed. Before feeding the reactor the pH is adjusted according to the pH used in the batch assay (6, 5.5 and 5) by HCl addition.

Batch assays conditions

Acidogenic fermentation of brewer spent grains was performed in a 500 ml reactor inoculated with 150mL anaerobic granules supplied by Unicer.

The operating conditions tested were organic load of 30 (was tested only at pH 5.0) and 50gCOD L^{-1} and pH of 6.0; 5.5; 5.0. All the assays were performed at 30ºC. These consitions were selected based on previous work developed in our laboratories.

The pH was controlled by addition 1M HCl and 1M NaOH.The fermentation assays lasted 142h and were monitored through periodic sampling.

The different tests were analyzed in terms of specific substrate consumption rate, specific FP production rate, degree of acidification, yield of FPs, VFAs composition and gaseous composition. For this, triplicate assays were performed and the values analyzed were average of the three tests.

3.2.Sequencing Batch reactor (SBR) and Accumulations assays

Feedstock material

The selection reactor was fed with a VFA-rich effluent from a solid anaerobic digestion reactor that was already operating in our laboratories under the scope of another project. The selection reactor was operated under two operational conditions: a HRT of 41 days, corresponding an OLR of 6.1 gCOD/l.d and a SRT of 41 days (Effluent 1), and a HRT of 16 days, which corresponded to an OLR of 21.2 gCOD/l.d and a SRT of 16 days (Effluent 2). The reactor was operated during 94 days without pH control. The effluent was stored in the freezer until it was used. The mineral nutrients ammonia and phosphate were present in the fermented and therefore no supplement was necessary. The C:N:P ratio varies slightly when the operational contitions were changed (Table 3.1).

Table 3.1: Average of C:N:P ratio in both effluents used in SBR

Effluent	C:N:P ratio
1	100 : 10.0 ± 0.7 : 1,9 ± 0.2
2	100 : 6.7 ± 0.7 : 2.4 ± 0.5

SBR operation

The reactor was inoculated with sludge from a WWTP. The selection of PHA accumulating microorganisms was conducted in a SBR with a working volume of 2L at room temperature and operated under feast and famine regime (Duque et al., 2014).

The operating conditions were 1 day of HRT, 12h of cycle length, SRT of 4 days (was kept by imposing a purge of 250 mL in each cycle) and an OLR of 41.3 ± 8.9, 78.5 ± 8.3 and 118.6 ± 13.5 C-mmol/L/Day (fermented effluent from an anerobic solid waste (BSG) fermentation reactor was diluated with tap water and allythiourea was also added (10mg/L) to avoid nitrification). pH was controlled to 8 through the automatic addition of 0.5 M HCl. Air was supplied with a flow between 1.5 and 2 L/min. Stirring was kept at 350 rpm. The SBR food to microorganisms ratio was 12.4 ± 3.9 C-mmol/gVSS.

The 12-hour cycles in the SBR consisted of six distinct periods: filling (5 min); aerobic reaction (feast and famine) (11.25 h); Biomass withdrawal (1min); settle (30min); decant (9 min) (Duque et al., 2014).

Dissolved oxygen (DO) and pH data were acquired by software, which allowed online monitoring of feast phase length. The SBR performance was monitored through routine sampling along the cycles.

The performance of the selected cultures was monitored over time in terms of fermentation products consumption rate (qS, C-mol-FP/ C-mol X.h); PHA storage rate (qPHA, C-mol-PHA/ C-mol X.h); storage yield (YPHA); growth rate (qx, C-mol-X/ C-mol X.h); growth yield (Yx); and Feast and Famine ratio (F/F ratio).

Accumulations assays conditions

The PHA accumulation assays were performed in the same reactor as the culture selection, but working at a volume of 1L and inoculated with an enriched SBR culture. At the end of the last phase of the cycle prior to the accumulation assay the biomass, corresponding to 4 purges, was withdrawn from the reactor (to ensure that it was in the endogenous state) and the SBR food to microorganisms ratio was maintained (Duque et al., 2014).

Prior to assays, the pH of the feed was adjusted to 7.5 by adding NaOH in order to avoid a pH decrease after the pulse feeding. Assays were carried out without pH control and at room temperature (16º - 24º)and the pH accumulation was carried out by feeding concentrated pulses of BSG fermented. The DO concentration was measured online, and when DO begins to stabilize, indicating that the carbon source was ending, a new pulse was added. This procedure was repeated until the culture reaches a state of saturation.

3.3.Analytical methods

Total suspend solids and Volatile suspend solids

Total and volatile suspend solids (TSS and VSS, respectively) were determined according to the standards methods (APHA, 1998).

Firstly, the glass-fiber filters disk (VWR, glass fiber 1,2µm, 47mm) was washed with distilled water and dried at 550°C for at least 30 minutes to remove volatile compounds. Each filter and the respective aluminum crucible were weighed, corresponding to the weight 0 (w0). Then, 2 mL of reactor sample (V) were filtrated and dried at 105°C overnight. After drying, the filter was remove from the oven to cool in a desiccator to room temperature and then weighted (w1). Then, the filter was dried at 550ºC during 2 hours and after cooling, the filter was weighted again (w2).

The TSS and VSS were determined with the following equations:

$$\mathrm{TSS}\left(\frac{\mathrm{g}}{\mathrm{L}}\right) = \frac{\mathrm{w1-w0}}{\mathrm{V}} * 1000 \qquad 1$$

$$VSS\left(\frac{g}{L}\right) = \frac{\mathrm{w1} - \mathrm{w2}}{V} * 1000 \qquad 2$$

Organic acids and ethanol analysis

The organic acids (Lactic, acetic, propionic, isobutyric, butyric, isovaleric and valeric acid) and ethanol concentration of filtered samples (Whatman® membrane filters nylon pore size 0.2 µm) was determined by high-performance liquid chromatography (HPLC) using VWR Hitachi Chromaster chromatographer with an RI detector, a Biorad Aminex HPX-87H column (300 x 7.8 mm) and a Biorad pre-column (125-0129 30 x 4.6 mm). The analysis was performed using sulphuric acid 0.01N as eluent at a 0.5 mL min^{-1} flow rate and 30ºC. The organic acids concentrations were calculated through calibration curves in the range of 3.9 to 1000 mg/L.

Protein analysis

Protein quantification was determined by the Lowry method (Lowry et al., 1951). This is a colorimetric method based on two main reactions: reaction of protein with copper in alkaline solution and the reduction of the phosphomolybdic-phosphotungstic reagent by the copper-treated protein.

The reagents used were: solution A (10 g Na_2CO_3 + 0.1 g $C_4H_4KNaO_6.4H_2O$ + 500 mL NaOH 0.1 M); solution B (0.5 g $CuSO_4.5H_2O$ + 1 drop H_2SO_4 + 100 mL H_2O); solution C (solution A + solution B in a proportion of 50:1); solution D (Folin 50% (v/v)). A bovine serum albumin (BSA) solution was used as standard in the range of 3 – 200 mg L^{-1}.

Firstly, 3 mL of solution C was added to 1 mL of the diluted samples, mixed using the vortex and incubated in the dark for 10 minutes at room temperature. After that, 300 µL of solution D was added, mixed in the vortex and incubated in the dark for 30 minutes at room temperature. Finally, the absorbance was measured at 750 nm in the spectrophotometer (Hach Lange DR 2800).

Sugar analysis

Sugar quantification was determined by the Anthrone method (Morris method with modifications by Koehler, Baily and Gaudy), a colorimetric method based in dehydration of carbohydrates with concentrated sulfuric acid which condenses with anthrone to form a yellow/green color complex. A glucose solution was used as standard (3 – 100 mg L-1).

All samples were diluted according their range of sugar content, and then 2mL of anthrone solution (0.125 g anthrone in 100 mL sulphuric acid) was added to 1mL of the diluted samples. The samples were mixed in a vortex. Then, the samples were digested at 100ºC during 14min and absorbance was measured at 625 nm in the spectrophotometer (Hach Lange DR 2800).

Phosphate and ammonium analysis

Ammonia and phosphate concentrations were determined by a colorimetric method implemented in a continuous flow analyser (Skalar San ++, Skalar Analytical, The Netherlands). Samples preparation included dilution with miliQ water in order to maintain the concentration of P and N in the range of the calibration curve of the equipament (4-20 mgN/L and 4-20 mgP/L) and filtration (Whatman® membrane filters nylon pore size 0.45 µm).

Chemical Oxygen Demand (COD)

The Chemical Oxygen Demand (COD) measurement allows the quantification of the amount of organic matter present in the sample. COD concentration was measured by a colorimetric method using *Hach Lange* kit LCK 914 (5 – 60 g L^{-1} O_2). Samples were digested (Hach Lange HT 200S) at 170ºC for 15 minutes. Finally, after cooling, COD concentration was measured using a spectrophotometer (Hach Lange DR 2800).

Gas analysis

Gas composition was analysed in a gas chromatography (GC) to evaluate CH_4, CO_2, O_2, N_2 and H_2 content. The GC (Agilent technologies, 7890B, GC system) was equipped with TCD detector and 25 meters of Agilent J&W PoraBOND Q PT column The mobile phase was argon with 10mL/min of flow rate with temperature programed runs during 7.4 min at 40º. The injector temperature was 150º.

PHA determination

Polyhydroxyalkanoates determination was performed according to Duque et al., 2014 . The samples were centrifuged at 11 000g during 3min, the supernatant was discarded and the pellet was lyophilized in a *CoolSafe* freeze-drier (ScanVac) overnight. The pellets were weighted (3–8mg) and 1 mL of acidic methanol (20% sulfuric acid) and 1 mL of chloroform were added. The samples were incubated in a thermoblock at 100°C for 3.5 hours and after digestion were cooled down in water and ice. To separate the organic from inorganic phase, 1 mL of distilled water was added and then mixed for 30 seconds. The organic phase that contains the PHA was extracted and analysed.

The sampls were injected (2 µL) and analyzed in a gas chromatograph with a Flame Ionization Detector (Gas Chromatograph 430-GC, Bruker), equipped with a Restek column (60 m, 0.53 mm internal diameter, 1 µm film thickness, Crossbond, Stabilwax). The carrier gas was helium, at a flow rate of 1 mL/min.

The standard used for PHB and PHV quantification was a copolymer of P(HB/HV) (88%/12% mol) (Sigma). Standards were also digested and extracted as samples. A calibration curve for HB and other for HV was used to determinate the concentration of hydroxybutyrate (HB) and Hydroxyvalerate (HV), respectively.

Nile Blue staining method

Nile blue staining was performed in one sample of each monitoring or accumulation assays. Briefly, 20 µL of nile blue were added to 1.5 mL of sample in the eppendorf. The sample was incubated at 55 °C for 10 minutes. After that time, the slides were prepared with 20 µL of sample and observed at the microscope (Olympus BX51 epifluorescence) under fluorescent light, which allows to observe accumulated PHA granules.

3.4.Calculations

Batch assays: Acidogenic Fermentation Stage

The volumetric substrate uptake rate (-rS in gCOD-S/L.h) and volumetric fermentation products (FP) production rate (rFP in gCOD/L.h) were determined by linear regression of substrate and FP concentrations, converted to COD units, plotted over time, respectively.

The specific substrate uptake rate (*-qS* in gCOD-S/gVSS.h) and FP production rate (qFP in gCOD-FP/gVSS.h) were determined by dividing –rS and rFP for the concentration of biomass (gVSS/L), respectively.

$$-qS = \frac{-rS}{[Biomass]} \qquad (3)$$

$$qFP = \frac{rFP}{[Biomass]} \qquad (4)$$

The degree of acidification (DA in % (gCOD-FP/gCOD-S)) was calculated by the sum of total FP produced ($\sum[FP]$) divided by the total COD concentration in the substrate (COD_{in}).

$$DA = \frac{\sum[FP]}{\mathrm{CODin}} \qquad (5)$$

The FPs yield (Y_{FP} in gCOD-FP/gCOD-S) was calculated by fraction the total FPs (TFP) in relation to the total substrate consumed ($TS_{in} - TS_{out}$).

$$Y_{FP} = \frac{TFP}{\mathrm{TSin} - \mathrm{TSout}} \qquad (6)$$

Sugar removal (%) was determined by the variation of concentration of sugar inlet ($[S]_{in}$) and sugar in the end of the reactor ($[S]_{out}$) divided by concentration of sugar inlet.

$$Sugar\ removal = \frac{[S]in - [S]out}{[\mathrm{S}]\mathrm{in}} * 100 \qquad (7)$$

Fermentation products profile (in %(gCOD-HA/gCOD-FP)) was obtained by dividing of each hydroxyl acid (HA) produced by total FP concentration.

$$\%HA = \frac{[HA]}{[\mathrm{FP}]} * 100 \qquad (8)$$

Gas profile (in %mol) was obtained by dividing of each gas produced by total gas concentration(Tgas). Where x represents CH_4, CO_2, O_2, N_2 or H_2.

$$\%gas = \frac{x}{\text{Tgas}} * 100 \qquad (9)$$

Sequencing Batch reactor (SBR) and Accumulations assays

The specific substrate uptake rate (*-qS* in C-mol-FP/C-mol-X.h), PHA storage rate (qPHA in C-mol-PHA/C-mol-X.h) and growth rate (qX in C-mol-X/C-mol-X.h) were determined by linear regression of substrate, PHA and active biomass (X) specific concentrations plotted over time, respectively.

The PHA content (PHA (%) in %(gPHA/gVSS)) was determined as percentage of VSS on mass basis, where PHA corresponds to sum of PHB and PHV.

$$PHA\ (\%) = \frac{PHA}{VSS} * 100 \qquad (10)$$

In SBR, active biomass (X) was estimated through equation 11 and based on ammonia uptake curve (equation 12), where mVSS corresponds to measured VSS, %VSS to the percentage of VSS in TSS, $NH_{4,n}$ e NH_{4n-1} are the concentration of ammonium (in Nmol /L); n and n-1 correspond to the period of sample and the period of the previous sample, respectively. In accumulations assays, active biomass was estimated through equation 11. To determine X in Cmol a general biomass chemical formula of $C_5H_7NO_2$ was considered.

$$X = mVSS * \frac{1 - \frac{PHA(\%)}{100}}{\%VSS} \qquad (11)$$

$$X_n = X_{n-1} + \left(NH_{4,n-1} - NH_{4,n}\right) * 5 \qquad (12)$$

The PHA storage yield and growth yield per substrate consumed ($Y_{PHA/FP}$, C-mol-PHA/C-mol-FP and $Y_{X/FP}$, C-mol-X/C-mol-FP) were determined by division of PHA storage rate and growth rate by the specific substrate uptake rate, respectively.

$$Y_{PHA/FP} = \frac{qPHA}{qS} \qquad (13)$$

$$Y_{X/FP} = \frac{qX}{qS} \qquad (14)$$

The percentage of HB and HV precursors were determinated as the ratio between the sum of HB and HV precursors concentration and the sum of total FP concentration.

$$\%Precusors\ HB = \frac{\sum([HLac]\{HAc][HBut][HIsobut])}{\sum[FP]} \qquad (15)$$

$$\%Precusors\ HV = \frac{\sum([HProp][HVal][HIsoval])}{\sum[FP]} \qquad (16)$$

The Feast/Famine ratio (Feast/Famine, h/h) was determinated by division between the length of feast divided and the length of the famine phase in SBR cycle.

4. Results and discussion

4.1. Batch assays: Acidogenic Fermentation Stage

In order to study the effect of the operational conditions on the reactor's performance, namely on the VFAs production, different OLR (30 and 50 gCOD/L) and pH (5; 5.5; 6) were tested in 142h batch assays.

Allong all the assays, it was observed the consumption of sugars derived from the hydrolysis of BSG and their conversion to fermentation products (FP) (Figure 4.1). The most abundant sugar present in the BSG hydrolysed was xylose, followed by arabinose and glucose. Although glucose was the sugar present in lower content, it was the first to be consumed, and the microorganisms only converted the other sugars when glucose was practically consumed. In fact, Silva et al., 2004 reported that when xylose and glucose were used as the carbon source, a preference for glucose was observed. This is true for many of the microorganisms studied, and the presence of glucose prevents the use of another carbon source through a catabolic repression of carbon, where the expression of enzymes for the use of a secondary carbon source such as other sugar is reduced by the presence of glucose (Gorke et al., 2008). Regarding the FP, in all assays, butyric acid was the most produced followed by acetic acid.

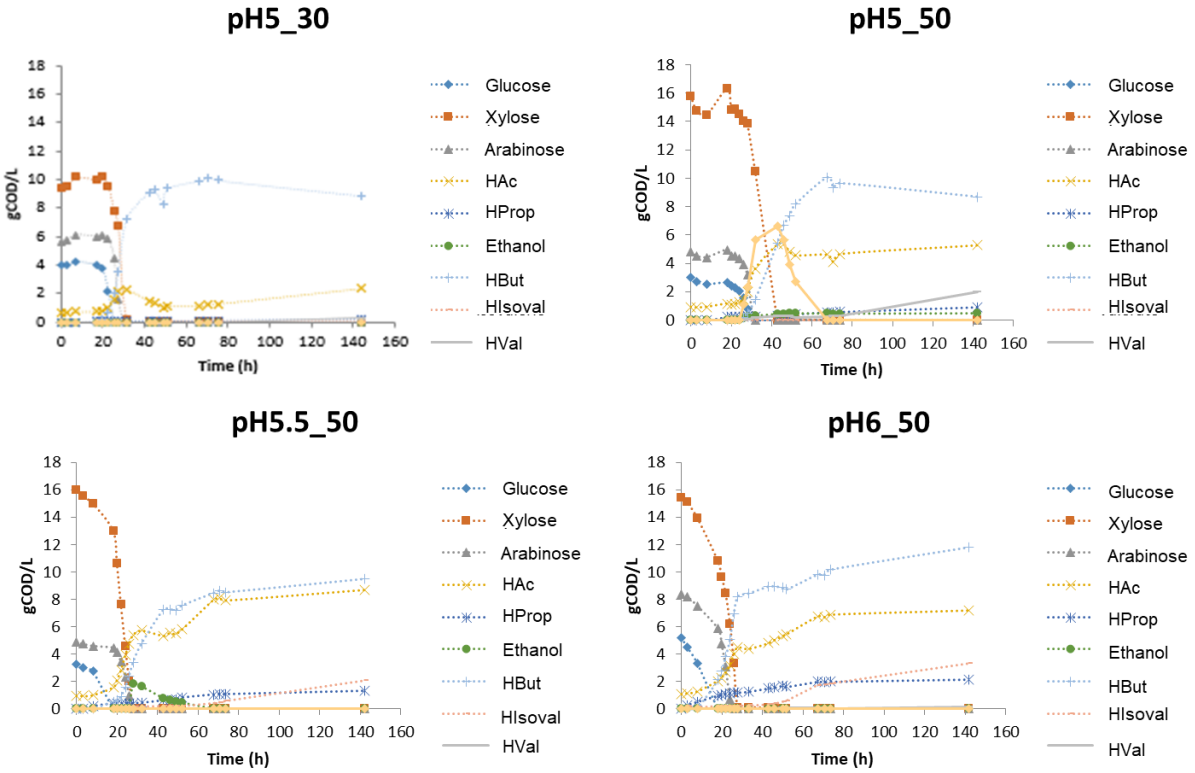


Figure 4.1: Consumption of sugars and production of FPs over time of different batch assays

The OLR applied to the microorganisms influences the production of VFAs, and it is known that high OLR (48gCOD/L) favor the acidogenesis and the accumulation of organic acids (Begum et al., 2017). For this reason, in this study an lower OLR of 30 gCOD/L and higher OLR of 50 gCOD/L at pH 5 were tested. However, it is important determined the maximum OLR that is not inhibitory for the production of VFAs.

Comparing the rate of substrate consumption and VFA production, was observed a slightly decrease when the organic load was increased from 30 gCOD/L to 50 gCOD/L at pH 5 (Table 4.1). In both conditions, butyric and acetic acid were the most produced representing about 78 – 89 %of total FP. This result is in accordance with Jiang et al., (2013), who have studied the effects of organic loading rate (OLR) on VFAs using food waste as substrate ,in which 60% were butyric and acetic acid in all studied OLRs. However with the OLR increase, the content of acetic, isovaleric and valeric acid increased (34, 77 and 45% respectively), while the content of butyric acid decreased (25%) (Table 4.2). Once again similar results were obteined by Jiang et al. (2013), using food waste, whereas a higher OLR producer a increased percentage of acetic and valeric acid and a decreased of butyric acid. Regarding the degree of acidification and yield of FPs similar results were obtained for both conditions, reaching a degree of 41.1 ± 1.68 and 39.6 ± 6.17 % and a yield of 0.65 ± 0.05 and 0.64 ± 0.07 gCOD FPs/gCOD S for an OLR of 30 and 50 gCOD/L, respectively (Table 4.1). For this reason, it can be assumed that with an OLR of 50 gCOD / L there was no inhibition of FPs production, but also did not improve the acidogenesis.

Considering gas production, there was a decrease in the H_2 and CO_2 percentage and an increase in the CH_4 percentage when the OLR was increased. Ren et al., 2006 showed that with the increase of OLR using molasses as subtract, there is an increase in H_2 production, which is the opposite of our case. However, Ren et al., 2006, found that when OLR is further increased (approximately 86gCOD/L), accumulation of PF inhibits H_2 and CO_2 production. As molasses has a different composition of BSG, increased OLR from 30 to 50 gCOD/L using BSG as substrate may be inhibitory in the production of both gases. In addition, decreasing CO_2 and increasing CH_4 in biogas means that acidifying microorganisms are decreasing and that methanogens are increasing, which can lead to decreased VFAs accumulation (Babaee et al., 2011) which may explain the slight decrease of DA and FP yield.

pH is one of the most important parameters in the production of VFAs, being responsible for the choice of active microorganisms in a mixed culture. At a lower pH the formation of VFAS is favored and at pH between 6 and 8, the most favored microorganisms are methanogenic (Begum et al., 2017). Moreover, the VFA produced varies according to the pH used (Tamis et al., 2015). For this reason, at higher OLR different pHs were tested in order to assess the pH influence on the production of VFAs.

Comparing the assays at pH 5.5 and pH 5, the substrate consumption rate increased from 0.112 gCOD/(gVSS.h) to 0.121 gCOD/(gVSS.h) and the FP production rate decreased about 50% from 0.093 to 0.059 gCOD/(gVSS.h) (table 4.1). Although the rate of FP production was lower, the degree of acidification and FPs yield were higher, reaching 53% and 0.89 gCOD-FP/gCOD-s, respectively. At pH 6 both rates, consumption and substrate uptake increased relative to pH

5.5, however the yield was lower. At the same time, there was an increase in the content of methane to about 26% (Table 4.3), which may indicate that, at pH 6, methanogenic microorganisms may have started to be favored, explaining the small decrease in the yield of VFAs. Albuquerque et al. (2007) who have studied the effect of pH (5, 6 and 7) on acidogenic fermentation using sugarcane molasses concluded that under more acidic pH, higher yiels were obtained. In our study higher FP yiels were obtained at pH 5.5 instead pH 5 as Albuquerque et al. (2007), which may be due to the use of anaerobic granules instead of sludge or due to the use of a different substrate, whose conversion to VFAs can be potentiated at different pHs.

As the pH was changed, the composition of FPs also changed, observing an increase of 46%, 44& and 6% in acetic, propionic and isovaleric acid, respectively and a decrease of 20% and 55% in butyric and valeric acid, respectively. The effect of pH on VFA concentration profiles had a similar trend as observed by Albuquerque et al., 2007. The impact of pH on VFA composition becomes an important factor to be studied when VFAs are used as precursors of PHA, since the composition and final characteristics of the biopolymer depend on the initial composition of the VFAs used (Albuquerque et al., 2007; Gouveia et al., 2016).

Regarding sugar removal, the assay with lower OLR, achieved a removal of 93%, while the assays at 50 gCOD/(L.d) achieved an average removal of 97.7 ± 0.41%, showing that sugar removal was independent of pH. Despite this small diference, it was observed an efficient consumption of sugars in all conditions tested.

It was observed that the increase on the OLR from 30 to 50 gCOD/L didn't improve stoichiometric and kinetic parameters. Since the main objective is to achieve the highest degree of acidification and FPs yield, pH 5.5 was considered to be the most effective.

Table 4.1: General performance of different batch assays. The values in parentheses represent the standard deviations.

	Specific substrate uptake rate (-qS)	Specific FP production rate (q FP)	Degree of acidification	FPs yield	Sugar removal
	(g COD/g VSS.h)	(g COD/g VSS.h)	% (g COD-FP/g CODin)	g-COD-FP/g-COD S	%
pH5_30	-0.177 (0.12)	0.129 (0.11)	41.1 (1.68)	0.65 (0.05)	93.2 (2.92)
pH5_50	-0.112 (0.22)	0.093 (0.22)	39.6 (6.17)	0.64 (0.07)	97.8 1.04)
pH5.5_50	-0.121 (0.17)	0.059 (0.24)	53.3 (2.08)	0.89 (0.07)	97.2 (1.94)
pH6_50	-0.13 (0.44)	0.079 (0.27)	60.2 (4.79)	0.87 (0.09)	98.2 (1.23)

Table 4.2: Organic acids concentration profile of the different conditions tested

	% FPs (gCOD/L)						
	HLac	HAc	HProp	Ethanol	HBut	HIsoval	HVal
pH5_30	0.0	18.7	2.5	0.3	70.5	6.3	1.6
pH5_50	0.0	25.1	5.4	3.3	52.7	11.1	2.4
pH5.5_50	0.0	28.9	6.8	0.0	53.2	10.0	1.1
pH6_50	0.0	36.6	7.8	0.0	42.8	11.9	1.1

Table 4.3: Gas composition profile of the different conditions tested

	%mol				
	H_2	O_2	N_2	CH_4	CO_2
pH5_30	41.9	0.0	3.6	0.7	53.8
pH5_50	0.2	8.6	58.4	6.0	26.7
pH5.5_50	2.9	3.4	20.6	13.0	60.0
pH6_50	0.3	4.6	24.0	26.2	44.9

4.2. Sequencing Batch reactor (SBR) and Accumulations assays

4.2.1. SBR

The main objective of this reactor was to produce PHA through the fermented BSG.

As mentioned above, in chapter 3, the selection reactor was operated with a VFA-rich effluent from an anerobic solid waste fermentation reactor. The anaerobic reactor was operated during 94 days under two conditions: HRT/SRT of 40.7 days and an OLR of 6.1 gCOD/l.d and HRT/SRT of 16 days and an OLR of 21.2 gCOD/l.d, producing two different effluents, Eff_1 and Eff_2, respectively (Figure 4.2 and table 1 in appendix 1). The first effluent showed a variation in the composition, which was related to the acclimatization of the fermentation reactor. After 24 days, the effluent presented a stable composition with proprionic, acetic and butyric acids as the major compounds present (39.9±2.7%, 28.7±2.1% and 19.9±1.0%, respectively).

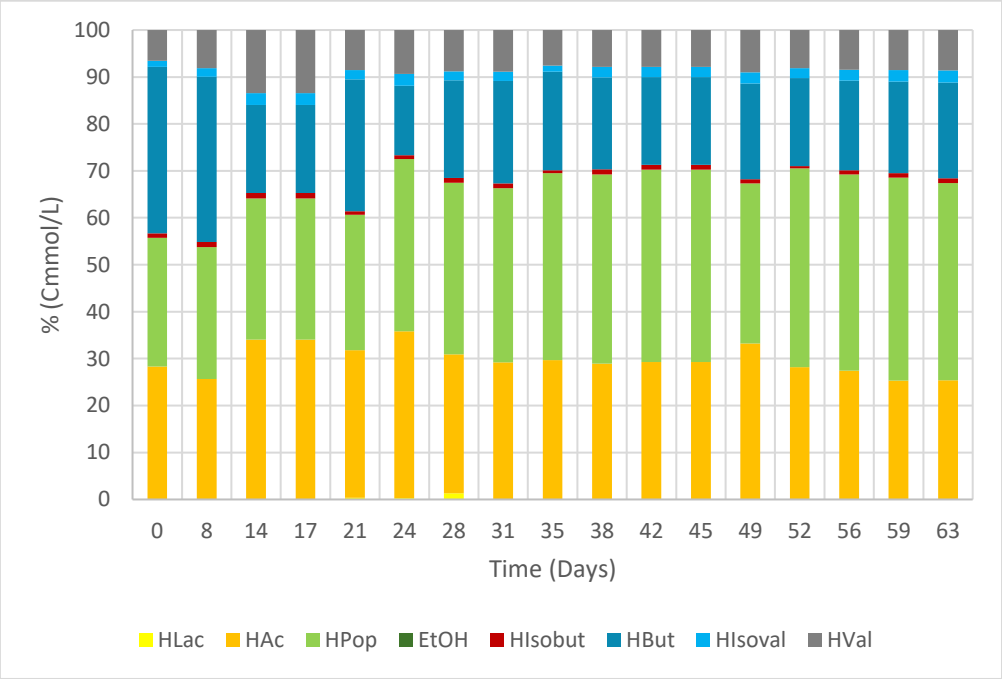


Figure 4.2: Composition of organic acids and ethanol in the effluent in percentage over time.

In the SBR start-up, a lower OLR (41 ± 8.9 C-mmol/L/Day) was applied in order to avoid carbon inhibition. After 20 days, the OLR was increased to 79 ± 8.3 C-mmol/L/Day and after day 52 was increased to 119 ± 13.5 C-mmol/L/Day.

With the first OLR the reactor took about 8 days to acclimatise reaching an F / F ratio which varies between 0.07 - 0.08. When the OLR was double there was an instability in the reactor and an acclimatization time of approximately 8 days was required (day 20 to 28). This can be observed through the variation of F/F ratio, which increases from 0.07 h/h to values between 0.22 - 0.34

h/h when the OLR changes occurs. This is because when OLR increases, it takes longer to consume the substrate, increasing the feast time and in turn the F/F ratio. After the days of acclimatization, even with the effluent change, the biomass remains relatively stable presenting an F/F ratio between 0.08 and 0.15 h / h. It is important to maintain the low F / F ratio (fast feast and long famine) to ensure that there is a long famine phase, in order to promote the selection of PHA accumulating microorganisms (Reis et al., 2011; Wang et al., 2017). In order to assure a stable system, the F/F ratio should not exceed 0.25 h / h (Hao et al., 2018). Between day 20 and 28 that corresponds to days that increased F/F ratio, a lower YPHA was observed (Figure 4.5).

After day 28 substrate concentration was approximately 42 Cmmol/L and the storage yield was about 0.54 Cmol-PHA/Cmol-FP and with time increased to 0.73 Cmol-PHA/Cmol-FP. Albuquerque et al., 2010, obtained identical results (0.70 Cmol-PHA/Cmol-FP), using sugar molasses as substrate and with influent concentration of 47 Cmmol/L. The authors also reported a decrease on the storage yield (0.52 Cmol-PHA/Cmol-FP), when the substrat concentration was higher (60 Cmmol/L), while in this study, when the substrate concentration increased to 59.3 ± 6.8 Cmmol/L, the storage yield also increased to 0.91±0.24 Cmol-PHA/Cmol-FP. This difference can be due to the limitation of micronutrients such as magnesium or phosphate by Albuquerque et al., 2010, making the feast phase selective not only by the carbon source but also by micronutrients. Thus it was reduced the selection capacity of PHA accumulating organisms and increased the pressure for the growth of microorganisms. Further it prevented PHA accumulators from using these nutrients to grow during the famine phase by decreasing competitive advantage. In our study phosphate concentration was higher and therefore was not a limiting micronutrient, which may explain high storage yield obtained.

The substrate consumption rate changed throughout the time. Initially, a -qS about 0.47 ± 0.12 C-mol-FP / C-mol X.h was obtained. With the OLR increase, the rate of substrate consumption decreased to approximately 0.29 ± 0.13 C-mol-FP / C-mol X.h. After the second OLR increase, an average value of 0.30 ± 0.04 C-mol-FP / C-mol X.h was obtained. Since the -qS varied, the PHA storage rate also changed. Initially, PHA storage rate varied between 0.1 and 0.4 C-mmol PHA / C-mmol X.h (average value of 0.32 ± 0.13 C-mmol PHA / C-mmol X), which may be due to the fact that the organisms were not completely selected and of the substrate was consumed by other organisms rather than PHA accumulating organisms. When the OLR changes, a decrease in storage capacity was observed, reaching a rate of 0.22 ± 0.13 C-mmol PHA / C-mmol X.h. However, after the second OLR increase 0.33±0.08 C-mmol PHA / C-mmol X.h was the PHA storage rate. Ben et al. (2016) noted that specific substrate uptake rate and polymer production rate increased on increasing the substrate concentration using brewery wastewater as substrate. However, in this study, this only happens when increasing the substrate concentration of 40 to 60 Cmmol/L. When increasing from 20 to 40 Cmmol/L there was no increase, probably due to the instability of the microorganism culture.

The storage can be visually checked through images obtained by microscopy using Nile Blue staining method in figure 4.6. Through the images we can corroborate the storage yiel values

obtained and verify that there was higher accumulation of PHA in the reactor after the first change of OLR and after day 28.

In terms of consumption of organic acids, it was observed that propionic acid was the faster acid to be consumed, followed by acetic and butyric acid. The isovaleric and isobutyric acid were only consumed when the remaining acids were practically consumed, which indicates that the microorganisms have preference for the other acids comparing to isovaleric and isobutyric acid.

The composition of the final polymer varies according to the composition of the substrate supplied (Albuquerque et al., 2007). Considering the composition of the feed (appendix 1), and assuming that lactic, acetic, butyric and isobutiric acidare precursors of HB and propionic, isovaleric and valeric acids are precursors of HV (Duque et al., 2014; Lemos et al. , 2006; Serafim et al. , 2004), it can be confirmed that in most instances a substrate having a 1:1 molar ratio composition of HB and HV precursors, resulting in a polymer composed of about 50% HB monomer and 50% HV monomer (table 4.5). This correlation has previously been shown by Beccari et al, 1998.

In terms og growth yield was obtained 0.16 ± 0.08, 0.06± 0.05 and 0.03 ± 0.02 Cmol-X/Cmol-FP for an initial substrate concentration of 20.7 ± 5.1, 39.3 ± 4.2 and 59.3 ± 6.8 C-mmol/L. This suggests that low substrate concentrations favor cell growth. In addition, Eff_1 had more ammonia than Eff_2 (table 3.1), which also contributes to decreased cellular growth. Ben et al. (2016) studied the effect of substrate concentration on PHA production using brewery wastewater as substrate and observed that when the substrate concentration increases from 57 to 109 Cmmol/L decreases the growth yield 0.1 to 0.06 Cmmol-X/Cmmol-FP.

Table 4.4: Fermentation products consumption rate (-qS); PHA storage rate (qPHA); Storage yield (YPHA); Growth rate (qx); Growth yield (Yx); and Feast and Famine ratio (F/F ratio) over the time of PHA enrichment culture selection in the SBR. The double line represents the OLR change. The blue zone represents the use of eff_1 and the pink zone using Eff_2. The values in parentheses represent the standard deviations.

Monitoring	Time	OLR (average)	Feast/Famine	FP consumption rate (-qS) (Cmmol-FP/(C-mmol-X . h)								PHA storage rate (qPHA)	YPHA	qX	YX
	(Days)	Cmmol/L.day	11.3 (h)	HLac	HAc	HProp	HIsobut	HBut	HIsoval	HVal	Total FP	(Cmmol-PHA / (C-mmol-X . h)	Cmol-PHA/Cmol-FP	(Cmmol-X / C-mmol-X . h)	Cmol-X/Cmol-FP
0	0		0.000												
1	8		0.088	0.0000	-0.1604 (0.013)	-0.1895 (0.032)	-0.0052 (0.002)	-0.2957 (0.066)	-0.0053 (0.002)	-0.0808 (0.012)	-0.5681 (0.074)	0.3904 (0.053)	0.5297 (0.099)	0.0239 (0.003)	0.0421 (0.008)
2	14	41.3 (8.9)	0.068	0.0000	-0.1750 (0.087)	-0.2149 (0.089)	-0.0084 (0.006)	-0.1198 (0.076)	-0.0089 (0.005)	-0.0991 (0.059)	-0.5326 (0.214)	0.4649 (0.128)	0.743 (0.361)	0.0905 (0.033)	0.1700 (0.092)
3	17		0.071	0.0000	-0.1039 (0.004)	-0.1152 (0.030)	-0.0048 (0.006)	-0.0678 (0.046)	-0.0051 (0.001)	-0.0561 (0.025)	-0.2979 (0.079)	0.4112 (0.133)	1.1654 (0.486)	0.0761 (0.019)	0.2553 (0.093)
4	21		0.215	0.0000	-0.0506 (0.031)	-0.0549 (0.030)	-0.0012 (0.001)	-0.0389 (0.009)	-0.0019 (0.001)	-0.0194 (0.005)	-0.1351 (0.055)	0.1036 (0.020)	0.621 (0.278)	0.0155 (0.002)	0.1148 (0.278)
5	24		0.215	0.0000	-0.0762 (0.037)	-0.0692 (0.041)	-0.0013 (0.002)	-0.0386 (0.013)	-0.0025 (0.002)	-0.0241 (0.009)	-0.1919 (0.059)	0.0908 (0.039)	0.429 (0.227)	0.0219 (0.007)	0.1141 (0.052)
6	28		0.343	0.0000	-0.0531 (0.023)	-0.0526 (0.037)	-0.0006 (0.001)	-0.0463 (0.005)	-0.0013 (0.001)	-0.0192 (0.003)	-0.1601 (0.049)	0.0701 (0.044)	0.4052 (0.281)	0.0279 (0.007)	0.1745 (0.069)
7	31	78.5	0.045	0.0000	-0.1400 (0.019)	-0.2227 (0.050)	-0.0049 (0.011)	-0.0952 (0.069)	-0.0062 (0.003)	-0.0673 (0.052)	-0.4299 (0.105)	0.2913 (0.280)	0.5430 (0.538)	0.0081 (0.003)	0.0187 (0.008)
8	35	(8.3)	0.056	0.0000	-0.1638 (0.012)	-0.2781 (0.033)	-0.0015 (0.005)	-0.2135 (0.183)	-0.0034 (0.002)	-0.0770 (0.064)	-0.5035 (0.122)	0.3731 (0.314)	0.5061 (0.444)	0.0120 (0.003)	0.0237 (0.008)
9	38		0.076	0.0000	-0.1553 (0.028)	-0.1959 (0.029)	-0.0057 (0.003)	-0.1702 (0.067)	-0.0058 (0.002)	-0.0328 (0.021)	-0.4374 (0.087)	0.3310 (0.080)	0.5852 (0.183)	0.0136 (0.003)	0.0310 (0.008)
10	42		0.097	0.0000	-0.1179 (0.007)	-0.1458 (0.001)	-0.0017 (0.001)	-0.1036 (0.055)	-0.0042 (0.001)	-0.0242 (0.021)	-0.3201 (0.048)	0.3703 (0.191)	0.9318 (0.501)	0.0092 (0.002)	0.0288 (0.008)
11	45		0.153	0.0000	-0.0914 (0.008)	-0.0947 (0.014)	-0.0020 (0.002)	-0.0765 (0.022)	-0.0043 (0.001)	-0.0349 (0.012)	-0.2193 (0.024)	0.2366 (0.050)	0.7789 (0.186)	0.0080 (0.002)	0.0366 (0.009)
12	49		0.149	0.0000	-0.0709 (0.013)	-0.1181 (0.007)	-0.0015 (0.002)	-0.0700 (0.027)	-0.0038 (0.001)	-0.0336 (0.011)	-0.2129 (0.043)	0.2161 (0.090)	0.7255 (0.336)	0.0065 (0.001)	0.0305 (0.008)
13	52		0.113	0.0000	-0.0483 (0.005)	-0.0772 (0.007)	-0.0010 (0.001)	-0.0580 (0.013)	-0.0025 (0.001)	-0.0246 (0.007)	-0.2404 (0.030)	0.2515 (0.052)	1.1889 (0.352)	0.0086 (0.003)	0.0616 (0.027)
14	56		0.106	0.0000	-0.0867 (0.006)	-0.1559 (0.015)	-0.0003 (0.001)	-0.1141 (0.070)	-0.0047 (0.002)	-0.0571 (0.032)	-0.2934 (0.066)	0.4437 (0.097)	1.0595 (0.331)	0.0074 (0.001)	0.0251 (0.007)
15	59	118.6 (13.5)	0.080	0.0000	-0.0924 (0.009)	-0.1701 (0.023)	-0.0015 (0.002)	-0.1185 (0.063)	-0.0063 (0.002)	-0.0573 (0.031)	-0.3286 (0.066)	0.3674 (0.038)	0.8237 (0.187)	0.0047 (0.001)	0.0144 (0.005)
16	63		0.088	0.0000	-0.0866 (0.003)	-0.1601 (0.012)	-0.0009 (0.002)	-0.1367 (0.024)	-0.0061 (0.002)	-0.0620 (0.011)	-0.3320 (0.050)	0.2501 (0.098)	0.5528 (0.231)	0.0049 (0.001)	0.0147 (0.004)

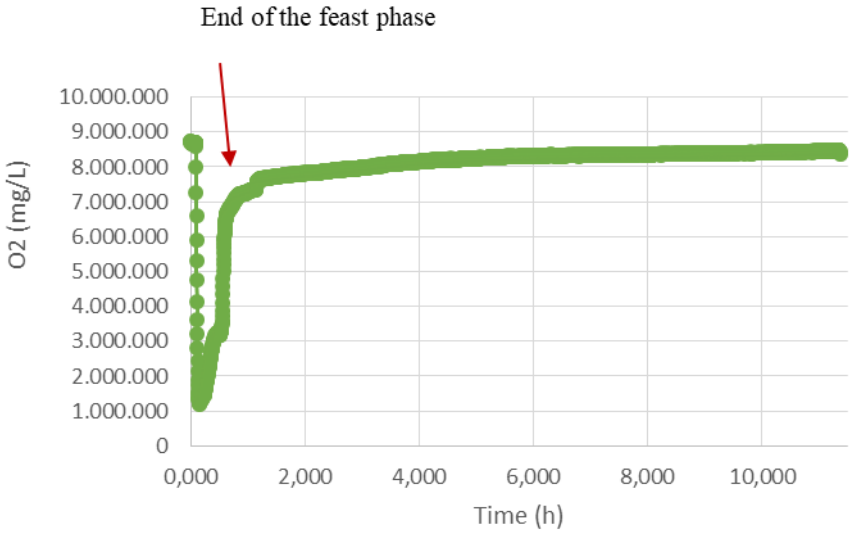


Figure 4.3: Concentration of DO in the SBR. representing the end of the feast phase.

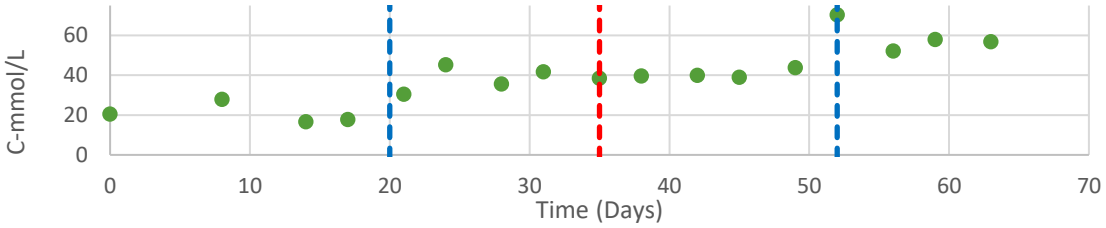


Figure 4.4: Substrate concentration at the inlet of the reactor. The blues dashed lines show the change of OLR. The red dashed line represents the change of conditions in the anerobic solid waste fermentation reactor

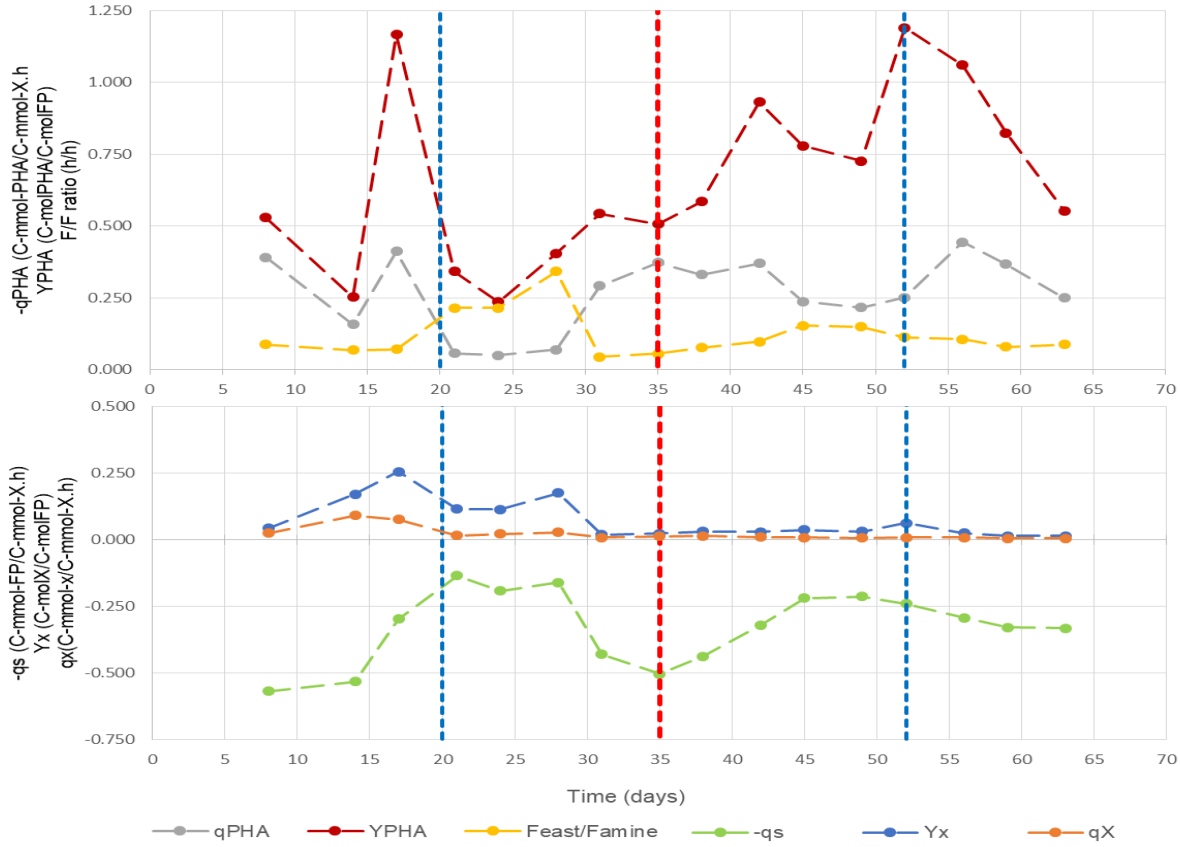


Figure 4.5: Fermentation products consumption rate (-qs); PHA storage rate (qPHA); Storage yield (YPHA); Growth rate (qx); Growth yield (Yx); and Feast and Famine ratio (F/F ratio) over the time of PHA enrichment culture selection in the SBR. The blues dashed lines show the change of OLR. The red dashed line represents the change of conditions in the anerobic solid waste fermentation reactor.

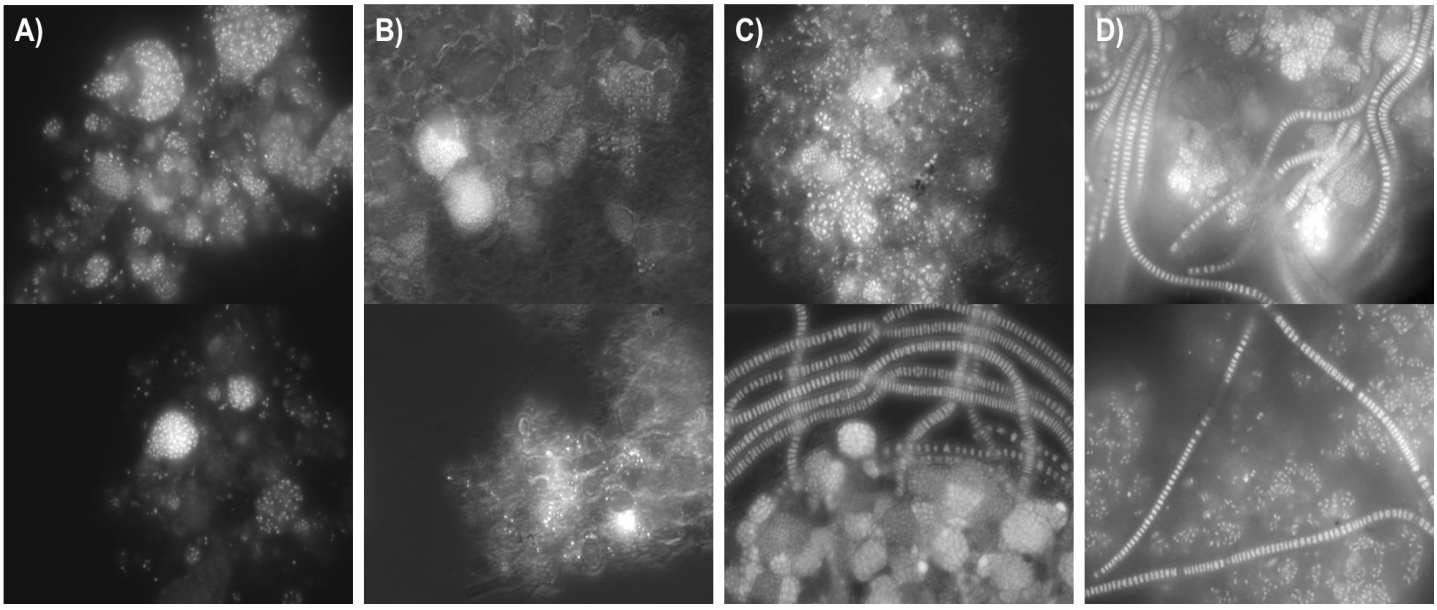


Figure 4.6: Nile Blue image of PHA accumulation in the monitoring days; A) represents the first days of the reactor with a lower OLR; B) Corresponds to the days of acclimatization after the OLR change; C) Corresponds to the days after acclimatization and change of effluent 1 to effluent 2; D) Corresponds to the last OLR change.

Table 4.5: Percentage of HB and HV precursors is in the substrate and percentage of HB and HV monomer is found in the polymer.The double line represents the OLR change. The blue zone represents the use of Eff_1 and the pink zone using Eff_2.

		PHA %		Precursors	
Monitoring	Time	%HB	%HV	Prec. HB	Prec. HV
		% Cmmol		% Cmmol	
1	8	56.21	43.79	61.07	38.93
2	14	54.06	45.94	54.56	45.44
3	17	64.59	35.41	55.98	44.02
4	21	53.93	46.07	53.32	46.68
5	24	84.95	15.05	51.08	48.92
6	28	50.86	49.14	52.47	47.53
7	31	61.24	38.76	53.20	46.80
8	35	49.93	50.07	51.54	48.46
9	38	44.34	55.66	49.95	50.05
10	42	51.73	48.27	49.72	50.28
11	45	48.53	51.47	52.43	47.57
12	49	48.46	51.54	48.60	51.40
13	52	48.04	51.96	47.35	52.65
14	56	46.21	53.79	47.51	52.49
15	59	34.99	65.01	45.88	54.12
16	63	38.96	61.04	46.74	53.26

4.2.2. Accumulations assays

The accumulation assays were performed in order to determined the maximum PHA accumulated by the enriched culture.

Two accumulation assays were performed, using Eff_1 and Eff_2 as carbon source (composition on table 4.6 and 4.8). The pulses volume were determined in order to maintain the ratio of food to microorganisms of 11.28 C-mmol / g-VSS.

Table 4.6: Composition of the feed used in the accumulation assay 1

Lactate	Acetate	Propionate	Ethanol	Isoutyrate	Butyrate	Isovaleric	Valerate	Total FP
(C-mmol/L)								
0.00	239.45	372.74	0.00	0.05	165.94	20.13	72.83	871.14
Lactate	Acetate	Propionate	Ethanol	Isoutyrate	Butyrate	Isovaleric	Valerate	Total FP
%								
0.00	27.75	41.43	0.00	1.00	19.53	2.16	8.13	100.00

According to the first assay (Eff_1), it was observed that the most concentrated acids (proprionic, acetic and butyric acids) were consumed at higher rates, 0.10 – 0.28 C-mmol FP/C-mmol X.h during the first 3 pulses (Table 4.7). In the 4th pulse, the consumption rates of the substrate increased, which can be related with the total consumption of FP in the 3rd, which triggered the stored PHA consumption since between the total consumption and the 4th pulse took 30 min.

PHA production and storage rate were stable and followed the same trend as the FP consumption rate in the first three pulses, with an average storage yield of 0.74 ± 0.04 C-molPHA/C-molFP In the fourth pulse the yield decreased to 0.47 C-molPHA/C-molFP.This reduction in the last pulse can be explained by the consumption of PHA observed in the 3rd pulse (Figure 4.8). In the second pulse, as the FP werealmost totally consumed, some PHA stored started to be consumed (Figure 4.8). However as the consumption of PHA was not significant it did not affect the results.

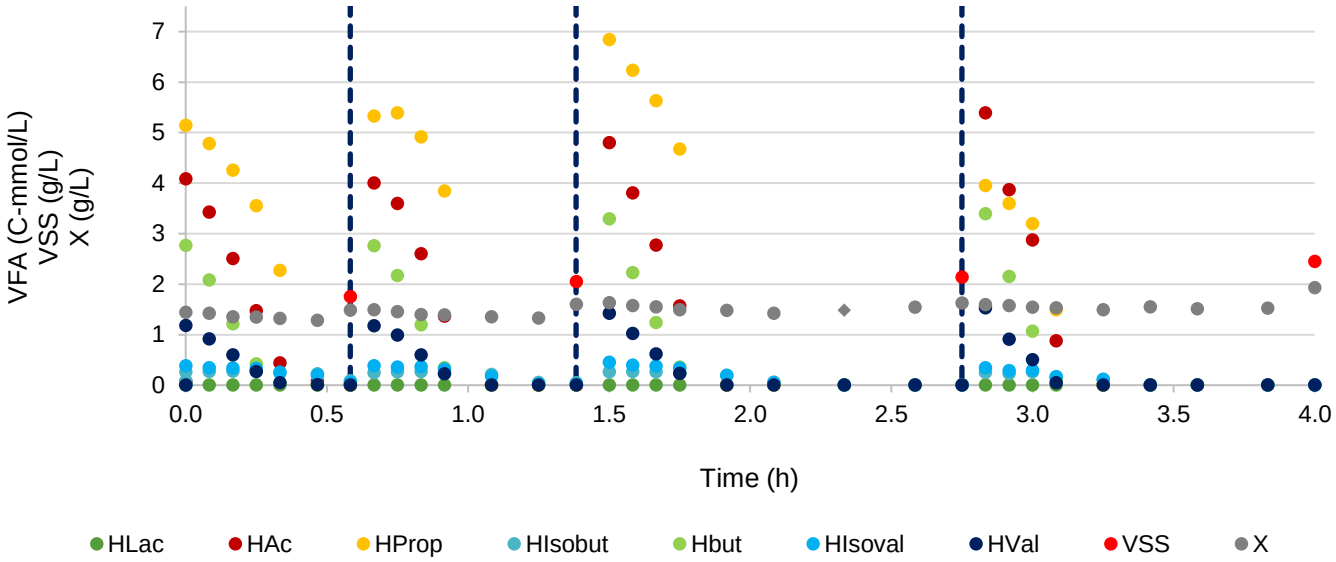


Figure 4.7: FPs consumption over time during the first accumulation assay using Eff_1. The blues dashed lines show the end of each pulse.

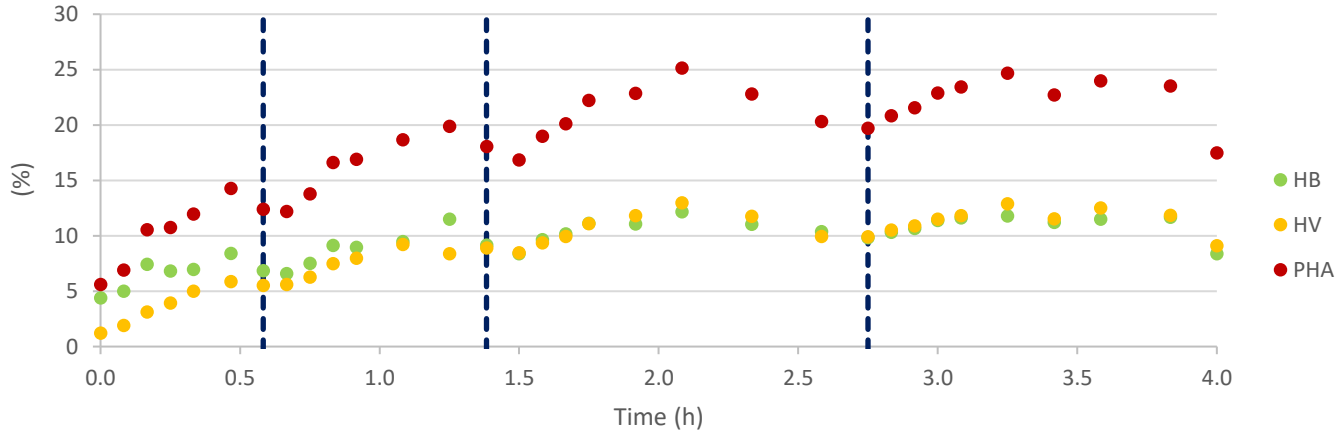


Figure 4.8: PHA production over time during the first accumulation assay using Eff_1. The blues dashed lines show the end of each pulse.

Table 4.7: Principal parameters obtained in each pulse in PHA accumulation assay 1. The values in parentheses represent the standard deviations.

	qHLac	qHAc	qHProp	qHIsobut	qHBut	qHIsoval	qHVal	qS	qX	qHB	qHV	qPHA	YX	YPHA
	Cmol/Cmol X.h								Cmol X/ Cmol X.h	Cmol HB/ Cmol X.h	Cmol HV/ Cmol X.h	Cmol PHA/ Cmol X.h	Cmol X/ Cmol FP	Cmol PHA/ Cmol FP
1st pulse	0.000	-0.147	-0.171	-0.009	-0.100	-0.013	-0.043	-0.466	-0.242	0.150	0.179	0.328	0.518	0.704
		(0.041)	(0.079)	(0.005)	0.046)	(0.004)	(0.016)	(0.088)	(0.390)	(0.028)	(0.022)	(0.036)	(0.843)	(0.154)
2nd pulse	0.000	-0.155	-0.262	-0.008	-0.107	-0.014	-0.046	-0.414	-0.194	0.147	0.179	0.327	0.469	0.789
		(0.043)	(0.177)	(0.003)	(0.057)	(0.007)	(0.019)	(0.135)	(0.277)	(0.062)	(0.060)	(0.086)	(0.687)	(0.330)
3rd pulse	0.000	-0.163	-0.275	-0.007	-0.112	-0.009	-0.049	-0.442	-0.220	0.139	0.187	0.326	0.496	0.736
		(0.017)	(0.169)	(0.002)	(0.066)	(0.002)	(0.022)	(0.158)	(0.091)	(0.059)	(0.039)	(0.071)	(0.272)	(0.308)
4th pulse	0.000	-0.189	-0.164	-0.005	-0.185	-0.008	-0.082	-0.514	-0.159	0.098	0.145	0.242	0.309	0.471
		(0.083)	(0.067)	(0.003)	(0.090)	(0.003)	(0.040)	(0.199)	(0.046)	(0.055)	(0.021)	(0.059)	(0.149)	(0.215)

Regarding the second accumulation assay, using Eff_2 as carbon source (table 4.8), the FP were consumed at uptake rates higher than the previous assay (0.13 to 0.46 C-mmol / C-mmol X.h) for propionic, acetic and butyric acid and lower uptakes rates for the remaining FP.

The -qS was constant in the first four pulses and decreased in the 5th pulse, and decrease more in the 6th pulse without total FP consumption, suggesting that the storage saturation state of PHA may have been reached (Table 4.9). The PHA production increased during the first four pulses, and stabilized from the fifth pulse.(Figure 4.10)

The fact that the total consumption of FP was not allowed to reach the next carbon pulse allowed the results obtained. Thus, there was no consumption of stored PHA and therefore it was possible to take the accumulation to its maximum, until the microorganisms reach a state of saturation of accumulation of PHA.

In figure 4.11 can be observed the PHA accumulation through images obtained by microscope using nile blue staining method on both accumulations. Both accumulations show ability to store large amounts of PHA in different microorganisms.

In table 4.10 the two accumulation tests can be compared. As accumulation 1 reached the total consumption of FPs and atart to consume the accumulated biopolymer from the third pulse, obtained a slower storage rate than the second accumulation, 0.31 and 0.46 Cmol PHA / Cmol X.h respectively. Due to the PHA consumption, Accumulation 1 not achieved the maximum of PHA. This explains why the maximum PHA accumulated in this assay was significantly lower than in the second assay. The maximum PHA content obtained in the first accumulation was 25%,while 41% was the maximum PHA content in accumulation 2. When Albuquerque et al., 2010, used the selected culture that had no micronutrient limitations obtained a FP consumption rate and a PHA storage rate (0.54 C-molFP/C-molX.h and 0.43 C-molPHA/C-molX.h, respectively) similar to those obtained in accumulation 2 (0.64 C-molFP/C-molX.h and 0.46 C-molPHA/C-molX.h, respectively). However the maximum PHA content obtained by these authors was higher, reaching 75%. This difference may be due to better optimization of the selection reactor achieved in the latter..

Other study, using glycogen accumulating organisms for PHA production using fermented sugar cane molasses as substrate reached 37% maximum PHA contente (Bengtsson et al., 2010). While in our study, using a different mixed culture selected from feast and famine regime, a higher maximum PHA content was obtained (in second accumulation). This result demonstrated that PHA accumulation is better when feast and famine cycles is used for culture selection.

Despite of the changing conditions in the fermentation reactor, both collected effluents have a similar composition.For this reason, the final composition of the polymers in both accumulations are similar, about 50% of each of the monomers, which would be expected since several authors stated that the composition of the polymer is proportional to the composition of the substrate used (Beccari et al., 1998).

This polymer should be analyzed at the level of meltin temperature, glass transition temperature, elongation to break and tensile strength in order to understand possible applications.

Table 4.8: Composition of the feed used in the accumulation assay 2

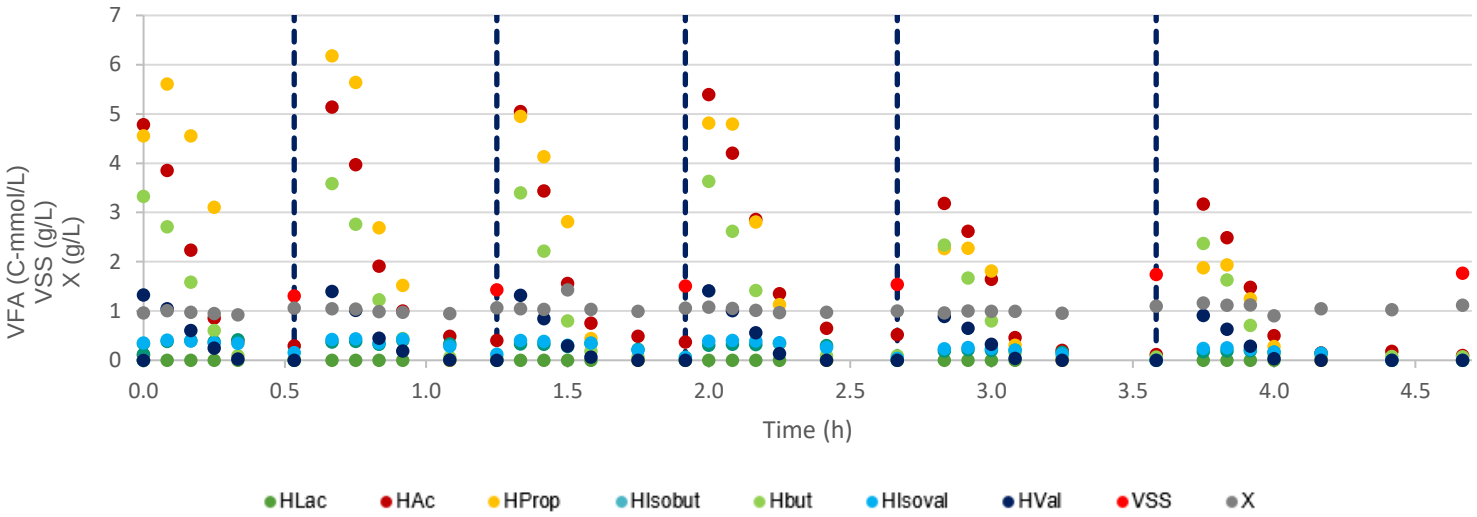
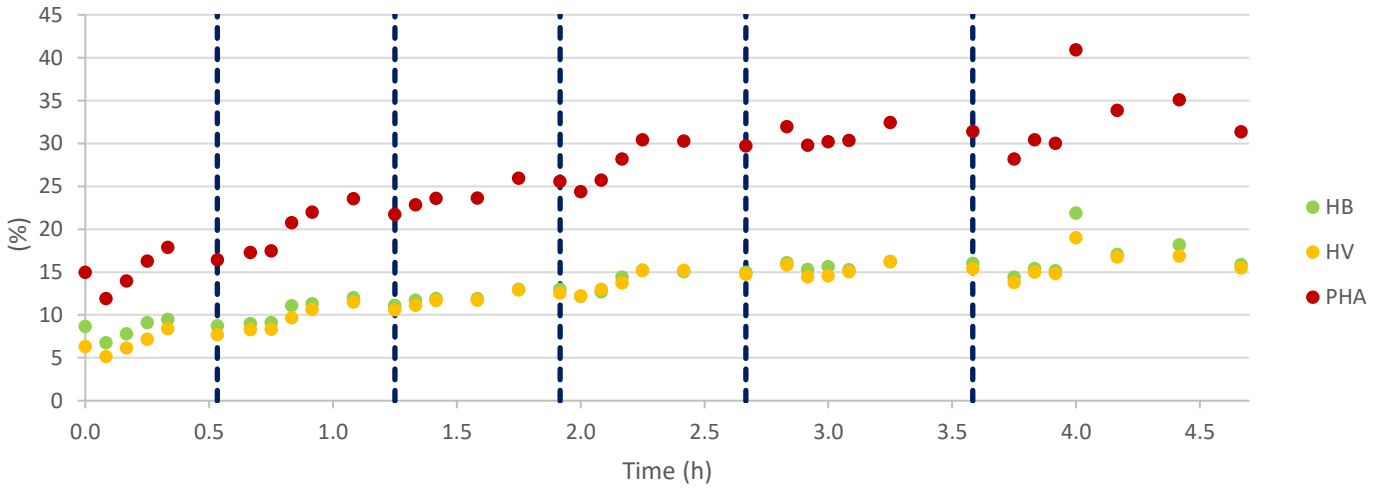


Figure 4.9: FPs consumption over time during the first accumulation assay using Eff_2. The blues dashed lines show the end of each pulse.



Lactate	Acetate	Propionate	Ethanol	Isoutyrate	Butyrate	Isovaleric	Valerate	Total FP
%								
0.00	24.54	43.20	0.00	0.00	21.59	2.47	8.19	100.00

Figure 4.10: PHA production over time during the first accumulation assay using Eff_2. The blues dashed lines show the end of each pulse.

Table 4.9: Principal para meters obtained in each pulse in PHA accumulation assay 2. The values in parentheses represent the standard deviations.

	qHLac	qHAc	qHProp	qHIsobut	qHBut	qHIsoval	qHVal	qS	qX	qHB	qHV	qPHA	YX	YPHA
	Cmol/Cmol X.h								Cmol X/ Cmol X.h	Cmol HB/ Cmol X.h	Cmol HV/ Cmol X.h	Cmol PHA/ Cmol X.h	Cmol X/ Cmol FP	Cmol PHA/ Cmol FP
1st pulse	0.000	-0.208 (0.134)	-0.459 (0.164)	-0.019 (0.021)	-0.238 (0.093)	-0.016 (0.010)	-0.094 (0.036)	-0.702 (0.299)	0.103 (0.526)	0.212 (0.038)	0.244 (0.101)	0.456 (0.108)	0.147 (0.752)	0.649 (0.317)
2nd pulse	0.000	-0.248 (0.123)	-0.345 (0.141)	-0.028 (0.015)	-0.192 (0.095)	-0.022 (0.009)	-0.108 (0.036)	-0.629 (0.369)	-0.019 (0.138)	0.172 (0.113)	0.192 (0.085)	0.364 (0.142)	0.031 (0.220)	0.580 (0.408)
3rd pulse	0.000	-0.158 (0.150)	-0.406 (0.139)	-0.022 (0.010)	-0.293 (0.112)	-0.013 (0.005)	-0.114 (0.044)	-0.772 (0.430)	-0.009 (0.092)	0.061 (0.062)	0.096 (0.081)	0.157 (0.102)	0.011 (0.119)	0.203 (0.174)
4th pulse	0.000	-0.245 (0.104)	-0.302 (0.117)	-0.020 (0.011)	-0.186 (0.094)	-0.016 (0.007)	-0.106 (0.036)	-0.782 (0.324)	-0.110 (0.184)	0.343 (0.190)	0.340 (0.165)	0.683 (0.252)	0.141 (0.243)	0.874 (0.484)
5th pulse	0.000	-0.179 (0.085)	-0.179 (0.071)	-0.008 (0.004)	-0.131 (0.070)	-0.008 (0.003)	-0.080 (0.027)	-0.517 (0.238)	0.133 (0.233)	0.093 (0.164)	0.162 (0.145)	0.255 (0.218)	0.256 (0.466)	0.493 (0.479)
6th pulse	0.000	-0.148 (0.048)	-0.117 (0.034)	-0.008 (0.003)	-0.177 (0.041)	-0.009 (0.002)	-0.069 (0.016)	-0.411 (0.134)	-0.037 (0.193)	0.171 (0.053)	0.160 (0.093)	0.331 (0.107)	0.090 (0.470)	0.806 (0.369)

Table 4.10: Principal para meters obtained in each pulse in PHA accumulation assay 2. The values in parentheses represent the standard deviations.

Acc	VSS	X	Lactate	Acetate	Propionate	Isobutyrate	Butyrate	Isovaleric	Valerate	qS	qPHA	YPHA	PHA max	HB:HV
	(g/L)	(g/L)	C-mmol/L							Cmol FP/Cmol X.h	CmolPHA /Cmol X.h	Cmol PHA/Cmol FP	%	%
1	1.99 (0.31)	1.49 (0.12)	0.00	239.45	372.74	0.05	165.94	20.13	72.83	-0.459 (0.04)	0.306 (0.04)	0.675 (0.12)	25	53:47
2	1.50 (0.20)	1.03 (0.09)	0.00	217.19	382.40	0.01	191.13	21.84	72.53	-0.636 (0.13)	0.459 (0.14)	0.727 (0.12)	41	50:50

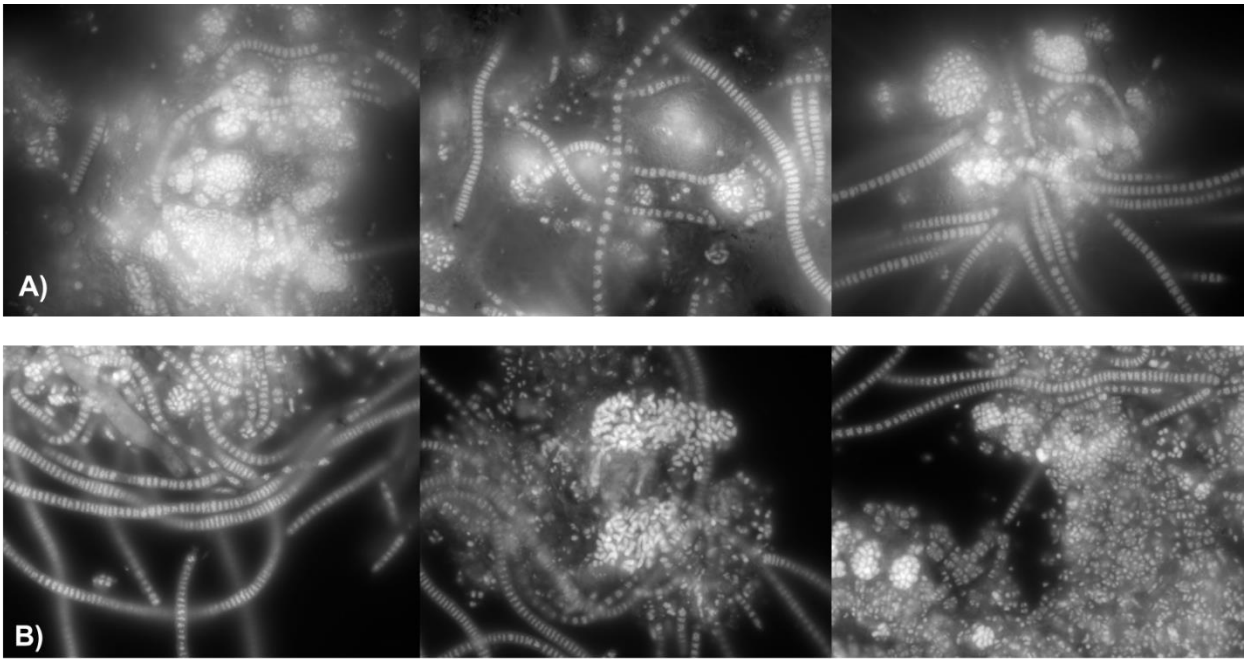


Figure 4.11: Nile Blue image of PHA accumulation in A) Accumulation 1 and B) Accumulation 2

5. Conclusions and future work

In this study the effect of OLR and pH on acidogenic fermentation using BSG as substrate was investigated. Two different OLRs, 30gCOD / L and 50gCOD / L, and three different pH,5.0, 5.5 and 6, were tested in order to study their effect on the production of organic acids.

The increase of OLR does not imply a better acidogenesis, because when the OLR was increased from 30gCOD / L to 50gCOD / L, the DA remained practically the same (41.1 ± 1.2 and 39.6 ± 6.2%, respectively). However, the OLR increase allowed the change in the FP composition, observing an increase in acetic, isovaleric and valeric acid and a decrease in butyric acid.

The pH study showed that with pH increase, higher DA was obtained (39.6 ± 6.2, 53.3 ± 2.1 and 60.2 ± 4.8 %, to pH 5, 5.5 and 6, respectively). However, the YFP slightly decreased from pH 5.5 to pH6 (0.89 ± 0.07 to 0.87 ± 0.09) and, at the same time, methane content increased, seeming that this pH may favored some methanogenic. The pH change showed a change in the FP profile, where the pH increase implied the increase of acetic, propionic and isovaleric acid and the reduction of butyric and valeric acid.

These results showed that the anaerobic granules were able to adapt to different conditions and obtained a good degree of acidification.

As the higher OLR was not inhibitory, it is possible to change the FP profile changing the OLR or pH. In this way, the profile of the monomer precursors can be altered, and PHA can be produced with a specific composition and specific characteristics depending on their application.

In the SBR it was observed that, with the increase of the OLR, the PHA storage rate increased, as well as, the storage yield, reaching a qPHA of 0.33±0.08 CmmolPHA/CmmolX.h and a YPHA of 0.91 ± 0.24 CmolPHA/CmmolFP using an OLR of 118.6±13.5 Cmmol/L.day. The faster consumed acids were propionic, acetic and butyric acid. Regarding to the composition of the polymer, it presented a predominant 50/50 ratio (%HB/%HV)

In the accumulation test, a storage yield of 0.73 CmolPHA/CmolFP was obtained, reaching a maximum PHA content of 41%. The composition of the polymer was similar to the selection reactor

This study allowed to conclude that valorization of BSG through the production of PHAs is possible, however the process still need optimization.

As future work, in acidogenic fermentation, tests with different combinations of OLR and pHs could be performed in order to study their effect on the profile of the VFAs. Also determine the maximum OLR which is inhibitory and reduces the acidogenic capacity of microorganisms. In the SBR, different OLR should be tested in order to understand the best operating conditons for the optimization of culture selection. It will also be important to optimize the accumulation process in order to reach the maximum PHA content. Finally study the characteristics of the polymer to find out the best applications.

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7. Appendix

Appendix 1

	Days	effluent	PO_4^{3-} (P-mmol/L)	NH4+ (NH4+-Nmmol/L)	FP															
					HLac	HAc	HProp	EtOH	HIsobut	HBut	HIsoval	HVal	Total FP	HLac	HAc	HProp	EtOH	HIsobut	HBut	HIsoval	Valerate
					(C-mmol/L)									%							
Mon 0	**0**	**31-01-2018**	8.3	49.7	0.0	122.9	119.0	0.0	4.3	153.8	5.7	28.3	**434.0**	0.0	28.3	27.4	0.0	1.0	35.4	1.3	6.5
Mon 1	**8**	**07-02-2018**	10.4	62.4	0.0	158.9	174.0	0.0	6.7	218.0	11.4	50.2	**619.2**	0.0	25.7	28.1	0.0	1.1	35.2	1.8	8.1
Mon 2	**14**	**14-02-2018**	12.1	67.6	0.0	198.7	175.5	0.0	6.7	109.5	15.0	78.3	**583.7**	0.0	34.0	30.1	0.0	1.1	18.8	2.6	13.4
Mon 3	**17**	**14-02-2018**	12.1	67.6	0.0	198.7	175.5	0.0	6.7	109.5	15.0	78.3	**583.7**	0.0	34.0	30.1	0.0	1.1	18.8	2.6	13.4
Mon 4	**21**	**28-02-2018**	14.7	71.6	3.3	268.7	247.2	0.0	6.4	240.3	16.9	73.3	**856.2**	0.4	31.4	28.9	0.0	0.7	28.1	2.0	8.6
Mon 5	**24**	**07-03-2018**	15.5	73.4	2.2	288.4	296.8	0.0	6.6	120.0	20.8	75.6	**810.4**	0.3	35.6	36.6	0.0	0.8	14.8	2.6	9.3
Mon 6	**28**	**14-03-2018**	2.2	28.5	1.3	30.5	37.6	0.0	1.1	21.4	2.0	9.1	**103.1**	1.3	29.6	36.5	0.0	1.1	20.8	1.9	8.8
Mon 7	**31**	**21-03-2018**	19.3	68.6	0.0	247.8	314.2	0.0	9.0	185.0	16.5	75.4	**847.9**	0.0	29.2	37.1	0.0	1.1	21.8	1.9	8.9
Mon 8	**35**	**28-03-2018**	21.6	61.9	0.0	215.5	288.5	0.0	4.4	152.8	8.9	55.2	**725.3**	0.0	29.7	39.8	0.0	0.6	21.1	1.2	7.6
Mon 9	**38**	**02-04-2018**	22.9	54.6	0.0	241.4	336.5	0.0	8.8	163.2	18.7	65.8	**834.4**	0.0	28.9	40.3	0.0	1.0	19.6	2.2	7.9
Mon 10	**42**	**06-04-2018**	21.5	51.5	0.0	213.3	298.8	0.0	7.1	136.1	16.3	57.0	**728.6**	0.0	29.3	41.0	0.0	1.0	18.7	2.2	7.8
Mon 11	**45**	**06-04-2018**	21.5	51.5	0.0	213.3	298.8	0.0	7.1	136.1	16.3	57.0	**728.6**	0.0	29.3	41.0	0.0	1.0	18.7	2.2	7.8
Mon 12	**49**	**09.04.2018 + 11.04.2018**	22.3	51.4	0.0	273.8	281.0	0.0	7.2	168.5	19.4	74.4	**824.3**	0.0	33.2	34.1	0.0	0.9	20.4	2.4	9.0

Mon 13	**52**	**13.04.2018**	25.0	48.9	0.0	236.6	355.3	0.0	4.1	157.7	17.6	68.1	**839.4**	0.0	28.2	42.3	0.0	0.5	18.8	2.1	8.1
Mon 14	**56**	**16.04.2018 + 18.04.2018**	24.9	47.1	0.0	246.9	376.6	0.0	7.6	172.5	20.8	76.0	**900.4**	0.0	27.4	41.8	0.0	0.8	19.2	2.3	8.4
Mon 15	**59**	**20.04.2018**	27.2	71.5	0.0	262.7	449.6	0.0	10.1	202.7	25.5	88.3	**1038.9**	0.0	25.3	43.3	0.0	1.0	19.5	2.5	8.5
Mon 16	**63**	**23.04.2018**	28.0	88.7	0.0	274.1	453.2	0.0	10.5	220.5	27.6	92.9	**1078.7**	0.0	25.4	42.0	0.0	1.0	20.4	2.6	8.6