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Study of Polyhydroxyalkanoates Production Processes

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M'agradaria donar les gràcies a tota les persones que han fet possible l'elaboració d'aquest treball de final de grau. Gràcies al meu tutor Dr. Joan Dosta Parras, per deixar-me treballar en aquest projecte, ensenyar-me, guiar-me i corregir-me sempre que ho he necessitat. Gràcies al Dr. Ricard Torres Castillo per haver-me ajudat a arrancar aquest projecte i per tots els consells. També vull donar-te les gràcies a tu, Carme Vidal Antich, que m'has ensenyat a moure'm en el laboratori d'investigació a més de tot l'esforç que has dedicat en aquest projecte que m'ha servit de suport en tot moment. Gràcies a tu també, Noemí Pérez i Esteban, per ajudar-me en tot el que has pogut.

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SUMMARY

The challenge due to the unsustainable situation caused by petroleum-based plastics, requires different alternative process and technologies to obtain biodegradable plastics. Polyhydroxyalkanoates (PHA) are a kind of biodegradable plastic.

PHA are polyesters mainly composed by two majority groups: Polyhydroxybutyrate (PHB) and Polyhydroxyvalerate (PHV). Those plastics can be obtained using biological processes which are composed by four phases: an anaerobic acidogenic fermentation, a PHA-producer biomass selection, an accumulation of PHA and the PHA extraction. These processes could use sustainable resources such as wastewaters and Organic Fraction of Municipal Solid Waste (OFMSW) as influent substrate.

In this final degree project, the selection and accumulation of bioplastics will be studied. On one hand in lab scale, where the characterization of different selection cycles are carried out based on profiles of Dissolved Oxygen (DO), pH, Reduction-Oxidation Potential (ORP), ammoniacal nitrogen concentration ($N\text{-}NH_4^+$) and Volatile Fatty Acids (VFAs). And on the other hand, different pilot scale production processes are described and discussed.

Regarding to lab experimentation, a synthetic influent, which simulates fermented OFMSW, was used applying an Organic Loading Rate (OLR) of 2.5 g COD L^{-1} d^{-1} in a 3.75 L Sequential Batch Reactor (SBR) with temperature control at 35 ºC. This reactor was initially inoculated with sewage sludge from a municipal wastewater treatment plant. The strategy used for the selection of biomass has been an aerobic Feast-Famine with uncoupled nitrogen feeding. Experimental results obtained from the lab study suggest an effective pseudo-stationary selection cycles, where DO profile follows the same trend. Moreover, the biomass adaptation at OLR working value indicates the possibility to increase the OLR to rise the PHA-accumulation and production potential. Accumulation could not have been studied but, based on previous research, PHA compositions are estimated nearby 14% (g PHA) $(g\ VSS)^{-1}$ and 44% (g PHA) $(g\ VSS)^{-1}$ in the selection and accumulation process, respectively.

After the analysis and description of the different pilot plants, it is concluded that different technologies are applicable depending on the main objective of the biorefinery. If nitrogen removal

is the principal objective, the use of nitrification and denitrification using part of the accumulated PHA could be used. Furthermore, if PHA production is desired, the use of OFMSW or biological sludges are suitable.

Therefore, PHA production is a viable alternative which generates environmental-friendly plastics using wastes as sources.

Keywords: Polyhydroxyalkanoates (PHA), Volatile Fatty Acids (VFAs), Feast-Famine, PHA production at pilot scales.

RESUM

Davant del repte que presenta l'actual situació insostenible causada pels productes plàstics provinents de derivats del petroli, es requereix de processos i tecnologies alternatives que permetin obtenir plàstics amb característiques biodegradables. Per això, s'estudien els Polihidroxialcanoats (PHA), que presenten aquest requisit.

Els PHA es tracten de polièsters on els dos grups majoritaris són el Polihidroxibutirat (PHB) i el Polihidroxivalerat (PHV). Aquests plàstics es poden obtenir a partir de processos biològics conformats, principalment, per quatre etapes: fermentació anaeròbica acidogènica, selecció de biomassa productora de PHA, acumulació de biomassa i extracció del PHA. En aquests processos es parteixen, com influents, de recursos sostenibles, com les aigües residuals i les Fraccions Sòlides Orgàniques dels Residus Municipals (FSORM).

En aquest treball de final de grau s'estudiarà la selecció i acumulació de bioplàstics. Per una banda, a escala laboratori, on es caracteritzaran diferents cicles de selecció basant-se amb el perfil d'Oxigen Dissolt (OD), de pH, del Potencial d'Oxidació-Reducció (POR), concentració de nitrogen amoniacal ($N\text{-}NH_4^+$) i dels Àcids Grassos Volàtils (AGVs). Per altra banda, descrivint diversos processos de producció de PHA existents a escala pilot per elaborar una discussió d'aquestes.

Per a l'experimentació al laboratori, s'ha dut a terme l'estudi amb influent sintètic que simula la FSORM amb una Càrrega Orgànica (CO) de 2,5 g COD L^{-1} d^{-1} en un Reactor Discontinu Seqüencial (RDS) de 3,75 L amb control de temperatura a 35 ºC. Aquest reactor va ser prèviament inoculat amb fangs de depuradora d'una planta de tractament d'aigües residuals. L'estratègia portada a terme per a la selecció de biomassa ha estat la de Sacietat-Fam aeròbiques juntament amb el desacoblament de nitrogen. Els resultats experimentals suggereixen uns cicles de selecció efectius i pseudo-estacionaris, on els perfils de OD segueixen la mateixa tendència. A més l'adaptació de la biomassa a la CO de treball indica la possibilitat d'augmentar-la per obtenir més potencial d'acumulació i producció de PHA. L'acumulació no s'ha pogut dur a terme, però, basant-se en estudis previs, s'ha estimat un contingut de PHA del voltant

del 14 % (g PHA) (g VSS)$^{-1}$ i del 44 % (g PHA) (g VSS)$^{-1}$ en el procés de selecció i d'acumulació, respectivament.

Després de l'anàlisi i descripció de les diferents plantes pilot, s'arriba a la conclusió de que, depenent de l'objectiu principal de la biorefineria, interessa l'ús d'una tecnologia diferent. Si es vol eliminar el nitrogen, es pot utilitzar l'ús de la nitrificació i desnitrificació, utilitzant part del PHA acumulat. En canvi, si es vol produir PHA, l'ús de FSORM o de fangs biològics és més adequat.

Així doncs, la producció de PHA es presenta com una alternativa viable de generació de plàstics que disminueixen l'impacte ambiental tot transformant els residus en matèria primera.

Paraules clau: Polihidroxialcanoats (PHA), Àcids Grassos Volàtils (AGVs), Sacietat-Fam, producció de PHA a escala pilot.

1. INTRODUCTION

Plastics have been produced for 100 years (González, 2009) due to its durability and their potential for diverse application. This material benefits and transform everyday life by using their variety of properties. Consequently, the use of that material is increasing, arriving on annual global production that exceeds 260 million tonnes nowadays (Thomson et al., 2009) and causing an environmental problem because of the slow degradation associated and the damage caused in all kind of wildlife which plastics arrive directly.

Plastics are inexpensive, strong, lightweight, durable, resistant to corrosion and can also have suitable thermal and electrical properties. Because of the diversity of polymers that exist and the different properties associated with each one, plastics are used to produce a vast array of products that brings technological advances, energy savings, medical uses and a lot of other social benefits (Andrady et al., 2009).

As consequence of the continuously increasing production of plastics over the last 60 years, the European industry has a combined turnover of about 300 billion euros and employs 1.6 million people (Plastics Europe, 2008) offering resources to investigate and improve the plastic industry and production. In daily life, almost every aspect requires the use of plastics as in telecommunications, clothing, packaging materials… So, it is understood that their use will be present in the future too as part of our lives (Thomson et al., 2009).

Plastics are mostly obtained by petrochemical industry as a result of petroleum distillation in different fractions composed by different hydrocarbon chains. These plastics are non-biodegradable material because of the stability that carbon links give that demands a lot of energy to be broken (PLASTISAX, 2020). Contamination is not the only problem caused by petroleum-based bioplastics. Another important question is the quantity and the price of petroleum as a prime resource. Nowadays, 5% of the world petroleum is used to produce 200 million tonnes of plastic per year but it is expected that the plastic demands will be 2000 million tonnes per year in the 2100 involving the 50% of world petroleum. So, this situation will increase considerably the petroleum costs requiring alternative resources as a better economical way to produce plastics (González et al., 2013).

Plastics waste supposes ecosystem destruction because of their direct presence in nature. Notable quantities of plastic have been accumulated in the environment and around 10% by weight of the municipal waste streams are plastic (Barnes et al., 2009). The non-treatment of these residual streams causes a wide range of damage in nature becoming waterborne waste circulating by aqua flows as a turbid mix of plastics that cause fatal consequences to water-individuals and constantly accumulates in oceans arriving at 2.41 million tonnes per year (Lebreton et al., 2017) and producing directly related deaths from about 260 species (Laist 1997; Derraik 2002; Gregory 2009).

Globally less than a fifth part of plastic is recycled. However, there is an obvious necessity in research and develop technologies to fix the problem. Biobased plastics, such as Polyhydroxyalkanoates (PHA) and PHA-based bioplastics, could represent an alternative to petroleum-based plastics for an array of potential applications (Res Urbis Project, 2017-2020).

1.1. **POLYHYDROXYALKANOATES (PHA)**

Polyhydroxyalkanoates (PHA) are bio-polyesters synthesized intracellularly by some microorganisms as a carbon and energy reservoir. This material is extracted from the inside of the cell and it has similar properties as the petroleum-based plastics. Since 1980 PHA have been studied intensively (González et al., 2013) resulting nowadays an important way for the investigation as petrochemical plastics substitutes because of their biodegradable property and the capability to be produced by renewable carbon resources. Moreover, these bioplastics have relevance for medical use, biodegradable commodity film, packaging interlayer film, speciality durables as electronics among other applications (Res Urbis Project, 2017-2020).

Although PHA are nowadays more expensive (2 to 5 € kg^{-1}) compared with derived petroleum plastics (nearby 0.30 € kg^{-1}), it is expected that in the near future its cost will be highly reduced due to the enhancement of existing technologies, for example developing microbial strains capable to use low cost substrates (Khanna and Srivastava, 2005). Nowadays, PHA investigations have obtained strains with more than 80 g L^{-1} concentration of bioplastic and with a 2 g L^{-1} h^{-1} productivity using batch or continuous systems that potentially could product 50 000

tonnes per year (Salehizadeh and Van Loosdrecht, 2004). Most economical resources are aimed at substrate required and PHA extraction from microorganisms (Dosta and Mata, 2017).

PHA are hydroxyalcanoic acids polymers which some bacteria, archaea and microalgae, accumulate intracellularly to be used as carbon and energy reservoir. The general structure and nomenclature of those compounds are shown in Table 1.1. The acid polymerization occurs via intracellular enzymes by the carboxylic group condensation from a monomer with the hydroxyl group of the next monomer, forming an ester link (Khanna and Srivastava, 2005). Then, proceeds the accumulation of that PHA as a liquid, mobile and amorphous as granules sited at the cytoplasm of the cell and wrapped by a phospholipid layer which contains polymerases and depolymerases, enzymes responsible of the biological synthesis and degradation (see Figure 1.1).

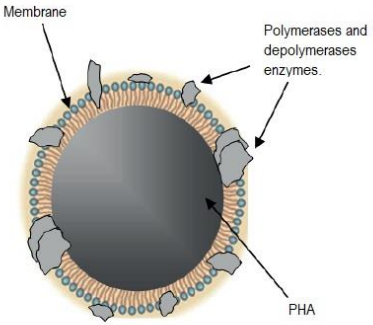
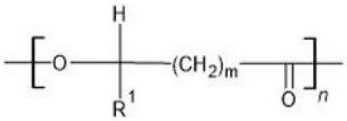


Figure 1.1. Representation microorganism cell with membrane, enzymes and PHA location. (González et al., 2013).

Table 1.1. General PHA stoichiometric formula and polymer type (Made by ChemSketch Freeware) and examples of nomenclature used for PHA based in the radical R^1 and the hydrocarbon length (m).



m	R^1	Polymer name	Symbol
1	Hydrogen	Poli (3-hydroxypropioanoate)	PHP
1	Methil	Poli (3-hydroxybutyrate)	P3HB
2	Hydrogen	Poli (4-hydroxybutyrate)	P4HB
3	Hydrogen	Poli (5-hydroxyvalerate)	P5HV

PHA can be classified based on the length of the hydroxycalcanoic acids in the monomeric structure as it is shown in Figure 1.2. Hence, there are classified in short-chain PHA (PHA-scl) if

the monomeric unities have from 3 to 5 carbon atoms and middle chain PHA (PHA-mcl) if monomeric chain is formed by 6 to 14 carbon atoms. Mixed PHA are those composed by PHA-scl and PHA-mcl monomers. The type of PHA synthesized depends on the microorganism producing mostly PHA-scl or PHA-mcl and a little part can produce mixed PHA. Moreover, PHA can present homopolymers or copolymers depending on the carbon sources and microorganisms. It is found more than 100 monomeric PHA constituents (González et al., 2013).

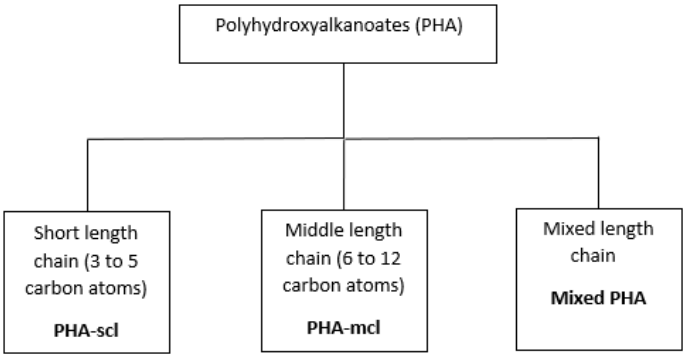


Figure 1.2. PHA type based on the numbers of carbon atoms in monomer. Adapted from González et al. (2013).

1.2. **POLYHYDROXYALKANOATES PRODUCTION**

The synthesis of PHA occurs under determined conditions. In general, this process is a response of a limitation of nitrogen (N), phosphorus (P), sulphur (S), magnesium (Mg) or oxygen (O_2) with an excess of carbon presence in the medium (Reddy et al. 2003) although some bacteria produces PHA under growth conditions (presence of all necessary nutrients) being an uncommon fact. There are more than 300 microorganisms species producers of PHA that use a wide variety of carbon resources as proteins, lipids, carbohydrates, aromatic compounds, gases, wastewater… Principal microorganisms and nutrient limitations capable to produce bioplastics are those shown in Table 1.2.

Table 1.2. Principal microorganisms and nutrient limitations producers of PHA. (González et al., 2013)

Microorganism	Limited nutrient
Alcaligenes latus	Nitrogen
Pseudomonas oleovorans	Nitrogen
Pseudomonas cepacian	Nitrogen
Ralstonia eutropha	Nitrogen
Rhodobacter sphaeroides	Nitrogen
Pseudomonas sp. K.	Magnesium
Azobacter vinelandii	Oxygen
Azobacter beijerinckii	Oxygen
Rhizobium ORS571	Oxygen
Rhodospirillum rubrum	Phosphorous
Rhodobacter sphaeroides	Phosphorous
Caulobacter crescentus	Phosphorous
Pseudomonas oleovorans	Phosphorous

Referring to PHA properties, those are different depending on the monomeric composition but, in general terms, those bioplastics are water insoluble compounds with lipidic character. Major PHA compounds are partially crystalline polymers and their mechanical and thermal properties are characterized by stained glass transition temperature (T_g) or fusion temperature (T_m) (Anderson and Dawes, 1990). In Table 1.3, a short length chain bioplastic P(3HB) homopolymer and copolymer with different fractions of 3HV properties are compared with polypropylene and polystyrene petroleum-based compounds. In this Table 1.3, it is observed that fusion temperature, young module and tensile force in PHA are similar to those reported for polypropylene and polystyrene and that the grade of copolymerization in the PHA has an impact in the values of these properties.

As stated by Anderson and Dawes (1990) and González et al. (2013), PHA-scl are usually thermoplastic polymers capable to be moulded treated in upper temperatures than T_m which is

relatively high (180 ºC) and have a value of T_g around -5 and 20 ºC but small length PHA copolymers are more versatile than homopolymers because of the capability to change particular properties by adding monomers. Although, PHA-mcl present high amorphous grade with a T_g surrounding -62 and -26 ºC) and a T_m around 42 to 58 ºC and they are classified as elastomers.

Table 1.3. Comparison of stained-glass transition and fusion temperatures and mechanical properties between biobased plastics and petroleum-based bioplastics. (Adapted from Babel and Steinbüchel, 2001)

Polymer	Fusion Temperature (T_m) [ºC]	Young Module [GPa]	Tensile force [MPa]	Elongation [%]	Stained glass temperature [ºC]
P(3HB)	179	3.5	40	5	4
P(3HB-co-3HV)					
3 mol % 3HV	170	2.9	38	*	*
14 mol % 3HV	150	1.5	35	*	*
25 mol % 3HV	137	0.7	30	*	*
Polypropylene	170	1.7	34.5	400	45
Polystyrene	110	3.1	50	*	21

*Indicates that the information is not available.

1.3. POLYHYDROXYALKANOATES PRODUCTION FROM MUNICIPAL WASTEWATER OR ORGANIC WASTES

Much attention has been developed to achieve efficient PHA production processes. Conventional PHA production involves the use of pure cultures and, in the majority of the cases, refined substrates where fed-batch fermentation is the most common strategy used (Kedia et al., 2014) and operation conditions as pH and temperature must be well-established and controlled.

The use of mixed microbial cultures for PHA production is an alternative process which is capable to produce bioplastics without sterility conditions required, thus reducing its operating costs while providing selective pressure to ensure PHA-storage capacity in the process through the use of Feast and Famine strategy based on the alternating exposure of presence and absence of carbon substrate with the finality to produce an enrichment of PHA-storing microorganisms.

Mixed cultures can produce PHA using pure substrates or industrial and municipal residual and waste streams as alternative substrates. So, using those wastewater streams, biopolymers can be generated as a value-added product using a biological treatment converting an end-of-pipe wastewater stream as a renewable resource into a biorefinery value-added and environmentally friendly product as bioplastic. Commonly this process is carried out through the enrichment of Volatile Fatty Acids (VFAs) into wastewater streams. This PHA producing potential enrichment is given by four sequential steps process as stated in Figure 1.3 (Reis et al. 2003; Dosta and Mata, 2017):

Step 1) Anaerobic fermentation of Organic Fraction of Municipal Solid Waste (OFMSW) or biological sludges:

First, the application of an acidogenic fermentation in organic fraction of municipal solid waste (OFMSW) as an effective bioprocess to produce short length aliphatic carboxylates compounds as VFAs and other organic low molecular weight compounds. The anaerobic fermentation can be applied to biological sludges as well. Operational parameters as HRT, SRT, pH, temperature and the Organic Loading Rate (OLR) applied requires regulation in order to maximize the VFAs production obtaining determined VFAs distributions with direct influence in PHA monomers that will be formed in the process. Recent studies are based on in-situ VFAs recovery by different unitary operations as adsorption, extraction, ionic exchange or membranes (Jones et al. 2015, Reyhanitash et al. 2016) to avoid fermentation inhibition due to high concentrations of VFA. Also, VFAs stream can be increased using industrial or agricultural streams that have necessary conditions to obtain VFAs-rich feedstocks.

Step 2) Biomass selection in a Sequencing Batch Reactor (SBR):

Secondly the Feast and Famine strategy is used to produce a selection pressure to enhance PHA-storage bacteria proliferation. During the Feast (external carbon source presence) some microorganisms can accumulate carbon and energy reservoirs in different ways as the case of PHA-accumulating bacteria using intracellular bioplastic as reservoir. Hence, Feast and Famine allows the growth of PHA-accumulating bacteria during Famine or non-carbon presence stage. On the other hand, if uncoupled carbon and nitrogen feeding is performed, those microorganisms non-capable to store carbon resources during Feast phase will not be able to grow because the

absence of an elemental nutrient as N in this stage. In order to obtain an effective PHA-storage biomass selection, the rate between the Feast and Famine should be from 20% to under values (Reis et al. 2011). If this process is applied using an enriched biodegradable organic wastewater as substrate in a Sequencing Batch Reactor (SBR), a biotreated water and selective PHA biomass could be obtained as a result.

Step 3) Bioplastic accumulation in a Batch Reactor (BR):

Once biomass has been selected with PHA-storing microorganisms, the accumulation stage is applied to obtain the highest PHA matter content using a Batch Reactor (BR) instead and using wastewater enriched with VFAs to maintain a Feast phase continuously. This operation is based on the application of a pulse-feed strategy. By a pulse feeding of carbon source while a continuous aeration is given in the mixed liquor. When Dissolved Oxygen (DO) profile has a maximum value (indicating the high presence of oxygen in liquor and so, the absence of carbon sources because of the microorganism's metabolism) another pulse of feeding carbon source is given. This process is repeated considering that the carbon degradation kinetic takes lower values in each pulse feeding. Conditions as VFA composition, operation batch reactor conditions, OLR and pH values will affect the content and composition of PHA (Dosta and Mata, 2017).

Step 4) Bioplastic extraction:

Finally, PHA extraction from biomass is done mostly by chloroform extraction as simple and effective way to separate PHA granules from biomass and then producing a precipitation by alcohol obtaining highly purified PHA (Kunasundari and Sudesh, 2010). Also, this extraction could be done by many ways such as causing a cellular lysis using UV rays (Divyashree, and Shamala, 2009).

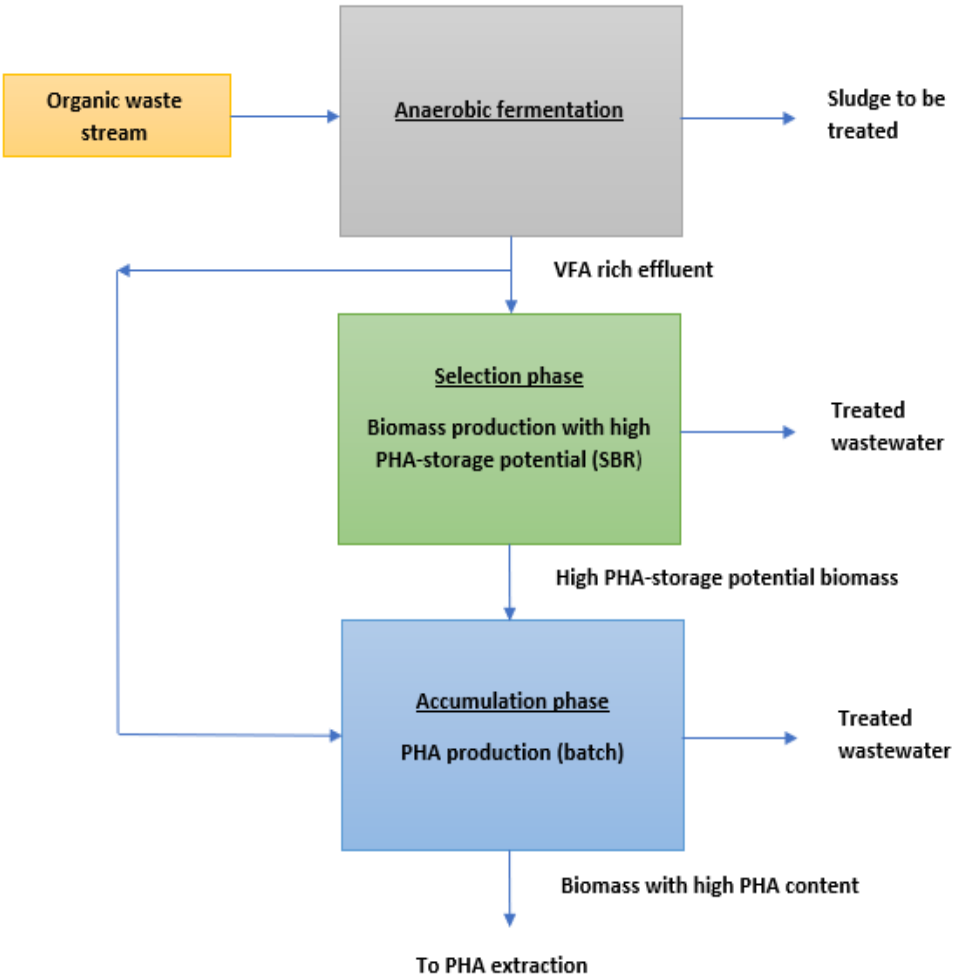


Figure 1.3. General PHA production process form wastewater. (Adapted from Reis et al., 2003).

2. JUSTIFICATION AND OBJECTIVES

This project has the intention to study the effectiveness of mixed cultures storing bioplastic (namely, PHA) in a sequential process composed by a selection reactor and an accumulation reactor. The operation mode will be based on synthetic feed simulating an Organic Loading Rate (OLR) of 2.5 g COD L^{-1} d^{-1}. Moreover, selection cycles will be characterized by recording experimental profiles of: Dissolved Oxygen (DO), pH, $N\text{-}NH_4^+$ concentration, VFAs concentration and Oxidation-Reduction Potential (ORP) including an estimation of the PHA concentration expected in both reactors.

Another important part of this final grade project is to describe different technologies implemented at industrial or pilot scale productions. A discussion will be done to compare the available bioplastic production technologies using mixed microbial cultures that have been implemented at industrial or pilot scale.

The objectives to be covered are specifically determinate in below:

- To characterize the performance of selection cycles in a SBR focused on the selection of PHA storing biomass and subsequent accumulation of this bioplastic.
- To estimate the bioplastic produced by biomass in both operation methods.
- To describe and compare industrial and pilot scale bioplastic production processes.

3. MATERIALS AND METHODS

In this chapter, the experimental set up to carry out this study is detailed. Basically, two lab-scale reactors were operated: a selection reactor, with the objective to select PHA-producer biomass, and an accumulation reactor, to increase the PHA content of the biomass. Moreover, the synthetic wastewater characteristics and inoculum used, as well as the standardized methods used in this work, are also presented.

3.1. EXPERIMENTAL SET-UP

As stated previously, two reactors were operated in this study:

Sequential Batch Reactor (SBR) for the selection of PHA storing biomass:

This digester was a glass reactor of 5 L (3.75 L of effective volume), equipped with a thermostat that warms water in a plastic bucket and circulates through the jacket of the reactor and then is returned to the initial recipient. The reactor also had an agitation system at 80 rpm conformed by a pallet shaker and a lid with six entrances for accessories in which the pH probe (Mettler Toledo HA405-DPA-SC-S8/225), the oxygen probe (CellOx 325, WTW) with a dissolved oxygen portable meter (Oxi 3310, WTW), ORP probe (Double Junction Redox Electrode 9086-10B) and tubes related with the bombs where connected. Figure 3.1 schematizes this reactor. Before the operation, a hydraulic test was taken to check the volumetric flow of pumps (see *Annex 3 Selection reactor pumps calibration*) and the different probes were tested seeing if different values measured were correct. Air checking was done by observing if bubbles diffusion worked properly avoiding the formation of big bubbles. Stationary operation was defined as a constant feast-famine ratio and $N\text{-}NH_4^+$ residual concentration in effluent.

Air supply to the reactor were performed by 12 air compressors (Moure air pump 5, Epsilon) and net-air system that derives in three tube connections into the reactor with three porous diffusors. Fill and draw operations were performed by 4 peristaltic pumps (PERCOM-I). 5 timers (Smartwares 10.047.65 with programable mechanical temporizer) were connected to each bomb and to the oxygen-agitation system to perform the SBR cycle.

Two feed streams were used in the SBR cycles of this reactor, one enriched in VFAs and the other in ammonium nitrogen. The VFAs rich feed was stored in two identical closed tanks of 10 L to prevent the degradation of organic mass because of light exposure, algae proliferation or microorganism growth. This tank was connected to a peristaltic pump (PERCOM-I) to add it in the digester. Ammonium chloride feeding solution was stored in two glass bottles (1 L capacity in each one) which were connected to the reactor with the same kind of pump as feed does and pumps used to extract volume and biomass connected reactor with some 12 L recipients.

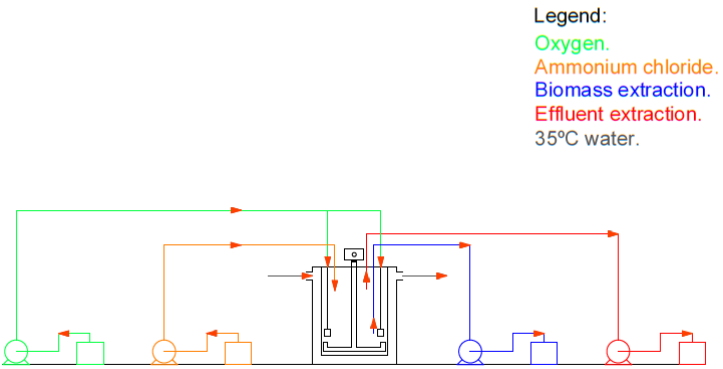


Figure 3.1. Flux diagram of selection reactor done by AutoCAD software.

The reactor worked as a Sequencing Batch Reactor (SBR) and followed cycles of 6 h (4 cycles per day) controlled by the timers (Smartwares 10.047.65 with programable mechanical temporizer). Table 3.1 shows the distribution of the SBR cycles in the working periods of this work.

Table 3.1 – Characteristics of the operating cycles of the selection reactor.

Parameter	Value	Units
Temperature	35	ºC
Hydraulic retention time (HRT)	1.12	d
Solid Retention Time (SRT)	4.21	d
Organic loading rate	2.51	g COD L^{-1} d^{-1}
Nitrogen loading rate	0.074	g NH_4^+-N L^{-1} d^{-1}
Cycle length	6	h
Time distribution in each operating cycle (steps 1 – 8)		
1 -VFAs rich wastewater feeding	15	min
2 - Agitation + air supply	135	min
3 – Agitation + air supply + sludge purge	7	min
4 - Agitation + air supply	8	min
5 - Agitation + air supply NH_4^+-N rich wastewater feeding	2	min
6 - Agitation + air supply	148	min
7 – Settling (no agitation nor air supply)	30	min
8 – Effluent withdrawal	15	min

As it could be seen in Table 3.1, each operating cycle is composed the following steps:

VFAs rich wastewater feeding: for 15 minutes, the synthetic feed composed by VFAs and nutrients (except N) is fed to the selection reactor.

Agitation + air supply: The duration of this part is 5 h by using a mechanical stirrer at 800 rpm with flat shovels to offer a soft homogenization in improving the contact between microorganisms and feed. At the same time, dissolved oxygen is supplied using air compressors and/or compressed air service connected to diffusors, to ensure the dissolved oxygen not being a limiting factor in the growth of microorganisms.

Sludge purge: as the reactor is being agitated, some purge of biomass is extracted to maintain the desired Solid Retention Time (SRT) in the reactor.

Ammonium rich wastewater feeding: during 2 minutes the necessary concentration of NH4+-N is given to the reactor as ammonium chloride (NH_4Cl).

Settling: After the reaction phase, the agitator and oxygen pumps turn off to settle the biomass in the reactor for 30 minutes.

Effluent: for 15 minutes, the desired volume of clarified effluent is removed of the system to establish an operating Hydraulic Retention Time (HRT).

Due to the monitorization of a selection reactor cycle 15 samples of 20 ml were extracted by using a syringe and a plastic tube connected. There were 20 minutes of difference between each sample extraction. The clarified was collected from the samples after a 5 minutes centrifugation, filtered with 45 μm syringe-filters (Simsi Syringe Filter with pore size of 0.45 μm and diameter of 0.25 mm) and stored into vials of 20 ml capability with lids into the fridge. It will be used for VFAs and N-NH_4^+ concentration measurements. Therefore, two PHA samples were taken, the first 20 minutes after feed was given and the second 20 minutes before NH_4Cl is given. Furthermore, DO profile was being recorded every minute using MultiLab Importer software and pH and ORP were being lectured every 20 minutes.

Batch Reactor (BR) for the accumulation of PHA in sludges:

The accumulation was a jacketed glass lab-scale reactor with a working volume of 1 L. Operating temperature was controlled by means of a heating system that maintains the temperature at 35 ºC. Stirring was performed by an agitation system at 80 rpm. The reactor was also equipped with a pH probe (Mettler Toledo HA405-DPA-SC-S8/225), an Oxygen probe (CellOx 325, WTW) connected to a dissolved oxygen portable meter (Oxi 3310, WTW) and the an ORP probe (Double Junction Redox Electrode 9086-10B). The reactor had a bottom system to empty all the working volume. Aeration was carried out using a net-air system connected with a porous diffusor by plastic tube. Operational parameters are shown in Table 3.2. The scheme of this experimental set-up is presented in Figure 3.2. Same test-checking as selection reactor was done before starting the operation.

Otherwise, the feed was prepared in a closed tank of 10 L in order to prevent the degradation of organic substrate to prevent the entrance of air and the exposition to external light (to avoid or at least minimize the proliferation of algae and microorganisms). Hence, the feed was added into

the reactor by pipette to control the volume. Ammonium chloride was not supplied to this reactor because that would benefit reproduction and the consume of PHA reservoirs.

Table 3.2 – Characteristics of the operating cycle of the accumulation reactor.

Parameter	Value	Units
Temperature	35	ºC
Feeding COD concentration	3.5	g COD L^{-1}
Nitrogen loading rate	0	g NH_4^+-N L^{-1} d^{-1}
Cycle length	7-8	h
Time distribution in each operating cycle		
- Agitation + air supply	continuously	-

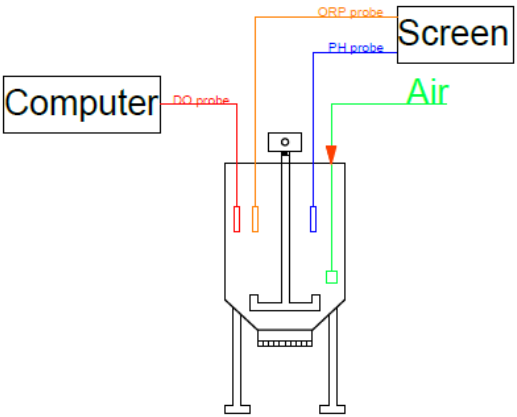


Figure 3.2. Accumulation reactor representation done by AutoCAD software.

The operating cycle of the accumulation reactor cycle was based in the addition of 450 ml biomass as the resulting volume of two purges from selection reactor involving 7:30 hours of continuous operation in SBR. Secondly a pulse-feed strategy is adopted with 150 ml of feed addition when dissolved oxygen increases due to the organic matter consumption and consequently the VFAs

depletion repeating that operation for 3 more times obtaining 4 feed additions. The approximate duration of the global process takes 7-8 hours (Pérez, 2019).

Cycle characterization was based on the monitored lecture of pH, ORP and dissolved oxygen profile (on-line measurements), as well as the analysis of VFAs, SS and PHA content (off-line analysis). The oxygen portable meter is connected to the computer via USB due to the monitorization of oxygen data. Hence, with MultiLab Importer software the data are exported every minute. The lecture of pH and ORP probes were done every 20 minutes. Therefore, at the start of the cycle biomass is characterized by suspended solids, volatile suspended solids and the %PHA analysis. After the addition of feed, VFAs sample is taken and when the degradation is completed to ensure the VFAs depletion (DO at maximum value) suspended solids, volatile suspended solids, %PHA and VFAs analysis are done and repeating this process in each feed putting.

3.2. **SUBSTRATE AND INOCULUM**

The substrate used for both the selection and accumulation reactor was synthetic substrate.

For the selection reactor, the substrate contained 3.5 g COD L^{-1} based on previous results of the research group (Pérez, 2019), since it is organic matter concentration that ensures a ratio Feast/cycle duration minor than 20% on the reactor. To obtain this COD concentration, synthetic feed is used with VFAs as C source. However, not only the total VFAs concentration is important but also the VFAs distribution. In this study, the synthetic mixture is based on 62.5% of acetic acid, 18.8% of propionic acid and 18.8% of butyric acid in COD. These percentages are chosen to represent the typical composition of fermented OFMSW (Dosta et al., 2018). Moreover, macronutrients and micronutrients are added in the mixture to ensure the growth of the microorganisms following an adaptation of Dapena et al. (2004). Table 3.3 summarizes the composition of the synthetic wastewater used in this study, where it should be noted that ammonium nitrogen was not included as a macronutrient, since uncoupled carbon and nitrogen feeding was carried out in the selection reactor.

Table 3.3. VFAs rich synthetic wastewater composition (the calculations carried out to define the wastewater characteristics are shown on Annex 1).

Compound	Concentration	Units	Compound	Concentration	Units
Propionic acid	0.51	g L^{-1}	$FeCl_3 \cdot 6H_2O$	1.50	mg L^{-1}
Butyric acid	0.51	g L^{-1}	H_3BO_3	0.15	mg L^{-1}
Acetic acid	1.69	g L^{-1}	$CuSO_4 \cdot 5H_2O$	0.03	mg L^{-1}
K_2HPO_4	0.58	g L^{-1}	KI	0.03	mg L^{-1}
KH_2PO_4	0.23	g L^{-1}	$MnCl_2 \cdot 4H_2O$	0.12	mg L^{-1}
$MgSO_4 \cdot 7H_2O$	0.09	g L^{-1}	$Na_2MoO \cdot 2H_2O$	0.06	mg L^{-1}
$CaCl_2 \cdot 2H_2O$	0.07	g L^{-1}	$ZnSO_4 \cdot 7H_20$	0.12	mg L^{-1}
EDTA	0.02	g L^{-1}	$CaCl_2 \cdot 2H_2O$	0.12	mg L^{-1}

*$NaHCO_3$ dosage of 25 g L^{-1} was required to ensure a buffer capacity into the reactor.

Using the heterotrophic ratio (Yobs) of the organic loading (g COD L^{-1} d^{-1}) destinated to the growth of microorganisms, the concentration of the nitrogen in form of ammonium chloride has been calculated considering the pump flowrate too as it is represented on the *Annex 1: VFAs composition and selection reactor concentration of ammonium chloride required per day in selection reactor*.

The concentration theoretically calculated and used in the first step was 3.77 g NH_4Cl L^{-1}. However, the concentration of ammonium ($NH4^+$) obtained after famine process was 0 mg N-$NH4^+$ L^{-1} so it was decided to increase this value by a 26% resulting on 5.00 g NH_4Cl L^{-1}. As previously explained, this external nitrogen addition is uncoupled from the feed and added independently.

The substrate used for the accumulation reactor was the same used at selection reactor (Table 3.1) to observe the PHA concentration obtained without the increasing of the OLR. In the accumulation reactor there was no addition of external nitrogen preventing the reproduction of the microorganisms and the reduction of PHA reservoirs.

The initial inoculum used to start-up the selection SBR came from a previously operated lab-scale reactor (Pérez, 2019) which consists in the set-up of bioreactors to produce bioplastic using organic waste and where the selection of biomass producer of PHA had been done. Approximately 750 ml of that sludge was putted on the reactor at the first start of the set-up

operation which has a concentration of 1.88 VSS. The inoculum for the accumulation reactor was the sludge purge of 0.5 days of operation of the selection reactor.

3.3. ANALYTICAL METHODS

The analytical methods used in this work were performed according to the *Standard Methods for the Examination of Water and Wastewater* (APHA, 2017). In the following section the analytical methods applied are briefly explained.

3.3.1. Total Suspended Solids (TSS)

Total Suspended Solids (TSS) are solid particles larger than 2 microns remaining in suspension into the solvent. Depending on the water or wastewater origin, the presence of SS could be related to inorganic materials and bacteria or algae (Fondriest Envoirmental Learning Center, 2020). The standard method followed to assess TSS was the method 2540D, which is explained at *Annex 2: Analytical methods procedures*.

$$\text{TSS}\ \left[\frac{g}{L}\right] = \frac{M_o - M_f}{V} \tag{3.1}$$

3.3.2. Volatile Suspended Solids (VSS)

Total Volatile Suspended Solids are the organic part of the SS, so it is a variable usable in order to characterize the concentration of biomass into the reactor or a stream. The process followed to do the analysis 2540E is showed in *Annex 2: Analytical methods procedures*.

$$\text{VSS}\ \left[\frac{g}{L}\right] = \frac{M_f - M_i}{V} \tag{3.2}$$

3.3.3. Total ammonium nitrogen (TAN or [N-NH_4^+])

The analysis of Total ammoniacal nitrogen (4500-NH_3 D) has been done with a selective ammonia probe in which using a logarithmic calibration based on conductivities values given by different patron concentrations of ammoniacal nitrogen (N-NH4+): 10, 25, 50 and 100 mg N L^{-1}. Before any measurement done even in calibrations it is required the use of few drops of NaOH of 10 M concentration in order to favour acid-base equilibrium to the formation of NH_3 and proton liberation which conductivity is measured and related with N-NH4+. Using this technique, the concentration unit obtained is ppm N-NH_4^+ or mg N-NH_4^+ L^{-1}.

It is important that before any use of the selective ammoniac probe samples requires previous 0.45 μm filtration done by a syringe and syringe-filter (Simsi Syringe Filter with pore size of 0.45 μm and diameter of 0.25 mm).

3.3.4. Volatile Fatty Acids (VFAs)

After the filtration of samples using a syringe-filter (Simsi Syringe Filter with a pore size of 0.45 μm and diameter of 0.25 mm), 1.5 ml of each one are put in different chromatography vials and 50 μl of H_3PO_4 is added on each vial to acidify the media under 2 pH values. Then VFAs concentration and distribution were analysed by gas chromatography technique (Shimadzu GC 2010 plus) equipped with a capillary column (Nukol™, 15 m x 0.53 mm x 0.5 μm) and a flame ionization detector (FID). The chromatograph uses helium as carrier gas, hydrogen as fuel gas and synthetic air as the oxidizing gas. The temperature of the capillary column starts at 80 ºC and is heated by 10 ºC·min^{-1} to 110 ºC. From then on, the temperature increases 15 ºC·min^{-1} until 145 ºC and, finally, it is increased 20 ºC·min^{-1} to 190 ºC. The chromatograph is calibrated to detect acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic and heptanoic acids. Concentrations obtained using this analytical method are given in mg VFA L^1.

3.3.5. Polyhydroxyalkanoates (PHA) content

PHA concentration analysis is done by solvent extraction technique as the most simple and rapid method used in laboratory (Kunasundari and Sudesh, 2010). It involves two steps mainly where the first is the modification of the cell membrane permeability allowing the solubilization of PHA and the following step is to non-solvent precipitation causing negligible degradation to the polymers.

PHA extraction is done by different solvents as chlorinated hydrocarbons or cyclic carbonates (Jaquel et al., 2008). In this analysis chloroform as a chlorinated solvent is used. Moreover, precipitation of PHA is mostly induced by non-solvent such as methanol and ethanol (Ramsay et al., 1994). In this case methanol is used as the non-solvent-cause precipitation. The analysis of standards and samples of the bioreactor are detailed in *Annex 2: Analytical methods procedures.*

4. EXPERIMENTAL RESULTS AND DISCUSSION

In this section, the experimental work carried out to produce PHA storing biomass with high PHA content will be discussed. For the selection SBR, monitored data (VFA concentration, N-NH_4^+ concentration, DO, pH and ORP) profiles obtained in different operational periods are shown and explained in this part. Consequently, several variables which had influence in PHA production are discussed deducing different operation improvements. Conclusive experimental characterizations of the accumulation reactor cycle and PHA concentrations in both reactors could not be obtained because of the Covid-19 pandemical situation. Moreover, by bibliography from different reviews and a last-year similar process and condition operation (Pérez, 2019) the expected PHA concentrations are estimated.

4.1. SELECTION REACTOR EXPERIMENTAL RESULTS

During the experimentation carried out in the laboratory, two operational periods were monitored to obtain information about the selection reactor operation. An operating SBR cycle was considered representative when the performance of the reactor was nearly the same during 3 times the solid retention time of the biomass. Despite of the initially operation period involving an inoculation, DO and ammonia concentration profiles stabilization resulting in a pseudo-stationary phase then the first operational period could be obtained. However, because of the continuously increasing biomass, a second operation period is observed. First and second periods were monitored by experimental data. In the tracking cycles, the following parameters were studied: DO, VFAs concentration, N-NH_4^+ concentration and pH. Moreover, the followed protocol can be seen at cycle monitoring part of the experimental set-up (section 3.1).

Representative SBR cycle when pseudo-stationary phase is achieved (first operational period):

Figure 4.1 shows the monitoring of a representative SBR cycle during the first operational period. Where a repetitive DO cycle can be demonstrated (see *Annex 5: DO repetitive patron*). In the following paragraphs, each state variable will be discussed separately.

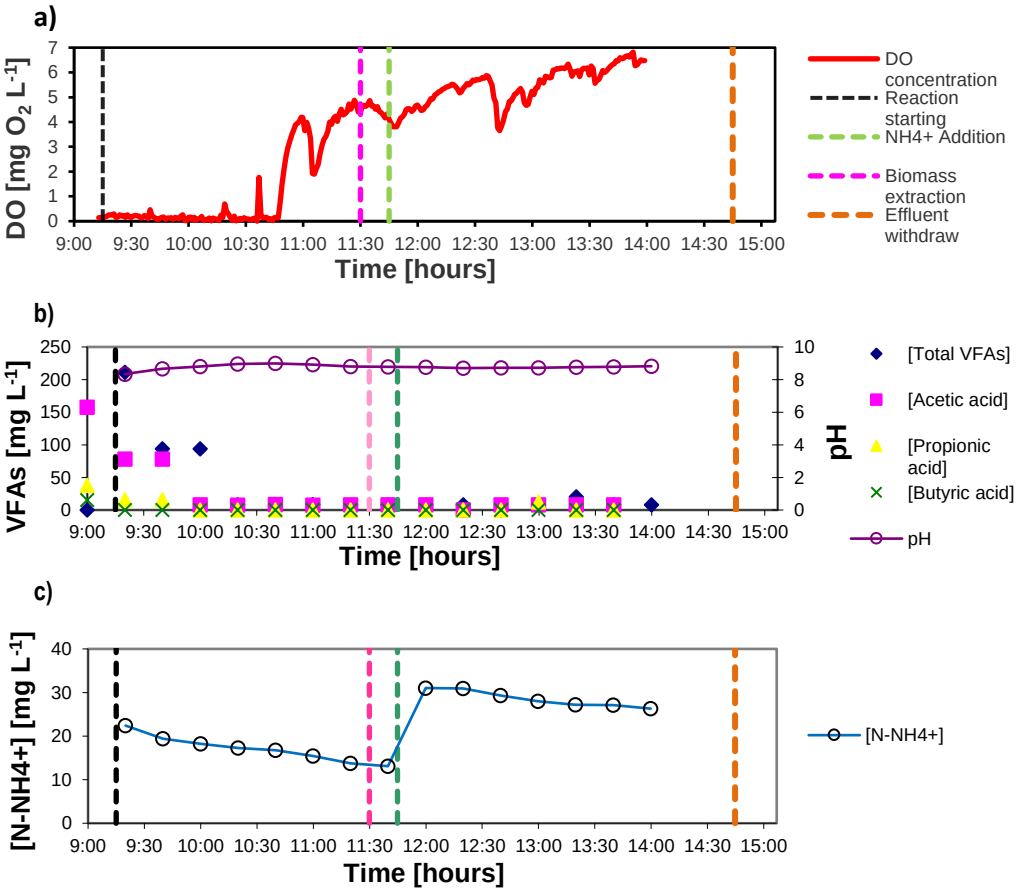


Figure 4.1. Monitoring of a representative SBR cycle of the selection reactor during the first operational period: Dissolved oxygen profile (a); pH and VFAs profile (b) and NH4+-N profile (c). (Monitoring day: 13/02/2020).

- ***Dissolved Oxygen (DO) profile.*** As it can be seen in Figure 4.1 (a), DO profile in reactor follows the typical trend of a selection reactor with both Feast-Famine phases with uncoupled nitrogen strategy:

  When feed is given at 9:00 h, DO is null because the feeding addition is carried out in anoxic conditions. Hence, at 09:15 h, the reaction phase starts with the oxygen supply. Even so, the DO presence is absent because of the consumption of carbon source by

mixed culture requiring the use of oxygen. Approximately at 10:48 h, DO starts to raise arriving on a maximum value at 10:57 h indicating an almost completely consumption of VFAs indicating the famine phase (see Figure 4.2.). As it is indicated, biomass extraction is done at 11:30 h coinciding with the maximum accumulation of PHA.

Moreover, at 11:45 h, the addition of NH_4^+ is done and DO starts showing lower concentration values because of the PHA will be consumed with the N for the microorganisms to grow. Since 11:50 h, the DO starts raising concentration levels until 14:15 h, when aeration is interrupted for the supernatant extraction done at 14:45 h. Lastly, at 15:00 h, another feed volume will be given, and the cycle will be repeated.

- ***pH profile***. Figure 4.2 (b) shows that pH values are nearby 8 to 9 and following a stable evolution into this range. In the initial time, the pH drops slightly due to the acidic feed added in the reactor.
- ***Volatile Fatty Acids (VFAs) profile.*** As it is represented in Figure 4.2 (b), VFAs total concentration is reduced from 211.22 mg L^{-1} at 9:20 h to 8.15 mg L^{-1} at 10:20 h and a total consumption of butyric and propionic acid is observed. Acetic acid maintained at approximately 8 mg L^{-1} over the cycle operation, becoming the controlling degradation acid with an average uptake rate of 1.51 mg L^{-1} min^{-1}. Even so, it is important to note that VFAs are consumed before biomass purge as have been commented before with the DO profile. This fact indicated that the biomass purge is well programmed ensuring the VFAs consumption in the feast phase.
- ***Total Ammonium Nitrogen (NH_4^+-N) profile.*** In Figure 4.1(c) it is observable a downward trend that N-NH_4^+ concentration follows until N-NH4+ is added at 11:45 h when this concentration increases to a 30.9 mg L^{-1} as the maximum value and decreases continuously remaining 26.31 mg L^{-1} before the next cycle starts. This residual NH_4^+-N ensures the that there is not any nitrogen limitation in famine phase. Although, these concentration values are elevated and some decrease in NH_4Cl added is required to have a most efficiently nitrogen uncoupling.

Representative SBR cycle when a substantial growth of the biomass is experimented (second operational period)

In Figure 4.2 a second operational period monitoring is shown. The following track was carried out in the last days of the laboratory work. Due to this, some data such as VFAs concentration could not be analysed. Even so, the behaviour of the microorganisms could be estimated with the DO and ORP profiles.

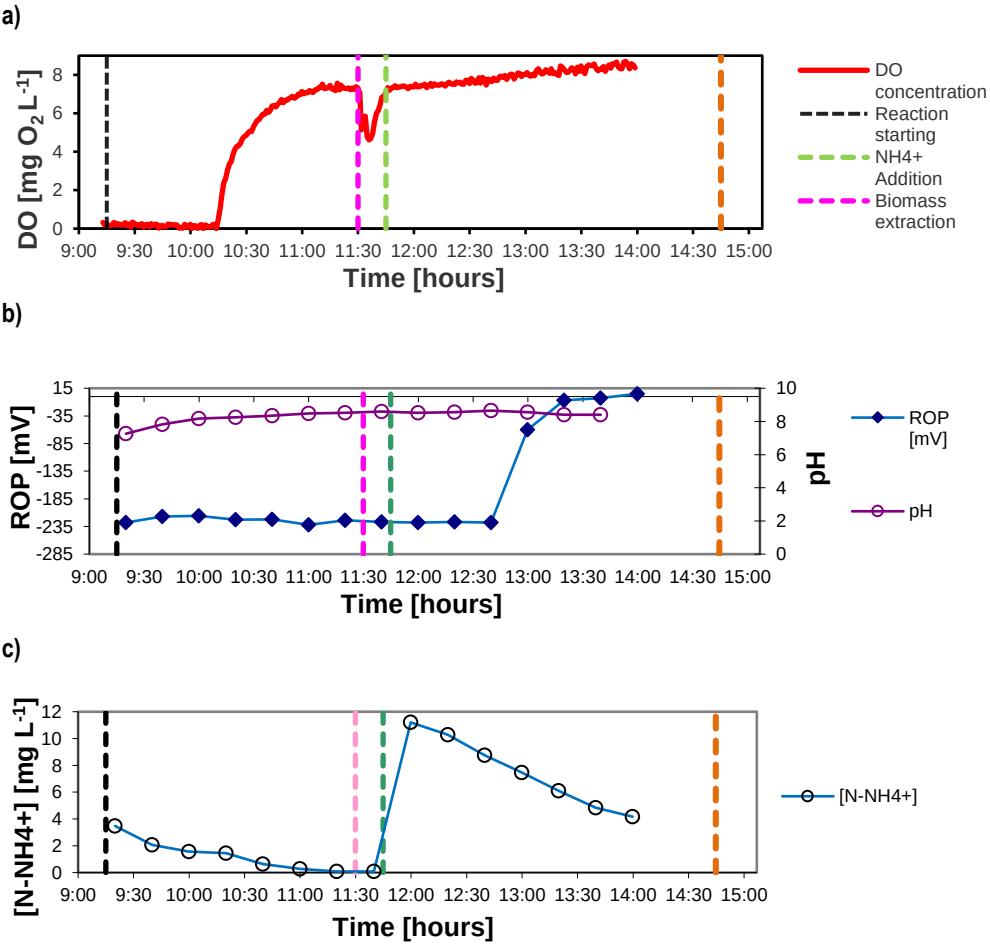


Figure 4.2. Monitoring of a representative SBR cycle of the selection reactor during the second operational period: Dissolved oxygen profile (a); pH profile (b) and NH4+-N profile (c). (Monitoring day: 27/02/2020).

- ***Dissolved Oxygen (DO) profile.*** It is seen in Figure 4.2 (a) that this DO profile shows a total VFAs consumption before nearby when the biomass extraction is effectuated, the maximum value of DO is observed at 11:30 h. So, despite no VFAs profile concentration could be done in this cycle characterization, VFAs consumption can be assumed as completed totally. It is observed that DO profile does not experiment any decrease after $NH4^+$ addition because of the rapidly consumption as it is showed in N-$NH4^+$ concentration profile.
- ***pH profile and ORP values.*** As Figure 4.2 (b) shows, pH is stable and maintained into the range of 7-9 values. Moreover, ORP values during Feast phase were nearby -228 mV and 5 mV at the end of famine phase indicating the changes from anaerobic conditions (negative values) to aerobic conditions (positive values).
- ***Total Ammonium Nitrogen (NH_4^+-N) profile.*** Figure 4.2 (c) indicates that N-NH_4^+ concentration decrease until NH_4^+ addition is given, and the residual concentration is nearly the 4 mg N-NH_4^+ L^{-1} value becoming lower than the 13/02/2020 cycle. This lower residual concentration value is possibly because of a major biomass concentration and the changes of the diffusor for others newer, that offers better oxygen supply (more diffused bubbles with less size).

4.2. BIOPLASTIC OBTAINED FROM SELECTION REACTOR OPERATION:

Because of the Covid-19 pandemic cause, the experimental work was suspended and there was not the possibility to analyse the PHA samples extracted from the selection cycle characterization, although these samples are frozen in the laboratory to be analysed. However, by reviewing the previous studies from Pérez, 2019, carried out in the laboratory and with similar profiles recorded, the expected concentration and composition of PHA was evaluated.

Depending on the feed compositions, different PHA polymers are obtained (Cheah et al., 2019). When the feed is composed by pair number of carbons as acetate and butyrate, hydroxybutyrate monomers are formed. Moreover, if feed is composed by non-pair number of carbons as propionate and valeric, hydroxyvalerate monomers production is favoured. The feed used in this experimental reactor is composed by acetic, butyric and propionic acids in which the carbon-pair

components (acetic (C_2) and butyric (C_4) acids) are predominant than non-pair carbon components (propionic (C_3) acid). Because of that it is expected that the resulting PHA composition should be mostly PHB as the predominant compound, and PHV. Even so, in Wen et al. (2012) it is seeing that working with an ADF and nitrogen depletion produces a higher PHB storing rate.

In the final degree project of Pérez (2019) it is shown that by operating with a selection reactor with 3.5 g DQO L^{-1} d^{-1} as OLR, the PHA percentage obtained has the value of 13.80% (g PHA) (g VSS)$^{-1}$ in which 99% was PHB and 1% was PHV. Becoming PHB the major PHA produced. Although, same nearby values could be expected in the selection reactor used in this project.

By evaluating the performance of the selection SBR some improvements could be carried out to enhance PHA storing biomass selection and production. A major productivity could be obtained in less working volume in selection reactor. It can be done by reducing the dilution of VFAs by a minor water volume or more VFAs concentration. Moreover, as Wijeyekoon et al. (2018) shows, taking more time feeding would allow a PHA yield increase. Otherwise, in ammonia stripped liquor, PHA yield is directly proportional to DO concentration, so, increasing DO concentration in media will increase PHA yield but the costs associated will be magnified too. Otherwise, maximal OLR at industrial plants in similar processes using OFMSW are nearby 3.4 g COD L^{-1} d^{-1} (Valentino et al. 2018) so experimental OLR could be raised by 1 g COD L^{-1} d^{-1} to produce more PHA. Lastly, oxygen is another controlling factor and operation requires an excess of air supply to ensure the end of Feast phase, experimentally it was fixed by using net-air plus compressors.

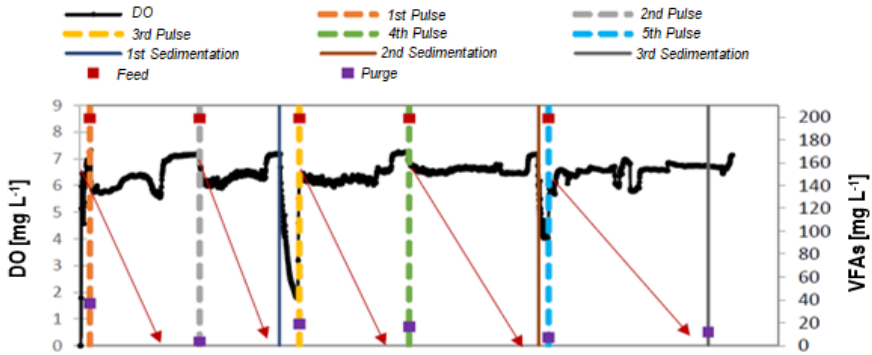
4.3. BIOPLASTIC OBTAINED FROM ACCUMULATION REACTOR OPERATION:

As it has been done in selection reactor, the evaluation of the bioplastic quantity obtained is done by bibliography analysis after observing similar tendencies in profiles from Pérez (2019). That project shows that by five additions of 100 ml and 5 g DQO L^{-1} of VFAs, a 44.09% in mass of PHA was obtained in which 90.67% was PHB and 9.33% and taking around 6.5 hours. PHA percentage experimented an approximately increasement of 30% in mass. If this accumulation is done with

5 g DQO L^{-1} of VFAs, the mass percentage of PHA value is 46.12% composed by 93.2% of PHB and 6.87% of PHV.

In Figure 4.3 the monitoring of an accumulation cycle using a synthetic feed with 5.0 g COD L^{-1} of VFAs concentration and five pulse feedings.

a)



b)

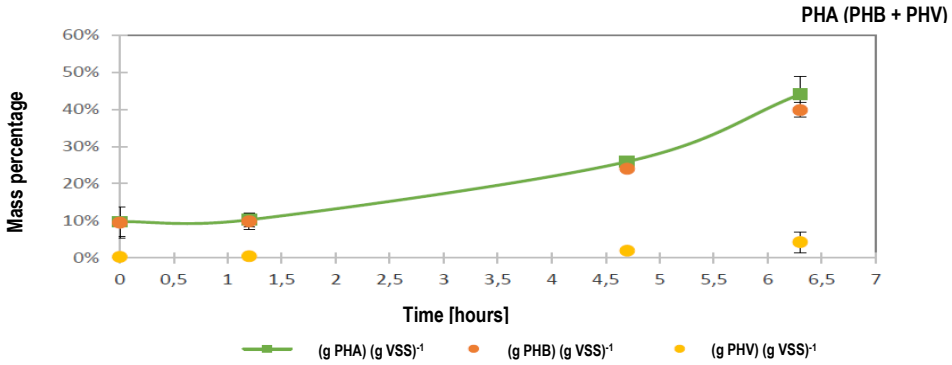


Figure 4.3. Monitoring of an accumulation cycle with 5.0 g COD L^{-1} of VFAs concentration and five pulse feedings. Extracted from Pérez, 2019); (a) DO and VFAs concentration profiles; (b) Mass percentage of PHA, PHB and PHV.

- ***DO profile and VFAs concentration.*** Figure 4.3 (a) shows DO profile and total VFAs concentration. It is seen that as DO are at a maximum value, a pulse feeding is added in the reactor. Moreover, total VFAs concentration in synthetic feed and in biomass

purge is apparently maintained at constant values with small differences in purge (about <10 mg VFAs L^{-1}). Important differences between the VFAs degradation speed (red lines) are seeing, slopes are major in first pulses (more degradation speed values) and lower as pulses are added into the system becoming less efficient operation (less VFAs degradation to PHA accumulation) over the time.

- ***Mass percentage of bioplastics.*** In Figure 4.3 (b) mass percentages of total PHA, PHV and PHB are represented. It can be observed that %PHA (g PHA) $(g\ VSS)^{-1}$ increases following an exponential trend. Same occurs in %PHB (g PHB) $(g\ VSS)^{-1}$ and PHV (g PHV) $(g\ VSS)^{-1}$. PHA is mostly composed by PHB.

5. REVIEW OF PHA PRODUCTION USING MIXED CULTURES IN PILOT OR INDUSTRIAL PLANTS

The possibility of PHA-storing biomass enrichment in wastewater treatment containing high concentration of VFA has been demonstrated by several authors (Bengtsson et al., 2007; Jiang et al., 2012; Albuquerque et al., 2010; Valentino et al., 2014). In the following sections different processes which are implemented at pilot scale plants are explained from the information available and obtained from the specified research papers.

*Each plant contains figures that explain the process in diagrams and schemes. Figures can be found at *Annex 4: Figures from the review of PHA production using mixed cultures in pilot or industrial plants*.

5.1. PRODUCTION OF PHA STORING BIOMASS WITH BIOLOGICAL NITROGEN REMOVAL OF MUNICIPAL WASTEWATER (BENGTSSON ET AL., 2017)

As it has been said, one well-established PHA production is the aerobic Feast and Famine application with nitrogen limitation as a double selective pressure strategy. Because of the common excess presence of nitrogen in municipal wastewater, usually it is necessary to include nitrogen removal stages to ensure the effectiveness of the process. So, a pre-denitrification stage must appear as influent pre-treatment in municipal wastewater treatment.

Bengtsson et al. (2017) shows the development of a process for PHA production integrated into a municipal wastewater treatment and reported the successful continuous operation for 225 days including the daily and seasonal variability in influent composition at Leeuwarden wastewater treatment plant (160,000 equivalent inhabitants, mainly treating domestic streams in Friesland, Netherlands). As stated in Figure IV.1, the process had different stages, including pre-denitrification, nitrification and post-denitrification followed by the accumulation of PHA in a pilot-scale plant (from 500 to 800 L equipment volumes). Period I of the Bengtsson et al. (2017) which involves only one SBR operation is dealt with in this section (see Figure IV.1).

The process consists in an influent wastewater stream entrance at 15-20 ºC from Leeuwarden municipality, corresponding to a population equivalent of 160 000 and mostly contribution from domestic households. This influent stream suffers a primary treatment by 800 L equalization tank operating and HRT< 30 min that remove grid and suspended solids. The following equipment is an SBR reactor in which the treatment of wastewater and the selection of biomass with PHA producing potential were performed. The main SBR operation parameters are shown in Table 5.1.

Table 5.1. Operational parameters of the SBR for Nitrogen removal and PHA accumulation described in Bengtsson et al. (2017)

Parameter	Value	Units
Active volume	500	L
Cycle time	180	min
Feeding stage	6	min
Active anoxic time	30	min
Active aerobic time	123	min
Settling time	15	min
Supernatant withdrawal	6	min
Feast / Famine ratio	0.24	-
Temperature	15	ºC
HRT	6.4	h
SRT	2.6	d
OLR	1.54	g COD L^{-1} d^{-1}

The SBR operating sequence consists in five steps:

- **Step 1)** At the beginning, 200 L of wastewater is filled into the reactor taking 6 minutes. So, the organic biodegradable matter is fed directly into the bacteria as carbon substrate with nutrients.
- **Step 2)** Secondly, anoxic Feast conditions are given for 30 minutes and denitrification is produced because of anoxic media conditions. Therefore, in order to support the nitrifying reaction (while the selection of PHA-accumulator biomass is being done in suspended biomass) an Integrated Film Active Sludge (IFAS) is used in a moving bed

biofilm reactor (MBBR) AnoxKaldes™ K5 (inoculum with established nitrifying biomass) biofilm carrier with 250 L, offering 200 m^2 biofilm growing area where that nitrifying activity is concentrated. As a result, NO_3-N and NO_2-N nitrogen presence in water is transformed into N_2 as SBR operates.

- **Step 3)** Thirdly, aerobic Famine conditions for 123 minutes are given enabling the complete application of Feast-Famine and nitrogen-uncoupled strategy and causing an enrichment of PHA-storage biomass. Nitrogen nutrient is added into the media as NH_4Cl according into double strategy selection criteria. Moreover, the aeration is given by a bottom diffusor offering to the media a Dissolved Oxygen (DO) of 4.3 mg L^{-1}.
- **Step 4)** In this step, 15 minutes of setting occurs by deactivation of agitation. Decantation of biomass is produced facilitating the supernatant withdrawing and rich PHA potential biomass stream separation to the accumulation reactor.
- **Step 5)** Finally, the supernatant withdrawing is produced for 6 minutes. Maintaining the volume reaction for the next income wastewater stream.

Enriched PHA accumulation potential biomass stream from the SBR reactor is led to an accumulation system composed by a 500 L volume batch reactor used as the accumulation reactor with an operating method governed by two sequential steps:

- **Step 1)** When initial biomass income from selection reactor is settled into accumulation reactor, from 100 to 200 mg COD L^{-1} substrate concentration additions are followed by the “feed-on-demand” control principles based on the changes in biomass respiration rates.

  Those accumulation feedstocks are VFAs enriched coming from fermented residues (each batch of fermented feedstock is prepared in 800 L pilot-scale of an acidogenic fermenter treating 150-200 L from greenhouses residues mechanically shredded and 500-550 L of water). This previous fermentation process takes about 7-8 days and the pH in the fermenter is maintained at 5.6 by NaOH additions. After the fermentation, 60-70% of the suspended solids are removed by centrifugation representing only the 5% of the COD. Soluble COD of the fermented feedstock was 9.7-12.4 COD L^{-1} of which 80-86% are VFA composed by acetic acid (34% of the COD), propionic acid (22-24%

of the COD), butyric acid (13-18% of COD) and caproic acids (9% of the COD). This fermented feedstock contains limited nutrient levels for the microorganism's growth.

- **Step 2)** The downstream treatment is required in order to maximize the biomass and PHA fraction by the use of different thickening process involving a thermal stabilization at 6 pH as the first operation and then a centrifugate dewatering drying and, as next step, a drying for 24 h at 70 ºC is applied. Lastly a mechanical granulation is done.

The conclusions obtained from the operation of this plant can be resumed in three main points:

- Total COD and N removals were achieved fulfilling European standard demands.
- Pilot plant was able to accumulate up to 49% (w/w) PHA of VSS in stable operation in PHA accumulation despite significant variable composition from influent wastewater.
- IFAS technology strategy can be readily integrated into COD and nitrogen removal from a municipal wastewater treatment plant.

5.2. PHA PRODUCTION WITH BIOLOGICAL NITROGEN REMOVAL OF SUPERNATANT FROM SEWAGE SLUDGE ANAEROBIC DIGESTION (CONCA ET AL., 2020)

In this pilot scale plant, integrated production of Polyhydroxyalkanoates (PHAs) via-nitrite nitrogen removal form anaerobic reject water is studied under long-term period. In this part period 3 of the study is explained because of the integration of a Rotating Belt Dynamic Filter (RBDF) and the smallest mesh size used of 210 μm involving the most separation between solid-liquid phases. This pilot plant is in Carbonera Wastewater Treatment Plant (WWTP) in Treviso, Italy. The concrete process studied involves six different units to treat around 10% of the daily produced anaerobic reject water (about 1.5-3.0 m^3 d^{-1}) and can be seen represented in Figure IV.2. This process and units are explained on below as a sequence of six steps:

Step 1) Cellulosic Primary Sludge (CPS) is separated from raw wastewater using a rotating belt dynamic filter. This CPS stream is sent into Sequencing Batch Fermentation Reactor (SBFR) and the liquid stream goes to main wastewater treatment line from the WWTP.

Step 2) Using CPS as a feedstock, the application of an anaerobic fermentation in SBFR to convert CPS into VFAs-rich stream as Cellulosic Primary Sludge Fermented Liquid (CPSFL). It is done using 2.6 m^3 working volume and a fixed temperature of 37 ºC using an electrical heating system. The HRT is maintained from 4 to 6 days. The control of pH is not required, and the mixed liquor is constantly agitated by a mixer at 20 rpm. By this process, the VFAs concentration generated is approximately about 9975 mg COD L^{-1}.

Step 3) The CPSFL is filtered by a solid-liquid separation system composed by 7 ceramic membranes with 8 tubular channels and a porosity of 0.2 μm and specific surface area of 0.2 m^2 in each membrane. Operating with an internal recycle into the SBFR unit. The permeate from this solid-liquid separation unit is stored in a 1 m^3 intermediate tank to ensure a constant flowrate to the following units.

Step 4) Nitrification is effectuated into a Sequencing Batch Reactor (N-SBR) where $N\text{-}NH_4^+$ is oxidized to $N\text{-}NO_3^-$. Reaching this objective demands four phases:

- **Idle**. The N-SBR is inoculated previously by sludge from the biological reactor of the main wastewater treatment line and adopting the strategy for Ammonium Oxidizing Bacteria (AOB) enrichment by maintaining a free ammonia level higher than 1.5 mg NH_3 L^{-1} to foster the stable Nitrite Oxidizing Bacteria (NOB) washout.
- **Feeding**. Using the anaerobic reject water from an anaerobic digestor as feedstock in the reactor after being pumped from an equalization tank of 90 m^3 volume where the effluent is withdrawn through a centrifugate pump into another equalization tank of 1.4 m^3 volume in order to feed the 1.1 m^3 working volume of the N-SBR.
- **Aerobic condition**. By four ultra-fine bubble diffusers at the bottom of the reactor and a centrifugate blower, dissolved oxygen is provided and controlled at 1.5 mg O_2 L^{-1} using a valve installed on the pipe for air supply. Allowing the nitrification reaction taking part in the mixed liquor**.**
- **Settling and discharge**. By stopping aeration, supernatant can be separated from biomass and then pumped into the selection reactor.

N-SBR consists in a stainless-steel SBR and over the operation pH is controlled at 7.5-8.0 values by automatic additions of 30% NaOH (w/v) and a heating system maintains the temperature at 22-25 ºC.

Step 5) Using another SBR as selection reactor (S-SBR) operating in aerobic-Feast and anoxic-Famine with a Feast/Famine ratio under 0.2 value as selection strategy. The operation process in reactor can be described as 4 different points:

- **Feast**. Firstly, the treated effluent in N-SBR arrives in anoxic conditions and it is mixed in all the operation points, except in settling and discharge points, by a mixer at 100 rpm. Then DO is given through ultrafine bubble diffusors maintaining the DO value at 2 mg O_2 L^{-1}. Although, the CPSFL as carbon source for Feast is pumped and fed into the reactor.
- **Famine**. At Famine conditions no air-supply is given, indeed mixed liquor in S-SBR has an anoxic condition. The presence of N-NO_3^- in the medium is used by microorganisms as electron acceptor for the biological denitrification with PHA-reservoirs. Because of that, the growing of PHA-producer biomass is done.
- **Settling and discharge**. With the agitation and air-supply deactivated the sedimentation of biomass allows the discharge by a pneumatic valve of the treated effluent in a hand and in the other hand sedimented selected biomass can be pumped as microorganism supply for the accumulation reactor.

The S-SBR is a stainless-steel SBR whit a 2.8 m^3 working volume where ORP and DO are monitored, the blower has a variable frequency drive to maintain DO concentration as a constant value and no pH control is required.

Table 5.2. Operational parameters of the SBR described in Conca et al. (2020).

Parameter	Value	Units
Active volume	2.8	m^3
Cycle time	480	min
Feast / Famine ratio	<0.2	-
Temperature	25-29	ºC
HRT	1.7	d
SRT	6-7	d
OLR	1.58	kg COD m^{-3} d^{-1}

Step 6) In a Sequencing Batch Reactor for the accumulation of PHA (A-SBR) and 1 m^3 of working volume. Biomass sludge from S-SBR is used to apply different batches of CPSFL as carbon source by following a feed-on demand and pulse-feeding strategy according to the dissolved oxygen profile. Aerobic conditions are maintained all over the operation time of the reactor (except from the settling and discharge point) by ultra-fine bubble diffusers through a centrifugate blower at the bottom of the reactor. Initial concentrations of COD_{VFA} L^{-1} are from 0.7-1.2 g COD_{VFA} L^{-1} range to prevent any substrate inhibition as it is reported by Valentino et al. (2019). This accumulation cycles take about 6-7 h duration per cycle.

By resuming the results of the production process followed by Conca et al. (2020) it can be seen those points:

- About the 80% of the influent ammonia was efficiently removed.
- Good PHA accumulation yields were observed by aerobic Feast and anoxic Famine conditions (0.58-0.68 (g COD_{PHA}) (g COD_{VFA})$^{-1}$).
- Revenue could be about 2.8 to 6.5 € PE^{-1} globally if anaerobic digestion with PHAs recovery is integrated in the sludge line.
- This process can produce about 1.2 kg PHA PE^{-1}.
- Co-production of biogas is obtained.

5.3. PHA PRODUCTION USING FERMENTED OFMSW (VALENTINO ET AL., 2018)

In this pilot-scale plant organic wastes as a feedstock to produce PHA is used in order to reduce the associated costs to the process. This plant becomes cost-effective by using as source of VFAs the Organic Fraction of the Municipal Solid Waste (OFMSW) and without a nitrogen removal treatment. A process scheme is shown in Figure IV.3. The production process consists in three steps and involves both anaerobic and aerobic conditions:

Step 1) Firstly, anaerobic fermentation is performed in a 200 L Continuous Stirred Tank Reactor (CSTR) operating in thermophilic conditions (55 ºC) with an OLR 20.0 kg VS $m^{-3} \cdot d^{-1}$, HRT 3.3 d in a pH of 5.0-5.6. As a result, significant COD conversion from OFMSW into VFAs with 80% presence of Acetic, Propionic and Butyric acids respect of total VFAs is obtained. This process operates sufficiently stable under steady state after a consistent production of VFAs. The separation between solid and liquid phases are carried out in downstream by using a coaxial filter bag with 5.0 μm porosity equipped centrifuge.

Step 2) This step involves the operation of a selection reactor in a SBR in aerobic conditions with 140 L of volume. The SBR was initially inoculated using sewage sludge from Treviso Wastewater Treatment Plant (WWTP). Operating for 90 days. HRT, SRT and cycle duration are maintained at 1 d, 1 d and 6 h respectively. The reactor has linear membrane blowers as oxygenation system. The pH and temperature are constantly measured but no control is required, pH had a value of 8 and nearly. Temperatures fluctuated between 28 ºC as the highest temperature and 16 ºC as the lowest depending on the external conditions and weather. OLR was 3.4 g of soluble COD $L^{-1} \cdot d^{-1}$ but then decreased to 2.00 g of soluble COD $L^{-1} \cdot d^{-1}$ to regulate OLR and maintaining a Feast-Famine ratio under 0.12. The SBR reactor performance is evaluated based on different measurements as biomass VSS and PHA concentrations. Operational parameters of the SBR are summarized in Table 5.3.

Table 5.3. Operational parameters summarized from SBR selection reactor (Valentino et al., 2018).

Parameter	Value	Units
Active volume	140	L
Cycle time	360	min
Feeding stage	6	min
Active anoxic time	0	min
Active aerobic time	360	min
Feast / Famine ratio	0.12	-
Temperature	16-28	ºC
pH	8	-
HRT	1	d
SRT	1	d
OLR	2.00-3.40	g COD L^{-1} d^{-1}

Step 3) Lastly, an accumulation reactor as a BR in aerobic conditions and using as method the fed-batch feed-on-demand based by the addition of enriched VFAs stream as fast biodegradable carbon resource. This strategy is based on Valentino et al. (2014) results, where it is observable that mixed microbial cultures express a reproducible PHA accumulation potential with the presence of nutrients or not. By controlling levels of OLR to adjust the time of the operation and the duration of the PHA accumulation.

The conclusions obtained from the operation of this plant can be summarized as:

- Production of PHA is possible in a process that uses as a prime resource the urban organic waste.
- Thermophilic fermentation of Organic Fraction of OFMSW produces a VFAs-rich stream usable for bioplastic synthesis as a source for selection and accumulation reactors.
- Aerobic processes do not require temperature and saving energy requirements.
- Capable accumulation of PHA was up to 49% (g PHA) (g VSS)$^{-1}$ in the accumulation reactor.

5.4. PHA PRODUCTION USING FERMENTED WASTE ACTIVATED SLUDGE AND OFMSW (MORETTO ET AL., 2019)

Another biorefinery technology chain has been developed at pilot scale in Treviso WWTP in Italy with a Technology Readiness Level (TRL) at level 5 showing the validation of the process in a real place environment. This biorefinery process consists in five steps producing biogas and bioplastic as products. The diagram of the process can be seen in Figure IV.4. It is described on below as step by step process:

Step 1) A mixture of biological sludge and OFMSW is fed into an anaerobic fermenter (this feeding is available in Treviso WWTP). The biological sludge comes from a static thickener after a process of biological nutrient removal applied in Treviso WWTP water line. OFMSW comes from a separate collection involving different districts from Treviso province. This waste goes into a dedicated plant for solid-liquid separation by a mechanical screw-press. The liquid fraction is sent to anaerobic co-digestion using biological sludge in Treviso WWTP. Moreover, solid fraction is sent to composting. The solid fraction from the waste and the biological sludge suffers a weekly collection from Treviso WWTP converted into biorefinery sources.

Those biorefinery sources are the fed for the anaerobic fermenter working in batch mode operation in a 380 L reactor equipped with a temperature control system jacket and a mechanical stirrer. A thermal pre-treatment at 72 ºC for 48 h to the feedstock mixture inside the reactor to maximize the organic matter solubilization and after the 48 h the temperature into the fermenter is decreased to 37 ºC for four days. In this step a fermented stream enriched in volatile fatty acids is obtained and sent a solid-liquid separation unit.

Step 2) A solid-liquid separation unit consisting in a coaxial centrifuge with 5.0 μm porosity nylon filter bag for solids removal and a 0.2 μm porosity ultrafiltration membrane is applied to the stream obtained in step 1 due to produce a solid removal before it is fed into the sequencing batch reactor for PHA selection and into the batch reactor for PHA accumulation.

Step 3) A SBR due to the selection of PHA-producer biomass in a 100 L working volume by using Feast-Famine in aerobic conditions. Dissolved oxygen is maintained at 8 mg O_2 L^{-1} using linear membrane blowers which allows a complete stirring of the mixed liquor. The pH and temperature are constantly monitored. Temperature is maintained at 25-28 ºC because of an immersion

heater. The Feast-Famine cycles were automatized and controlled by a Programmable Logic Controller (PLC). General SBR characteristics are shown in Table 5.4. The feedstock used is the liquid VFAs-rich stream from the anaerobic fermentation after the solid-liquid separation is done. The PHA-producer biomass stream from the SBR is pumped into the accumulation reactor.

Table 5.4. Operational parameters summarized from SBR selection reactor (Moretto et al., 2019).

Parameter	Value	Units
Active volume	100	L
Cycle time	360	min
Active anoxic time	0	min
Active aerobic time	360	min
Feast / Famine ratio	*	-
Temperature	25-28	ºC
HRT	1	d
SRT	1	d
OLR	3.10-5.90	g COD L^{-1} d^{-1}

* No information available.

Step 4) By the use of fed-batch mode and the same equipment described by the selection reactor the accumulation of PHA is induced in this unit ranging working volumes between 80 L and 120 L and using the same feedstock as the selection reactor but maintaining an initial relation between VFAs and selected biomass under 2.0 on COD basis to prevent substrate or pH inhibition.

Step 5) Anaerobic co-digestor is applied to obtain biogas using solid-rich streams from the solid-liquid separation unit and thickened biological sludge as feed. It is done in stainless steel CSTR (AISI-304) with a working volume of 230 L equipped with mechanical stirrer and temperature is maintained between 37 ºC and 55 ºC because of hot water recirculation in external jacket. The inoculum used comes from a full-scale digester of the Treviso WWTP and suffers an acclimatization for at least 2 HRT.

Therefore, the production process described in Moretto et al. (2019) leads to:

- An overall PHA yield obtained was about 59% (g COD_{PHA}) (g COD_{VFA})$^{-1}$ and 51% (g PHA) (g VSS)$^{-1}$.
- The co-production of biogas and PHA.

5.5. **PHA PRODUCTION IN A SINGLE STAGE MEMBRANE BIOREACTOR FOR SELECTION AND ACCUMULATION (JIA ET AL., 2014)**

This pilot-scale plant operates in a continuous production of a fermented liquid and PHA (see Figures IV.5 and IV.6). The system comprised different stages involving membrane systems to effective separation between solid and liquid phases:

Step 1) Continuous Anaerobic Fermentation Reactor (AFR) producing the hydrolytic-acidogenic fermentation to the excess sludge. A waste-sludge stream coming from WWTP that uses an anaerobic-anoxic-oxic strategy to treat residual water is pumped into an AFR unit with 4000 L working volume, an HRT of 2 d and working at 50 ºC. This generates a high-quality Sludge Fermentation Liquid (SFL) containing a 65 % in mass of (mg COD_{VFAs}) (mg COD_{total})$^{-1}$.

Step 2) A Ceramic membrane system (CMS) is applied to treat the SFL getting as result a high-quality SFL due to the separation of water and giving a membrane outcome flow of 100 to 120 L h^{-1}. This filtrated liquid is stored in an intermediate tank and then it is pumped into the bioreactor (described in step 3).

Step 3) By operating in Feast-Famine cycles, at 30 ºC, with an agitation of 150 rpm and maintaining aerobic conditions without the requirement of pH control, the selection of PHA biomass and accumulation either are carried out in an SBR reactor because of Feast-Famine strategy. At the end of the Feast phase about 46% of the total working volume of the PHA-enriched bacteria (46 L) is collected for PHA extraction unit and the rest (24 L) goes into the bioreactor as microorganism supply during Famine phase and overflew goes into the AFR through a CMS obtaining dewatered fresh sludge. Lastly, PHA extraction is done at the same pilot plant obtaining PHA as a final product.

Therefore, the conclusions obtained from the operation of this plant can be summarized as:

- PHA production could be done by VFAs-enriched liquid stream from anaerobic fermentation of waste sludge coming from a wastewater treatment plant.
- Ceramic Membrane System obtains a high-quality sludge fermentation liquid that used to obtain high quality PHA products.
- No treatment of nitrogen removal is required to produce bioplastic effectively.
- Possibility to obtain high levels of bioplastic accumulation as 59.47% (w/w) of PHA from total dry cell weight in four accumulation operation cycles.

5.6. COMPARASION OF THE PROCESSES REVIEWED FOR PHA PRODUCTION FROM ORGANIC WASTES

In the previous section, five pilot-scale plants are described. Each one includes an anaerobic acidogenic fermentation process to obtain VFAs as carbon sources from the influent. Various kinds of influents are used in the pilot-scale plants being the major process conditioners and determining the principal operation parameters.

If the PHA production process is implemented in a municipal WWTP with the aim to couple this bioplastic production treatment with biological nitrogen removal the PHA-storing biomass selection can be achieved. In the selection process, the Feast stage, where VFAs are consumed and converted to PHA, is typically carried out under aerobic conditions while Famine stage could be destined to denitrification under anoxic conditions using the previously stored PHA. However, it can be used an anoxic Feast, producing denitrification and PHA, followed by aerobic Feast, which uses nitrification to produce the bacteria growth. Nevertheless, an enriched carbon stream from an acidogenic fermentation of sewage sludge with a relatively high C/N ratio is typically used as a feed stream to the accumulation process, which is carried out under aerobic conditions. Maximum PHA production yields resulting from this kind of operation are nearby 68% (g COD_{PHA}) (g COD_{VFA})$^{-1}$.

The use of biological sludges or OFMSW as influent, demands an anaerobic acidogenic fermentation to obtain VFAs as carbon sources from those influents to select and accumulate PHA. Nonetheless, if the biological nitrogen removal process is not integrated in the PHA

production process, a full aerobic Feast-Famine operation in selection reactor is usually required elevating the general cost of operation. Moreover, all plants operations had Feast/Famine ratios lower than 0.2 or closer to this value. The major values of PHA yield are 59% (g COD_{PHA}) (g COD_{VFA})$^{-1}$.

Referring to OLR, major values of OLR requirements (2.00-5.90 g COD L^{-1} d^{-1}) are used in OFMSW and biological sludges influents rather than wastewater influent plants and anaerobic digestion supernatant, which use around 1.50 g COD L^{-1} d^{-1}, becoming the plants with less values of OLR required.

Furthermore, general PHA production yields from accumulation tests are nearby 50% (g COD_{PHA}) (g COD_{VSS})$^{-1}$. Namely, for the pilot plants studied, the PHA production ranges between 49% (g PHA) (g VSS)$^{-1}$, which was obtained in Bengtsson et al. (2017) and Valentino et al. (2018), to a maximum value of 68% (g COD_{PHA}) (g COD_{VSS})$^{-1}$, which was obtained by Conca et al. (2020). Even so, major scale operation plant is done in Conca et al. (2020) working with a 2.8 m^3 active volume in S-SBR and 1 m^3 working volume in A-SBR. Other plants work with 500 L to 100 L of active volumes.

In conclusion, choosing a PHA production technology will depend on the biorefinery objectives. If nitrogen removal is the principal problem, PHA production by using nitrification and denitrification could be suitable. However, most complexed operation is required. Instead, if PHA production is the main cause, a treatment using OFMSW or biological sludges could be the best option. Offering a possibility to eliminate those solid wastes while obtaining high levels of PHA accumulation.

6. CONCLUSIONS AND RECOMMENDATIONS

From laboratory experimentation, different conclusions are obtained:

- VFAs as a carbon source could promote the accumulation of PHA.
- Aerobic Dynamic Feeding with nitrogen uncoupled in Feast phase is an effective selection PHA-producer biomass strategy showing the capability to operate easily in a pseudo-stationary phase.
- Total ammonium nitrogen consumption during Famine is important to ensure PHA accumulation under feast conditions due to microbial growth limitation of this nutrient.
- Suitable biomass adaptation with a 2.5 g COD L^{-1} d^{-1} offers the possibility to study higher organic loads improving PHA accumulation potential, although the oxygen supply should be highly increased.
- When compared with butyric and propionic acids, acetic acid had the lowest uptake speed.

By the comparison of the different pilot scale PHA production processes it is seen these different points:

- PHA production yields are similar nearby the 50% (g PHA) $(g\ VSS)^{-1}$.
- Feast-Famine ratios are nearby 0.2 or lower indicating a good selection of PHA-producer biomass.
- In the processes in which municipal wastewaters are influents, nitrification and denitrification could be used to do the selection phase by alternating anoxic and aerobic conditions.
- OLR values in the range of 2.00-5.90 g COD L^{-1} d^{-1} are required (if OFMSW or biological sludges are used as feedstock). However, lower values of OLR (around 1.50 g COD L^{-1} d^{-1}) are recorded in wastewater influent plants and anaerobic digestion supernatant influents.

Recommendations

- By seeing the efficiency and variety of technology in the different PHA productions processes at pilot scale, different industrial scale process should be studied and implemented.
- Real effluent as carbon source in lab studied process could be done to see PHA selection and accumulation results.

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ACRONYMS

ADF, Aerobic Dyinamic Feeding; **AOB**, Ammonium Oxidizing Bacteria; **A-SBR**, Accumulation Sequencing Batch Reactor; **BR**, Batch Reactor; **CMS**, Ceramic Membrane System; **COD**, Chemical Oxygen Demand; **CPS**, Cellulosic Primary Sludge; **CSTR**, Continuous Stirred Tank Reactor; **DO**, Dissolved Oxygen; **F-F**, Feast-Famine; **HB**, Hydroxybutyrate; **HRT**, Hydraulic Retention Time; **HV**, Hydroxyvalerate; **IFAS**, Integrated Film Active Sludge; **NOB**, Nitrite Oxidizing Bacteria; **N-SBR**, Nitrification Sequencing Batch Reactor; **OFMSW**, Organic Fraction of Municipal Solid Waste; **ORP**, Oxidation-Reduction Potential; **PE,** Population Equivalent; **PHA**, Polyhydroxyalkanoates; **PHA-mcl**, Polyhydroxyalkanoates middle chain length; **PHA-scl**, Polyhydroxyalkanoates small chain length; **PHB**, Polyhydroxybutyrate; **PHV**, Polyhydroxyvalerate; **PLC**, Programable Logic Controller; **RBDF**, Rotating Belt Dynamic Filter; **SBR**, Sequencing Batch Reactor; **S-SBR**, Selection Sequencing Batch Reactor; **SBFR**, Sequencing Batch Fermentation Reactor; **SRT**, Solid Retention Time; **SS**, Suspended Solids; $\mathbf{T_g}$, Stained glass temperature; $\mathbf{T_m}$, Fusion temperature; **TRL**, Technology Readiness Level; **TSS**, Total Suspended Solids; **VFA**, Volatile Fatty Acid; **VFAs**, Volatile Fatty Acids; **VS,** Volatile Solids; **VSS**, Volatile Suspended Solids; **WWTP**, Wastewater Treatment Plant.

ANNEXES

ANNEX 1: VFAS COMPOSITION AND SELECTION REACTOR CONCENTRATION OF AMMONIUM CHLORIDE REQUIRED PER DAY

Volume required of each VFA in the synthetic feed of 3.5 g COD L^{-1}:

1) Volume required by each acid depending on mass proportions (x) exposed on (Dosta et al. 2018):

a. Acetic acid (HAc):

$$\frac{0.625\ x\ g\ HAc}{L}\ \frac{1\ mol\ HAc}{60.05\ g\ HAc}\ \frac{2\ mol\ O_2}{1\ mol\ HAc}\ \frac{32\ g\ O_2}{1\ mol\ O_2} = 0.670\ x \frac{g\ O_2}{L}$$

b. Propionic acid (HPr):

$$\frac{0.188\ x\ g\ HPr}{L}\ \frac{1\ mol\ HPr}{74.04\ g\ HPr}\ \frac{3.5\ mol\ O_2}{1\ mol\ HPr}\ \frac{32\ g\ O_2}{1\ mol\ O_2} = 0.284\ x \frac{g\ O_2}{L}$$

c. Butyric acid (HBt):

$$\frac{0.188\ x\ g\ HBt}{L}\ \frac{1\ mol\ HBt}{88.11\ g\ HBt}\ \frac{5\ mol\ O_2}{1\ mol\ HPr}\ \frac{32\ g\ O_2}{1\ mol\ O_2} = 0.342\ x \frac{g\ O_2}{L}$$

2) COD balance to obtain x (mass proportion):

$$\frac{3.5 g\ COD}{L} = (0.670 + 0.284 + 0.342)\ x \frac{g\ O_2}{L}$$

$$\frac{3.5 g\ COD}{L} = 1.296\ x\ \frac{g\ O_2}{L}$$

$$x = 2.70$$

3) Acid volume required with x=2.70 for 10L of volume:

a. Acetic acid (HAc):

$$\frac{0.625\ x\ g\ HAc}{L} = \frac{0.670\ x\ g\ O_2}{L} = \frac{0.670\ 2.70\ g\ O_2}{L} = 1.69\ \frac{g\ HAc}{L}\ \frac{1\ L}{1050\ g\ HAc}\ 10\ L$$
$$= 0.016\ L\ HAc = 16.10\ ml\ HAc$$

b. Propionic acid (HPr):

$$\frac{0.188\ x\ g\ HPr}{L} = \frac{0.188\ x\ g\ O_2}{L} = \frac{0.188\ 2.70\ g\ O_2}{L} = 0.51\ \frac{g\ HPr}{L}\ \frac{1\ L}{990\ g\ HPr}\ 10\ L =$$
$$= 0.00515\ L\ HPr = 5.15\ ml\ HPr$$

c. Butyric acid (HBt):

$$\frac{0.188\ x\ g\ HBt}{L} = \frac{0.188\ x\ g\ O_2}{L} = \frac{0.188\ 2.70\ g\ O_2}{L} = 0.51\ \frac{g\ HBt}{L}\ \frac{1\ L}{960\ g\ HBt}\ 10\ L =$$
$$= 0.00531\ L\ HBt = 5.31\ ml\ HBt$$

Concentration of ammonium chloride required per day:

1) COD destinated to the bacteria growth:

$$Y_{obs} = \frac{Y}{1 + k_d\ SRT} = \frac{0.035}{1 + 0.1\ 4.21} = 0.25\ \frac{g\ DQO\ to\ celular\ growth}{g\ DQO}$$

Where:

$Y_{obs}\ is\ heterothropic\ performance\ on\ anoxic\ conditions.$

k_d *is the death constant.*

2) COD concentration required per day:

a. Acetic acid (HAc):

$$\frac{0.670\ x\ g\ O_2}{L}\ \frac{1}{HRT} = \frac{0.670\ 2.70\ g\ O_2}{L}\ \frac{1}{1.12\ d} = 1.61\frac{g\ DQO}{L\ d}$$

b. Propionic acid (HPr):

$$\frac{0.188\ x\ g\ O_2}{L}\ \frac{1}{HRT} = \frac{0.188\ 2.70\ g\ O_2}{L}\ \frac{1}{1.12\ d} = 0.45\frac{g\ DQO}{L\ d}$$

c. Butyric acid (HBt):

$$\frac{0.188\ x\ g\ O_2}{L}\ \frac{1}{HRT} = \frac{0.188\ 2.70\ g\ O_2}{L}\ \frac{1}{1.12\ d} = 0.45\frac{g\ DQO}{L\ d}$$

d. Total (OLR):

$$(1.61 + 0.45 + 0.45)\frac{g\ DQO}{L\ d} = 2.51\frac{g\ DQO}{L\ d}$$

3) NH_4Cl mass required:

$$2.51\frac{g\ DQO}{L\ d}\ 3.75\ L\ \frac{0.25\ g\ DQO\ cell\ growth}{1\ g\ DQO}\ \frac{1\ g\ C_5H_7O_2N}{1.42\ DQO}\ \frac{1\ mol\ C_5H_7O_2N}{113\ g\ C_5H_7O_2N}\ \frac{1\ mol\ N}{1\ mol\ C_5H_7O_2N}$$

$$\frac{1\ mol\ NH_4Cl}{1\ mol\ N}\ \frac{53.5\ g\ NH_4Cl}{1\ mol\ NH_4Cl} = 0.785\ \frac{gNH_4Cl}{d}$$

$$\frac{1}{52\frac{ml}{cycle}\ 4\frac{cycle}{1\ d}\ \frac{1L}{1000ml}\ \frac{1\ d}{0.785\ gNH_4Cl}} = 3.77\frac{gNH_4Cl}{L}$$

ANNEX 2: ANALYTICAL METHODS PROCEDURES

Total suspended solids (TSS) (2540D)

1. A quantitative paper filter (MilliPore of 0.45 μm) is put on a metal plate and then goes in a stove at 100 ºC for 24 h in order to eliminate possible impurities.
2. After the 24 hours, the plate with filter weight (M_o [g]) is measured and wrote using scientific balance with a +/-0.0001 precision.
3. Then some volume (V [L]) of the biomass purge is vacuum filtered by using the filter put into the metal plate and using a Kitasato and vacuum bomb.
4. The filter goes into a 100 ºC stove for 24 hours to eliminate the water presence in the sample.
5. The next step is to measure the weight passed the 24 hours (M_f [g]) and wrote it.
6. Using the difference between the weight of the plate with biomass and the weight without it and divided by the volume added at point 3 the TSS concentration is obtained (see Equation 3.1).

Volatile suspended solids (VSS) (2540E)

1. The starting point is the sixth step of the TSS analysis. The metal plate goes into a muffle at 500 ºC for 2 hours producing the elimination of the organic particles.

2. Then the weight of the plate (M_l [g]) is measured and wrote. The difference between the weight of the sixth step plate in SS analysis and this plate divided by the volume added at step 3 of the TSS process is the concentration of the biomass into the reactor.

Polyhydroxyalkanoates (PHA) content

a) Standards preparation:

1. 6.0 g of the PHA patron which contains 88% in mass of PHB and 12% in mass of PHV is dissolved into 1 L of chloroform with constant concentration of benzoic acid as an internal patron and indicator of chromatogram peaks because the peak caused by this acid will be a constant and then the PHB and PHV peaks will change peaks area (y label in chromatogram with intensity as unit) in function of the concentration but distances (x label in chromatogram in minutes) between benzoic acid, PHB and PHV are maintained. Moreover, if some changes in benzoic acid area peaks are experimented it indicates dilutions or some sample changes in composition invalidating the results of the sample.

2. In order to prepare the patrons, a Hamilton 1000 μL syringe cleaned using the point 1 and under into a vitrine. Different volumes (100 μl, 200 μl, 300 μl, 400 μl, 500 μl and 600 μl) of the solution described at the point 1 are added into different test tubes and after, some chloroform is added arriving to 1 ml volume.

3. Next step consists in adding 1 ml of methanol (20% sulphuric) as non-solvent in each tube resulting in 2 ml volume and producing the bacterial wall. Then test tubes must be closed in order to avoid chloroform and methanol evaporation.

4. Those test tubes are put into a COD digestor for 5 hours at 100 ºC. After that time, samples are cooled with water and ice for 30 minutes. After those 30 minutes, 0.5 ml of water are added into test tubes producing two phases one aqueous phase which water is the dissolvent and the other is the organic phase where methanol is the dissolvent compound. Bottom phase is the aqueous phase which contains chloroform and PHA dissolved. Then the tubes suffer a vortex centrifugation for a minute.

5. About 2-3 molecular sieves are used as water adsorbent and put into chromatography vial. Using a Pasteur pipe, 0.5 ml of the bottom phase commented at point 4 are taken and put into those chromatography vials and closed hermetically.

It is important to say that when the analysis could not be done at the same day closed vials can be saved into a fridge.

6. Analysis of the samples are done by gas chromatography technique (Shimadzu GC 2010 plus) equipped with a capillary column (Nukol™, 15 m x 0.53 mm x 0.5 µm) and a flame ionization detector (FID). The chromatograph uses helium as carrier gas, hydrogen as fuel gas and synthetic air as the oxidizing gas. The temperature of the capillary column starts at 80 ºC and is heated by 10 ºC·min^{-1} to 110 ºC. From then on, the temperature increases 15 ºC·min^{-1} until 145 ºC and, finally, it is increased 20 ºC·min^{-1} to 190 ºC.

7. Using an integration of the peaks given by chromatography due to the different concentrations of PHA two lineal calibrations can be done by the relation of the area (abscise label) and concentration of PHV and PHB (ordinate axis).

b) Samples analysis:

1. Biomass due to analysis of PHA samples are frozen during 24 h at -80 ºC producing a biomass deactivation state. After that time lyophilization of the samples are done taking 24 h at -52 ºC and by the decrease of the pression water is sublimated.

2. After the lyophilization process, 2 mg of biomass samples are taken using a scientific balance with +/- 0.0001 precision and this weight is put on a test tube.

3. Next step consists in adding 1 ml of methanol (20% sulphuric v/v) as non-solvent and as bacterial wall rupture. Then 1 ml of chloroform with the constant benzoic acid

concentration is put on the tube obtaining 2 ml total volume and PHA solubilization. It is important to close the tubes hermetically.

4. Those test tubes are put into a COD digestor for 5 hours at 100 ºC. After that time, samples are cooled with water and ice for 30 minutes. After those 30 minutes, 0.5 ml of water are added into test tubes producing two phases one aqueous phase which water is the dissolvent and the other is the organic phase where methanol is the dissolvent compound. Bottom phase is the aqueous phase which contains chloroform and PHA dissolved. Then the tubes suffer a vortex centrifugation for a minute.

5. Analysis of the samples are done by gas chromatography technique (Shimadzu GC 2010 plus) equipped with a capillary column (Nukol™, 15 m x 0.53 mm x 0.5 µm) and a flame ionization detector (FID). The chromatograph uses helium as carrier gas, hydrogen as fuel gas and synthetic air as the oxidizing gas. The temperature of the capillary column starts at 80 ºC and is heated by 10 ºC·min^{-1} to 110 ºC. From then on, the temperature increases 15 ºC·min^{-1} until 145 ºC and, finally, it is increased 20 ºC·min^{-1} to 190 ºC.

6. By using the calibration done at point 7 in standards preparation PHB and PHV mass compositions can be obtained from the area given by the chromatography.

ANNEX 3: SELECTION REACTOR PUMPS CALIBRATION

Pumps are calibrated to know the real flow that is given by them. The process followed is to measure the volume whit different capacity test tubes (25 - 250) ml given by the pumps in different times and speeds to obtain a linear expression. Hence a straight gauge is obtained to ensure that the real values follow these calibrations.

The volumetric flows corresponding on the duration phases of the cycle obtained for each pump are the following ones:

NH_4Cl flow per cycle:

$$q_{NH4Cl}\left[\frac{ml}{s}\right] = 0.0138 * x + 0.0214 \quad \text{(III.1)}$$

$$Where\ x\ [-]\ is\ the\ velocity\ of\ the\ bomb.$$

In this experiment the x has the value of 30 and that gives a 0.43 ml s^{-1} flow corresponding into 52 ml $cycle^{-1}$.

Purge flow per cycle:

$$q_{purge}\left[\frac{ml}{s}\right] = 0.008 * x - 0.255 \quad \text{(III.2)}$$

$$Where\ x\ [-]\ is\ the\ velocity\ of\ the\ bomb.$$

Pump velocity is fixed at 98 and the volumetric flow obtained is 0.53 ml s^{-1} and that means 222.18 ml $cycle^{-1}$.

Feed flow per cycle:

$$q_{feed}\left[\frac{ml}{s}\right] = 0.04 * x + 0.133 \quad \text{(III.3)}$$

$$Where\ x\ [-]\ is\ the\ velocity\ of\ the\ bomb.$$

In this case x is 20 and that gives 0.93 ml s^{-1} flow which is 837 ml $cycle^{-1}$.

Effluent flow requirements per cycle:

This pump works different because it works by tube level different in order to extract 837 ml before the feed is given to maintain the working volume at the valued fixed of 3.75 L so the tube connected to this must be putted at 2.9 L level on the reactor.

The minimal volumetric flow required to pump off the reactor this volume is:

$$q_{min}\left[\frac{ml}{s}\right] = \frac{837\ ml}{1cycle\ 15\ \frac{min}{cycle}} = 55.8\ \frac{ml}{min} \quad \text{(III.4)}$$

The flow obtained for this pump at pump speed of 70 is 148.75 ml min^{-1} so it is enough to satisfy the objective.

ANNEX 4: FIGURES FROM THE REVIEW OF PHA PRODUCTION USING MIXED CULTURES IN PILOT OR INDUSTRIAL PLANTS

Production of PHA storing biomass with biological Nitrogen Removal of municipal wastewater (Bengtsson et al., 2017)

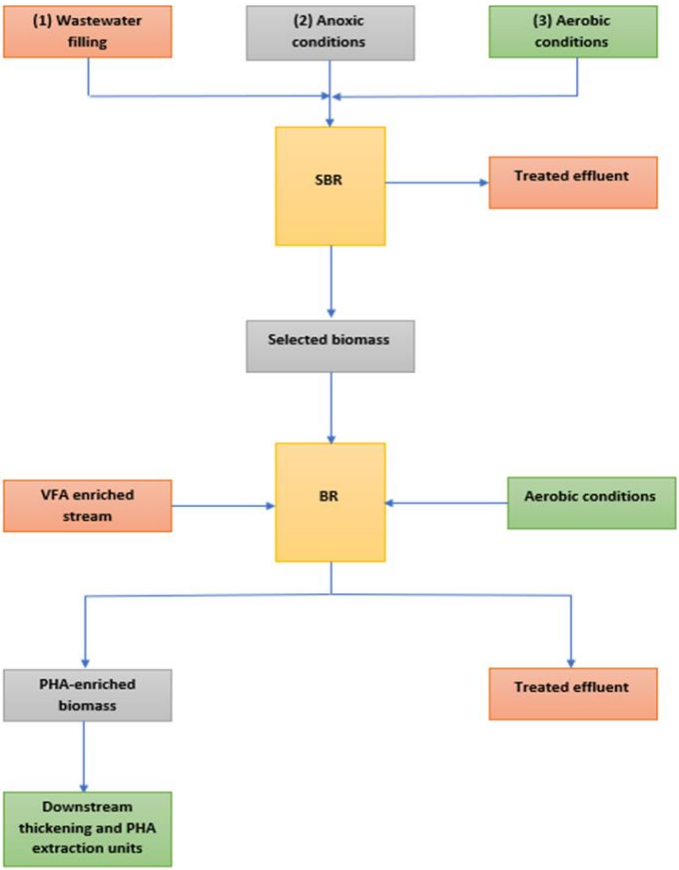

Figure IV.1. Operating diagram of pilot-scale plant from Bengtsson et al. (2017) based in aerobic Feast and Famine and N as limited nutrient. (SBR: Sequencing Batch Reactor; BR: Batch Reactor).

PHA production with biological Nitrogen Removal of supernatant from sewage sludge anaerobic digestion (Conca et al., 2020)

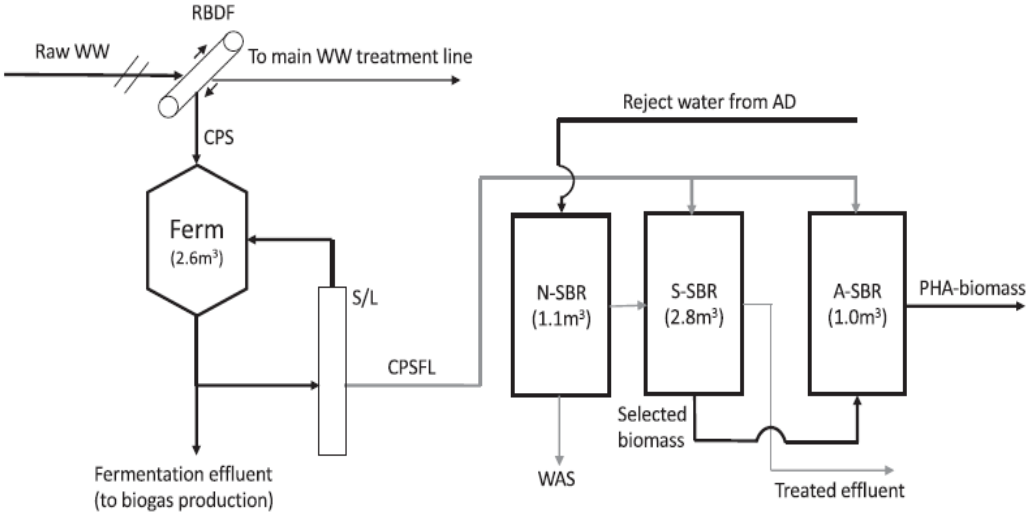

Figure IV.2. Process schematization from the pilot-scale plant described in Conca et al. (2020). Extracted from Conca et al. (2020).

PHA production using fermented OFMSW (Valentino et al. 2018)

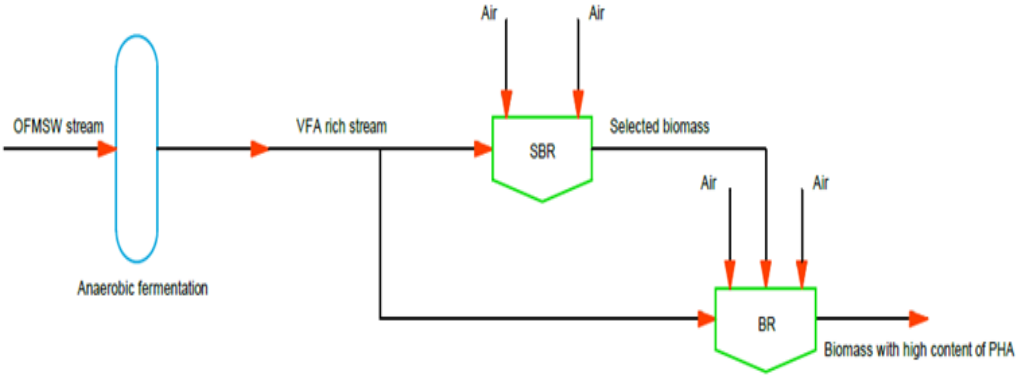

Figure IV.3. Flow diagram of the pilot-scale plant based on the description of Valentino et al. 2018. (Made with AutoCAD software).

PHA production using fermented Waste Activated Sludge and OFMSW (Moretto et al. 2019)

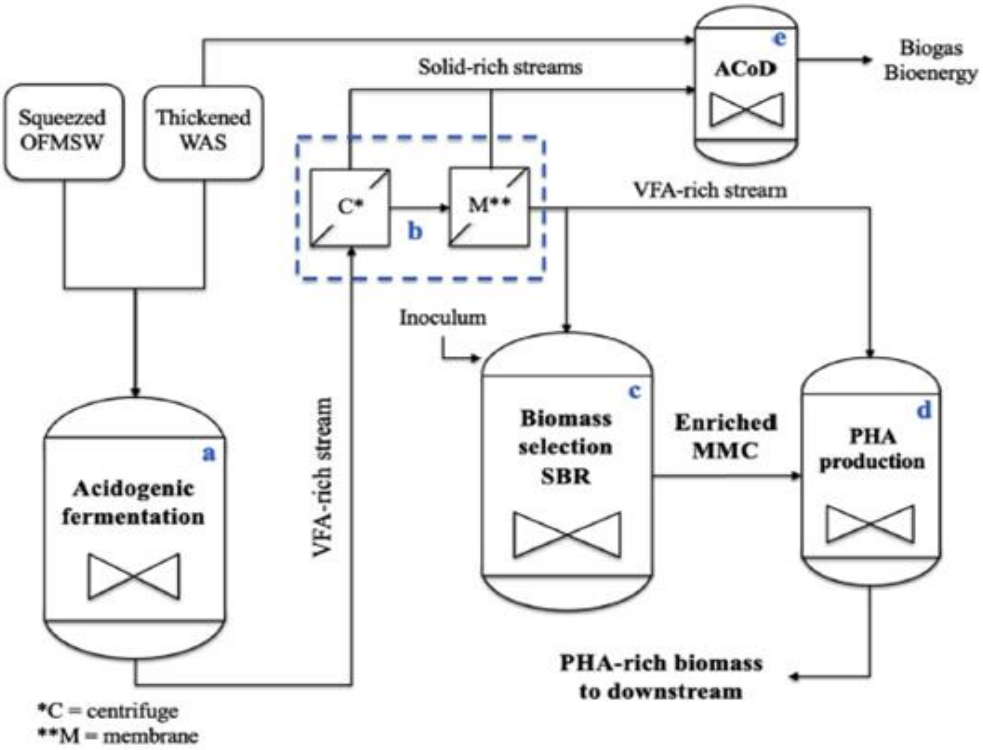

Figure IV.4. Flow diagram of the process at pilot scale of Moretto et al. (2019). Extracted from Moretto et al. (2019).

PHA production in a single stage membrane bioreactor for selection and accumulation (Jia et al. 2014)

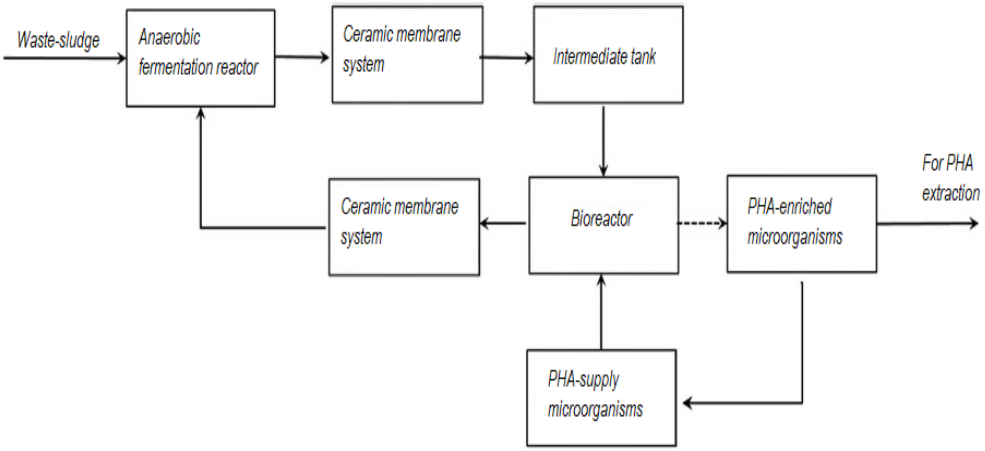

Figure IV.5. Schematization of the processes that takes part in Jia et al. 2014 pilot scale plant. Adapted from Jia et al. (2014).

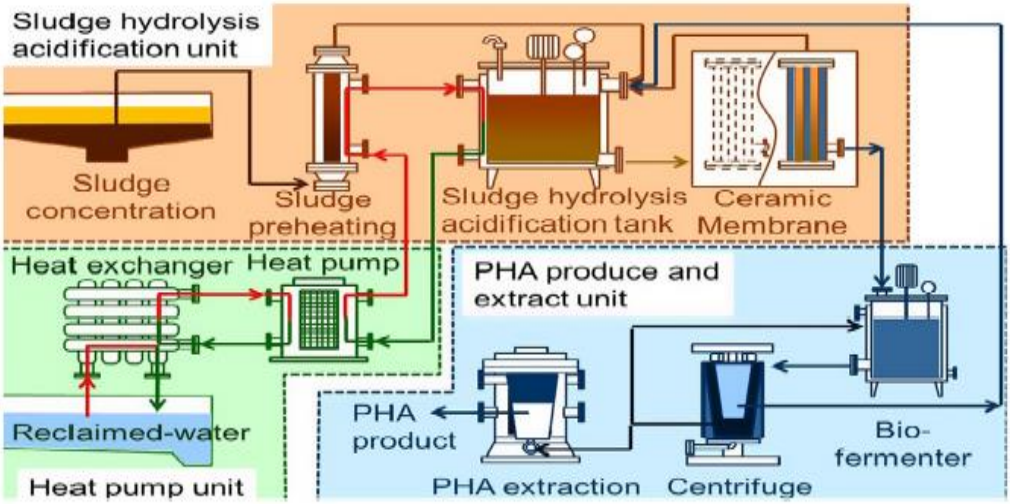

Figure IV.6. Flow diagram of the incorporated membrane system process from Jia et al. 2014. Extracted from Jia et al. (2014).

ANNEX 5: DO REPETITIVE PATRON

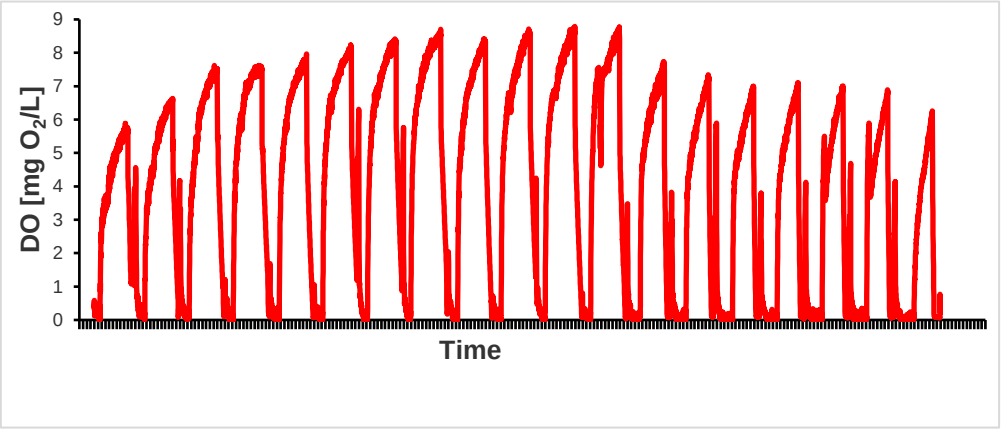

Figure V.1. DO profiles from day 07-02-20 to 12-02-20.

As it can be seeing in the graphic above that contains DO data from day 7-2-20 to 12-2-20, a repetitive DO profile is experimented during the operation. There are little differences in maximum DO value because of diffusors dirtying because of growing bacteria cultures. Moreover, a pseudo-stationary state can be observed in this experimental data.