Universitat Autònoma de Barcelona

Polyhydroxyalkanoates production alongside wastewater treatment by mixed microbial cultures

Gabriela Montiel Jarillo

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Universitat Autònoma
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Escola d´Enginyeria
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Environmental Science and Technology studies

Polyhydroxyalkanoates production alongside wastewater treatment by mixed microbial cultures

PhD Thesis

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Que la llicenciada en química i màster en ciències ambientals **GABRIELA MONTIEL JARILLO** ha realitzat sota la meva direcció el treball amb títol "Polyhydroxyalkanoates production alongside wastewater treatment", que es presenta en aquesta memòria, i que constitueix la seva tesi per a optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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ABSTRACT

The idea of implementing a circular economy model has enabled the development of new biotechnological processes that maximize the valorisation of wastes using them as raw materials to obtain value-added products. The polyhydroxyalkanoates (PHA) are a group of biodegradable polymers that are biologically synthesized by a wide range of bacteria. There is increasing attention on PHA production because they are fully biodegradable materials and may be produced by using wastes. These biopolymers may be used in different industrial fields because their mechanical and physical properties are very similar to that of petroleum-based plastics. Nevertheless, the industrial PHA production is mainly limited due to the high production costs associated with the use of pure cultures and specific feedstocks and also because of the high downstream process costs.

The scope of this thesis is to demonstrate the feasibility of biological wastewater treatment alongside PHA production as value-added product. For that purpose, the performance and effectiveness of a three-stage process was investigated including: (1) the acidogenic fermentation of different industrial wastes; (2) the enrichment of a mixed microbial culture with PHA-accumulating organisms; and (3) the accumulation step to improve the PHA content of the enriched-biomass. Finally, the PHA synthetized was extracted and characterized.

The use of inexpensive wastes as substrates for PHA production will help to diminish the overall process costs by ca. 50%. In this sense, the acidogenic fermentation was used for the bio-conversion of different wastes into volatile fatty acids (VFA)-rich streams that can be potentially used as precursors for PHA synthesis. In this thesis, different waste activated sludge with variable sludge residence times (SRT), an olive mill wastewater, glycerol, a winterization oil cake and apple pomace were evaluated under batch acidogenic fermentation experiments to determine their degree of acidification (DA). The higher DA was observed for the waste activated sludge with the lower SRT (69% ±1) followed by the olive mill wastewater (48% ±1). The fermented liquid of each waste was also analysed in terms of total VFA content. H_2 production was also assessed. The composition of the produced VFA rich stream was analysed in regard to the odd-to-even ration as it is a crucial parameter to determine their suitability for the production of biopolymers with different properties. A higher odd-to-even ratio is obtained when high amount of propionic or valeric acids (odd-chain carboxylic acids) are produced. The higher odd-to-even ratio was obtained for the winterization oil cake (0.86) with propionic acid as the majoritarian VFA followed by acetic acid. The rest of wastes fermented liquor were mainly composed by acetic and butyric acids.

The research work presented herein also includes the start-up of a 16L sequencing batch reactor for the enrichment of a mixed microbial culture able to accumulate PHA. The process was operated under full aerobic conditions using a feast/famine regimen that alternates periods of presence and absence of carbon source. The enrichment step was evaluated at controlled pH (7.5) and without pH control (oscillating in a range of 8.8 to 9.2). The process performance, the biopolymer content and the depletion of the organic fraction was monitored over 120 days. In this case, acetic acid was used as sole carbon source to attain a better understanding of the PHA accumulation mechanisms. The enriched culture obtained without pH control, exhibited a higher PHA content (36%, gPHA g^{-1}VSS) and thus, it was further used in the fed-batch experiments for the PHA accumulation step. The effect of different pHs and nutrients (nitrogen and phosphorus) concentrations on the PHA maximum content was evaluated. The PHA accumulation was found to be higher without using pH control achieving a PHA content up to 44 % gPHA g^{-1}VSS. The effect of nutrients concentrations was evaluated without using a pH control and a maximum PHA content of 51% gPHA g^{-1}VSS was attained under nitrogen limitation.

Following, a synthetic acidified olive-mill-wastewater (OMW) was used as carbon source for the enrichment of a mixed microbial culture to establish its feasibility to produce PHA. The OMW was selected as low-cost substrate that currently represents an environmental concern. The presence of a phenolic fraction in the OMW represent a problem as it may inhibit the PHA accumulation process. In the present study a strategy based on the feeding the OMW into pulses was implemented to achieve both, the wastewater treatment and the enrichment of the mixed microbial culture in PHA-accumulating microorganisms. Around a 90% of organic matter depletion was achieved and the maximum PHA content in the enriched biomass was 33% gPHA g^{-1}VSS. The PHA accumulation capacity of the enriched biomass was evaluated under different split-fed strategies based on the dissolved oxygen (DO) profile. The PHA content and biopolymer composition were also investigated using different carbon sources and a maximum PHA content of 73 % gPHA g^{-1}VSS was achieved using a mixture of 75:25 acetic to propionic acid.

Finally, the PHA extraction was performed using dimethyl carbonate (DMC) as a green solvent and the recovery efficiency was compared with that obtained using chloroform as solvent. The impact of using a NaOCl pre-treatment or an ethanol precipitation post-treatment on the recovery was also explored. A recovery of 25% ±4 of the copolymer P(HB-co-HV) was attained using DMC and NaOCl which was very similar to the result obtained for chloroform extraction (23% ±2). The structure, thermal properties and molecular weights of the extracted biopolymers was determined. PHB exhibited a higher degree of crystallinity in comparison with the copolymer (35.8% and 14.7%, respectively). The decomposition temperature of both biopolymers were around 240°C.

RESUMEN

El concepto de economía circular ha dado lugar al desarrollo de nuevas procesos biotecnológicos que tienen como objetivo valorizar el uso de residuos como precursores en la obtención de productos de valor añadido. Los polihidroxialcanoatos (PHAs) son polímeros sintetizados de manera biológica por una gran variedad de microorganismos. Existe un gran interés en los procesos de producción de PHAs ya que son materiales completamente biodegradables que pueden obtenerse a partir de residuos. Las propiedades mecánicas y físicas de estos materiales son muy similares a las de los plásticos convencionales derivados del petróleo por lo cual, pueden emplearse en diversos sectores industriales. Sin embargo, actualmente la producción industrial de PHAs está limitada por los altos costos de producción relacionados con el proceso de extracción y el uso de cultivos puros y de fuentes de carbono específicas.

El objetivo de esta tesis es evaluar la factibilidad de realizar el tratamiento biológico de aguas residuales y en paralelo obtener PHAs como productos de valor añadido. Para ello, se evaluó el funcionamiento y la viabilidad de un proceso de tres etapas dividido en: (1) la fermentación acidogénica de residuos industriales, (2) el enriquecimiento de un cultivo bacteriano mixto con microorganismos acumuladores de PHA y (3) el proceso de acumulación para maximizar el contenido de PHA en el cultivo bacteriano mixto enriquecido. Finalmente se realizó la extracción y la caracterización del PHA obtenido.

En primer lugar, se propone el uso de residuos como sustratos de bajo costo, para la producción de PHA. Para ello se evaluó la producción de ácidos grasos volátiles (AGV) mediante fermentación acidogénica (FA). Se determinó el grado de acidificación de diferentes lodos activos con tiempos de residencia celular (TRC) diferentes, aguas residuales de almazara, glicerol, residuo de winterización y pulpa de manzana mediante experimentos anaeróbicos en discontinuo. El grado de acidificación más alto se obtuvo de la fermentación del lodo activo con menor TRC (69% ±1), seguido por las aguas residuales de almazara (48% ±1). Los residuos fermentados se evaluaron en cuanto al contenido de AGV e hidrógeno (H_2), sin embargo se hizo un mayor énfasis en determinar la composición de los AGV ya que es crucial para definir el tipo de PHA que se producirá así como sus propiedades. En este sentido el ratio par-impar fue calculado. Una mayor proporción de ácido propiónico y valerico (ambos AGVs de cadena impar) resultara en ratios más altos. El ratio más alto se obtuvo de la fermentación del residuo de winterización, el AGV mayoritario fue ácido propiónico seguido de acido acético. En el resto de residuos, el ácido acético fue producido como componente mayoritario después de la fermentación.

Este trabajo también incluye la puesta en marcha de un reactor secuencial por lotes para el enriquecimiento de un cultivo bacteriano mixto capaz de acumular PHAs. El proceso se operó en condiciones completamente aerobias con alternancia de presencia y ausencia de sustrato (festín/hambruna). En esta fase de enriquecimiento se evaluó el efecto de trabajar a un pH controlado (pH = 7.5) o en ausencia de control de pH (en un intervalo de 8.8 a 9.2). Se realizó el seguimiento de la operación del reactor durante 120 días. El funcionamiento del reactor se evaluó en función de la producción de PHA y eliminación de materia orgánica. El cultivo enriquecido sin control de pH alcanzó un contenido de PHA de 36%, gPHA g^{-1}VSS. Para tener una mejor comprensión del mecanismo de acumulación de PHAs se empleó ácido acético como única fuente de carbono. Posteriormente, el cultivo bacteriano mixto enriquecido se utilizó para promover el máximo almacenamiento de PHAs y evaluar el efecto de diferentes pH, así como de la concentración de nutrientes (nitrógeno y fosforo) sobre el contenido máximo de PHAs. Se observó una mayor acumulación de PHA en ausencia de control de pH (44%, gPHA g^{-1}VSS). La acumulación incrementó al trabajar en condiciones de limitación por nitrógeno (51%, gPHA g^{-1}VSS) y sin necesidad de emplear un control de pH.

Posteriormente, se evaluó la factibilidad de emplear un agua residual fermentada de aceite de oliva (OMW) como sustrato para la etapa de enriquecimiento. El OMW tiene una fracción fenólica que puede resultar inhibitoria en proceso de producción de PHAs. En esta tesis, se propuso una estrategia basada en la distribución de la alimentación del sustrato en diversos pulsos a lo largo del tiempo de reacción, para minimizar el efecto causado por los compuestos fenólicos. Se consiguió la eliminación de un 90% de la materia orgánica y el cultivo enriquecido acumuló un 33%, gPHA g^{-1}VSS. Se determinaron las condiciones óptimas para lograr una máxima acumulación de PHA tomando como base el perfil de oxígeno disuelto

(OD). Así mismo, se evaluó la composición del PHA en función del uso de fuentes de carbono con diferentes composiciones. Se logró una acumulación máxima de PHA de 73%, gPHA g^{-1}VSS empleando una mezcla 75:25 de ácido acético y ácido propiónico.

Finalmente, se realizó la extracción del PHA sintetizado empleando dimetil carbonato (DMC) como “solvente verde”. La eficacia de recuperación se comparó con los resultados obtenidos al emplear cloroformo. A la par, se evaluó el efecto de emplear un pre-tratamiento de lisis celular con hipoclorito de sodio (NaOCl) para favorecer la recuperación del PHA. También se evaluó la precipitación del biopolímero con etanol. Se logró una recuperación del 25% ±4 de P(HB-co-HV) empleando el pretratamiento con NaOCl. Este resultado fue muy similar en comparación con lo obtenido de la extracción con cloroformo (23% ±2). El PHA extraído, se caracterizó en función de su peso molecular, propiedades térmicas y estructura. El grado de cristalinidad fue mayor para el homopolíymero PHB que para el copolímero (35.8% and 14.7%, respectivamente). La temperatura de descomposición de ambos polímeros fue similar (aproximadamente 240°C).

List of Acronyms

ΔH_m	Enthalpy of fusion
AD	Anaerobic digestion
AF	Acidogenic fermentation
AnMBR	Anaerobic membrane biorectors
AP	Apple pomace
APBR	Anaerobic packed-bed reactor
ATU	Allylthiourea
B	Butyrate signals assignment in NMR spectra
BESA	Bromoethanesulphonic acid
BOD	Biochemical oxygen demand
C/N	Carbon/nitrogen ratio
CAS	Conventional activated sludge
CCD	Comonomer composition distribution
$CDCl_3$	Deuterated chloroform
CF	Chloroform
COD	Chemical oxygen demand
CSTR	Continuous stirred-tank reactor
CW	Cheese whey
D	Degree of randomness on the dyad level
DA	Degree of acidification
DE	Dichloroethane
DMC	Dimethyl carbonate
DO	Dissolved oxygen
DSC	Differential scanning calorimetry
EBPR	Enhanced biological phosphorus removal
EU	European Union
F/F	Feast/famine ratio
F_{BB}	Butyrate-butyrate dyad sequence
F_{BV}	Butyrate-valerate dyad sequence
FF	Feast/famine regime
FID	Flame ionization detector
F_{VB}	Valerate-butyrate dyad sequence
F_{VV}	Valerate-valerate dyad sequence
FW	Food waste
GAOs	Glycogen-accumulating organisms
GC	Gas chromatography
GPC	Gel-permeation chromatography
HA	Hydroxyacid

HRT	Hydraulic retention time
HAc	Acetic acid
HB	Hydroxybutyrate
HBt	Butyric acid
HBz	Benzoic acid
HCn	Cinnamic acid
HFIP	Hexafluoroisopropanol
HHx	Hydroxyhexanoate
HiBt	Iso-butyric acid
HiVc	Iso-valeric acid
HPAA	4-hydroxyphenylacetic acid
HPLC	High-performance liquid chromatography
HPr	Propionic acid
HRAS	High-rate activated sludge
HSQC	Heteronuclear single quantum coherence
HV	Hydroxyvalerate
HVc	Valeric acid
k	Ratio of hydroxybutyric and hydroxyvaleric proportions
MCB	Mixed cultures biotechnology
MCL	Medium-chain length
MMC	Mixed microbial cultures
$\mathbf{M_n}$	Number average molecular weight
$\mathbf{M_w}$	Average molecular weight
N	Nitrogen
NMR	Nuclear magnetic resonance
OLR	Organic loading rate
OMW	Olive mill wastewater
P	Phosphorous
PAOs	Polyphosphate-accumulating organisms
PBBR	Packed-bed biofilm reactor
PBAT	Poly(butylene adipate-co-terephtalate
PBS	Poly(butylene succinate)
PBT	Poly(butylene terephthalate)
PCL	Poly(Ɛ-caprolactone)
PDBR	Periodic discontinuous batch reactor
PDI	Polydispersity index
PE	Polyethylene
PET	Poly(ethylene terephthalate)
PF	Phenolic fraction
PGA	Polyglycolate

PH2MV	Polyhydroxy-2-methylvalerate
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyric acid
P(HB-co-HV)	Copolymer Poly(hydroxybutyrate-co-hydroxyvalerate)
PHV	Polyhydroxyvaleric acid
PLA	Polylactide
PMM	Poly(methylmethacrylate)
PNP	*p*-nitrophenol
POME	Palm oil mill effluents
PP	Polypropylene
PS	Polystyrene
PTT	Poly(trimethylene terephthalate)
PVA	Polyvinylalcohols
PVC	Poly(vinyl chloride)
q	Rate
R	Degree of randomness on the triad level
SBR	Sequencing batch reactor
SCL	Short-chain lenght
SCM	Sugar cane molasses
sCOD	Soluble Chemical oxygen demand
SDS	Sodium dodecyl sulfate
sOMW	Synthetic fermented olive mill wastewater
SRT	Solids retention time
SSF	Solid state fermentation
SVI	Sludge volumetric index
T	Temperature
$\mathbf{T_d}$	Decomposition temperature
TGA	Thermal gravimetric analysis
$\mathbf{T_m}$	Melting point temperature
TSS	Total suspended solids
V	Valerate signals assignment in NMR spectra
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WOC	Winterization oil cake
WWTP	Wastewater treatment plant
X	Biomass
$\mathbf{X_c}$	Degree of crystallinity
Y	Yield

Chapter 1

Introduction

1.1 Wastewater treatment evolution

All over the years, wastewater treatment has been an evolving challenge from the technological and political point of view, and the way it is conceived nowadays is the result of many key events through human history. The first human civilizations were small nomadic communities with no waste management problems mainly because the waste was discharged to land and decomposed following a natural cycle. Nevertheless, when permanent settlements were established, waste management became a sanitary and hygienic issue (Lofrano and Brown, 2010). Ancient civilizations began to use latrines and cesspits and employed drainage systems to carry away wastes to a vessel or to be discharged into the street, desert sand or rivers. The Greeks were recognized to substantially improved the water management by using sewers to drain the latrines wastes as well as stormwater in a basin out of the city and later, the model was also employed, enhanced and perfected by the Romans. The advances on wastewater technologies achieved until this period, were lost because of the deterioration of the aqueducts and constructions as a consequence of the Roman Empire collapse which lead to the beginning of the namely “Sanitary dark age” (Cooper, 2001; Lofrano and Brown, 2010; van Lier et al., 2008).

"Sanitary Dark Age" lasted for more than a thousand of years, wastes were again thrown into the streets, the constructions for water transport were abandoned, the facilities were assaulted and diseases were spread. It was until the XIX century that the importance of waste and wastewater management began to be considered again due to the increment of industry and urbanization. Sewers and pump systems were installed again and wastewater dilution was seen as the solution and thus, it was discharged in rivers without considering the importance of a prior removal of pollutants (Lofrano and Brown, 2010; van Lier et al., 2008; Wiesmann et al., 2006).

During the XX century, the rivers were recognized as part of the treatment process and the relation between chemical water pollution and toxicity was recognized. The government, society, and science began to be focused on pollution and had been established concepts like biochemical oxygen demand (BOD). Also, mathematical modelling started to be applied to avoid rivers pollution. In the 1950´s water quality standards emerged in parallel with waste management policies. With the eutrophication problem due to nitrogen and phosphorus present in rivers, removal of nutrients was implemented. Furthermore, the development of analytical equipment such as gas chromatography and atomic absorption spectrophotometry in 1970, spread the characterization of pollutants (Lofrano and Brown, 2010; van Lier et al., 2008). During the 20th century, wastewater treatment has significantly evolved leading to the development of biotechnological processes aiming to the removal of contaminants; aerobic and anaerobic wastewater treatments have been implemented and enhanced, and new technologies have been emerged such as biological aerated filters, fluidized bed reactors, granular sludge processes, moving bed reactors, etc. (van Lier et al., 2008).

Wastewater treatment has been a challenge throughout history and is incessantly evolving, seeking to adapt to the new demands of a society in constant growth and development. In fact, nowadays the concept of wastewater treatment has shifted by the recognition of its potential to become not only a removal process but also a sustainable process for nutrients recovery and the obtaining of value-added products (Kleerebezem and van Loosdrecht, 2007).

1.1.1 Circular economy and bio-economy concepts

The "linear economy" that has been employed in our society for years is not possible anymore, the "take-make/use-dispose" model resulted in the scarcity of resources and the degradation of the environment because it does not have into account the long-term damage of the technological innovations. In contrast (Figure 1.1) a “circular economy” concept has been adopted to face the current situation, searching for an economic growth but respectfully and sustainably with the environment (Maina et al., 2017).

It is interesting to look at the circular economy not only as a zero-emissions process but also as a regenerative system that valorises products that have fulfilled their function into a resource for the production of another, closing loops of the product lifecycle and minimizing the wastes (Korhonen et al., 2018).

Bioeconomy is stated in the Europe 2020 Strategy as a key for sustainable economic growth. It involves the re-valorisation of a waste by its conversion and recognition as a raw material for the production of a new bio-based material. The bioeconomy does not attain by itself the final goal of the circular economy but can be considered as a technological step that facilitates the development of a resource efficient society (EuropeanComission, 2012; Maina et al., 2017; Székács, 2017).

In this context, wastes and wastewater generated from the current linear model of the industry can be used as resources in the development of the new "circular economy" concept.

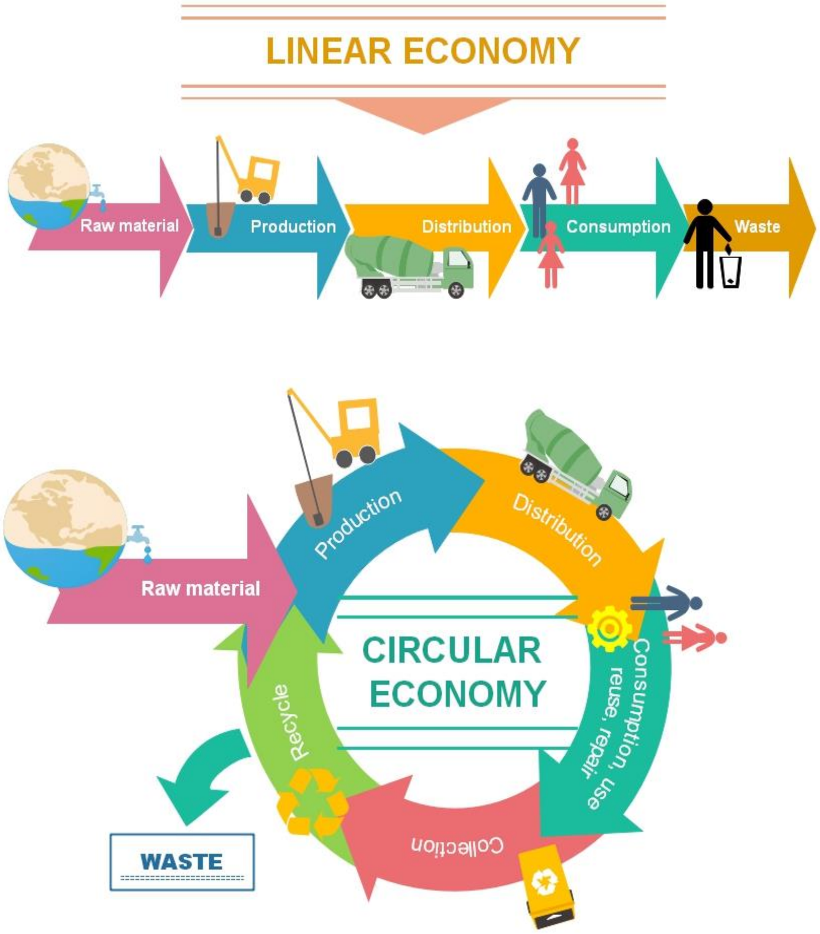


Figure 1.1 Linear economy model versus circular economy (Adapted from EuropeanComission (2014))

1.1.2 Wastewater role in the circular economy model

The world population is continuously increasing, and it is estimated that by the year 2050 is going to increase up to 9.2 billion which means 2.2 billion tons of solid waste per year because it makes sense to link the population growth with an increase in the demand for services and goods that consequently arose in a greater production of wastes (Hoornweg and Bhada-Tata, 2012; United Nations, 2013).

Wastes and wastewaters management is playing an important role in the transition from a linear economy to a circular economy, the production of clean water is expected to be achieved alongside resource recovery and energy production (Nielsen, 2017). Thus "mixed cultures biotechnology" (MCB) is as a key piece to achieve a circular bio-economy as it represents the jointure between environmental biotechnology, which aim is to "clean" water, with industrial biotechnology, that seeks for product maximization; thereby MCB re-valorizes residues from waste to raw materials for the production of a variety of new products, closing loops and boosting the implementation of a circular economy (Kleerebezem and van Loosdrecht, 2007; Nielsen, 2017; Nizami et al., 2017).

1.1.3 Resources that can be recovered from wastewater

Since its implementation, wastewater treatment looks for the elimination of contaminants such as carbon, nitrogen, phosphorus, micropollutants, and pathogens; however, resource recovery has attracted attention as a way to reduce operational costs, to reduce energy consumption and to diminish the risks of gas and odours emissions, metals discharge, etc. (Holmgren et al., 2014). Resource recovery from wastewater is not new and so, the more widespread example is anaerobic digestion (AD) for energy (biogas) production however, with the increasing interest on resource recovery during the past years, several processes has emerged and are under development because there are a lot of recoverable components available in wastewaters and the recovery of products like alcohols, biopolymers, organic acids, enzymes, fertilizers, hydrogen, biochar, fuels, cellulose, proteins, biosurfactants, cellulose fibers etc. have been reported (Maina et al., 2017; Puyol et al., 2017; Van Der Hoek et al., 2016; WERF, 2011).

Nowadays, the idea of wastewater treatment is evolving from what have to be **removed** to what can be **recovered** (Holmgren et al., 2014). Biological processes are promising for efficiently achieve resource recovery in an environmental and economically way (Puyol et al., 2017). The worth of wastewater as a resource is evident, the point now, is to define where to focus since a lot of materials can be recovered from wastewaters and thus, there are a broad range of available strategies. The International Water Association (IWA) resource recovery cluster established three main groups of recoverable resources from water: water, energy and components (Fig. 1.2) (Holmgren et al., 2014; Van Der Hoek et al., 2016; WERF, 2011).

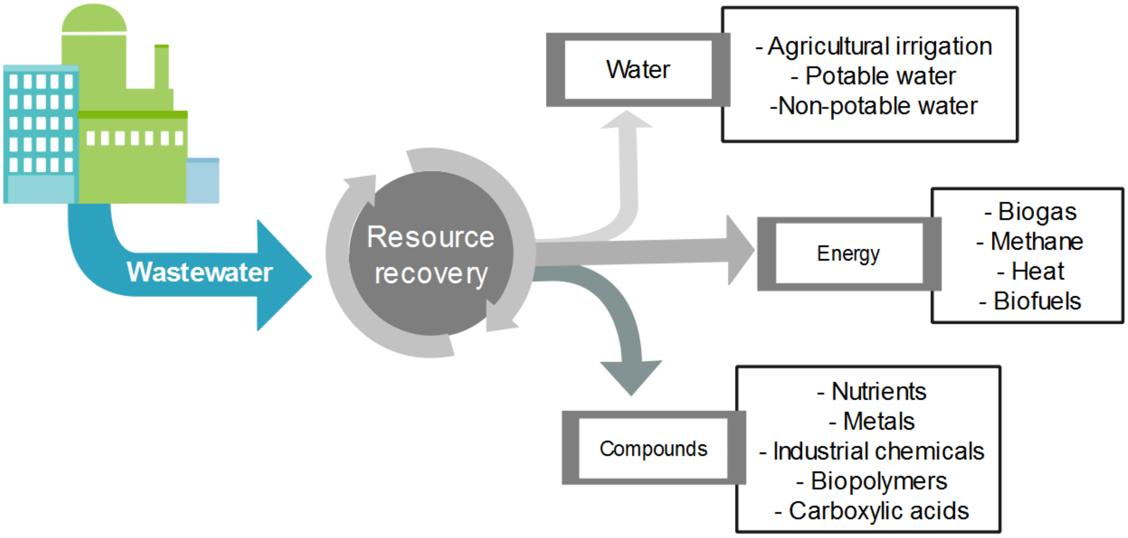


Figure 1.2 Resource recovery from wastewaters

Water recovery by itself has been implemented since many years ago (Van Der Hoek et al., 2016; WERF, 2011). Depending on the quality of the recovered water it could be used for different purposes such as agricultural irrigation (after the organic matter and suspended solids removal during the secondary water treatment), municipal and domestic use for example for toilet flushing but it can also be use as potable human use. The last, requires advanced processes to achieve the desired quality (Holmgren et al., 2014). There are several examples of successful systems reusing water for potable use (Guest et al., 2009).

Commonly, **energy** is recovered as biogas during AD (Guest et al., 2009) however the thermal energy from the wastewater can be also used. Besides, many other technologies have been implemented to convert organic-rich fraction of wastewaters into biohydrogen, biodiesel, bioethanol or directly into electricity using microbial cell fuels (Holmgren et al., 2014; Puyol et al., 2017).

Some other **components** can be recovered such as metals, enzymes, hormones, bioplastics and fertilizers. Nutrients recovery (specially nitrogen (N) and phosphorous (P)) technologies has been extensively used, it is estimated that around a 20% of the manufactured N and P is found in wastewater and can be successfully recovered (Holmgren et al., 2014; Puyol et al., 2017). The P recovery has been studied more thoroughly mainly for obtaining struvite. However more efforts have been done in developing techniques for N removal than for their recovery and mainly techniques like stripping, precipitation, adsorption and desorption have been used for the recovery of nitrite/nitrate species (Holmgren et al., 2014). Metals are also present in wastewaters and represent a human and environmental concern, however they can be recovered by ion exchange, leaching, adsorption, electrolysis and photocatalysis (Holmgren et al., 2014; Puyol et al., 2017). Other materials may be recovered from wastewaters such as polyhydroxyalkanoates (PHA) that are biologically produced bioplastics. Some other process for recovery of cellulose fibers, proteins, enzymes, hormones and industrial chemicals (like carboxylic acids, alcohols hydrogen peroxide and sulphate) are emerging (Holmgren et al., 2014; Puyol et al., 2017; Van Der Hoek et al., 2016)

1.1.4 Mixed cultures biotechnology for resource recovery

Actually, conventional wastewater treatments could be well integrated and optimized in the circular economy due to the implementation of MCB. The main approach of MCB is to enrich the mixed culture only by natural/ecological selection, so this kind of processes take advantage of microorganisms' metabolism in order to achieve contaminants removal but also energy production and resource recovery (Kleerebezem and van Loosdrecht, 2007).

In fact, the recognition of the importance and value of wastewaters as nutrient-rich streams and as a potential resource for obtaining value-added products, has led to rebrand "Waste Water Treatment Plants (WWTPs)" as "Water Resource Recovery Facilities (WRRFs)" (Coats and Wilson, 2017). Despite of this, most of the treatment plants are focus on the recovery of one resource but few approaches of integrated systems are well implemented (Holmgren et al., 2014).

A wholesome example of an integrated MCB process is illustrated in figure 1.3. A biological wastewater treatment alongside polyhydroxyalkanoates (PHA) production using mixed cultures is shown. This process involves, on the one hand, the anaerobic fermentation of a wastewater for the production of a volatile fatty acids (VFA)-rich stream, and on the other hand, the production of PHA using an enriched mixed culture that is a raw material for bioplastics production.

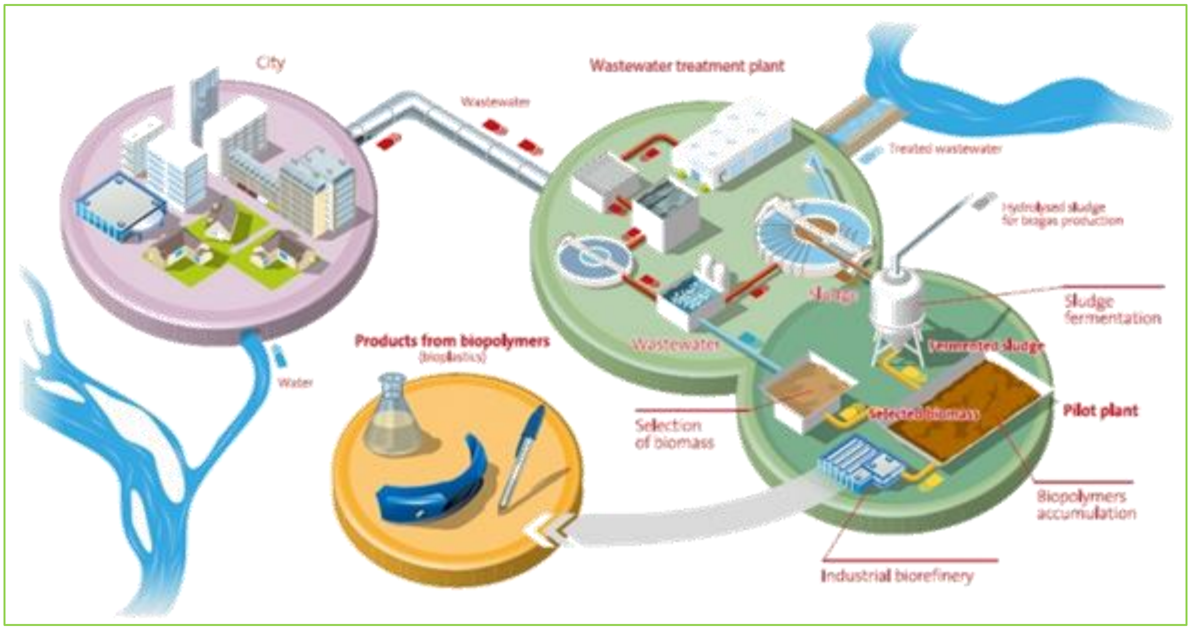


Fig 1.3 PHA production alongside wastewater treatment (Taken from Hansen et al. (2017) - Veolia Water Technologies: https://biovalue.dk/media/Poster-7.pdf)

1.2 POLYHYDROXYALKANOATES

The overproduction and accumulation of petrochemical plastics have become of environmental concern, and in consequence, there is an increasing interest in the development of more sustainable and renewable alternatives to replace their use. Plastic represents more than an 80% of the total marine litter and in the year 2010, it was estimated that plastic could cover more than 40% of the surface of the oceans and 25% of the Earth's surface (Albuquerque and Malafaia, 2018; Penca, 2018).

Since its origin, plastics have been considered as an amazing material that facilitates most of the advances in our society and are considered as useful materials in human’s daily-life due to its low costs, versatility, utility, and durability. They can be produced in different shapes and are highly valued because of their desirable mechanical, thermic and chemical properties (Albuquerque and Malafaia, 2018; Nkwachukwu et al., 2013). Plastics have existed for less than a hundred years and its production has severely increased from 1.5 Mt in the 1950´s to 335 Mt in 2016 (Fig. 1.4) and it is expected to increase in the overcoming years (PlasticsEurope, 2017; Thompson et al., 2009). Plastics are mostly used as packaging materials like bottles and bags, but their application is also extended in other fields such as building and construction, automotive, electrical and electronic equipment, sports materials, furniture, agriculture and medical devices.

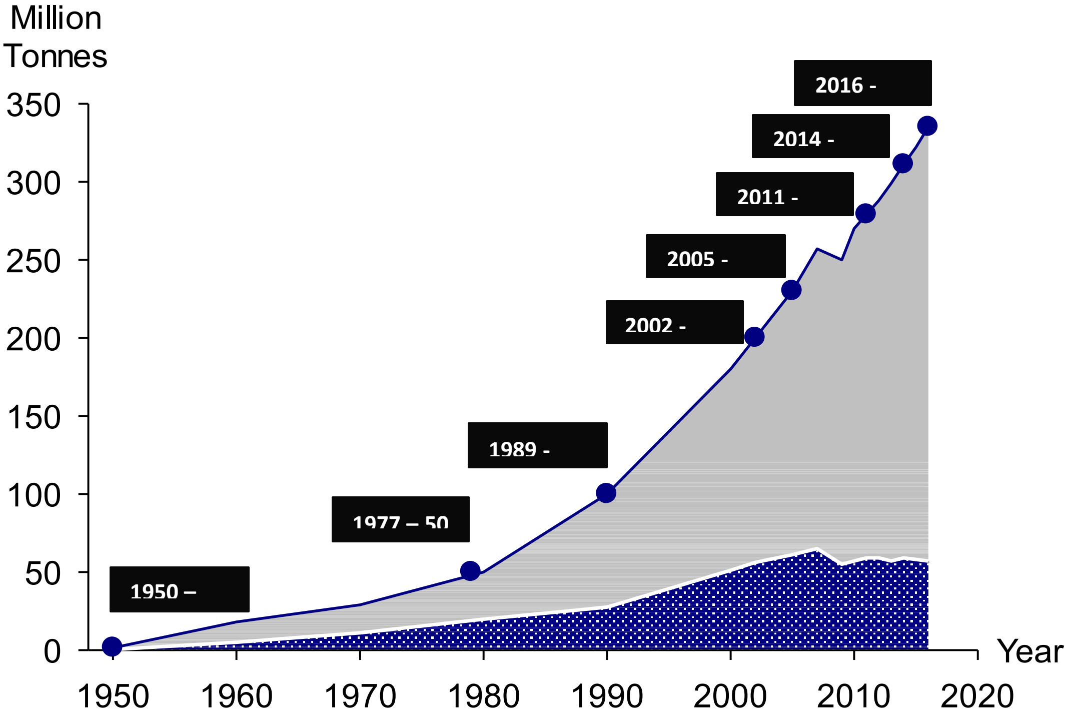


Figure 1.4 Annual growth of plastics production from 1950 to 2016 (PlasticsEurope, 2017)

However, the main problem of plastics is that are produced from fossil resources and are not biodegradable materials persisting in the environment for a long time. Around 50% of plastic has single-use disposable applications and after being used, are discharged and converted into a waste challenge. It has been generated 6300Mt of plastic waste only since 2015 and merely a 9% had been recycled, a 12% incinerated and the remaining 79% was accumulated in landfills and natural environments (Geyer et al., 2017; Nkwachukwu et al., 2013).

An efficient plastic waste management is needed. Currently, the options for their treatment includes reuse, recycling, incineration and landfilling none of which represent an efficient and fully sustainable option. For example, the reuse of plastic waste are expensive and involve high-energy consumption; on the other hand, recycling only delay the plastics disposal instead of solving the problem, and what is more, the recycle can be done only 2-3 times because it causes the polymer thermal degradation affecting their mechanical properties and also, the mixing of different kind of polymers may reduce the processability and thus, the economic value of the recycled polymer and finally, not all the plastics are recyclable. The incineration has environmental drawbacks such as CO_2 emissions and the generation of ash and slag with toxic compounds content and finally, landfilling, needs large areas of land, generates odors and release hazardous chemicals in the leachate (Albuquerque and Malafaia, 2018; Geyer et al., 2017; Nkwachukwu et al., 2013; Singh et al., 2017).

The European Union (EU) global balance of 2017 shows a general improvement in the plastic waste treatment between 2006 and 2016 with an increase by 79% for recycling and 61% for energy recovery and a 43% decrease on landfill. However, the situation is very irregular in Europe (Fig 1.5). Many EU countries still apply the conventional landfill as the first or second plastic waste treatment option (Emadian et al., 2017; PlasticsEurope, 2017).

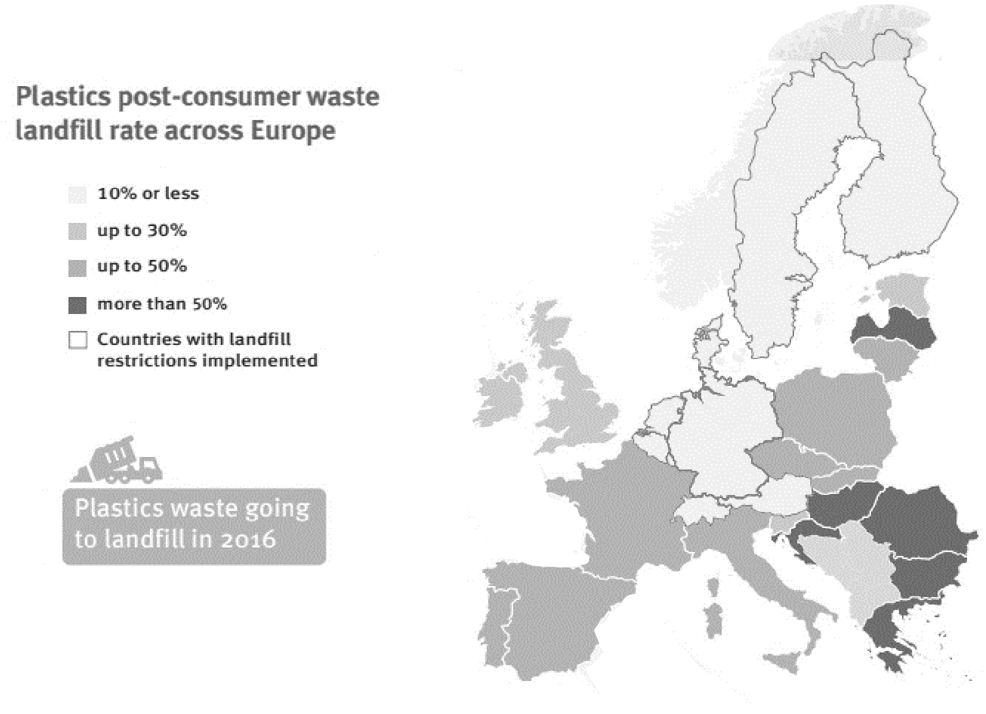
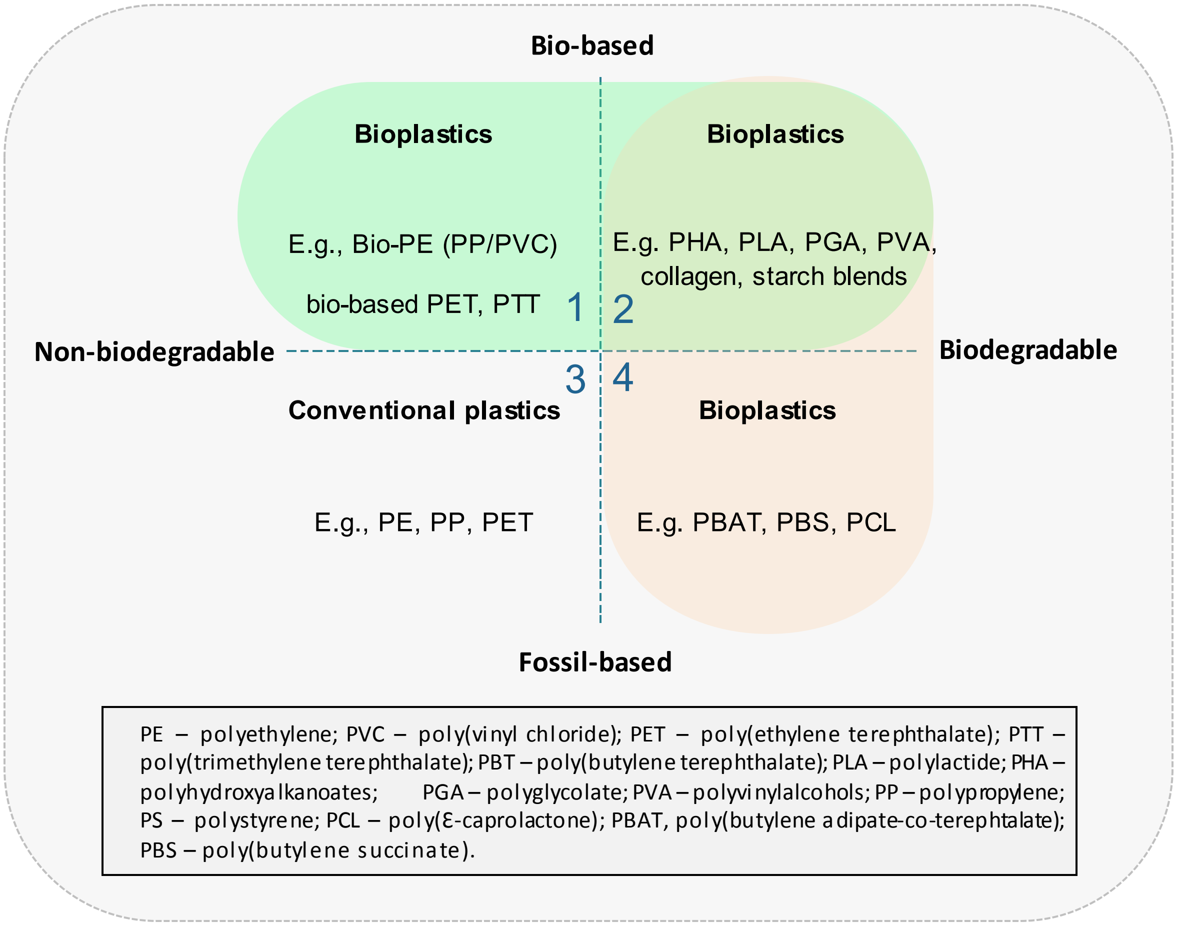


Figure 1.5 Distribution of plastics waste landfill rate in Europe (PlasticsEurope, 2017)

1.2.1 Bioplastics

According to European Bioplastics definition, "bioplastics are plastic materials that can be either bio-based, biodegradable or feature both properties". The biodegradability is not dependent on the resource origin of a material and in this way, not all the bio-based plastics are biodegradable and not all biodegradable plastics are bio-based. Figure 1.6 represents the different plastics materials categories, three of which corresponds to bioplastics: the non-biodegradable (1), the bio-based and also biodegradable (2) and the biodegradable but fossil-based bioplastics (4) (Pathak et al., 2014; Ross et al., 2016).



PE – polyethylene; PVC – poly(vinyl chloride); PET – poly(ethylene terephthalate); PTT – poly(trimethylene terephthalate); PBT – poly(butylene terephthalate); PLA – polylactide; PHA – polyhydroxyalkanoates; PGA – polyglycolate; PVA – polyvinylalcohols; PP – polypropylene; PS – polystyrene; PCL – poly(Ɛ-caprolactone); PBAT, poly(butylene adipate-co-terephtalate); PBS – poly(butylene succinate).

Figure 1.6 Plastic materials classification (adapted from Pathak et al. (2014))

Bioplastics were introduced in the 1980s to overcome the growing use of conventional plastics and from its implementation, they were widely accepted among the society as they save fossil resources by using biomass and also, due to their high resource efficiency, the reduction of carbon footprint and greenhouse emissions (Rivero et al., 2017). The versatility of bioplastics allows their use for the production of many different materials by employing the conventional plastics technologies. The outlook of the bioplastics market is very promising among the searching of a more sustainable circular economy. Their production capacity has increased from 1.5 Mt in 2012 to 2.05 Mt in 2017 and is expected to reach 2.44 Mt production by 2022 (Fig 1.7) (EuropeanBioplastics, 2017; Rivero et al., 2017).

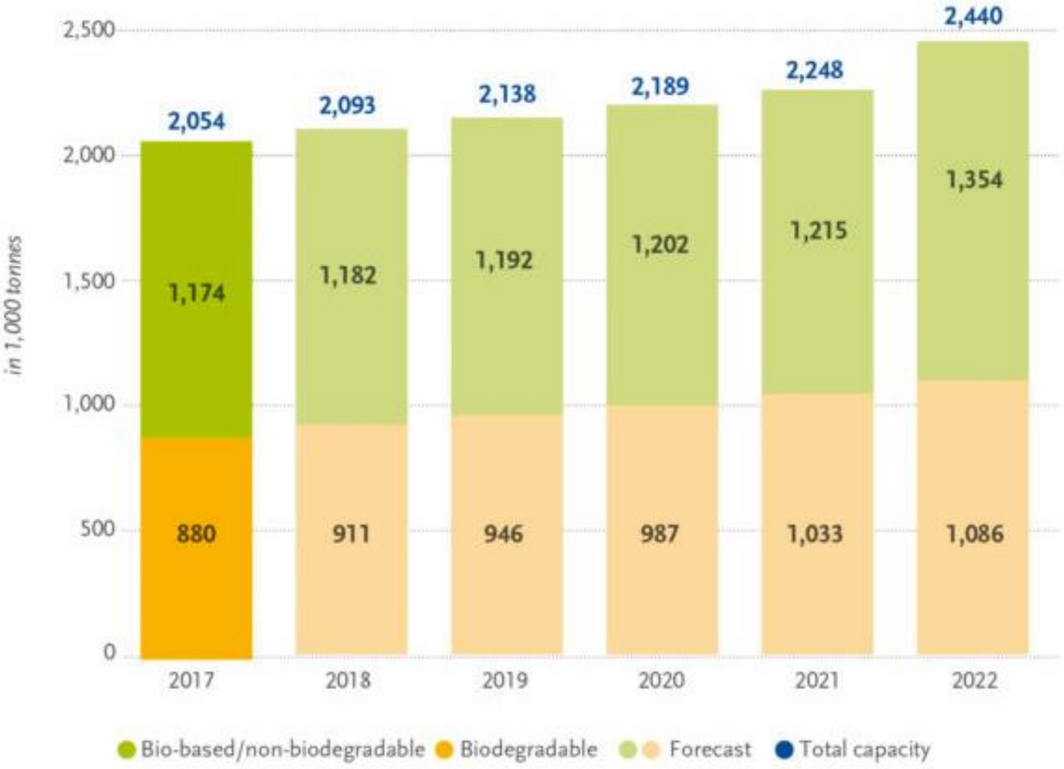


Figure 1.7 Global production capacities of bioplastics (Source: European Bioplastics, 2017)

By now, the market is led by the bio-based but non-biodegradable plastics, nevertheless, the market perspective for the group of bio-based and biodegradable plastics shows a constant increase (Fig 1.7) and is forecasted to reach a production of 1.1 Mt for the year 2022. Bioplastics are sharply used in the manufacture of packaging materials; however, their use is widespread in several fields to name, the medical, agricultural, textile and building (EuropeanBioplastics, 2017; Soroudi and Jakubowicz, 2013). The main problems that are limiting bioplastics production are on one side their limited mechanical properties and on the other side their high production costs. The high production costs of bioplastics are often associated with the use of expensive carbon sources as precursors for their production and so, bioplastics industry and many researchers are developing technologies to produce them from low cost available and renewable carbon sources such as agroindustrial wastes (Albuquerque and Malafaia, 2018; Emadian et al., 2017).

Within the different biodegradable and bio-based plastics that are currently available, PHA are one of the most well-known bioplastics that emerge as possible candidates to replace the conventional plastics due to their biodegradability, non-toxicity and tuneable mechanical and physical properties (Dietrich et al., 2017; EuropeanBioplastics, 2017; Możejko-Ciesielska and Kiewisz, 2016).

PHA were described for the first time in 1923 and the polymer hydroxybutyric acid (PHB) was extracted with chloroform in 1927. The PHB commercial production was explored at the beginning of the 1960s and used for the fabrication of medical devices like sutures and prosthesis but then their production was limited as a consequence of the low yields and also because their extraction was costly. However, with the oil crisis in the 1970s, PHA were considered again as alternative plastics but only until oil prices went low again. Nevertheless, owing to the great potential of PHA, many works kept on this line of research, trying to improve the process and also developing new copolymers with better characteristics (Dietrich et al., 2017; Philip et al., 2007).

1.2.2 PHA characteristics

PHA are biologically synthesized by many Gram-positive and Gram-negative bacteria from around 75 different genera. It has been reported more than 300 species of bacteria that can accumulate PHA in their cytoplasm as carbon and energy storage sources under unfavorable conditions. Bacteria can accumulate up to 80% dry cell weight of PHA as granular lipid inclusions of around 0.2 – 0.5 µm of diameter (Keshavarz and Roy, 2010; Khanna and Srivastava, 2005; Philip et al., 2007).

Structurally, PHA are thermoplastic polyesters of hydroxyacid (HA) monomers that are connected by an ester bond; their general structure is shown in Fig. 1.8. The pendant alkyl group of PHA (as R in Fig 1.8) can vary from methyl (C_1) to tridecyl (C_{13}) and it has been isolated over 150 different hydroxyalkanoate units with different R-pendant groups (Akaraonye et al., 2010; Q. Chen et al., 2013). The polymer chain can contain between 100 and 35000 monomer HA units all of which are found with R(-) configuration due to the stereospecificity of the PHA synthase. The number of methylene groups in the monomer´s backbone can range from 1 to 4 (represented as *x* in Fig. 1.8). Commonly, the HA monomers are in the form of 3, 4 or 5 hydroxyalkanoates (3-HA, 4-HA, or 5-HA) (Albuquerque and Malafaia, 2018). The length of the side chain and the functional group are going to influence the physical properties of the PHA and thus, its final application.

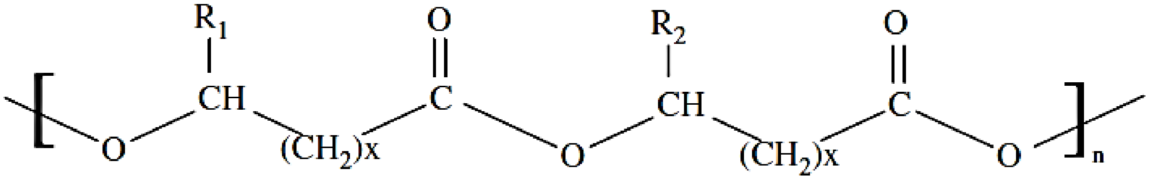


Figure 1.8 General structure of polyhydroxyalkanoates. R_1 and R_2 represent the alkyl groups from C_1 to C_{13}, x = 1 - 4 and n = 100 - 35000 (Akaraonye, 2010)

Depending on the number of carbon atoms in the monomers, PHA can be classified as short-chain length (SCL), medium-chain length (MCL). SCL and MCL PHA are synthesized by different bacteria and have different thermal and mechanical properties. The SCL polymers have 3-5 carbon atoms with a high degree of crystallinity (60-80%). *Alcaligenes latus* and *Cuprivadus necator* can synthesize this kind of SCL PHA. The MCL polymers, consist of 6-14 carbon atoms and have elastomeric properties, are more flexible. This PHA can be synthesized by *Pseudomonas putida, Pseudomonas mendocina*, and *Pseudomonas oleovorans*. Both SCL and MCL polymers can be found as homopolymers (the same monomer units) or as copolymers (more than one type of monomer units). PHA with a non-alkyl group can also be synthesized. The functional groups may include halogen, esters, epoxides, phenyl, phenoxy, cyano, carboxyl and allyl groups and also aromatic rings. The synthesis of either SCL, MCL, PHA homopolymers or PHA copolymers is dependent on the type of bacteria, the process conditions and also the substrate used for feeding the bacteria.

(Akaraonye et al., 2010; Albuquerque and Malafaia, 2018; Chodak, 2008; Kosseva and Rusbandi, 2018).

PHA can be obtained by chemical synthesis or using genetically modified plants, however the use of bacteria for their biological synthesis is a most attractive process (Chodak, 2008). PHA synthesis via microorganisms can follow three different metabolic pathways depending on the carbon source used that may be "related" source (synthesize structurally identical monomers to that used source) or "un-related" source (give rise to monomers completely different from the given carbon source). These pathways are depicted in Fig. 1.9 (Anjum et al., 2016; Philip et al., 2007).

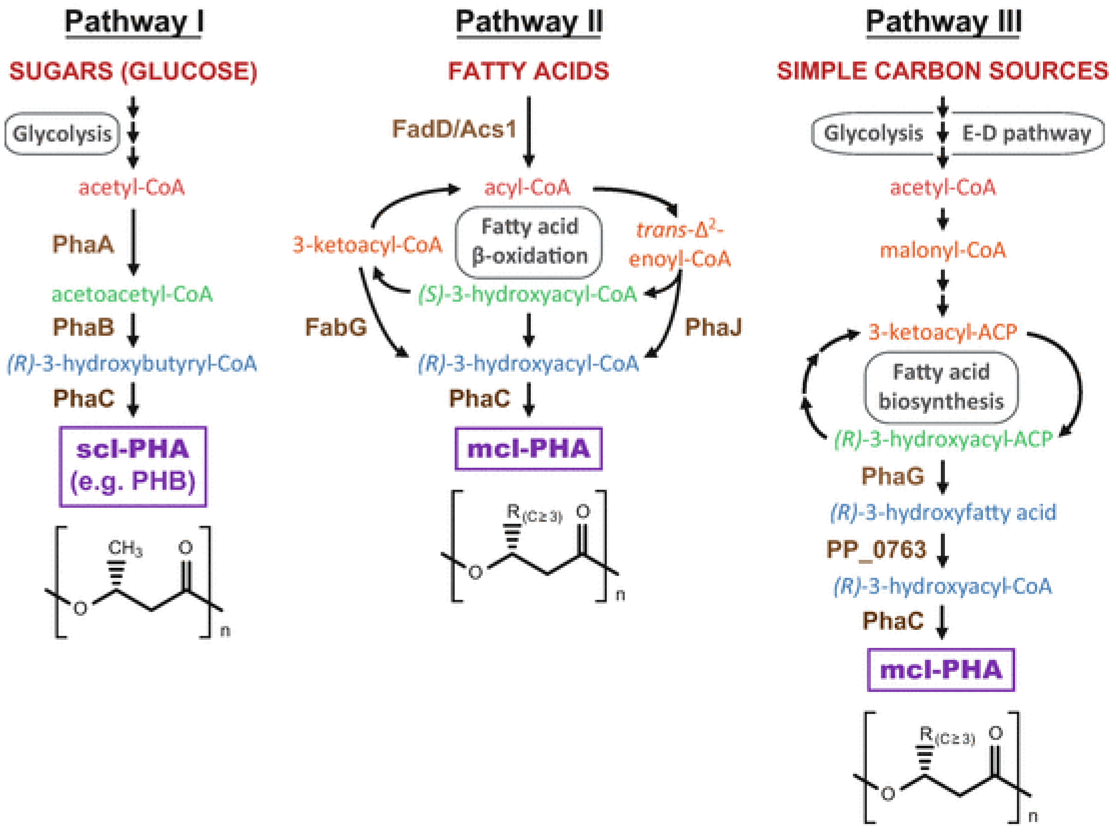


Figure 1.9 Metabolic pathways for the synthesis of PHA (Kniewel et al., 2017)

When "related" sources are used, bacteria may follow the Pathways I and II. **Pathway I** is the better-known pathway and SCL PHA are synthesized. It involves three different enzymes for PHB production. Firstly, two acetyl-CoA molecules from the tricarboxylic acid (TCA) cycle, are condensate into acetoacetyl-CoA by β-ketothiolase (PhaA). Then in a second reduction reaction by NADPH-dependent reductase (PhaB), acetoacetyl-CoA is reduced to (R)-3-hydroxybutyryl-CoA. Finally (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHA synthase enzyme (PhaC) (Reddy et al., 2003; Yu, 2007). Pathways II and III commonly produce MCL PHA. The **pathway II** occurs by the β-oxidation of fatty acids that are polymerized by the enzyme PHA synthase. The intermediate *trans* 2-enoyl-CoA is converted by a specific enoyl-CoA hydratase (PhaJ) into (R)-3-hydroxyacyal-CoA that us incorporated into PHA polymers by PhaC. PHA synthesis through **pathway III** results attractive as PHA monomers from unrelated sources can be obtained. In this pathway, the carbon source is oxidize into acetyl-CoA and converted into malonyl-CoA and finally into the intermediate (R)-3-hydroxyacyl of the fatty acid pathway that is converted from their acyl carrier protein (ACP) form to the CoA form by action of the acyl-ACP-CoA transacylase (PhaG) (Anjum et al., 2016; Kniewel et al., 2017; Philip et al., 2007).

Notwithstanding the significant benefits of use PHA, their industrial production and commercialization is still being limited due to their high costs. Currently, PHA production technology relies on the use of pure microbial cultures which had been reported to accumulate high PHA contents. However, their use involves high costs associated with the fermentation process that requires sterile conditions and specific expensive substrates and also due to the downstream processing (Albuquerque et al., 2011; Serafim et al., 2008). Recombinant strains had been proposed as a cost-effective alternative to the use of pure cultures. The advantages of their use include a rapid growth, high cell density and the possibility of using inexpensive substrates. However, the need for aseptic conditions is also a drawback of this technology. Both scenarios, are disadvantageous for the competitiveness of PHA against to the conventional plastics (Dias et al., 2006).

The production of PHA by using mixed microbial cultures (MMC), has attracted much attention during the last years. The MMC are recognized as potential PHA producers that do not need sterile conditions either expensive process control but the more important advantage of using MMC, is the possibility of employ a huge range of inexpensive feedstocks including industrial and agricultural wastes (Chanprateep, 2010; Pakalapati et al., 2018).

1.2.3 PHA production using MMC

The PHA production using MMC is based on the natural principles of selection and competition of the microorganisms with a PHA storage ability against the microorganisms that are not able to accumulate PHA (Kosseva and Rusbandi, 2018). When using MMC, a desire metabolism can be enforced by varying the operational conditions of the biological system, so it can be say that the ecosystem is engineered instead of the microorganisms (Dias et al., 2006; Koller, 2017). The key to achieving a successful mixed culture PHA production process relies on the enrichment of PHA-accumulating bacteria. To that aim two main enrichment strategies has been used: i) under **anaerobic/aerobic** conditions and ii) alternating **availability/unavailability** of a carbon source (Reis et al., 2003).

PHA intracellular storage was first observed during a biological phosphorus removal process (EBPR). These systems alternate **anaerobic/aerobic** cycles and the PHA accumulation occurs during the anaerobic phase where two microorganisms can store carbon source: the polyphosphate-accumulating organisms (PAOs) and the glycogen-accumulating organisms (GAOs). Both microorganisms under **anaerobic** conditions take up the substrate and use it towards PHA synthesis. The energy required for this process is provided by the internally stored polyphosphate (PAOs) or glycogen (GAOs). Then, under **aerobic** conditions, the stored PHA is used for growth, maintenance and glycogen/polyphosphate replenishment (Koller et al., 2011; Salehizadeh and Van Loosdrecht, 2004; Serafim et al., 2008).

PHA-storing microorganisms with high and stable PHA productions can be selected and enriched under fully aerobic conditions by using a strategy known as **feast/famine** that alternates an external substrate availability phase (feast) with a large period of carbon source absence (famine). During the feast, the carbon source is taken up and stored as PHA granules. Once the external carbon source is depleted, the PHA accumulated is used as a carbon and energy source. The PHA-storing microorganism's selection occurs by the competitive advantage of some microorganisms to accumulate PHA over the microorganisms without this ability as PHA-storing microorganisms may grow during the famine and while non-storing PHA microorganisms will starve (Albuquerque et al., 2010b; Dias et al., 2006; Reis et al., 2003; Salehizadeh and Van Loosdrecht, 2004) .

Usually, the PHA production by MMC take place in a two or a three stages process (Fig 1.10) depending on the substrate used as feedstock (Amulya et al., 2015; Serafim et al., 2008).

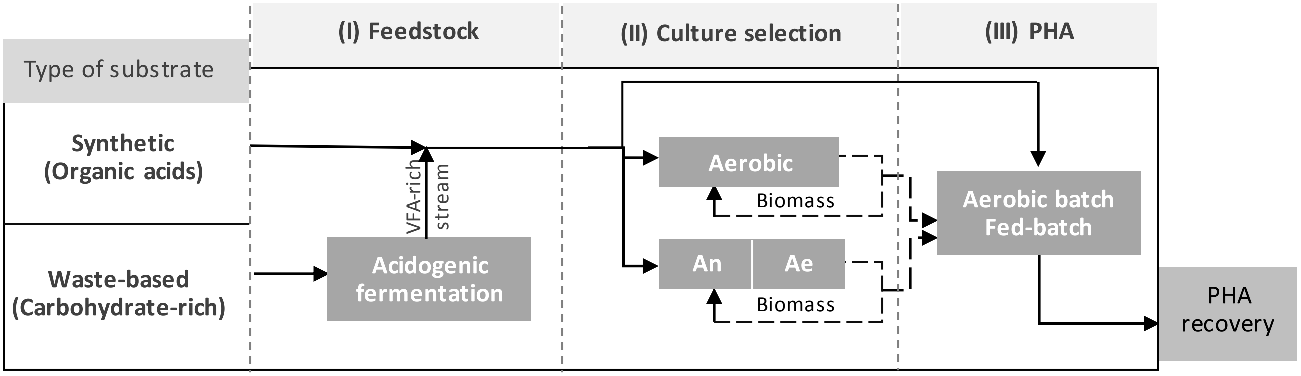


Figure 1.10 Schematic representation of the three-step PHA production by using MMC (Adapted from Serafim et al. (2008))

The two-stage process is applied when pure organic acids are used as substrates. Nevertheless, one of the main advantages of using MMC is the possibility of use wastes as precursors for PHA production and so, a first stage is needed to generate a VFA-rich stream under substrate acidogenic fermentation (AF) (feedstock production in Fig 1.10). The second step is the culture selection of PHA-storing microorganisms that may be achieved under aerobic/anaerobic strategy or by feast/famine regime and finally during the third stage, known as the "PHA production" step, the enriched biomass is fed with the VFA of the first stage to maximize the PHA production capacity (Duque et al., 2014; Serafim et al., 2008).

By using cheap carbon sources, PHA production costs can be reduced in around 40 - 50% (Akaraonye et al., 2010). The use of MMC for the production of PHA has allowed the valorization and recycle of a large number of agricultural and industrial fermented wastes and wastewaters for the PHA synthesis (Kosseva and Rusbandi, 2018). These wastes and wastewaters can be used as zero cost feedstocks for PHA production, which ensures the sustainability of the process since anyhow a previous management is required before their disposal thus reducing costs of both, the PHA and the waste management (Akaraonye et al., 2010; Kosseva and Rusbandi, 2018; Ntaikou et al., 2014).

1.2.4 Potential of wastes as PHA precursors

In the past decades, the maximization of VFA production using wastes has been evaluated. There are several reports showing the use of waste materials towards VFA production with different yields (Dahiya et al., 2015; Lee et al., 2014). The composition of wastes is crucial to consider it as a possible acidogenic fermentation (AF) feedstock and in general, it should have high biodegradability and carbon load and has to be available in great amounts.

The use of such VFA streams for the synthesis of biodegradable polymers, such as polyhydroxyalkanoates (PHA), has attracted a lot of attention as a promising alternative to replace petroleum-based plastics. In that context, the liquid fermentation of a wide range of residues has been assessed as feedstocks for PHA synthesis by using MMC (Pakalapati et al., 2018). To date several reports in the literature explore the use of wastes and by-products, for PHA production, such like: **food waste (FW)** (Amulya et al., 2015; H. Chen et al., 2013; Colombo et al., 2017; Jiang et al., 2013; Venkateswar Reddy and Venkata Mohan, 2012; Zhang et al., 2014), **sugar cane molasses (SCM)** (Albuquerque et al., 2011, 2010b, 2010a, 2007, Bengtsson et al., 2010a, 2010b; Duque et al., 2014), **Olive mill wastewater (OMW)** (Beccari et al., 2009; Campanari et al., 2014; Dionisi et al., 2005; Ntaikou et al., 2014; Silva et al., 2013; Villano et al., 2010; Yarimtepe et al., 2017), **Waste activated sludge (WAS)** (Huang et al., 2015; Jiang et al., 2009; Silva et al., 2013; Yu et al., 2018)**, paper mill wastewater** (Bengtsson et al., 2008a, 2008b; Chen et al., 2015; Jiang et al., 2012), **Cheese whey (CW)** (Colombo et al., 2016; Duque et al., 2014; Gouveia et al., 2017; Silva et al., 2013; Valentino et al., 2015a, 2015b)**, Palm oil mill effluents (POME)** (Din et al., 2012; Lee et al., 2015; Salmiati et al., 2007) as well as some other wastes for instance, pulp and paper mill effluents, glycerol, soapy residues, winery effluents, leachates, milk whey, and dairy, ice-cream and milk wastewaters (Bosco and Chiampo, 2010; Chakravarty et al., 2010; H. Chen et al., 2013; Chen et al., 2015; Queirós et al., 2014; Silva et al., 2013). Table 1.1 shows the VFA production performance of some wastes reported in the literature and their PHA production. Only few wastes have been used unfermented towards PHA production. **Glycerol** has been used as carbon source during PHA production process, a content of 61 – 80 % is reported to be attained (Dobroth et al., 2011; Moralejo-Gárate, 2014; Moralejo-Gárate et al., 2011); **hardwood sulphite spent liquor (HSSL)** was use by (Queirós et al.,

2016) but only a merely 6.6% of PHA content was obtained. **Olive mill waste** was also used without fermentation and a PHA content of 43% was achieved using pure cultures (Alsafadi and Al-Mashaqbeh, 2017)

Table 1.1 VFA and PHA production from acidogenic fermentation using wastes

Waste	AF reactor	VFA yield	Units	PHA (%)	Reference
Paper mill wastewater	Continuously	0.74	g VFA g sCOD^{-1}	48	Bengtsson et al. (2008a)
		0.74		42	Bengtsson et al. (2008b)
	Batch-mode	0.60		77	Jiang et al. (2012)
Olive mill waste	PBBR	0.32	gVFA gCOD^{-1}	35	Beccari et al. (2009)
		0.42 – 0.50		30	Campanari et al. (2014)
	Batch-mode	0.22 – 0.44		54	Dionisi et al. (2005)
Food waste	PDBR	4.0	g VFA L^{-1}	24	Amulya et al. (2015)
	Batch-mode	0.52 – 0.61	g VFA g sCOD^{-1}	51 – 65	H. Chen et al. (2013)
	APBR	7.5	g HAc L^{-1}	48	Colombo et al. (2017)
	Continuous	3.8	g VFA L^{-1}	35	Venkateswar Reddy and Venkata Mohan, (2012)
Sugar cane molasses	CSTR	0.63 – 0.75	CmolVFA Cmol$_{Sugars}^{-1}$	30	Albuquerque et al. (2007)
		0.70		56	Albuquerque et al. (2011)
		0.70		32 -61	Albuquerque et al. (2010a)
		0.70		75	Albuquerque et al. (2010b)
		0.75		20	Bengtsson et al. (2010a)
		0.75		37	Bengtsson et al. (2010b)
	AnMBR	0.80	g COD g COD$_{sugars}^{-1}$	56	Duque et al. (2014)
Waste activated sludge	Batch-mode	0.26	g VFA g VSS^{-1}	73	Jiang et al. (2009)
Cheese whey	Batch-mode	0.4 – 0.6	C-mol VFA C-mol^{-1}	24 – 42	Colombo et al. (2016)
	CSTR	0.42 – 0.61	gCOD gCOD^{-1}	ND	Gouveia et al. (2017)
	AnMBR	0.65	g COD g COD$_{sugars}^{-1}$	65	Duque et al. (2014)

PBBR – Packed-bed biofilm reactor; PDBR – Periodic discontinuous batch reactor; APBR – Anaerobic packed-bed reactor; CSTR – Continous strirred-tank reactor; AnMBR – Anaerobic membrane bioreactors; sCOD – soluble COD; PHA content is reported as % of g PHA g^{-1} VSS; ND – not determined

The operational conditions of AF of wastes towards VFA production, as well as the composition of the waste will produce different VFA mixtures in different amounts (Dahiya et al., 2015; Yin et al., 2016a). For this purpose, it is essential to consider the composition of the VFA mixture after acidogenic fermentation as it is going to govern the properties of the resulting PHA (Gouveia et al., 2017).

1.2.5 Volatile fatty acids production from wastes

A well-known technology for waste valorisation is the anaerobic digestion (AD) that is industrially well implemented for the production of bioenergy in form of methane and also, for the use of the residue as a natural fertilizer (Dahiya et al., 2018; Jankowska et al., 2015; Khan et al., 2016). Among methane, the AD has been proved as a way to produce other value-added products with great potential such as hydrogen and VFA (Khan et al., 2016). The overall AD process involves biochemical reactions distributed in four interdependent steps that include hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig. 1.11) for the degradation of organic material to basic constituents.

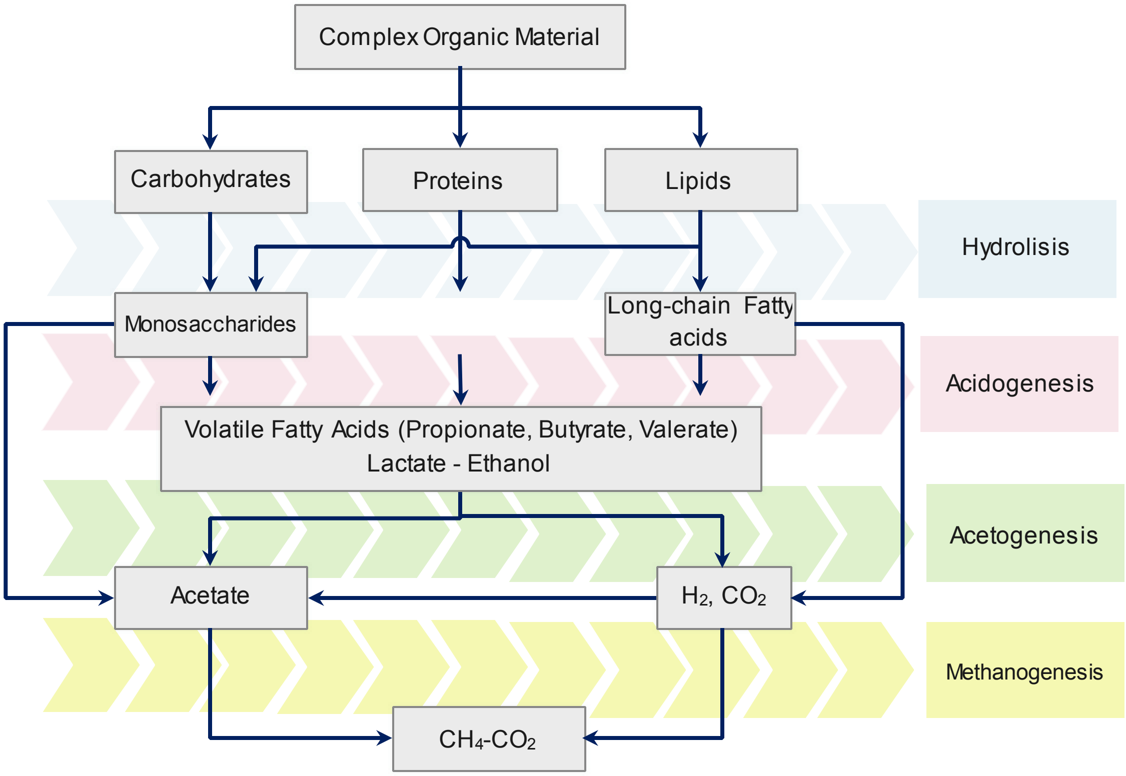


Figure 1.11 Steps on the anaerobic digestion process (adapted from Ersahin et al (2011))

Currently, the production of VFA using AD has attracted increasing interest due to its high potential for a wide range of end uses. Although methane production has already been established as an industrial process, VFA production is still being investigated (Perimenis et al., 2018).

A two-phase AD process has been proposed in which two different reactors perform two different phases: acidogenesis and methanogenesis (Pohland and Ghosh, 1971). The first phase is known as **acidogenic fermentation (AF)** and involves the bioconversion of the monomers produced from hydrolysis into hydrogen (H_2) and a mixture of VFA and also CO_2 (Dahiya et al., 2015). H_2 and VFA are valuable by-products of acidogenic fermentation and their production turn acidogenic fermentation into an environmentally sustainable and economically viable process.

When mixed cultures are used for the anaerobic production of VFA by using wastes, a lot of microorganisms are involved and so, several biochemical reactions take place and it can be expected the formation of different intermediates and by-products such as ethanol, butanol, acetate, propionate, butyrate, H_2 and CO_2 (Fig 1.12) (Ghimire et al., 2015; Zhou et al., 2018).

The biochemical pathways involved in acidogenic fermentation of complex wastes, can be mediated by many microorganisms that could be i) anaerobes, as Clostridia, methylotrophs, methanogenic bacteria, archaea, etc. ii) facultative anaerobes: Escheria coli, Enterobacter, Citrobacter and iii) aerobes: Alcaligens and Bacillus (Ghimire et al., 2015). Despite acetate and butyrate are the most commonly reported by-products of acidogenic fermentation, the production of one or another VFA as well as their yields, depends on the type of substrate used, the operational parameters such as loading rate, pH and temperature, the inocula, as well as environmental conditions. Then, the distribution of soluble by-products can be used as a guide to identify the preferred metabolic pathways (Ghimire et al., 2015; Silva et al., 2013; Zhou et al., 2018).

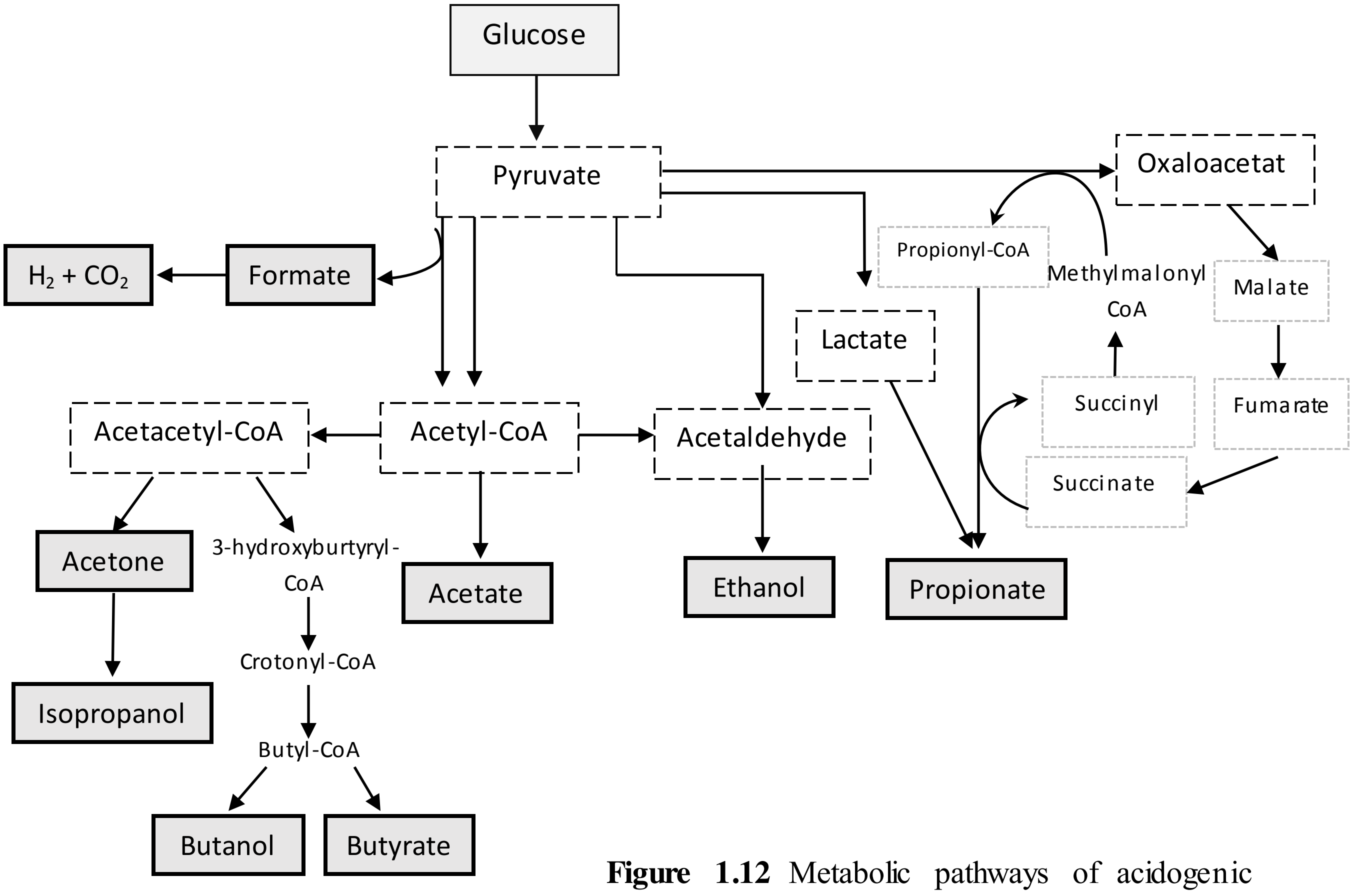


Figure 1.12 Metabolic pathways of acidogenic fermentation (adapted from Zhou et al. (2018))

1.2.5.1 Promoting acidogenic fermentation

In order to achieve high levels of VFA it has been proposed that a) hydrolysis must be improved in order to produce soluble products for their further fermentation, b) acidogenesis process must be promoted and c)acidogens inhibition should be avoided by inhibiting, for example, methanogens activity (Wang et al., 2014; Zhou et al., 2018).

Hydrolysis is the rate-limiting step throughout acidogenic fermentation, their improvement may lead to an increase in the readily available carbon of the substrate and consequently increase the VFA production yields. (Lee et al., 2014; Zhou et al., 2018). For that reason, many waste pretreatments have been proposed to enhance hydrolysis (Bougrier et al., 2006; Devlin et al., 2011). The most popular pretreatments are the chemical acid or alkali hydrolysis, biological pretreatments that make use of enzymes, pre-fermentation with mature compost and activated sludge and physical pretreatments that include thermal, microwave and ultrasound methods (Dinesh et al., 2018; Zhou et al., 2018). It is important to consider that the use of these pretreatments methods can affect the final composition of VFA due to the production of different solubilized compounds after pretreatment and it must also be considered the economic feasibility as well as the environmental impact of the selected pretreatment methods.

To **promote the acidogenic process** is important to consider factors like pH, temperature, retention time, organic loading rate (OLR) and the substrate/inoculum used, as all of it will directly affect the quality, distribution and VFA production yields. The pH has been reported to be a critical factor during acidogenic fermentation due to its strong influence over the metabolic pathways and effect on the predominance of different microbial populations. Unless it has been found to be inhibitory for acidogens at pH below 3 and over 12, the optimal pH ultimately depends on the nature of the waste employed (Lee et al., 2014; Temudo et al., 2007).

The temperature effect is less critical compared with the pH, however, is well-known to influence the microorganisms' growth, the enzymes activity and the hydrolysis of the organic matter (Zhou et al., 2018) and what is more important, it has been recognized to directly affects VFA composition (Jiang et al., 2013). Their effect against VFA production yield has also been studied at psychrophilic, mesophilic, thermophilic and even at extreme-thermophilic environments (Lee et al., 2014). The mesophilic range (20-50°C), specially at 35°C, was considered as the most beneficial and economical temperature for a successful VFA production, and in almost all the researches, an increment in temperature has resulted in an increment in VFA production yields (Lee et al., 2014; Zhou et al., 2018).

The hydraulic retention time (HRT) and solids retention time (SRT) are both important parameters during acidogenic fermentation. On one side, HRT is going to define the reactor volume and on the other hand, the SRT will control the selection of predominant microorganisms (Khan et al., 2016).

Low retention times (around 4-6 days) has been observed to enhance VFA production because the acidogens growth rate is higher than that of the methanogens and so is not enough for methanogens to consume VFA towards CH_4 and CO_2 production. However other studies found that high retention time may exhibit a positive effect over VFA production as it promotes waste hydrolysis promoting the availability of soluble proteins and carbohydrates for its fermentation (Jankowska et al., 2015; Khan et al., 2016; Lee et al., 2014).

The availability of substrate in the reactor increases with an increase in the OLR which leads to a higher production of VFA, however, several reports conclude that this linear correlation remains only until reaching the optimum range and above this, an ORL increase will have a negative impact on the system. Likewise, it was observed that the OLR significantly influences the distribution of the VFA (Lee et al., 2014; Zhou et al., 2018). Jiang et al. (2013) reported a rise of the acetate and valerate content with a low percentage of propionate and butyrate as ORL increases.

As mentioned above, the waste characteristics are one of the most important parameters affecting VFA production yields and composition. The content of carbohydrate-rich substances enhances proteins conversion into VFA (Yin et al., 2016a). On the other hand, the lack of some nutrients can reduce production yields, and the presence of toxic compounds such as phenols may inhibit acidogenic fermentation. However, co-digestion has been used to face these problems by the dilution of high organic contents and also, the dilution of toxic compounds, the regulation of moisture content and by the addition of nutrients that result helpful to achieve suitable carbon-nitrogen ratios (Ren et al., 2018; Zhou et al., 2018).

Finally, the inoculum is a key parameter of acidogenic fermentation. The use of mixed cultures for VFA production represents a great economic approach against the use of pure cultures, however, VFA production yields have been reported to be different depending on the use of either anaerobic granular sludge or aerobic sludge as inoculum (Wang et al., 2014; Yin et al., 2016b). It is also well documented that in order to achieve high VFA production methanogens activity must be inhibited. For that aim, several methods have been proposed to inactivate undesirable microorganisms from the inoculum(Dinesh et al., 2018; Zhou et al., 2018).

To promote a better acidogenic fermentation conditions, the **acidogens inhibition factors** may be avoided**.** One of the most studied inhibition during acidogenic fermentation is the presence of methanogens in the inoculum. There are many methods reported in the literature to avoid their presence that includes heat-shock treatment, pH control (acid/alkali environments) and the addition of chemical inhibitors (bromoethanesulphonic acid (BESA) and chloroform) (Zhou et al., 2018). All these strategies take advantage of the spore-forming characteristics of H_2-producing bacteria that can sporulate under adverse conditions and then germinate again in more favorable environments (Ghimire et al., 2015). Other factors may inhibit VFA production, for example, VFA accumulation that may cause the acidogenic reactions thermodynamically unfavorable and so the VFA overloading may be avoided (Zhou et al., 2018); also the presence of high amounts of other compounds like sodium chloride (NaCl) can negatively affect VFA production as reported by Wang et al. (2014).

1.2.5.2 Other VFA applications

Apart for their use as PHA precursors, VFA are valuable products by itself as useful chemicals, but can also be used as raw materials in various industries. Depending on their use, the fermented VFA-rich streams can be directly used or, in some cases, recovery technologies could be necessary (Agler et al., 2011). VFA are used in food industry as additives or preservatives and in livestock feed as antibacterial and preservative agents. In the pharmaceutical industry can act as buffer solutions and coating agents. Moreover, VFA is used in the chemical industry for the production of pesticides, solvents, esters and in the fabrication of cosmetics are employed as moisturizers and anti-acne agents (Agler et al., 2011; Dahiya et al., 2018; Zacharof and Lovitt, 2013). More recently waste-derived VFA has been recognized as a source for energy generation either as bioelectricity through bioelectrochemical systems, H_2 through the use of photofermentation, electrohydrolysis or microbial electrolysis cell or as biogas under anaerobic digestion(Lee et al., 2014).

1.3 Factors influencing the PHA contents and composition

A thoughtful selection of the culture, the substrate and the media composition will result in successful processes towards PHA production (Kosseva and Rusbandi, 2018). The PHA content and composition is a response of different parameters such as the kind of culture and substrate used, the SRT, the ORL, pH, temperature, nutrients concentration (N and P), feeding frequency and regime, dissolved oxygen (DO) and fest/famine ratio (F/F) (Albuquerque et al., 2010b; Kosseva and Rusbandi, 2018) (Figure 1.13).

The most important factor to effectively produce PHA is the strain as it is going to determine the process kinetics of growth and production (Albuquerque and Malafaia, 2018).

Commercialized PHA are normally produced by pure cultures or by genetically modified strains however the use of mixed cultures has arisen as an alternative to overcome the high production costs and also because their use allow the use of wastes as precursors for PHA production (Dias et al., 2006).

The substrate is crucial for determining the composition of the synthesised biopolymer and unless waste streams are promising substrates for PHA production, their use still limited to lab-scale (Albuquerque and Malafaia, 2018). By choosing the substrate, it is possible to tailor-made the desired characteristics of the final product (Koller et al., 2014)

Feast/famine strategy has exhibited better PHA accumulation results in comparison with Anaerobic/Aerobic systems and more recently microaerophilic conditions has been evaluated to enhance PHA contents (Moralejo-Gárate, 2014).

Short SRT drive to high cell growth rates with low use of the substrate for PHA storage and contrary, long SRT were reported to induce higher PHA contents (Dias et al., 2006). Increasing T has been found to negatively affect the PHA accumulation rates (Salehizadeh and Van Loosdrecht, 2004) and the ammonium concentration, F/F ratio, and OLR were reported as crucial factors for polymer storage (Albuquerque et al., 2010b). It was observed that a nitrogen limitation results in better PHA accumulations and as the carbon to nitrogen ratio (C/N) increases, PHA accumulation does with a decrease in the cell growth rate (Raza et al., 2018). The F/F ratio may affect PHA storage, low F/F ratios drive to an internal growth limitation that favors PHA storage (Albuquerque et al., 2010b; Villano et al., 2014). It was demonstrated that when working at high OLR, higher biomass volumetric productivities can be achieved however the system might be unstable (Dionisi et al., 2006).

One of the most interesting advantages of PHA is that are "tuneable" biopolymers which mean that their structure may vary by changing the substrate composition. The most well-known PHA is the PHB that is usually synthesized by using acetic acid as feedstock, however, when using mixtures of acetic and propionic acid, hydroxyvaleric (HV) monomer units can be introduced into the biopolymer structure enhancing the material properties (Philip et al., 2007). Once the substrate composition is a crucial factor governing the final PHA structure, the microbial community composition was also found to have an effect on the polymer structure (Carvalho et al., 2014).

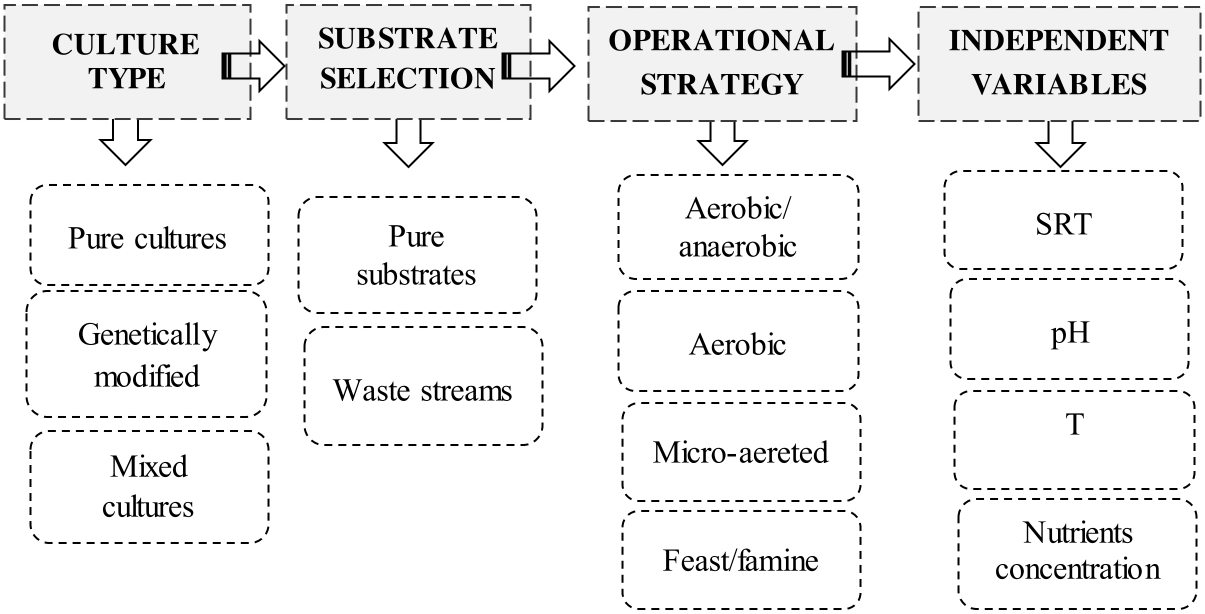


Figure 1.13 Factors influencing PHA content and composition

1.4 PHA Downstream processing

The overall PHA production costs not only relies on the feedstock nor the use of pure/mixed cultures but also on the downstream process. A suitable recovery method must be selected by considering the PHA producer, biopolymer composition, and purity requirements of the final product, impact of the extraction process on the PHA properties as well as the cost and environmental impact because the main goal is to maintain the polymer properties achieving high purity and good recovery levels keeping low the production costs (Kosseva and Rusbandi, 2018). In general, the biopolymer recovery involves three steps: i) pretreatment, ii) extraction and iii) purification (Fig 1.13).

The goal of applying a pretreatment is to facilitate the further PHA recovery. The first and most common method applied to recover PHA from microbial cells is the extraction using organic and halogenated solvents like chloroform and dichloromethane. Unless good recovery yields are achieved the need of using large quantities of hazardous solvents represents an enormous drawback and then, the use of environmentally friendly and efficient solvents have been investigated in parallel with the development of non-solvent recovery strategies, for example digestion with sodium hypochlorite, sodium hydroxide, enzymes or acids and the chemical disruption using detergents or chemicals (e.g. Sodium dodecyl sulfate (SDS) and sodium hypochlorite (NaOCl)) (Koller et al., 2014; Kosseva and Rusbandi, 2018; Lee et al., 2011). The supercritical fluid extraction is the newest technology developed for PHA recovery, and apart from CO_2, ammonia and methanol had also been investigated. It had been described as an effective process with no-impurities PHA extraction (Raza et al., 2018). Finally, a purification step may be employed if a high pure PHA wants to be obtained. Commonly hydrogen peroxide is used but due to their effect on the molecular weight of the PHA and the instability and high temperatures requirements, it can be substituted by the use of ozone (Kosseva and Rusbandi, 2018).

Downstream processing is still a bottleneck and a cost-determining factor in PHA production that needs further research to overcome the current high demands of energy and chemicals (Koller et al., 2014).

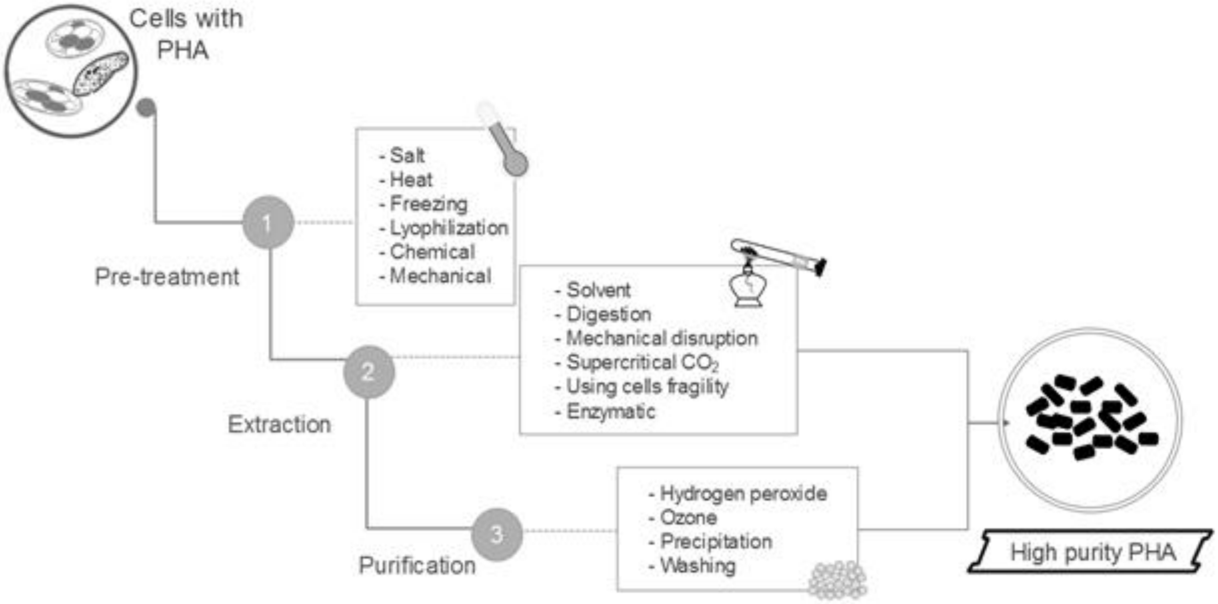


Figure 1.13 PHA downstream process (Modified from Kosseva and Eusbandi, 2018)

1.5 Properties and applications of the PHA

As mentioned above, the polymer properties can be tailored by "tunning" the biopolymer composition which leads to a significant number of different types of PHA with a wide range of physical properties and thus applications(Koller et al., 2014).

PHA are very competitive against polypropylene (PP) due to their similar properties. The key properties of the PHA are their non-toxicity, biocompatibility, and biodegradability. In general, their properties include high polymerization degree and crystallinity, are isotactic and optically active materials piezoelectric and insoluble in water (Raza et al., 2018). The PHB is a brittle and stiff material with high crystallinity; their mechanical properties are closely like to those of PP. The brittleness of PHB represents an obstacle for its application. HV monomer units are added to the backbone of the homopolymer PHB for the synthesis of the copolymer PHBV; by the addition of HV, PHA properties can be manipulated and enhanced, and different polymer characteristics may be achieved depending on the HV percentage. In general, stiffness and brittleness decrease and the elongation to break, tensile strength and toughness increase. Molecular weights (M_W) of PHA using MMC are very similar to that reported for PHA obtained by pure cultures being in the range of 1.7×10^5 to 4.5×10^6 (Dias et al., 2006; Laycock et al., 2014; Ross et al., 2016).

The first PHA application was in the packaging market for the fabrication of a wide range of products like cosmetic containers, milk cartons, shampoo bottles, bags, and pens and as conventional plastic products for example sanitary towels, razors, and diapers. However, with the development of new technologies and taking advantage of the stereospecificity of PHA, it can also be used as precursors for the synthesis of optically active compounds such as antibiotics, pheromones, herbicides and vitamins (Keshavarz and Roy, 2010; Khanna and Srivastava, 2005). In 2009 the esterification of PHA to hydroxyalkanoate methyl ester using ethanol was recognized to have a high potential to drive the use of PHA as biofuel (Gao et al., 2011). Biocompatibility of PHA is another of their great characteristics that allow their use for medical applications for example in the tissue engineering, to produce sutures, bone implants, surgical mesh and implants among others (Pakalapati et al., 2018). PHA has also been reported as suitable materials for the manufacture of bio-composites used in nanotechnology and drug delivery (Raza et al., 2018). PHA are also used as raw materials for the production of paints; in agriculture can be employed as mulch films, as bacterial inoculants to enhance nitrogen fixation (Philip et al., 2007; Reddy et al., 2003).

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Chapter 2

Objectives

GENERAL OBJECTIVE

The main objective of this thesis is to assess the feasibility of the integration of a three-stage process for PHA production into the circular economy model

SPECIFIC OBJECTIVES

- Use the acidogenic fermentation to promote wastes bio-conversion into volatile fatty acids production-rich streams
    - Compare the effect of different inoculum pre-treatment strategies over the acidogenic potential
    - Elucidate the degree of acidification of different wastes under anaerobic batch experiments
    - Determine the composition of the VFA matrix of each waste
- Evaluate the effect of the pH on the enrichment step of a PHA-accumulating mixed culture
- Evaluate the enrichment of a PHA-accumulating culture using a synthetic fermented-OMW as substrate
- Assess the effect of the pH, nutrients concentration, feeding regimen and substrate composition over the PHA accumulation
- Investigate the feasibility of using dimethyl carbonate (DMC) for PHA extraction
    - Evaluate the effect of a pre-treatment with NaOCl over the PHA recovery efficiency
    - Evaluate the effect of the ethanol precipitation over the PHA recovery efficiency
- Determine the structure, thermal properties and molecular weight of the extracted PHA

Chapter 3

Bio-conversion potential of waste into volatile fatty acids (VFA) through acidogenic fermentation

Summary

Polyhydroxyalkanoates (PHA) are naturally occurring biopolymers that are considered as potential substitutes of the petrochemical plastics. Notwithstanding, the main problem of PHA commercialization is due to their high production cost which is largely a consequence of the use of expensive carbon sources. The valorisation of wastes through biological processes has gained a lot of interest as a variety of value-added products may be obtained. Under wastes acidogenic fermentation (AF), is possible to obtain volatile fatty acids (VFA)-rich streams that can be used as low-cost carbon sources for PHA production.

In the first part of this study, the effect of three pre-treatment strategies on the inoculum were assessed with respect to the degree of acidification (DA) using glucose and cellulose as substrates. The pre-treatment strategies evaluated were the heat-shock pre-treatment, acid pre-treatment and alkaline pre-treatment. The higher DA was obtained for the heat-shock (39 % ± 2) pre-treatment. Subsequently, the pre-treated sludge was used as inoculum to evaluate the acidogenic potential of different wastes under batch acidogenic fermentation experiments. The wastes evaluated were: the sludge of two high-rate activated sludge system (WASa and WASb, respectively), a WAS of a common biological treatment (WASc), an olive mill effluent (OMW), a winterization Oil Cake (WOC), Glycerol and Apple pomace (AP). The higher DA was observed for WASa (69 %) followed by the OMW (43 %), while the lowest was observed for glycerol (16 %). The odd-to-even ratio was calculated as a parameter to determine the potential of the fermented liquid for the synthesis of PHA polymers. The odd-to even ratio for the OMW (0.01) indicates the acetic acid as the dominant acid which leads to the synthesis of the homopolymer polyhydroxybutyric acid (PHB). However WAS exhibited a higher odd-to even ratio (0.28) which means that a PHA copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV) (HB-co-HV) can be synthesized. To the best of our knowledge it was the first time that WOC was evaluated in terms of its acidogenic potential and the results elucidates a high content of propionic acid after fermentation.

3.1 Introduction

Polyhydroxyalkanoates (PHA) are bio-based and biodegradable polymers that may be produced by many microorganisms (Agustín Martinez et al., 2015). PHA thermoplastic properties are very similar to that of petroleum-based plastics and so, have been considered as interesting materials to replace the use of synthetic plastics (Amulya et al., 2015). The main obstacle for PHA widespread is their high production costs compared to the petroleum-based plastics. The use of mixed microbial cultures (MMC) has been widely investigated as an alternative to reduce the final PHA cost processes (Agustín Martinez et al., 2015). The implementation of MMC allow the use of a wide range of inexpensive wastes, as precursors for PHAs production, such as molasses, cheese whey, olive mill wastewaters, glycerol, paper mill wastewater, food waste, etc.(Amulya et al., 2015; Gouveia et al., 2017; Jiang et al., 2012). The production of PHA using wastes as feedstocks normally occurs in a three-stage process that involves: i) acidogenic fermentation, ii) culture selection and iii) PHA production (Alsafadi and Al-Mashaqbeh, 2017; Gouveia et al., 2017).

Waste valorisation has gained a great interest in the last decade due to their potential for energy and value-added chemicals recovery, which represents a green and valuable alternative to their disposal. The organic fraction of industrial and agro-industrial wastes can be fermented into volatile fatty acids (VFA), alcohols, hydrogen (H_2), methane (CH_4) and carbon dioxide (CO_2) (Perimenis et al., 2018; Yin et al., 2016a). The most widely applied treatment for industrial and agro-industrial wastes is anaerobic digestion (AD) which can be divided in two steps: acidogenic fermentation (AF) and methanogenesis. Commonly AD has been mainly used for the production of methane. However it has been recognised the presence of intermediates with high industrial value during AF such as H_2 and VFA (Jiang et al., 2007; Perimenis et al., 2018; Zhou et al., 2018).

H_2 is a potential, clean and renewable alternative to fossil fuels with a high-energy yield (142kJ/g) and only water production after its combustion (Silva et al., 2017, 2018). Biological hydrogen production reduces greenhouse emissions by 57-73% and AF has shown to be a sustainable process for biohydrogen production as it can use different substrates and is a simpler technology (Silva et al., 2018). Among H_2, the production of VFA can also be achieved via AF (Ghimire et al., 2015). It is reported than a 10-20% of organic wastes are converted into H_2 and CO_2 and around an 80-90 % is turned to soluble metabolic products that includes not only VFA, but also alcohols (Silva et al., 2018). VFAs are considered as promising by-products due their potential application as versatile precursors for example for the generation of electricity and bio-based solvents, as preservatives in food industry, synthesis of pharmaceuticals and chemicals, for biological nitrogen removal, biodiesel production and as precursors for the synthesis of biopolymers and alcohols (Dahiya et al., 2015; Perimenis et al., 2018; Zhou et al., 2018).

VFAs are well-known precursors for PHA synthesis and thus, AF can be integrated in the PHA production process which enhance, from an environmental and economic point of view, the overall process (Bengtsson et al., 2008; Campanari et al., 2014; Dahiya et al., 2018). The composition and properties of the synthesized PHA depends on the VFA chain-length and composition. In general, the presence of even number VFA as acetic and butyric acids, resulted in the synthesis of hydroxybutyrate (HB) and with odd-number VFA (propionic and valeric acids) hydroxyvalerate (HV) is produced. The polyhydroxybutyrate (PHB) polymer is the most widely known biopolymer, however their applications are limited because is stiff and brittle. On the other hand, the incorporation of HV leads to the synthesis of a copolymer P(HB-co-HV), which is more interesting from an industrial point of view as it is more elastic and flexible (Bengtsson et al., 2008; Lee et al., 2014; Silva et al., 2013).

During AF many parameters such as the carbon/nitrogen ratio (C/N), pH, temperature and feedstock composition, influence the yields of H_2 and VFAs production and composition (Jung et al., 2015; Sarkar et al., 2017). It is feasible to use an anaerobic mixed culture as inoculum for AF, but methanogens activity must be supressed (Kumari and Das, 2017; Singhania et al., 2013). For this goal, there are many pre-treatments reported in literature like the use of chemical inhibitors such as 2-bromoethanesulfonate acid (BESA), methyl chloride and lumazine (O-thong et al., 2009; Valdez-Vazquez and Poggi-Varaldo, 2009; Zhou et al., 2018), chemical pre-treatments such as acid, alkali and organic-shocks addition, and also, some physical strategies like heat, freezing and thawing, ultrasound, among others that effectively inhibit methanogens (Chang et al., 2011; Kumari and Das, 2017). The proposed pre-treatments take advantage of the capacity of the hydrogen-producing bacteria to produce protective end spores when exposed to harsh conditions (Hernández-Mendoza and Buitrón, 2014; Ruggeri et al., 2015).

A variety of solid and liquid wastes have been used in this research to appraise their acidogenic potential, namely: three waste activated sludge (WAS), three industrial wastes: Olive mill wastewater (OMW), Winterization Oil Cake (WOC) and Apple pomace (AP) and a by-product of biodiesel production: glycerol. Biological wastewater treatment processes, produces high volumes of waste activated sludge (WAS) which treatment and disposal represents around 60% of the total wastewater treatment plant (WWTP) costs (Wang et al., 2018). Commonly WAS is treated under anaerobic fermentation to produce methane, however, WAS is rich in carbohydrates and proteins and it has been considered as a suitable substrate to produce H_2 and VFAs. Nevertheless WAS requires pre-treatment methods to enhance their acidogenic yields that are usually low (Cai et al., 2004; Ghimire et al., 2015; Jiang et al., 2007; Wang et al., 2003). Besides WAS, the acidogenic potential of industrial and agro-industrial wastes has been also studied. On one hand, the OMW is an abundant bio-waste derived from olive oil production mostly in the Mediterranean region and represents an environmental problem due to the high amounts of production and because of its chemical characteristics (Campanari et al., 2014; Gameiro et al., 2015). Acidogenic fermentation to obtain VFAs, has been proposed as an alternative to treat OMW (Agustín Martinez et al., 2015; Beccari et al., 2009; Gameiro et al., 2015). On the other hand, the WOC is an industrial waste generated from the process to remove waxes from sunflower oil, safflower oil, canola oil and corn oil and is mainly composed by filtering aid, oil and waxes (Chalapud et al., 2017; Gupta, 2017). WOC is reported to be used as a substrate for solid-state fermentation (SSF) for the production of lipases (El-Bakry et al., 2015; Santis-Navarro et al., 2011) and sophorolipids (Jiménez-Peñalver et al., 2016) and to our best knowledge it is the first time to be explored in terms of its acidogenic potential. AP is highly biodegradable waste resulting from fruit juice industry that contains saccharides, proteins, vitamins and organic acids (Feng et al., 2010; Piwowarek et al., 2016). AP has been used to obtain value added products such as pectins, enzymes, phenolic compounds, organic acids, ethanol, antioxidants and as animal feed (Ajila et al., 2011) and some studies exploited its potential for H_2 and VFA production (Feng et al., 2010; Wang et al., 2010). Finally, Glycerol is a by-product generated during biodiesel production (Chong et al., 2009) that is considered as a waste due to their excess availability and thus, novel uses for glycerol has to be developed to avoid its environmental accumulation (Selembo et al., 2009). Glycerol acidogenic potential has been previously assessed as it is considered a versatile raw material for this purpose (Chookaew et al., 2012; Schwengber et al., 2016; Silva et al., 2013).

The acidogenic potential for all the residues described above was assessed with the main standpoint of being used as potential precursors for PHA production. For that aim, the experiments were strongly focused on the bioconversion of each substrate into VFAs due to their recognition as favourable substrates for PHA production. Moreover, the influence of three inoculum pre-treatment strategies (heat-shock, acid and alkaline) were assessed in order to select the one that most enhance acidogenic conditions.

3.2. Materials and methods

3.2.1 Inoculum and pre-treatment strategies

Anaerobic biomass used as inoculum was collected from an anaerobic reactor of a wastewater treatment plant (WWTP) (Rubi, Catalonia, Spain), with 6.3 g L^{-1} of volatile solids (VS). The biomass was stored under anaerobic conditions until the beginning of the experiments. The inoculum was treated by heat-shock, acid and alkali strategies in order to inhibit methanogenic activity. For the heat-shock, the sludge was heated at 104˚C for 2 h. Then, the dried sludge was broken with a mortar and pestle and the resulted powder was used as inoculum (Buitrón and Carvajal, 2010). The acid and alkali treatments were performed as described by (Zhang et al., 2011). The sludge pH was adjusted to 3.0-4.0 (acid) or 12.0 (alkali) by adding 1M HCl or 1M NaOH. The sludge were mixed and stirred for 24h and the re-adjusted back to pH 7.0.

3.2.2 Assessment of the pre-treatment strategies

Batch experiments were conducted to assess the inhibition degree of methanogens by the different pre-treated strategies applied to the inoculum using glucose as model substrate. The experiments were performed anaerobically in 150 mL glass reactors with a working volume of 140 mL. The initial concentration of VS was 6 gVS L^{-1} and the substrate concentration was 9 gCOD L^{-1}. The nutrients medium was prepared as described by Angelidaki et al. (2009)

and consists of adding 10 mL of stock solution A, 2 mL of B and 1 mL of C, D and E (stock solutions are detailed in Table 1) to 975 mL of distilled water. The mixture was sparkled with nitrogen gas (N_2) for about 5 minutes. A solution of 0.5 g of cysteine hydrochloride and 2.6 g of $NaHCO_3$ were prepared in 10 mL of distilled water and added to the nutrients medium. 20 mL of the nutrient medium was added to each bottle and distilled water was added to achieve the working volume. The pH was measured and fixed to pH 7 by the addition of 1M NaOH. Finally, each bottle were purged with nitrogen gas during 2 minutes and sealed. The reactors were incubated at 37 ˚C using a thermostatic incubator (MEMMERT® IN75).

Table 3.1 Stock solutions composition for the mineral medium used in the acidogenic fermentation batch experiments

Stock solution	Units	Composition
A	g L-1	NH4Cl (100); NaCl (10); MgCl2·6H2O (10); CaCl2·2H2O (5)
B	g L-1	K2HPO4·3H2O (200)
C	g L-1	Resarzurin (0.5)
D	g L-1	FeCl2·4H2O (2); H3BO3 (0.5); ZnCl2 (0.5); CuCl2·2H2O (0.038); MnCl2·4H2O (0.5); (NH4)6Mo7O24·4H2O (0.05); AlCl3 (0.05); CoCl2·6H2O (0.05); NiCl2·6H2O (0.092); EDTA (0.5); conc. HCl (1mL); Na2SeO3·5H2O (0.1)
E	mg L-1	Biotin (2); folic acid (2); pyridoxine acid (10); riboflavin (5); thiamine hydrochloride (5); cyanocobalamine (0.1); nicotinic acid (5); P-aminobenzoic acid (5); lipoic acid (5); DL-pantothenic acid (5).

3.2.3 Application of the selected pre-treated sludge to assess the acidogenic potential of different wastes

Once the best pre-treatment strategy was established, the pre-treated inoculum was used to determine the acidogenic potential of different wastes: three different waste activated sludge (WAS), an Olive mill wastewater (OMW), glycerol, apple pomace (AP) and winterization oil cake (WOC). In all experiments, the inoculum to substrate ratio was maintained ca. 1.

The WAS were collected from the high-rate activated sludge (HRAS) reactor from the SAVING-E pilot plant and from a WWTP (Rubi, Catalonia, Spain) performing organic matter removal by a conventional activated sludge (CAS) system. In the HRAS reactor, the hydraulic retention time (HRT) was set-up at 2 (WASa) and 7 days (WASb), while HRT was of 14 days at the CAS system of the WWTP. The OMW has been obtained from an olive oil producer (Lleida, Spain). Glycerol came from biodiesel production (Montmeló, Spain) the WOC was provided by an oil refinery (Barcelona, Spain) and the AP was collected from a cider industry (Girona, Spain). The characterisation of each waste is shown in Table 3.2.

Table 3.2 Characterization of wastes used as substrate for anaerobic fermentation

Waste	TS (g L-1)	VS (g L-1)	Organic content
WASa	3.90 ± 0.21	2.77 ± 0.15	3.68 ± 0.20[a]
WASb	7.44 ± 0.05	6.20 ± 0.03	9.25 ± 0.10[a]
WASc	8.8 ± 0.30	7.4 ± 0.36	6.02 ± 0.21[a]
OMW	0.24 ± 0.05	0.20 ± 0.03	1.4 ± 0.06[a]
Glycerol	750 ± 100*	625 ± 75*	1000 ± 0.33[a]
WOC	-	-	63.1 ± 1.61[b]
AP	-	-	97.0 ± 3.00[b]

*results presented in g Kg^{-1}; [a]organic content as g COD L^{-1}; [b]presented as % of dry basis

3.2.4 Acidogenic potential of wastes

Batch experiments were performed in the same way as described in section 2.2 for WASa, WASb, WASc, OMW, glycerol and WOC. In the case of the AP, the experiments were performed at a working volume of 50 mL and only 20 mL of mineral medium was added without any other source of water.

Samples were taken at regular interval times directly from the bottles in order to analyse the biogas composition and the VFAs profile. pH has been measured at the beginning and at the end of each experiment.

3.2.5 Calculations

The cumulative H_2 production (mmol H_2) was calculated as the total gas production (CH_4, CO_2 and H_2) and the concentration of H_2 in the headspace. The H_2 production yield was calculated as the quotient of the H_2 volume (mL) produced by the VS concentration (in grams) of the substrate (Noparat et al., 2011).

The degree of acidification (DA) was used as the parameter to evaluate the acidogenic potential. It was calculated by the sum of each VFA in COD divided by the substrate total initial COD as described by (Gameiro et al., 2015). The productivity of VFA was also calculated (in gCOD $gCOD^{-1}$) as the quotient of the amount of VFA by the substrate consumed and the volumetric VFA production rate was calculated by linear regression of the total VFA concentration versus the time (mgCOD L^{-1} h^{-1}), as reported by Bengtsson et al. (2008). As the VFAs composition represents an important parameter for further uses, the quality of the acidified waste for was determined in terms of the odd-to-even VFA ratio. It is defined as the sum of odd-equivalent carboxylic acids (propionic acid (HPr) and valeric acid (HVc)) divided by the sum of even-equivalent carboxylic acids (Acetic (HAc), butyric (HBt), iso-butyric (HiBt) and iso-valeric (HiVc) acids). VFA concentrations are expressed as theoretical equivalents of Chemical oxygen demand (COD) (HAc, 1.06; HPr, 1.51 mgCOD L^{-1}; HBt and HiBt, 1.81; HVc nd HiVc, 2.04) (Naresh Kumar and Venkata Mohan, 2018).

3.3 Results and discussion

3.3.1 Comparison of pre-treatment methods effect over VFA bioconversion and H_2 production

In order to achieve favourable conditions for acidogenic fermentation, H_2 and VFA consuming bacteria such as methanogens must be inhibited. Pre-treatment methods such as heat-shock, acid and alkali have shown to effectively repress H_2-consuming bacteria (O-thong et al., 2009; Rafieenia et al., 2018). Therefore these three pre-treatments were evaluated to identify the better and use it for the acidogenic potential tests. Glucose and cellulose were used as substrates.

In some cases, volatile fatty acids are considered as an indicator of a favourable environment for acidogenic fermentation; their production is coupled with H_2 production (Kumari and Das, 2017; Zhang et al., 2011). Fig 3.1 shows the DA for each pre-treatment. The higher DA was obtained for the heat-shock (39 % ± 2) followed by the alkali pre-treatment (35 ± 2) such as previously observed by O-thong et al. (2009) and Zhang et al. (2011). The lowest 22 % was for the acid pre-treatment. Ren et al. (2008), also reported the lower VFA production when using the acid pre-treatment. On the other hand, when cellulose was used as substrate, lower DA were obtained as it represents a complex substrate and their hydrolysis by anaerobic microflora is more difficult than when using glucose (Jiang et al., 2015). However among the different pre-treatment methods assessed similar results were observed (Fig 3.1) achieving a higher DA for the heat-shock pre-treatment (26 % ± 1) followed by the alkali pre-treatment (22 % ± 1).

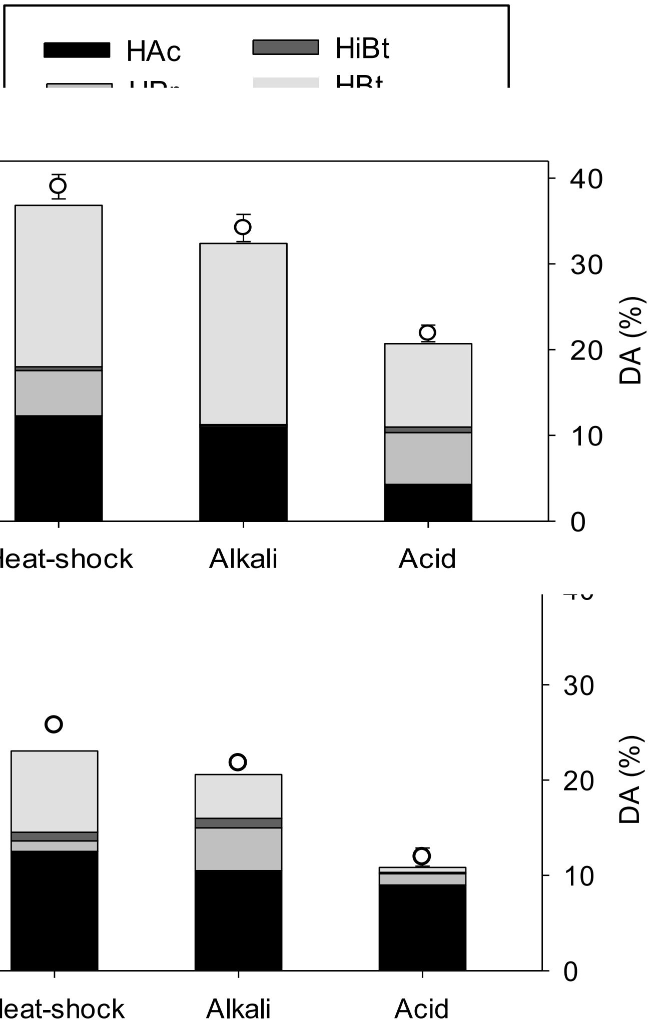

Figure 3.1 VFA concentrations after acidogenic fermentation using different pre-treatment methods. White dots represent the degree of acidification (DA) as %. A) Glucose as and B) Cellulose

Fig 3.1 also depicted the VFA composition after fermentation for both substrates. In all cases changes in the distribution of VFA in the fermented liquid was observed depending on the pre-treatment method. For the heat-shock and alkali pre-treatments a higher amount of HAc and HBt was obtained with almost negligible concentrations of HiBt and HVc acids when glucose is used. The high acetate and butyrate bioconversion was consistent with literature statements that reports high acetate and butyric production when using heat-shock and alkali treatments (Jiang et al., 2015; Kumari and Das, 2017; Zhang et al., 2011). On the case of cellulose, the major liquid metabolite, was HAc for all cases such as previously reported (Jiang et al., 2015; Lin and Hung, 2008). HPr was observed for the heat-shock and acid pre-treatments but not for the alkali pre-treatment when using glucose. On the contrary, a significant fraction of HPr was obtained for the acid pre-treatment using cellulose.

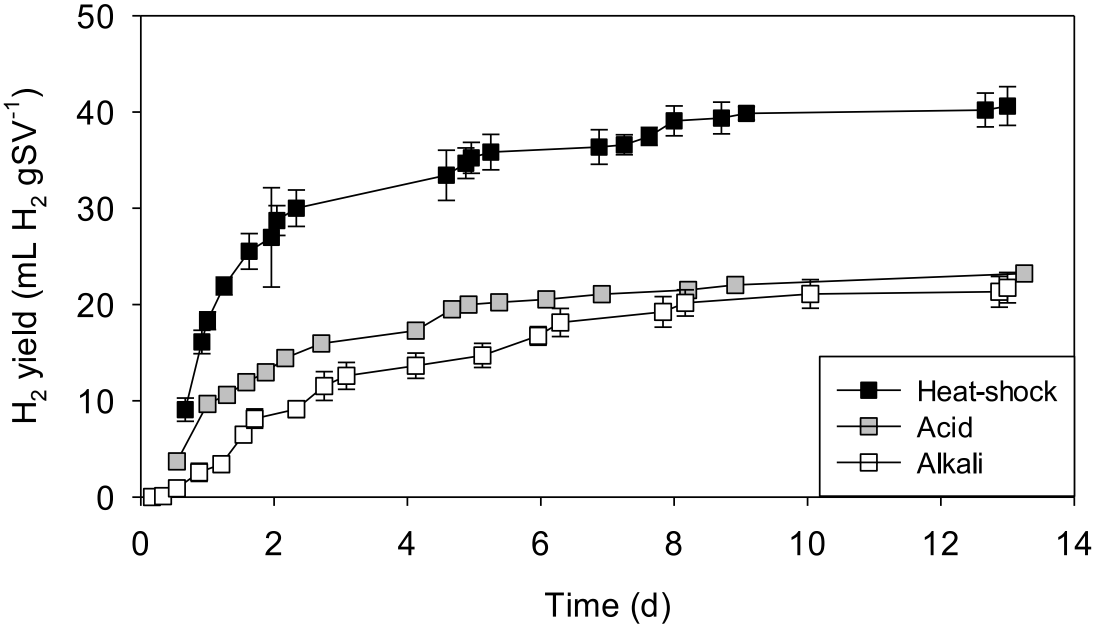

Figure 3.2 H_2 production yields over the time for the different pretreatment methods using glucose

The H_2 production yields were calculated to compare the effect of the different inoculum pre-treatments. Fig 3.2 depicted the time-course profiles for H_2 production yields. As it can be observed the higher H_2 yield was obtained with the heat-shock pre-treated inoculum (40.6 mL H_2 gVS^{-1}) followed by the acid and alkali pre-treatments (23.2 and 21.7 6 mL H_2 gVS^{-1}). Kumari and Das, (2017) reported similar results when comparing the H_2 production of glucose using different pre-treated inoculums (heat-schock, acid, alkali and freeze drying), achieving the maximum H_2 production for the heat-shock treatment. Also O-thong et al. (2009) reported better H_2 yields for the heat-shock pre-treatment against alkali and acid using sucrose as substrate. There are several reports that compare the effect of inoculum pre-treatment methods and most of the results are conclude that the heat-shock ptretreatment seems to be a preferable method to inhibit methanogens and prepare a H_2-producing inoculum using different substrates (O-thong et al., 2009; Ren et al., 2008; Zhang et al., 2011).

3.3.2 Acidogenic potential of different wastes under acidogenic fermentation

Batch experiments were performed aiming to evaluate the acidogenic potential of different wastes. Figure 3.3 depicted the evolution of the VFAs production yields for all substrates in time. It can be appreciated that the time to achieve the maximum VFAs is different for each waste and in all cases bioconversion of wastes into VFAs increases after inoculation. Three different WAS with 2, 7 and 14 days of sludge residence time (SRT), were assessed. Many researchers investigate the effect of different WAS pre-treatments to enhance VFAs production like the addition of a bio surfactant (Huang et al., 2016; X. Huang et al., 2015), alkaline and acidic conditions (Naresh Kumar and Venkata Mohan, 2018; Wang et al., 2018), heat (Cai et al., 2004; Yu et al., 2018) or nitrite pre-treatment (C. Huang et al., 2015) as well as the mixtures of different pre-treatments. In the present study, high rate WAS (low SRTs) was obtained from a novel biological wastewater treatment process and was assessed under acidogenic fermentation. It is supposed that the least the SRT the highest the biodegradability of the sludge and thus, better acidogenic potential (Xin et al., 2018). In line with the aforementioned, the objective was to determine the effect of different SRT over WAS fermentation. WAS biodegradability was also boosted by heat pre-treatment in order to pre-hydrolyse the organic fraction. The highest VFA yield was obtained for WASa (SRT=2d) (0.75 ± 0.08 g COD gVS^{-1}) which is higher compared to the results obtained by (C. Huang et al., 2015) (527.9 mg COD gVS^{-1}) by adjusting the pH to 10 and using the nitrite pre-treatment and Naresh Kumar and Venkata Mohan, 2018 using alkaline pre-treated WAS at pH 10. The results herein are also higher than the VFAs yields obtained using bio-surfactants or only alkaline pre-treatments (Huang et al., 2016; X. Huang et al., 2015; Li et al., 2017). On the other hand, the VFA yields for WASb and WASc were much lower in comparison with WASa. The maximum VFA yield was observed between days 4-6 of fermentation (ca. 0.30 g COD gVS^{-1}).

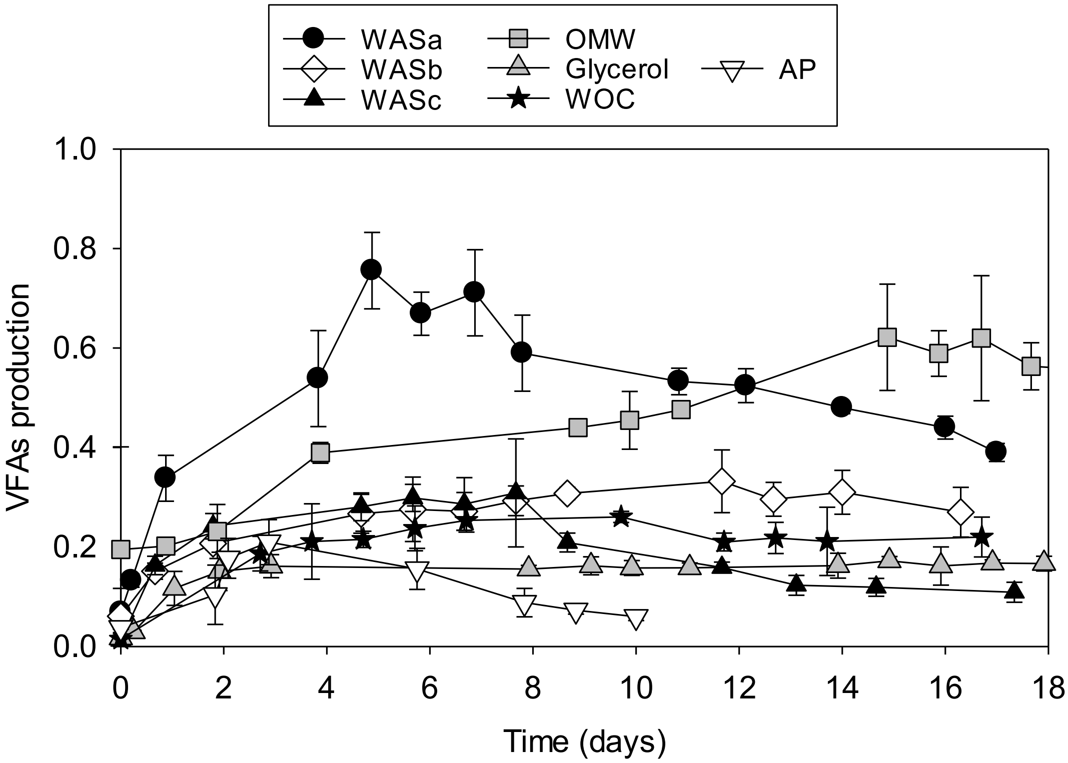

Figure 3.3 VFA conversion yields (in mgCOD $mgCOD^{-1}$) over the time for different wastes after fermentation

A high VFA yield were obtained from the OMW fermentation (0.62 ± 0.11 g COD g VS^{-1}) which yield was similar to that obtained for WASa. In the case of OMW, degradability was also boosted by heat pre-treatment and the results were higher compared with a 0.36 reported by Dionisi et al. (2005). The results obtained are very similar to the VFAs conversion yields reported by Campanari et al. (2014), however it is important to note that in that study a previous step of solid liquid separation was applied and then fermentation under different dilutions were performed. In this study that previous steps were not necessary as the characteristics of the OMW used are favourable for acidogenic fermentation because during the OMW disposal it is mixed with the urban waste of the facilities.

AP and WOC exhibited a very similar VFA yield, 0.21 ± 0.05 and 0.24 ± 0.02 g COD g VS^{-1}, respectively. To our best knowledge, this is the first time WOC is assessed as a substrate for acidogenic fermentation and regarding to AP only few studies are reported about the use of AP towards VFA production as nowadays AP, as well as WOC fermentation are used for the production of enzymes, aromas, animal feed, polyphenols, etc. This study suggests their use as substrates for its bioconversion into VFAs under acidogenic fermentation. Glycerol fermentation resulted in the lower VFA bioconversion yield (0.17 ± 0.02 g COD g VS^{-1}) and is very similar to the results reported by Silva et al. (2013). A higher conversion yield for glycerol was found in the work published by Forrest et al. (2010) (0.29 g total acids g^{-1} glycerol), but this study the fermentation was performed using iodoform as a methanogenic inhibitor and under thermophilic temperature (55°C) which will boost the production of VFA; it was also considered the bioconversion of glycerol into other carboxylic acids (caproic and heptanoic).

The Figure 3.4A depicted the results of the net DA for each fermented waste. The highest net DA was observed for the WASa (69 % ± 1) and is higher compared with the results of Li et al. (2017) (55-40%), Naresh Kumar and Venkata Mohan, (2018) reported a DA of 37.9 % and the 31 % reported by Silva et al. (2013). However, the DA for WASb and WASc which SRT corresponds to that of a typical biological WWTP, are in the range of previous studies (27 % ± 1 and 29 % ± 1, respectively). This difference could be due to a high biodegradability of the sludge with a low SRT (WASa) as more fermentable organic carbon availability favors the conversion into VFAs. The net DA for the OMW was 48 % ± 1, which is 2-fold high in comparison with WASb and WASc. The results obtained herein are significantly higher than the results presented by Silva et al. (2013) (13.6%), however in that study, no pretreatment was applied to the OMW. There are also higher DA reported by other authors (between 53 – 68%) in comparison with the results presented here (Gameiro et al., 2015; Yarimtepe et al., 2017), but different pre-treatments methods like electro-coagulation and dilution were employed to decrease the phenolic compounds which are supposed to inhibit the bioconversion of the organic matter into VFA at concentrations over 5 g L^{-1}. Glycerol was found to be the waste with the lowest DA (16 % ± 1) and this value is in line with previous reports (Silva et al., 2013; Tokumoto and Tanaka, 2012). However in this study only the bioconversion of glycerol into VFAs is considered but it had been found that under AF, glycerol may be fermented mainly into other soluble metabolites such as ethanol and 1,3-propandion (Chookaew et al., 2012).

Interestingly, WOC shown a higher net DA (22 % ± 1) compared with glycerol and very similar to that obtained for AP (19 % ± 1). In this study, any pretreatment was employed to

WOC and so, further investigations could be focused in enhance WOC acidogenic potential for its valorization into other value added products, different from lipases and sophorolipids.

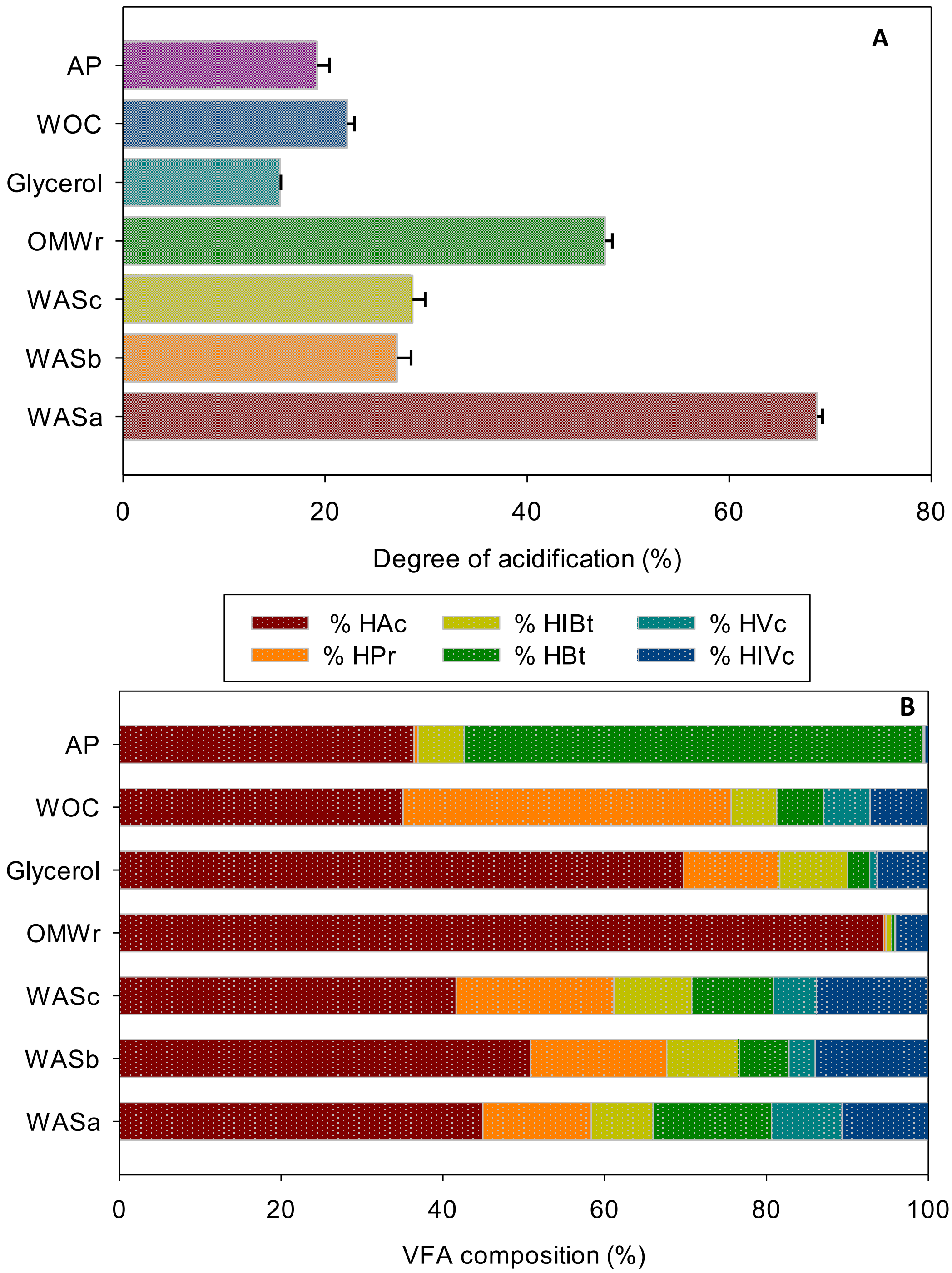

Figure 3.4 Acidogenic fermentation of different wastes. A) Net maximum degree of acidification (DA) for the fermented wastes, in %. B) VFAs profile (in %) for each waste

At this point, is very important to identify the composition of the VFA matrix obtained after fermentation (Fig. 3.4B). The valorisation of each waste is strongly dependent on VFA somposition as it is going to determine their final use (Gameiro et al., 2015). Taking into account that the goal of this research is to evaluate the potential of different wastes fermentation towards PHA production, VFA composition represents the most determinant parameter and so special attention must be pointed to the odd-to-even ratio. High odd-to-even ratios are more interesting because it means a higher HPr and/or HVc production. The presence of this VFAs will be profitable for the production of PHAs co-polymers such as P(HB-co-PHV) which is a more attractive bioplastic with better properties and possible applications (Gameiro et al., 2015). Table 3.3, collect the odd-to-even results obtained for the each waste fermentation at the maximum DA point.

Table 3.3 Acidogenic fermentation results for each waste

Substrate	VFA yield	Net DA	Odd-to-even
WASa	0.75 ± 0.08	69 ± 01	0.28
WASb	0.33 ± 0.06	27 ± 1	0.25
WASc	0.29 ± 0.03	29 ± 1	0.33
OMW	0.62 ± 0.11	48 ± 1	0.01
Glycerol	0.17 ± 0.01	16 ± 1	0.15
WOC	0.24 ± 0.02	22 ± 1	0.86
AP	0.21 ± 0.05	19 ± 1	0.01

When analyzing the results of each waste in terms of the VFA composition, we found that the AP and OMW resulted in the less interesting fermentation liquid if it is considered to be further used as a precursors for PHA production. Both substrates VFA were composed almost entirely by HAc that had been described to synthesize the PHB monomer. PHB is the most widely known PHA, however it has been demonstrated that copolymer P(HB-co-HV) has better mechanical properties (Gameiro et al., 2015). In the OMW fermentation liquid, the dominant and almost sole VFA produced was HAc (98.4 %) (Fig. 3.4A). Despite some other authors reported a great amount of HAc during OMW fermentation (Campanari et al., 2014; Gameiro et al., 2015; Ntaikou et al., 2009; Yarimtepe et al., 2017), the results presented in here are higher than in any other report. The presence of HPr, HBt, HiBt and Hvc were almost negligible (<1 %) while in other studies it accounts to around a 20 %.

Unless the odd-to-even ratio for AP was the same as OMW (0.01), their fermented liquid composition were very different. AP was composed by a mixture of HBt and HAc which are even-carbon fatty acids. The major VFA was HBt followed by HAc (56.6 and 36.4 %, respectively) (Fig 3.4A). HPr, HVc and HiVc were almost neglible (< 1 %) and only a 6 % of HiBt acid was observed. These results are similar to the VFA composition reported by Doi et al. (2010) using rhizosphere microflora, in which study HBt was also found to be the predominant fatty acid followed by HAc. Feng et al. (2010) reported also the presence of HAc and HBt as the main liquid end-products, with the difference that HAc was the main fatty acid synthesized (65 %) with very low concentration of HPr.

The main product of glycerol fermentation was found to be HAc (Fig 3.4A) as previously found by other authors (Forrest et al., 2010; Silva et al., 2013), but there are contrasting results for the HPr fraction. We found a 12 % of HPr such as the described by Silva et al. (2013), but Forrest et al. (2010) found it in a low amount (< 1 %) and Yin et al. (2016b) describe it as the dominant VFA.

The results for all WAS were very similar with an odd-to-even ratio between 0.25 and 0.33. The majoritarian VFA was found to be the HAc in a 40-60 %, followed by the HPr (15-20%) (Fig.3.4A). Such results are similar to previous studies that identified that HAc accounted to the highest percentage of the total VFAs produced after WAS fermentation (Cai et al., 2004; Huang et al., 2016; Li et al., 2017; Xin et al., 2018; Yu et al., 2018). The presence of HBt, HiBt and HVc have also been detected but in a low percentage. Naresh Kumar and Venkata Mohan, (2018) reported different VFAs composition when working at different pH conditions, concluding that at higher alkaline pH, higher chain fatty acids (as valeric) production is boosted; however when the initial pH is 7 (as in our study) the composition achieved was such as the presented here, with the acetic and propionic acids as the dominant VFAs. That results suggest that WAS may be a potential feedstocks for PHAs copolymer P(HB-*co*-HV) production.

In an interesting way, WOC was proved to be an attractive precursor for PHAs production as their VFA composition exhibited the higher odd-to-even ratio among all the evaluated wastes and so the presence of HP may result in the enhancement of PHA characteristics due to the with composition and properties as HV content can be enhance due to the incorporation of HV units. HPr was the dominant VFA (43.7 %) followed by HAc (37.8 %) and HBt, HiBt and HVc in the same low proportion (~ 6 %) (Fig 3.4A).

The production of VFA as soluble metabolites during AF are coupled to the production of H_2; nevertheless, the presence of VFA directly influences the H_2 yields. It is reported that, either dissociated or undissociated, VFA affect bacterial growth; its undissociated form can diffuse into the cell where due to the intracellular pH is shifted into their dissociated form releasing H^+ in the cytoplasm creating an imbalance that affect the metabolic activity leading to an increase of energy required for cellular maintenance which means lower energy for cell growth. On the other hand, dissociated form of acids increases the ionic strength of the medium producing cell lysis (Bundhoo and Mohee, 2016; Ciranna et al., 2014; Zheng and Yu, 2005).

In general, it is supposed that low VFA production can be favourable for H_2 production, but under high production, the process may be inhibit suppressing H_2 yields (Bundhoo and Mohee, 2016). Under such consideration it could be expected that the substrates that exhibited a lower bioconversion into VFA might result into a higher H_2 yield, however as it can be observed in Figure 3.5 it was not true in all cases. In this study Glycerol was the substrate with the lowest DA and also with the highest H_2 yield (10.6 mL H_2 gVS^{-1}), notwithstanding the VFA produced by using AP were very low and its H_2 yield was very similar to that obtained for OMW and WASc. It could be due to its high bioconversion into HBt followed by HAc. Zhao et al. (2010) reported that concentrations of HBt close to 4.0 g L^{-1} cause the inhibition of bacterial H_2 production and in our experiments, HBt accounted to 3.8 g L^{-1}. It was also observed previously, that the presence of HBt has a stronger effect against H_2 production in comparison with HAc (Bundhoo and Mohee, 2016; van Ginkel and Logan, 2005) and that the stoichiometric yield of H_2 is higher for HAc than for HBt as 4 mol of H_2 can be produced for 1 mol of glucose when HAc is the final product and on the other hand only 2 mol of H_2 are synthesised when HBt is produced (Ghimire et al., 2015). A low H_2 yield (3.6 mL H_2 gVS^{-1}) was measured for WOC despite its DA was very similar to that for AP. It could be a consequence of the high production of HPr as end-product as it was previously demonstrated that HPr can decrease H_2 yields due to its H_2-consuming pathway (Ghimire et al., 2015; Zhang et al., 2011).

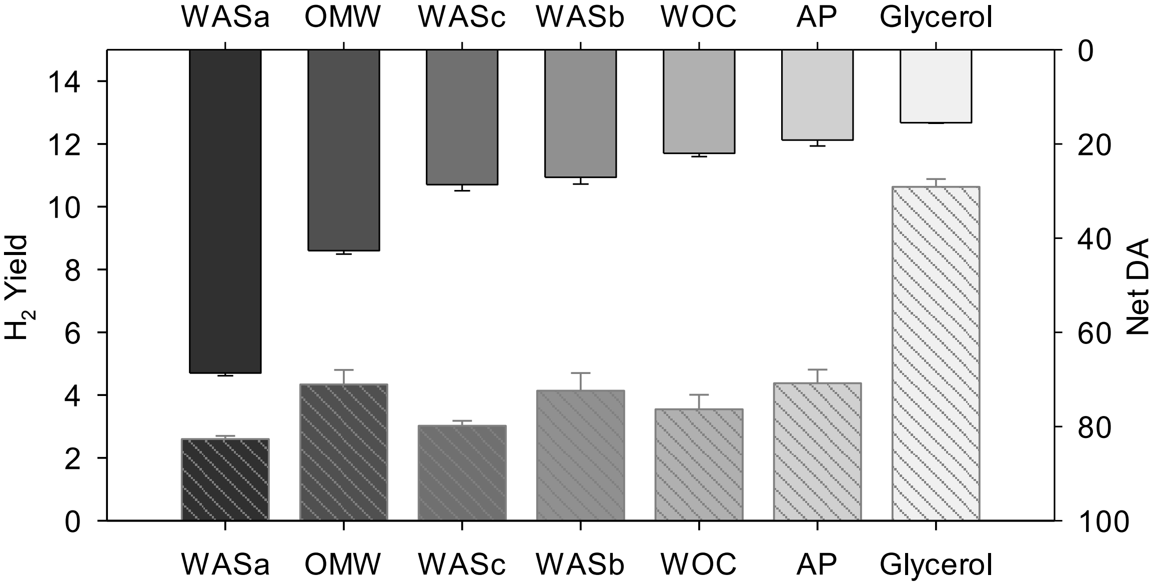

Figure 3.5 H_2 production yields (mL H_2 gVS^{-1}) and net degree of acidification (DA) (VFA produced, in %) for each waste

The bioconversion of WASb and WASc into VFA were very similar, however a slight difference on their H_2 yields was observed (4.1 and 3.0 mL H_2 gVS^{-1}, respectively) (Fig. 4.5). Such difference could be due to the high amount of HPr produced after WASc fermentation whicht might decrease of H_2 production as mentioned above. WASa was the substrate with the highest DA and therefore its H_2 yield was the lowest (2.6 mL H_2 gVS^{-1}), however a discrepancy was observed regarding the results obtained for the OMW. Despite its bioconversion in VFA was high, we observed a H_2 yield higher than the expected (4.3 mL H_2 gVS^{-1}) which is very similar to the result obtained for AP even though the DA of the AP is twice lower compared to the OMW. Such result could be due to the almost completely production of HAc as fermentation product.

3.4 Conclusions

A high VFA production was obtained during acidogenic fermentation using a pre-treated anaerobic inoculum. The heat-shock pre-treatment show higher DA in comparison with the alkali and acid pre-treatments.

The bio-conversion of wastes into VFA has attracted a lot of attention due to their potential use as precursors for PHA production. The batch acidification experiments of the wastes evaluated herein, exhibited different degrees of acidification (DA) as well as VFA compositions. The higher DA were attained for WAS collected from a high-rate activated sludge reactor operated at and HRT of 2 days and for an OMW (69 and 48 % gVFA (as COD) $gCOD^{-1}$, respectively). The lower DA was observed for the glycerol (16 %). To our best knowledge, it was the first time that WOC was evaluated for their bio-conversion into VFA under acidogenic fermentation and a 22% of DA was observed. The odd-to-even ratio was calculated to identify the feasibility of using the fermented liquid waste as precursor for PHA production. Higher odd-to-even ratios are desired as it means higher amounts of HV monomers in the biopolymer. WOC exhibited the higher odd-to-even ratio (0.86) and contrary, OMW and AP exhibited the lower value. For all the WAS evaluated, an odd-to-even ratio around 0.30 was obtained. The main soluble metabolites obtained in this study were acetic, butyric and propionic acids.

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Chapter 4

Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: effect of pH and N and P concentrations

Summary

Polyhydroxyalkanoates (PHA) are biopolymers that can be an alternative against conventional plastics. The study reported herein evaluated the enrichment of a mixed microbial culture (MMC) operated under feast/famine regime and different pHs in a sequencing batch reactor (SBR) using acetate as sole carbon source to produce polyhydroxyalkanoates (PHAs). The enrichment step was evaluated at controlled pH of 7.5 and also without pH control (averaged value of 9.0). The acetate uptake rate ($-q_S$) of both enrichments at the end of the experimental period exhibited similar behaviour being about 0.18 C-mol Ac C-mol X^{-1} h^{-1} and 0.19 C-mol Ac C-mol X^{-1} h^{-1} for SBR-A and SBR-B, respectively. However, the PHA-storing capacity of the biomass enriched without pH control was better, exhibiting a maximum PHA content of 36% (gPHA g^{-1}VSS) with a PHA production rate (q_{PHA}) of 0.16 C-mol PHA C-mol X^{-1} h^{-1}. Batch experiments were performed to evaluate PHA-storing capacity of the enriched culture at different pHs and nutrients concentrations. In the pH experiments (without nutrient limitation), it was found that in the absence of controlled pH, the enriched biomass exhibited a PHA content of 44% gPHA g^{-1}VSS with $-q_S$ and PHA to substrate yield ($Y_{PHA/Ac}$) of 0.57 C-mol Ac C-mol X^{-1} h^{-1} and 0.33 C mmol PHA Cmmol Ac^{-1}, respectively. Regarding the experiments at variable nutrients concentration (pH ranging 8.8 to 9.2), the results indicate that the PHA content in the enriched biomass is significantly higher being around 51% gPHA g^{-1}VSS under nitrogen limitation. This work demonstrated the feasibility of the enrichment of a MMC with PHA storage ability without pH control. Results also suggest that better PHAs contents and substrate uptake rates are obtained without controlling the pH in the accumulation step. Finally, this work also highlights the importance of understanding the role of nutrients concentration during the accumulation step.

4.1 Introduction

Polyhydroxyalkanoates (PHA) represent a group of biopolymers that could be accumulated as carbon and energy sources by several of bacterial genera. Their thermoplastic properties are comparable with those of petroleum-based plastics, converting them into a potential alternative to overcome environmental issues derived from the current plastics market (Basak et al., 2011; Salehizadeh and Van Loosdrecht, 2004). PHA research is attracting an increasing interest to understand and to improve their production technologies. Nowadays, most of PHA industrial production is achieved by the use of pure or recombinant cultures which requires sterile conditions and the use of specific carbon sources resulting in high operational costs between 4-9 times higher than that of conventional plastics production. Additionally, the final PHA production costs are strongly affected by the recovery processes, therefore efforts must be conducted to improve biopolymer content of biomass (which affects the efficiency of the recovery processes) and also, to develop new recovery methods (Hand et al., 2016; Reddy and Mohan, 2012).

The use of a mixed microbial culture (MMC) is an interesting alternative in order to reduce the current PHA production costs (Wen et al., 2010); activated sludge is a well-known MMC found to be able to accumulate PHA under unsteady conditions (Liu et al., 2011; Salehizadeh and Van Loosdrecht, 2004). In contrast with PHA production by pure cultures, the use of MMC would reduce the costs because a simpler control process is needed, non-sterile conditions are required, and furthermore MMC can easily adapt to changes in the carbon source, therefore a variety of waste substrates can be used for this purpose (Cavaillé et al., 2016; Reddy and Mohan, 2012). The overall process for PHA production using MMC is commonly accomplished in a three-step process (Bengtsson et al., 2008a; Johnson et al., 2009; Lee et al., 2015). A first step, where organic matter is rendered into a rich volatile fatty acids (VFA) feedstock via acidogenic fermentation. Secondly, a culture enrichment step is needed to select PHA-storing bacteria using the VFA produced previously. Finally, the enriched biomass is harvested and is used in the third step where certain operational conditions are applied with the main objective of maximising the polymer content in the biomass.

To achieve PHA accumulation in MMC, a specific selection pressure strategy must be imposed in the culture selection step, by applying, for example, an intermittent feeding regime or by varying the availability of an electron acceptor (Liu et al., 2011; Valentino et al., 2015). A well-known method is the use of the Feast and Famine (FF) regime, which consists in alternating short periods of C-source availability with long periods of unavailability (Dias, et al., 2006; Albuquerque et al., 2013). In such transient conditions, only the microorganisms able to store PHA will withstand the starvation period due to their competitive advantage over the other microorganisms (Villano et al., 2010).

Metabolic processes for PHA production are normally affected by changes in certain parameters, such as: carbon source concentration and type, nutrient availability, temperature, pH (Keshavarz and Roy, 2010) and electron acceptor availability (Morgan-Sagastume et al., 2014), among others. According to Wei et al. (2011), pH which is one of the main factors influencing PHAs production. However, studies about the effect of pH on aerobic PHA-production processes with MMC are contradictory. For example, Wei et al. (2011) suggested a pH range of 6.0 to 7.5 for PHAs production and cell growth. While Mohan and Reddy (2013) concluded that a neutral pH would be better as enzymes involved in PHAs production are active at pH 7. On the contrary, other studies reported a PHAs content to be higher at a pH range of 8.0-9.0 (Chua et al., 2003; Liu et al., 2011; Serafim et al., 2004; Villano et al., 2010).

Among pH, other decisive factors must be taken into account to achieve higher PHA contents of enriched biomass. As mentioned before, nutrients content (mainly phosphorus (P) and nitrogen (N)) is an important parameter to be considered (Silva et al., 2016) as a way to optimize PHA-accumulation.. It is suggested on literature, that C source is used for biomass growth instead of being used for PHA accumulation, when excess of nutrients is available (Morgan-Sagastume et al., 2010). On the contrary, under N and/or P limitation, the metabolic pathway for PHA accumulation is promoted (Albuquerque et al., 2007). In most of the cases, higher PHA content (Dionisi et al., 2005; Johnson et al., 2010) and accumulation yields (Basak et al., 2011; Marang et al., 2013; Reddy and Mohan, 2012) were found under N-limitation than under N-excess. However, PHA specific storage rates and productivities are suggested to be improved when working at non-limiting N levels (Dionisi et al., 2005).

Concerning P-limitation, it restrains the Krebs cycle favouring the accumulation of PHA (Liu et al., 2011; Cavaillé et al., 2013; Reddy and Mohan, 2012).. Nevertheless, Jiang et al. (2012) have concluded that PHA contents are not improved when increasing nutrients concentration). Therefore, seems that rather than working with an specific N and/or P limiting concentration it seems that adequate levels of these nutrients must be found to achieve a high PHA storage capacity.

According to Fang et al. (2014), the influential priority of parameters affecting the overall PHA production rate using activated sludge is the following: pH>N concentration>P concentration>COD concentration. Therefore, in this study, on the one hand the pH effect was selected to be assessed firstly as the absence of a pH control would result in a robust and more cost-effective process. On the other hand, considering that different type of wastewaters could be used, the study of the influence of nutrients concentrations must shed some light in the improvement of the overall PHA-production process.

To evaluate the feasibility of MMC for PHA production, activated sludge from a municipal wastewater treatment plant was enriched under Feast/Famine conditions to evaluate the effect of pH during this step. Consequently, to assess PHA accumulation capacity of the enriched biomass, batch experiments were conducted varying pH conditions from 4.5 to 8.5 and also at free pH (ranging from 8.8 t 9.2). Moreover, it was also performed batch experiments under different conditions of nutrients from starvation to excess.

4.2 Materials and methods

4.2.1 Biomass enrichment

The selection and maintenance of a biopolymer-producing bacterial culture was performed using two Sequencing Batch Reactors (SBRs) with a working volume of 15L (SBR-A) and 20L (SBR-B). The reactors were operated at room temperature (ca. 22-24°C), as non-sterile MMC in 12-h cycles under FF regime. Each cycle consists of the following phases: idle (12 min), feeding (3 min), reaction (640 min), settling (60 min) and effluent withdrawal (5 min). The medium was aerated and stirred continuously during the reaction time. The sludge retention time (SRT) and hydraulic retention time (HRT) were maintained at 2 and 1 day, respectively in both reactors. For SBR-A, the pH was controlled at 7.5 ± 0.1

by adding 1M HCl or 1M NaOH. In the case of SBR-B no pH control was used with values ranging around 8.8 to 9.2.

Both reactors were continuously operated for 120 days and were inoculated using activated sludge from a municipal wastewater treatment plant (Granollers, Catalonia, Spain). Acetate was used as the sole carbon source at a concentration of 1.2 g COD L^{-1} with and organic loading rate of 1.2 g COD L^{-1} d^{-1} calculated as the inlet flowrate (L d^{-1}) multiplied by the initial concentration of the substrate in the SBRs divided by the reactor volume. In each cycle the volumetric exchange ratio was of 50%. The medium solution was composed of (concentrations are expressed in mg L^{-1}): KH_2PO_4 (54.4), K_2HPO_4 (43.6), NH_4Cl (129.4), $MgSO_4$ (43.9), $MgCl_2$ (160), $CaCl_2{\cdot}2H_2O$ (42), $NaHCO_3$ (30), allylthiourea (ATU, 50) to prevent nitrification and 30 mL of a trace elements solution consisting in (in µg L^{-1}): $FeCl_3{\cdot}6H_2O$ (1500), H_3BO_3 (150), $CuSO_4{\cdot}5H_2O$ (30), KI (180), $MnCl_2{\cdot}4H_2O$ (120), $Na_2MoO_4{\cdot}4H_2O$ (60), $ZnSO_4{\cdot}7H_2O$ (120), $CoCl_2{\cdot}6H_2O$ (150) and 68.5 mL EDTA 0.5M (Tayà et al., 2015). This gave a carbon to nitrogen to phosphorus (C/N/P) weight ratio of 100/10.3/6.1 (taking 100 g of C as basis) that is equivalent to a molar ratio of 100/8.8/2.4 (taking 100 mol of C as basis), i.e. reactors were operated without nutrients limitation.

Cycle measurements were conducted throughout SBRs operation in terms of biomass, acetate and dissolved oxygen (DO) concentration. Once stable operational performance was achieved, SBRs cycles where characterised by measurements of biomass, acetate, PHA and DO concentrations. Stable operation was considered when the suspended solids (VSS) concentration and the length of the feast phase were constant for at least 5 cycles.

4.2.2 Batch experiments for PHA-accumulation depending on pH

To evaluate the PHA storage capacity of the enriched culture, batch accumulation experiments were performed under different pH conditions: 4.0, 5.5, 6.5, 7.5, 8.5 and free pH (ranging between 8.9 - 9.2). At the end of each cycle, 0.5L of biomass was collected from the SBRs and it was mixed with 0.5L of the same fresh medium as the one used in the enrichment step. The experiments were carried out in successive days to minimize the impacts related to fluctuations on biomass composition.

For each experiment, 1L glass reactors were used. During the whole experiment biomass was aerated and magnetically stirred. Reactors were operated at room temperature (ca. 22ºC) and pH was controlled using 0.6M HCl and 0.6M NaOH at different pH values. Each experiment started by the addition of a feed pulse of acetate at an initial concentration of 1.2 gCOD L^{-1}. Biomass samples were taken regularly to follow acetate and PHA concentration, as well as, the volatile suspended solids (VSS).

4.2.3 Batch experiments for PHA-accumulation capacity at different nutrients concentration

Similar accumulation experiments were performed to assess PHA storage capacity of the enriched culture under different nutrients concentration. In this case, 0.5L of biomass was collected from the SBR-B and it was mixed with 0.5L of the same fresh medium as before, but changing the concentrations of nitrogen or phosphorus to have limited or excess conditions in the C/N/P molar ratio as follows: a) 100/4.1/2.4 (N-limited experiment), b) 100/65.9/2.4 (N-excess experiment), c) 100/8.8/1.3 (P-limited experiment), d) 100/8.8/90.5 (P-excess) and e) 100/4.1/1.3 (N and P limited experiment).

As in the previous experiments, 1L glass reactors were used and in the whole experiment biomass was aerated and magnetically stirred. Reactors were operated at room temperature (ca. 22°C) and in this case, pH was left free. The experiments started by the addition of a feed pulse of acetate (1.2 gCOD L^{-1}) and then, biomass samples were taken to analyse the evolution of acetate and PHA concentration, as well as VSS.

4.2.4 Analytical methods

Mixed liquor samples from SBR-A and SBR-B were taken to determine total suspended solids (TSS) according to method 2540D (APHA, 2005), VSS concentrations were done following the method 2540E (APHA, 2005). Sludge volumetric index (SVI) was conducted based on the method 2710D (APHA, 2005).

For acetate analysis, samples taken from the reactor were filtered with a 0.45 µL pore size filters and transferred to 2 mL septum-capped vials. Acetate concentration in the supernatant was measured using an Agilent Technologies 7820 A gas chromatographer equipped with a DB FFAA column (length: 30 m, internal diameter: 0.25 mm and film thickness: 0.25 µm) and a flame ionization detector (FID). A sample of 1 µL was injected at 275°C using helium as carrier gas with a split ratio of 10:1 at 2.9 mL min^{-1}. Oven temperature was initially set at 85°C and maintained for 1 min, followed by an increase of 3°C min^{-1} until reach 130°C. A second ramp of 35°C min^{-1} was maintained to reach 220°C.

For PHA analysis, sludge samples were mixed with 0.6 mL of formaldehyde to inhibit biological activity. Subsequently, samples were lyophilized. Copolymer poly (3-hydroxybutyric acid-co-3Hydroxyvaleric acid) and polyhydroxy-2-methylvalerate (PH2MV) were used for calibration and as standard during the analysis, so it was treated in the same way as the biomass samples. Lyophilized samples were weighted and transferred into glass tubes with screw caps. Benzoic acid was added as internal standard in all tubes. 1.5 mL of butanol and 0.5 mL of hydrochloric acid were added to each tube and incubated at 100°C for 8h. After cooling, 2.5 mL of hexane and 4 mL of MilliQ grade water were added. The tubes were vortex mixed and the organic phase was transferred to 15 mL clean tubes. A second 4 mL aliquot of MilliQ grade water was added and the tubes were centrifuged at 2500xg for 10 min. The organic phase was extracted and transferred into GC vials and analyzed by injecting 1 µL of sample in an Agilent Technologies (7820 A) gas chromatograph equipped with a FID detector and a HP-InnoWax column (30 m x 0.53 mm x 1 µm). Helium was used as carrier gas at 54 mL min^{-1}. The temperature of the injector was 220°C and the temperature of the detector was 275°C. The oven temperature was set to 70°C for 2 min, increased at 10°C/min to 160°C and held for 2 min (Werker et al., 2008).

4.2.5 Calculations

PHA contents in the biomass were calculated as a percentage of VSS on mass basis (%PHA = gPHA/gVSS *100), where VSS includes active biomass (X) and PHA. Active biomass (X) was calculated by subtracting the PHA content from the VSS (gX = gVSS – gPHA).

The yields for PHA production ($Y_{PHA/Ac}$ in C-mol PHA/C-mol Ac) and active biomass produced ($Y_{Xp/Ac}$ in C-mol X_p/ C-mol Ac), were calculated by dividing the amount of PHA produced or active biomass produced by the total amount of acetic acid consumed.

Acetate uptake rate ($-q_{Ac}$ in C-mol Ac/C-mol X) and PHA production rate (q_{PHA} in C-mol PHA/ C-mol X), were calculated by linear regression of the concentrations divided by the average of active biomass at the end of the feast phase.

Finally, the feast to famine ratio (F/F ratio) was calculated as the length of the feast phase divided by the length of the famine phase.

4.3. Results and discussion

4.3.1 Enrichment of activated sludge

Laboratory-scale SBRs namely as SBR-A and SBR-B were operated for 120 days to understand the effect of pH alongside the biomass enrichment step. Biomass concentration in both reactors is depicted in Figure 4.1.

Total solids concentration was higher in SBR-B compared to SBR-A working at pH 7.5 (2.2 g L^{-1} and 1.0 g L^{-1}, respectively). These results are consistent with the SVI measured in both reactors. For SBR-A, the SVI was maintained at around 180 mL g^{-1} while for SBR-B, SVI was of 152 mL g^{-1} during the first 50 days of and then, it improved to 84 mL g^{-1}, which has a positive effect on the content of biomass in the reactor. The improvement on SVI could be related with a good selection of PHA-storage biomass; hypothetically three different types of bacteria exists 1) strong PHA accumulating ability bacteria, 2) weak PHA accumulating ability bacteria, and 3) non-PHA accumulating bacteria. Considering that PHA is stored as intracellular granules it could be assumed that cell density increase as more PHA granules are accumulated and as a consequence, a fast settling would occur (Chen et al., 2015).

Figure 4.2 shows the concentration profiles of acetate, PHA and DO in the reactors obtained at the end of period I. It was observed that acetate was available only for 3 to 1.5 h of the 12-h cycles. As observed in Figure 4.2, the metabolic behaviour of the PHA-accumulating organisms, in both reactors, was achieved as the typical feast/famine profiles were established. During the feast phase and after substrate addition, the DO decreases until acetate was completely depleted. Before acetate depletion, the beginning of the famine was identified due to the sudden DO increase, returning to similar values as those observed before carbon source addition. In the case of SBR-B a pH value variation was observed with increasing values after substrate addition from 8.8 to 9.2 because of the decarboxylation and the utilization of acetate (Chua et al., 2003; Serafim et al., 2004). In both reactors, the length of the feast phase was shorter than the famine and the overall acetate removal efficiencies were up to a 95% ((Initial acetate concentration-remaining acetate concentration)/ (initial acetate concentration)·100) and remained constant during the whole system operation.

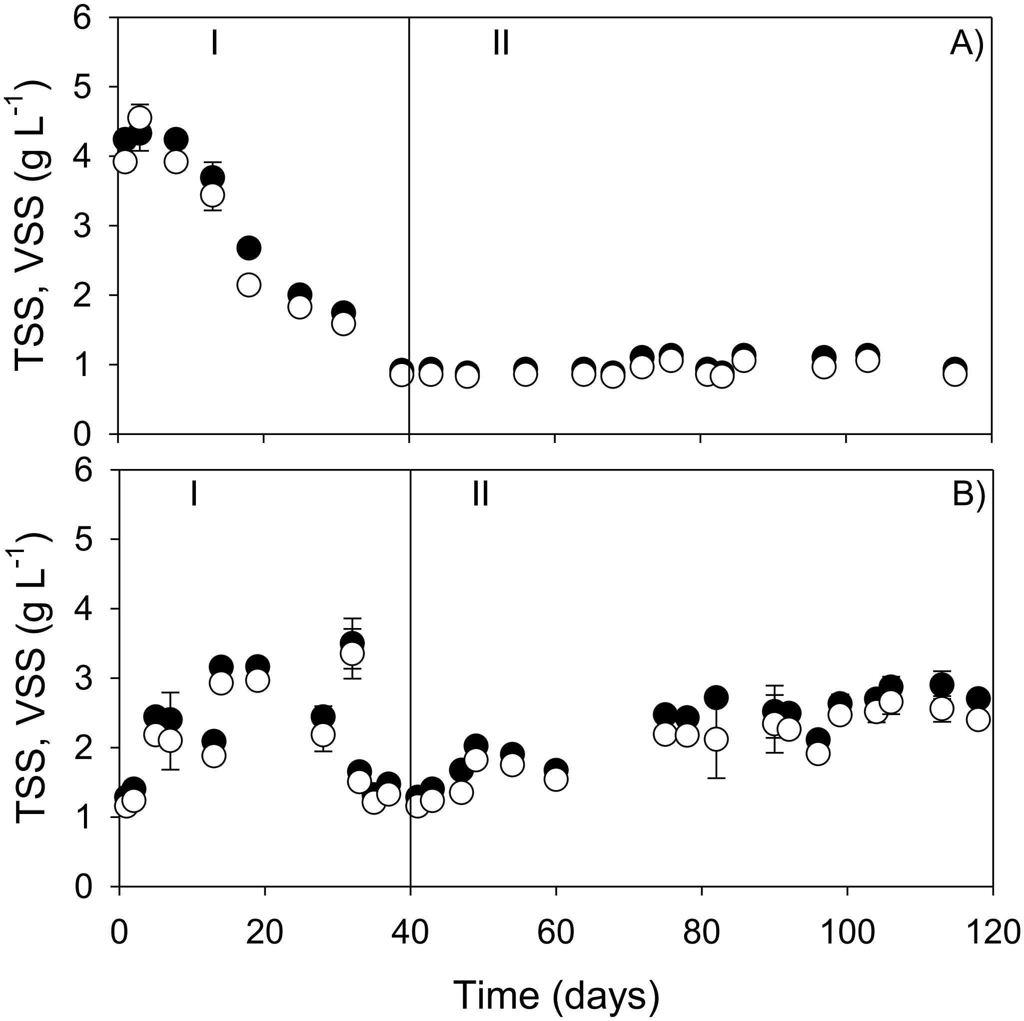

Figure 4.1. Solids performance of activated sludge enrichment under feast/famine conditions. a) SBR-A and b) SBR-B. TSS are represented as filled circles and VSS are depicted as empty circles

An improvement on PHA biomass content was observed at the end of the feast phase throughout the operation days. For SBR-A, PHA content at the beginning was 8% (gPHA g^{-1}VSS) and increases to 20% (gPHA g^{-1}VSS) at day 115. A similar trend was observed in SBR-B, which begins with a PHA content of 19 % (gPHA g^{-1}VSS) and increase it to reach a content of 36% (gPHA g^{-1} VSS) on day 111. Such increments on polymer content confirm the success on the selection of PHA-accumulating microorganisms in the activated sludge under feast/famine regime.

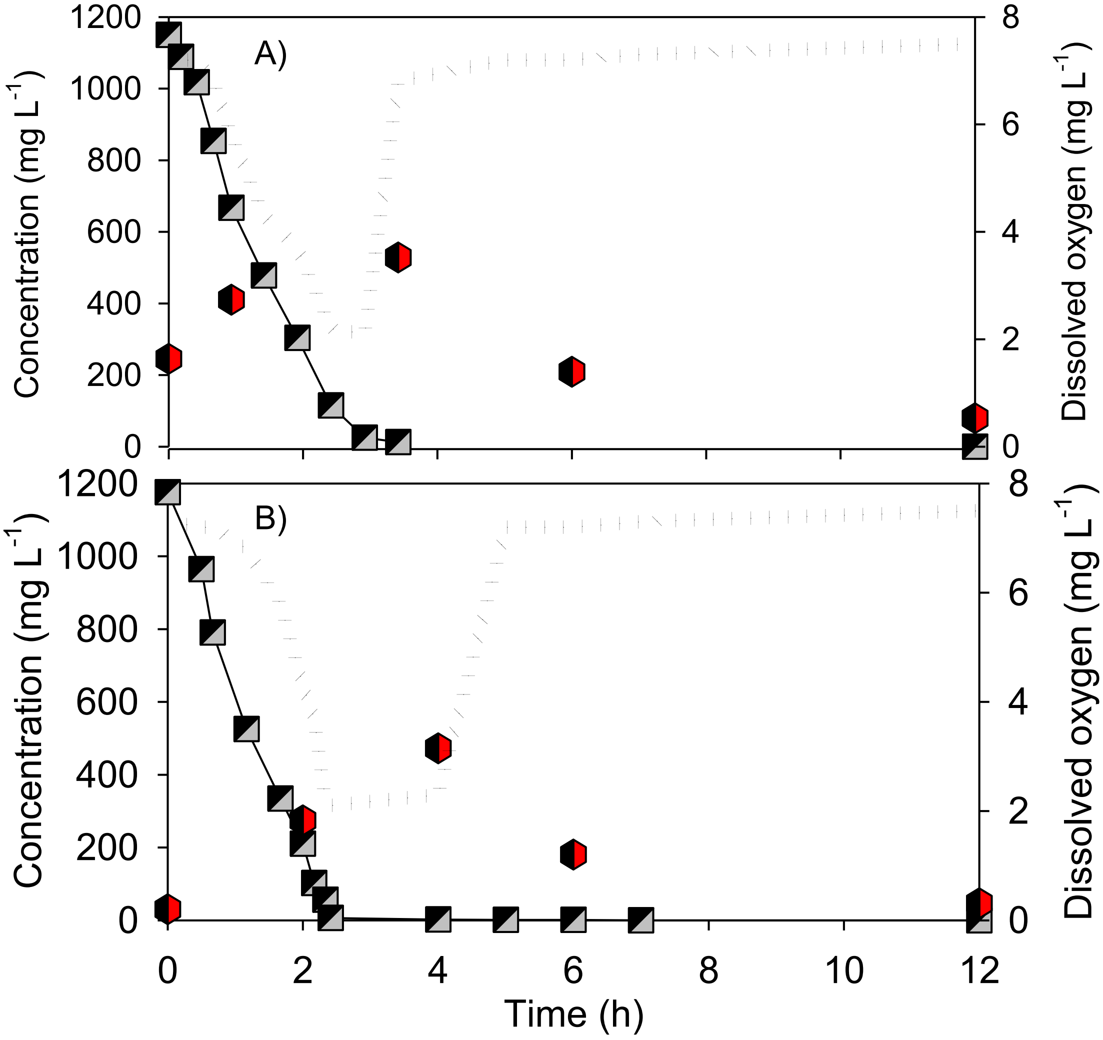

Figure 4.2 Cycle profiles for sequencing batch reactors. Concentration profiles of acetate in the reactor (filled squares), PHA concentration (filled circles) and DO profile (dotted line) for a) SBR-A and b) SBR-B. The data reported correspond to end of period I of Figure 4.1.

The feast/famine ratio (F/F ratio) is an important parameter helping to increase the PHA-storage biomass capacities. It is defined as the length of the feast phase divided by the length of the famine phase (Chen et al., 2015). In SBR-A, the averaged F/F ratio was of 0.28 at the beginning of period II. In the case of SBR-B, the averaged F/F ratio at the beginning of period II was around 0.22 and at the end of the second experimental period (indicated as II in Fig 4.1), F/F ratio decreased to 0.19. In the case of SBR-B when F/F ratio decreased (Fig. 4.3a), a clear improvement on biomass storage capacity was observed (Fig. 4.3). A low F/F ratio of up to 0.25 is preferred to allow biomass selection as non PHA-accumulating biomass death because of the long absence of external carbon source while PHA-accumulating biomass are able to survive the famine by using the stored PHA for metabolic purposes (Lee et al., 2015; Silva et al., 2016).

The evolution of biopolymer content throughout the SBRs operation is shown in Fig 4.3. After 80 days of operation, a PHA content of biomass of 20% gPHA g^{-1}VSS was reached in SBR-A (Fig. 4.3b). For the case of SBR-B, PHA content gradually increased through operation days. During period I, a PHA content of 21% gPHA g^{-1}VSS was achieved and then, a higher PHA content in biomass of around 36% gPHA g^{-1}VSS was observed in period II (Fig 4.3c). Biomass accumulation capacity of SBR-A working with controlled pH at 7.5 is lower (20% gPHA g^{-1}VSS) than that of SBR-B in period I (23% gPHA g^{-1}VSS) working without pH control. Removing pH control during the enrichment step may represent a reduction in both, the complexity and costs of the process, as not chemicals for pH control are needed.

Besides, it was observed that PHA yields ($Y_{PHA/Ac}$) were enhanced in both reactors along the operation days (Table 4.1) but a higher $Y_{PHA/Ac}$ of 0.35 (C-mol PHA C-mol^{-1} Ac) was observed in SBR-B than that of SBR-A. Also, in the same period, a high PHA production rate (q_{PHA}) of 0.16 (C-mol PHA C-mol^{-1} X h^{-1}) was attained. Moreover, in both reactors the acetate uptake rate was maintained 0.18 (SBR-A) and 0.19 (SBR-B) C-mol Ac C-mol^{-1} X h^{-1}, being these results similar to the reported by Albuquerque et al. (2013) where a PHA storage rate of 0.22 C-mol PHA C-mol^{-1} X h^{-1} and an acetate uptake rate of 0.34 C-mol Ac C-mol^{-1} X h^{-1} were obtained.

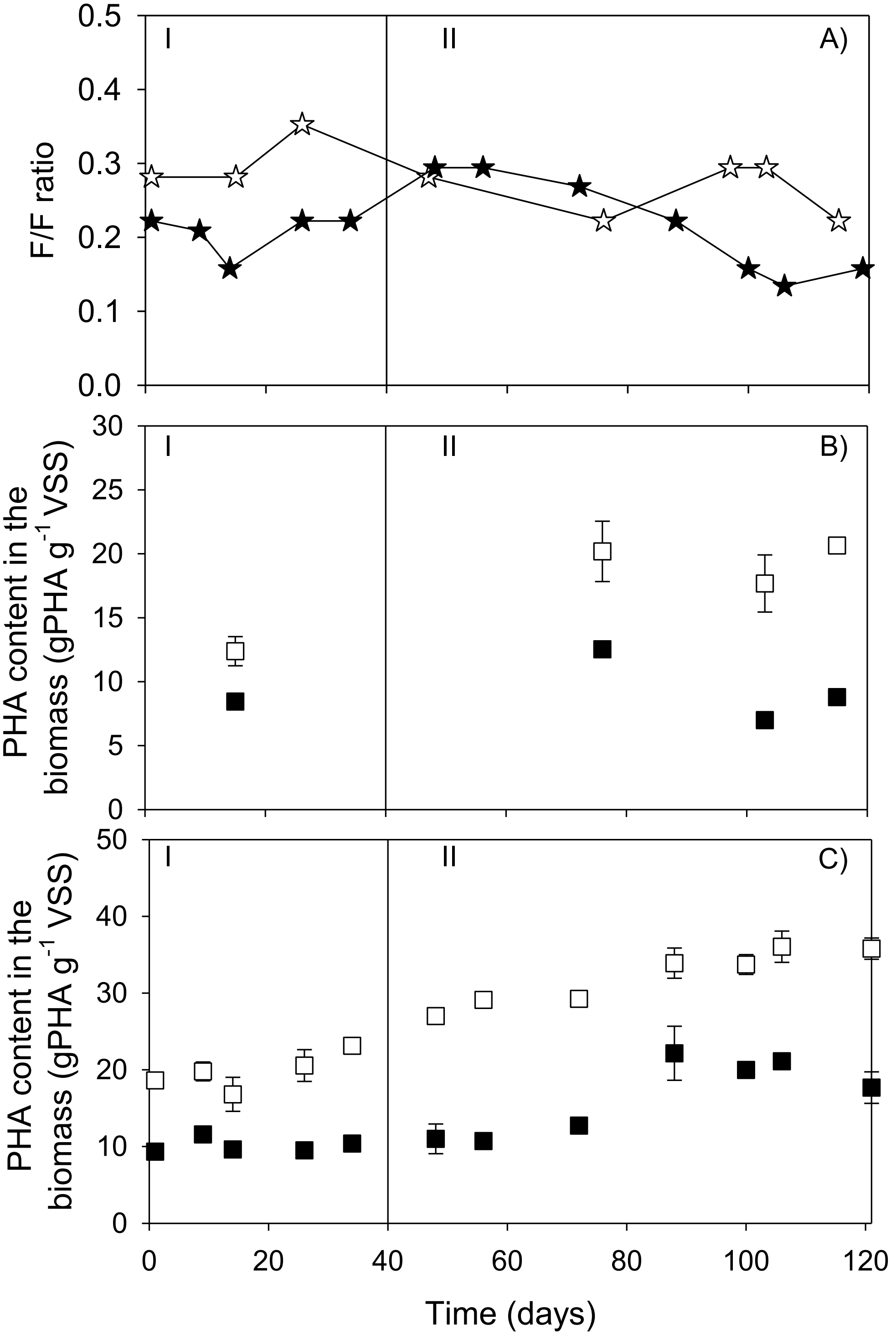

Figure 4.3 a) Feast/famine ratio for SBR-A (empty stars) and SBR-B (filled stars) during biomass enrichment.. PHA content in the biomass for b) SBR-A and c) SBR-B. PHA content at the beginning are represented on filled squares and PHA content at the end of the feast are represented on empty squares.

Table 4.1 Kinetic parameters of PHA production for SBR-A and SBR-B performance

	pH	%PHA	YPHA/Ac	YXp/Ac	qPHA	-qs
SBR-A (period I)	7.0	12 ± 1	0.09 ± 0.01	0.38 ± 0.01	0.01 ± 0.01	0.05 ± 0.01
SBR-A (period II)	7.0	20 ± 2	0.19 ± 0.02	0.36 ± 0.01	0.05 ± 0.01)	0.18 ± 0.02
SBR-B (period I)	8.8-9.2 (No control)	21 ± 2	0.23 ± 0.03	0.48 ± 0.02	0.09 ± 0.01	0.16 ± 0.02
SBR-B (period II)	8.8-9.2 (No control)	36 ±2	0.35 ± 0.02	0.42 ± 0.02	0.16 ± 0.03	0.19 ± 0.01

Standard deviation as ± for each value; %PHA (gPHA g^{-1}VSS); $Y_{PHA/Ac}$: PHA to substrate stoichiometric yield (C-mol PHA C-mol^{-1} Ac); $Y_{Xp/Ac}$: active biomass produced to substrate stoichiometric yield (C-mol Xp C-mol^{-1} Ac) -q_s: Acetate uptake rate (C-mol Ac C-mol^{-1}X h^{-1}); q_{PHA}: PHA production rate (C-mol PHA C-mol^{-1}X h^{-1})

4.3.2 Influence of pH on PHA accumulation of the enriched biomass

Experiments using biomass enriched on SBR-A were conducted and no significant changes were obtained when varying the pH (data not shown). However, when using the biomass of period I of SBR-B, PHA accumulation (Fig. 4.4) was 44% gPHA g^{-1}VSS (no control, pH ranging from 8.8 to 9.2), 39% gPHA g^{-1}VSS (pH 7.5) and 31% g PHA g^{-1}VSS (pH 8.5) all of them higher that that achieved in the enrichment step (23% gPHA g^{-1}VSS). Similar results were obtained by Chua, et al. (2003) and Serafim et al. (2004) whose demonstrate that pH≥8 was beneficial for PHA production when they use biomass enriched at pH 8.5. However, Lee et al. (2105) and Liu et al. (2011) reported higher PHA content at pH 7.0 (around 64% gPHA g^{-1}VSS) and at pH of 6.0 (around 60%), respectively. Such discrepancies must be due to the differences in the microbial characteristics of the sludge and also on the differences during biomass enrichment conditions (aeration mode, feeding pattern, nutrients concentration, etc). In this study the microbial community of the SBR-B was assayed by pyrosequencing adapting the protocol followed by Reino et al. (2016) with the primer 939F-1492R. It was found that biomass was dominated by *α-proteobacteria* and *β-proteobacteria* which have been previously reported in PHA production systems (Carvalho et al., 2014; Cavaillé et al., 2016; Jiang and Chen, 2009; Lee et al., 2015). It was also identified the presence of reported PHA-storing organisms: *Flavobacteriia*, *Verrucomicrobiae* and *δ-proteobacteria* (Jiang and Chen, 2009; Majone et al., 2006; Pieja et al., 2011; Wattanaphon et al., 2011).

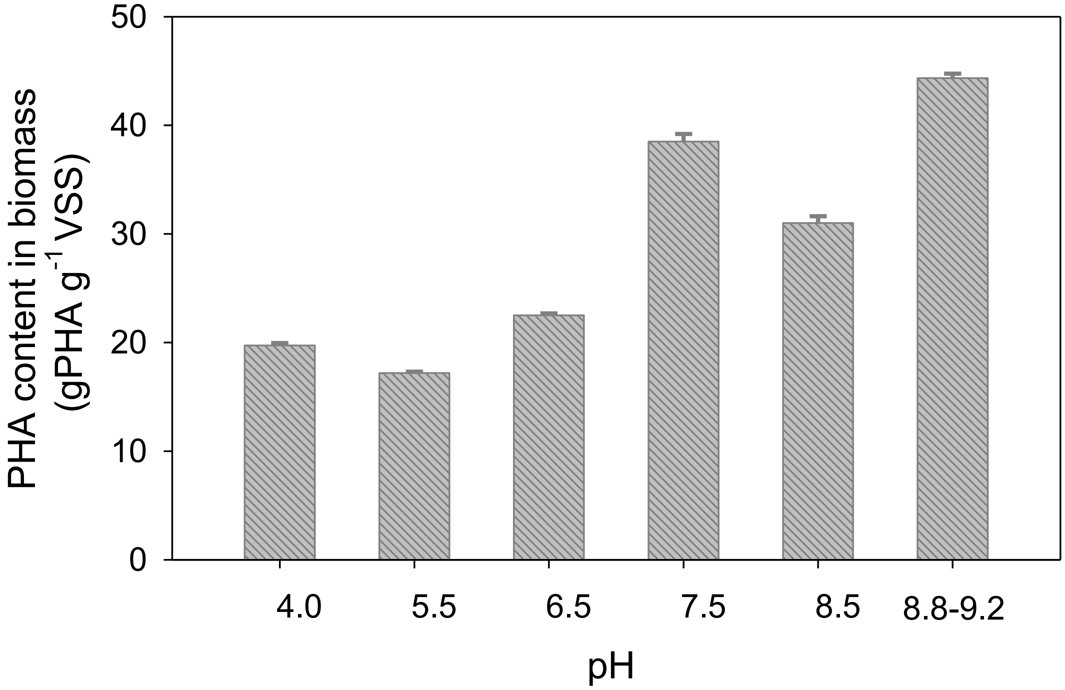

Figure 4.4. Effects of pH values on PHA maximum accumulation for batch experiments

The lowest PHA accumulations were found at acidic pH values of 4.0, 5.5 and 6.5, in which no significant accumulation was observed in comparison with the PHA content of enriched biomass. These trends could be explained, as Chua et al., 2003 stated, because at low pH conditions, acetate remains as un-dissociated form (acetic acid); this un-dissociated acetic acid rapidly diffuses into bacterial cells, then it dissociates and imposes an intracellular proton load lowering the intracellular pH. This decrease in internal pH could be the reason of the decrease on PHA production.

Moreover, PHA content of biomass at pH 8.8-9.2 could be related with the higher acetate uptake rate (0.57 C-mol Ac C-mol^{-1} X h^{-1}) compared with the rest of pH values evaluated (Table 4.2). In the range of pH >7.0 PHA production rates were found to be significantly higher than those of acidic pH values. Besides, the polymer substrate yield at pH 8.8-9.2 was higher (0.33 C-mol PHA C-mol^{-1} Ac) compared with the rest of pHs evaluated demonstrating that carbon source is being used towards PHA accumulation.

Table 4.2 Kinetic parameters of PHA production under different pH

pH	%PHA	YPHA/Ac	YXp/Ac	qPHA	-qs
4.0	20 ± 1	0.10 ± 0.01	0.56 ± 0.02	0.20 ± 0.01	0.48 ± 0.02
5.5	17 ± 1	0.11 ± 0.01	0.44 ± 0.01	0.08 ±0.00	0.24 ± 0.01
6.5	23 ± 1	0.08 ± 0.01	0.32 ± 0.03	0.18 ± 0.01	0.77 ± 0.04
7.5	39 ± 1	0.25 ± 0.02	0.30 ± 0.02	0.35 ± 0.02	0.77 ± 0.01
8.5	31 ± 1	0.26 ± 0.01	0.28 ± 0.01	0.36 ± 0.01	0.84 ± 0.05
8.8-9.2 (No control)	44 ± 1	0.33 ± 0.01	0.30 ± 0.02	0.39 ± 0.03	0.83 ± 0.02

Standard deviation as ± for each value; %PHA (gPHA g^{-1}VSS); $Y_{PHA/Ac}$: PHA to substrate stoichiometric yield (C-mol PHA C-mol^{-1} Ac); $Y_{Xp/HAc}$: active biomass produced to substrate stoichiometric yield (C-mol Xp C-mol^{-1} Ac) -q_s: Acetate uptake rate (C-mol Ac C-mol^{-1}X h^{-1}); q_{PHA}: PHA production rate (C-mol PHA C-mol^{-1}X h^{-1})

4.3.3 Influence of nutrients concentrations on PHA accumulation of enriched biomass

To assess the PHA storage capacity when varying the nutrients concentration, a second set of batch experiments were carried out with the enriched biomass of the SBR-B. No pH control was used in these experiments, as previous results (section 4.2) showed that control of pHs was unnecessary for maximizing PHA storage capacity. Typical batch test profiles were observed in all experiments (Figure 4.5): After substrate addition, DO showed a sharp decrease and then, it remained constant until complete acetate depletion, moment in which it returned back to the initial concentration. Regarding PHA concentration, it increased over the time going in parallel with acetate depletion.

The results indicate that limitation of one nutrient, N or P, enhance PHA biomass content (Fig. 4.6). Under N-limitation, almost three times higher PHA content (51% gPHA g^{-1}VSS) was obtained compared with the 21% gPHA g^{-1} VSS obtained when N was supplied in excess. A similar trend was obtained in previous reports under N-limitation conditions reaching PHA contents of 45% (Reddy and Mohan, 2012), 39% (Serafim et al., 2004) and 66% (Marang et al., 2013). This tendency could be explained as N-limitation decrease enzymatic activity and as a result, the potential for biomass growth is restricted (Bren et al., 2013).

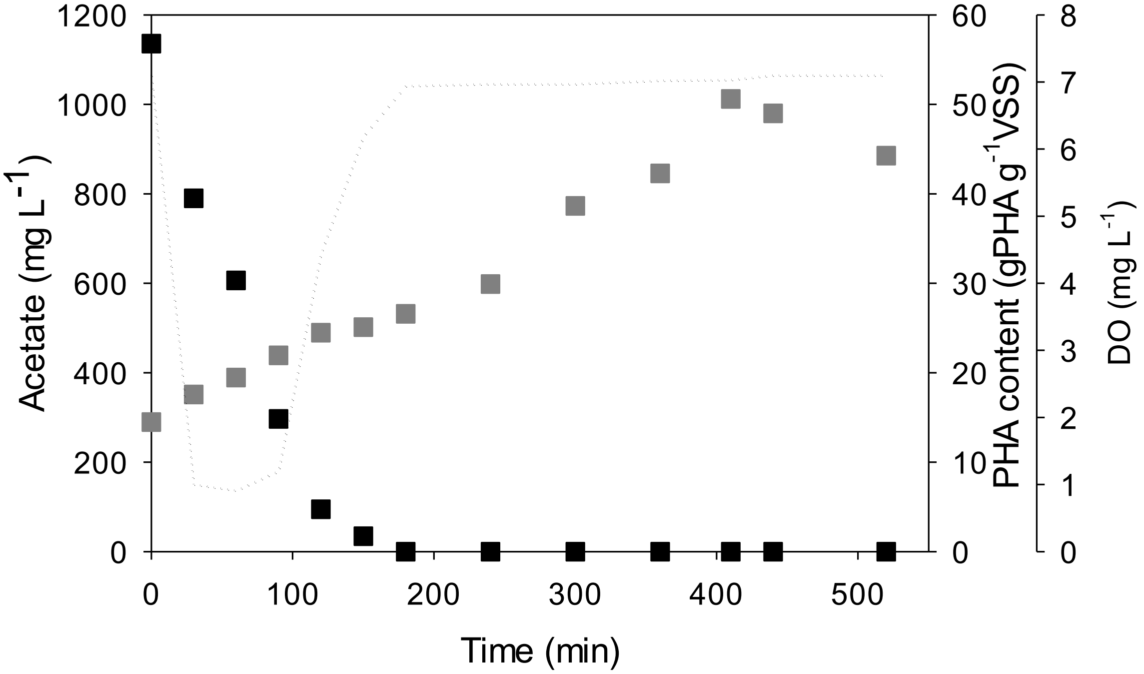

Figure 4.5 Typical profile for the batch experiments of PHA accumulation. The results depicted herein, are for the experiment under N-limitation conditions. Acetic acid concentration (black squares), PHA accumulation (gray squares) and DO profile (dotted line).

A similar trend for PHA content was observed for P availability experiments (Fig. 4.6). For P-limitation, a 42% gPHA g^{-1} VSS of PHA was achieved and a low PHA accumulation of 13% (gPHA g^{-1} VSS) was obtained when P was in excess, demonstrating that P concentration could be also a key factor for PHA maximisation. High PHA contents on biomass when N or P are limited has been reported as lower nutrients concentrations stimulates the metabolic pathway towards PHA accumulation rather that for biomass growth (Reddy and Mohan, 2012). By comparing the results of this research, it is clear that PHA accumulation is higher under N-limited condition, such as previously reported by Wen et al. (2010). However Reddy and Mohan (2012) obtained higher PHA content for P-limitation than for N-limitation conditions (54% and 42%, respectively). Such difference could be due, on the one hand, to the fact that PHA accumulation experiments were performed under anoxic microenvironment, or, on the other hand, because P effect over PHA maximum accumulation was evaluated by adjusting OLR and N concentration according to optimal values previously obtained in that work (4.54 kg COD L^{-1} d^{-1} and 100 mg L^{-1}, respectively).

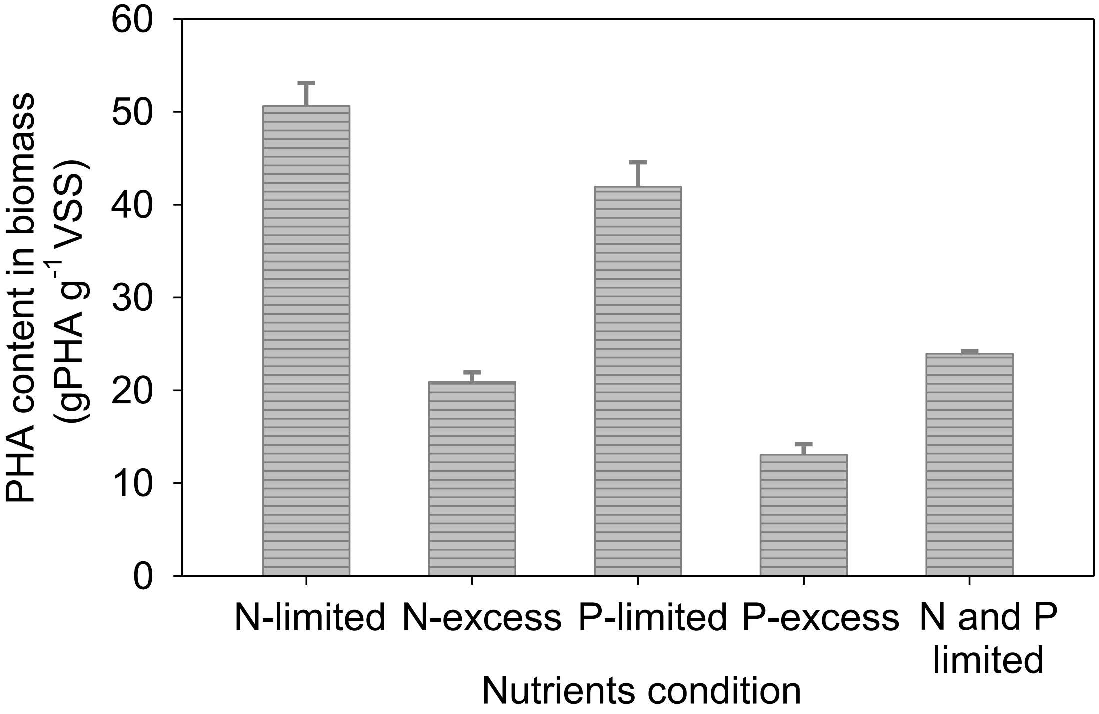

Figure 4.6 Effects of nutrients limitation or excess on PHA maximum accumulation for batch experiments

Yields and substrate and PHA rates were calculated for each nutrient condition, as shown in Table 4.3. The PHA storage yields ($Y_{PHA/Ac}$) under limitation of N or P (0.78 C-mol PHA C-mol^{-1} Ac and 0.75 C-mol PHA C-mol^{-1} Ac, respectively) were significantly higher than those for N or P excess conditions (0.21 C-mol PHA C-mol^{-1} Ac and 0.12 C-mol PHA C-mol^{-1} Ac, respectively). Similar results have been reported with polymer yields into the range from 0.32 to 0.65 when N is limited (Basak et al., 2011; Liu et al., 2011; Serafim et al., 2004). Turning to the growth yield ($Y_{Xp/Ac}$) it was observed that it increases under N-excess or P-excess conditions (0.29 C-mol X_p C-mol^{-1} Ac and 0.40 C-mol X_p C-mol^{-1} Ac, respectively) being almost two higher than the results for N-limited or P-limited conditions (0.14 C-mol

X_p C-mol^{-1} Ac and 0.27 C-mol X_p C-mol^{-1} Ac, respectively). Regarding specific storage rate (q_{PHA}), it was higher for N-limitation that for N-excess conditions (0.12 C-mol PHA C-mol^{-1} X h^{-1} and 0.09 C-mol PHA C-mol^{-1} X h^{-1}, respectively) this trend was also observed for P experiments, where under P-limitation, higher PHA storage rate was observed (0.11 C-mol PHA C-mol^{-1} X h^{-1}) compared with that obtained for P-excess conditions (0.05 C-mol PHA C-mol^{-1} X h^{-1}). The results reported herein, match with previous conclusions reported by other authors which suggest that is possible to enhance polymer storage rates by using nutrients limitation as an external metabolic limitation together with the imposed transient conditions during the feast/famine (Dionisi et al., 2005).

Concerning the specific acetate uptake rate ($-q_S$), it was found that high N and also high P levels, exhibited a better performance (0.20 C-mol Ac C-mol^{-1} X h^{-1} and 0.30 C-mol Ac C-mol^{-1} X h^{-1}, respectively). It was previously reported that under nutrient excess, higher volatile fatty acids uptake rates are achieved and it could be due to the growth of non-PHA storing biomass. Such conclusion agrees with the results obtained herein as better acetate uptake rates were obtained when P or N where in excess (Bengtsson et al., 2008b; Johnson et al., 2010).

Table 4.3 Kinetic parameters of PHA production under different N and P concentrations

C/N/P (taking 100 mol of C as basis)	%PHA	$Y_{PHA/Ac}$	$Y_{Xp/Ac}$	qPHA	-qs
100/4.1/2.4 (N-limited)	51 ± 2	0.78 ± 0.04	0.14 ± 0.03	0.42 ± 0.02	0.26 ± 0.06
100/65.9/2.4 (N-excess)	21 ± 1	0.21 ± 0.02	0.29 ± 0.09	0.09 ± 0.01	0.20 ± 0.01
100/8.8/1.3 (P-limited)	42 ± 3	0.75 ± 0.07	0.27 ± 0.09	0.11 ± 0.02	0.26 ± 0.03
100/8.8/90.5 (P-excess)	13 ± 1	0.12 ± 0.01	0.40 ± 0.02	0.05 ± 0.01	0.30 ± 0.01
100/4.1/1.3 (N and P-limited)	27 ± 1	0.19 ± 0.01	0.38 ± 0.05	0.08 ± 0.04	0.19 ± 0.02

Standard deviation as ± for each value; %PHA (gPHA g^{-1}VSS); $Y_{PHA/Ac}$: PHA to substrate stoichiometric yield (C-mol PHA C-mol^{-1} Ac); $Y_{Xp/Ac}$: active biomass to substrate stoichiometric yield (C-mol X C-mol^{-1} Ac) $-q_s$: Acetate uptake rate (C-mol Ac C-mol^{-1}X h^{-1}); q_{PHA}: PHA production rate (C-mol PHA C-mol^{-1}X h^{-1})

An additional batch experiment was performed with simultaneous limitation of N and P concentrations. In this case, the PHA content obtained was 27% gPHA g^{-1} VSS. Although the PHA biomass content is lower than that when N or P are individually limited, it is still slightly higher than that for N or P excess conditions. Similar trend but with higher PHA content, was reported by Villano et al. (2015) reporting a PHA content of 66% when combining N and P limitation.

Storage PHA yield (Table 4.3) was similar with that of N or P excess conditions (0.19 C-mol PHA C-mol^{-1} Ac), however, the highest biomass growth yield was obtained in such conditions (0.38 C-mol X C-mol^{-1} Ac), demonstrating that when both nutrients are limited, acetate uptake is preferable used for cell maintenance. Acetate uptake rate was similar with those under N or P limitation (0.19 C-mol Ac C-mol^{-1} X h^{-1}). In this case, the low acetate uptake performance was not related with a higher PHA biomass content but could be due to the poor biomass behaviour because of the lack of nutrients.

In line with this result, Reddy and Mohan (2012) suggest that a higher PHA accumulation could be reached by combining a N-limitation with P-excess, as growth limitation by just one essential nutrient reduces the complexity of cell metabolism and so, carbon is used just for PHA production (Valentino et al., 2015; Reddy and Mohan, 2012).

4.4 Conclusions

The results of this work have demonstrated that no pH control is needed to enrich a MMC able to accumulate up to 36% of PHA. The amount of PHA stored on the enriched biomass during the batch tests, was shown to be affected also by the pH of the system. In this study, pHs≥7.5 exhibited a high PHA accumulation, demonstrating the importance of this parameter to enhance the performance of the enriched biomass. It was also demonstrated the important role of nitrogen and phosphorus concentration on PHA accumulation. Higher PHA contents were achieved at limited nitrogen or phosphorus concentrations.

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Chapter 5

Feasibility of using a fermented olive mill wastewater as precursor for polyhydroxyalkanoates production

Summary

Olive Mill Wastewater (OMW) is a seasonally environmental problem in the Mediterranean countries due to its high organic levels and the presence of phenolic compounds. The possibility of using OMW as a low-cost substrate towards polyhydroxyalkanoates (PHA) production, represents an interesting approach for its valorization and disposal. The present study investigates the feasibility of a process for PHA production using a synthetic fermented-OMW liquid (sOMW) as a carbon source that includes (1) the enrichment of a PHA-producing mixed microbial culture (MMC) in a sequencing batch reactor operated under feast/famine conditions and (2) the cellular PHA accumulation capacity of the enriched biomass in a fed-batch mode using different feeding strategies and substrates.

During the PHA-producing microbial community enrichment, a fed distribution of the sOMW alongside the reaction time was applied to avoid the process performance due to the presence of phenolic compounds. A ca. 90 % of the sOMW was successfully depleted and a PHA production of 33 % (gPHA $gVSS^{-1}$) was obtained and maintained during the enrichment step. The enriched biomass was used for further accumulation batch experiments. The effect of different feeding regimes on the maximum PHA content was evaluated. It was observed that the split-fed substrate added immediately the substrate was depleted (assumed as a markly decrease in the dissolved oxygen (DO) profile was observed) lead to high PHA storage rates and accumulation rates (0.77 Cmol-C-mol^{-1} and 0.40 Cmol-C-mol X^{-1} h^{-1}, respectively). A maximum PHA content of 73 % was achieved by using a mixture of acetic and propionic acids (85:15) as carbon source. A copolymer of polyhdroxybutyric acid (PHB) and polyhydroxyvaleric acid (PHV) was synthesized with a significantly high content of HV up to 57 %.

5.1 Introduction

Petroleum-based plastics market is considered as an important environmental problem due to their complex biodegradation. In 2016, 335 million tonnes of plastics had been produced, and it is estimated that only 7% is recycled, being the rest disposed in landfills and oceans (Pathak et al., 2014). As more sustainable and renewable alternatives are needed to replace conventional plastics, biopolymers production has been investigated as a very promising alternative. Among all biopolymers available nowadays, polyhydroxyalkanoates (PHAs) attracted significant interest as they have shown to have similar properties compared with fossil fuel-based polymers like polypropylene (PP). PHAs are biologically synthesized and have the advantage of being completely biodegradable (Kosseva and Rusbandi, 2018), therefore they are truly a biodegradable bio-based plastic. These biopolymers can be used as raw material for the production of packaging materials and furthermore, can be produced from waste sources. All that reasons make PHAs being considered as a circular economy product (Agustín Martinez et al., 2015; Dietrich et al., 2017).

By now, PHAs industrial production is limited mainly because of their production costs which are still much higher compared with that of the fossil fuel-based plastics and for this reason research work on PHAs are mainly focused on decreasing their production costs by i) replacing the use of pure cultures, ii) using waste streams as substrates and iii) improve downstream processes. These three factors are the main ones influencing the overall production costs. Following these guidelines, the use of expensive pure cultures has been proposed to be replaced by the use of mixed microbial cultures (MMCs) by taking advantage of the ecological role of PHAs as a microbial storage material (van Loosdrecht et al., 1997). Overall, the use of MMCs reduces PHAs production costs as no sterilization is needed and complex feedstocks can be used as substrates and so, the substitution of expensive carbon sources by the use of low-cost agricultural and industrial wastes have been explored (Kosseva and Rusbandi, 2018).

Olive Mill Wastewater (OMW) is an effluent generated from olive oil extraction by the traditional or three-phase methods. It represents an environmental problem especially for Mediterranean countries where is concentrated the 90% of the global production of which Spain is one of the leading producers. The most important environmental issues are associated with their high and seasonally levels of production, and also, with their chemical composition that includes different amounts of organic matter, sugars and oil additionally to polyphenols (Campanari et al., 2017; Yarimtepe et al., 2017). The presence of polyphenols in the OMW is a barrier for their direct biological treatment and so, different pre-treatments to reduce their organic load must be used before their use as feedstock (Campanari et al., 2017). According to this, a multi-stage process for OMW valorization towards PHA production is proposed by different authors including, in some cases, a first stage for polyphenols removal (Agustín Martinez et al., 2015; Campanari et al., 2017). Then, OMW acidogenic fermentation is performed to obtain a Volatile Fatty Acids (VFAs)-rich stream to be fed to a following reactor for the enrichment of microorganisms towards PHAs production. Commonly, PHAs-storing microorganisms are selected under aerobic dynamic feeding conditions performed in sequencing batch reactors (SBR) by alternating feed-starvation cycles, a strategy that is better known as "feast and famine" (F/F) (Campanari et al., 2014; Kosseva and Rusbandi, 2018). Then, the enriched biomass is harvested and used for maximizing PHA contents. Finally, this biomass is recovered and PHAs extraction is performed.

In this work, the bioconversion of a fermented OMW effluent into PHAs by using a MMC system was assessed. For that aim, a SBR was inoculated with activated sludge from a municipal wastewater treatment plant and enriched under feast/famine conditions. We also evaluated the capacity of the system to deplete the organic matter which includes acetic and propionic acids (HAc and HPr, respectively) and a polyphenols fraction composed by benzoic acid (HBz), 4-hydroxyphenylacetic acid (HPAA) and cinnamic acid (HCn). After obtaining the PHA-accumulating biomass, different fed-pulse strategies were assessed under a series of batch experiments to evaluate the PHA accumulation capacity of the biomass. On each fed-pulse strategy, the Dissolved Oxygen (DO) was used to determine the boundary on the feast/famine period; thus, the next pulse of the substrate was performed just before the famine period start. It was also compared the PHA productivities of the different feeding regimens to identify the most effective feeding strategy. The finally selected feeding strategy was used to assess the effect of different carbon sources towards PHA production with the aim of maximizing PHA accumulation capacity of the enriched biomass.

5.2 Materials and methods

5.2.1. Olive mill wastewater composition

A concentrated synthetic fermented OMW (sOMW) was used as the carbon source in this study with the following composition, expressed as chemical oxygen demand (COD) (gCOD L^{-1}): HAc (40.5), HPr (8.1), HPAA (1.8), HBz (1.8) and HCn (1.8). The proportion of each compound in the sOMW was established based on a typical composition described in the literature for an OMW. For example, Torrecilla (2010) described that the OMW effluents might be composed of a phenolic fraction between 5 to 15%; three main phenolic families are commonly found as part of this fraction an so, a representative of each one was used as model substrates in this study, representing a 10% of the total COD.

5.2.2 Biomass Enrichment

The selection of the biopolymer-producing MMC was performed in a SBR equipped with a pH, DO and T sensors for process monitoring. The reactor was inoculated with activated sludge from a full-scale municipal wastewater treatment plant (WWTP) located at Rubí (Catalonia, Spain). A total influent concentration of 1.2 g COD L^{-1} was added to the reactor by using the concentrated sOMW described in section 5.2.1 as the carbon source and the same medium composition described by Montiel-Jarillo et al. (2017). The reactor was operated under a feast/famine regime under no-sterile conditions and at room temperature (ca. 23°C). No pH control was implemented as it was previously reported that pH control is not necessary to enrich a PHA-accumulating MMC (Montiel-Jarillo et al., 2017). DO concentration was in the range 7.0 – 8.0 mg O_2 L^{-1} although a closed control loop for DO was not implemented.

The whole experimental time (240 days) was divided in different periods according to the operating conditions applied. The Period I (comprised from the 1-15 days) was operated in cycles of 12h distributed in the following phases: settling (60 min); withdrawal (10 min); idle (15 min); mineral medium feeding (7 min), single substrate feeding (3 min) and aerobic reaction phase (625 min). The exchange volume in each cycle was 50% of the total volume. The hydraulic retention time (HRT) in this period was 1 day and the sludge residence time (SRT) was maintained at 4 days.

From the days 16 to 38 (Period II) the operation was performed in 24 h cycles divided into settling (60 min); withdrawal (10 min); idle (15 min); mineral medium feeding (7 min), a single substrate feeding (3 min) and aerobic reaction phase (1345 min). The exchange volume was maintained at the 50% of the total volume and therefore, the HRT in this period was 2 days. The purge was calculated for maintaining a SRT of 4 days.

Finally, the Period III (days 39 to 240) was operated in 24 h cycles divided into the same phases as the Period II, but with the difference that the carbon source feeding was divided into 3 pulses throughout the whole aerobic phase (1 pulse every 7.5 h).

The SBR was monitored three times per week during the overall experimental period in terms of the total suspended solids (TSS), volatile suspended solids (VSS) HAc, HPr, HBz, HPAA and HCn and COD concentrations in the influent and in the effluent. DO concentration was continuously recorded. Stable operation was considered to be attained when the VSS concentration and the length of the feast phase were constant for at least five cycles.

5.2.3 Batch experiments for determining the PHA storage capacity of the enriched biomass

Batch experiments were performed in order to evaluate the PHA storage capacity of the enriched biomass. Each experiment was performed in 1L glass reactors at room temperature (ca. 23ºC) and without pH control. The enriched biomass was collected from the 16L SBR and washed twice with tap water, then it was left to settle down and 0.5L of settled biomass was mixed with 0.5L of a fresh medium with the same composition as the one of the enrichment step, with the only difference that no ammonium was added. In general, it is suggested, that under nutrients restriction (nitrogen in this case), higher PHAs content are obtained. Nitrogen has shown to have a strong influence on maximum PHAs biomass capacities (Bengtsson et al., 2008; Dionisi et al., 2004; Johnson et al., 2010; Montiel-Jarillo et al., 2017; Valentino et al., 2015; Wen et al., 2010).

In all the experiments, the biomass was continuously aerated and magnetically stirred. The DO, pH, and temperature (T) were monitored online, and biomass samples were taken regularly to follow COD consumption, PHA and VSS concentrations.

5.2.3.1 Fed-pulse strategy assessment in batch experiments

Different fed-pulse strategies based on the DO profile were evaluated. The PHA accumulation capacity of the enriched biomass was assessed by feeding the sOMW in 3 or 6 pulses (split-fed) up to a total concentration of 1.2g COD L^{-1} in each experiment. All the substrate additions were triggered by the increase in the biomass respiration rate.

The fed addition on each split-fed strategy assessed were done as shown in Fig. 5.1. For the batch experiment A, 3 or 6 pulses (3A and 6A) were added every time DO rose up to the saturation value and remains constant (around 7.0 mg O_2 L^{-1}). In batch B, 3 or 6 pulses (3B and 6B) were added immediately when the DO return close to the initial value (around 6.7 mg O_2 L^{-1}) and without waiting for its stabilization. Finally, for experiments C, 3 or 6 pulses were added (3C and 6C) the fed pulse was added once a sharply increase in the DO profile was observed (~ 3.5 – 4.0 mg O_2 L^{-1}).

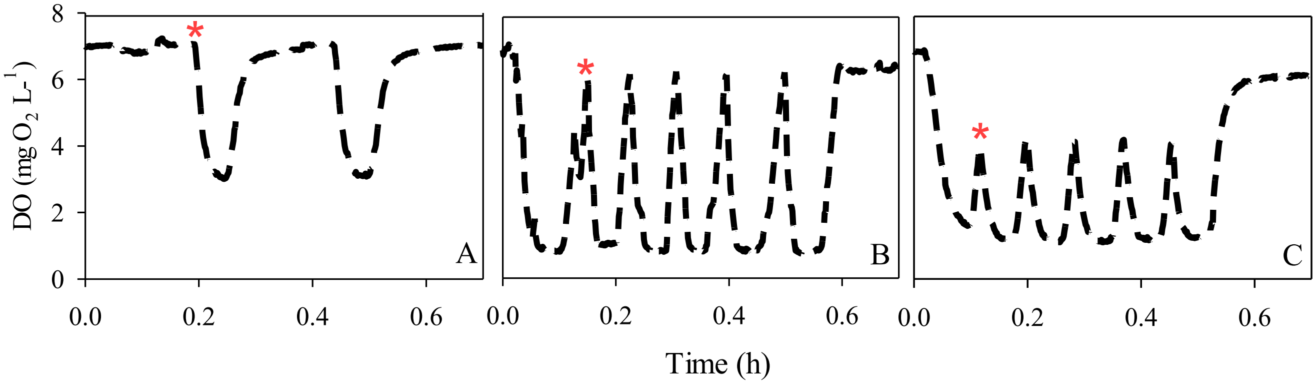

Figure 5.1 Split-fed strategies applied to the batch experiments

5.2.3.2 Accumulation capacities of the MMC with different substrates

Batch experiments were used to assess the PHA accumulation capacity of the enriched MMC with different substrates. In all cases, a total concentration of 1.2 g COD L^{-1} had been fed in 6, 12 or 8 pulses as detailed in Table 5.1. Finally, two other experiments were performed but adding a two-fold higher total COD concentration (Experiments HAc and HAc-HPr) in order to assess the maximum PHA-accumulation capacity of the enriched biomass.

Table 5.1 Experimental setup of the batch experiments using different substrates

Exp	**HAc**	**HPr**	**HBz**	**HCn**	**Number of pulses**	**Pulse Concentration (g COD L^{-1})**
1	100 %	-	-	-	6	0.2
2	-	100 %	-	-	12	0.1
3	100 %	-	-	-	4	0.2
	-	100 %			4	0.1
4	75 %	25 %	-	-	6	0.2
5	75 %	15 %	10 %	-	6	0.2
6	75 %	15 %	5 %	5 %	6	0.2
HAc	100 %	-	-	-	12	0.2
HAcHPr	75%	25 %	-	-	12	0.2

5.2.4 Analytical methods

Biomass samples from the SBR were analysed using the Standard Methods procedures to determine the TSS (2540D), VSS (2540E) and the sludge volumetric index (SVI) using the method 2710D (APHA, 2005).

Acetic and propionic acid concentrations were determined from filtered samples using an Agilent Technologies 7820 A gas chromatographer equipped with a DB FFAA column (length: 30 m, internal diameter: 0.25 mm and film thickness: 0.25 µm) and a flame ionization detector (FID). Phenolic compounds were measured following the protocol reported by Ramos et al. (2015) by High-Performance Liquid Chromatography (HPLC), using an UltiMate 3000 (Dionex Corporation) with an Agilent Zorbax SB-C18 (4.6 mm x 100 mm x 3.5µm) column and a UV detector set at 254 mm. The flow rate was set at 1.875 ml min^{-1} with a column temperature of 30°C. Acidified Ultrapure water (pH 1.41, H_2SO_4) and HPLC grade methanol were used as mobile phases; the injection volume was 20 µL. For the calibration, external standards of each phenol were used.

For the PHA analysis, 0.6 mL of formaldehyde were added to sludge samples in order to inhibit biological activity, and then, they were lyophilized. After, 50 mg of lyophilized samples were was analyzed following the method described by Werker et al. (2008).

5.2.5 Calculations

PHA contents in the biomass were calculated as a percentage of VSS on mass basis (%PHA = gPHA/gVSS *100), where VSS includes active biomass (X) and PHA. Active biomass (X) was calculated by subtracting the PHA content from the VSS (gX = gVSS – gPHA).

The yields for PHA production ($Y_{PHA/S}$ in C-mol PHA/C-mol substrate) and active biomass produced ($Y_{Xp/S}$ in C-mol Xp/ C-mol substrate), were calculated by dividing the amount of PHA produced or active biomass produced by the total amount of acetic acid consumed.

Substrate uptake rate ($-q_S$ in C-mol/ C-mol X) and PHA production rate (q_{PHA} in C-mol PHA/ C-mol X), were calculated by linear regression of the concentrations divided by the average of the active biomass at the end of the feast phase.

Finally, the feast to famine ratio (F/F ratio) was calculated as the length of the feast phase divided by the length of the famine phase.

5.3. Results and discussion

5.3.1 Biomass Enrichment

An acidified synthetic OMW was used as carbon source for the enrichment of a MMC. The SBR was inoculated with conventional activated sludge from a WWTP and was operated for 350 days under a feast/famine regime and without pH control. These conditions have been previously reported to effectively achieve a PHA-accumulating culture (Montiel-Jarillo et al., 2017).

The operational conditions in the SBR throughout 340 days of operation are shown in Table 5.2. The total and suspended solids and the COD in the influent and the effluent, are shown in Figure 5.2. The total COD corresponds to the sum of the phenolic fraction (PF) and the VFA (HAc and HPr). Initially, the reactor was operated on 12 h cycles (Period I), with a single pulse of substrate addition in a concentration of 1.2 g COD L^{-1}. The maximum PF loading rate was achieved at this stage resulting in a progressive decrease in the solids (Fig 5.2) and a COD accumulation in the effluent; this accumulation corresponds to the PF due to substrate inhibition. Besides, a poor PHA accumulation was observed (13%) as a consequence of the biomass deterioration (Table 5.2).

In a first attempt to overcome the SBR deterioration, a reduction of the OLR was imposed by establishing a 24 h cycle (Period II) with an increase of the HRT up to 2 days. The PF loading rate was reduced (Table 5.2) and thus, a progressive improvement was observed in the reactor; the TSS and VSS gradually increased and stabilized around 1.2 g VSS L^{-1} and the PHA accumulation increases up to 19%. Nevertheless, the organic fraction depletion was not totally achieved, remaining a PF in the effluent of ca. 35 mg L^{-1}(Fig 5.2).

During the Period III, a feeding strategy previously developed in our group was implemented from day 38 onwards. This strategy consists of the distribution of the fed in different equal additions along the aerobic reaction phase Martín-Hernández et al. (2009). The strategy was developed for the biological treatment of a high-strength *p*-nitrophenol (PNP) wastewater. Under these conditions, the substrate inhibition is minimized. The fed was added in three pulses of 0.4 gCOD L^{-1} every 7.5 hours, and the PF loading rate was lowered (Table 5.2). Steady-state conditions were achieved about 65 days after (Fig. 5.2) as it was observed by the constant TSS and VSS average concentrations of 3.1 and 2.9 g L^{-1}, respectively. The SVI also remain constant at around 112 ± 36 mL g^{-1} and the average length of the feast phase for each pulse under the steady-state conditions was 55 ± 5 min which represents a 13% of the reaction time for each pulse agreeing with other results that indicates that percentages of F/F ratio below 20% are indicative of the presence of PHA-accumulating microorganisms (Albuquerque et al., 2013). A progressive decrease of the COD in the effluent was observed achieving a COD depletion of around 90%. This indicates that the enriched biomass was able not only to accumulate PHAs but also to deplete the easily assimilated C sources (HAc and HPr) as well as the phenolic compounds. As it was previously mentioned, under such conditions, there is a long period where no C source is added (famine phase), and this limitation of C can be advantageous to enrich a biomass capable of consuming the phenolic fraction, as it is reported by Saha et al. (2017). By adding the C source in three different pulses, we ensure that no inhibition effect due to the phenolic fraction (Martín-Hernández et al., 2009) will negatively affect the PHA accumulation performance. It was reported by Zhang et al. (2018) that the addition of the overall phenol dosage divided into different phenol additions, enhance the degradation time, and also the final PHA content.

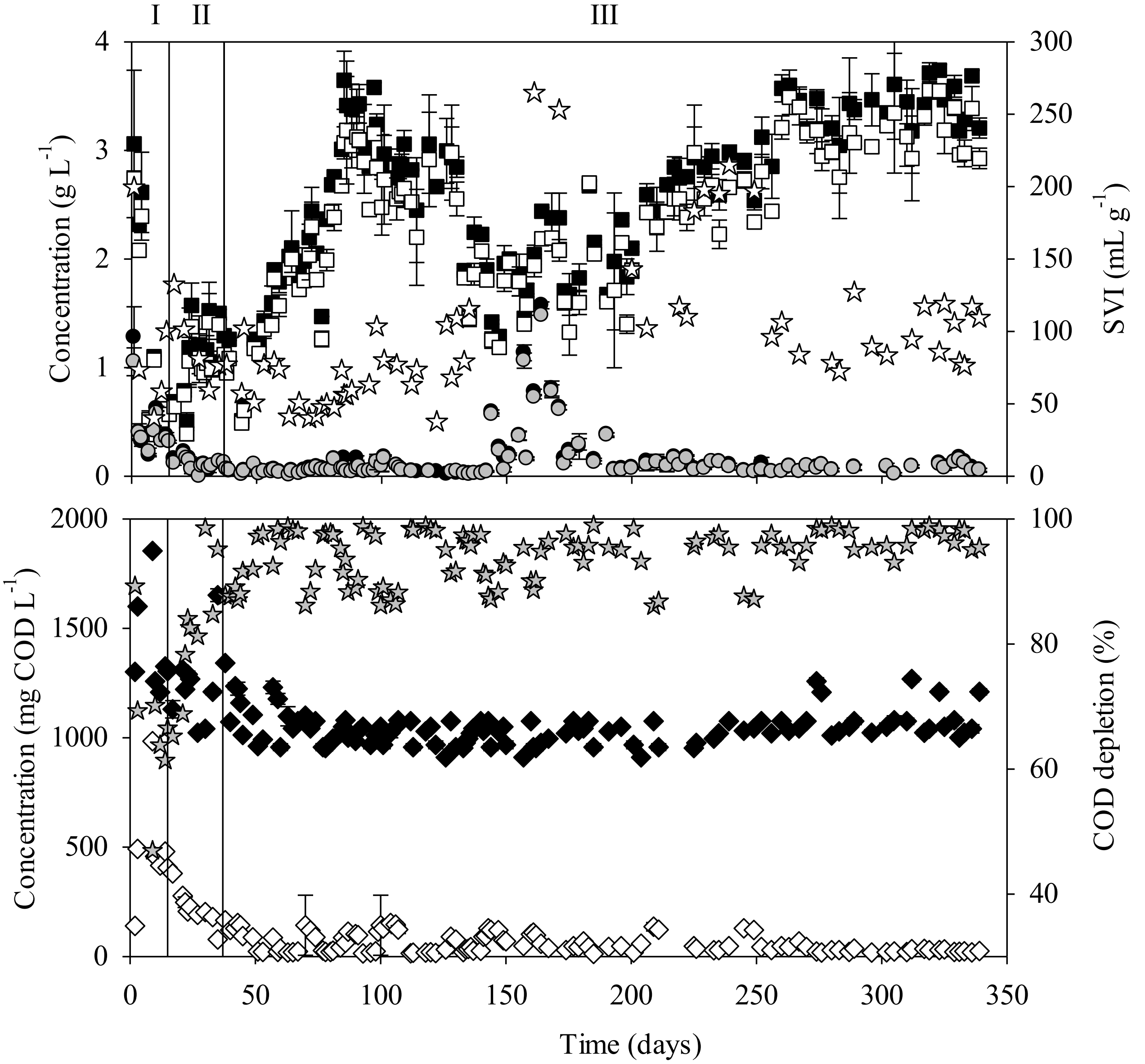

Figure 5.2. SBR performance. A) Solids performance of MMC under feast/famine conditions. TSS (in g L^{1}) are represented as filled squares for SBR, and empty circles for the effluent and VSS (in g L^{-1}) are depicted as empty squares for the SBR and empty circles for the effluent. Empty stars represent the SVI (in mg g^{-1}). B) COD consumption during the overall SBR performance. The filled diamond represents the COD at the beginning of each cycle (in g COD L^{-1}) and empty diamonds represent the COD concentrations in the effluent (in g COD L^{-1}). The grey stars represent the percentage of COD depletion

Table 5.2. Operational conditions for the SBR enrichment using sOMW as substrate

Period	Days	VSS	HRT	ORL	Specific PF loading rate	Specific COD loading rate	PHA
I	1-15	0.5	1	2.4	0.48	4.8	13 ± 2
II	15-37	1.1	2	1.2	0.10	1.1	19 ± 3
III	38-340	2.9	2	1.2	0.04	0.4	33 ± 2

Standard deviation as ±; VSS in g L^{-1}; HRT in days, OLR – organic loading rate of each period in g COD L^{-1} d^{-1}; Specific PF and COD loading rate is the phenolic and organic fraction loading rate in the reactor as COD (gCOD $gVSS^{-1}$ d^{-1}); %PHA (gPHA g^{-1}VSS).

Once the steady-state conditions were achieved, cycle measurements were performed to evaluate the accumulation of PHAs throughout the operation days. Fig 5.3 shows a typical profile of a feast/famine MMC enrichment towards PHA accumulation. As it is observed (Fig 5.3) a better overall production rate (0.45 and 0.56 Cmmol $Cmmol^{-1}$ X L^{-1}, respectively) and PHA accumulation (20 and 33 %, respectively) is observed for the third pulse in comparison with the first C source addition.

The VFAs fraction (HAc and HPr) was firstly depleted. VFAs which are well documented to be favourable substrates for PHA production (Cerrone et al., 2014; Kosseva and Rusbandi, 2018). A higher consumption rate was observed for acetate than for propionic acid (table 5.3). Acetate has been previously reported to be consumed in a higher consumption rate when compared to propionic acid, and it is related with their different metabolic pathways. The propionic acid consumption is regulated by the rate of its decarboxylation to acetyl-CoA as it can be converted in both, acetyl-CoA and propionyl-CoA; on the contrary, acetic acid is directly converted to acetyl-CoA (Fradinho et al., 2014; Kourmentza and Kornaros, 2016). Once VFAs are consumed, the phenolic fraction is depleted, but at a slower consumption rate than VFA as shown in table 5.3.

The DO profile exhibits a typical behaviour of the process: after the sOMW pulse, the concentration of oxygen decrease till the carbon source is consumed, after which, it increases progressively until reaching its initial value. The PHA accumulation performance is also depicted in figure 5.3 and exhibit a typical feast/famine tendency as previously reported (Albuquerque et al., 2011; Moita and Lemos, 2012; Oliveira et al., 2017). PHA accumulation increases as substrate concentration decrease, reaching a maximum accumulation when

VFAs are completely depleted, then when there is no substrate, and as the DO increase, microorganisms use the accumulated PHA as a carbon source

Table 5.3. Kinetic parameters of SBR performance and PHA production

# pulse	Pulse concentration	%PHA	$Y_{PHA/S}$	$Y_{X/S}$	q_{PHA}	$-q_{HAc}$	$-q_{HPr}$	$-q_{PF}$
1	400 mg COD L^{-1}	20 ±3	0.45 ±0.03	0.42 ±0.04	0.12 ±0.02	0.36 ±0.02	0.07 ±0.02	0.06 ±0.02
3	400 mg COD L^{-1}	33 ±2	0.56 ±0.02	0.32 ±0.02	0.20 ±0.01	0.29 ±0.03	0.05 ±0.01	0.04 ±0.01

Standard deviation as ± for each value; %PHA (gPHA g^{-1}VSS); $Y_{PHA/S}$: PHA to substrate stoichiometric yield (C-mol PHA C-mol^{-1}); $Y_{X/S}$: active biomass produced to substrate stoichiometric yield (C-mol X C-mol^{-1}); q_{PHA}: PHA production rate (C-mol PHA C-mol^{-1} Xh^{-1}); -q_{HAc}: acetic acid uptake rate (C-mol C-mol^{-1} X h^{-1}); -q_{HPr}: propionic acid uptake rate (C-mol C-mol^{-1} X h^{-1}); -q_{PF}: phenolic fraction uptake rate (C-mol C-mol^{-1} X h^{-1}).

In this study, a copolymer of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) was synthesized; a high proportion of hydroxybutyric (HB) content was obtained (ca. 63 %, in Cmmol) in comparison with the hydroxyvaleric (HV) content (ca. 37 %, in Cmmol). The polymer composition is a result of the presence of the HAc and HPr in the carbon source. It is well documented that using HAc as carbon source PHB is synthesized and that the presence of odd number VFA, such as the HPr in this research, lead to the synthesis of PHV (Albuquerque et al., 2011; Kourmentza and Kornaros, 2016). The major proportion of HB units in the copolymer is due to the higher fraction of HAc in the sOMW used in this study as carbon source.

5.3.2 Effect of the feeding regimen on the maximum PHA-accumulation capacity of the enriched biomass

The PHA accumulation capacity of the enriched MMC was evaluated under different feeding strategies (Fig 5.1). The substrate addition in all the batch experiments was triggered by the DO-profile in a split-fed mode. For all the feeding strategies (A, B and C) the total COD (1.2 g COD L^{-1}) was divided into 3 pulses (0.4 g COD L^{-1} per pulse) or 6 pulses (0.2 g COD L^{-1} per pulse). In all batch experiments, the DO was used as in indirect parameter to identify the substrate depletion.

Figure 5.3 shows the results of this set of experiments and the kinetic parameters are presented in Table 5.4. In the first feeding strategy (A) the 3 or 6 pulses of the fed were added before the DO rose up to the initial saturation value and remains constant (3A and 6A, respectively). A high PHA accumulation (32 %) is attained when the substrate was added in a 6-pulses mode (6A) in contrast with 26 % in the 3-pulse substrate addition. The feeding strategy "B" was performed by adding the substrate pulse immediately after the DO rose up to the saturation value. Once again, when the total COD was divided into 6 pulses, a better PHA accumulation was observed (33 %) compared with the 30 % of PHA in the 3B experiment. In the final strategy, name as “C”, the time for each pulse addition was determined when a sudden increase in the DO was observed. The 6-pulse feeding strategy (6C) accumulates a higher PHA content in comparison with the 3-pulses (3C) strategy (35 and 32 %, respectively). When compared the 3 pulse against the 6-pulses fed, we observed that the more the pulse numbers, the highest the PHA content. It has been previously reported, that PHA accumulation is higher when the carbon source was added in various pulses rather than when was fed in one-pulse (Ahn et al., 2016; Albuquerque et al., 2011). However, none of these strategies improved the PHA accumulation of the MMC in comparison with the SBR operation.

From the three different strategies assessed, the feeding strategy “C” exhibited a higher PHA accumulation. It may be due to the minimization of the time between pulses. Albuquerque et al. (2011), observed that the PHA synthesized is consumed between pulses and it may be allowed by reducing the stop time between substrate additions. The maximum PHA accumulation obtained in this set of experiments was 35% (Experiment 6C). However, this PHA content is lower than that reported by Agustín Martinez et al. (2015) (49 % ± 2) using a similar synthetic OMW with a 10% of polyphenols, but a *Cupriavidus necator* pure culture was used in their study. Agustín Martinez et al. (2015) also reported that the volumetric productivity decreases as the stop time between feeding pulses are high which is in concordance with the results obtained in this study.

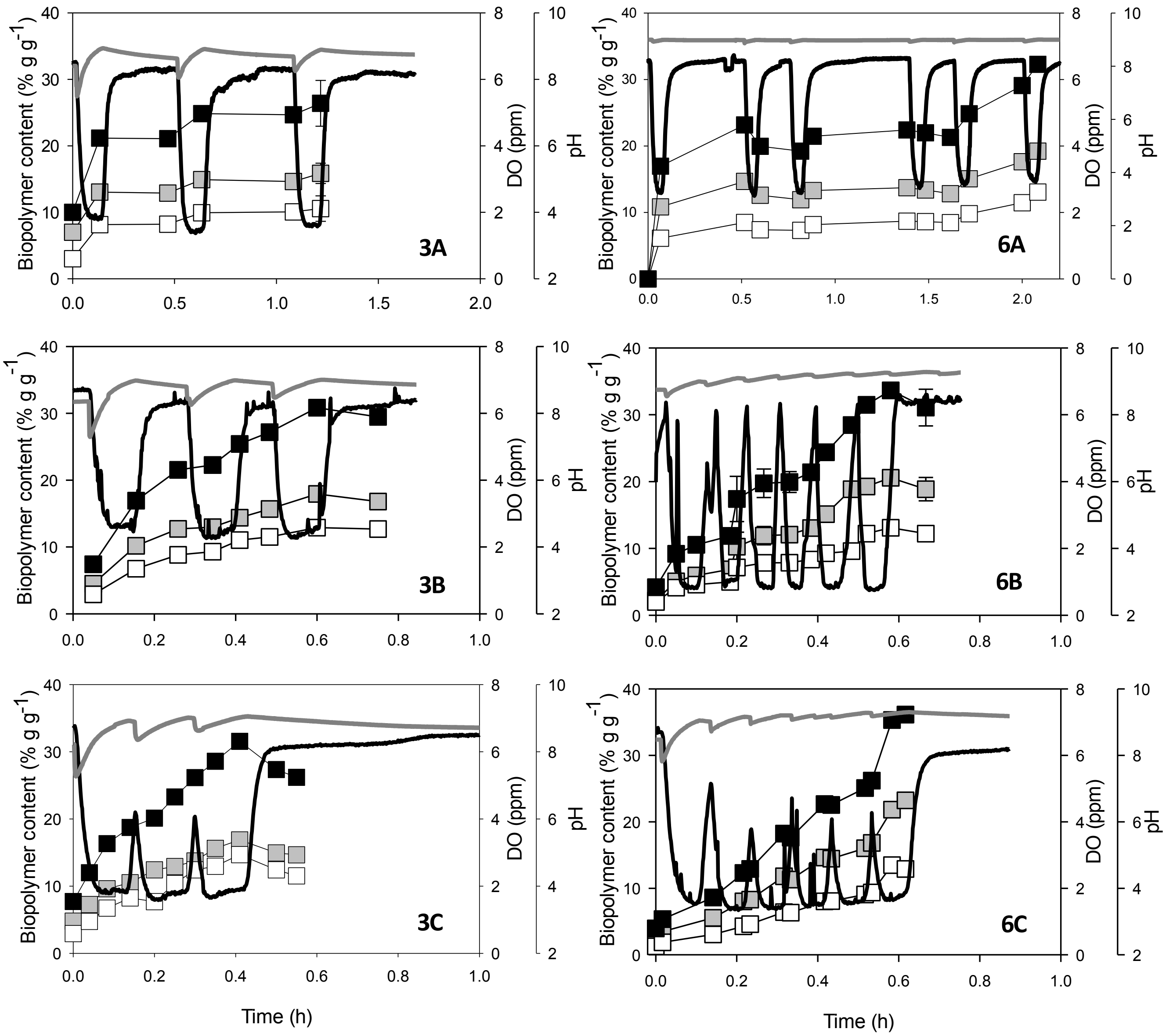

Figure 5.3. Batch experiments profiles for different split-fed strategies. DO (in ppm) is depicted as a black line. The grey line represents the pH. PHB, PHV and total PHA are represented as gray, white and black squares, in % of gPHA gVSS-1. 3A, 3B and 3C are for the experiments where the COD was added in 3 pulses of 0.4 ppm for each one. 6A, 6B and 6C are for the experiments were the COD was added in 6 pulses of 0.2 ppm for each one

The PHA volumetric productivity is defined as the amount of PHA produced per unit of volume of reactor and time (gPHA L^{-1} h^{-1}). Volumetric productivity still a bottleneck in the MMCs PHA production process although it is an important parameter to ensure the competitiveness of the biopolymer as it is going to enhance the overall costs (Możejko-Ciesielska and Kiewisz, 2016; Oehmen et al., 2014; Valentino et al., 2017; Villano et al., 2014). A PHA volumetric productivity of 1.84 and 1.69 for 3C and 6C respectively, was obtained, which was higher than the volumetric productivities for the "A" experiments (0.43 ± 0.2 (3A) and 0.35 ± 0.4 (6A) gPHA L^{-1} h^{-1}). The productivities observed for the batch experiments "C" were higher than that obtained for the SBR in the period III (0.91 gPHA L^{-1} h^{-1}), calculated at the point of maximum PHA accumulation (third pulse). The productivities reported herein are slightly over the range of the overall PHA volumetric productivities previously reported (in the range from 0.5 – 1.50 g PHA L^{-1} h^{-1}) (Albuquerque et al., 2011; Campanari et al., 2014; Valentino et al., 2017; Villano et al., 2014). However this values still lower compared with those of pure cultures also when using low-cost carbon substrates and lower than the reported by Oehmen et al. (2014) using MMC (6.9 and 10.8 gPHA L^{-1} h^{-1}). It can be noted that higher volumetric productivities are attained during the accumulation experiments in comparison with the enrichment step. It may be a result of the higher OLR applied during the batch experiments 3C and 6C (60 and 41.4 gCOD L^{-1} d^{-1}, respectively) compared with the 1.2 g COD L^{-1} d^{-1} in the SBR. Previous researchers also observed a detrimental effect of the PHA productivity as the ORL increases in the range from 8.5 to 40 g COD L^{-1} d^{-1} (Campanari et al., 2014; Dionisi et al., 2006; Valentino et al., 2017).

Regarding to the biopolymer composition, we observed in all the experiments, that a copolymer with HB and HV units was synthesised. As previously described, the HV fraction may be a result of the presence of HPr in the fed (Albuquerque et al., 2011; Bengtsson et al., 2010). In all the feeding strategies, the polymer was mainly composed of HB units (Table 5.3). Among the batch experiments, we observed a difference regarding to the HB to HV ratio. It can be observed (Fig 5.3) that when the substrate was added in 3 pulses (batch experiments 3A, B and C) the HV fraction remains almost constant during the whole batch experiment, however a constant decrease of the HV to HB ratio was observed when the fed was added in 6 pulses (Fig 5.3, 6A, 6B and 6C). The higher HV content (around 47 – 49 %) was attained for the 3-pulses experiments 3B and 3C; on the contrary, for the 6-pulses experiments, the HV fraction was around 40 %. Some other authors had reported changes on the HV fraction depending on the feeding strategy for PHA accumulation (Ahn et al., 2016;

Campanari et al., 2014; Chen et al., 2013; Duque et al., 2014). It is essential to select the better feeding strategy as it is going to be a critical parameter not only for a maximum PHA accumulation but also to determine the polymer end-use. The HV contents obtained herein for the copolymer (HB-co-HV) are higher if it is considered that the HPr represents only a 15% of the total COD in the substrate. Such HV contents has been previously found using HAc:HPr mixtures of (50-50 %) (Albuquerque et al., 2007; Montano-Herrera et al., 2017) as the molar content of HB respect to HV has been found to be directly proportional to the proportion of VFAs with even number of carbon atoms (acetic and butyric acids) and odd number of carbon atoms (propionic, valeric and lactic acids) present in the fed (Albuquerque et al., 2007). HPr contents in the feedstock around 10- 20 % had been reported to produce copolymers with HV contents of around 14 – 22 % (Campanari et al., 2017), odd content of 21% lead to a low 31% of HV fraction in the study reported by Albuquerque et al. (2007); The polymer obtained by Colombo et al. (2016) using a 84:16 even to odd VFA ratio in the fed, resulted in a 100% HB content and a higher 40% HV was observed when using a higher even VFA proportion in the fed (up to 29%). Also Campanari et al. (2014) found low HV contents (ca. 10%) when using a mixture composed of a 20% of VFA with odd carbon number.

Table 5.4 shows the kinetic results for the batch experiments. As stated, the lower PHA content achieved was 26 % for the experiment 3A and the PHA accumulation in all the other experiments were ca. 30 % with the higher PHA content of 35 % for experiment 6C. The uptake rates for all the batch experiments (Table 5.4) were around 0.33 and 0.56 Cmol Cmol X^{-1} h^{-1}; the higher PHA storage rate was observed for the experiment 6C (0.40 ± 0.03 Cmol Cmol X^{-1} h^{-1}) and the lower was for the experiment 3A (0.12 ± 0.02 Cmol Cmol X^{-1} h^{-1}). Similar PHA storage rates was found by other authors during batch experiments were the fed was added in a pulse-wise mode. Albuquerque et al. (2013) report a PHA storage rate of 0.27 Cmol Cmol X^{-1} h^{-1}; also Duque et al. (2014) reported storage rates around 0.30 Cmol Cmol X^{-1} h^{-1} during the addition (controlled by the DO profile) of fermented cheese whey as substrate. Besides, the batch experiments carried out by Colombo et al. (2016) also by applying a pulse-wise mode, exhibited storage yields of 0.29 Cmol Cmol X^{-1} h^{-1} when using sodium acetate as carbon source and 0.2 – 0.4 Cmol Cmol X^{-1} h^{-1} using fermented cheese whey. Despite the contents obtained during the batch experiments were very similar to the results obtained during the enrichment step (33 %), the PHA production rate attained at the experiment 6C was found to be 2-folds higher in comparison with that of the SBR (0.20 Cmol PHA Cmol X^{-1} h^{-1}).

Storage yields between 0.48 and 0.58 C-mol PHA C-mol^{-1} substrate were observed for almost all experiments, except for the 3A batch experiment that exhibited the lower PHA storage yield (0.22 Cmol $Cmol^{-1}$); such result indicate that the substrate may be consumed for other processes (such as growth) rather than for PHA synthesis and this is consistent with the high biomass growth yield (0.63 C-mol C-mol^{-1}). On the other hand, the higher storage yield was observed for the batch experiment 6A (0.77 Cmol $Cmol^{-1}$). This higher storage yield and is comparable to those results reported by Jiang et al. (2012) using as substrate a fermented and a synthetic paper mill wastewater (0.7 – 0.8, in gCOD $gCOD^{-1}$). Polymer srorage yields of 0.7 – 0.8 C-mol C-mol^{-1}) are also reported by Colombo et al. (2016) using fermented cheese whey and acetate. Campanari et al. (2014) used an OMW fed in pulses and observed that the storage yield increases within the pulses from 0.51 to 1.61 Cmol C-mol^{-1}).

Table 5.4 Kinetic parameters for batch experiments under different feeding strategies

Pulse number	Strategy	PHA content	PHB fraction	PHV fraction	$-q_S$	q_{PHA}	$Y_{PHA/S}$	$Y_{X/S}$
3	A	26 ± 3	66 ± 1	34 ± 1	0.56 ± 0.02	0.12 ± 0.01	0.22 ± 0.01	0.63 ± 0.02
	B	30 ± 1	53 ± 2	47 ± 2	0.40 ± 0.02	0.19 ± 0.01	0.48 ± 0.02	0.50 ± 0.03
	C	32 ± 1	51 ± 2	49 ± 2	0.52 ± 0.03	0.34 ± 0.02	0.51 ± 0.01	0.27 ± 0.01
6	A	32 ± 1	56 ± 2	44 ± 2	0.46 ± 0.03	0.22 ± 0.01	0.49 ± 0.02	0.45 ± 0.02
	B	33 ± 2	60 ± 2	40 ± 2	0.33 ± 0.03	0.20 ± 0.03	0.58 ± 0.03	0.37 ± 0.01
	C	35 ± 1	64 ± 1	36 ± 1	0.51 ± 0.03	0.40 ± 0.03	0.77 ± 0.04	0.18 ± 0.01

Standard deviation as ± for each value; PHA content as % (gPHA g^{-1}VSS); $-q_s$: Substrate uptake rate (C-mol C-mol^{-1} X h^{-1}); q_{PHA}: PHA production rate (C-mol PHA C-mol^{-1} Xh^{-1}); $Y_{PHA/S}$: PHA to substrate stoichiometric yield (C-mol PHA C-mol^{-1} substrate); $Y_{X/S}$: active biomass produced to substrate stoichiometric yield (C-mol X C-mol^{-1}).

5.3.3. Effect of the substrate composition on the maximum PHA-accumulation capacity of the enriched biomass

Using the 6A approach, a set of batch experiments were performed to assess the effect of different substrates (Table 5.1) over the PHA accumulation performance of the enriched biomass. The experiments were done without pH control and under nitrogen limitation.

A total of 1.2 gCOD L^{-1} were added in all experiments by adding 0.2 gCOD L^{-1} on each pulse; nevertheless, as a very low uptake rate when HPr were fed as sole carbon source was observed (data not shown) lower PHA concentration pulses were added in Exp. 2 and Exp. 3, accounting to up to 12 and 8 fed pulses to reach the desired total COD. Figure 5.4 depicted the profiles obtained during this set of experiments. As it was expected and in concordance with previous studies, the PHA composition was found to vary depending on the type of substrate used as the amount of energy (ATP) for bioconversion of VFA into PHA is different for each carbon source (Anjum et al., 2016; Wang et al., 2017). The polymer composition is of great importance as it is going to determine their industrial use. A copolymer composed by HB and HV in similar proportions is preferable rather than higher contents of only HB or HV (Chang et al., 2012) and it has been demonstrated that may be regulated by varying the odd to even VFA proportions in the fed composition (Takabatake et al., 2000). When acetic acid is used as a sole carbon source, the homopolymer PHB is mainly produced (Kourmentza and Kornaros, 2016; Lemos et al., 2006). In this study, HAc was used as sole carbon source in the Exp. 1 and so, the synthesized biopolymer was mainly composed of PHB (98.6% of the total PHA). On the other hand, a copolymer of the monomers HB and HV is obtained (Jiang et al., 2011; Kourmentza and Kornaros, 2016; Lemos et al., 2006) when propionic acid is used as the substrate (Exp. 2) but it is also reported that theoretically, propionic acid leads to polyhydroxymethylvalerate (PHMV). Nevertheless, in this study, only the copolymer P(HB-co-HV) was obtained with a major fraction of HV (88 Cmol %HV) (Table 5.4). This result was very similar to that obtained by Dias et al. (2008) that reported a 20 Cmol % HB and 80 Cmol % HV. As in our study, (Jiang et al., 2011)no PHMV production was observed by Jiang et al. (2011) and such results are supposed to be due to the microbial community characteristics. PHMV monomer is more frequently synthesized in systems that alternate anaerobic/aerobic conditions, where Polyphosphate-accumulating organisms (PAOs) or Glycogen-accumulating organisms (GAOs) are found (Dai et al., 2007; Oehmen et al., 2006). When alternating an acetate pulse plus a propionate pulse (Exp. 3), a copolymer of HB and HV was produced as well as on the other experiments were a mixture of acetic and propionic acids were added (table 5.5). For the case in which the alternating pulses were added, a lower PHV fraction was obtained (around 20 Cmol %). However, when the both VFAs were added as a mixture (Exp. 4, 5 and 6), the PHV fraction percentage increases (between 30 and 48

Cmol %) as stated in table 5.5. A copolymer of HB and HV has been previously reported by Kourmentza, 2016 when using a mixture of acetic and propionic acid, however, in this study a higher HV fraction was achieved which is directly related with the difference of the ratio of acetic to propionic acid used.

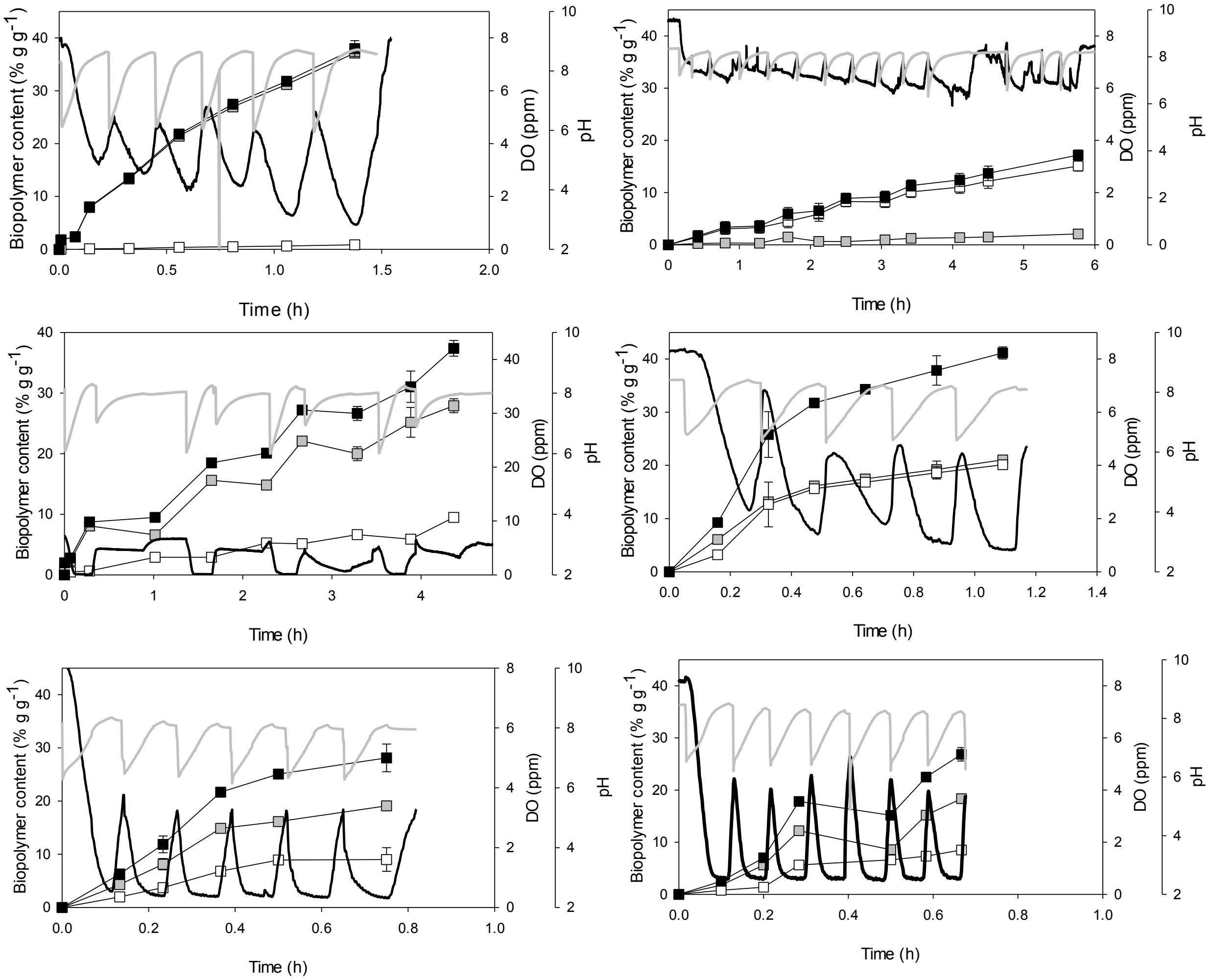

Figure 5.4. Batch experiments profiles using different substrates. DO (in ppm) is depicted as a dotted line. The gray line represents the pH. PHB, PHV and total PHA are represented as gray, white and black squares, in % of gPHA gVSS-1. The letters correspond as follow A – Exp. 1; B – Exp. 2, C – Exp. 3; D – Exp. 4; E – Exp. 5 and F – Exp. 6

The highest PHA accumulation (44.5 %) was achieved when the mixture of HAc:HPr was feed (Exp. 4), followed by the experiment of acetic acid as the sole carbon source (Exp. 2). The lower PHA accumulations for our research, and contrary to previous reports, was achieved when the propionic acid was fed alone (17%) and this could be due to the enrichment step, in which the proportion of propionic acid was always lower than the acetic acid and the enrichment step had shown to influence the PHA accumulation in the accumulation stage (Chang et al., 2012).

We found that the presence of a phenolic fraction in the substrate (Exp. 5 and 6) directly affect the total PHA accumulation of the enriched biomass, a decrease of around a 20% of PHA with respect to the experiment with the maximum PHA content (Exp. 4) was observed demonstrating that phenols in the substrate inhibit PHA accumulation.

As the higher PHA contents were obtained when HAc and the mixture of HAc-HPr were used (Exp. 1 and 4), two more batch experiments (1-HAc and 2-HAc:HPr) were performed in order to evaluate the maximum PHA accumulation capacity. Both experiments were performed up to a 2-fold high total COD concentration, divided into 12 pulse additions. We observed that the PHA accumulation in both cases increases resulting in a PHA content of 56 % and 73 % for the experiments 1-HAc and 2-HAc:HPr, respectively (Table 5.5). The maximum PHA content obtained in this study (73 %) is a comparatively high value for a MMC PHA production system. Up to now, the highest PHA production using MMC is 89 % using acetate as the sole carbon source (Johnson et al. 2009); Albuquerque et al. 2011 reported a 77 % using synthetic fermented molasses fed. In this study, a lower PHA content was observed when HAc was used as sole carbon source. Some authors suggest a higher PHA content when acetate is used as sole carbon source, however, in our study, a higher PHA content was obtained when the mixture of HAc and HPr (75:25) was used. As previously mentioned that could be due to the enrichment step which determines the microbial composition and, in our study, MMC had been enriched using both VFAs (Kourmentza et al., 2016).

As shown in Table 5.5 the substrate uptake rates were very similar for all the experiments (~ 0.55 Cmol Cmol X^{-1} h^{-1}) where HAc is used in the fed composition. The uptake rate for the Exp. 2, in which HPr was used as sole carbon source, is considerably low (0.09 Cmol Cmol X^{-1} h^{-1}). For the experiments 1-HAc and 2-HAc:HPr a lower substrate uptake rate was observed in comparison with the Exp. 1 and Exp. 4. The reason for such behaviour may be due to the higher PHA content (77%). Albuquerque et al. 2011, has previously reported that the proximity to the saturation PHA level may cause a decrease in the uptake rates and PHA storage rates.

Table 5.5 Kinetic parameters for batch experiments using different substrates

Pulse #	Exp.	Total COD	PHA	PHB fraction	PHV fraction	$-q_s$	q_{PHA}	$Y_{PHA/S}$	$Y_{X/S}$
6	1	1.2	38 ± 2	99 ± 1	1 ± 1	0.56 ± 0.01	0.40 ± 0.02	0.72 ± 0.03	0.24 ± 0.02
12*	2		17 ± 1	12 ± 5	88 ± 5	0.09 ± 0.01	0.04 ± 0.01	0.37 ± 0.01	0.50 ± 0.03
8**	3		37 ± 1	79 ± 3	21 ± 3	0.31 ± 0.02	0.12 ± 0.01	0.39 ± 0.01	0.47 ± 0.02
6	4		45 ± 1	51 ± 6	49 ± 6	0.61 ± 0.04	0.51 ± 0.02	0.84 ± 0.03	0.12 ± 0.01
	5		28 ± 3	67 ± 4	33 ± 4	0.58 ± 0.02	0.29 ± 0.01	0.51 ± 0.01	0.20 ± 0.01
	6		27 ± 1	69 ± 8	32 ± 8	0.60 ± 0.02	0.26 ± 0.03	0.49 ± 0.02	0.26 ± 0.01
12	1-HAc	2.4	56 ± 2	97 ± 1	3 ± 1	0.41 ± 0.04	0.21 ± 0.01	0.52 ± 0.01	0.33 ± 0.02
	2-HAc:HPr		73 ± 1	47 ± 2	53 ± 2	0.43 ± 0.03	0.28 ± 0.02	0.65 ± 0.02	0.18 ± 0.01

*12 pulses of 0.1 g COD L-1; **4 pulses of HAc (0.2 g COD L-1) and 4 pulses of HPr (g COD L-1); Standard deviation as ± for each value; Total COD in g COD L-1; %PHA (gPHA g-1VSS); -qs: Substrate uptake rate (C-mol C-mol-1 X h-1); qPHA: PHA production rate (C-mol PHA C-mol-1 X h-1); YPHA/S: PHA to substrate stoichiometric yield (C-mol PHA C-mol-1); YX/S: active biomass produced to substrate stoichiometric yield (C-mol X C-mol-1).

The results obtained for the specific PHA production rate were higher when the mixture of acetic and propionic acid was added (Exp. 4) (0.51 Cmol Cmol X^{-1} h^{-1}) than when HAc was used as the sole carbon source (Exp. 1). When the PHA production rates of Exp. 1 and Exp. 4 are compared with that for the experiments 1-HAc and 2-HAc:HPr (0.21 and 0.28 C-mol PHA C-mol^{-1} X h^{-1}) a 2-folds decrease was noted which is in agreement with the previous observation of Albuquerque et al. 2011 that suggest a decrease in the storage rates when the PHA content is close to the saturation level.

Acetic acid (Exp. 1) as well as the mixture of HAc and HPr (Exp. 4) have shown to be better substrates for the production of PHA in comparison with HPr as sole carbon source (Exp. 2) as it could be determined by the differences between their PHA to substrate yields

(Table 5.5). It was also obtained better PHA yields for the VFA mixture than for the HAc; Kourmentza and Kornaros, (2016) also reported better production yields when both acids are fed as a mixture than in the case where they are fed alone. The production yields for Exp. 5 and 6 were around 0.28 Cmol C-mol^{-1}, demonstrating that the presence of phenols affects PHA accumulation. That could be due to the accumulation of the phenolic fraction because at the end of each experiment the COD accounted to up to ca. 0.08 gCOD L^{-1} corresponding only to the presence of phenolic compounds. That value corresponds to a 67% of the total PF (0.12 g L^{-1}, as COD) added, demonstrating that only a low amount of PF can be depleted as the time between each pulse is not enough to for its complete depletion. By following the experiments performances (Fig 5.4), it could be observed, a PHA accumulation increment during the three first substrate additions but, for the next pulses, a sightless increment is noted. During the experiments also a yellow colour of the sludge and foaming were observed confirming that the phenolic fraction might be accumulated, as previously reported for such characteristics (Zhang et al., 2018)). In general, an increase in phenol concentration could negatively affect PHA accumulation performances because of its toxicity, which affects microorganism's cellular activity (Zhang et al., 2018).

5.4 Conclusions

The enrichment of a PHA-accumulating microorganisms was successfully performed using a synthetic olive mill wastewater (sOMW) as feedstock under feast/famine conditions. The inhibitory effect of the sOMW phenolic fraction was effectively overcome by distributing the fed in three different spikes along the reaction time. The enriched culture exhibited a good performance for PHA accumulation under batch experiments using the sOMW. It was found that the implementation of a split-fed strategy based on the DO profile, enhance the overall PHA production yields directing the substrate uptake towards PHA synthesis. A copolymer of P(HB-co-HV) was observed and in all cases high amounts of HV units was observed. Finally, the enriched biomass PHA accumulation capacities were assessed using different substrates. The use of a mixture of acetic acid and propionic acid (85:15) as carbon source lead to a high PHA content with HV contents up to 50% when the split-fed strategy is used. The results obtained represents a promising outlook for the valorization of OMW.

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Chapter 6

Extraction and characterisation of polyhydroxyalkanoates biologically synthesised using mixed microbial cultures

Summary

Bacterially synthesized polyhydroxyalkanoates (PHA) are biodegradable and biocompatible materials that can replace conventional plastics. The mechanical properties of PHA may be tailored for their final application in many fields such as medical, domestic and industrial. Currently, PHA recovery technologies represents one of the major drawbacks for PHA commercialization, not only for the high costs but also because of the use of chlorinated solvents, such as chloroform (CF), that are hazardous materials.

In this work, a polyhydoxybutyrate (PHB) homopolymer and a polyhydroxybutyrate-co-polyhydroxyvalerate P(HB-co-HV) were produced using an enriched PHA-producing mixed culture. The extraction of both PHAs were performed using dimethyl carbonate (DMC) as a green solvent and the recovery efficiencies were compared with those obtained when using CF. We found that biomass pre-treatment with NAOCl increase the polymer recovery efficiencies due to improved cell lysis. In general, the recovery of P(HB-co-HV) was higher than PHB recovery with both solvents. A higher P(HB-co-HV) recovery (30 %) was achieved using DMC than CF (27 %). However, the extraction of PHB was better using CF (22%) than DMC (11%).

The extracted PHA structure were analysed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and their thermal properties were determined by thermal gravimetric analysis (TGA) and Differential scanning calorimetry (DSC). The average molecular weight was determined using gel-permeation chromatography (GPC). Based on a dyad and triad analysis, a "blocky" structure of P(HB-co-HV) was determined based on the degree of randomness (D and R) obtained (D = 4.06 and R = 0.65). The decomposition temperature (T_d) of both polymers were very similar (~ 240 °C) however the melting point (T_m) and degree of crystallinity (X_c) were lower for the copolymer (78°C and 14.7 %, respectively) than for the homopolymer (154,6 °C and 35.8%, respectively). The average molecular weights (M_w) obtained in this study were lower compared with those reported in the literature (4.53 and 3.49 x 10^4 Da for PHB and P(HB-co-HV)) and we hypothesised that it could be due to the NaOCl pre-treatment.

6.1 Introduction

There is a growing environmental concern as a result of the problems driven from the overuse of petroleum-based plastics that has urged an enormous interest in the research of new biobased and biodegradable materials that can replace them. One of such biobased biodegradable plastics is the family of polyhydroxyalkanoates (PHA).

PHA are fully bio-based and biodegradable plastics, synthesized by more than 300 hundred of bacteria as intracellular granules and has gained interest due to its plastic-like properties that can be manipulated depending on the polymer composition (Moralejo-Gárate, 2014; Samorì et al., 2015a; Yasotha et al., 2006). However, the overall PHA production costs still higher compared with petroleum-based plastics which limits their competitiveness in the plastics market. In fact, the current PHA price are between 2.2 and 5.0 €/Kg which is around three times higher compared with the price of conventional plastics such as polystyrene (PS), polypropylene (PP) or polyvinyl chloride (PVC) (< 1.0 €/kg) (Gholami et al., 2016). The main factors affecting the PHA costs are on the one hand, the use of expensive carbon sources as feedstocks and on the other hand the PHA downstream processing (Gahlawat and Soni, 2017; Jacquel et al., 2008; Samorì et al., 2015a). In the former, many efforts had been made to reduce the PHA production costs by using mixed microbial cultures (MMC) instead of pure or genetically modified cultures and also, by using agricultural and industrial wastes as inexpensive carbon sources (Kosseva and Rusbandi, 2018; Kunasundari and Sudesh, 2011). In the last, downstream processing represent a challenge and is necessary to focus on the development of inexpensive and non-toxic eco-friendly processes.

The PHA granules are produced and accumulated intracellularly and in fact this is the main drawback that limit the extraction processes (Madkour et al., 2013). PHA granules need to be released from the cells and applying a pre-treatment methods such as: heat, alkali, salt and freezing/thawing cycles will be helpful to make more easily the cell disruption (Gholami et al., 2016; Jacquel et al., 2008; Madkour et al., 2013). However, rather if a pre-treatment is used or not, many approaches has been developed to recover PHA from the cells. The selection of an appropriate method depends on several factors like the PHA producer, the type and composition of the synthesized PHA, the production scale, the impact of the extraction method over the biopolymer properties and of course the end-use of the PHA that is going to determine the purity requirements (Kosseva and Rusbandi, 2018; Madkour et al., 2013).

According to several authors (Fiorese et al., 2009; Neves and Müller, 2012; Patel et al., 2009; Rosengart et al., 2015), the main factors that affect the recovery of PHAs are: 1) the mixing conditions between the biomass and the extraction solvent, 2) the solubility of PHAs in the solvent, 3) the boiling point of the solvent, and 4) the type biomass (pure or mixed cultures). As a general rule, solvents in which PHAs are more soluble may favor the recovery of the polymers. Additionally, solvents with a high boiling point allows extractions under higher temperature conditions, favoring the cell lysis and therefore, enhancing the polymer recovery.

One of the most widespread methods for PHA recovery is the solvent extraction which normally involves the use of chlorinated solvents such as chloroform (CF) and 1,2 dichloroethane (DE). Unless a high PHA purity is achieved using chlorinated solvents their use is limited to lab-scale because are considered as undesirable solvents at industrial scale due to their harmful effect on humans and on the environment (Gahlawat and Soni, 2017; Kosseva and Rusbandi, 2018; Madkour et al., 2013; Samorì et al., 2015b). The use of "green solvents" such as dimethyl carbonate (DMC), has emerged as a promising alternative. DMC is a non-toxic and biodegradable solvent that has not been extensively studied for the extraction of PHA. Righi et al. (2017) evaluated the potential life cycle environmental impacts related to the extraction of P(3HB) with DMC compared to the use of 1,2-dichloroethane, demonstrating that the environmental performance of DMC is much better than the use of halogenated hydrocarbons.

In comparison with PHA extraction from pure strains, the PHA recovery from MMC is a more complex process. Some authors suggested the presence of a complex cellular matrix that surrounds the PHA granules difficulting their extraction because the cells are more resistant to hydrolysis (Patel et al., 2009; Samorì et al., 2015a). The polymer extraction from MMC commonly is carried out using CF (Albuquerque et al., 2011; Bengtsson et al., 2010; Dai et al., 2007; Duque et al., 2014; Serafim et al., 2008) and to our best knowledge DMC had been previously explored only by Samorì et al. (2015a) achieving a 1.7 times lower PHA recovery (50 %) compared with the extraction from pure *Cupriavidus necator* confirming that the extraction from MMC is more challenging in mixed cultures. Characterization

The polymer extraction must be considered as a key-parameter of the overall process as it is going to deeply influence the final PHA properties. Specially, the use of chemicals for the pre-treatment step, has been reported to degrade the PHA negatively affecting the molecular weight (M_w) and also, affecting their thermal stability properties (Jiang et al., 2015; Rivero et al., 2017; Samorì et al., 2015a). Mechanical properties of PHA are crucial parameters as it is going to determine the final use of the biopolymer (Arcos-Hernández et al., 2013). The homopolymer polyhydroxybutyrate (PHB) is the most widely known PHA, but their industrial use is limited due to their poor mechanical and physical properties (Gahlawat and Soni, 2017). However, the copolymerization of PHB with hydroxyvalerate (HV) units can improve the mechanical properties of the biopolymer. The copolymer P(3HB-co-3HV) has better elasticity and thermal processability and as HV units increase in the copolymer, the flexibility and ductility are improved, the melting temperature decreases and brittleness is reduced (Arcos-Hernández et al., 2013; Gahlawat and Soni, 2017). The type of PHA synthesised can be tailored by the substrate composition; the presence of propionic acid (HPr) in the feedstock leas to the copolymerisation of PHA (Kosseva and Rusbandi, 2018).

Therefore, this study aims to evaluate the PHA extraction process using DMC as a green solvent alternative. The extraction using CF was also evaluated to compare the results. The effect of a biomnass pre-treatment with sodium hypochlorite (NaClO) and the effect of the PHA precipitation with ethanol were investigated. A PHA production platform based on MMC was used for the biopolymer synthesis. Acetic acid (HAc) and a mixture of acetic and propionic acid (HPr) was employed as substrates during the batch PHA-accumulation experiments for the synthesis of the homopolymer PHB and the copolymer P(HB-coHV). Finally, the extracted biopolymers were characterised in terms of polymer structure, molecuar weight and thermal properties.

6.1Materials and methods

6.2.1 PHA production

The enriched-biomass used in the present study, was harvested from a seuqencing batch reactor (SBR) fed with a synthethic olive mill waste (OMW). The SBR is operated under fully aerobic conditions and under a feast/famine regimen. The biomass was collected at the end of the feast phase and settle down. The supernatant was removed and the biomass was resuspended with the medim mineral described by Montiel-Jarillo et al. (2017).

Batch PHA-accumulation experiments were performed using HAc (Batch A) and a mixture of 75:25 HAc:HPr (Batch B) as substrates. For each experiment a 1L glass reactor was used. Biomass was arated and magnetically stirred. No pH control was used an the reactors were operated at room temperature (ca. 23°C). In each experiment, the substrate was added in a spli-fed strategy triggered by the dissolved oxygen profile (DO). Each time a DO increase was observed, a 0.2 g COD L^{-1} pulse of substrate was added. Biomass samples were regularly taken to follow the substrate depletion and the PHA concentration. At the end of each batch experiment, drops of formaldehyde were added to inhibit biological activity. The mixed liquor was lyophilized and stored at room temperature until further use for analysis.

6.2.2 PHA extraction

PHA were extracted from the MMC using DMC and CF were tested as extraction solvents according to the method described by Samorì et al. (2015b). At the end of each batch experiment, 6 mL of formaldehyde (37% v/v)) was added to the harvested biomass to inhibit biological activity. The biomass was centrifuged at 8500 rpm for 45 minutes. The supernatant was discharged and the sedimented biomass was stored overnightat at -80° and then lyophilized during 24h. The samples were weighted (25, 50 or 100 mg) and transferred to eppendorf vials. The samples were resuspended in 1 mL of Milli-Q water and mixed with 2 mL of solvent. The vials were incubated at the boiling temperature of each organic solvent (90°C for DMC or 60°C for CF) for 1 or 3 h. and were periodically stirred using a vortex. The resulted mixtures were centrifuged at 8000 rpm for 15 min at room temperature and three phases were obtained: 1) the supernatant phase, composed by water and water-soluble compounds; 2) the intermediate phase that corresponds to the organic fraction containing the solubilized PHAs; and 3) a biomass debris precipitate. The organic fraction (2) was recovered and filtered through 0.45 µm pore size cellulosic membranes (Millipore®) to remove any cell debris. Finally, the sovent was removed by evaporation to obtain the dried PHA sample.

The samples that exhibited a better recovery efficiency were submitted to a pre-treatment and an ethanol precipitation to enhance the PHA extraction process. The process is detailed below:

(A) Precipitation of PHAs with ethanol (Gahlawat et al., 2017). The filtered organic phase containing PHAs (2 mL) was mixed with 6 mL of cold absolute ethanol (96%). Then, the mixture was incubated at room temperature with periodic stirring during 1 h and finally, PHAs was obtained by volatilizing the solvents.

(B) A biomass chemical digestion was done with sodium hypochlorite (NaClO) (Samorì et al., 2015a). A total of 50 mg of lyophilized biomass were resuspended in 5 mL of NaClO 5% (wt/v) and were incubated at 100 °C during one h. The digested samples were centrifuged at 4000 rpm for 10 min at room temperature. The pellets were washed three times with Milli-Q water and resuspended in 1 mL of Milli-Q water. Finally, PHAs were extracted according to the previously detailed procedure.

For a clear understanding, Figure 6.1 schematically represents the process flow of the different variables assessed during the PHA extraction method.

6.2.3 Soxhlet extraction of PHAs

To obtain higher amounts of PHA a larger scale extraction using a Soxhlet device (Buchi, E-816 SOX) was performed. In this case, CF was used as the extraction solvent and 2.2 g of lyophilized biomass was treated with 220 mL of NaOCl 5%(w/v) at 100 °C during 1 h. Then, the digested sample was centrifuged at 4000 rpm for 20 min. The obtained pellet was centrifuged and washed three times with milliQ water and then, introduced into the Soxhlet cartridge. PHA was extracted using 90 mL of CF and the recovered PHAs was used for further characterisation.

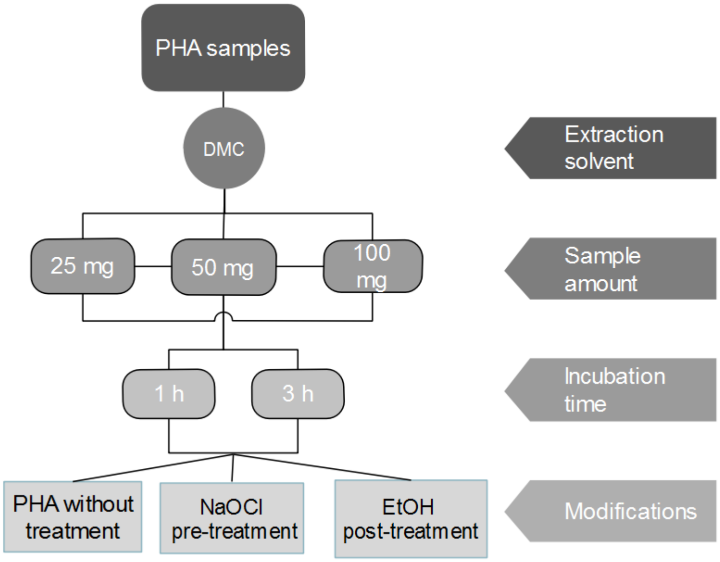

Figure 6.1 Schematic representation of the PHA extraction process

6.2.4 PHA composition

PHA composition was determined by the gas chromatography protocol previously described (Montiel-Jarillo et al., 2017). The copolymer P(HB-co-HV) was used for calibration and benzoic acid was added as the internal standard in all tubes. Butanol and hydrochloric acid were added to the sample and incubated at 100°C for 8h. Then the PHA fraction was extracted using hexane and transferred into GC vials. 1 µL of sample was injected and analysed in an Agilent Technologies (7820 A) gas chromatograph equipped with a FID detector and a HP-InnoWax column (30 m x 0.53 mm x 1 µm) (Werker et al., 2008).

6.2.5 Nuclear magnetic resonance (NMR)

The PHA chemical structure was (PHA chemical structure were determined by quantitative ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra using a BRUKER DRX-500 spectrometer. Between 30-40 mg of each sample were dissolved in deuterated chloroform ($CDCl_3$). To the entire sample dissolution, biomass was heated and sonicated. The Bruker TopSpin3.5pl7 software was used to process the NMR spectra. Also, the gradient-selected ^{1}H/^{13}C heteronuclear single quantum coherence (HSQC) spectra were acquired.

The comonomer composition distribution (CCD) of the copolymer P(HB-co-HV) was determined by ^{13}C NMR spectra. A dyad and triad sequence distribution was used to estimate the degree of randomness *D* on the dyad level as proposed by (Kamiya et al., 1989) and *R* on the triad level (Laycock et al., 2014; Zagar et al., 2006) as given by the following equations:

$$D = \frac{F_{BB}+F_{VV}}{F_{BV}+F_{VB}} \qquad \text{(eq. 1)}$$

Where F_{BB}, F_{VV}, F_{BV}, F_{VB} represents the intensities of the dyads sequences: butyrate-butyrate, valerate-valerate, butyrate-valerate and valerate-butyrate, respectively.

$$R = \frac{L_B^R}{L_B^E} = \frac{L_V^R}{L_V^E} \qquad \text{(eq. 2)}$$

L_V^E is the experimental number average length of HV blocks in the copolymer and is calculated by:

$$L_V^E = \frac{(F_{VVV}+F_{VVB} + F_{BVV}+F_{BVB})}{(F_{BVB}+F_{VVB})} \qquad \text{(eq. 3)}$$

L_V^R represents the number average length of HV blocks of statistically random distribution of HB and HV units in the copolymer and is defined by:

$$L_V^R = \frac{(k+1)}{k} \qquad \text{(eq. 4)}$$

so *k* is the ratio of the HB and HV proportions in the copolymer:

$$k = \frac{[HB]}{[HV]} \qquad \text{(eq. 5)}$$

6.2.6 Molecular weight

The average molecular weights (M_w), number average molecular weight (M_n) and polydispersity index (PDI) of PHAs, were estimated by Gel Permeation Chromatography (GPC) on a Waters equipment provided with RI and UV detectors. The polymer samples were diluted (0.1% w/v) and filtered. 100 μL of the sample solution was injected and chromatographed using 1,1,1,3,3.3-hexafluoro-2-propanol (HFIP) as mobile phase with a flow of 0.5 ml min^{-1}. HHR5E and HR2 Waters linear Styragel columns (7.8 mm x 300 mm, pore size 103-104 Å) packed with crosslinked polystyrene and protected with a pre-column were used. The polymer molecular weight was calibrated using as reference the Poly (methyl methacrylate) (PMMA).

6.2.7 Thermal properties

Differential scanning calorimetry (DSC) was performed to assess the melting temperature (T_m), enthalpy of fusion (ΔH_m) and degree of crystallisation (X_c) following the protocol described by Arcos-hernández et al. (2013).

The crystallinity degree (%) was calculated as described by Dai et al. (2008) using the melting enthalpies in the equation:

$$X\ (\%) = \frac{\Delta H}{\Delta H^{\circ}_{PHB}} * 100 \qquad \text{(eq. 6)}$$

Where ΔH is the measured melting enthalpy of each PHA and ΔH°_{PHB} is 146 J g^{-1} (Rosengart et al., 2015) which is the melting enthalpy per a gram of a fully crystalline PHB.

TGA analysis was conducted to determine the degradation temperature (T_d) under a nitrogen atmosphere (Arcos-Hernández et al., 2013).

6.2.8 Recovery efficiency of PHAs

In all cases, the recovery of PHAs was determined by a gravimetric method. The evaporation residue (PHAs) was dried at 105 °C to constant weight. Then, the recovery (%) was calculated as follows:

$$\text{Recovery} = \frac{W_{PHA}\ x\ Purity}{W_{cell} \times A} \times\ 100 \qquad \text{(eq. 7)}$$

where W_{PHA} (gPHA) is the amount of recovered PHAs from a weight $_{cell}$ (g dry-cell) of lyophilized biomass, and A is the specific content of PHAs of the cells (gPHA/g dry-cell). These values were obtained by determining the content of PHA by GC. This constant was 0.57 gPHA/g dry-cell for the biomass enriched with PHB, and 0.69 gPHA/g dry-cell for the biomass enriched with P(HB-co-HV). The extracted polymer was quantified by GC analysis to determine their purity. Measured purity was 0.79 gPHA g^{-1}dry-cell for the PHB and 0.85 gPHA g^{-1}dry-cell for the copolymer. All the results reported in the present work were obtained from triplicate experiments.

6.2Results and discussion

6.2.1 PHA accumulation experiments

A set of batch experiments (Batch A and Batch B) were performed in triplicates using a previously PHA-enriched MMC under a feast/famine regime. Both experiments were performed to accumulate PHA-rich biomass for its further characterisation. For Batch A acetic acid (HAc) was used as a sole carbon source to produce PHB polymer while for batch B a mixture of volatile fatty acids (VFA) of HAc:HPr (propionic acid) (75:25) was used as source aiming to favour the synthesis of a copolymer with HV units P(HB-co-HV). A fed-pulse strategy was applied as previously described. In both experiments 6.0 g COD L^{-1} of were added. At the end of each batch experiment, a biomass sample was lyophilized and analyzed to determine their monomeric composition and total content using the GC as previously described by (Montiel-Jarillo et al., 2017).

The evolution of PHA accumulation over the time for Batch A is shown in figure 6.2. As it can be observed, a new fed pulse was added immediately when an increase in the DO profile was observed as it indicates the substrate depletion. It is also observed that the pH profile follows the same tendency as that observed for the DO profile. The COD content at the end was negligible. PHA content increases over the time at a specific production rate (qPHA) of 0.37 C-mol PHA C-mol X^{-1} h^{-1} and a consumption rate (-qS) of 0.52 C-mol HAc C-mol X^{-1} h^{-1}). This results are The PHA yield (Y_{PHA}) was found to be higher than the biomass yield (Y_X) (0.69 and 0.26 C-mol Cmol HAc^{-1}, respectively), indicating that acetate uptake is preferably used towards biopolymer synthesis. As expected, a homopolymer of

PHB was obtained and a maximum PHA content of 57% (g gVSS^{-1}) of PHA was achieved. The polymer was composed of 95% of PHB on average as determined by GC.

On the other hand, figure 6.3 depicted the time-lapse PHA accumulation during Batch B. The same pH and DO profiles were observed with a constant increase of the PHA production and the substrate was completely depleted. Due to the addition of a mixture of acetic and propionic acids, it can be observed that similar amounts of PHB and PHV were accumulated. A better performance was achieved using the acids mixture than in batch A with higher q_{PHA} and $-q_S$ (0.47 and 0.55 C-mol C-mol X^{-1} h^{-1}, respectively) being acetic acid the preferred substrate. A higher Y_{PHA} was attained in comparison with the batch A (0.80 C-mol Cmol VFA^{-1}) and the Y_X was found to be lower (0.12 C-mol Cmol VFA^{-1}). A higher PHA content of 72% (g g^{-1}) was accumulated, with a polymer composition of 52% HB and 48% of HV on average.

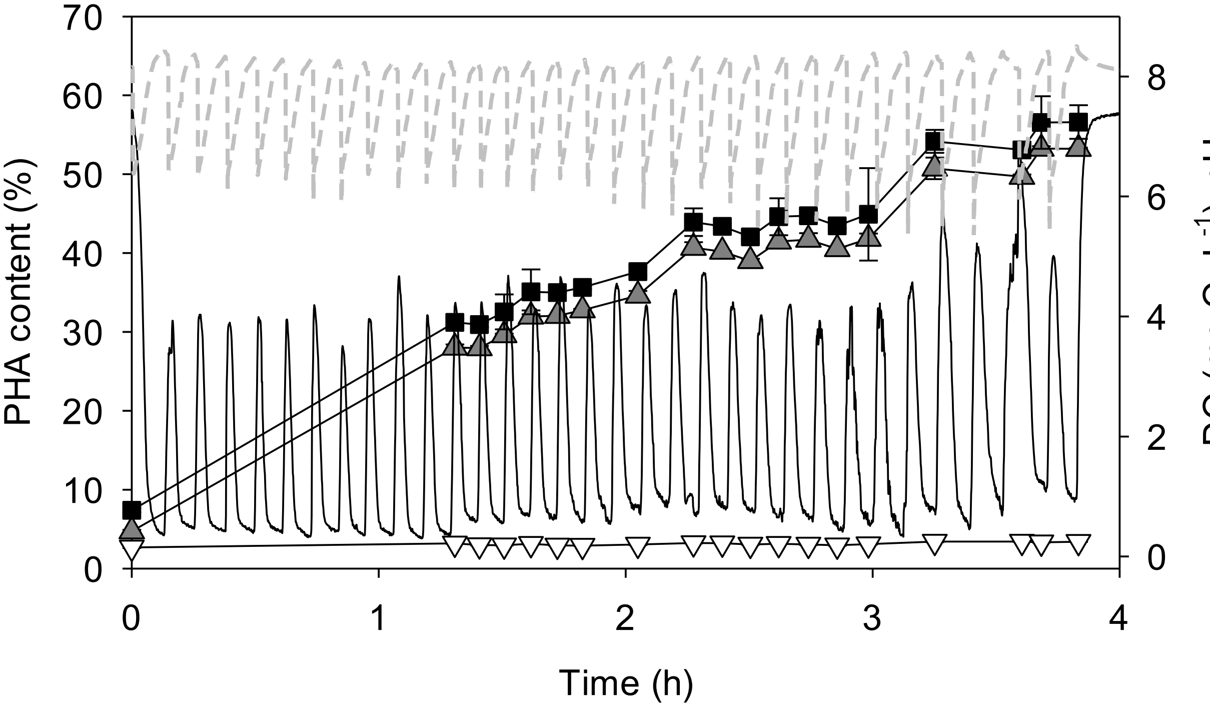

Figure 6.2 Cummulative production of PHB over the time using acetate as sole carbon source. Total PHA are depicted as black squares; PHB and PHV are represented as gray and white triangles, respectively; The black line represents the DO profile and the gray dotted line corresponds to the pH.

As expected, the difference in the substrate composition, changes the PHA composition. HAc is used for the synthesis of the homopolymer PHB (Ciesielski and Przybylek, 2014). It is well known that the biopolymer composition can be manipulated by changing the substrate composition. Particularly, to the presence of HV monomer units for the synthesis of a P(HB-co-HV) copolymer can be achieve by adjusting the presence of odd-chain VFA with respect to the even-chain VFA (Montano-Herrera et al., 2017). Due to the use of a mixture of acetic and propionic acid in Batch B, the synthesis off a copolymer was observed. It can be noted in figure 6.3, that the polymer composition was very stable during the whole batch experiment and that the production yields, total content and production rate of batch B exhibited a better performance.

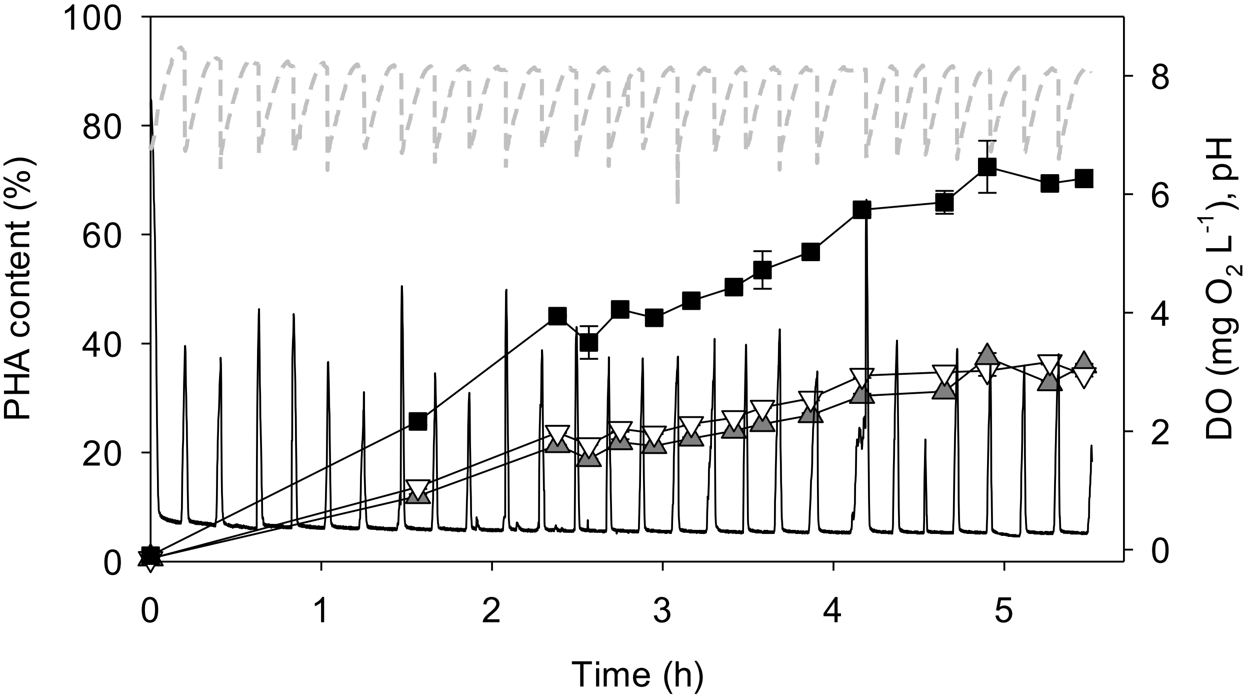

Figure 6.3 Cummulative production of the copolymer P(HB-co-HV) over the time using a mixture of acetatic and propionic acids. Total PHA are depicted as black squares; PHB and PHV are represented as gray and white triangles, respectively; the black line represents the DO profile and the gray dotted line corresponds to the pH.

The kinetic parameters obtained for both experiments where in the range of previos results reported for PHA production using MMC and different synthetic substrates such as acetate, propionic or mixtures of them (Colombo et al., 2016; Montano-Herrera et al., 2017; Woraittinun and Suwannasilp, 2017), fermented sugar cane molasses, chesse whey and waste activated sludge (Duque et al., 2014; Morgan-Sagastume et al., 2010).

6.3.2 Extraction of PHAs using DMC

In a first set of experiments, the effect of the initial biomass concentration on the extraction and recovery of PHAs using DMC at 90 °C was studied. Biomass for both experiments, Batch A and Batch B were used. When the biomass concentration was 25 g dry-cell/L, the amount of recovered PHB was negligible in comparison with the initial content of PHB measured by GC. A similar result was obtained for samples corresponding to PHB/PHV enriched biomass samples. Table 6.1 shows that the increase of the biomass concentration also had a negligible effect on the PHB recovery. Conversely, the recovery of the copolymer PHB/PHV reached 21 ± 1 % when 50 g dry-cell/L was tested. It must be pointed out that when 100 g dry-cell/L was used, the suspension became very viscous and difficult to deal with it, leading to a quite low recovery of the copolymer P(HB-co-HV). Table 6.1 also shows that the increase of the extraction time from 1 to 3 h did not improved the recovery of the polymers. For this reason, a biomass concentration of 50 g dry-cell/L and 1h of extraction time were employed in all the subsequent experiments.

Table 6.1 Recoveries of PHAs using DMC at 90 °C.

Sample	Biomass concentration (g dry-cell/L)	Precipitation with ethanol	Pretreatment with NaClO	Extraction time (h)	Recovery (%)
Biomass enriched in PHB	25	no	no	1	≈ 0
	50	no	no	1	4 ± 3
	100	no	no	1	2 ± 1
	50	no	no	3	5 ± 2
	50	yes	no	1	8 ± 2
	50	no	yes	1	8 ± 3
Biomass enriched in PHB-PHV	25	no	no	1	≈ 0
	50	no	no	1	17 ± 1
	100	no	no	1	5 ± 1
	50	no	no	3	12 ± 3
	50	yes	no	1	7 ± 5
	50	no	yes	1	25 ± 4

Standard deviation as ± for each value

Rosengart et al. (2015) performed the extraction of PHB from *Burkholderia sacchari* DSM 17165 using anisole, phenetole and cyclohexanone at 60 and 120 °C. The three tested solvents showed quite low recovery performances at 60 °C (2.8, 0.9 and 0.3%, respectively). However, when the extraction temperature was 120 °C these authors reported that the recovery of PHB increased substantially (99.6, 96.8, and 94.7%, respectively). Yabueng and Napathorn, (2018) studied the extraction of PHB from *Cupriavidus necator* strain A-04 by 1,3-dioxolane. These authors demonstrated that using extraction temperatures of 30 and 60 °C no recovery of the biopolymer was obtained, while at 80 °C the PHB recovery was about 92%. It must be noted that in the present work the extraction temperature was the boiling temperature of DMC (90 °C). For this reason, we could not performed extraction experiments at higher temperatures.

Samorì et al., (2015b) studied the recovery of PHAs from *Cupriavidus necator* using DMC at different temperatures. While a low recovery of PHB (11 ± 1 %) at 50 °C was reported, the recovery of PHB at 90 °C was 88 ± 6 %. These recovery values are higher than those depicted in Table 6.1. However, it must be taken into account that in that study, pure cultures was used while in the present work a PHA prouction was achieved using an enriched MMC. According to several authors (Patel et al., 2009; Samorì et al., 2015a) MMC are generally more resistant than pure cultures to chemical or thermal lysis. Thus, this effect would hinder the solubilization of the intracellular granules of PHAs on the extraction solvent.

Increasing the recovery efficiencies of PHA from MMC is a crucial factor to an economically viable PHA recovery process. For that reason, two modifications of the extraction technique were studied. The simplest modification (B), consisted in the precipitation of the PHA using ethanol (Gahlawat and Soni, 2017). However as it can be observed in Table 6.1, it did not improve the PHA recovery.

PHAs are intracellular biological polyesters produced by certain bacteria as cell inclusions. To boost PHA recovery, a cell lysis is required prior the extraction step (Jacquel et al., 2008). There are different lysis methods (e.g., physical, chemical or enzymatic) reported the literature (Divyashree and Shamala, 2009; Gobi and Vadivelu, 2015; Neves and Müller, 2012), being the digestion by sodium hypochlorite (NaClO) one of the most employed (Samorì et al., 2015a; Sindhu et al., 2015). Table 6.1 shows that NaClO digestion successfully enhance the PHB and PHB-HV recovery when DMC was used as the extraction solvent.

6.3.3 Extraction of PHAs using chloroform (CF)

Because CF is the one of the most widely used solvents for PHA extraction, it was also assessed as extraction solvent. The results are presented in Table 6.2 ; it can be observed that NaClO digestion pre-treatment clearly improved the recovery of PHA. Extraction using CF was performed at 60 °C, and thus, the low recovery of PHB without using the NaClO pre-treatment could be due to the fact that there was no cell thermolysis, leading to poor contact conditions of the solvent with the intracellular granules of PHB. These results demonstrate that the chemical digestion step is a crucial pretreatment to enhance the recovery of PHAs from MMC.

Table 6.2 PHA Recovery efficiencies using chloroform at 60 °C.

Sample	Pretreatment with NaClO	Recovery** (%)
Biomass enriched in PHB*	no	2 ± 1
	yes	17 ± 1
Biomass enriched in PHB-PHV*	no	5 ± 2
	yes	23 ± 2

* In all cases the biomass concentration was 50 g L^{-1}, and the extraction time 1 h. ** The recovery was performed without precipitation with cold ethanol.

Rosengart et al. (2015) reported recoveries of PHB from *Burkholderia sacchari* close to 97% using CF at 4 °C. Those authors state that this solvent is an aggressive chemical that do not need heating to cause cell disruption. Samorì et al. (2015b) achieved PHB recoveries of 39% using CF directly in contact with wet cells at room temperature. However, these studies were carried out by extracting PHB from pure cultures of *Burkholderia sacchari* and *Cupriavidus necator*. It must be noted that the high recovery reported by Rosengart et al. (2015) and Samorì et al. (2015b) may be due to an intrinsic feature of the tested strains, rather than a property of CF to cause cell lysis.

From the comparison between results depicted in Tables 6.1 and 6.2 it can be concluded that DMC and CF had similar performances regarding to the PHA recovery. As a general rule, for both tested solvents the recovey of the co-polymer P(HB-co-HV) was higher than those corresponding to PHB. The results obtained herein suggest that DMC can be used as "green solvent" alternative for PHA extraction. Nevertheless a better understanfing of the process is necessary for their further optimization to achieve a higher PHA recovery efficiency for both PHB and P(HB-co-HV).

Samorì et al. (2015a) reported that the extraction of PHA from MMCs was about 1.7 times lower than that obtained from pure cultures of *Cupriavidus necator under similar extraction conditions*. Similar findings were reported by Patel et al. (2009) when the extraction was performed from MMC in comparison with pure cultures. Those authors suggest that in the mixed cultures a strong and complex cell matrix creates a barrier around the granules of the biopolymer.

6.3.4 Soxhlet extraction using chloroform

In order to extract higher amounts of both polymers, PHB and P(HB-co-HV), a large scale Soxhlet extraction was performed using 2.2 g of lyophilized biomass and CF as extraction solvent. A recovery of 49 ± 7 % of PHB and 40 ± 4 % of P(HB-co-HV) was attained.This samples were used for further biopolymers characterization.

6.3.5 Biopolymers characterization

The composition of the homopolymer PHB and the copolymerPHB-HV can be analysed by ^{1}H and ^{13}C NMR spectra (Sindhu et al., 2015). The spectra obtained for both biopolymers agreed with those reported in the literature and so, their composition were determined based on previous published results (Arcos-hernández et al., 2013; Divyashree and Shamala, 2009; Gobi and Vadivelu, 2015; Ivanova et al., 2009; Liu et al., 2018; Patel et al., 2009).

The ^{1}H spectrum for PHB and PHB-HV are depicted in Figure 6.4a and 6.4b. Typical signals for PHB are observed (Fig. 6.4a). There are three characteristic signals described for the PHB, a doublet for the methyl group, a doublet of quadruplet for the methylene group and a multiplet for the methyne group (Sindhu et al., 2015). All these signals were observed in the ^{1}H spectra for PHB. The first doublet signal at 1.28 ppm corresponds to the methyl group (B4 in Fig 6.4a). For the methylene group (B2) the doublet of quadruplet is observed at 2.50 – 2.58 ppm and finally the multiplet in 5.25 ppm for the methine group (B3). The small signals observed may be due to the presence of almost neglible amounts of PHV.

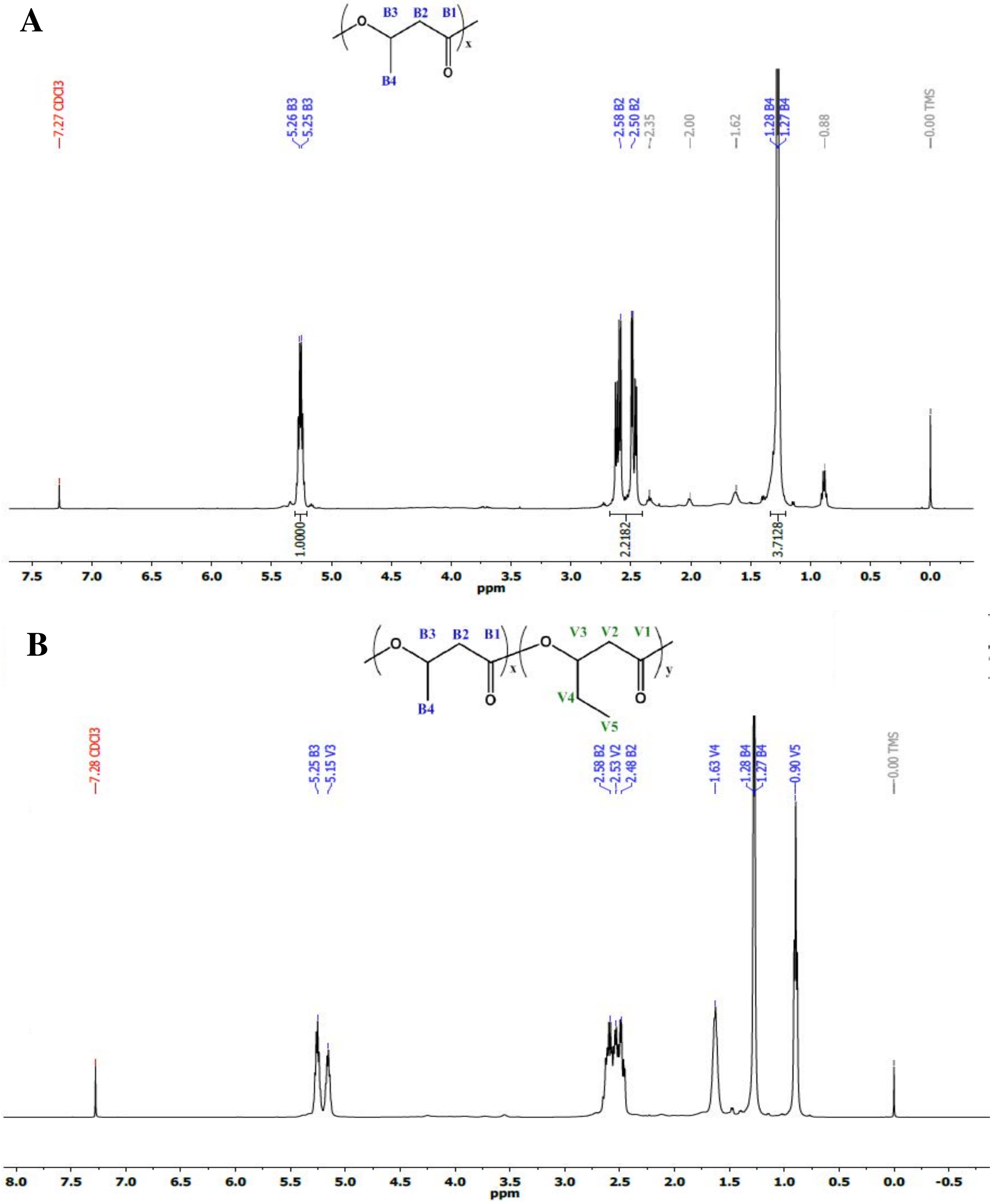

Figure 6.4 ^{1}H NMR spectrums for (A) PHB and (B) PHB-HV extracted from MMC

It can be observed in Figure 6.4b, characteristic signals for P(HB-co-HV) ^{1}H spectrum. The first resonance at 0.90 ppm corresponds to a triplet of the methyl group of the HV (V5). The doublet in 1.28 ppm represents the HB methyl group (B4). Resonance in 1.63, the doublet of quadruplets is assigned to the HV methylene protons (V4) and in the signals observed in the range 2.48-2.58 correspond to the HV methylene protons (2.53 ppm, V2) and the HB methylene group (2.48 ppm and 2.58 ppm, B2). The resonance signal at 5.15 is a double doublet of triplets and is characteristic for the methine proton of HV (V3), similar to the signal at 5.25 that represents the methine proton of HB (B3). The molar fraction for HB and HV in the copolymer can be estimated by the intensity of the B3 with respect to V3 signals and from the intensity of the resonance signals for V5 respect to B4 (Ivanova et al., 2009). The molar fraction with respect to the methine groups (B3 and V3) gives a 58.5 % of HB and a 41.5 % of HV; a very similar molar fraction is inferred from the intensity of the methyle groups (58.7% and 41.2% of HB and HV, respectively). This results are very similar to the HB and HV determined by GC, however the difference may be because NMR is a more precise technique.

Figure 6.5 depicted the resonance ^{13}C spectra for PHB (6.5A) and P(HB-co-HV) (6.5B). The assignment of each carbon peak were deterrmined by data published in literature (Doi et al., 1986a; Irorere et al., 2014; Ivanova et al., 2009; Patel et al., 2009; Zagar et al., 2006).

In the homopolymer PHB ^{13}C spectrum (Fig 6.5A), only four clearly discernible peaks are observed which corresponds with the carbonyl (B1) at 169.12 ppm, the methyl carbon (B4) in 19.77, the methylene (B2) and the methine (B2) at 40.78 and 67.62 ppm, respectively.

Fig 6.5B display the ^{13}C spectrum for the copolymer P(HB-co-HV) and the assignment of each carbon signal is shown in Table 6.3. An expanded NMR spectrum is shown in Fig 6.6, it can be observed the sensitivity of the carbon nuclei of the carbonyl (~169 ppm) and methylene groups (between 26 to 41 ppm) of HB and HV units, which signals are observed to be spplited in several peaks as previously identified and described (Dai et al., 2008; Doi et al., 1986b; Kamiya et al., 1991; Patel et al., 2009; Zagar et al., 2006).

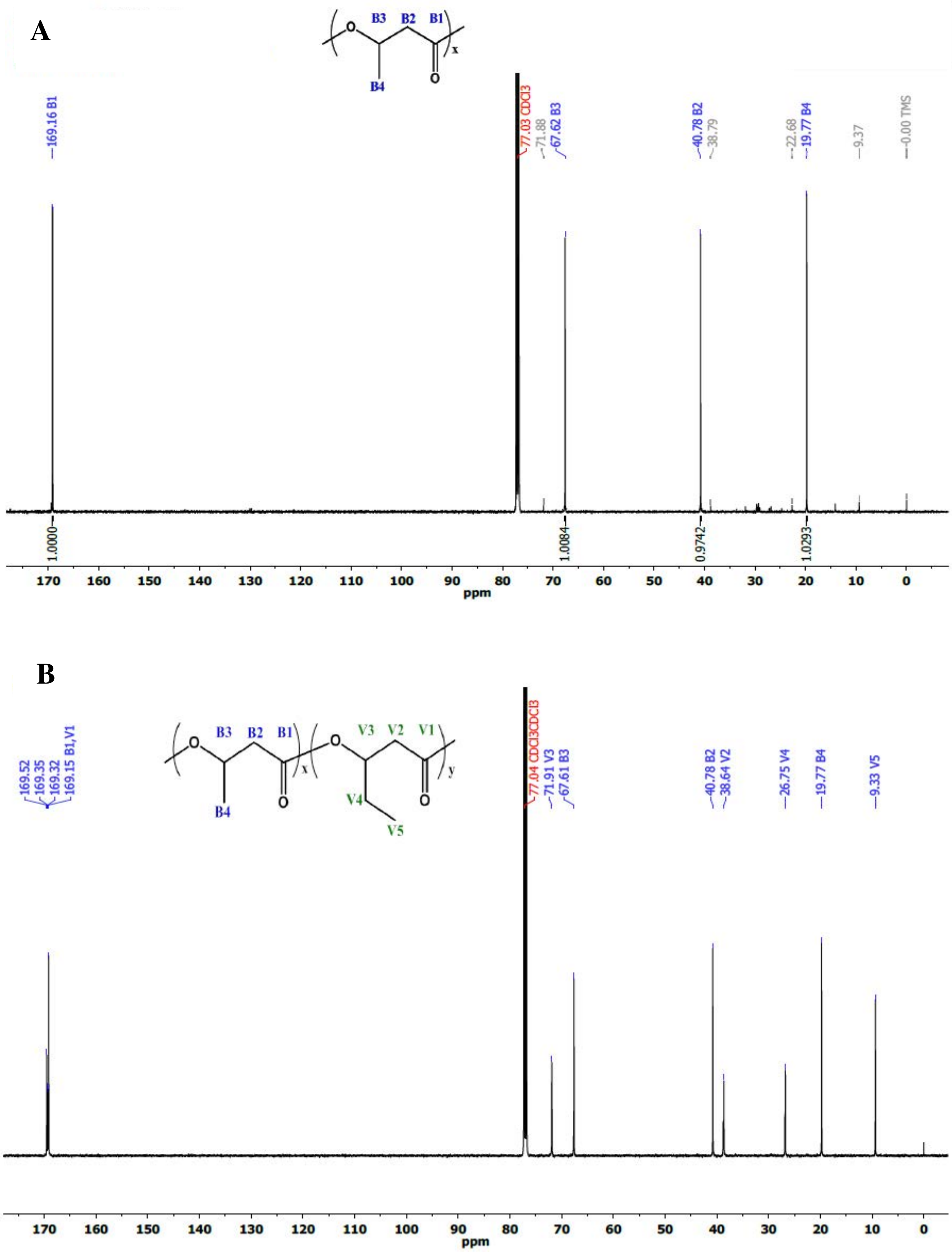

Figure 6.5 ^{13}C NMR spectrums for (A) PHB and (B) PHB-HV extracted from MMC

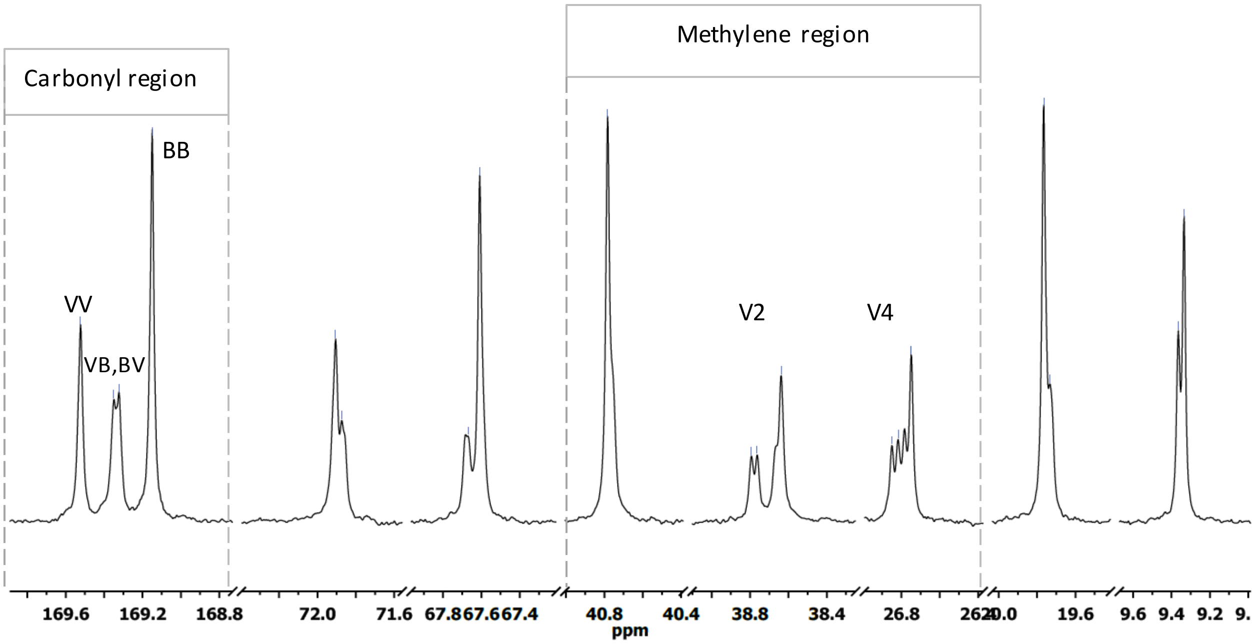

Figure 6.6 expansion of the ^{13}C NMR spectrum for P(HB-co-HV) extracted from MMC

The signals in the carbonyl region contribute to diads of HB and HV units, namely BB, BV, VB, or VV (B corresponds to butyrate and V to valerate); on one hand the peak at 169.15 ppm is consistent with the carbonyl resonance for PHB and so it was assigned to the BB sequence, and as also described by Doi et al. (1986b) the VV sequence was assigned for the peak that appear with a difference of 0.38 ppm (169.52). The intermediate peaks with low intensities are assigned to the BV and VB sequences. Peaks in the methylene region were found to be consistent with literature. First, an almost neglible split of two peaks for the signal in 40.78 ppm was observed and it corresponds to the methylene of the HB unit (B2); the split is due to the diades sequences BV and BB. For the HV unit, the signals for the side-chain methylene group (V4) and main-chain methylene group (V2) may be differentiated, unless both signals are split in four peaks and has almost similar intensities. The main-chain methylene signal is observed between 26.75 and 26.85 ppm and the shift for side-chain methylene is around 38.64-38.79 ppm. The peaks were assigned for triad sequences of VVV, BVV, VVB and BVB in both cases. spectrum, 58.4 % of HB and 41.6 % of HV.

Table 6.3 Assignment of resonance peaks for ^{13}C NMR spectra of copolymer P(HB-co-HV)

Position	Diad/triad	^{13}C (ppm)
V5	VV	9,33
	VB	9.36
B4	BB	19.73
	BV	19.77
V4	VVV	26.75
	BVV	26.77
	VVB	26.82
	BVB	26.85
V2	VVV	38.64
	BVV	38.65
	VVB	38.76
	BVB	38.79
B2	BV	40.77
	BB	40.78
B3	VB	67.61
	VV	67.67
V3	BB	71.87
	BV	71.91
B1	BB	169.15
	BV	169.32
V1	VB	169.35
	VV	169.52

B and Vrepresent butyrate and valerate units

The molar ratio of HV with respect to HB in the copolymer may be determine by the comparison of the peak areas between the signals of the methine carbons (Patel et al., 2009) that appeared at 67.61 ppm for PHB (B3 in Fig 6.5A) and at 71.91 ppm for PHV (V3 in Fig 6.5B). The calculated composition was in agreement with that obtained from the ^{1}H spectrum, 58.4 % of HB and 41.6 % of HV.

Finally, HSQC spectra was analysed to confirm the assignment of resonance peaks of the carbons belonging with their corresponding hydrogen signals. The C-H couplings in HSQC are shown in figure 6.7 for the homopolymer PHB and on figure 6.8 for the copolymer P(HB-co-HV).

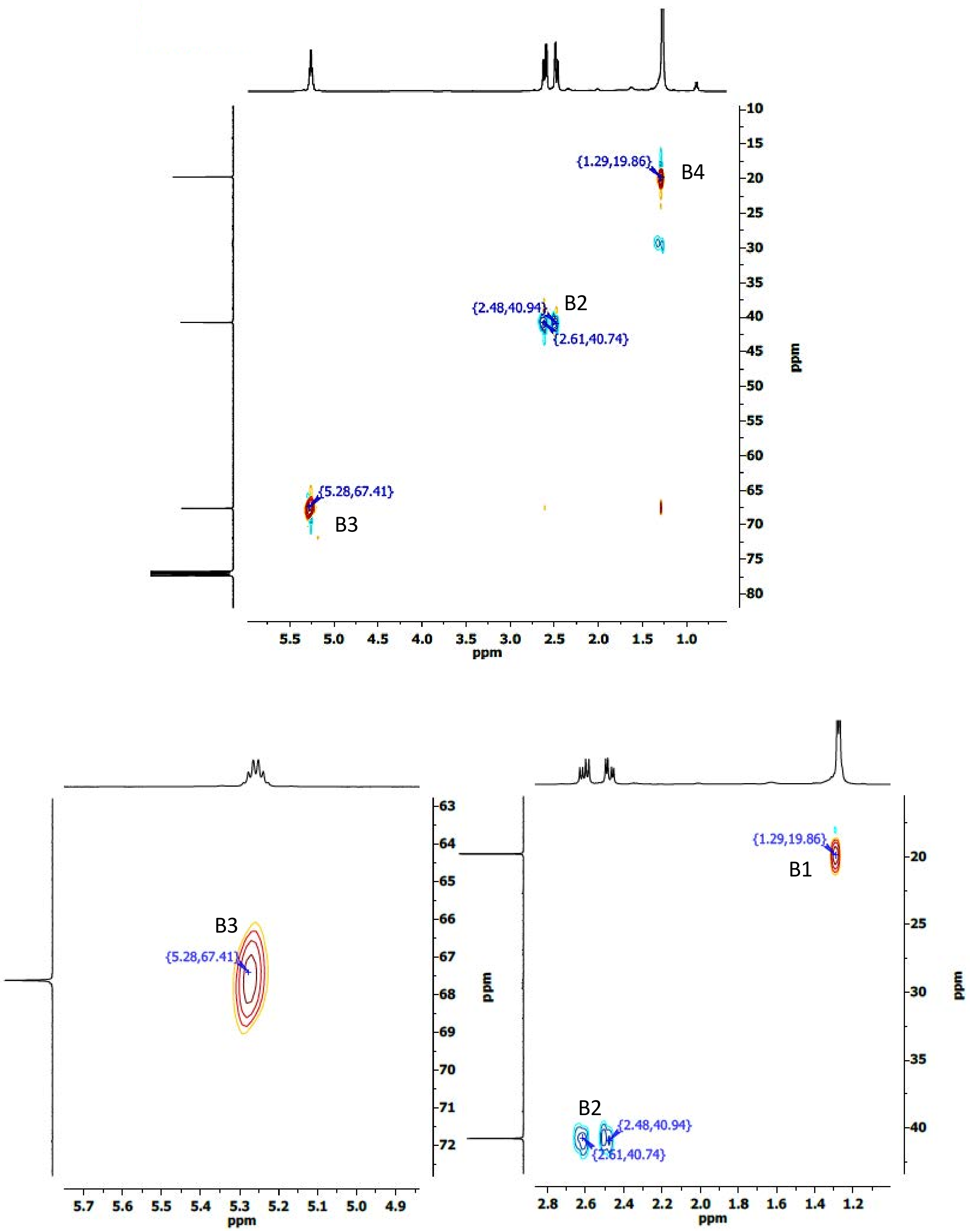

Figure 6.7 HSQC spectra for the homopolymer PHB

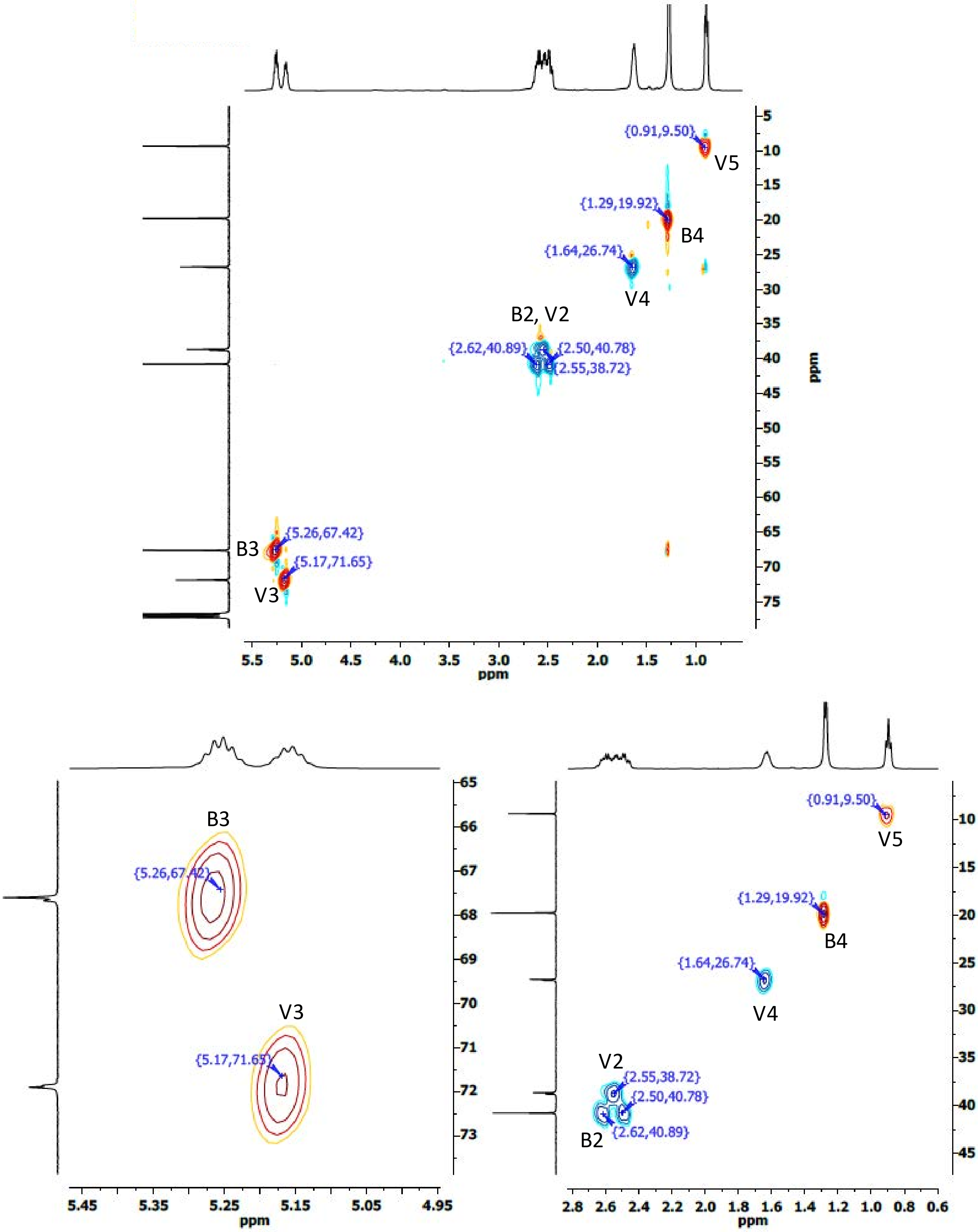

Figure 6.8 HSQC spectra for the copolymer P(HB-co-HV)

The comonomer composition distribution (CCD) of a copolymer have been identified as a key factor determining the physicial properties of a PHA (Zagar et al., 2006). To determine if the P(HB-co-HV) extracted corresponds to a random or a non-random copolymer the ^{13}C NMR spectrum was used. The relative peak intensities of ^{13}C NMR spectra are presented in Table 6.4 and was was used to calculate the parameter *D* and *R* as previously detailed in section 6.2.5.

Table 6.4 Experimental monomer, dyad and triad sequence mole fractions for the copolymer P(HB-co-HV) determined from ^1H NMR spectra^a and ^{13}C NMRspectra^b

Monomer	F_B	*0.587*^{a} *0.584*^{b}
	F_V	*0.413*^{a} *0.416*^{b}
Dyad	F_{BB}	*0.409*
	F_{BV}	*0.176*
	F_{VB}	*0.151*
	F_{VV}	*0.264*
Triad	F_{VVV}	*0.181*
	F_{VVB}	*0.081*
	F_{BVV}	*0.075*
	F_{BVB}	*0.075*

B represents butyrate unit; V represents valerate unit. ^{a}determined by ^1H NMR spectra and ^{b}determined by ^{13}C NMR spectra

In general values of *D* = 1 (in the range 0.99 to 1.5) are obtained for a statistically random compolymers, *D* >1 or D<1 define non-random copolymers; a *D* value close to 0 is obtained for alternating nature copolymers while *D*>1 indicates "blocky" copolymers (Arcos-Hernández et al., 2013; Kamiya et al., 1989; Mitomo et al., 1995). Nevertheless when the *D* value is greater than 1, the copolymer may be: i) a true block copolymer; ii) a mixture of ranadom copolymers; or iii) a mixture of HB and HV homopolymers (Kamiya et al., 1989; Laycock et al., 2014). For the copolymer P(HB-coHV) synthesised and extracted in this study, the calculated value of *D* was 4.06, so it can be surely concluded that the microestructure corresponds to a "blocky" copolymer character but no other information can be elucidated. Considering the reports found in literature, the P(HB-co-HV) may be considered as a mixture of random copolymers or a simple block structure (Arcos-Hernández et al., 2013; Kamiya et al., 1989; Zagar et al., 2006). To get a better insight about the nature of the copolymer, the parameter *R* was estimated as it was described to be a more sensitive parameter of the degree of randomness in a copolymer (Laycock et al., 2014; Zagar et al., 2006). A value of $R = 0$ indicates a diblock copolymer while a *R* value of 1 corresponds to a "completely random distribution" (Arcos-Hernández et al., 2013; Laycock et al., 2014; Zagar et al., 2006). The *R* obtained herein was 0.65. This value support the hyphothesis of P(HB-co-HV) to be more a block copolymer rather than a random copolymer. "Blocky" materials has shown higher elongation to break (larger Young´s module and tensile strength) than a random polymer (Li et al., 2011; Pederson et al., 2006).

The determination of the molecular weights of both biopolymers, were performed by GPC analysis. The results for the weight average molecular weight (M_W), number average molecular weight (M_n) and polidispersity index (PDI) are presented in Table 6.5. Such parameters, are of great importance as they are responsible of the end-use suitability of a given polymer for specific applications (Fiorese et al., 2009; Laycock et al., 2014; Sim et al., 1997).

Even though the M_W obtained in this study are in the range of the molecular weights reported in literature ($1.0 \times 10^4 – 4.0 \times 10^6$ Da) (Fiorese et al., 2009; Rosengart et al., 2015; Sudesh et al., 2000) it still differing in around one order of magnitude from the lowest PHA molecular weights commonly reported in literature (10^5 Da) (Fiorese et al., 2009). The reason for such low molecular weights may be a consequence of the effect of different conditions during the extraction process. On one hand, the extracted biopolymers were pre-treated using NaOCl for 1 hour at 100°C. The NaOCl is a strong oxidizer and so, is a perfect agent for disrupting the cellular wall (Gobi and Vadivelu, 2015; Samorì et al., 2015a) and this is the same property that may be responsible of their strong effect on the molecular weight decrease of the polymer. It has been described that amorphous structure polymers are weak to alkaline saponification which causes their degradation into monomers and oligomers with a molecular weight decrease of around 50 - 70% when using NaOCl (Jacquel et al., 2008; López-Abelairas et al., 2015). However as NaOCl is a helpful agent for PHA extraction, several authors have suggested their use in CF mixtures as their co-presence minimize the PHA degradation because once PHA is released, it may be immediately dissolved in the CF (Gobi and Vadivelu, 2015) as well as the use of surfactants and mixtures with chloromethane (López-Abelairas et al., 2015). On the other hand, the temperature and time of thermal conditions, have shown to influence the molecular weights. Temperature increase the PHA solubility but there is a point of inflection of the temperature at which the solvent starts to degrade the PHA (Berger et al., 1989; Fiorese et al., 2009; Jacquel et al., 2008). Finally, when the Soxhlet method was used, lower molecular weights were obtained (around 3-times lower) and it was supposed to be due to a intrinsenc degradation caused by the long extraction time at high temperatures (Dai et al., 2007; Samorì et al., 2015a). Aditionally some other conditions such as the extraction from dried or wet cells and the fermentation conditions (mainly pH, type and concentration of C source and nutrients) can affect the molecular weight of biopolymers obtained (Laycock et al., 2014; Patel et al., 2009; Sudesh et al., 2000; Yabueng and Napathorn, 2018). There are several factors that affect the molecular weight of the extracted PHA, but their effect is not clearly defined and so further efforts must be employed to well-stablish the effect of each extraction condition affecting the final PHA characteristics. Despite the molecular weights obtained for the PHB and P(HB-co-HV) are low, the values obtained for the molecular weight distribution index (defined by the PDI) are in the range of the PDI reported in the literature (Aoyagi et al., 2002; Arcos-Hernández et al., 2013; Dai et al., 2008; Fiorese et al., 2009; Khanna and Srivastava, 2005; López-Abelairas et al., 2015; Rosengart et al., 2015; Xie and Chen, 2008). The PDI is a hint to known the heterogeneity of the polymer chain lenghts (Fiorese et al., 2009). However, narrow PDI (close to 1.0) are preferred, as it reflects a higher homogeinity of the polymer, which enables their use into a huge range of applications (Gobi and Vadivelu, 2015).

Table 6.5 Molecular weights for the recovered PHB and PHB-HV

PHA	M_w (x10^4 Da)	M_n (x10^4 Da)	PDI
PHB	4.53	1.69	2.7
PHB-HV	3.49	1.23	2.8

M_w, weight average molecular weight; M_n, number-average molecular weight; PDI, polidispersity index

The results obtained for the thermal properties of the PHB and P(HB-co-HV) extracted from a MMC, are depicted in Table 6.6. DSC analysis were performed to determine the melting points (T_m) of the synthesised biopolymers. The PHB has been described as a brittle, crystalline and stiff polymer with a melting point arounf 175°C that is only a little bit lower than the temperature at which it starts to degrade into crotonic acid. The PHB DSC melting curve obtained in the present study, exhibited a single peak at 154.6 °C which is in the range of results reported in several studies (Bengtsson et al., 2010; Gahlawat and Soni, 2017; Irorere et al., 2014; Khanna and Srivastava, 2005; López-Abelairas et al., 2015; Serafim et al., 2008; Sudesh et al., 2000; Xie and Chen, 2008; Yabueng and Napathorn, 2018). As summarized in Table 6.6, multiple melting point were observed for the copolymer at 78.3 and 152.9 °C. This results are similar to the data estimated for similar HB-HV copolymers. Arcos-hernández et al. (2013) found similar results for the melting points (77.5 and 159.2 °C) of a P(HB-co-HV) obtained using the same feeding strategy and a similar carbon source as in the experiments reported herein. The range of melting points reported are in agreement with the results obtained in this study (Arcos-Hernández et al., 2013; Doi et al., 1986b; Gahlawat and Soni, 2017; Serafim et al., 2008; Silva et al., 2013). As explained above, the results of the ^{13}C NMR spectra analyisis, the P(HB-coHV) seems to be as a blocky copolymer, with $D > 1.5$ which is expected to have 2 or 3 peaks representing multiple melting temperatures as it should be the result of the presence of two different polymers (Arcos-hernández et al., 2013; Kamiya et al., 1989; Zagar et al., 2006). Based on the melting entalpy for each biopolymer, the degree of crystallinity (X_c) was determined as previously detailed in section 6.2.7. The results are presented in Table 6.6 and as it can be compared, the degree of cristallinity for the copolymer is 2-times lower compared with that of the PHB; it demonstrated that as HV units in the polymer chain increase, the T_m and X_c decrease, improving flexibility and tensile strenght of the polymer leading to a grater range of applications (Dai et al., 2008; Gahlawat and Soni, 2017; Rosengart et al., 2015; Serafim et al., 2008).

Table 6.6 Thermal characterisation parameters calculated for the recovered polymers

PHA	$T_{d\text{-}1\%}$	$T_{d\text{-}5\%}$	$T_{d\text{-}max}$	T_{m1}	ΔH_{m1}	T_{m2}	ΔH_{m1}	X_c (%)
PHB	183.8	244.9	250.1 – 302.5	154.6	47.2	-	-	35.8
PHB-HV	104.5	242.3	256.0 – 304.7	78.3	15.9	152.9	3.44	14.7

$T_{1\%}$ is the degradation temperature correspondig to 1 % weight loss in the sample; $T_{5\%}$ is the degradation temperature correspondig to 5 % weight loss in the sample; T_{max} is the range of the maximum degradation rate; all the T values are presented in °C and the ΔH in J g^{-1}; X_C crystallinity

The results from the decomposition temperatures (T_d) give an overview of the range of temperatures in which biopolymers may be processed (Bengtsson et al., 2010; Xie and Chen, 2008) and TGA analysis were performed for their determination. The TGA profiles obtained for PHB and P(HB-co-HV) are depicted in Figure 6.9. It can be observed that the thermal stability behavoir of both samples is very similar. However PHB is thermally stable up to 184 °C and PHB-HV up to 105°C (weight loss <1 wt% at this temperatures). The degradation temperature for a 5% weight loss obtained for both polymers is around 240 °C which is very similar to the results reported by Arcos-Hernández et al. (2013) for PHB-HV copolymers (in the range of 240 – 270 °C), Xie and Chen, (2008) for HB and copolymers of HB-HHx (hydroxyhexanoate) in the range of 235-257 °C and some other authors (Aoyagi et al., 2002; López-Abelairas et al., 2015; Rosengart et al., 2015). Degradation of both polymers occurs in a single step with the maximum degradation rate ($T_{d\text{-}max}$) around 250 and 300 °C in both cases. The detailed thermogravimetric data for both biopolymers is shown in table 6.6.

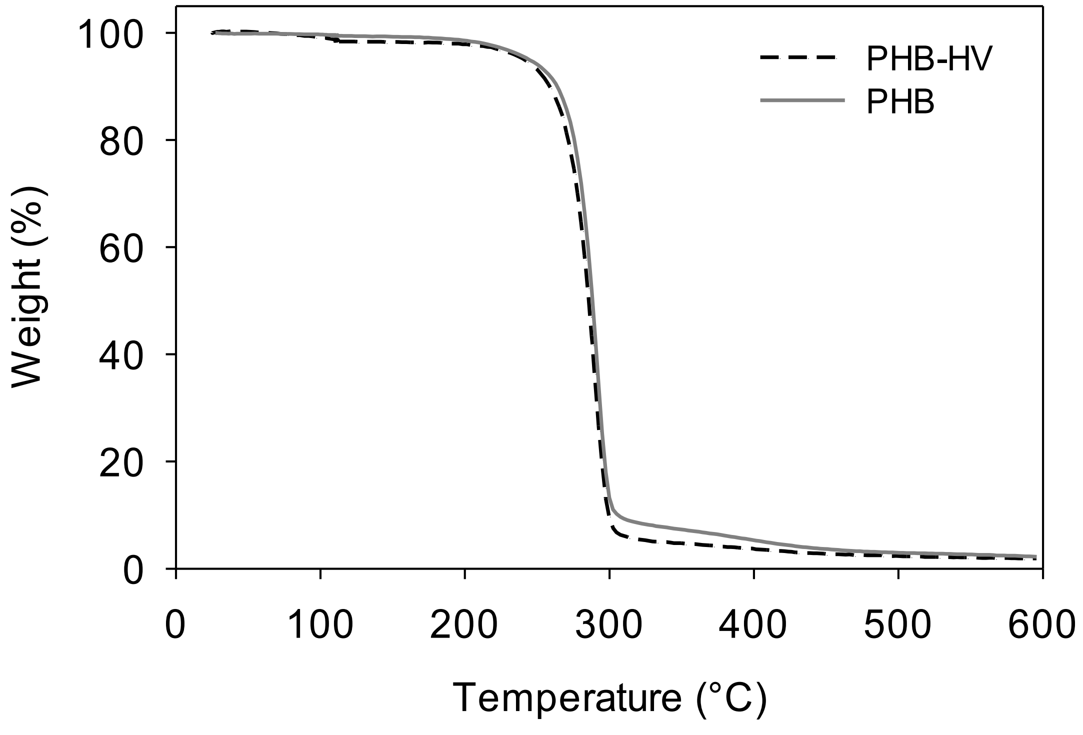

Figure 6.9 TGA curves for the recovered PHB (gray line) and P(HB-co-HV) (dotted line)

6.3Conclusions

In this study, the biopolymer composition was effectively manipulated by the substrate. A PHB homopolymer and a copolymer P(HB-co-HV) were effectively synthesized by using an enriched-MMC. A extraction using a NaOCl pre-treatment coupled to DMC or CF solvent extraction was evaluated. The results for the PHB extraction using DMC exhibited a lower recovery efficiency compared with the results attained when CF was used as solvent. Nevertheless, the copolymer extraction was effectively recovered using DMC and the results were very similar in comparison with the CF recovery. The NMR ^{1}H and ^{13}C spectra obtained are in agreement with previous reports, corroborating the biopolymers structure. The *D* and *R* parameters calculated suggest the synthesis of a "blocky" copolymer, however further analysis such as copolymer fractionated methodo may be needed to obtain a detailed comonomer composition. The thermal properties elucidated in this study, suggest that the presence of HV units in the polymer has desirable thermal characteristics for further PHA processability. The molecular weights of both biopolymers were very low and it may be consequence of the pre-treatment step, but a more detailed study is necessary to determine if the effect of NaOCl is the only parameter influencing the biopolymer degradation.

6.4 References

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Chapter 7

General conclusions and future work

7.1 General conclusions

This thesis was focused on evaluating the feasibility of the biological production of polyhydroxyalkanoates (PHA) using mixed microbial cultures (MMC) and wastes and wastewaters as substrates. A three-stage process was used to achieve a waste-based PHA bioproduction. The main conclusions of this research work, are detailed below:

→ **Bio-conversion of wastes into VFA through acidogenic fermentation**

When a heat-shock pre-treatment was applied to the anaerobic inoculum, the maximum degree of acidification (DA) of 39 % ± 2 was observed in the acidogenic fermentation of glucose. Also, the higher H_2 yield (40.6 mL H_2 gVS^{-1}) was attained using the heat-shock.

Waste activated sludge (WAS), olive mill wastewater (OMW), glycerol, winterization oil cake (WOC) and apple pomace (AP)) were evaluated for assessing VFA production in acidogenic fermentation. It was found that the WAS with low sludge residence time (2 days) and the OMW are suitable precursors for VFA production with maximum degree of acidification of 69 % ± 1 for WAS and 48 % ± 1 for the OMW. By contrast, a lower H_2 yield (2.6 6 mL H_2 gVS^{-1}) was obtained for the fermentation of WAS.

The VFA composition was determined to calculate the odd-to-even ratio of each fermented waste. For almost all the wastes evaluated herein, the odd-to-even ratio was ca. 0.30, being acetic acid (HAc) the VFA produced in a higher proportion, followed by propionic (HPr) and butyric acids (HBt). Conversely, the AP and the OMW exhibited the lower odd-to-even ratio (0.01) as there were almost entirely composed of even-chain VFA (HAc and HBt). Interestingly, WOC was the only waste that produces HPr in higher amounts followed by HAc, resulting in an odd-to-even ratio of 0.88.

→ **Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: effect of pH and N and P concentrations**

A mixed microbial culture was enriched under fully aerobic feast/famine regimen using HAc as the sole carbon source. The enrichment performance was evaluated under control of pH at 7.5 and without pH control (pH fluctuated in the range 8.8. - 9.2). It was demonstrated that working without pH control lead to a high PHA content (36 % gPHA $gVSS^{-1}$).

The enriched-MMC achieved a biopolymer yield of 0.35 Cmol PHA $Cmol^{-1}$ VFA and a production rate of 0.19 Cmol PHA $Cmol^{-1}X$ h^{-1}) and was found to be dominated by the presence *α-proteobacteria, β-proteobacteria*, *Flavobacteriia*, *Verrucomicrobiae* and *δ-proteobacteria*, all of which had been previously described as PHA-storing microorganisms.

Batch experiments were performed to evaluate the storage capacity of the enriched-MMC. The effect of different pH values over the maximum PHA content was determined and the lower PHA content was observed at a pH of 5.5 (20% gPHA $gVSS^{-1}$) while without pH control the highest PHA content of 44% gPHA $gVSS^{-1}$ was achieved.

A second set of experiments were performed to elucidate the effect of nitrogen (N) and phosphorus (P) concentrations over the PHA storage capacity. Higher PHA contents were achieved under limitation nutrients with a higher PHA content (51 % gPHA $gVSS^{-1}$) when N was limited (C/N/P = 100/4.1/2.4, taking 100 mol of C as basis).

→ **Feasibility of using an acidified oil mill wastewater (OMW) as a precursor for PHA production**

The selection of a PHA-producing MMC was assessed under a full aerobic feast/famine regimen using a synthetic acidified OMW as carbon source composed by HAc (75 % of total COD), HPr (15 % of total COD) and a phenolic fraction (10 % of total COD). The enrichment performance was successfully achieved with 90 % of COD removal. The phenolic fraction inhibition was minimized by using a strategy of distributing the feeding in different additions during the reaction time. A maximum PHA content of up to 33 % gPHA $gVSS^{-1}$ was observed with a biopolymer yield of 0.56 Cmol PHA $Cmol^{-1}$ VFA and a production rate of 0.20 Cmol PHA $Cmol^{-1}X$ h^{-1}.

The enriched-MMC was used to perform batch accumulation experiments with the aim of maximising the PHA accumulation inside cells. The effect of different split-fed strategies based on the dissolved oxygen profile were evaluated. Similar PHA content was observed for the batch experiments compared to the one of the enrichment step, but an important improvement on the biopolymer yield and production rate was attained (0.77 Cmol PHA $Cmol^{-1}$ S and 0.40 Cmol PHA $Cmol^{-1}X$ h^{-1}, respectively).

Using the same MMC, another group of batch experiments were made to evaluate the monomer PHA composition using different substrates. The HV content (calculated on a mol basis) in the obtained copolymers ranged from 21 to 53 %. The highest PHA accumulation 73% gPHA $gVSS^{-1}$) was achieved using a mixture of HAc and HPr (75:25) fed in split-pulses of 0.2 gCOD L^{-1} for the synthesis of the copolymer P(HB-co-HV)

→ Extraction and characterisation of the biopolymers PHB and P(HB-co-HV) biologically synthesized using a MMC

A green extraction method using dimethyl carbonate (DMC) was assessed for the recovery of synthesized PHB and P(HB-co-HV) polymers from a MMC using acetic acid as sole carbon source and a mixture of acetic and propionic acid. In general, the biopolymers recovery was enhanced when a pre-treatment using NaOCl was used to promote cell lysis.

Similar recovery efficiency of the copolymer P(HB-co-HV) were obtained when DMC and chloroform were used as extraction solvent (ca. 30 %). However, a low recovery performance resulted when PHB was extracted using DMC (11 %).

Nuclear magnetic resonance spectra of ^{1}H and ^{13}C were used to determine the composition of the polymers and in both cases, typical signals previously reported in the literature were observed. The molar ratio and the comonomer distribution of the copolymer were determined by ^{13}C. The content for the HB and the HV was found to be 58 % and 42 %, respectively and the randomness parameters D and R calculated, suggest that the P(HB-co-HV) is a "blocky" copolymer.

Nevertheless, the molecular weights calculated by gel permeation chromatography (GPC) were in the low range of some other previous results found in the literature (PHB $M_w = 4.53$ and P(HB-co-HV) $M_w = 3.49$, x 10^4 Da). It was assumed to be a consequence of the NaOCl pre-treatment; however, a more in-deep study is necessary to elucidate the effect of the extraction conditions over the PHA extraction from MMC.

The thermal properties were also analysed, and the results obtained for the degradation temperature (T_d), the melting temperature (T_m) and the crystallinity degree (X_c) were found to be very close to previous reports; confirming that the copolymer thermal properties are better that those of pure PHB.

7.2 Future work

In this thesis, each stage of a three-step PHA bioproduction process were assessed, however the performance of each phase was individually evaluated. One of the challenges is to integrate all the process for continuous PHA synthesis.

The acidogenic fermentation of several wastes was evaluated at lab-scale in batch experiments. Their bioconversion of these substrates into VFA must be evaluated at larger scale and also in continuous systems. Because the composition of the waste can vary according to the season in which it is discharged, an imperative objective must be to identify the key parameters that are crucial to carry out the AF towards a stable composition of VFA. Achieving a stable fermented waste will lead to the production of PHAs with stable composition, characteristics and properties regardless of whether the composition of the waste varies or not.

Another of the forthcoming directions to focus the following work, is the evaluation of the enrichment step and the accumulation step using a real acidified OMW. The effect of a real matrix of phenolic compounds over the process performance and accumulation capacity has to be well-determined.

Finally, the development of a cheap, efficient and environmentally friendly downstream process is needed. An extraction process with high recovery yields and without negative effects on PHA properties is desired and so, the effect of different extraction methods has to be investigated to find the optimal one. The large scale of the extraction method also represents a challenge that is essential for both, their use at industrial scale, and to obtain more material for further mechanical properties analysis. Such analysis needs bigger polymer quantities than those produced at lab-scale and must be statistically validated.