Strategies for production and purification of polyhydroxyalkanoates using mixed microbial consortia: Study on fermented crude glycerol as a substrate

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Polyhydroxyalkanoates (PHA) are natural polymers produced by bacteria as storage granules. They present a broad range of applications as biodegradable and renewable plastics, but their high production costs remain a major obstacle for a wider presence in the market.

The use of mixed microbial consortia (cultivated under non-sterile conditions) and second-generation feedstocks (waste streams) are strategies of interest to reduce costs, as well as life-cycle impacts. The main goal of this thesis was to advance the knowledge on PHA production from second-generation feedstocks using mixed microbial consortia. The study was based on crude glycerol as a feedstock, an abundant by-product from the biodiesel industry. Yet, the research provided insights beyond this substrate, regarding both the production and purification of PHA.
Title: Strategies for production and purification of polyhydroxyalkanoates using mixed microbial consortia. Study on fermented crude glycerol as a substrate

PhD thesis

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Date: February 2020

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Associate professor Anders E. Daugaard
Preface

This PhD dissertation is submitted to the Department of Chemical and Biochemical Engineering of the Technical University of Denmark (DTU) in partial fulfilment of the requirements for the degree of Doctor of Philosophy. All work has been performed at the Department of Chemical and Biochemical Engineering of DTU.

The PhD studies were supervised by Associate Professor Hariklia N. Gavala, and co-supervised by Associate Professors Ioannis V. Skiadas and Anders E. Daugaard. The research has been funded by the GRAIL project - Glycerol Biorefinery Approach for the Production of High Quality Products of Industrial Value (FP7 Grant Agreement no 613667 - European Commission) and the Department of Chemical and Biochemical Engineering at DTU (PhD fellowship).

Anna Burniol Figols
30th January 2020
Kgs. Lyngby
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Last but not the least, I would like to thank Brian. Thank you for your invaluable support and patience during this period.
Abstract

Strategies for production and purification of polyhydroxyalkanoates using mixed microbial consortia. Study on fermented crude glycerol as a substrate

Polyhydroxyalkanoates (PHA) are a group of natural polyesters synthesised as storage polymers in prokaryotic microorganisms, and with applications as biodegradable and biobased plastics. Nowadays, commercial production is based on first-generation feedstocks, and is associated with high production costs and limited sustainability benefits over conventional fossil-based plastics. One of the strategies to reduce operational demands is the use of mixed microbial consortia (MMC), which are cultivated under non-sterile conditions. Similarly, the use of second-generation feedstocks could lower the costs and life-cycle impacts related to the raw materials. The main goal of this thesis was to advance the knowledge on PHA production from second-generation feedstocks using MMC. The study was based on crude glycerol as a feedstock, an abundant by-product from the biodiesel industry with current limited market applications. Yet, the research provided insights beyond this substrate, regarding both the production and purification of PHA.

Crude glycerol can be directly converted to PHA in MMC. Nonetheless, it presents some limitations, such as reduced PHA yields derived from the co-production of other storage polymers (glycogen). The strategy of the project was to perform a fermentation of crude glycerol prior to its use as a substrate for PHA production. Hence, the conversion of fermentation products - volatile fatty acids (VFA) and 1,3-propanediol (1,3-PDO) - to PHA was investigated. The research showed for the first time in MMC, that 1,3-PDO could be converted to PHA. However, several limitations were identified, such as the relatively low yield of this conversion. Some of these limitations might be overcome with further research, but from the results obtained here, the use of 1,3-PDO and VFA did not show a clear advantage over a direct conversion of crude glycerol to PHA.

On the basis of these observations, an alternative approach was considered, where only the VFA fraction was converted to PHA, while 1,3-PDO was recovered as an additional high value product. This approach led to much higher PHA yields from the carbon consumed. Given that VFA represented a minor fraction of the fermentation products, the overall PHA yield from glycerol was not higher than by directly converting glycerol to PHA, but the combined process showed an overall higher carbon recovery to valuable products. This fact, together with the higher rates of the process, could make this approach an interesting alternative to the conversion of crude glycerol to only PHA. Some of the possible limitations are discussed in this dissertation.

The results above were obtained by investigating different culture enrichment strategies with distinctive selective pressures, which are the key to direct the metabolism to desired products when using MMC. In the case of PHA, this is generally achieved with repeated cycles of availability and absence of substrate, which favour microorganisms that can store the carbon in the form of PHA and use it during fasting periods. An additional selective pressure was investigated in this study, consisting in limiting the nitrogen during substrate availability. The application of the second strategy led to a net production of PHA from 1,3-PDO, while this substrate was mostly derived to growth under the first enrichment strategy. In a similar manner, selective conversion of VFA to PHA with 1,3-PDO recovery was attained by adaptation to the microbial community to only VFA. Such observations might be applicable for other substrates besides 1,3-PDO.
A major barrier during PHA production from second-generation substrates is their dilute carbon concentration, which leads to low values of productivity due to increased reactor volumes. In this PhD, membrane bioreactors were tested as a way to allow an exchange of bioreactor broth while keeping the cells in the bioreactor. More specifically the study evaluated immersed pressure-driven and diffusion-based membrane bioreactors (iMBRs and dMBRs). In the dMBR configuration, the membranes tested did not provide enough VFA diffusion to meet the substrate consumption of the culture. Possible research directions to increase substrate diffusion are suggested. On the other hand, iMBRs using hollow fibers and ceramic filters resulted in very high values of PHA productivity compared to current operations. The two iMBR filters offered similar results during fed-batch operation, but presented different limitations and advantages.

In relation to the PHA purification, ammonia digestion was investigated as a method to solubilise non-PHA cell material. Given the possibility to reuse ammonia as a nitrogen source for PHA production, this method had previously been recognised as an interesting alternative to present practices, which involve expensive solvents or generate large amounts of wastewater. However, earlier research showed high levels of PHA degradation. The results obtained here demonstrated that the outcome of the digestion is very dependent on digestion conditions (especially in regards to the temperature), and that high levels of PHA purity, PHA recovery and thermal stability can be obtained with this method.
Dansk sammenfatning

Strategier til produktion og oprensning af polyhydroxyalkanoater under anvendelse af mikrobiele blandingskulturer. Undersøgelse af fermenteret rå glycerol som et substrat

Polyhydroxyalkanoater (PHA) er en gruppe naturlige polyestere syntetiseret som depotpolymerer i prokaryotiske mikroorganismer og med anvendelser som bionedbrydelig og biobaseret plast. I dag er kommersiel produktion baseret på første generations råmateriale og er forbundet med høje produktionsomkostninger og begrænsede bæredygtighedsfordele i forhold til konventionel fossilbaseret plast. En af strategierne for at reducere de operationelle krav er brugen af blandet mikrobiel kulturer (MMC), der dyrkes under ikke-sterile forhold. Tilsvarende kan brugen af anden generation af råmateriale reducere omkostningerne og livscyklus-påvirkningerne i forbindelse med råmaterialene. Hovedmålet med denne afhandling var at fremme viden om PHA-produktion fra anden generation af råmateriale ved hjælp af MMC.

Undersøgelsen var baseret på rå glycerol som råstof og et rigeligt biprodukt fra biodieselindustrien med aktuelle begrænsede markedsanvendelser. Ikke desto mindre gav forskningen indsigt ud over dette substrat, hvad angår både produktion og oprensning af PHA.

Rå glycerol kan omdannes direkte til PHA i MMC. Imidlertid udviser det nogle begrænsninger, såsom reducerede PHA-udbytter, der stammer fra co-produktion af andre depotpolymerer (glycogen). Projekternes strategi var at udføre en gæring af rå glycerol inden det blev anvendet som et substrat til PHA-produktion. Derfor blev omdannelsen af fermenteringsprodukter - flygtige fedtsyrer (VFA) og 1,3-propanediol (1,3-PDO) - til PHA undersøgt. Forskningen viste for første gang i MMC, at 1,3-PDO kunne omdannes til PHA. Der blev imidlertid identificeret flere begrænsninger, såsom det relativt lave udbytte af denne omdannelse. Nogle af disse begrænsninger må kunne overvindes med yderligere forskning, men ud fra de opnåede resultater viste anvendelsen af 1,3-PDO og VFA ikke en klar fordel i forhold til direkte omdannelse af rå glycerol til PHA.

På baggrund af disse observationer blev en alternativ fremgangsmåde overvejet, hvor kun VFA-fraktionen blev konverteret til PHA, mens 1,3-PDO blev udvundet som et yderligere produkt med høj værdi. Denne tilgang førte til meget højere PHA-udbytter fra det forbrugte kulstof. I betragtning af at VFA repræsenterede en mindre fraktion af fermenteringsprodukterne, var det samlede PHA-udbytte fra glycerol ikke højere end ved direkte omdannelse af glycerol til PHA, men den kombinerede proces viste den samlet højere carbonudvinding til værdifulde produkter. Denne kendsgerning sammen med de højere hastigheder i processen kunne gøre denne fremgangsmåde til et interessant alternativ til omdannelse af rå glycerol til PHA. Nogle af de mulige begrænsninger diskuteres i denne afhandling.

Resultaterne ovenfor blev opnået ved at undersøge forskellige kulturberigelsessstrategier med distinkte selektive tyk, som er nøglen til at dirigere metabolismen til de ønskede produkter ved anvendelse af MMC. I tilfælde af PHA opnåes dette generelt med gentagne cykler med tilgængelighed og fravær af substrat, som favoriserer mikroorganismen, der kan opbevare kulstoffet i form af PHA og bruge det i faste perioder. Et yderligere selektivt tyk blev undersøgt i denne undersøgelse, der bestod i at begrænse nitrogenet under substratliglægnelage. Undersøgelsen af den anden strategi førte til en nettoproduktion af PHA fra 1,3-PDO, mens dette substrat stort set var afledt af vækst under den første berigelsessstrategi. På en lignende måde
blev selektiv omdannelse af VFA til PHA med 1,3-PDO-opsving opnået ved tilpasning den mikrobielle blandingskultur til kun VFA. Sådanne observationer kan være anvendelige for andre substrater udover 1,3-PDO.


**Abbreviations**

1,3-PDO: 1,3-propanediol  
3HB: 3-hydroxybutyrate  
3HP: 3-hydroxypropionate  
3HV: 3-hydroxyvalerate  
ADF: Aerobic dynamic feeding  
$C_{\text{mol}}$: Carbon mol  
COD: Chemical oxygen demand  
CDW: Cell dry weight  
CSTR: Continuous stirred tank reactor  
dMBR: Diffusion-based membrane bioreactor  
E3HB: Ethyl-3-hydroxybutyrate  
HRT: Hydraulic retention time  
iMBR: Pressure-driven immersed membrane bioreactor  
LCA: Life Cycle Assessment  
lcl-PHA: long-chain-length PHA  
MBR: Membrane Bioreactor  
mcl-PHA: medium-chain-length PHA  
MMC: Mixed microbial consortia  
$M_w$: Weight-average molar mass  
$N_{\text{mol}}$: Nitrogen mol  
NMR: Nuclear magnetic resonance  
NPCM: Non-PHA cell material  
P(HB-co-HV): Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)  
PE: Polyethylene  
PET: Polyethylene terephthalate  
PHA: Polyhydroxyalkanoates  
PHB: Poly(3-hydroxybutyrate)  
PHP: Poly(3-hydroxypropionate)  
PHV: Poly(3-hydroxyvalerate)  
PLA: Polylactic acid  
$pO_2$: Oxygen saturation (as % of maximum concentration in a liquid)  
PP: Polypropylene  
PSF: Polysulphone  
PVC: Polyvinyl chloride  
PVDF: Polyvinylidene fluoride  
$q_s$: Specific substrate consumption rate ($C_{\text{mol}} S / C_{\text{mol}} X / h$)  
$q_p$: Specific product formation rate ($C_{\text{mol}} PHA / C_{\text{mol}} X / h$)  
$r_s$: Substrate consumption rate ($C_{\text{mol}} S / h$)  
$r_p$: Product formation rate ($C_{\text{mol}} PHA / h$)  
S: Substrate  
SBR: Sequential batch reactor  
scl-PHA: short-chain-length PHA  
SRT: Solids retention time  
TCA cycle: tricarboxylic acid cycle  
TGA: Thermogravimetric analysis  
TSS: Total suspended solids  
VFA: Volatile fatty acids  
X: active biomass (cells excluding PHA)
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Part 1
Chapter 1: Motivation and scope

1.1. Motivation

Over the last century, fossil-based plastics have become an abundant and essential part of our society. But their exponential increase has also contributed to important environmental impacts, such as plastic pollution and CO₂ emissions. This has led to an interest in biobased and biodegradable plastics, which have the potential to mitigate these impacts, as well as to bring new applications. In this scenario, bacterial polyhydroxyalkanoates (PHA) have attracted considerable attention, given their high versatility, biodegradability and the possibility to produce them from multiple renewable sources. Despite PHA are already a commercial product, their market is still limited, which relies to a big extent, on their high production costs. Hence, strategies to reduce operational costs are needed, such as the use of non-sterile conditions and second-generation carbon sources, the increase of productivity and the improvement of the purification process. Such strategies are also expected to translate into reduced life-cycle impacts, which is key to achieve a supportive legal framework in the current European policy on plastics.

1.2. Objectives and scope

The ultimate objective of this PhD project was to contribute to the development of more efficient and viable strategies for PHA production and purification processes from second-generation feedstocks using mixed microbial consortia (MMC). The work was based on crude glycerol given the recent availability of this feedstock. Specifically, on crude glycerol derived from second-generation biodiesel production (slaughterhouse waste fats). Yet, many of the investigations are relevant beyond this substrate.

Previous literature on the topic has identified several limitations of the direct conversion of crude glycerol to PHA. One of the main goals of this thesis was to evaluate an alternative strategy, where crude glycerol would be fermented prior to its use as a substrate for PHA production in MMC. The fermentation step was not within the scope of this thesis, and was based on the results obtained by Varrone et al. (2018, 2017, 2015) within the frame of the same project (GRAIL). According to that work, 1,3-PDO (1,3-propanediol) was always the main fermentation product, a compound that had rarely been studied as a substrate for PHA production either in pure or mixed cultures. Thus, the study of the metabolism of this compound in MMC was one of the main objectives of this thesis. This was done in relation to the other compounds in the fermentation effluent: volatile fatty acids (VFA). The research focused on the conversion of all fermentation products (VFA and 1,3-PDO) to PHA (Chapter 3), or alternatively, on a selective conversion of only the VFA fraction, enabling a recovery of 1,3-PDO (Chapter 4).

Another goal of this PhD was to investigate strategies to increase the productivity of the PHA accumulation step, which is one of the main challenges for a cost-effective PHA production from second-generation
substrates. More specifically, this thesis focused on the role of nitrogen limitation or starvation and the use of cell-retention systems (Chapter 5).

Besides PHA production, PHA purification was also covered within this thesis (Chapter 6), given the important implications of this step on the sustainability and commercial viability of PHA. The scope was focused on the use of dilute ammonia to digest non-PHA cell material, and more specifically on the effect of some parameters (time, ammonia concentration and temperature) on the digestion effectiveness and the thermal stability of the resultant PHA.

The main objectives and specific objectives can be summarised as follows:

**Main objectives**

1) Assess the suitability of fermented crude glycerol as a substrate for PHA production in MMC.

2) Develop strategies to improve the PHA production and purification using MMC, applicable beyond crude glycerol.

**Specific objectives**

1) Evaluate 1,3-PDO as a possible substrate for PHA production, by the application of different MMC enrichment strategies based on nitrogen availability (Chapter 3).

2) Test the possibility of enriching MMC with selective consumption of VFA over 1,3-PDO, with the objective of recovering 1,3-PDO while converting VFA into PHA (Chapter 4).

3) Explore the use of cell-retention systems to increase the PHA productivity (Chapter 5).

4) Investigate dilute ammonia digestion as a method for PHA recovery and purification (Chapter 6).

**1.3. Thesis structure**

This dissertation is arranged in two parts.

Part II contains the scientific papers and manuscripts that describe in detail the research carried out during this PhD.

Part I is structured in seven chapters, and is meant to provide the reader with a more cohesive idea of how the different manuscripts contributed to the scope of the thesis, which is presented here (Chapter 1). A more detailed background on the motivation and scope of the thesis is provided in Chapter 2. Chapters 3 to 6 present the main findings within each specific objective, followed by a discussion on the significance of the results in relation to the main objectives of the thesis and possible future perspectives. These chapters are only a summary of the research performed within this PhD, and the reader is referred to the articles and manuscripts in Part II for a more complete description of the methodology and argumentation of results. The overall conclusions and outlook are presented in Chapter 7.
The articles and manuscripts enclosed in Part II of this dissertation are the following, referenced by their respective roman numbers throughout this document.


IV. **Burniol-Figols, A.** Pinelo, M., Skiadas, I. V. & Gavala, H. N. High polyhydroxyalkanoate productivity using cell-retention membrane bioreactors. (To be submitted).

Chapter 2: Introduction

2.1. Crude glycerol and the GRAIL project

Glycerol (or glycerine) is a commodity chemical with an exceptional range of uses due to its unique set of physical and chemical properties (Ayoub and Abdullah, 2012; Quispe et al., 2013). It is non-toxic, odourless, colourless hygroscopic, clear and easy to handle, what enables many applications in the food, pharmaceutical and cosmetic industry. Moreover, it contains three hydroxyl groups, which make it interesting as a platform chemical for catalysis, giving many products of interest for the chemical and polymer industries (Ayoub and Abdullah, 2012). Already in 1990, glycerol was quoted to have over 1500 uses (SDA, 1990). Until 2000, most of the glycerol was produced as a by-product from the fatty acids industry, or by chemical synthesis from propylene oxide (Figure 2-1). However, since the mid-2000s, the market has been inundated with glycerol generated as a by-product of the biodiesel industry (Ayoub and Abdullah, 2012). In 2018, the latter represented 62 % of the glycerol in the market (Vantage Oleochemicals, 2018).

The biodiesel market experienced an exponential increase in the last decades (Figure 2-2), mostly driven by political mandates to increase the biofuels percentage (Ayoub and Abdullah, 2012; Flach et al., 2019). In Europe, this coincided with a boom in the diesel sector in general pushed by political support, justified by the higher fuel efficiency and lower CO₂ emissions attributed to diesel engines at the time (Helmers et al., 2019). The EU is (and has been) the main producer of biodiesel, with a 38 % of the global production in 2017 (36 million tonnes worldwide) (UFOP, 2019a).

Biodiesel (fatty acid methyl esters) is produced by transesterification of vegetable oils or animal fats (triglycerides) with methanol in the presence of an acid or base catalyst (most commonly NaOH) (Figure 2-3). The main by-product of this process is glycerol, which is separated in a stream called crude glycerol. About 1 Kg of crude glycerol is produced for every 10 Kg of biodiesel (Ayoub and Abdullah, 2012). Glycerol corresponds to 60-88 % of this stream, which contains as well residual water (3-15 %), fatty acids (<1-5 %) and salts (5-
Methanol is usually recovered and reused within the biodiesel process, leaving about 1 % residual methanol. However, this is not always the case, and some crude glycerol streams can contain up to 20 % methanol (Quispe et al., 2013). Given the low glycerol purity of this stream, together with its dark colour and smell, crude glycerol has to undergo expensive purification steps to meet the requirements for most conventional uses of glycerol (Ayoub and Abdullah, 2012; Quispe et al., 2013).

The increased presence of glycerol in the market due to growth of the biodiesel industry was not accompanied with a corresponding growth in the demand, which led to a situation of market saturation and a dramatic decrease of market prices. This affected specially the price of crude glycerol, which decreased from 330 US$/tonne in 2001 to 44 US$/tonne in 2006 (Ayoub and Abdullah, 2012). Prices for refined glycerol also decreased from 600 to 350 US$/tonne from 2001 to 2006, what compromised the economic viability of crude glycerol purification (Ayoub and Abdullah, 2012). Since then, prices have remained low, but with significant fluctuations (ICIS, 2018; Quispe et al., 2013; Vantage Oleochemicals, 2018).

Low prices and market surplus justified the use of crude glycerol for low value applications, such as biogas production or heat production (Ayoub and Abdullah, 2012). Moreover, it incentivised the research for new high-value applications, which would be very desirable from the perspective of the biodiesel producers, whose viability is affected by the revenue obtained from crude glycerol (Ayoub and Abdullah, 2012; Plácido and Capareda, 2016; Quispe et al., 2013). This led to an exponential increase on the number of scientific publications on the topic during the last decade (Figure 2-4). Besides research on new uses for refined glycerol, and more efficient ways of obtaining it from crude glycerol, there has been an increasing interest in...
finding applications where crude glycerol can be utilised without any further purification (Quispe et al., 2013). Given the current interest on biotechnology and a biobased economy, a lot of research is focused on biological conversions to a wide range of products (e.g. lipids, biopolymers, 1,3-propanediol, citric acid, succinic acid, enzymes, ethanol, etc.) (Clomburg and Gonzalez, 2013; da Silva et al., 2009; Kumar et al., 2014; Plácido and Capareda, 2016; Yang et al., 2012).

In this framework, the EU granted financing to the GRAIL project (2013-2017), with the aim to develop a set of technologies converting glycerol derived from the biodiesel industry into high value products (European Comission and CORDIS, 2013). The work presented in this PhD thesis was part of this project, which besides polyhydroxyalkanoates (PHA), also investigated the production of 1,3-propanediol, fatty acid glycerol formal esters, hydrogen and ethanol, synthetic coatings, powder coating resins or cyanocobalamin (vitamin B12), among others.

![Number of scientific publications with the words "crude glycerol" from 1990 to 2019. Data from scopus.com.](image)

2.2. CONVENTIONAL PLASTICS

2.2.1. The plastic industry

The term _plastics_ refers to a wide group of materials, which can be easily moulded or shaped. In other words, they have a high plasticity. They are organic polymers of high molar mass, typically synthetic and made from natural gas or crude oil.

The plastic industry experienced an extensive development during the 20th century, enabling to produce materials with very versatile properties at a very low cost. Nowadays, plastics play a vital role in all sectors of economy, with virtually infinite applications. The main use of plastics corresponds to packaging (40% of the demand), followed by building and construction (20%) and automotive (10%) (Plastics Europe, 2019). About 400 million tonnes of plastics were produced in the world in 2015 (Geyer et al., 2017), a figure that has been growing exponentially since the 1950s (Figure 2-5) and is expected to quadruple by 2050 (World Economic Forum, 2016). The most common plastics produced nowadays are polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR) and polyethylene terephthalate (PET) (Figure 2-5). These five types of plastics together represent about 65% of the world plastics production (Plastics Europe, 2019).
2.2.2. Negative impacts of conventional plastics

It is unquestionable that plastics have contributed enormously to the development of modern societies. However, there are many concerns in regards to the environmental impacts and sustainability of plastics. Externalities related to plastics have been calculated to represent a cost of about $75 billion (UNEP, 2014). In Europe, this cost was estimated in $22 billion (UNEP, 2014), a number that is very close to the contribution of this sector to the public finances in the EU (30 billion €) (Plastics Europe, 2019). Some of the most important aspects are explained in the following paragraphs, namely: use of fossil resources and CO$_2$ emissions, chemical additives, waste management and plastic pollution.

Use of fossil resources and CO$_2$ emissions

Conventional plastics are predominantly made from crude oil and natural gas - non-renewable resources. It is estimated that about 6-8 % of the crude oil and natural gas are used for plastics production. This value is expected to increase to 20 % by 2050 due to the increased production of plastics, as well as the lower demand in the transportation section, which is currently the main consumer of fossil reserves (British Plastics Federation, 2019; World Economic Forum, 2016).

In terms of CO$_2$ emissions, a recent study estimated that the plastic sector was responsible for about 3.8 % of the global emissions in 2015 (Zheng and Suh, 2019). The highest impact of these emissions are generated during the manufacturing stage (60 %), followed by the conversion processes (30 %). End-of-life emissions

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corresponded to less than 10% of the total CO₂ emissions from the sector, although this percentage could increase considerably with the forecasted increases in the incineration rates (Zheng and Suh, 2019).

It is sometimes argued, that plastics contribute to a reduction of the CO₂ emissions and use of fossil resources (British Plastics Federation, 2019; Mülhaupt, 2013; Plastics Europe, 2019). One of the arguments for this position is that incinerating plastic waste reduces the use of other fossil fuels to generate energy. The validity of this argument is conditioned to the energy mix of the specific country and the incineration technology. Plastic incineration would only represent a reduction when compared to less efficient conversion technologies also employing fossil based sources (Eriksson and Finnveden, 2009). Another justification of this perspective is that the lightweight plastics used in planes, trucks or cars help reduce the CO₂ emissions related to costs. Likewise, plastic packaging can prevent food waste, and thus the concomitant environmental impacts (Plastics Europe, 2019).

Chemical additives

Plastics usually contain additives such as stabilisers, plasticisers, fillers, colorants, flame retardants, antimicrobial agents, etc. On average, plastics contain 7% additives by mass (Geyer et al., 2017). Many of these compounds have been identified as toxic, carcinogenic, mutagenic or endocrine disruptors, both for humans and wildlife (Galloway et al., 2019; Tyler et al., 2019). Some examples of concerning additives are bisphenol-A (BPA), phthalates, polybrominated diphenyl ethers (PBDEs) or heavy metals. All these compounds are not chemically bound, so they can leach out from the plastic structure, representing a risk for human exposure or for leakage into the environment. Plastic additives are commonly detected in freshwater, saltwater, soils and sediments (Avio et al., 2017; Tyler et al., 2019), but also in animals and human urine (Rochester and Bolden, 2015; Tyler et al., 2019).

Extensive research has been performed for compounds like BPA, resulting in a very strict regulation in most countries. However, the list of possible chemical additives in plastics contains thousands of compounds, and in many cases, little information is known about their chronic exposure effects, synergistic effects with other chemicals or fate in nature (Tyler et al., 2019). In many cases, these studies occur after their approval as chemical additives, which leads to cases like the Bisphenol-S (BPS). The use of this compound has increased over the last years, as a substitute of BPA in “BPA-free” products. Nonetheless, recent studies have shown that it presents similar endocrine-disrupting effects to BPA (Rochester and Bolden, 2015).

The waste management challenge

Among plastic applications, single-use plastics occupy a notorious percentage, especially within the packaging industry. A recent study estimated that in 2015 nearly 302 million tonnes of plastics ended its user phase, a close value to the amount of plastics produced the same year (407 million tonnes) (Geyer et al., 2017). Hence, the rate of plastic disposal is almost the same as the rate of plastic production, which poses a serious challenge to waste management systems worldwide.

At their end of life, some plastics can be recycled. The flows of recycled plastics have been increasing a lot in the past decades (Figure 2-6). Yet, in 2016, only 18% of the global plastic waste was recycled (32% in the
EU in 2019) (Plastics Europe, 2019). The intensification of recycling rates has recently become even more difficult, given that countries which used to process a lot of these streams (e.g. China) have now banned plastic waste imports (Parker, 2019).

Today, plastic recycling is almost exclusively done by mechanical recycling (Ragaert et al., 2017). The process presents varying complexity depending on the level of sorting and cleanliness of the original plastic mix, and its economic feasibility highly depends on the price of virgin crude oil (Hundertmark et al., 2019). Because of the added complexity, some plastics are simply not suitable for mechanical recycling. Moreover, due to degradation of polymer properties during the recycling process (and during the plastic lifetime), the number of times a plastic can be recycled is limited (Ragaert et al., 2017). Recycled plastics are of lower quality than virgin plastics and often contain non-intentionally added compounds, which limits market demand and applications (Geyer et al., 2017; Zero Waste Europe, 2019). For all these reasons, mechanical recycling has a limited capacity to solve the increasing challenge of plastic waste.

Besides mechanical recycling, chemical recycling strategies are emerging, including chemical depolymerisation, gasification or pyrolysis. These technologies aim at the recovery of plastic monomers or plastic building blocks (Ragaert et al., 2017; Zero Waste Europe, 2019). They are all still at low levels of maturity, and their full potential, limitations, economic profitability, and possible environmental and health impacts are still under evaluation (Ragaert et al., 2017; Zero Waste Europe, 2019). Some studies foresee that chemical recycling will be able to provide a very significant fraction of the feedstock for plastics in the future (Hundertmark et al., 2019). But even these optimistic reports still foresee virgin crude oil as the main feedstock for plastic production and a total plastic recycling rate of 50 % by 2050.

Another important waste management option for plastics is incineration, which enables energy production through combustion. About 24 % of the plastic waste was treated by incineration in 2016, a value that has been increasing in recent years (Figure 2-6). In the European Union, the value is higher, reaching a percentage of about 42 % in 2019 (Plastics Europe, 2019). This technology is in principle flexible enough to handle complex mixes of plastics or other substrates, and can effectively eliminate plastic waste. However, it results in fossil CO₂ emissions, which in many scenarios are higher than by landfilling plastics and producing energy elseway (Bernardo et al., 2016; Eriksson and Finnveden, 2009). Other air pollutants are also produced, such as carbon monoxide (a product of incomplete combustion), dioxins (from halogenated plastics like PVC), furans (aromatic hydrocarbons), heavy metals or particulate matter. All of them are serious concerns for human health (Mattiello et al., 2013). The level at which these compounds are generated and released, strongly
depends on the efficiency of the combustion process, as well as on the off-gas cleaning technologies and control of emissions. Studies on health risks of incineration plants before 2000 systematically reported increased risk of cancer in surrounding populations (Mattiello et al., 2013). Modern technologies and restrictive legislations have drastically reduced these emissions, and the risk for human health is in many studies found non-significant. (Mattiello et al., 2013; Ritchie and Max Roser, 2018). Nevertheless, there are still reports of state-of-the-art facilities exceeding permitted limits, or claims about the unsuitability of current monitoring and legislative measures (ToxicoWatch, 2018). Moreover, such technologies and legislations are not present in many parts of the world, where incineration can still represent a risk for human health (Ritchie and Max Roser, 2018). Hence, there is still controversy around incineration as a disposal option. Incineration enables only energy recovery, and is generally discouraged over other recycling options enabling material recovery (Innovation Fund Denmark and McKinsey & Company, 2019; Zero Waste Europe, 2019).

Despite increasing rates of plastics recycling and incineration in recent decades, landfilling is still the fate of about 60% of the plastics produced worldwide (Figure 2-6). This practice is considered a non-sustainable waste disposal option, as it represent a major loss of resources and land (European commission DG ENV, 2011).

**Plastics and microplastics pollution**

Plastics are designed to be durable and resistant to environmental conditions, but the same reasons that make them suitable for a wide range of applications, also make them highly recalcitrant to degradation in nature. Indeed, the vast majority of polymers currently commercialised are not biodegradable (or only under very specific conditions not occurring in nature), and will persist in nature for hundreds of years (Chin and Fung, 2019).

An important percentage of the plastic waste is inadequately disposed (e.g. in open landfills) or littered. In certain regions of the world, mainly corresponding to developing countries of south-east Asia and Africa, the percentages of mismanaged plastic waste are above 80% (Jambeck et al., 2015). The total estimate of plastic waste entering the marine environment in 2010 was between 4.8 and 12.7 million tonnes, corresponding to 1.7-4.6% of the global plastic waste (Figure 2-7, Jambeck et al., 2015). Independently of the accuracy of these complex estimations, the presence of massive plastic marine litter in the oceans and environment is undeniable. Once in the environment, they can cause several negative impacts in the economy (such as costs of cleaning or reduced yields in the fishery industry) (UNEP, 2014). Moreover, plastics convey important impacts for the ecosystems due to their physical structure (e.g. entanglement or effects in the digestion system), the release of chemical additives, or even as a hydrophobic carriers of other contaminants (Aldridge, 2015; Avio et al., 2017; Horton et al., 2017; UNEP, 2014; Wright and Kelly, 2017).

Despite the lack of significant biodegradation in nature, plastics are susceptible to fragmentation when exposed to UV radiation, high temperatures or other natural forces, and lead to small plastic debris, generally referred as microplastics (Horton et al., 2017). Microplastics in nature can also originate from direct release of microparticles (such as microbeads present in cosmetics and toothpastes (Horton et al., 2017)), tyre
deterioration, outdoor paints or synthetic fibers on clothing (Sundt et al., 2014). According to recent estimates, over 200,000 tons of microplastic particles are present in our oceans (Avio et al., 2017), representing a recognised pollutant in marine, terrestrial and freshwater environments. The presence of microplastics and associated chemicals in the human food chain has been widely proven, although the extent of their effects on ecosystems and human health are still largely unknown (Wright and Kelly, 2017).

2.3. **BIOBASED AND BIODEGRADABLE PLASTICS**

2.3.1. Definitions

Biobased plastics (made from biomass) and biodegradable plastics (that can be decomposed by microbial/biological activity) are often put together under the category of **Bioplastics** (European Bioplastics, 2019a). Hence, bioplastics can include materials that are biobased and not biodegradable, biobased and biodegradable, and biodegradable but not biobased (Figure 2-8). This term should be used with caution, as it contains materials with very different properties and offering different opportunities to tackle the impacts of conventional plastics.

The term **Biopolymers** is sometimes used to refer to biobased materials. However, according to the IUPAC, this terminology should be used only to refer to materials that are made by living organisms (Vert et al., 2012). Out of all bioplastics, only PHA, polysaccharide-based polymers (e.g. cellulose or starch), or protein-based plastics (e.g. soy protein films) should be considered as biopolymers, as they are the only ones produced by living organisms. The rest of bioplastics are synthetic despite the possibility of producing them from renewable carbon sources or being biodegradable.
2.3.2. The bioplastics market

The market of bioplastics is young, and in constant evolution. In 2019, the global production of bioplastics was 2.11 million tons (Figure 2-9 A), equivalent to only 1 % of the global plastic demand.

Within the category of biobased non-biodegradable plastics, the predominant materials are the so-called “drop-in” plastics such as bio-PE, bio-PET and bio-PA (Figure 2-9 B). These materials are identical to their petrochemical analogues, but made from renewable sources. For example, bio-PE is polymerised from ethylene produced by catalytical conversion of bio-ethanol. Drop-in polymers have a clear market advantage over new polymers, as they can be used, processed and recycled with the same technologies and equipment as their fossil-counterparts. It is important to note though, that not all biobased materials are 100 % biobased. For instance, current production of bio-PET is based on biobased ethanol, but fossil-based terephtalic acid. Thus, the product is approximately 30 % biobased.

Until 2017, bio-PET clearly dominated the market of bioplastics, representing over 30 % of the market (Figure 2-9 B). This polymer was forecasted to more than double its presence in the market by 2018 (Florence and Mechael, 2015), but instead, its estimated global production has been decreasing since 2017 to represent a 10 % of the bioplastics production in 2019. On the other hand, other drop-in polymers are rising, such as bio-PP and bio-PTT (Polytrimethylene terephthalate). Likewise, it is expected that PEF (Polyethylene furanoate) - a 100 % biobased alternative to PET - will be present in the market at a significant percentage in the upcoming years (Figure 2-9 B) (European Bioplastics, 2019c).
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Non-biodegradable plastics used to dominate the bioplastic market, but since 2018, biodegradable plastics have increased their presence, and now represent 55.5 % of the total (Figure 2-9 A). Within this category, starch blends are the major players (21.3 %) (Figure 2-9 C). They are followed by three kinds of biodegradable synthetic polyesters: PLA (polylactic acid) (13.9 %), PBAT (polybutylene adipate terephthalate) (13.4 %) and PBS (polybutylene succinate) (4.3 %). These polyesters can be produced from bio-based sources or from fossil derivatives. PHA represent a very small proportion of the market (1.2 %), with a total production of 26230 metric tons in 2019. This figure is expected to increase to about 160000 by 2024 (Figure 2-9 C).
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The main sector where bioplastics find applications is within the packaging industry (about 50% of the global production), but new applications are arising for both biodegradable and non-biodegradable polymers in many sectors (Figure 2-10).

2.3.3. Bioplastics as a solution to the plastics challenges?

Biodegradable plastics

By definition, biodegradable plastics can be decomposed by microorganisms. However, this term does not have implications about the biodegradation rate or the conditions needed for biodegradation to occur. In Europe, biodegradable materials can be certified and labelled as compostable according to the EN 13432/14995 standard, which establishes that the material has to disintegrate after 3 months, and a minimum of 90% mineralisation in 6 months. Moreover, they can cause no negative impacts on the composting process or the quality of the compost, measured by the presence of heavy metals and ecotoxicity tests on plant growth (European Bioplastics, 2019b). These evaluations are done under controlled conditions of humidity, temperature and nutrient availability, etc., replicating the conditions found in industrial composting facilities.

The fact that a material is certified as compostable is not a synonym of biodegradability in natural environments, which can present unfavourable conditions for microbial activity. The biodegradation rate is largely dependent on the specific environmental conditions (e.g. pH, temperature, abundance of microbes,
salinity, presence of oxygen), polymer type, plastic shape (e.g. films or pellets), degree of crystallinity, presence of additives, etc. (Emadian et al., 2017; Karamanlioglu et al., 2017; Lambert and Wagner, 2017). In terms of material types, PLA is widely reported to undergo very limited degradation in unfavourable conditions, in many cases comparable to conventional non-biodegradable plastics (Bátori et al., 2018; Greene, 2018; Karamanlioglu et al., 2017). On the other extreme, PHA has been repeatedly found to be highly degradable in several environments (both aerobic and anaerobic), with many examples showing complete disintegration in a few months (Bátori et al., 2018; Emadian et al., 2017; Eubeler et al., 2010; Greene, 2018, 2012; Wang et al., 2018).

An equivalent to the European standard for compostable plastics does not exist for degradation in natural environments. Some standards and test procedures have been proposed for aquatic environments and soils, but they have been considered insufficient to assess the rate of biodegradation in uncontrolled natural environments or to provide a guarantee of the lack of effects on the ecosystems during their biodegradation (Harrison et al., 2018). Most biodegradable plastics are regarded as biocompatible, meaning that they decompose in the human body without leading to immune responses (Brigham and Sinskey, 2012; Marques et al., 2002; Nair and Laurencin, 2007). However, some studies have reported (for example) negative effects of the ingestion of PLA particles in animal species (Avio et al., 2017; Green et al., 2016; Straub et al., 2017). For all these reasons, a specific regulation on biodegradable plastics is still missing (section 2.4).

Biodegradable plastics are often advertised as a remedy to plastic pollution. Such statements are discouraged from many public and private agencies (e.g. European Bioplastics, 2019b; European Comission, 2018), as biodegradable plastics include a wide variety of materials and products with different properties in terms of biodegradability. As discussed above, some of them are likely to present minimal or reduced impacts on marine and terrestrial pollution compared to conventional plastics if released to the environment. Nevertheless, there is a general fear that biodegradable plastics would increase littering rates if generally perceived as “safe for the environment” (e.g. Surfrider Foundation Europe et al., 2018).

In terms of chemicals impacts, it is important to ensure that biodegradable plastics do not contain harmful additives. This is unlikely given that biodegradable plastics have to pass ecotoxicity tests to be certified as compostable, but biodegradable should not be considered a synonym of safe (European Bioplastics, 2019b).

Biodegradable plastics can also offer some opportunities and challenges in regards to their end-of life treatment. Compostable plastics can be treated in industrial composting facilities. Moreover, some biodegradable plastics (such as PHA) can also be degraded anaerobically at sufficient rates to be considered as an additional carbon source during biogas production from organic municipal solid waste (Bátori et al., 2018). In the case of PHA, organic municipal solid waste could also be a substrate for PHA production (Korkakaki et al., 2016), leading to very interesting waste-production cycles. However, given that biobased plastics still represent a minor fraction of the plastic streams, sorting schemes and treatment infrastructures are not ready (Dilkes-Hoffman et al., 2019). Further development and consumer education is needed to ensure that biodegradable plastics are recycled and do not end-up in landfill or contaminating conventional plastic recycling streams (European Comission, 2018).
Biobased plastics

Biobased plastics are made from biomass, and they offer the opportunity of being carbon neutral. In other words, the CO₂ released during degradation is equivalent to the CO₂ captured during plant growth. For materials that are only partially biobased (as 30% biobased PET), this could offer a reduction in the CO₂ footprint.

However, when taking into account the manufacturing process, calculations of the total CO₂ emissions of biobased plastics are not always favourable to biobased plastics. As an example, Tsiropoulos et al. (2015) recently estimated the greenhouse emissions of bio-PE to be 140% lower than for conventional PE. On the other hand, bio-PET and conventional PET had a similar level of emissions. A recent review compared the results of global warming potential and non-renewable energy use of the major types of biobased and fossil-based plastics (Spierling et al., 2018). The compilation of data showed lower minimum emissions for biobased plastics (-0.3 vs. 1.6 CO₂-eq/Kg material), but also a higher maximum (11.9 vs. 6.4 CO₂-eq/Kg material). In terms of non-renewable energy use, biobased plastics presented a lower range (1.1-92 MJ/kg material) than fossil-based plastics (55.5-155.9 MJ/kg material). Similar conclusions have been reported in reviews looking specifically at PHA vs. fossil-based polymers (Narodoslawsky et al., 2015). Thus, biobased plastics have the potential to lower the global warming emissions and use of non-renewable sources, but the specific results are very much dependent on the context and considerations of the study (Narodoslawsky et al., 2015; Spierling et al., 2018). For example, Kho et al. (2010) reported that emissions from PHA carrier bags could be 69% higher or 80% lower than for PP bags depending on the energy mix considered during their production.

The raw material is another important factor affecting the results of LCA studies. In this regard, lower greenhouse gas emissions have been generally attributed to PHA produced from agricultural or industrial feedstocks compared to dedicated crops (Kendall, 2012; Kim and Dale, 2008; Narodoslawsky et al., 2015). Moreover, dedicated crops usually present higher effects in other impact categories such as acidification or eutrophication, largely derived from the use of pesticides, fertilisers and intensive agricultural practices (Narodoslawsky et al., 2015; Spierling et al., 2018).

The debate about the sustainability of biobased plastics is just an extension of the on-going controversy about the sustainability of biofuels. Biobased materials and fuels can present lower levels of greenhouse gas emissions, but they present negative environmental impacts in categories different to fossil derivatives (Bisinella et al., 2018; Spierling et al., 2018). Besides the already mentioned acidification and eutrophication, decreased biodiversity, soil erosion or water resource depletion are often reported. Moreover, there is a general concern about the possible competition with food crops, and the indirect effects that this might cause in food prices and CO₂ emissions, commonly referred as ILUC (Indirect Land Use Change) (Mohr and Raman, 2013). These impacts are mostly associated with the use of dedicated crops or first-generation feedstocks, which are currently dominating the market. The use of second-generation feedstocks, obtained from non-food biomass such as agricultural or industrial by-products, is still under development, but they are anticipated to minimise such impacts and improve the overall environmental performance of biofuels and biobased plastics (Mohr and Raman, 2013; Solomon, 2010).
Biobased materials are not intrinsically sustainable. Further advancements in terms of use of second-generation feedstocks, atom conversion efficiency, process productivity, minimisation of process wastes and energy consumption would put bioplastics (and PHA) into a more clear advantage over petrochemical plastics in regards to sustainability (Álvarez-Chávez et al., 2012; Narodoslawsky et al., 2015).

2.3.4. Current European policies

The European Commission released in January 2018 the European Strategy for Plastics in a Circular Economy (European Commission, 2018). The major emphasis of this plan is on advancing sorting and recycling technologies, increasing recycling rates, as well as on reducing plastic consumption and plastic littering. Both biobased and biodegradable plastics are mentioned in the document as interesting opportunities, but with certain scepticism. The use of “alternative feedstocks” is recognised as a possible way to reduce greenhouse emissions and dependency on fossil fuels, but the text acknowledges the need to prove their sustainability over conventional plastics. The only concrete measure, in relation to biobased plastics, is to increase research on their life-cycle impacts. In regards to biodegradable plastics, the document states that “innovations on applications where biodegradable plastics can represent advantages are welcomed”. Nonetheless, it also recognises risks, mostly related to inadequate certification and labelling schemes that can lead to consumer confusion, contamination of recycling streams, aggravated littering or leakage of plastics into the environment. In this regard, there is a commitment to create harmonised rules to define and label biodegradable plastics, and to develop assessments to identify the conditions under which biodegradable plastics can be beneficial. A similar approach has been adopted in the Danish strategy for plastics, released on December 2018 (Miljø og Fødevareministeriet, 2018).

Some organisations, such as the European Biopolymers association, have already claimed the EU plastics strategy to be insufficient (European Bioplastics, 2019a). This organisation has emplaced the EU to define a specific target of biobased materials and to define sustainability criteria for biobased plastics, similar to the current framework on biofuels defined in the Renewable Energy Directive (RED II - 2018/2001).

To date, the EU plastics strategy has only been translated into one European Directive, the Single-use plastics directive (2019/904). This document puts forward bans on single-use plastics where a clear alternative already exists (e.g. straws, plastic cutlery and plates), and suggests measures to reduce the presence of other items where a readily available alternative is still not in place. The directive affects as well biobased and biodegradable plastics, but sets an exception for “natural polymers that have not been chemically modified”. Thus, the directive would affect biodegradable polymers so far dominating the market, such as cellulose acetate, PLA or modified starch. Whether PHA are going to be exempt of prohibition seems to be a debatable issue that would certainly affect the future perspectives of this material in the market (Laird, 2019; Nova Institute, 2019).

In conclusion, the current legislation does not specifically incentivise biobased nor biodegradable plastics, although the scenario is likely to change considerably within the next decade. What seems clear is that both biobased and biodegradable plastics will need to prove clear advantages over conventional plastics to benefit
from any incentives or a positive legal framework. Hence, technological developments improving the sustainability of bioplastics are likely to be highly beneficial for their future market developments.

### 2.4. Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHA) are biobased and biodegradable polymers produced naturally by microorganisms. They are synthesised by bacteria and archaea in situations of unbalanced growth, such as lack of nutrients (nitrogen or phosphorous), and stored in the cytoplasm as carbon and energy storage granules (Anderson and Dawes, 1990; Dias et al., 2006; Kourmentza et al., 2017). In many cases, PHA can represent more than 80% of the dry weight of the cells (Chen, 2009a).

![Figure 2-11: PHA granules inside the cells. Reproduced from Higuchi-Takeuchi et al., 2016.](image)

PHA are polyesters of hydroxy fatty acids (Figure 2-12). The most common polymers are poly-3-hydroxybutyrate (PHB) and poly-3-hydroxyvalerate (PHV), although more than 150 types of monomers have been described, with different positions of the hydroxyl group or side chains (Dias et al., 2006; Steinbüchel and Valentin, 1995) (Figure 2-12). PHA can exist as homopolymers (formed by a single type of monomer) or as heteropolymers, formed with different types of monomers, such as the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) here abbreviated as P(HB-co-HV). According to its monomer composition, PHA can be classified into short-chain-length PHA (scl-PHA) with 3-5 carbons in the repeating units, medium-chain-length PHA (mcl-PHA) with 6-14 carbons in the monomers and long-chain-length PHA (lcl-PHA) with more than 14 carbons (Luengo et al., 2003).

The ability to produce PHA is widespread among bacteria and archaea, including extremophiles, anaerobic bacteria or photosynthetic bacteria (Koller et al., 2017; Luengo et al., 2003). However, a lot of the research and commercial production has been focused on *Cupriavidus necator* (previously *Ralstonia eutropha*), *Alcaligenes latus* and *Pseudomonas* strains, as well as recombinant *Escherichia coli* (Chen, 2009a; Dias et al., 2006; Kourmentza et al., 2017).

The first observation of PHA was in the early 20th century, but it was not until the late 1970s when the research started to intensify, due to the increase in oil prices, and the fact that PHA present properties similar to some fossil-derived polymers (Luengo et al., 2003). As a general trend, scl-PHA have similar properties to PE and PP, while mcl-PHA present properties more similar to elastomers (Laycock et al., 2014; Rai et al., 2011). Between these extremes, polymer properties largely depend on the monomer composition, what gives the possibility to modulate their properties and obtain very different materials (Bugnicourt et al., 2014; Laycock et al., 2014). For example, copolymers of P(HB-co-HV) are known to be less brittle and to have a lower melting temperature than pure PHB.
Given their diversity, PHA can have applications in many fields (Chen, 2009a). They are insoluble in water, and their oxygen and water barrier properties are close to that of PET, for what they are good candidates for the packaging sector (Bugnicourt et al., 2014). They also have applications in general plasticware products such as cutlery or catering articles (Ashter, 2016), or in the textile industry as biodegradable alternative to synthetic polyesters (MangoMaterials, 2017). Thanks to their high biodegradability, the agricultural and fishery sectors have also been attracted to the use of PHA, with products like biodegradable mulch films and pottery or fertiliser release systems (Malinconico, 2017). Another major field of applications is the biomedical industry, which has found endless uses for PHA thanks to their biocompatibility: drug delivery systems, implants, scaffolding for tissue and bone reparation, sutures, etc (Kundu et al., 2015; Rai et al., 2011). Besides polymer applications, PHA monomers (hydroxy acids) can be used as precursors or intermediates for many fine chemicals such as vitamins, antibiotics or pheromones (Gao et al., 2011). Additionally, monomers can be esterified with methanol to give hydroxyalkanoate methyl esters, which are a biofuel with similar properties to ethanol (Gao et al., 2011). New PHA applications are in continuous development, not only for natural polymers, but also through chemical modification (Chen, 2009a; Steinbüchel and Füchtenbusch, 1998).

Already in the 1980s, some companies started producing PHA at commercial scale (Chen, 2009a). Some of them are still in business, such as Biomer® (Germany), and many others have ceased or sold business. A sound example of that is the relatively recent sell of Metabolix®, one of the major players in the PHA industry since the early beginning (c&en, 2016). About a dozen manufacturers are currently active worldwide (Kourmentza et al., 2017; Levett et al., 2016), with an estimated total production of 26230 tonnes in 2019 (Figure 2-9) (section 2.3.2). Production is carried out using axenic cultures of natural bacteria or engineered strains, and using first generation feedstocks (Kourmentza et al., 2017; Levett et al., 2016). Yet, some companies are suggesting activities based on second generation feedstocks such as waste methane (Newlight Technologies LLC, USA) or other waste materials (Bio-on, Italy and VEnvirotech, Spain).
2.4.1. General challenges of PHA production

One of the current challenges preventing PHA to have a wider presence in the market are their high production costs. Market prices of PHA are between 1.5 and 6 €/kg (Alibaba.com; Chanprateep, 2010; Kourmentza et al., 2017; Levett et al., 2016; Metabolix, 2012), which is much higher than for petrochemical plastics (0.8 – 2 €/kg for PE, PP and PET) (Plastics Technology). This price difference certainly limits the adoption of PHA for low value applications. One of the strategies to reduce production costs is to avoid sterilisation by using mixed microbial consortia (MMC) or halophilic microorganisms instead of axenic cultures (Blunt et al., 2018; Chen, 2009b; Kourmentza et al., 2017; Reis et al., 2003). In the same line, advancements in production and purification strategies to increase productivity, reduce inputs of energy and chemicals are needed, especially for second-generation substrates (Blunt et al., 2018; Koller et al., 2017; Kourmentza et al., 2017). Besides reducing the costs, all these aspects would also improve the sustainability of the process, which would be of great advantage considering the current European policies (section 2.3.4). As exposed in Chapter 1, the scope of this thesis revolves around some of these aspects.

Another major limiting factor is the difficulty of processing PHA (Ashter, 2016; Chen, 2009b; Laycock et al., 2014; Niaounakis, 2015). For the most common types of PHA, degradation starts occurring close to the melting temperature, which is aggravated already at low levels of humidity. Thus, they allow a very narrow window of operation and require careful control of processing conditions (Ashter, 2016; Niaounakis, 2015). At the same time, PHA have low viscosity and strength at the molten state (which limits the range of processing techniques), and slow crystallisation rates (which can make them stick to metal during cooling from the melt) (Niaounakis, 2015). Crystallisation is also an issue for the mechanical properties of PHA, which usually suffer from ageing and brittleness (Laycock et al., 2014; Niaounakis, 2015). Improvements in the processability and mechanical properties have been achieved by optimisation of the operating conditions or the introduction of additives (Ashter, 2016; Niaounakis, 2015). Yet, further investigation is required to provide plastic converters with similar processing robustness to petrochemical plastics.

2.5. PHA production in mixed microbial consortia (MMC)

Sections 2.5, 2.5.2 and 2.5.3 have been adapted from paper I: Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H. N., & Reis, M. A. (2017). Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering, 4(2), 55.

This approach uses open mixed cultures and ecological selection principles, where bacteria are selected by the operational conditions imposed on the biological system. Thus, the principle is to engineer the ecosystem rather than the strains, combining the methodology of environmental biotechnology with the goals of industrial biotechnology (Kleerebezem and van Loosdrecht, 2007). As it operates under non-sterile conditions, this strategy brings a considerable simplification of the process requirements and costs (Gurieff and Lant, 2007; Reis et al., 2003). Moreover, due to the inherent diversity of these cultures, they present high adaptability to waste streams used as substrates or to operational changes (Kleerebezem and van Loosdrecht, 2007).
Mixed culture biotechnology has its roots in anaerobic biotechnology, but has expanded to other fermentation products, such as lactate, ethanol or butyric acid. In the latter case, the main challenge is to find operating conditions that achieve a selective pressure to the desired metabolite (Kleerebezem and van Loosdrecht, 2007). In the case of PHA, a selective pressure can successfully be achieved by different techniques, mostly based on application of dynamic or limiting growth conditions (section 2.5.2).

Processes for PHA production in mixed cultures are usually structured in two steps (Figure 2-13). In the first step, SBRs (sequential batch rectors) are used to select and maintain a population with high PHA production capacity by applying transient conditions. In the second step, the culture from the SBR is subjected to conditions maximizing the PHA accumulation, from where cells are harvested for PHA extraction and purification at the moment of maximum PHA content (Johnson et al., 2009; Serafim et al., 2008). The main characteristics and current state of art of these two steps are described in sections 2.5.2 and 2.5.3.

![Figure 2-13: PHA production process by mixed microbial cultures. CSTR: continuous stirred tank reactor, SBR: Sequential Batch reactor. Reproduced from paper I - Kourmentza et al., 2017.](image)

Unlike pure cultures, where sugars have been the predominant substrate for PHA production, volatile fatty acids (VFA) are preferred substrates for MMC. When carbohydrates are used directly as a substrate in MMC, they tend produce glycogen rather than PHA from carbohydrates (Dircks et al., 2001; Serafim et al., 2004) (section 2.5.1). Hence, a previous step is generally included (Figure 2-13), where sugars are fermented into VFA, generally in continuous mode (Albuquerque et al., 2007; Bengtsson et al., 2008; Serafim et al., 2008; Shen et al., 2014). This is also applied to complex substrates, such as olive mill waste (Dionisi et al., 2005) or food waste (Amulya et al., 2015), in order to obtain a more homogeneous and readily available feed for PHA production. By modifying operational conditions of the fermentation step, it is possible to alter the VFA distribution, which determines the PHA monomer composition and affects the biopolymer properties (Serafim et al., 2008).

### 2.5.1. Metabolic pathways and feedstocks

PHA production pathways in mixed cultures are generally assumed to be the same as described for pure cultures, although the mechanisms that trigger PHA production over growth might be different in both production strategies (Serafim et al., 2008) (section 2.5.2). Metabolic pathways vary among substrates and strains, but they always have as intermediates hydroxyacyl-CoA molecules, which polymerise to PHA. The precursors for hydroxyacyl-coA are acyl-coA molecules such as acetyl-coA, a key metabolite in the central metabolism of bacteria and intermediate from many metabolic pathways. Thus, PHA production has been
observed from many substrates, including VFA, carbohydrates, glycerol, fatty acids and even C1 substrates such as methane, CO₂ and syngas (Dürr and Eikmanns, 2015; Steinbüchel and Füchtenbusch, 1998; Tan et al., 2014). Acetyl-coA is also the branching point to biomass generation reactions and the citric acid cycle (TCA cycle) to produce ATP and reduced cofactors. This section describes PHA production from VFA and glycerol (which are the ones related to the scope of this dissertation), and focusing mostly on MMC.

2.5.1.1. Volatile fatty acids (VFA)

When using VFA as substrates, the pathway to PHA is relatively simple, as they are structurally related to the PHA monomers (Figure 2-14). The first step for all VFA is cellular uptake and activation by incorporation of a coenzyme A, which is generally considered to consume 2 mol ATP/mol of VFA (Pardelha, 2013). From this point, acetate forms acetyl-coA, which condenses with another molecule of acetyl-coA to form acetoacetyl-coA. The latter is reduced to 3-hydroxybutyryl-coA (at the expense of NADPH) and polymerised to PHB. A net consumption of ATP and NADPH occurs in the pathway from acetate to PHA. A parallel process occurs to propionate to produce PHP via propionyl-coA. Alternatively, propionyl-coA can also decarboxylate to acetyl-coA. If a molecule of acetyl-coA and propionyl-CoA condense, the hydroxyacyl monomer will be 3-hydroxyvaleryl-coA and polymerise into 3HV monomers (Lemos et al., 2006, 2003). Other combinations between acetyl and propionyl-coA molecules could also lead to the formation of other monomers: 3-hydroxy-2-methylbutyrate, 3-hydroxy-2-methylvalerate or 3HB (Lemos et al., 2006, 2003; Satoh et al., 1992). Conversion of propionate to PHA involves consumption of ATP, but can also produce reducing equivalents depending on the monomer composition.

Butyrate is assumed to enter the β-oxidation pathway, which eventually leads to acetyl-coA and PHB. However, a more direct conversion to 3-hydroxybutyryl-coA has also been described (indicated in dotted lines in Figure 2-14) (Yamane, 1993). This does not affect the overall redox balance, but would affect the NADPH/NADP and NADH/NAD pools, which are assumed to have an important role on the maximum theoretical yield from this compound (Grousseau et al., 2013; Shi et al., 1997; Yamane, 1993). Valerate would follow a similar pathway, but leading to the same distribution of monomers as described above for propionate (Lemos et al., 2006). Thus, as a general trend, VFA with odd number of carbons tend to give other monomers besides 3HB. An exception to this, is lactate, which results in PHB besides being a three-carbon acid, as its pathway goes through pyruvate and acetyl-coA (Jiang et al., 2011b).

Internally stored PHA can be cycled back to acetyl-coA to be used for respiration or growth in expense of reducing equivalents and ATP (Reis et al., 2003).
Among VFA, butyrate seems to be the preferred substrate for PHA production due to its high PHA yields, rates and achieved PHA content (Albuquerque et al., 2013; Marang et al., 2013; Shi et al., 1997). It has a maximum theoretical biochemical yield\(^2\) of \(0.98 \frac{C_{mol\text{ PHB}}}{C_{mol\text{ S}}}\) (carbon mol of PHB per carbon mol of substrate) (Yamane, 1993), considerably higher than the calculated for acetate and propionate (0.67-0.84 \(\frac{C_{mol\text{ PHB}}}{C_{mol\text{ S}}}\)) depending on culture conditions and monomer distribution (Dias et al., 2008)). One of the reasons is the lack of decarboxylation to its path to acetyl-CoA. Moreover, while other VFA need reducing equivalents in its path to PHA, butyrate has a net production of reduced cofactors. This enables the production of ATP to cover the needs for activation, which is already lower than in shorter VFA in terms of ATP per mol of carbon (Marang et al., 2013; Pardelha, 2013).

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\(^2\) The term *theoretical biochemical yield* is used during this thesis to refer to the maximum yield calculated from metabolic pathways occurring in microorganisms taking into account the reactions needed to produce energy and recycle cofactors. This can be culture dependent as it depends on specific pathways and the efficiency of the oxidative phosphorylation. The term *theoretical stoichiometric yield* is used to refer to yield calculated from the chemical stoichiometric equation for the formation of PHA from the compound (Yamane, 1993).
Chapter 2: Introduction

All of this translates into a lower need to derive carbon to the TCA cycle to generate ATP and reduced cofactors, and ultimately a higher carbon yield. Higher PHA yields and conversion rates have also been observed experimentally, with a maximum of 0.89 Cmol PHB/Cmol S (Albuquerque et al., 2013; Marang et al., 2013). Preferential uptake of butyrate over acetate and propionate has been observed in several cultures (Albuquerque et al., 2013; Dionisi et al., 2005; Jiang et al., 2012; Marang et al., 2013). A maximum PHA content of 88 % of the cell dry weight (% CDW) has been reported when using synthetic butyrate (Marang et al., 2013).

VFA have been extensively studied as substrates for PHA production in mixed cultures, with many examples of feedstocks submitted to fermentation to produce VFA as a substrate for the PHA production according to the scheme in Figure 2-13: food waste, sugar cane molasses, paper mill effluent, tomato cannery wastewater, brewery wastewater, municipal wastewater, cheese whey, etc. (Serafim et al., 2008; Valentino et al., 2016).

2.5.1.2. Glycerol

Glycerol can be transformed to dihydroxyacetone-phosphate and enter the glycolysis to give acetyl-coA and eventually PHB (Figure 2-15) (Zhu et al., 2013). The capability to convert glycerol to PHB has been observed in many microbial strains (Koller and Marsalek, 2015). PHB production has also been studied from crude glycerol, which commonly results in inhibition problems due to the presence of methanol, salts or fatty acids (Zhu et al., 2013).

In MMC, limited research has been conducted regarding the use of glycerol, but all the studies coincide in the observation of glycogen production besides PHA, lowering the PHB yield (Freches and Lemos, 2017; Moita et al., 2014; Moralejo-Gárate et al., 2011). This fact also occurs when sugars are fed to MMC, what has been attributed to glycerol being a more energetically efficient storage polymer than PHB and its faster kinetics of polymerisation and depolymerisation (Albuquerque et al., 2007; Dircks et al., 2001). In synthetic glycerol, Moralejo-Gárate et al., (2013b) suggested that glycogen is not energetically more favourable than PHB, but that glycogen could still be favoured due to its faster kinetics. Indeed, glycogen production over PHB was especially notorious in conditions promoting the selection of fast substrate uptake rates, such as low food to microbe ratios. In another study, the same authors reported a maximum PHB content of 80 % CDW, and a specific productivity of 0.36 Cmol PHB/Cmol X/h (Moralejo-Gárate et al., 2011). Despite glycogen production was observed, the authors reported a PHB yield of 0.57 Cmol PHB/Cmol glycerol (0.4 g PHB/g glycerol), close to its theoretical stoichiometric yield (0.67 Cmol PHB/Cmol glycerol - 0.47 g PHB/g glycerol) (Moralejo-Gárate et al., 2011). Both the yield and the specific productivity considerably surpassed values previously achieved with pure strains.

The study of Dobroth et al. (2011) was the first one attempting a conversion of crude glycerol to PHA in MMC. However, their cultures only metabolised methanol and did not consume glycerol. Later on Moita et al. (2014) reported a preferential use of glycerol over methanol, coinciding with previous observations in pure cultures (Zhu et al., 2013). According to this study, the crude glycerol matrix did not have a negative impact over the culture, with similar performance to synthetic glycerol. A final PHB content of 47 % CDW and a yield of 0.46 Cmol HB/Cmol glycerol were obtained. A similar yield (0.36-0.51 Cmol HB/Cmol glycerol), but a higher PHB content...
(59 % CDW) was attained after optimisation of the enrichment conditions (Freches and Lemos, 2017). But still, the specific productivity (0.085-0.164 Cmol HB/Cmol X/h), was lower than previously reported for VFA: up to 3.64 Cmol PHB/Cmol X/h in synthetic medium (Marang et al., 2013), and often in the range of 0.26-0.49 Cmol PHB/Cmol X/h in real fermented effluents (Albuquerque et al., 2011, 2010, 2007). To the extent of our knowledge, these are still the best results obtained with crude glycerol in MMC.

Besides the observation of lower yields and rates compared to VFA, and concomitant production of glycogen, direct production of PHA from glycerol has other limitations. One of them is that to date, no other short-chain PHA other than PHB has been reported when using glycerol as a sole carbon source in native strains (Zhu et al., 2013). This has implications, given that copolymers such as P(HB-co-HV) have more desirable properties compared to pure PHB (Kumar et al., 2014; Laycock et al., 2014; Zhu et al., 2013). Another common drawback reported in several studies is that glycerol-fed cultures tend to produce PHB of low molar mass, because the hydroxyl groups of glycerol can incorporate into the PHB molecule and act as chain terminators (Ashby et al., 2005; Zhu et al., 2013).

Figure 2-15: Schematic representation of the pathway leading to PHA production from glycerol and other carbohydrates, using glucose as example. Abbreviation 2[H]=NADH/NADPH/FADH2. Constructed from pathways described in Berg et al., 2002; Dircks et al., 2001; Moralejo-Garate et al., 2013a; Zhu et al., 2013.

**Fermented glycerol and 1,3-PDO**

A possible way to overcome the limitations described above for the direct conversion of glycerol to PHA would be to ferment glycerol to VFA, as commonly done for carbohydrate-like substrates (Figure 2-13). However, besides VFA, glycerol fermentation tends to produce 1,3-propanediol (1,3-PDO), generated as a way to balance the electron equivalents (Clomburg and Gonzalez, 2013) (Figure 2-16). Production of PHA from 1,3-PDO had never studied in MMC. This conversion had only been reported in recombinant bacteria (e.g. Meng et al., 2012) and in two defined strains producing a mixture of mcl-PHA and poly(3-hydroxybutyrate-co-3-
hydroxpropionate) (Kimura et al., 2002; Lee et al., 1995) (Burniol-Figols et al., 2018a), but no conversion yields had been reported.

The study of the conversion of 1,3-PDO to PHA in MMC was one of the objectives of this thesis, covered in Chapter 3.

![Glycerol fermentation pathways to VFA and 1,3-PDO. Abbreviation 2[H]=NADH/NADPH/FADH₂. Constructed from pathways reported in Barbirato et al., 1997; Clomburg and Gonzalez, 2013.](image)

**2.5.2. The enrichment step**


First observations of PHA production in mixed microbial consortia were in phosphate removal steps in waste water treatment plants, where aerobic and anaerobic steps alternate (Van Loosdrecht et al., 1997). In these systems, Polyphosphate Accumulating Organisms (PAOs) and Glycogen Accumulating Organisms (GAOs) were described to accumulate PHA during the anaerobic phase, where the electron acceptor becomes limiting. In the aerobic phase, these microbes consume the internally stored PHA using the available oxygen, obtaining a higher ATP yield than if the substrate was metabolised anaerobically (Dias et al., 2006; Reis et al., 2003). Nevertheless, as substrate activation and PHA formation during the anaerobic phase requires ATP and reducing equivalents, these microbes would also depend on the accumulation of glycogen or polyphosphate from the accumulated PHA during respiration, which limited the PHA yield of the cultures. Maximum PHA contents of around 20 % CDW had been described for these consortia when the aerobic dynamic feeding
enrichments (described in the following paragraph) appeared in the research scenario in the late 1990s with much higher PHA contents (Dias et al., 2006; Reis et al., 2003; Salehizadeh and Van Loosdrecht, 2004). Thus, research in this field has been rather limited. However, more recent developments with anaerobic/aerobic enrichments demonstrated that bacteria enriched in these cultures can also accumulate PHA aerobically, without depending on glycogen and phosphate reserves, and high PHA storage capacities (up to 60 % CDW) could be obtained (Bengtsson, 2009).

During the mid 1990s, a new strategy called aerobic dynamic feeding (ADF) was developed, where the limiting factor promoting the PHA accumulation was not the electron acceptor but the substrate availability (Reis et al., 2003; Serafim et al., 2008). These enrichments rely on repetitive feast-famine cycles (generally operated in SBR), where the cultures are supplied with excess carbon source, and then submitted to starvation, always under aerobic conditions (Figure 3-1 A). Bacteria that are able to store PHA from the substrate during the feast phase have a competitive advantage in these conditions, as they can utilise it as carbon and energy source during the famine phase, allowing them to overgrow non-PHA storing microorganisms (Albuquerque et al., 2007; Reis et al., 2003). Moreover, a limitation of internal factors such as RNA and enzymes required for growth seems to be playing a role in the selection of PHA accumulators (Dias et al., 2006). In order for cells to grow, a considerable amount of RNA and enzymes are needed, which might not be available after a period of starvation. Nevertheless, PHA synthesis enzymes are believed to be active both during PHA formation and degradation, generating a futile cycle that wastes ATP but enables the PHA machinery to be ready when a sudden addition of carbon occurs (Frigon et al., 2006; Ren et al., 2009). In this way, a new competitive advantage of PHA producers arises, given that PHA-accumulators have a higher responsiveness to substrate addition, and can use PHA as a buffer between substrate consumption and growth (Reis et al., 2003; Ren et al., 2009). PHA contents up to 90 % CDW have been reported using this strategy (Jiang et al., 2011b; Johnson et al., 2009).

Most of the research in the last 10 years has been based on ADF enrichments. Apart from proving the feasibility of the strategy in a variety of substrates, the main focus has been in evaluating the impact of different parameters on the enrichment. Namely, the hydraulic retention time (HRT), the solids retention time (SRT), the pH, the temperature, the nitrogen concentration, the dissolved oxygen concentration, the cycle length, the influent concentration, the feast/famine ratio or the food/microbe ratio, all of which are known to affect the process performance (Dias et al., 2006; Pardelha, 2013).

Among these factors, only the role of nitrogen is within the scope of this thesis (Chapter 1). The availability of nitrogen during the famine phase has been considered crucial for PHA-accumulating microorganisms to proliferate during the famine phase and overgrow non-accumulating bacteria (Albuquerque et al., 2007) (Figure 3-1 A). However, as described in the next section, nitrogen-limiting conditions are generally regarded to lead to higher PHA yields during PHA accumulation. This discrepancy, together with the incapacity of certain bacteria to accumulate PHA in the presence of nitrogen (Lee, 1996), inspired an alternative enrichment strategy consisting in limiting the nitrogen only during the feast phase (developed in Chapter 3).

Other types of selection strategies based on the transient presence of substrate or lack of external electron acceptors have been developed during recent years. For example, the enrichment of mixed photosynthetic
cultures (which avoid the use of aeration) (Fradinho et al., 2016, 2014, 2013a, 2013b) or halophilic mixed cultures (which could simplify the purification costs due to their tendency to lyse in distilled water) (Cui et al., 2016). Various authors have also explored the possibility of coupling the nitrification/denitrification process to PHA production schemes (Anterrieu et al., 2014; Basset et al., 2016). Moreover, some authors have suggested the elimination of a separate enrichment step as presented in Figure 2-13, as it has a considerable negative influence on the PHA productivity and costs of the process (Cavailé et al., 2016, 2013; Mengmeng et al., 2009). These and other enrichment strategies developed during recent years (which are not within the scope of the thesis) are described in more detail in Paper I included as attachment in this dissertation.

Regarding the process configuration, a continuous system was proposed by Albuquerque et al. (2010), where instead of an SBR with two phases, the feast and the famine phases were successfully operated in separate CSTRs (Continuous Stirred Tank Reactors).

2.5.3. The PHA accumulation step

As in the enrichment step, several operational parameters such as temperature (Johnson et al., 2010b) and pH play a role in PHA accumulation (Albuquerque et al., 2011; Chen et al., 2015; Villano et al., 2010). Nonetheless, the most critical aspect of the PHA accumulation is the feeding strategy. High substrate concentrations supplied in batch mode are generally regarded to limit the rate of the process, possibly due to an inhibitory effect of VFA (Albuquerque et al., 2007; Serafim et al., 2004). Pulsed fed-batches have given good results when applied using synthetic VFA as a substrates (Serafim et al., 2004), however it implies an increase in the working volume after each addition, which makes it feasible just when a very concentrated feed is available. This is rarely the case with fermentation effluents, which usually do not exceed 20 g COD/L (Valentino et al., 2016). When using such substrates, a discharge of the exhaust supernatant has been suggested as an alternative (Albuquerque et al., 2011; Chen et al., 2013; Pardelha et al., 2012). Nevertheless, this approach requires a settling step between pulses, which severely limits the productivity (Albuquerque et al., 2011).

Continuous feeding processes have shown until now the best results, given that they can attain a sustained productivity (Albuquerque et al., 2011; Chen et al., 2013; Jiang et al., 2012; Johnson et al., 2009). Control of the substrate addition has been successfully attained using the pH control (given the pH rise provoked by VFA consumption) (Albuquerque et al., 2011; Chen et al., 2013; Jiang et al., 2012; Johnson et al., 2009). Less successful results have been obtained when the substrate has been supplied based on previously observed substrate uptake rates, which resulted in either accumulation of substrate (Moita et al., 2014) or too low carbon in the system (Serafim et al., 2004). Alternatively, an on-demand continuous addition of substrate based on change in the dissolved oxygen has been proven efficient to maintain optimal amounts of carbon in the reactor (Valentino et al., 2015; Werker et al., 2011). High productivities (up to 1.2 g PHA/L/h) combined with high PHA yields (0.8 Cmol PHA/Cmol S) have been attained with continuous-feeding systems (Albuquerque et al., 2011). However, similarly to the pulsed-fed-batch, such figures were reported only when synthetic
substrates with high concentration were used in the feed. Much lower values (generally below 0.5 g/L/h) are reported in real substrates due to (among other factors) the dilution imposed with the feeding (Valentino et al., 2016).

Regarding the nutrient availability, nitrogen limitation or starvation is usually reported to improve the PHA yield and content (Albuquerque et al., 2007; Johnson et al., 2010a; Serafim et al., 2004; Venkateswar Reddy and Venkata Mohan, 2012). Nevertheless, some studies have observed no important role of the presence of nitrogen during the accumulation step, where PHA storage response was preferred over growth with nitrogen consumption, regardless of the nitrogen concentration (Jiang et al., 2012; Moralejo-Garate et al., 2013b). Marang et al. (2014) recently suggested that more than stimulating the accumulation in PHA bacteria, the main role of limiting the nitrogen was in preventing the growth of non-PHA-accumulating bacteria. Hence, different observations from different cultures do not imply contradictions but rather that the requirement for nitrogen limitation to obtain high PHA contents is largely dependent on the culture and the type of substrate. In terms of productivity though, a nutrient limitation rather than starvation showed to provide better results (Valentino et al., 2015). According to the discussion of the authors, complete nutrient starvation leads to a saturation of the PHA content of the cells, while only a limitation allows the cells to duplicate, which prolongs the PHA accumulation without enabling an excessive growth response. The best PHA productivities were obtained from limitation, but not starvation, of both nitrogen and phosphorous. The role of nitrogen limitation and starvation on PHA productivity was also covered in this thesis in the frame of PHA production in membrane bioreactors (Chapter 5).

PHA process brought to industrial scale using pure substrates and axenic cultures have broadly surpassed cell densities of 150 g/L with PHA contents of 80 % CDW and productivities in the range of 1 to 3 g PHA/L/h in synthetic media (Chen, 2009a). Despite much lower cell densities are usually attained in MMC (generally not surpassing 10 g/L), these cultures generally present higher specific substrate consumption rates due to the kinetic competition stablished during the enrichment. Comparable productivities (up to 1.2 g PHA/L/h) (Albuquerque et al., 2011) and PHA contents (up to 90 % CDW) (Jiang et al., 2011b) have already been attained from MMC using synthetic substrates.

The challenge of productivity and the use of membrane bioreactors (MBRs)

Values of volumetric PHA productivity obtained with second-generation feedstocks have rarely surpassed values of 1 g PHA/L/h, neither in pure cultures or MMC (Blunt et al., 2018; Koller et al., 2017; Valentino et al., 2016). Hence, there is a need for developing efficient cultivation techniques to produce PHA from these substrates. One of the main challenges is the low carbon concentration in such feedstocks, which causes volume increase during fed-batch cultivations (lenczak et al., 2013; Koller et al., 2017; Kourmentza et al., 2017). A way to circumvent this issue is the use of MBRs with cell-retention systems, which enable removal and addition of feed while keeping the cells in the bioreactor.
MBRs have been extensively applied in wastewater treatment (Judd, 2010) and increasingly in other biotechnological applications (Carstensen et al., 2012), but little research has been performed on the frame of PHA production. A few studies have explored the use of external loop MBRs in PHA production bioreactors with pure cultures (Ahn et al., 2001; Haas et al., 2017; lenczak et al., 2016; Schmidt et al., 2016). In such systems, the fermentation broth is recirculated through an external cross-flow filtration module, where part of the supernatant is removed (Figure 2-17). High PHA productivities were reported, up to 4.6 g PHA/L/h in Ahn et al. (2001). However, external loop MBRs rely on high cross-flow velocities to control membrane fouling, which result in high operational costs (Gander et al., 2000; Judd, 2010). Moreover, cell recirculation can lead to shear stress or oxygen limitation, resulting in cell death or reduced cell growth (Carstensen et al., 2012; de Andrade et al., 2014; Judd, 2010).

Operational costs are generally lower in immersed MBR configurations (iMBR), in which the filtration modules are submerged in the cell broth and aeration can provide fouling control (Carstensen et al., 2012; Gander et al., 2000) (Figure 2-17). To the extent of our knowledge, iMBRs have only been tested for PHA production in the study of Kumar et al., (2018), resulting in a 2 fold increase in the cell concentration. Yet, the productivity of the system was low (< 0.1 g PHA/L/h) due to the low PHA yield of the strain.

Both immersed and external loop MBRs are based on the application of a pressure differential as the driving force to achieve liquid permeation through a membrane. More recently, an alternative MBR strategy has been suggested, using concentration gradients as the driving force (Judd, 2010; Mahboubi et al., 2016) (Figure 2-17). In such systems, the microorganisms are separated from the feed medium by means of a membrane, which allows diffusion of the substrate to the cell compartment due to the difference of concentration. Experience on these systems is very limited, and so far only focused on soluble products and anaerobic processes (Mahboubi et al., 2016). Membrane fouling - the main challenge of MBRs - can still occur, but it is less likely, as the magnitude of the force bringing the cells and other foulants to the surface of the membrane is smaller.
The use of immersed pressure-driven and diffusion-based configurations during PHA accumulation in MMC was investigated in Chapter 5. Despite they are both immersed systems, the abbreviation iMBR is kept for conventional immersed pressure-driven MBRs, while dMBR is used to refer to diffusion-based MBR configurations.

2.6. PHA PURIFICATION

This section (2.6 and 2.6.1) has been adapted from manuscript V: Burniol-Figols, A., Skiadas, I. V., Daugaard, A. E. & Gavala, H. N. (2020) Polyhydroxyalkanoate (PHA) purification through dilute aqueous ammonia digestion at elevated temperatures. Journal of Chemical Technology & Biotechnology. ‘in press’.

After PHA production, the granules have to be extracted from the cells and purified from non-PHA cell material (NPCM), a step with significant impact on the overall costs and sustainability (Jacquel et al., 2008; Koller et al., 2013; Kourmentza et al., 2017). The methods studied so far for PHA recovery and purification can be divided into two main categories based on their basic approach:

- **Solubilisation of the PHA.** This approach uses solvents in order to break the cell membranes and solubilise the PHA granules. Pre-treatments or further purification steps are generally applied to eliminate other dissolved components.

- **Solubilisation of the NPCM.** With this approach, PHA remain as insoluble solids, while the non-PHA cell material (NPCM) is digested and solubilised.

Solubilisation of the PHA has been typically attained with carcinogenic chlorinated solvents like dichloromethane or chloroform. Subsequently, the polymer is precipitated by the addition of a non-solvent (e.g. methanol). Although lower efficiencies have been described for MMC compared to pure strains (Patel et al., 2009; Samori et al., 2015), these processes usually result in high PHA recovery and purity, while maintaining the integrity of the polymer. Nonetheless, these methods present obvious concerns in terms of safety and sustainability, and imply significant costs due to the large amounts of solvent and non-solvent utilised. Substantial efforts are therefore being directed towards finding non-hazardous and easily recyclable organic solvents (such as ionic liquids), as well as towards identification of other more environmentally friendly processes (Jacquel et al., 2008; Koller et al., 2013; Kourmentza et al., 2017).

Both enzymatic and chemical methods have been applied for the second approach aiming at disintegrating the NPCM. The main advantage of enzymatic methods is the mild conditions generally applied and the specificity of the enzymes, which prevent degradation of the PHA during the process. However, the high costs of enzymes are considered a limiting factor (Kourmentza et al., 2017). In the line of biological treatments, innovative approaches making use of bacteriophages or predatory bacteria to lyse bacterial cells and release PHA granules have recently been tested (Hand et al., 2016; Martínez et al., 2016). The use of such approaches would be risky in the case of non-sterile MMC processes, as these agents could easily infect the PHA production reactors.

The major advantage chemical digestion methods is the reduction of the processed volumes (compared to the use of solvents), as well as lower reagent costs. Several chemicals have been tested including alkalis (e.g.
NaOH, KOH or NH₃, acids (e.g. H₂SO₄) surfactants (e.g. sodium dodecyl sulfate (SDS)) and oxidising agents (e.g. NaOCl). High PHA purity and recovery (> 90 %) have been attained with some of these reagents (or combinations of them) both in pure strains and MMC (Gobi and Vadivelu, 2015; Heinrich et al., 2012; Jiang et al., 2015; López-Abelairas et al., 2015; Villano et al., 2014; Yu and Chen, 2006). Nevertheless, the ester bonds of PHA are susceptible to hydrolysis and PHA degradation (reduction of molar mass) is usually reported as a significant drawback of these processes (Heinrich et al., 2012; Jiang et al., 2015; López-Abelairas et al., 2015; Villano et al., 2014). In this sense, one of the most promising treatments so far is the use of dilute H₂SO₄ followed by NaOCl. Limited PHA degradation (around 50 % molar mass reduction) has been reported for this treatment, while achieving high PHA purity (up to 99 %) and a high recovery (up to 79 %) (López-Abelairas et al., 2015; Yu et al., 2005; Yu and Chen, 2006). Besides PHA degradation, another disadvantage of the chemical digestion methods is the generation of significant volumes of wastewater containing chemicals.

2.6.1. Dilute ammonia digestion

In regards to the wastewater generation, ammonia could bring important benefits to the process, given that NH₃ (and other nutrients derived from NPCM digestion) could be used as a nitrogen source during the PHA production steps (and as such would not be considered a waste stream) (Figure 2-18).

Ammonia digestion was first suggested in 1993 (Page and Cornish, 1993), when a PHA purity up to 94 % was reported with no decrease in the molar mass by using relatively high ammonia concentrations (1 N) at 45 °C. In addition, the authors proved that NH₃ could be successfully recycled as a nitrogen source for the PHA production. However, the strategy was only successful in strains with compromised cell membranes. Later on, a patent was published in which ammonia digestion at high concentrations (5-10 N) followed by NaOCl (4-6 %) washing reached 92 % PHA purity. No values of PHA recovery or polymer degradation were reported (Godbole et al., 2009). Despite these encouraging results, little research has been dedicated to this digestion method. Some studies included ammonia in initial screenings, which generally resulted in lower PHA recoveries and purities compared to other alkalis (such as NaOH and KOH), after which NH₃ was excluded from further experiments (Anis et al., 2012; Choi and Lee, 1999; Jiang et al., 2015; Mannina et al., 2019). To the
extent of our knowledge, only two studies have reported the molar mass of PHA after NH$_3$ digestion (Page and Cornish, 1993; Samori et al., 2015).

Up to date, there has not been a comprehensive evaluation of the potential of NH$_3$ digestion as a PHA purification method. There is lack of knowledge about the effect of digestion conditions on the PHA purity and polymer degradation. Likewise, there are no studies on the impact of NH$_3$ digestion on the thermal stability under different conditions.

One of the chapters of this study was dedicated to investigate ammonia digestion with a focus, not only on PHA purity and recovery, but also on the effects on the polymer integrity (reduction of molar mass and thermal stability), which are commonly overlooked in PHA recovery studies (Chapter 6).
This chapter is based on the following published article:


The contents have been adapted to provide a summary of the study and a discussion in the context of this thesis.

### 3.1. Background, Scope and Main Hypotheses

As presented in section 2.5.1.2, direct conversion of glycerol to PHA has some limitations, such as the production of glycogen besides PHA. The strategy defined by the project was to incorporate a previous fermentation step, where crude glycerol was transformed to VFA. As the latter are preferred substrates for PHA production, the hypothesis was that higher PHA yields could be obtained. Moreover, this would allow the incorporation of other monomers besides 3HB on the polymer structure. However, the work performed within the group on glycerol fermentation (Varrone et al., 2017, 2015), always led to 1,3-PDO as a main product of the fermentation. The main objective of this chapter was to evaluate the PHA production from a crude glycerol fermented effluent containing 1,3-PDO and VFA. Given that the conversion of 1,3-PDO to PHA had rarely been studied before (section 2.5.1.2) this compound was the main focus of the study.

Figure 3-1: Schematic representation of the hypothetical behaviour and selective pressure applied on cultures submitted to: A) An enrichment with nitrogen availability during the whole cycle. B) An enrichment where nitrogen is provided only during the famine phase (B).

The first hypothesis was that 1,3-PDO could be converted to PHA by a classical aerobic dynamic feeding enrichment strategy, where nitrogen is supplied during the whole enrichment cycle (Figure 3-1 A). As explained in section 2.5.2, the selective pressure for PHA accumulators in these enrichments is the transient presence of substrate, which results in a kinetic advantage for PHA accumulators, given their higher responsiveness when substrate is provided to the medium. Moreover, these bacteria can grow from the accumulated PHA during the famine phase.
Chapter 3: Conversion of 1,3-PDO to PHA

The second hypothesis was that 1,3-PDO could be converted to PHA when nitrogen limiting conditions are applied during the feast phase (Figure 3-1 B). Under these conditions, there would be an additional selective pressure for PHA accumulation, as growth is limited when substrate is available. Nitrogen provided during the famine phase, would allow growth from internally stored PHA.

### 3.2. EXPERIMENTAL OUTLINE

Two enrichment strategies based on aerobic dynamic feeding were evaluated (Figure 3-2). Both enrichment reactors (SBR) were fed with crude glycerol fermentation effluent containing 1,3-PDO (4.2 g/L), butyrate (1.7 g/L), propionate (0.8 g/L) and acetate (0.2 g/L).

In the first reactor (SBR_N), nitrogen was available during the whole cycle. In the second reactor (SBR_noN), nitrogen was present only during the famine phase. A separate PHA accumulation under nitrogen limiting conditions was performed for SBR_N. This was not done for SBR_noN, as nitrogen limitation was already present in the SBR.

![Figure 3-2: Schematic representation of the enrichment strategies tested for PHA production from 1,3-PDO and VFA. Reproduced from paper II - Burniol-Figols et al., 2018.](image)

To support the observations on the SBRs, the culture from these reactor was used in batch experiments with synthetic substrates (1,3-PDO or butyrate) in absence and presence of nitrogen.

Analysis of the microbial communities (by next generation 16SrRNA gene sequencing) were performed on the enriched cultures (SBR), as well as after the batch experiments on synthetic 1,3-PDO to investigate connections between the microbial population and the metabolic activities observed.

### 3.3. KEY FINDINGS

- In the conventional aerobic conventional aerobic dynamic feeding strategy with nitrogen availability during the whole cycle (SBR_N), no PHA production from 1,3-PDO was observed during the enrichment cycles. During PHA accumulation (in the absence of nitrogen), this community produced $0.56 \text{ Cmol PHA/Cmol S}$ when 1,3-PDO and VFA were being consumed, but no net PHA production when 1,3-PDO was the only substrate available (Figure 3-3 A).
Chapter 3: Conversion of 1,3-PDO to PHA

Figure 3-3: Substrate consumption and product formation during: (A) PHA accumulation in nitrogen limiting conditions with the inoculum from SBR_N; (B) a cycle in SBR_noN. Modified from paper II - Burniol-Figols et al., 2018a.

- When feeding this culture (SBR_N) with synthetic 1,3-PDO, PHA production was observed under nitrogen limiting conditions, but not when nitrogen was available (Figure 3-4). This fact proved the relevance of nitrogen for this conversion. Moreover, it evidenced that this enrichment was not carried out at conditions favouring PHA production from 1,3-PDO.

Figure 3-4: PHA accumulation experiments with synthetic substrates with the culture enriched with nitrogen during the feast phase (SBR_N). Modified from paper II - Burniol-Figols et al., 2018a.

- With the culture enriched without nitrogen during the feast phase (SBR_noN), 1,3-PDO conversion to PHA was observed in the fermented effluent, with an overall yield of 0.42 Cmol PHA/Cmol S (including both VFA and 1,3-PDO) (Figure 3-3 B). The yield when 1,3-PDO was the only substrate was 0.24 Cmol PHA/Cmol S, which was much lower than obtained during the VFA consumption phase (0.74 Cmol PHA/Cmol S). The PHA content was 23 % CDW, although maximum storage capacity of the cells was not evaluated.
• The change of enrichment strategy did not only change the physiology of the culture, but also led to important differences in the microbial community composition.

• Besides PHA, 1,3-PDO also led to the accumulation of glycogen (0.1 Cmol glycogen/Cmol S) (Figure 3-3 B). Moreover, indications of lipid accumulation were found. Thus, multiple storage responses seemed to be competing in the culture, similar to what occurs when MMC are fed with glycerol.

• 3-hydroxypropionate (3HP) was detected in the supernatant as intermediate of 1,3-PDO metabolism in the absence of nitrogen (Figure 3-3 B).

• Despite 1,3-PDO is a three-carbon substrate, and its metabolism goes through 3HP, 3HB was the main monomer in the PHA produced from 1,3-PDO, and only traces of 3HV and 3HP were detected. This fact limited the overall yield of 1,3-PDO to PHA, as there was a decarboxylation step to produce acetyl-coA (Figure 3-5).

• The molar mass of the obtained polymer was not measured, but NMR (Nuclear Magnetic Resonance) spectra did not show signs of 1,3-PDO incorporated into the PHA, which is the main reason for low molar mass polymers obtained from glycerol.

Figure 3-5: Schematic representation of the possible pathways involved in the conversion of 1,3-propanediol, butyrate and propionate into PHA, glycogen and other lipid inclusions. Reproduced from paper II - Burniol-Figols et al., 2018a. (Thomson et al., 2010)

3.4. Significance of the study and perspectives

The main contribution of this study was the fact that it showed for the first time, that 1,3-PDO could be converted to PHA in MMC, but only under certain enrichment conditions. Moreover, it was demonstrated that nitrogen limitation had a key role on the conversion.

3.4.1. On fermented crude glycerol as a substrate for PHA

The main hypothesis behind the application of a fermentation step on crude glycerol before PHA production, was that higher yields would be obtained compared to a direct conversion. The overall yield of fermentation products to PHA was 0.42 Cmol PHA/Cmol S, which would correspond to 0.36 Cmol PHA/Cmol glycerol when the
stoichiometry of the fermentation was taken into account. Based on these results, a previous fermentation step did not provide an advantage to the process in terms of the overall yield PHA/glycerol, as it is in the same range of maximum yields reported from a direct conversion of crude glycerol (0.36-0.51 Cmol PHB/Cmol S in Freches and Lemos (2017)). In terms of rates, our process presented a lower specific PHA production (0.06 Cmol PHA/Cmol X/h) than previously reported for crude glycerol (0.085-0.164 Cmol PHA/Cmol X/h) in Freches and Lemos (2017)), again not providing an advantage. Moreover, glycerol fermentation would imply an additional step on the process, increasing the overall costs. The only advantage of the use of fermented crude glycerol observed here, was the fact a copolymer of P(HB-co-HV) was formed, which is generally considered to have more interesting properties compared to PHB (Kumar et al., 2014; Laycock et al., 2014; Zhu et al., 2013).

The main reason behind the moderate yields obtained with fermented glycerol in this study was the fact that 1,3-PDO was the main component, and that low yields of PHB were attained when this compound was the only substrate left in the effluent (0.24 Cmol PHA/Cmol S). One of the factors limiting the carbon yield of PHA from 1,3-PDO was that, even though 3HP was identified as intermediate, it produced mostly PHB, losing two carbons for each 3HB monomer formed. Moreover, the yield was also limited by a concomitant production of glycogen, and possibly, lipid inclusions. Further research into these factors might provide a more optimistic scenario. In this regard, it would be important to elucidate the competition between PHA and other storage polymers, and study the operational parameters that maximise PHA production. Such parameters have been observed to have a very important influence on the carbon distribution between glycogen and PHA in other studies (Freches and Lemos, 2017; Moralejo-Garate et al., 2013b).

Another possible consideration to improve the yields from fermented crude glycerol would be to focus on the fermentation products. As mentioned in (section 2.5.1.1), butyrate is the VFA presenting the highest PHA yields (theoretical biochemical yield of 0.98 Cmol/Cmol) and conversion rates. However, the fermentation pathway from glycerol to butyrate has a decarboxylation step (Figure 2-16), which decreases the overall carbon yield PHA/glycerol to a theoretical stoichiometric yield of 0.67 Cmol PHB/Cmol glycerol, equivalent to the one of glycerol (section 2.5.1.2). Moreover, the production of butyrate from glycerol is not an electron balanced reaction, and requires the production of 1,3-PDO or other reduced alcohols unless additional electron acceptors are provided (Figure 2-16).

Among short-chain VFA, propionate is the only acid that would enable an electron-balanced fermentation from glycerol while maintaining the carbon atoms (Barbirato et al., 1997; Clomburg and Gonzalez, 2013). The theoretical stoichiometric yield of propionate to PHA is 0.67 Cmol 3HB/Cmol S and 0.83 Cmol 3HV/Cmol S. Hence, directing the fermentation to propionate could potentially increase the overall carbon yield compared to the direct conversion of glycerol to PHB, with the added value of producing copolymers. Nevertheless, this strategy should also overcome limited yields obtained experimentally with propionate (up to 0.43 Cmol/Cmol (Jiang et al., 2011a)), usually affected by a higher carbon flux to cell growth (Jiang et al., 2011a; Lemos et al., 2003).

Previous to the start of this project, only one study had reported the use of fermented crude glycerol for PHA production (Shen et al., 2014), who used a fermentation effluent with propionate as a main component. Propionate was mostly converted to PHV, with an overall yield of 0.25 g COD PHA/g COD glycerol.
Later on, Ntaikou et al. (2018) studied PHA conversion from crude glycerol, as well as from a fermented effluent containing hexanoate in addition to 1,3-PDO, butyrate, acetate and propionate. 1,3-PDO was not converted to PHA, most likely because the culture was not adapted to this substrate. The results on this fermented effluent showed lower yields than obtained here (0.15 g PHA/g COD consumed), but the PHA presented 3-hydroxyhexanoate monomers, which enhanced the mechanical properties of the polymer.

In conclusion, with current results, a fermentation step prior to conversion of crude glycerol to PHA would not have clear advantages over a direct conversion besides the incorporation of monomers other than 3HB into the PHA structure.

### 3.4.2. Beyond crude glycerol

The fact that 1,3-PDO was only produced in the enrichment with nitrogen limitation during the feast phase could have importance beyond the case of 1,3-PDO. The results showed that a selective pressure only based on transient presence of substrate might not be enough to produce PHA from certain substrates. In the present case the additional limitation of nitrogen led to the observation of PHA from 1,3-PDO in fermented crude glycerol, but it could also contribute to reduce the non-PHA-accumulating population in cultures where PHA is already observed. To the extent of our knowledge, such enrichment strategy had not been suggested before the start of the research, but later articles also reported an increase in the PHA yield from VFA when this strategy was applied (Oliveira et al., 2016).
Chapter 4: Selective conversion of VFA to PHA with 1,3-PDO recovery

This chapter is based on the following published article:


The contents have been adapted to provide a summary of the study and a discussion in the context of this thesis.

4.1. Background, scope and main hypotheses

1,3-PDO, the main product of glycerol fermentation in the current study, showed reduced PHA yields compared to VFA in Chapter 3. Given these results, and the fact that 1,3-PDO is a high value product per se, let to the suggestion of an alternative strategy, where only VFA were converted to PHA. With this strategy, the PHA yield per carbon consumed could increase.

To achieve this objective, the hypothesis was that by only providing VFA but not 1,3-PDO during the enrichment step, the culture would develop a selective consumption of VFA. In this way, only VFA would be converted to PHA during the accumulation step and 1,3-PDO would remain in the supernatant at a higher purity (Figure 4-1).

4.2. Experimental outline

An enrichment reactor (SBR) was established under aerobic dynamic feeding. The feed for the reactor was a synthetic medium of VFA (butyrate, propionate and acetate) and crude glycerol (added to adapt the culture to possible inhibitors). The culture enriched in these conditions was tested in a PHA accumulation with crude glycerol fermentation effluent (containing VFA and 1,3-PDO) in absence of nitrogen. PHA accumulation was tested at different effluent concentrations, to observe possible inhibition phenomena. The robustness of the process was studied in 12 batch accumulation processes, and by analysing the changes in the microbial community.
4.3. KEY FINDINGS

- Selective consumption of VFA over 1,3-PDO was attained by using a microbial culture enriched in the absence of 1,3-PDO. During PHA accumulation, VFA were converted to PHA and 1,3-PDO was not consumed (average recovery of 99%).

- No signs of inhibition (decrease of the specific substrate consumption or specific product formation) were observed when increasing the VFA and 1,3-PDO concentration to the one present in the fermentation effluent (Figure 4-2).

- The MMC presented preference for butyrate over acetate and propionate, which led to two distinctive phases during the PHA accumulation, clearly identified by the oxygen saturation profile (Figure 4-2). When butyrate was present in the medium, the yield was 0.91 Cmol PHA/Cmol S and the rate was 26.8 Cmol PHA/h. The yield decreased to 0.22 Cmol PHA/Cmol S during the acetate/propionate phase, reducing the overall yield to 0.81 Cmol PHA/Cmol S and the rate to 18.4 Cmol PHA/h. Given this considerable reduction of the process performance, it was considered appropriate to stop the process after butyrate depletion. At this point, 1,3-PDO would represent an 80 % of the COD in the supernatant, while the purity would increase up to 85 % if all the VFA were allowed to consume.

- In successive PHA accumulation batches, highly reproducible values were obtained in terms of PHA yield and maximum PHA concentration, with average values of 0.99 ± 0.07 Cmol PHA/Cmol S (0.84 g COD PHA/g COD S), 1.48 ± 0.14 g PHA/L and a PHA productivity of 0.41 g PHA/L/h (1.13 Cmol PHA/Cmol X/h). The PHA content was 76 ± 3.1 % CDW.

- The PHA obtained was a copolymer, P(HB-co-HV), with an average content of 16 wt% 3HV and a molar mass of 529 kg/mol, similar to other MMC based processes and a priori acceptable for thermoplastic applications.

- The SBR reactor presented a steady behaviour despite changes in the relative percentage of the main genera of the MMC: Thauera and Amaricoccus.

- Small variations in the substrate uptake rates and PHA formation rates during the PHA accumulation were observed. These changes were not caused by variations in the specific substrate consumption of the cells, but by the initial cell concentration, which presented changes due to differences in the settling behaviour of the culture. The reason for those changes could not be verified in the present
study, but did not correlate with the abundance of filamentous bacteria identified in the microbial analysis.

- Taking into account the fermentation and the PHA accumulation steps, 1 C mol of glycerol would produce 0.5 C mol of 1,3-PDO and 0.23 C mol PHA, resulting in an overall carbon recovery of 0.73 C mol/C mol.

- Taking into account the three steps of the process (crude glycerol fermentation, enrichment and PHA accumulation), the overall COD recovered as products was 0.61 g COD/g CODin, where 0.19 g were in the form of PHA and 0.42 g in the form of 1,3-PDO. The synthetic VFA included in the enrichment represented about 12% of the total COD fed to the process.

Figure 4-2: PHA accumulation experiments with crude glycerol fermentation effluent at increasing initial concentration of VFA. Each experiment corresponds to a column of graphs. *Undefined COD refers to the difference between the COD measured in the supernatant and the sum of theoretical COD of metabolites measured by HPLC. Reproduced from paper III • Burniol-Figols et al., 2018b.
4.4. Significance of the study and perspectives

The main contribution of this study was the demonstration of the possibility of selective conversion of VFA to PHA with recovery of 1,3-PDO in open mixed cultures.

A selective conversion of 1,3-PDO to PHA by a co-culture in axenic conditions was also reported in literature during the course of this work (Pan et al., 2019, 2016), leading as well to a complete recovery of 1,3-PDO, but lower PHA productivity (0.13 g PHA/L/h) and PHA content (44.5 % CDW) than obtained here.

4.4.1. On fermented crude glycerol as a substrate for PHA

The average PHA yield (0.99 Cmol PHA/Cmol S), PHA content (76 % CDW) and rate (1.13 Cmol PHA/Cmol X/h or 0.41 g PHA/L/h) obtained in this study during the PHA accumulation were in the very high range of results previously reported in the literature for PHA production in MMC (Valentino et al., 2016). These values were much higher than obtained when transforming all the fermentation products to PHA as described in Chapter 3 (0.42 Cmol PHA/Cmol S), and also much higher than obtained when transforming directly crude glycerol into PHA (0.36-0.51 Cmol PHB/Cmol S and a PHA content of 59 % CDW in Freches and Lemos (2017)). Thus, looking exclusively at the PHA accumulation step, this strategy would represent a more efficient process. Moreover, in contrast to the direct conversion of crude glycerol to PHB, it led to the production of P(HB-co-HV).

Considering as well the stoichiometry of the fermentation, this process would produce 0.50 Cmol 1,3-PDO and 0.23 Cmol PHA per Cmol glycerol, representing a total carbon recovery of 0.73 Cmol/Cmol glycerol. Hence, this strategy would produce significantly less PHA than by directly converting crude glycerol to PHB, but it would enable a higher carbon recovery into high value products (even considering the theoretical stoichiometric yield of glycerol - 0.67 Cmol PHB/Cmol glycerol). These results, combined with the abovementioned high PHA production rates, could make this strategy an interesting alternative to the conversion of crude glycerol to only PHA.

On the other hand, the consideration of two products instead of only PHA would also imply two purification processes (and possibly higher process costs), as well as a lower economy of scale than by producing only PHA. As described above, maximum PHA productivity was attained if the process was stopped after butyrate depletion. But different strategies could be formulated on an overall analysis, considering as well the costs of further 1,3-PDO purification. Further considerations regarding PHA production rates and 1,3-PDO concentration are discussed in Chapter 5.

Future development of the strategy should also consider avoiding the use of synthetic substrates in the enrichment reactor. Despite the input being minor compared to the total COD (12 %), its substitution would probably benefit the economics of the process. The use of other fermentation effluents containing only VFA or crude glycerol alone could be tested.
4.4.2. Beyond crude glycerol

The selective conversion of VFA to PHA, could have applications beyond the case of 1,3-PDO in crude glycerol fermentation. VFA are common by-products of biological conversion process (e.g. ethanol or butanol fermentations). The strategy developed here enabled to convert these by-products into a high-value polymer (PHA) that can easily be separated from the broth. Thus, it could contribute to alleviate the efforts in the recovery steps while producing an additional revenue.
Chapter 5: The use of Membrane Bioreactors to increase PHA productivity

This chapter is based on the following manuscript:

IV. Burniol-Figols, A., Pinelo, M., Skiadas, I. V. & Gavala, H. N. High polyhydroxyalkanoate productivity using cell-retention membrane bioreactors.

The contents have been adapted to provide a summary of the study and a discussion in the context of this thesis.

5.1. BACKGROUND, SCOPE AND MAIN HYPOTHESES

The results of Chapter 4 showed that it was possible to convert the VFA fraction of fermented crude glycerol to PHA at a high yield and specific productivity. PHA accumulation was performed in batch mode, where after about 3 hours, the substrate was exhausted. As the cycles of the SBR reactor producing inoculum were longer (12 h), this would result either in a situation of long inactive periods in the PHA accumulation reactor, or in the need of multiple SBR reactors to provide inoculum for the PHA accumulation. Given the low concentration of VFA in the fermentation effluent, providing more substrate to extend the PHA accumulation would have resulted in an important increase in the volume of the reactor (lower productivity), as well as a dilution of 1,3-PDO.

The main objective of the study was to provide a proof of concept of the utilisation of Membrane Bioreactors (MBRs) to overcome the volume limitations imposed by diluted feed solutions, such as fermented crude glycerol. The hypothesis was that the use of MBRs during PHA accumulation would allow to attain high PHA productivities. To verify this hypothesis, two conditions had to be met:

1) that the PHA-accumulating culture was able to sustain PHA production after cell saturation (observed at 3 h during batch cultivation in Chapter 4). The hypothesis was that this could be achieved by providing nitrogen limiting (but not starving) conditions, which would allow cell division, but without taking over the PHA-accumulation response. This hypothesis was based on previous results obtained in MMC (section 2.5.3).

2) that the MBR configuration was able to operate for the whole PHA accumulation and meet the requirements of the culture. Two immersed MBR configurations - pressure-driven MBR (iMBR) and diffusion-based MBR (dMBR) - were evaluated for this purpose.

5.2. EXPERIMENTAL OUTLINE

Given that the main objective was to provide a proof of concept of the utilisation of MBRs during PHA accumulation, the experiments were performed on a synthetic medium of VFA, avoiding possible
interferences from 1,3-PDO. The feed simulated the concentration of VFA obtained after optimisation of crude glycerol fermentation (Varrone et al., 2018), which was about 4 times higher than in the effluent used for the experiments in Chapter 3 and 4: butyrate (6.4 g/L), propionate (1.8 g/L), acetate (1.1 g/L) and lactate (0.75 g/L).

To evaluate the first point above (section 5.1), the effect of different nitrogen to carbon ratios (N/C) during fed-batch PHA accumulations was tested. As this was done without the use of MBR, a 10-times concentrated feed solution was used to overcome reactor volume limitations.

The second point above (section 5.1) was evaluated in different iMBR and dMBR setups, summarised in Table 5-1. For the dMBR system, VFA diffusion across different membranes was measured. In iMBR configurations, critical and limiting flows were determined at different cell concentrations expected during the fed-batch process.

Finally, the use of loop hollow fibers and ceramic filters was tested during a fed-batch PHA accumulation in synthetic VFA medium at the concentrations of the model effluent. In these experiments, about 1/5th of reactor supernatant was removed after butyrate exhaustion (manifested by an 8 % increase in the pO₂ saturation), and replaced with fresh medium.

Table 5-1: Summary of MBR configurations tested within the study

<table>
<thead>
<tr>
<th>Configuration</th>
<th>Driving force</th>
<th>Membrane</th>
<th>Variants</th>
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</thead>
<tbody>
<tr>
<td>Diffusion-based immersed MBR (dMBR)</td>
<td>Concentration gradient</td>
<td>Microfiltration membranes</td>
<td>PVDF (Polyvinylidene fluoride) - 0.15 and 0.45 µm</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>PSf (Polysulphone) - 0.2 µm</td>
</tr>
<tr>
<td>Pressure-driven immersed MBR (iMBR)</td>
<td>Pressure gradient</td>
<td>Hollow fibers</td>
<td>Rod configuration</td>
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<td></td>
<td></td>
<td></td>
<td>Loop configuration</td>
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<tr>
<td></td>
<td></td>
<td>Ceramic filters</td>
<td></td>
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</tbody>
</table>

Figure 5-1: A: Diffusion membrane support. B: Schematic representation of the diffusion membrane inside the bioreactor. Blue arrows indicate diffusion across the membrane. Reproduced from manuscript IV.
Chapter 5: The use of Membrane Bioreactors to increase PHA productivity

5.3. Key findings

- The N/C ratio present in the fermented effluent (1.8 N\text{mmol}/C\text{mmol} - nitrogen starvation) led to cell saturation and an important decrease in the PHA production rate after approximately 6 h of fed-batch. Higher N/C ratios allowed to sustain PHA production for a 24 h period, although a decrease of PHA productivity was observed for all cases, caused by a decrease in the specific substrate consumption. The best balance in terms of PHA content, cumulative PHA yield and PHA productivity was attained with an N/C ratio of 15 N\text{mmol}/C\text{mmol}, which represented a situation of nitrogen limiting (but not starving) conditions.

- In the dMBR configuration, substrate was provided in a cell-free compartment, separated from the cell culture by a microfiltration membrane. VFA diffusion across the membranes tested (PSf and PVDF) was not enough to meet the substrate requirements of the culture. More specifically, the VFA diffusion rates were about half of the substrate consumption rate of the culture. Hence, the designed setup would not have been able to provide a high PHA productivity.

- Comparing the different membranes PSf provided a higher VFA diffusion than PVDF at a similar pore size. The first was more hydrophilic and less negatively charged compared PVDF.
Within iMBRs, loop hollow fibers and ceramic filters resulted in a critical flow of 13 and 19 mL/min, respectively. These flows were considered high enough to provide a fast removal of exhaust broth (and replacement with fresh medium) during PHA accumulation. The rod hollow fibers configuration was more prone to fouling, likely justified by the compact structure of the fibers, and could not sustain flows over 10 mL/min.

During fed-batch PHA accumulation, ceramic filters and loop hollow fibers presented very similar results. They both allowed to circulate over 3.5 L of feed through the reactor, while keeping the working volume at 0.75 L in a 24 h fed-batch. This would correspond to an 82 % reduction on the working volume compared to a scenario where these experiments would be performed without a cell-retention system (Figure 5-4).

The use of submerged filtration during PHA accumulation resulted in high values of productivity (0.87-1.44 g PHA/L/h) (Figure 5-4). However, a decrease of PHA productivity was observed during the fed-batch, especially after 12 h. At this point, the productivity was 1.20 and 1.27 g PHA/L/h, for hollow fibers and ceramic filters, respectively. At 24 h, the values decreased to 0.89 and 0.87 g PHA/L/h, respectively.
Despite the two iMBR systems presented very similar results in terms of productivity, several advantages and disadvantages were identified. Hollow fibers allowed to operate at higher flows, due to their lower ratio of surface/footprint. Nonetheless, they presented a risk of cell deposition, resulting in a decreased metabolic activity and PHA production rate. Ceramic filters were less prone to cell deposition and fouling, but they had a higher footprint, leading to a lower critical flow with the surface available in the bioreactor.

### 5.4. Significance of the study and perspectives

#### 5.4.1. Beyond crude glycerol

The main objective of this study was to perform a proof of concept of the utilisation of MBR to increase the productivity during PHA accumulation in MMC. In this sense, iMBR configurations (loop hollow fibers and ceramic filters) proved valuable to overcome the limitations of dilute feeding solutions, which are common in most second-generation substrates used for PHA production. These systems reached PHA productivities between 0.87 and 1.44 g PHA/L/h (depending on the cultivation time considered). To the extent of our knowledge, the highest productivity achieved in MMC corresponds to 1.2 g PHA/L/h, reported for a continuous-fed system with synthetic VFA in Albuquerque et al. (2011). The latter value could not be maintained for more than 6 h despite a relatively concentrated feed solution used in that study (approximately 2.5 times higher than used here). In real fermentation effluents with concentrations of substrate similar to the ones use here, values of productivity are generally below 0.5 g/L/h (Valentino et al., 2016). In the present study, a productivity of 1.27 g PHA/L/h could be obtained even after 12 h, showing the great potential of the use of MBRs to increase the PHA productivity.

Besides being valuable for fed-batch cultivations, MBRs could also be an asset in PHA production in continuous mode, as they could offer the possibility of uncoupling the hydraulic retention time from the cell-retention time and cell growth.

Experiments in real fermented effluents should be performed to fully understand the potential and limitations of this strategy during PHA accumulation, as these feedstocks are likely to increase membrane fouling. Moreover, the fouling behaviour should be studied during successive fed-batch processes in order to determine sustainable fluxes (referring to the ones enabling a balance between productivity and costs of membrane maintenance). In this respect, it would be interesting to consider the integration of membrane cleaning and PHA purification. In recent years, PHA purification methods based on the use of chemicals to digest and solubilise non-PHA material have gained interest. Such processes involve the same type of chemicals used for membrane cleaning (alkalis, acids or oxidizing agents) (section 2.6 and Chapter 6). Therefore, integration of both process could be envisioned. In this regard, ceramic membranes would offer an additional advantage, given their higher resistance to chemicals and robustness (Carstensen et al., 2012).

Diffusion-based setups did not provide a sufficient diffusion to be considered for a high PHA productivity process. Nonetheless, the strategy could still be valid if employing membranes enabling higher VFA diffusion
rates. Based on the results obtained here, a possible direction to achieve this would be to study positively charged or neutral hydrophilic membranes, as they would minimise charge repulsion with ionised VFA.

5.4.2. On fermented crude glycerol as a substrate for PHA

The experiments in this study were performed on a synthetic VFA mixture simulating the VFA concentration in fermented crude glycerol. The results obtained showed that the use of MBRs enabled a high productivity (1.2-1.27 g/L/h) for periods matching the duration of the cycle length in the SBR (12 h). Moreover, this value represented a threefold increase compared to the productivity obtained in batch mode (0.41 g/L/h) in Chapter 4. The PHA concentration in the reactor increased from 1.5 g PHA/L to 14 g PHA/L.

PHA accumulation could be extended for up to 24 h, which would represent a reduction in the inoculum need and consequently the volume of the SBR reactor. Hence, although lower productivities were observed in the PHA accumulation reactor after 24 h (0.9 g/L/h), an overall process analysis might provide a different perspective on the choice of the fed-batch duration.

As mentioned above, the use of MBRs should be further evaluated in real fermentation effluents to assess the effect of the matrix on the fouling behaviour. But for the case of fermented crude glycerol, an additional factor could be influencing the behaviour: the presence of 1,3-PDO. iMBRs enabled a sustained fed-batch process for up to 24 h, corresponding to about 24 feedings. Given that 1,3-PDO is not consumed by the culture, this would imply a progressive build-up of the concentration of 1,3-PDO in the reactor, eventually reaching the concentration in the fermented effluent, which was 21.8 g/L in the model effluent used during this study (Figure 5-5). Concentrations of 1,3-PDO up to 4.5 g/L did not represent inhibitory effects for the culture in Chapter 4. However, preliminary studies performed by others in this culture (Moukanakis, 2019) showed that concentrations of 10 g/L of 1,3-PDO already derived in a reduction of about 30% in the initial substrate consumption and PHA accumulation rate. Hence, the effects of increasing 1,3-PDO concentrations in extended PHA accumulation batches should be evaluated.

On the other hand, the increase in 1,3-PDO concentration derived from the use of MBRs would be an important advantage when considering the feasibility of 1,3-PDO purification in the frame of a combined production of 1,3-PDO and PHA. The concentration of 1,3-PDO in the exhaust broth collected after successive feedings would increase with the duration of the batch (Figure 5-5). This fact, together with the discussion above, provided indications that the optimisation of 1,3-PDO concentration and PHA production would not go in parallel, and that a combined process might require a balance between these two parameters.
Figure 5-5: 1,3-Propanediol (1,3-PDO) expected within the bioreactor and in the accumulated collected exhaust broth vs. time of fed-batch operation.
Chapter 6: PHA purification through ammonia digestion

This chapter is based on the following accepted manuscript:


The contents have been adapted to provide a summary of the study and a discussion in the context of this thesis.

6.1. BACKGROUND, SCOPE AND MAIN HYPOTHESES

The main objective of this chapter was to provide a comprehensive evaluation of the potential of NH₃ digestion as a PHA purification method, introduced in section 2.6. The focus was not only set on the PHA purity and recovery, but also on the effects on the polymer integrity (reduction of molar mass and thermal stability).

The main hypothesis was that high PHA purity and recovery could be achieved by studying the effect of digestion parameters (time, ammonia concentration and temperature). At the same time, the high volatility of ammonia could lead to polymers with high thermal stability.

6.2. EXPERIMENTAL OUTLINE

Ammonia digestion was studied on a freeze-dried cell culture with P(HB-co-HV) content of 64 wt%, here referred as crude PHA. After digestion, PHA was recovered as an insoluble product in the pellet, while impurities (NPCM) and PHA degraded into soluble oligomers and monomers would be transferred to the supernatant.

The digestion parameters included in the study were incubation time (30-60 min), NH₃ concentration (0-1 M) and temperature (30 - 140 °C). Moreover, sonication was evaluated as a pre-treatment. The outcome of these experiments was mainly evaluated in terms of PHA purity, PHA recovery and molar mass \( (M_w) \) on the solids recovered after digestion. The polymer recovered with the best conditions from this study was compared in terms of thermal stability with PHA obtained through other suggested methods for PHA purification (by thermogravimetric analyses (TGA) and molar mass reduction during melting).

The mechanisms behind the effect of temperature on ammonia digestion were further investigated by: I) quantification of PHA monomers lost in the supernatant (3HB); II) evaluating how the equilibrium constants were affecting the hydrolysis rate at different temperatures with a simple ester (ethyl 3-hydroxybutyrate (E3HB)); and III) studying possible conformational changes occurring on PHA with different treatment conditions.
6.3. **Key Findings**

- No increase in the purity (around 64 %) and low PHA recovery (from 65 to 80 %) were obtained at mild temperature conditions, with little changes upon increased incubation time or ammonia concentration.

- Treatments performed between 75 °C and 140 °C led to an increase in the PHA recovery (up to 90 %) and increased PHA purity (up to 83 %) (Figure 6-1). However, elevated temperatures also led to a decrease in the molar mass of the polymer, which was especially detrimental above 115 °C. At these temperatures, the outcome of the digestion was highly sensitive to changes in the digestion time.

A pre-treatment by sonication proved valuable to increase the PHA purity and maintain the PHA recovery at temperatures not leading to severe reduction of the molar mass. With a treatment at 115 °C (0.2 M NH₃ 30 min) with previous sonication, the values of PHA purity and recovery were 86 % and 92 %, respectively. The polymer presented a molar mass of 200 kg/mol.

PHA samples purified through ammonia digestion with PHA purities over 68 %, presented a maximum temperature of decomposition comparable to chloroform-extracted PHA (about 300 °C). Conversely, PHA purified through NaOH or combined H₂SO₄ and NaOCl, presented a temperature of decomposition equal or below the starting material before digestion (270 °C), despite presenting higher values of purity (up to 98 %).

PHA samples with purities above 70 % obtained after ammonia digestion presented a low molar mass reduction during melting at 170 °C, comparable to the observed for chloroform-extracted PHA.
 Conversely, PHA purified through NaOH or combined H\textsubscript{2}SO\textsubscript{4} and NaOCl, led to severe reduction of the molar mass and polymers below 100 Kg/mol.

Figure 6-2: Molar mass ($M_w$) change during melting of the purified PHA (5 min 170 °C). White bars indicate the $M_w$ after purification (before melting), and grey bars represent the $M_w$ after melting. Reproduced from paper V - Burniol-Figols et al., 2020.

- Reduction in molar mass and PHA loss presented opposite trends with the temperature, with an inflection point at 45 °C (Figure 6-3).

- Reduction of the molar mass increased at high temperatures, and correlated with the concentration of crotonic acid in the supernatant. This trend had been previously described to occur in PHA, and to be caused by a higher rate of chain scission reactions at high temperatures, occurring typically in the middle of the chains.

- PHA loss correlated with the concentration of 3HB monomers (product of PHB hydrolysis) in the supernatant. Thus, PHA loss was associated with hydrolysis reactions, leading to soluble monomers and oligomers. The rate of these reactions decreased with temperature, which was contradictory with the general trend of hydrolysis reactions increasing at elevated temperatures. Given that the same trend did not occur in simple esters (E3HB), the lower rates of hydrolysis at elevated temperatures were hypothesised to occur due to conformational changes in the polymer. This hypothesis was also based on the observation of a secondary glass transition of the polymer at about 60 °C, which could be associated with the glass transition of the rigid amorphous fraction typically occurring on PHA after drying. Hence, incubating PHA above 60 °C, could lead to a mobilisation of the rigid amorphous fraction and an increase in the crystallinity of PHA, making the polymer more resistant to hydrolysis.
6.4. SIGNIFICANCE OF THE STUDY AND PERSPECTIVES

6.4.1. On fermented crude glycerol as a substrate for PHA

The results obtained in this manuscript were obtained with a PHA-accumulating culture fed with fermented crude glycerol, however the strategy could have equal potential with any other substrate. Thus, the main contributions of this study are explained in the following paragraph.

6.4.2. Beyond crude glycerol

Most of the previous studies had only tested NH₃ digestion in a screening phase, and discarded the method due to lower initial PHA purity and recovery compared to other alkalis (such as NaOH, KOH or NH₃-laurate) (Anis et al., 2012; Choi and Lee, 1999; Jiang et al., 2015; Mannina et al., 2019). Nonetheless, the whole picture of the results obtained here and reported previously, suggested that NH₃ digestion efficiency largely depends on the digestion conditions (and possibly on the PHA-accumulating culture), and that adjustment of the parameters can lead to relatively pure PHA and high PHA recovery.

Additionally, the study showed for the first time that NH₃ digestion can result in higher thermal stability than achieved using other digestion methods, even with PHA of lower purity. These observations are of crucial importance for polymer applications.

The results showed that a high degree of thermal stability was obtained with PHA purities above 70 % (obtained at temperatures between 75 °C and 115 °C). Higher purities and thermal stability could be achieved at treatments above 75 °C and including a sonication pre-treatment, but as a trade-off, they led to higher loss of molar mass and would be expected to present higher energy costs. Thus, further optimisation should take into consideration the required purity level and molar mass, which were seen to be very sensitive to operational conditions at elevated temperatures.

Based on previous literature on the effect of molar mass on the mechanical properties of P(3HB-co-3HV), a threshold of 112 kg/mol was established as a limit for acceptable molar mass (Kanesawa and Doi, 1990). However, mechanical properties vary according to the monomer composition and the polymer purity. Thus, a
limit of acceptable molar mass should be established with a characterisation of a range of mechanical parameters, as well as taking the life-span degradation of the polymer into account.

Moreover, it would be crucial to study the digestion on fresh cells (not freeze-dried). This step was included here to not introduce a bias on results obtained on different experiments, but freeze-drying has a high influence on the crystallinity of the polymer, and could be expected to have a strong effect on the outcome of ammonia digestion.
One of the main objectives of this thesis was to assess the suitability of fermented crude glycerol as a substrate for polyhydroxyalkanoates (PHA) production in mixed microbial consortia (MMC). This was studied in a fermentation effluent where 1,3-propanediol (1,3-PDO) represented about 60 wt% of the fermentation products, followed by volatile fatty acids (VFA): butyrate, propionate and acetate. The results demonstrated for the first time that 1,3-PDO could be transformed to PHA in MMC. However, the PHA yields obtained from 1,3-PDO were low (0.24 C mol PHA/C mol S) and led to an overall PHA yield of 0.36 C mol PHA/C mol glycerol, which was in the range of previously reported by direct conversion of crude glycerol to PHA (0.36-0.51 C mol PHA/C mol glycerol). Hence, on the basis of the results obtained here, a fermentation step of crude glycerol prior to PHA conversion did not provide a clear advantage, although further research into the mechanisms behind 1,3-PDO metabolism towards cell growth and storage polymers might provide more positive perspectives.

An approach where only VFA were transformed to PHA, while 1,3-PDO was recovered as an additional product, led to much higher PHA yields from the VFA fraction (up to 0.99 C mol PHA/C mol VFA). Compared to a direct conversion of crude glycerol to PHA, this strategy produced significantly less PHA (0.23 C mol PHA/C mol glycerol), but enabled a full recovery of 1,3-PDO (0.5 C mol 1,3-PDO/C mol glycerol), resulting in a higher carbon recovery to valuable products (0.73 C mol/C mol glycerol). As an additional advantage, it led to PHA with 3-hydroxybutyrate and 3-hydroxyvalerate monomers. Thus, this combined process to 1,3-PDO and PHA could be considered as an alternative to the conversion of glycerol to only PHA. The productivity (0.41 g PHA/L/h) was already high in batch mode, but would have the potential to increase even more (to 0.87-1.44 g PHA/L/h) with the use of membrane bioreactors (developed as well within this PhD). The research provided some indications that 1,3-PDO concentration and purity could compromise PHA productivity and vice versa. Hence, further process development should have both products into consideration.

Besides the study of fermented crude glycerol as a substrate, this PhD also aimed at developing strategies to improve the PHA production and purification using MMC applicable beyond this substrate. In regards to the PHA enrichment step, the limitation of nitrogen during the feast phase in aerobic dynamic feeding enrichments proved valuable to obtain microbial communities able to produce PHA from substrates (in here 1,3-PDO) that were mostly utilised for growth when nitrogen was not limited. Also in the enrichment step, it was shown that it was possible to develop cultures with targeted substrate selectivity (in here VFA over 1,3-PDO). Both cases illustrate how the metabolic activity of MMC can be modulated by means of the operational conditions of the enrichment.

In the PHA accumulation step, the use of cell-retention systems proved useful to overcome one of the main limitations of second-generation substrates: their low carbon concentration. Immersed pressure-driven membrane bioreactors (IMBRs) allowed to perform repeated substrate additions without increasing the reactor volume. Their use led to high values of PHA productivity (0.87-1.27 g/L/h) during extended fed-batch
processes (12-24 h) using a dilute synthetic VFA feed, which represented a considerable improvement of current MMC operations (generally not exceeding 0.5 g PHA/L/h). These systems should be tested in real fermentation effluents and complemented with techno-economic analyses to further understand their potential in the frame of PHA production.

In the purification step, this PhD provided a detailed study of the use of ammonia digestion as a method for PHA purification. The results allowed to conclude that the effectiveness of this method is highly dependent on digestion conditions (especially in regards to the temperature) and that a relatively high PHA purity and high PHA recovery could be obtained at elevated temperatures (75 °C - 115 °C), particularly when combined with a sonication pre-treatment. Additionally, the method could lead to polymers with higher thermal stability than obtained with previously suggested digestion chemicals (e.g. H$_2$SO$_4$). Overall, the study provided the grounds for further optimisation of the method, which should consider the required purity level and molar mass, as well as the use of fresh cultures.
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Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production

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Abstract: Sustainable biofuels, biomaterials, and fine chemicals production is a critical matter that research teams around the globe are focusing on nowadays. Polyhydroxyalkanoates represent one of the biomaterials of the future due to their physicochemical properties, biodegradability, and biocompatibility. Designing efficient and economic bioprocesses, combined with the respective social and environmental benefits, has brought together scientists from different backgrounds highlighting the multidisciplinary character of such a venture. In the current review, challenges and opportunities regarding polyhydroxyalkanoate production are presented and discussed, covering key steps of their overall production process by applying pure and mixed culture biotechnology, from raw bioprocess development to downstream processing.

Keywords: polyhydroxyalkanoates; biopolymers; renewable feedstock; mixed microbial consortia; enrichment strategy; pure cultures; synthetic biology; downstream processing

1. Introduction

Polyhydroxyalkanoates (PHAs) are a class of renewable, biodegradable, and bio-based polymers, in the form of polyesters. Together with polylactic acid (PLA) and polybutylene succinate (PBS), they are considered the green polymers of the future since they are expected to gradually substitute conventional plastics with similar physicochemical, thermal, and mechanical properties such as polypropylene (PP) and low-density polyethylene (LDPE) [1,2]. While PLA and PBS are produced upon polymerization of lactic and succinic acid respectively, PHA polymerization is performed naturally by bacteria.

A wide variety of bacteria are able to accumulate PHAs in the form of intracellular granules, as carbon and energy reserves. PHA accumulation is usually promoted when an essential nutrient for growth is present in limited amount in the cultivation medium, whereas carbon is in excess. Although, several bacteria are able to produce PHAs during growth and do not require growth-limiting conditions. This carbon storage is used by bacteria as an alternate source of fatty acids, metabolized under stress conditions, and is the key mechanism for their survival [3]. Up to 150 different PHA structures have been identified so far [4]. In general, PHAs are classified into two
groups according to the carbon atoms that comprise their monomeric unit. Short-chain-length PHAs (scl-PHAs) consist of 3–5 carbon atoms, whereas medium-chain-length PHAs (mcl-PHAs) consist of 6–14 carbon atoms. PHB, the most well-known scl-PHA member, is characterized as a stiff and brittle material and is difficult to be processed due to its crystalline nature. The incorporation of 3-hydroxyvalerate (HV) units in PHB, results in the production of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), or else PHBV. PHBV is a material that becomes tougher, more flexible, and broader to thermal processing when its molar fraction in the copolymer increases [5]. scl-PHAs are mostly used for the production of disposable items and food packaging materials. On the other hand, mcl-PHAs are characterized as elastomers and they are suitable for high value-added application, such as surgical sutures, implants, biodegradable matrices for drug delivery, etc. [4].

Table 1. Pilot and industrial scale PHA manufacturers currently active worldwide

<table>
<thead>
<tr>
<th>Name of Company</th>
<th>Product (Trademark)</th>
<th>Substrate</th>
<th>Biocatalyst</th>
<th>Production Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomatera, Canada</td>
<td>PHA resins (Biomatera)</td>
<td>Renewable raw materials</td>
<td>Non-pathogenic, non-transgenic bacteria isolated from soil</td>
<td></td>
</tr>
<tr>
<td>Biomer, Germany</td>
<td>PHB pellets (Biomer®)</td>
<td>Sugar (sucrose)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bio-On Srl., Italy</td>
<td>PHB, PHBV spheres (minerv®-PHA)</td>
<td>Sugar beets</td>
<td>Cupriavidus necator</td>
<td>10,000 t/a</td>
</tr>
<tr>
<td>BluePHA, China</td>
<td>PHB, PHBV HHx, PHV, P3HP3HB, P3HP4HB, P4HB synthesis</td>
<td>Cold pressed canola oil</td>
<td>Development of microbial strains via synthetic biology</td>
<td></td>
</tr>
<tr>
<td>Danimer Scientific, USA</td>
<td>mcl-PHA (Nodax® PHA)</td>
<td>Cold pressed canola oil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaneka Corporation, Japan</td>
<td>PHB-PHHx (AONILEX®)</td>
<td>Plant oils</td>
<td>Newlight’s 9X biocatalyst</td>
<td>3500 t/a</td>
</tr>
<tr>
<td>Newlight Technologies LLC, USA</td>
<td>PHA resins (AirCarbon™)</td>
<td>Oxygen from air and carbon from captured methane emissions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHB Industrial S.A., Brazil</td>
<td>PHB, PHBV (BIOCYCLE® PHA)</td>
<td>Saccharose</td>
<td>Alcaligenes sp.</td>
<td>3000 t/a</td>
</tr>
<tr>
<td>PolyFerm, Canada</td>
<td>mcl-PHA (VersaMer™ PHA)</td>
<td>Sugars, vegetable oils</td>
<td>Naturally selected microorganisms</td>
<td></td>
</tr>
<tr>
<td>Shenzhen Ecomann Biotechnology Co. Ltd., China</td>
<td>PHA pellets, resins, microbeads (AmBio®)</td>
<td>Sugar or glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIRIM Bioplastics Pilot Plant, Malaysia</td>
<td>Various types of PHA</td>
<td>Palm oil mill effluent (POME), crude palm kernel oil</td>
<td></td>
<td>2000 t/a</td>
</tr>
<tr>
<td>TianAn Biologic Materials Co. Ltd., China</td>
<td>PHB, PHBV (ENMAT™)</td>
<td>Dextrose deriving from corn of cassava grown in China</td>
<td>Ralstonia eutropha</td>
<td>10,000 t/a, 50,000 t/a by 2020</td>
</tr>
<tr>
<td>Tianjin GreenBio Material Co., China</td>
<td>P (3, 4HB) films, pellets/foam pellets (Sogreen®)</td>
<td>Sugar</td>
<td></td>
<td>10,000 t/a</td>
</tr>
</tbody>
</table>

PHB, P3HB: poly(3-hydroxybutyrate); PHBV: poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PHBVHHX: poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate); PHV: poly(3-hydroxyvalerate); P3HP3HB: poly(3-hydroxypropionate-co-3-hydroxybutyrate); P3HP4HB: poly(3-hydroxypropionate-co-4-hydroxybutyrate); P3HP: poly(3-hydroxypropionate); P4HB: poly(4-hydroxybutyrate); mcl-PHA: medium chain length PHA; P(3,4HB): poly(3-hydroxybutyrate-co-4-hydroxybutyrate).

Taking into account the recalcitrance of conventional plastics in the environment, replacement of synthetic plastics with PHAs would have huge benefits for the society and the environment [6]. Wide commercialization and industrialization of PHAs is still struggling due to their high production cost, resulting in higher prices compared to conventional polymers. While the price of polymers such as PP and PE is around US$ 0.60–0.87/lb, PHA biopolymer cost is estimated to be 3–4 times higher, ranging between US$ 2.25–2.75/lb [7,8]. Although several companies have initiated and industrialized the production of PHAs, as presented in Table 1, there are still major issues that need
to be addressed in an effort to reduce the overall production cost. The main reasons for their high cost is the high price of high purity substrates, such as glucose, production in discontinuous batch and fed-batch cultivation modes, and large amount of solvents and/or labor regarding their downstream processing. The increasing availability of raw renewable materials and increasing demand and use of biodegradable polymers for bio-medical, packaging, and food applications along with favorable green procurement policies are expected to benefit PHA market growth. According to a recent report, published in 2017, the global PHA market is expected to reach US$ 93.5 million by 2021, from an estimated US$ 73.6 million within 2016, characterized by a compound annual growth rate (CAGR) of 4.88% [9].

In the following sections, the advantages and drawbacks of PHA production employing both pure and mixed culture biotechnology are presented and discussed, as well as several approaches regarding their downstream processing in order to identify bottlenecks and opportunities to leverage PHA production.

2. PHA Production by Pure Bacterial Cultures

Pure culture biotechnology is implemented on an industrial scale, since a wide variety of food, pharmaceutical, and cosmetic agents derive as metabolic compounds from certain bacterial strains. Within the last few decades, research has been focused on finding ways to decrease the high production cost of PHAs. One of the main contributors to their high cost is the use of high purity substrates, which can account for 45% of the total production cost [10]. Instead, renewable feedstocks are being explored and researchers have been developing bioprocesses for the valorization of waste streams and by-products. In addition, current legislation and policies promote biodegradable waste management solutions other than disposal in landfills. Since every type of waste stream or by-product has different composition, selecting the appropriate biocatalyst is of great importance. In cases where the raw material is rich in carbon and nutrients, a growth-associated PHA producer would be selected, such as *Alcaligenes latus* or *Paracoccus denitrificans*. Conversely, in cases where the feedstock lacks an essential nutrient for growth such as nitrogen, phosphorous, etc., PHA accumulation using non-growth-associated bacteria would be ideal, i.e., *Cupriavidus necator*.

Apart from well-known species involved in industrial PHA production such as *Alcaligenes latus*, *Cupriavidus necator*, and *Pseudomonas putida*, bacteria need to combine several features in order be selected and regarded as promising PHA producers. Such features include their performance utilizing renewable feedstocks and/or environmental pollutants, seawater instead of fresh water, possibility of PHA production under open, non-sterile conditions, and their potential to develop contamination-free continuous bioprocesses. The use of agricultural byproducts and forest residues as an abundant and renewable source of lignocellulosic material for PHA production, is mainly considered after its physicochemical or biological hydrolysis. However, a few microorganisms possess the ability to saccharify cellulose and simultaneously produce PHAs. Moreover, PHA producers isolated from contaminated sites may be regarded for combined PHA production and bioremediation of toxic pollutants and post-consumer plastics. In addition, microorganisms isolated from hypersaline environments are considered the most promising ones, since they combine several benefits with a huge potential for reducing PHA production cost, namely in the downstream step. Last but not least, within recent years synthetic biology tools are continuously being developed in order to provide solutions to industrial challenges such as maximizing cellular capacity to ‘make more space’ for PHA accumulation, manipulating PHA composition to design polymers for high value-added applications and enhancing PHA efficiency.
2.1. Lignocellulose Degraders

2.1.1. Saccharophagus Degradans

*Saccharophagus degradans*, formerly known as *Microbulbifer degradans*, refers to a species of marine bacteria capable of degrading complex marine polysaccharides, such as cellulose of algal origin and higher plant material [11–13]. So far, the only strain reported is *S. degradans* 2-40, that was isolated from decaying marsh cord grass *Spartina alterniflora*, in the Chesapeake Bay watershed [14]. It is a Gram-negative, aerobic, rod-shaped, and motile γ-proteobacterium and is able to use a variety of different complex polysaccharides as its sole carbon and energy source, including agar, alginate, cellulose, chitin, β-glucan, laminarin, pectin, pullulan, starch, and xylan [11,13,15–17].

The key enzymes involved in PHA biosynthesis—β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase—have been identified in the genome of *S. degradans* 2-40 [12,18]. Preliminary studies have been performed in order to evaluate the feasibility of *S. degradans* to produce PHAs from D-glucose and D-cellobiose as the sole carbon source in minimal media comprised of sea salts [19]. In addition, the authors evaluated the capability of the strain to degrade lignocellulosic material in the form of tequila bagasse (*A. tequilana*). According to the results obtained, it was shown that *S. degradans* successfully degraded and utilized cellulose as the primary carbon source to grow and produce PHB. However, PHB yields were not reported, so as to evaluate the efficiency of the process, but it became evident that prior hydrolysis of the lignocellulosic material is not required. This is considered positive since it can contribute to up-stream processing cost reduction and thus encourage further research employing the certain strain. In another study, Gonzalez-Garcia et al. [20], investigated PHA production using glucose as the sole carbon source and a culture medium designed according to bacterial biomass and seawater composition. Experiments were performed using a two-step batch strategy, where in the first step bacterial growth was performed under balanced conditions for 24 h, whereas in the second step cells were aseptically transferred to a fresh nitrogen deficient medium and incubated for 48 h. Under these conditions PHA content reached up to 17.2 ± 2.7% of the cell dry weight (CDW).

PHB biosynthesis from raw starch in fed-batch mode was also investigated and the results were compared to the ones obtained using glucose as the carbon source under the same conditions [21]. When starch was used PHA yield, content, and productivity reached up to 0.14 ± 0.02 g/g, 17.5 ± 2.7% of CDW and 0.06 ± 0.01 g/L·h, respectively. In the case where glucose was fed the respective values were higher but still low compared to other PHA producers. However, only a few microorganisms have been reported to directly utilize raw starch for PHA production [21,22]. During the experiments, the authors observed the simultaneous production of organic acids and exopolymers and this was the main reason for the low PHB accumulation. Higher PHA efficiency could be achieved by optimizing cultivation parameters to drive carbon flux towards PHA biosynthesis and also by applying genetic engineering to knock out genes responsible for the production of side products such as exopolymers.

PHA production in aquarium salt medium supplemented with 1% of different types of cellulosic substrates such as α-cellulose, avicel PH101, sigmacell 101, carboxymethyl cellulose (CMC), and cellobiose have also been studied [23]. In flask experiments, PHB production was 11.8, 14.6, 13.7, and 12.8% of the DCW respectively. Fed-batch cultivation strategy resulted in increased PHB contents reaching up to 52.8% and 19.2% of the DCW using glucose and avicel respectively, as carbon sources.

Recently, another approach towards PHA biosynthesis from *S. degradans* was proposed. During their experiments, Sawant et al. [24] observed that *Bacillus cereus* (KF801505) was growing together with *S. degradans* 2-40 as a contaminant and had the ability of producing high amounts of PHAs [25]. In addition, the viability and agar degradation potential of *S. degradans* increased with the presence of *B. cereus*. Taking those into account, they further investigated the ability of co-cultures of *S. degradans* and *B. cereus* to produce PHAs using 2% w/v agarose and xylan without any prior treatment. PHA contents obtained from agarose and xylan were 19.7% and 34.5% of the DCW respectively when co-cultures were used compared to 18.1% and 22.7% achieved by pure cultures of
S. degradans. This study reported for the first time the production of PHAs using agarose. Moreover, according to the results, the highest PHA content from xylan was obtained using a natural isolate. So far, only recombinant *Escherichia coli* has been reported to produce 1.1% PHA from xylan, which increased to 30.3% and 40.4% upon supplementation of arabinose and xylose, respectively [26].

These unique features of *S. degradans* open up the possibility to use it as a source of carbohydrates in order to saccharify lignocellulosic materials. Thus, coupled hydrolysis and fermentation is a promising alternative for the production of PHAs using carbon sources that may derive from biomass residues of different origin (Table 2). However, saccharification and coupled PHA production need to be studied in detail in order to understand their potential and find ways to increase the rates of their processes.

2.1.2. Caldimonas Taiwanensis

*Caldimonas taiwanensis* is a bacterial strain isolated from hot spring water in southern Taiwan in 2004 [27]. Researchers had been searching for thermophilic amylase-producing bacteria since those enzymes are of high industrial importance for the food and pharmaceutical sector. In addition, since starch hydrolysis is known to be faster at relatively high temperatures, thermophilic amylases are usually preferred [28]. Upon morphological and physiological characterization, it was shown that this Gram-negative, aerobic, rod shaped bacteria can form PHB granules.

A few years later, Sheu et al. [22] investigated PHA production from a wide variety of carbon sources. At first cultivation of *C. taiwanensis* on a three-fold diluted Luria-Bertani medium supplemented with sodium gluconate, fructose, maltose, and glycerol as the sole carbon sources under optimal nitrogen limiting conditions, C/N = 30 was performed. PHB contents reached up to 70, 62, 60, and 52% of the CDW at 55 °C in shake flask experiments. In the next step, fatty acids were tested as sole carbon sources for growth and PHA production. It was observed that the strain did not grow at a temperature between 45 °C or 55 °C while no PHA was formed. Nevertheless, when mixtures of gluconate and valerate were provided bacterial growth was feasible and PHA cellular content reached up to 51% of its CDW. The presence of valerate induced the presence of HV units in the polymer resulting in the production of a PHBV copolymer constituting of 10–95 mol% HV depending on the relative valerate concentration in the mixture. Moreover, mixtures of commercially available starches and valerate were evaluated for PHA production at 50 °C. The carbon source mixture consisted of 1.5% starch and 0.05% valerate. Starch types examined were cassava, corn, potato, sweet potato, and wheat starch. After 32 h of cultivation PHBV copolymer was produced in all cases, composed of approximately 10 mol% HV. PHA contents of 67, 65, 55, 52, and 42% of its CDW were achieved respectively.

Despite the fact that biotechnological process using thermophilic bacteria need to be performed at high temperatures, they reduce the risk of contamination. Another advantage is the fact that thermophiles grow faster compared to mesophiles, therefore less time is needed to achieve maximum PHA accumulation [22]. Moreover, employing *C. taiwanensis* for PHA production using starch-based raw materials is extremely beneficial, in economic terms, since no prior saccharification is required. On the other hand, as mentioned before, *C. taiwanensis* cannot grow on fatty acids but when a mixture of valerate and gluconate/or starch is supplied bacterial growth and PHA accumulation occurs in the form of PHBV copolymer. However, the concentration of fatty acids may result in toxicity for bacterial cells, that up to a point can be overcome by the fast growth of cells. In addition, since amylose, amyllopectin, and nitrogen contents vary between types of starch, prior characterization needs to be performed. The feasibility of enzymatic degradation of amylose and amyllopectin is considered a key factor as it regulates the amount of sugars present in the medium. Last but not least, nitrogen content should be also controlled as high amounts favor biomass growth instead of PHA accumulation.

2.2. Bioremediation Technologies Allowing PHA Production

One of the major causes of environmental pollution is the presence of volatile aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene (BTEX) that are found in crude oil
and petroleum products. In addition, huge amounts of starting materials for the production of petrochemical based plastics, such as styrene, are released annually [29]. Moreover, chemical additives in plastics, which are accumulated in the environment due to their recalcitrance, can leach out and are detectable in aquatic environments, dust and air because of their high volatility [30]. Also, textile dyes and effluents are one of the worst polluters of our precious water bodies and soils. All the above are posing mutagenic, carcinogenic, allergic, and cytotoxic threats to all life forms [31].

Since PHAs are known to have a functional role in bacterial survival under stress conditions, toxic environments characterized by poor nutrient availability are proven to be important sources of PHA producers [32]. Several attempts have been made within the last decade to explore contaminated sites as a resource of microorganisms that are expected to advance biotechnological production of PHAs. Employment of such bacteria combines bioremediation with the production of a high value-added material. So far, bacterial strains that belong to the genus of *Sphingobacterium*, *Bacillus*, *Pseudomonas*, and *Rhodococcus* have been isolated and studied regarding their PHA production potential degrading environmental pollutants, as summarized in Table 2 [3,29,33–39].

*Pseudomonas* species are characterized by their ability to utilize and degrade a variety of carbon sources due to their wide catabolic versatility and genetic diversity. For these reasons, they are a natural choice regarding techniques of in situ and ex situ bioremediation [40]. Several *Pseudomonas* strains have been isolated from hydrocarbon-contaminated soils and together with other *Pseudomonas* sp. have been examined regarding their ability to produce PHA from hydrocarbons. In their study, Nikodinovic et al. [41], investigated PHA accumulation in several *Pseudomonas* strains from single BTEX aromatic substrates and mixed aromatic substrates as well as mixtures of BTEX with styrene. It was reported that when *P. putida* F1 was supplied with 350 μL of toluene, benzene, or ethylbenzene it accumulated PHA up to 22, 14, and 15% of its CDW respectively, while no growth was observed when p-xylene and styrene were supplied as the sole carbon source. In the case of *P. putida* mt-2 no growth was obtained with benzene, ethylbenzene, or styrene but when toluene and p-xylene were used its PHA content was 22% and 26% respectively. *P. putida* CA-3 efficiently degraded styrene but could not metabolize any of the other hydrocarbons investigated. However, a defined mixed culture of *P. putida* F1, mt-2, and CA-3 was successfully used for PHA production from BTEX and styrene mixtures, where the highest biomass concentration was achieved and PHA content reached up to 24% of the CDW. In another study, strains from petroleum-contaminated soil samples were screened on their ability to degrade toluene and synthesize mcl-PHA [42]. Among them *P. fluva* TY16 was selected to be further investigated. It was shown that the highest PHA content of 68.5% was achieved when decanoic acid was used as the carbon source. In the case of benzene, toluene, and ethylbenzene PHA contents reached up to 19.1, 58.9, and 28.6% respectively, using a continuous feeding strategy. *Pseudomonas* sp. TN301 was isolated from a river sediment sample from a site in a close proximity to a petrochemical industry [43]. Both monoaromatic and polyaromatic hydrocarbons were examined as PHA precursors and cellular mcl-PHA contents varied between 1.2% and 23% of its CDW, while this study was the first one on the ability of a bacterial strain to convert polyaromatic hydrocarbon compounds to mcl-PHA. Moreover, *Pseudomonas* strains isolated from contaminated soil and oily sludge samples from Iranian southwestern refineries accumulated 20–23% of their CDW to mcl-PHA using 2% v/v crude oil as the sole carbon source [32].

As mentioned before, styrene—used for the synthesis of polystyrene—is a major and toxic environmental pollutant. Ward et al. [29], has reported that *P. putida* CA-3 was capable of converting styrene, its metabolic intermediate phenylacetic acid and glucose into mcl-PHA under nitrogen limited conditions, characterized by conversion yields of 0.11, 0.17, and 0.22 g/g, respectively. However, higher cell density and PHA production, characterized by a conversion yield of 0.28 g/g, were observed when cells were supplied with nitrogen at a feeding rate of 1.5 mg/L/h [37]. Moreover, in a recent study the key challenges of improving transfer and increasing supply of styrene, without inhibiting bacterial growth, were addressed [38]. It was shown that by changing the feed from gaseous to liquid styrene, through the air sparger, release of styrene was reduced 50-fold, biomass concentration was five times...
higher, while PHA production was four-fold compared to previous experiments, with a PHA content reaching up to 32% in terms of CDW and a conversion yield of 0.17 g/g.

A two-step chemo-biotechnological approach has been proposed for the management of post-consumer polystyrene, involving its pyrolysis to styrene oil and subsequent conversion of the styrene oil to PHA by *P. putida* CA-3 [44]. According to their results, after 48 h 1.6 g of mcl-PHA were obtained from 16 g of oil with a cellular content of 57%. Following the same approach, the solid fraction of pyrolyzed polyethylene terephthalate (PET) was used as feedstock for PHA production by bacteria isolated from soil exposed to PET granules at a PET processing plant [45]. The isolated strains were identified and designated as *P. putida* GO16, *P. putida* GO19, and *P. frederiksborgensis* GO23 and they were able to accumulate mcl-PHA up to 27, 23, and 24% of their CDW respectively, using 1.1 g/L sodium terephthalate as the sole carbon source under conditions of nitrogen limitation. Recently, conversion of polyethylene (PE) pyrolysis wax to mcl-PHAs was investigated employing *P. aeruginosa* PAO-1 [46]. Addition of rhamnolipid biosurfactants in the growth medium had a positive impact on bacterial growth and PHA accumulation. Substitution of ammonium chloride with ammonium nitrate led to faster growth and earlier PHA accumulation that reached up to 25% of its CDW.

A series of studies has been focused on the degradation of textile dyes for PHA production using *Sphingobacterium*, *Bacillus*, and *Pseudomonas* species. When the dye Direct Blue GLL (DBGLL) was used, *Sphingobacterium* sp. ATM completely decolorized 0.3 g/L in 24 h, while simultaneous polyhydroxyhexadecanoic acid (PHD) occurred reaching up to 64% of its CDW [38]. The potential of *B. odyssey* SUK3 and *P. desmolyticum* NCIM 2112 was also investigated. It was shown that both strains were able to decolorize 0.05 g/L DBGLL by 82% and 86% and produce PHD up to 61 and 52% of their CDW, respectively. In another study, 82% decolorization of 0.8 g/L of the textile dye Orange 3R was feasible, employing *Sphingobacterium* sp. ATM which resulted in the production of 3.48 g/L of PHD and a cellular PHA content of 65% after 48 h [39]. In addition, full decolorization of 0.5 g/L of the textile dye Direct Red 5B (DR5B) was accomplished when the medium was supplemented with glycerol, glucose, starch, molasses, frying oil, and cheese whey. In those cases PHD accumulation was 52, 56, 55, 64, 46, and 10% of its CDW respectively [47].

### 2.3. Halophiles

Halophiles are microorganisms that require salt for their growth and are categorized, according to their halotolerance, in two groups: moderate (up to 20% salt) and extreme (20–30% salt) halophiles [48]. Their name comes from the Greek word for ‘salt-loving’ [49] and can be found in the three domains of life: Archaea, Bacteria, and Eukarya. They thrive in marine and hypersaline environments around the globe such as the saline lakes, salt marshes, and salterns [50,51]. With the use of halophiles the risk for contamination is reduced and/or eliminated since non-halophilic microorganisms cannot grow in media containing high salt concentrations. This is of great importance since their use combines the advantages of low energy requirements under unsterile conditions, minimal fresh water consumption, due to its substitution with seawater for medium preparation, and the possibility of operating contamination free continuous fermentation processes that are much more efficient. In addition, downstream processing cost can be reduced by treating the bacterial cells with salt-deficient water in order to cause hypo-osmotic shock [52]. The above, together with the valorization of low cost substrates, bring halophilic bacteria a step closer to being used as biocatalysts for industrial PHA production.

PHA accumulation by halophilic archaea was first observed in 1972 by Kirk and Ginzburg [53]. So far, the most well-known and best PHA halophilic archaeon producer is *Haloferax mediterranei*, which was first isolated from seawater evaporation ponds near Alicante in Spain [54]. Several studies have shown their ability to accumulate high PHA contents utilizing low cost feedstocks. Among them, vinasse, a byproduct of ethanol production from sugarcane molasses, has been utilized [55]. After pre-treatment, via adsorption on activated carbon, 25% and 50% v/v of pre-treated vinasse led to cellular PHA contents of 70 and 66%, respectively. Maximum PHBV (86% HB–14% HV) concentration reached up to 19.7 g/L, characterized by a volumetric productivity of 0.21 g/L·h and a conversion yield of 0.87 g/g, for the case where 25% pre-treated vinasse was used. In
another study, stillage derived from a rice-based ethanol industry, was investigated [56]. PHA accumulation was 71% of its CDW that led to 16.4 g/L of PHBV (85% HB-15% HV) with a yield coefficient of 0.35 g/g and a volumetric productivity of 0.17 g/L-h. Moreover, cheese whey hydrolysate—obtained upon acid pre-treatment—has been used for the production of PHBV with low HV fraction, 1.5 mol% [57]. Batch cultivation of *H. Mediterranei* led to the production of 7.54 g/L of biomass, with a PHA content of 53%, and a volumetric productivity of 0.17 g/L-h. Olive mill wastewater (OMW), which is a highly polluting waste, was also utilized recently as the sole carbon source for PHA production [58]. Using a medium containing 15% of OMW up to 43% of PHBV/CDW was produced consisting of 6 mol% HV in a one-stage cultivation step.

Halophilic bacteria belong to γ-Proteobacteria and they can grow on a wide range of pH, temperature, and salinity concentrations up to 30% (w/v NaCl) and possess the ability to accumulate PHA [52]. *Halomonas* TD01 has been isolated from Aydingkol Lake in China. This strain was investigated regarding its PHA production potential under unsterile and continuous conditions [59]. Glucose salt medium was used and initial fed-batch cultivation resulted in the production of 80 g/L of biomass with a PHA content of 80%, in the form of PHB, after 56 h. A continuous and unsterile cultivation process was developed that lasted for 14 days, and that allowed cells to grow to an average of 40 g/L containing 60% PHB in the first reactor. Cells were forwarded by continuous pumping from the first to the second reactor that contained nitrogen-deficient glucose salt medium. In the second reactor PHB levels ranged from 65 to 70% of its CDW and a conversion yield of 0.5 g/g was achieved. This was the first attempt for continuous PHA production under non-sterile conditions from a halophilic bacterium. In addition, Yue et al. [60] explored the potential of *Halomonas campaniensis* LS21, isolated from the Dabancheng salt lake in China, to produce PHA in a seawater-based open and continuous process. The strain utilized a mixture of substrates mainly consisting of cellulose, starch, animal fats and proteins. Instead of fresh water fermentation was performed using artificial seawater composed of 26.7 g/L NaCl, among others under a pH around 10 and 37 °C. PHB accumulation reached up to 26% during 65 days of continuous fermentation without any contamination. Through this study the benefits of long-lasting, seawater-based, and continuous processes for PHA production under unsterile conditions were demonstrated.

*Bacillus megaterium* has recently drawn attention since several studies have isolated such stains from salterns. *Bacillus megaterium* H16, isolated from the solar salters of Ribandar Goa in India, was shown to accumulate up to 39% PHA in the presence (5% w/v) or absence of NaCl using glucose [61]. In another study, a mangrove isolate that was found to belong to *Bacillus* sp. could tolerate salinity up to 9% w/v [62]. The certain strain was able to utilize a wide variety of carbon sources such as monosaccharides, organic acids, acid pre-treated liquor, and lignocellulosic biomass reaching cellular PHA contents of up to 73% of its CDW. Furthermore, *Bacillus megaterium uyuni* S29, isolated from Uyuni salt lake in Bolivia, was examined in terms of its salinity tolerance and impact on biomass and PHB production [63]. It was observed that the strain could grow at 10% w/v NaCl while PHB production was observed even at high salinity levels of 25% w/v. Optimum results for biomass and PHB production were achieved in medium containing 4.5% w/v NaCl and were 5.4 and 2.2 g/L characterized by a yield coefficient of 0.13 g/g and a volumetric PHB productivity of 0.10 g/L-h.

The results obtained from the studies described above are very promising and demonstrate the remarkable potential of halophiles for biotechnological production of PHAs. Although, processes performed under high salinity concentrations present disadvantages such as the corrosion of stainless steel fermenters and piping systems [51,52]. However, since no sterilization is required when halophiles are used, other types of low cost materials, such as plastics and ceramics, may be used to design and construct fermentation and piping systems in order to overcome corrosion issues. In addition, the number of halophilic bacteria that their genome is being sequenced is constantly increasing throughout the years. Subsequently, in the near future, molecular biology techniques will result in metabolically engineered strains with better performances regarding their industrial application [48,51].
Table 2. Characteristic parameters describing PHA production from different types of bacteria.

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<tr>
<th>Strain</th>
<th>Carbon Source</th>
<th>PHA</th>
<th>Cultivation Mode</th>
<th>DCW (g L⁻¹)</th>
<th>PHA (g L⁻¹)</th>
<th>PHA (%)</th>
<th>YP/S</th>
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<td>Fed-batch</td>
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<td>Sweet potato + Val⁺</td>
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<td>PHBV (80–10)</td>
<td>1.6</td>
<td>0.83</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat starch + Val⁺</td>
<td></td>
<td>PHBV (80–10)</td>
<td>4.1</td>
<td>1.72</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Cont.

<table>
<thead>
<tr>
<th>Polyhydroxyalkanoates and Bioremediation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. putida F1</strong></td>
</tr>
<tr>
<td>Benzene</td>
</tr>
<tr>
<td>Tolune (mcl-PHA)</td>
</tr>
<tr>
<td>Ethylbenzene</td>
</tr>
<tr>
<td>0.34 0.05 14</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>P. putida mt-2</strong></td>
</tr>
<tr>
<td>Tolune (p-Xylene)</td>
</tr>
<tr>
<td>Ethylbenzene</td>
</tr>
<tr>
<td>0.37 0.08 22</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>P. putida CA-3</strong></td>
</tr>
<tr>
<td>Styrene</td>
</tr>
<tr>
<td>0.79 0.26 33</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>P. putida CA-3</strong></td>
</tr>
<tr>
<td>Styrene pyrolysis oil</td>
</tr>
<tr>
<td>2.80 1.60 57</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Sphingobacterium sp. ATM</strong></td>
</tr>
<tr>
<td>Benzene</td>
</tr>
<tr>
<td>3.48 65</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>B. odysseyi SUK3</strong></td>
</tr>
<tr>
<td>Ethylbenzene</td>
</tr>
<tr>
<td>2.10 61</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>P. desmolasticum NCIM 2112</strong></td>
</tr>
<tr>
<td>Ethylbenzene</td>
</tr>
<tr>
<td>1.12 52</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Halophiles</strong></td>
</tr>
<tr>
<td>H. mediterranei DSM 1411</td>
</tr>
<tr>
<td>25% pre-treated vinasse</td>
</tr>
<tr>
<td>PHBV (86–14)</td>
</tr>
<tr>
<td>16.4 71 0.35</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Hydrolyzed cheese whey</td>
</tr>
<tr>
<td>PHBV (85–15)</td>
</tr>
<tr>
<td>19.7 70 0.87</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>15% v/v olive mill wastewater</td>
</tr>
<tr>
<td>PHBV (98.5–1.5)</td>
</tr>
<tr>
<td>7.54 54 0.78</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>PHBV (94–6)</td>
</tr>
<tr>
<td>0.2 43</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Halomonas campaniensis LS21</strong></td>
</tr>
<tr>
<td>Glucose salt medium</td>
</tr>
<tr>
<td>65 0.51</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Halomonas campaniensis LS21</strong></td>
</tr>
<tr>
<td>Mixed substrates (mostly comprised of kitchen waste)</td>
</tr>
<tr>
<td>PHB</td>
</tr>
<tr>
<td><strong>B. megarherium H16</strong></td>
</tr>
<tr>
<td>Glucose salt medium</td>
</tr>
<tr>
<td>5.42 2.22 41</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>B. megaterium uyuni 529</strong></td>
</tr>
<tr>
<td>Mixed substrate</td>
</tr>
<tr>
<td>99.5% HB, 0.5% HHx; Mixtures consisting of 1.5% starch type + 0.05% Valerate; PHBV (%HB–%HV).</td>
</tr>
</tbody>
</table>

2.4. Synthetic Biology of PHA Producers

Synthetic biology tools may aid in developing competitive bioprocesses by engineering biocatalysts with the potential of being employed at industrial scale, producing large amounts of PHA at low prices [64]. Industrial biotechnology requires non-pathogenic, fast growing bacteria that do not produce toxins and their genome is easily manipulated. Utilization of cellulose and fast growth under a wider range of temperature and pH are considered a plus [65]. The effort of minimizing PHA production cost focuses mainly on engineering strains that show higher PHA production efficiency from raw waste material, require less energy consumption during PHA production, simplify downstream processing, and produce tailored functional polymers for high value-added applications.

In order to achieve high PHA volumetric productivities high cell densities need to be obtained of up to 200 g/L, characterized by high cellular PHA contents, above 90% g PHA/g CDW. Manipulation of genes related to the oxygen uptake, quorum sensing, and PHA biosynthetic mechanisms may enhance PHA production [65]. For example, oxygen limitation may occur, after obtaining high cell densities, in order to initiate/promote PHB production. In a relevant study, anaerobic metabolic pathways were designed in E. coli (over-expressing hydrogenase 3 and acetyl-CoA synthetase) to facilitate production of both hydrogen and PHB. In that way, the formation of toxic compounds such as formate and acetate was avoided by driving carbon fluxes towards the production of PHB. The engineered strain showed improved hydrogen and PHB production. In addition, PHB pathway optimization has been also investigated in E. coli by adjusting expression levels of the three genes phbC, phbA, and phbB [66]. phbCAB operon was cloned from the native PHA producing strain *Ralstonia eutropha*. Rational designed Ribosomal Binding Sites (RBS) libraries were constructed based on high or low copy number plasmids in a one-pot reaction by an
Oligo-Linker Mediated Assembly method (OLMA). Bacterial strains accumulating cellular contents of 0 to 92% g PHB/g CDW were designed and a variety of molecular weights ranging between 2.7–6.8 × 10^6 was achieved. The certain study demonstrated that this semirational approach combining library design, construction, and proper screening is an efficient tool in order to optimize PHB production.

Another example where synthetic biology has been implemented are halophilic bacteria, which allow for PHA production under continuous mode and unsterile conditions. These features increase the competitiveness of industrial PHA production. In addition, halophilic bacteria have been proven easy for genetic manipulation, thus allowing for the construction of a hyper-producing strain [65,67]. For example, both recombinant and wild type *Halomonas campaniensis* LS21 were able to grow on mixed substrates (kitchen wastes) in the presence of 26.7 g/L NaCl, at pH 10 and temperature of 37 °C continuously, for 65 days, without any contamination. Recombinant *H. campaniensis* produced almost 70% PHB compared to wild type strain in which PHA accounted for 26% of its CDW [60].

Engineering the morphology of bacteria, in terms of cell size increase, has been recently investigated. Apart from PHA granules, several bacteria may accumulate polyphosphates, glycogen, sulfur, or proteins within their cells that limit cell space availability. In order to increase cell size, approaches such as deletion or weak expression of an actin-like protein gene mreB in recombinant *E. coli* resulted in increasing PHB accumulation by 100% [68–70]. In addition, manipulating PHA granule-associated proteins leads to an increase in PHA granule size allowing for easier separation [71].

Intracellular accumulation of PHA necessitates several extraction and purification steps. Synthetic biology approaches have been developed to control and facilitate the release of PHA granules to the medium. For example, the programmed self-disruptive strain *P. putida* BXHL has been constructed in a recent study, deriving from the prototype *P. putida* KT2440 which is a well-known mcl-PHA producer [72]. This was based on a controlled autolysis system utilizing endolysin Ejl and holing Ejh isolated from EJ-1 phage and in order to improve the efficiency of the lytic system this was tested in *P. putida* tol-pal mutant strains with alterations in outer membrane integrity. According to results, it was shown that the engineered lytic system of *P. putida* BXHL provided a novel approach to inducing controlled cell lysis under PHA producing conditions, either produce PHA accumulating cells that were more susceptible to lytic treatments. The certain study demonstrated a new perspective on engineered cells facilitating PHA extraction in a more environmentally friendly and economic way.

PHA structures include PHA homopolymers, random and block copolymers, and also different monomer molar fractions in copolymers. Block copolymers have been reported regarding their resistance against polymer aging. This is of crucial importance since slower degradation of polymer occurs leading to better performance and consistent polymer properties [73]. It has been observed that downregulating of β-oxidation cycle in *P. putida* and *P. entomophila* may be used for controlling PHA structure when fatty acids are used as precursors for PHA production [73–76]. In the case of fatty acids, containing functional groups are consumed by bacteria, introduction of those functional groups into PHA polymer chains occurs [77]. In addition, recombinant strains of *E. coli* have been constructed for the synthesis of block polymers with superior properties [78–81]. PHA diversity is possible by engineering basic biosynthesis pathways (acytoacetyl-CoA pathway, in situ fatty acid synthesis, and/or β-oxidation cycles) as well as through the specificity of PHA synthase [82].

### 3. PHA Production by Mixed Microbial Consortia (MMC)

Currently, industrial PHA production is conducted using natural isolates or engineered strains and pure substrates [83,84]. An alternative scenario that would contribute to the reduction of the PHA production cost is to employ mixed culture biotechnology [85,86]. This approach uses open (under non-sterile conditions) mixed microbial consortia (MMC) and ecological selection principles, where microorganisms able to accumulate PHA are selected by the operational conditions imposed on the biological system. Thus, the principle is to engineer the ecosystem, rather than the strains, combining the methodology of environmental biotechnology with the goals of industrial biotechnology [87]. The cost reduction derives mainly from operations being performed under
non-sterile conditions, and their consequent energy savings, and the higher adaptability of MMC to utilize waste streams as substrates.

Processes for PHA production in mixed cultures are usually performed in two steps (Figure 1). In the first step, SBR reactors (sequential batch reactors) are used to select and enrich a microbial population with high PHA production capacity by applying transient conditions. In the second step, the culture from the SBR is subjected to conditions maximizing the PHA accumulation, from where cells are harvested for PHA extraction and purification when they reach maximum PHA content [88,89].

Unlike pure cultures, where glucose is mostly used as a substrate for PHA production, mixed culture biotechnology makes use of volatile fatty acids (VFA) as the precursors for PHA production [90]. The main reason is that carbohydrates in MMCs, as well as other substrates such as glycerol, tend to form glycogen besides PHA [91,92]. For those substrates, a previous step is generally included (Figure 1), during which they are fermented into volatile fatty acids (VFA) in continuous mode (CSTR). Moreover, this is also applied to complex substrates, such as olive mill wastewater [10,93,94], cheese whey [95], and other food wastes [96] in order to obtain a more homogeneous readily available feed for the PHA production. VFA conversion into PHA require few steps, and usually presents very high yields and rapid uptake rates [88,97]. This is especially the case for butyric acid, which has now been reported in many studies as the VFA presenting the highest yields (up to 0.94 C mol PHA/Cmol S) and being the one preferably up-taken by MMCs [2,98–100]. As a matter of fact, butyric acid preference has been observed even in cultures that were not exposed to it during the enrichment [93].

It is worth mentioning that the distribution of VFA is known to affect the PHA monomer composition, where VFA with an even number of carbon atoms tend to produce PHB while VFA with odd carbon atoms tend to produce PHBV copolymers with different % HV molar fractions [101]. Based on this fact, many studies have suggested the possibility of regulating the PHA composition by manipulating the fermentation conditions in the preceding acidogenesis step [2,95,100].

![Figure 1. PHA production process by mixed microbial cultures. Modified from [88]. CSTR: continuous stirred tank reactor, SBR: Sequential Batch Reactor.](image)

This section provides an overview of the different types of existing enrichment techniques, performed within the last 10 years. Each one of the enrichment strategies presents different advantages; either related to the cost of the process or to increased cellular PHA content. Thus, they all present opportunities to further improve economic and sustainable PHA production. Such opportunities are commented for each of the enrichment techniques. This section is followed by a compilation of recent advances regarding the PHA accumulation stage, aiming at increasing the productivity. Finally, recent attempts to bring MMC to pilot scale are described, followed by a section highlighting the main challenges and bottlenecks of the MMC.

3.1. Types of Enrichments

Until the late 2000s, two types of enrichments dominated the research panorama related to PHA mixed culture biotechnological production, namely the anaerobic/aerobic selection and aerobic dynamic feeding. These types of enrichments have already been previously reviewed in other articles [88,102–104], thus apart from a general description of the mechanisms, only the recent trends are described in the respective Sections 1.1.1 and 1.1.2. The following sections are dedicated to recent (and still less widespread) types of enrichments developed within the last decade. The main characteristics of all types of enrichments described are summarized in Table 3.
Table 3. Summary on the main characteristics of the enrichment techniques applied for MMCs

<table>
<thead>
<tr>
<th>Anaerobic-Aerobic Enrichment (AN/AE) (Section 3.1.1)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feast phase</strong></td>
<td><strong>Famine phase</strong></td>
</tr>
<tr>
<td>Aeration</td>
<td>No</td>
</tr>
<tr>
<td>e⁻ acceptor</td>
<td>-- (PHA)</td>
</tr>
<tr>
<td>Energy source</td>
<td>Glycogen / polyphosphate</td>
</tr>
<tr>
<td>Carbon source</td>
<td>External substrate</td>
</tr>
<tr>
<td>Driving force for PHA accumulation</td>
<td>• Lack of electron acceptor *</td>
</tr>
<tr>
<td></td>
<td>• Transient presence of substrate ***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aerobic Dynamic Feeding (ADF) (Sections 3.1.2 and 3.1.3)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feast phase</strong></td>
<td><strong>Famine phase</strong></td>
</tr>
<tr>
<td>Aeration</td>
<td>Yes</td>
</tr>
<tr>
<td>e⁻ acceptor</td>
<td>Oxygen</td>
</tr>
<tr>
<td>Energy source</td>
<td>Oxidation of substrate</td>
</tr>
<tr>
<td>Carbon source</td>
<td>External substrate</td>
</tr>
<tr>
<td>Nitrogen availability</td>
<td>Yes **</td>
</tr>
<tr>
<td>Driving force for PHA accumulation</td>
<td>• Transient presence of substrate ***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aerobic Dynamic Feeding (ADF) with Intermediate Settling Phase (Section 3.1.3)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feast phase</strong></td>
<td><strong>Famine phase</strong></td>
</tr>
<tr>
<td>Aeration</td>
<td>Yes</td>
</tr>
<tr>
<td>e⁻ acceptor</td>
<td>Oxygen</td>
</tr>
<tr>
<td>Energy source</td>
<td>Oxidation of substrate</td>
</tr>
<tr>
<td>Carbon source</td>
<td>External substrate</td>
</tr>
<tr>
<td>Nitrogen availability</td>
<td>Yes</td>
</tr>
<tr>
<td>Driving force for PHA accumulation</td>
<td>• Transient presence of substrate ***</td>
</tr>
<tr>
<td></td>
<td>• Higher settling capacity of PHA rich cells</td>
</tr>
<tr>
<td></td>
<td>• Elimination of residual COD after feast phase prevents growth of non-PHA accumulating bacteria</td>
</tr>
</tbody>
</table>
### Aerobic Dynamic Feeding (ADF) with Nitrogen Limitation in the Feast-Phase (Section 3.1.3)

<table>
<thead>
<tr>
<th></th>
<th>Feast phase</th>
<th>Famine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeration</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>e− acceptor</strong></td>
<td>Oxygen</td>
<td>Oxygen</td>
</tr>
<tr>
<td><strong>Energy source</strong></td>
<td>Oxidation of substrate</td>
<td>Oxidation of PHA</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td>External substrate</td>
<td>PHA</td>
</tr>
<tr>
<td><strong>Nitrogen availability</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Driving force for PHA accumulation</strong></td>
<td>• Transient presence of substrate ***&lt;br&gt;• Nitrogen limitation during the feast phase</td>
<td></td>
</tr>
</tbody>
</table>

### Photosynthetic Enrichment (Section 3.1.5)

**Photosynthetic Enrichments—Illuminated SBR**

<table>
<thead>
<tr>
<th></th>
<th>Feast phase</th>
<th>Famine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeration</strong></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>e− acceptor</strong></td>
<td>-- (PHA)</td>
<td>Oxygen produced by algae</td>
</tr>
<tr>
<td><strong>Energy source</strong></td>
<td>Light</td>
<td>Oxidation of PHA + Light</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td>External substrate</td>
<td>PHA</td>
</tr>
<tr>
<td><strong>Driving force for PHA accumulation</strong></td>
<td>• Lack of external electron acceptor with presence of light&lt;br&gt;• Transient presence of substrate ***</td>
<td></td>
</tr>
</tbody>
</table>

**Photosynthetic Enrichment—Dark Feast Phase**

<table>
<thead>
<tr>
<th></th>
<th>Feast phase</th>
<th>Famine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeration</strong></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>e− acceptor</strong></td>
<td>-- (PHA)</td>
<td>Oxygen produced by algae</td>
</tr>
<tr>
<td><strong>Energy source</strong></td>
<td>Glycogen</td>
<td>Oxidation of PHA + Light</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td>External substrate</td>
<td>PHA</td>
</tr>
<tr>
<td><strong>Driving force for PHA accumulation</strong></td>
<td>• Lack of external electron acceptor with presence of light&lt;br&gt;• Transient presence of substrate ***</td>
<td></td>
</tr>
</tbody>
</table>

**Photosynthetic Enrichment—Permanent Feast Phase**

<table>
<thead>
<tr>
<th></th>
<th>Feast phase</th>
<th>Famine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeration</strong></td>
<td>No</td>
<td>No famine phase</td>
</tr>
<tr>
<td><strong>e− acceptor</strong></td>
<td>-- (PHA)</td>
<td>No famine phase</td>
</tr>
<tr>
<td><strong>Energy source</strong></td>
<td>Light</td>
<td>No famine phase</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td>External substrate</td>
<td>No famine phase</td>
</tr>
<tr>
<td><strong>Driving force for PHA accumulation</strong></td>
<td>• Lack of external electron acceptor with presence of light</td>
<td></td>
</tr>
</tbody>
</table>
### Aerobic-Anoxic Enrichment (Section 3.1.6)

<table>
<thead>
<tr>
<th></th>
<th>Feast phase</th>
<th>Famine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeration</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>e⁻ acceptor</strong></td>
<td>Oxygen</td>
<td>NO₃/NO₂</td>
</tr>
<tr>
<td><strong>Energy source</strong></td>
<td>Oxidation of substrate</td>
<td>Oxidation of PHA</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td>External substrate</td>
<td>PHA</td>
</tr>
<tr>
<td><strong>Driving force for PHA accumulation</strong></td>
<td>• Transient presence of substrate ***</td>
<td></td>
</tr>
</tbody>
</table>

### Anoxic-Aerobic Enrichment (Section 3.1.7)

<table>
<thead>
<tr>
<th></th>
<th>Feast phase</th>
<th>Famine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeration</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>e⁻ acceptor</strong></td>
<td>NO₃/NO₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td><strong>Energy source</strong></td>
<td>Oxidation of substrate</td>
<td>Oxidation of PHA</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td>External substrate</td>
<td>PHA</td>
</tr>
<tr>
<td><strong>Driving force for PHA accumulation</strong></td>
<td>• Transient presence of substrate ***</td>
<td></td>
</tr>
</tbody>
</table>

### Microaerophilic Enrichment (Section 3.1.8)

<table>
<thead>
<tr>
<th></th>
<th>Feast phase</th>
<th>Famine phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aeration</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>e⁻ acceptor</strong></td>
<td>Oxygen</td>
<td>Oxygen</td>
</tr>
<tr>
<td><strong>Energy source</strong></td>
<td>Oxidation of substrate</td>
<td>Oxidation of PHA</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td>External substrate</td>
<td>PHA</td>
</tr>
<tr>
<td><strong>Driving force for PHA accumulation</strong></td>
<td>• Transient presence of substrate ***</td>
<td>• Limitation of electron acceptor</td>
</tr>
</tbody>
</table>

* Even though the lack of electron acceptor is the driving force of the enrichment, this limitation is not mandatory for these cultures to produce PHA, which can also be produced aerobically. ** Various C/N ratios have been applied resulting in a limitation of nitrogen in the famine or late feast phase. Nevertheless, most wide-spread configuration provides nitrogen in both phases *** Transient presence of substrate leads to the following effects in all cases mentioned in the table: Growth during famine phase consuming the PHA accumulated; Limitation of internal growth factors; Higher responsiveness of PHA producers to substrate addition.
3.1.2. Aerobic Dynamic Feeding (ADF)

In enrichment under ADF conditions the limiting factor, promoting PHA accumulation, is carbon substrate availability rather than the electron acceptor [104]. This process relies on subsequent feast/famine cycles where the culture is subjected initially to an excess of carbon source, and then submitted to carbon deficiency under aerobic conditions. Bacteria that are able to convert carbon to PHA during the feast phase have a competitive advantage towards the rest of the microbial population, as they utilize PHA as a carbon and energy reserve during the famine phase, allowing them to grow over non-PHA storing microorganisms [104,105]. Moreover, a limitation of internal factors, such as RNA and enzymes required for growth, seems to be crucial [102]. In order for cells to grow, a considerable amount of RNA and enzymes are needed, which might not be available after long starvation periods. Nevertheless PHA synthase, the key enzyme for PHA polymerization, is active during PHA production and degradation, generating a futile cycle that wastes ATP but enables the PHA mechanism to be ready when a sudden addition of carbon occurs, providing them with higher responsiveness [106–108]. In this way, a new competitive advantage of PHA producers arises, given that they can use PHA to regulate substrate consumption and growth [106]. PHA contents up to 90% of the dry cell weight have been reported using this strategy [89,109], higher than the ones reached following AN/AE enrichment [102–104].

Most of the studies performed within the last 10 years have been based on ADF enrichment. Apart from investigating the feasibility of this strategy using a variety of substrates, recently reviewed by Valentino and colleagues [97], the main focus has been on evaluating the impact of different parameters during the enrichment process. An overview on how those parameters influence PHA production from MMCs has been recently reported on, including the effect of hydraulic retention time (HRT), solids retention time (SRT), pH, temperature, nitrogen concentration, dissolved oxygen concentration (DO), cycle length, influent concentration, feast/famine ratio, and food/microbe ratio [110]. Regarding process configuration, a continuous system has been proposed where instead of an SBR, the feast and the famine phases were operated in separate CSTR [111]. Although no significant improvements were observed with respect to the conventional SBR configuration, the study demonstrated that successful enrichment was also possible in continuous mode, which is considered advantageous in the case of putative coupling to the following PHA accumulation step under continuous mode.

3.1.3. Variations of the ADF Enrichments

Even though microbial communities with high PHA-storing capacity have been obtained with several parameter combinations, the presence of non-PHA accumulating microorganisms is still not completely avoided. This is partially due to the presence of organic content other than VFA present in waste streams, allowing the growth of non-PHA accumulating bacteria [112]. A possible way to overcome the presence of such bacteria has been recently proposed where the culture was settled and the supernatant was discharged just after VFA depletion. In this way, consumption of the remaining organic matter—measured as chemical oxygen demand (COD)—and growth of side-population was prevented while the fraction of PHA-producers was considerably increased as verified by molecular techniques [113]. An increase in the PHA content from 48% to 70% was observed by applying this strategy. The authors suggested that, apart from the role of the remaining COD, also the increased cell density of PHA packed cells enhanced the enrichment, as those cells would have a higher tendency to settle after the feast phase. This observation coincided with the results obtained using only acetate as a carbon source, where the settling after the feast phase also increased the PHA accumulation capacity of the culture from 41% to 64–74% [114]. As no residual COD was present in those experiments, the effect could be entirely attributed to differences in the cell density that led to an additional physical selection.

Similarly, the growth of such non-PHA accumulating bacteria was observed to be restricted by applying nitrogen limitation [112]. Following this reasoning, a variation of the ADF was recently proposed, where also a nitrogen deficiency was imposed during the feast phase, while providing
nitrogen during the famine phase to enable growth from the accumulated PHA. Thus, an uncoupling of the carbon and nitrogen took place in the SBR [2,86,115]. This strategy resulted in higher PHA contents at the end of the feast phase using synthetic VFA [2,115], cheese whey [86], and 1,3-propanediol from fermented crude glycerol [116] as substrates. When a separate PHA accumulation was performed with the cultures of such enrichments, higher PHA yields and productivities were obtained with this strategy compared to carbon-nitrogen coupled ADF [86]. This strategy also allowed for a more stable system for long term operation. Nevertheless, given that the enrichment is already performed at conditions maximizing PHA accumulation, it was also suggested that a separate PHA accumulation might no longer be required if part of the biomass is already harvested after the feast phase, something that would significantly contribute towards the reduction of the costs of the process [115]. Moreover, given that there is already a selective pressure in the feast phase, the duration of the famine phase would be less important, so as to achieve an effective selection, and this could enable a reduction of its duration leading to enhanced process productivity [86].

3.1.4. ADF Enrichments in Halophilic Conditions

As previously discussed, the use of halophilic bacteria comes with various advantages. PHA production using halophilic bacterial populations can be performed using seawater instead of fresh or distilled water or using high salinity wastewater produced by several industries, namely food processing industries. In addition, halophilic bacteria can be lysed in distilled water thus reducing downstream processing costs due to lower quantities of solvents required. Enrichment of a halophilic PHA accumulating consortium under ADF conditions has been recently investigated using different carbon sources as substrate, which resulted in cellular PHA contents reaching up to 65% and 61% PHA using acetate and glucose respectively, demonstrating the potential of this strategy in MMC [117]. Recently, a previously enriched MMC fed with a mixture of VFAs containing 0.8 g/L Na+ was examined regarding its PHA accumulation capacity under transient concentrations of 7, 13, and 20 g/L NaCl [118]. Since the particular MMC was not adapted to saline conditions, PHA accumulation capacities and rates decreased with higher NaCl concentrations while biopolymer composition was affected in terms of HB:HV ratio.

3.1.5. Mixed Photosynthetic Consortia

A new approach relying on the photosynthetic activity of mixed consortia has been explored recently [119–122]. Based on the previous observation of PHA production in photosynthetic strains, an illuminated SBR operating without aeration was proposed, eliminating the costly need for aeration during ADF enrichments. In such a system, photosynthetic bacteria uptake an external carbon source, in the form of acetate, during the feast phase using light as an energy source. PHB was produced at the same time as a sink of NADH, given that no electron acceptor was present. During the famine phase PHB was consumed using oxygen as an electron acceptor, which was not provided though aeration but produced by algae also present in the SBR. Under these conditions, up to 20% PHB was attained during the PHA accumulation step [119], which was also possible by utilizing other VFAs such as propionate and butyrate [121]. However, it was observed that a dark feast phase could also be envisioned, given that similarly to AN/AE enrichments, glycogen accumulation occurred during the famine phase, which was subsequently used as a complementary energy source to uptake acetate. With this SBR configuration, which would considerably reduce the need of illumination, a 30% PHA was accomplished [122].

The best PHA productivity though, was obtained in a system operating in a permanent illuminated feast phase (instead of successive feast-famine cycles) without oxygen supply [120]. This was based on the fact that photosynthetic accumulating bacteria out-compete other bacteria and algae without the need of transient presence of carbon source. Therefore, productivity was significantly increased due to the elimination of the famine phase and since there was no need for a separate PHA accumulation reactor. However, considerable input of light was required in order to obtain cultures with high PHA content (up to 60% of the dry weight), so the economic advantages of
such systems should be further explored. Nevertheless, this process will allow significant savings in energy, since no sterilization and aeration are required, which will have an impact on the final price of the polymer.

3.1.6. Aerobic–Anoxic Enrichment Coupled with Nitrification/Denitrification

Basset and colleagues [123] developed a novel scheme for the treatment of municipal wastewater integrating nitrification/denitrification with the selection of PHA storing biomass, under an aerobic/anoxic and feast/famine regime. The process took place in a SBR (where NH$_4^+$ is converted into NO$_3^-$ with a simultaneous selection of PHA storing biomass—and with COD being converted to PHA) and the subsequent PHA accumulation in a batch reactor (where PHA is consumed to allow denitrification, under famine–anoxic conditions, without the need of external addition of organic matter). The carbon source added during the selection and accumulation steps consisted of fermentation liquid from the Organic Fraction of Municipal Solids Waste (OFMSW) and primary sludge fermentation liquor.

The advantage of this approach is that the potential for recovering biopolymers from wastewater presents particular interest, when the latter is integrated within the normal operation of the plant. An important benefit of this strategy is that anoxic denitrification usually requires a carbon source, which at that point is usually low and already consumed in a wastewater treatment plant (WWTP). In this case however, it can occur without external addition of carbon source by using those stored internally in the form of PHA.

Results showed that during SBR operation ammonium oxidation to nitrite reached on average 93.4 ± 5.25%. The overall nitrogen removal was 98% (resulting in an effluent with only 0.8 mg NH$_4^-$ N L$^{-1}$). Similar results were obtained by Morgan-Sagastume et al. [124]. Denitrification efficiency and rate did not seem to be affected by the carbon source. When sufficient PHA amount was available, denitrification of all available nitrate was observed. COD removal reached up to 70% when DO level was higher (2–3 mg L$^{-1}$). PHA content decreased during nitrification due to the lack of external COD. However, after complete nitrification, there was enough PHA to carry out the denitrification process. Even though biomass was rich in PHA storing bacteria, PHA accumulation reached only 6.2% during the feast phase (first 10 min) and it was progressively consumed before the initiation of the anoxic phase to 2.3%, which was enough to complete the subsequent denitrification. Nevertheless, the selection of PHA storing biomass under feast (aerobic)–famine (anoxic) conditions required less DO compared to the typical feast–famine regime carried out under continuous aerobic conditions, leading to a reduction of 40% of the energy demand.

The PHA accumulation capacity of the biomass, previously selected in the SBR, was further evaluated in accumulation batch reactors with the use of OFMSW, and primary sludge fermentation liquid. After 8 h of accumulation with OFMSW, the stored PHA was 10.6% (wt.). In the case where fermented sludge liquid and OFMSW was used as carbon source, the contribution to growth was higher, due to the elevated nutrient content, and the lower VFA/COD ratio but only 8.6 % PHA was accumulated after 8 h. The carbon source was proven to play an important role in the PHA accumulation step as the presence of non-VFA COD contributed to the growth of non-PHA-storing biomass [124,125]. PHA storage yields could be potentially improved with a more efficient solid–liquid separation after the fermentation process.

3.1.7. Anoxic–Aerobic Strategy Coupled with Nitrification/Denitrification

Anoxic–aerobic enrichments coupled with denitrification, where nitrate is used as electron acceptor during PHA accumulation, has been already explored using synthetic VFA since early 2000s [103]. A recent study applying this strategy has been reported which investigated the use of the condensate and wash water from a sugar factory [126]. Furthermore, they considered that, in combination with harvesting enriched biomass from the process water treatment, side-streams could be exploited as a substrate for PHA accumulation. This approach (together with the aerobic–anoxic strategy shown in Section 1.1.6) would have the big advantage of significantly reducing the PHA
production costs, through the integration of already existing full-scale WWTP and reduction of aeration needs.

In that study [126], they used parallel SBRs fed alternatively with condensate and wash water, developing a microbial consortium that removes inorganic nitrogen by aerobic and anoxic bioprocess steps of nitrification and denitrification. Alternating bioprocess conditions of anoxic feast (supporting denitrification) and aerobic famine (supporting nitrification) in mixed open cultures was expected to furnish a biomass with stable PHA accumulating potential characterized by its ability to remove carbon, nitrogen, and phosphorus in biological processes. In more detail, one laboratory SBR was operated with suspended activated sludge (AS) and long SRT, similar to the full-scale (SRT > 6 days), while the other SBR was a hybrid suspended activated sludge and moving bed biofilm reactor (MBBR) with short SRT of 4–6 days. MBBR technology employs thousands of polyethylene biofilm carriers operating in mixed motion within an aerated wastewater treatment basin. Therefore, the MBBR-SBR was used as a means for maintaining nitrifying activity while enabling enrichment of biomass at relatively low SRT.

The results showed a COD removal performance of 94 and 96% for AS- and MBBR-SBRs, respectively. Full nitrification was achieved in both systems, with exception of periods showing phosphorous or mineral trace element limitation. Soluble nitrogen removal reached 80 ± 21% and 83 ± 11% for AS- and MBBR-SBRs, respectively. MBBR-SBR showed more stable performance under lab-scale operation. The process achieved a PHA content of 60% g PHA/g VSS in both cases. A significant advantage was the possibility of lowering the SRT while maintaining a robust nitrification activity and improving the removal of soluble phosphorus from the process water.

3.1.8. Microaerophilic Conditions

In 1998, Satoh and colleagues [127] investigated the feasibility of activated sludge (from laboratory scale anaerobic–aerobic reactors) for the production of PHA, by optimizing the DO concentration provided to the system. They were able to obtain a PHA content of around 20% in anaerobic conditions and 33% under aerobic conditions. When applying a microaerophilic–aerobic process, by supplying a limited amount of oxygen into the anaerobic zone, they were able to increase the PHA accumulation to 62% of sludge dry weight.

PHA production using palm oil mill effluent (POME) was investigated by Din and colleagues [128], using a laboratory SBR system under aerobic feeding conditions. The microorganisms were grown in serial configuration under non-limiting conditions for biomass growth, whereas in the parallel configuration the nutrient presence was controlled so as to minimize biomass growth in favor of intracellular PHA production. PHA production under aerobic, anoxic, and microaerophilic conditions was investigated and it was shown that PHA concentration and content increased rapidly at the early stages of oxygen limitation while the production rate was reduced at a later stage implying that oxygen limitation would be more advantageous in the PHA accumulation step.

Another interesting study was published by Pratt and colleagues [129], where the effect of microaerophilic conditions was evaluated during the accumulation phase, using an enriched PHA culture, harvested from a SBR fed with fermented dairy waste. Batch experiments were conducted to examine the effect of DO on PHA storage and biomass growth. The results showed that in microaerophilic conditions a higher fraction of substrate was accumulated as PHA, compared to high DO conditions. Also, the intracellular PHA content was 50% higher during early accumulation phase. Interestingly, the accumulation capacity was not affected by the DO, despite its influence on biomass growth. The PHA content in both low and high DO concentrations reached approximately 35%. However, the time needed to achieve maximum PHA content at low DO level was three times longer than in the case of high DO concentration. The reason why PHA accumulation was proven to be less sensitive to DO, compared to its effect on biomass growth, was explained by the fact that low DO levels limit the availability of ATP, while high DO supply provides surplus ATP and high growth rates (and consequently reduced PHA yield). In addition, when MMCs were fed with multiple VFAs (acetate, propionate, butyrate, and valerate) it was also shown that, during PHA
accumulation, high DO concentration is required to reach maximum PHA accumulation rates due to low specific VFA uptake rates under low DO levels [130].

The effect of dual nitrogen and DO limitation has been also investigated in MMCs fed with a VFA mixture of acetate, propionate, and butyrate and acidified OMW [10,99]. As discussed above, it was shown that during the PHA accumulation step, under batch mode, lower substrate uptake and PHA production rates were obtained compared to assays performed under nitrogen limitation. Moreover, PHA accumulation percentages and the yield coefficient $Y_{PHA/S}$ was lower in the case of dual limitation, while the accumulation of non-PHA polymers within the cells was indicated.

Those reports demonstrate that manipulating oxygen concentration could influence growth and PHA storage. Manipulating DO instead of limiting nitrogen or phosphate availability could represent a significant opportunity for PHA production processes that utilize nutrient rich feedstocks. A major advantage of operating at low DO is the reduced aeration requirements leading to reduction of operating costs. However, this advantage can be countered by the fact that PHA accumulation in low DO environments can be significantly slower.

3.1.9. PHA Accumulation without Previous Enrichment

The three-step process described in Figure 1, consisting in an enrichment step followed by an accumulation step, has been proven efficient to obtain cultures with high PHA contents. Nevertheless, several authors have put in doubt the alternative of having a separate enrichment step. The main reason is that during the enrichment, PHA is produced, but it is also allowed to be consumed to drive the selection. Thus, this step consumes substrate without leading to any net production of PHA, lowering the overall PHA/Substrate yields of the system [97,131]. Thus, skipping this step could imply considerable improvements on those parameters.

Already in 2002, PHA accumulation without a previous enrichment step using activated sludge was reported to obtain PHA contents up to 30% [132]. Later on, fed-batch cultivation under nitrogen limiting conditions was reported to obtain up to 57% PHA [133]. Cavaillé and colleagues performed fed-batch PHA accumulation experiment as well using activated sludge without previous enrichment, but applying phosphorous limitation and achieved up to 70% PHA [134]. Substrate to PHA yield reached up to 0.2 Cmol PHA/Cmol S using acetic acid. This yield was considerably lower than that obtained in PHA accumulation steps submitted to a previous enrichment step (up to 0.9 Cmol PHA/Cmol S), but comparable to the overall yields of enrichment and accumulation strategies summed up [134]. Moreover, they further developed the system into a continuous process, and attained a stable operation where the cells in the effluent contained 74% PHA [135]. Their findings also evidenced that the continuous system was not stable at severely phosphorous limited experiments, because the growth rate could not be maintained and the cells were washed out of the reactor. The key was that differently from when a separate enrichment is performed, the continuous PHA accumulation without previous enrichment relied on the occurrence of both growth and storage responses. Moreover, the authors suggested that phosphorous limitation might offer more flexibility than nitrogen limitation when both PHA formation and growth are a goal, given that phosphorous is less needed than nitrogen for growth-related metabolism [135].

3.2. PHA Accumulation

As in the enrichment step, several operational parameters such the temperature [136] and the pH have an impact on PHA accumulation [2,137–139]. Nonetheless, the most critical aspect during PHA accumulation experiments is the cultivation strategy employed. High substrate concentrations supplied under batch mode should be avoided since they can cause inhibition and thus limit PHA productivity [105,140]. In order to circumvent that, several fed-batch strategies have been suggested. Pulsed fed-batch cultivation has been suggested when synthetic VFA mixtures are used [140,141]. However, due to an increase occurring in the working volume after the addition of substrate the feed should be very concentrated. Nevertheless, this is rarely the case with fermentation effluents, since they usually do not exceed 20 g COD/L [97]. Discharge of the exhaust supernatant has been
suggested as an alternative [137,142,143] yet, this approach requires a settling step between batches, which severely limits the productivity [137].

Continuous feeding processes have shown the best results until now, given that they can attain a sustained productivity [89,137,143,144]. Pulsed fed-batch production may result in high PHA productivity but as the substrate is being consumed, PHA productivity eventually decreases. This phenomenon might be avoided by supplying substrate in a continuous manner. Continuous substrate addition has been successfully performed using the pH as an indicator, given the pH increases with VFA consumption [89,137,143,144]. On the other hand, less successful results have been obtained when the substrate was supplied, taking into account previously observed substrate uptake rates resulting in either accumulation or limitation of substrate in the reactor [140,141]. Alternatively, an on-demand continuous addition of substrate, based on change of DO, has been proven efficient to maintain optimal amounts of carbon substrate in the reactor [145].

High productivities up to 1.2 g PHA/L·h combined with high PHA yields, 0.8 Cmol PHA/Cmol S, have been achieved with continuous-feeding systems [137]. However, similarly to the pulsed fed-batch, such values have been reported only when synthetic substrates of high concentration were present in the feed. Much lower values are reported in real substrates due to the diluted nature of these substrates and the consequent increment in reactor volumes [97]. A way to overcome this could be the development of a continuous feeding scheme for PHA accumulation under low biomass loading rates (3.5–5.5 Cmol VFA/Cmol X/d). So far, this venture has only been investigated once using MMC [139]. The authors suggested a PHA accumulation reactor operating under continuous mode, were the effluent was allowed to settle and the resulting sludge recycled back to the reaction vessel. The system worked with a rather diluted effluent in the feed (around 100 Cmol VFA) and was shown to obtain higher specific productivities than the pulse-fed-batch. Nevertheless, overall productivity of the system was not reported in that study. It is worth noting that the system was not coupled to an SBR operating in continuous mode, so the operation of the reactor was also for a limited period of time.

Regarding the nutrient availability, nitrogen limitation or deficiency is usually reported to improve the PHA yield and content [2,105,146,147]. On the other hand, several studies have concluded that the role of nitrogen during the accumulation step is secondary since PHA storage was preferred over growth regardless of the nitrogen concentration [144,148]. Moreover, in another study it was observed that nitrogen limitation did not enhance the PHA accumulation [149]. As a matter of fact, the main reason for limiting the amount nitrogen is to prevent bacterial growth of non-PHA-accumulating bacteria [112]. Hence, different observations from different cultures do not imply contradictions but highlight the fact that the requirement for nitrogen limitation, in order to obtain high PHA contents, is highly dependent on the composition of the enriched culture and the type of substrate fed. In terms of productivity though, nutrient limitation rather than deficiency was reported to show higher productivities [145]. According to a certain study, the absence of an essential nutrient for growth leads to cellular PHA saturation, while nutrient limitation allows cells to duplicate prolonging PHA accumulation without enabling excessive growth response. In addition, it was shown that the best productivities were obtained from dual limitation of nitrogen and phosphorous.

3.3. Pilot Scale Experiences

Several industrial/agro-industrial effluents and residues have been investigated so far as potential feedstocks for PHA production. Effluents, rich in sugars, glycerol, and/or fatty acids were either directly used for the selection of PHA accumulating MMCs or were previously fermented for the conversion of carbohydrates to VFAs, the preferable precursors for PHA production using MMCs. Numerous studies on PHA production from industrial effluents have been performed, that have been recently reviewed by Valentino et al [97]. On the other hand, studies on PHA production in pilot-scale by MMCs are rather scarce and relevant efforts have recently started, in 2010s, while no full-scale production of PHA by MMC exists yet.
A common feature in all pilot-scale studies is that effluents/feedstocks were always fermented prior to PHA production. Also, most efforts on PHA production in pilot-scale focus on integrating and combining PHA production with existing processes in wastewater treatment plants so as to reduce the production cost by exploiting the available infrastructure as much as possible. In this context, anoxic/aerobic MMC selection regimes can be coupled to nitrification and denitrification activities despite the fact that the highest PHA yields and cell content in PHA are usually reported for aerobic dynamic feeding selection regimes. Another tendency in pilot-scale studies is the use of a different effluent for the MMC enrichment than the one fed during the PHA accumulation step. The first pilot-scale study, concerning MMC PHA production, was performed from pre-fermented milk and ice-cream processing wastewater, as reported by Chakravarty et al. [150], with a PHA content of 43% and a PHA yield of 0.25 kg PHA/kg COD being obtained. In 2014, Jia et al. [125] studied the production of PHA in pilot-scale with pre-hydrolyzed and fermented raw excess sludge. In both studies, activated sludge was used as the raw material for its enrichment to PHA accumulating microorganisms. A series of pilot-scale studies was conducted and published with the participation of Anoxkaldnes and Veolia Water Technologies [126,151,152]. In the study of Morgan-Sagastume et al. [151], the potential of waste sludge, generated in wastewater treatment plants as a feedstock for PHA production was evaluated. This was done in the context of integrating PHA production in existent WWTP valorizing at the same time the excess sludge that in general represents a burden for further treatment and disposal. A very interesting point in the study of Bengtsson et al. [152] is that denitrifying microbial biomass was also selected towards high PHA producing potential by applying an anoxic-feast and aerobic-famine selection pattern and therefore the process comprised nitrification and denitrification steps followed by accumulation of PHA. Tamis et al. [153] investigated PHA production from fermented wastewater deriving from a candy bar factory. Activated sludge was enriched in PHA accumulating microorganisms under aerobic feast and famine regime and the obtained PHA content was the highest reported so far in pilot-scale at 70–76%. Table 4 summarizes the main features of the pilot-scale studies published so far. Overall, as it regards pilot-scale studies, their performance cannot really be compared to respective lab-scale studies but they provide valuable information on PHA formation under variable feedstock characteristics and allow production of significant amounts of polymer that can be processed for a full characterization. The variation of feedstock composition combined with the oxygen mass transfer limitations occurring at a larger scale, could be the main reason why PHA yields and cell content in pilot-scale studies are in general lower than the ones reported in lab-scale experiments.
Table 4. Main characteristics of PHA production in pilot-scale

<table>
<thead>
<tr>
<th>Pilot Plant, Location</th>
<th>Feedstock</th>
<th>Origin of MMC and Enrichment Strategy</th>
<th>Y_pH (g/g) *</th>
<th>PHA % (%mol HB: %mol HV)</th>
<th>mg PHA/g X/h</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagpur, India</td>
<td>Pre-fermented milk and ice cream processing wastewater</td>
<td>Activated sludge</td>
<td>0.425 *</td>
<td>39-43</td>
<td>[150]</td>
<td></td>
</tr>
<tr>
<td>Lucun WWTP in Wuxi, China</td>
<td>Hydrolyzed and acidified raw excess sludge</td>
<td>Activated sludge/ synthetic mixture of VFA, ADF feast famine with carbon limitation and inhibitor of nitrification</td>
<td>0.044-0.29 *</td>
<td>2.06-39.31</td>
<td>[125]</td>
<td></td>
</tr>
<tr>
<td>Eslöv, Sweden</td>
<td>Beet process water, 38% in VFA</td>
<td>PHA producing MMC from pre-fermented effluent of Procordia Foods</td>
<td>60 (85:15 HB:HV)</td>
<td>[126]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brussels North WWTP (Aquiris, Belgium)</td>
<td>Pre-hydrolyzed and fermented WWTP sludge</td>
<td>Sludge fed with municipal WW under aerobic feast famine</td>
<td>0.25-0.38</td>
<td>27-38 (66-74:26-34 HB:HV)</td>
<td>100-140</td>
<td>[151]</td>
</tr>
<tr>
<td>Leeuwarden WWTP, Friesland, Netherlands</td>
<td>Fermented residuals from green-house tomato production</td>
<td>Sludge fed with municipal WW under anaerobic feast/anaerobic famine</td>
<td>0.30-0.39</td>
<td>34-42 (51-58:42-49 HB:HV)</td>
<td>28-35</td>
<td>[152]</td>
</tr>
<tr>
<td>Mars company, Veghel, Netherlands</td>
<td>Fermented wastewater from a candy bar factory</td>
<td>Activated sludge from a WWTP fed with the fermented wastewater under aerobic feast/famine with inhibitor of nitrification</td>
<td>0.30</td>
<td>70-76 (84:16 HB:HV)</td>
<td>[153]</td>
<td></td>
</tr>
</tbody>
</table>

* Yield calculated on a COD basis by using the coefficients: for HB: 1.67 g COD PHA/g PHA and for HV: 1.92 g COD PHA/g PHA.

3.4. Challenges and Perspectives Regarding PHA Production by Mixed Microbial Consortia

PHA process brought to an industrial scale using pure substrates and strains has broadly surpassed cell densities of 150 g/L with PHA contents up to 90% and productivities in the range from 1 to 3 g PHA/L-h [4]. Comparable productivities, of up to 1.2 g PHA/L-h, combined with high PHA yields, up to 0.8 Cmol PHA/Cmol S, have already been attained from MMCs using synthetic substrates [137]. Likewise, PHA contents up to 90% of the CDW have been reported [109]. Thus, MMC have proven to be able to achieve comparable results to pure cultures in synthetic media. Nevertheless, PHA productivities could be further increased if higher cell densities were obtained, a parameter that is usually below 10 g/L in MMC. In pure cultures, the PHA accumulation phase is preceded by a biomass growth phase in order to achieve high biomass densities [154]. In the accumulation phase, the feeding strategy is modified accordingly (usually limiting the nitrogen) to obtain a high PHA content. Nevertheless, in processes for PHA production from MMC, the biomass generation step is also the enrichment step. Thus, two objectives, which might not have the same optimal conditions, are combined in the same process unit. A future direction could be to test if adopting a microbial biomass generation step—leading to higher cell densities before the PHA accumulation—could maintain the high PHA content of the cells in MMC.

In order to achieve a sustainable production of PHA, both in economic and environmental terms, high productivities should also be obtained in waste substrates. In the current state of art, neither pure strains nor MMC have achieved high productivities when waste streams are used as substrates [83,97,155]. Thus, the challenge is common.

One of the main issues that compromises productivity when using waste streams is their diluted nature [155,156]. This applies for the case of MMC where an anaerobic fermentation step is usually performed in order to convert sugars to fatty acids. However, it is also the case with other industrial wastes used both in pure and mixed cultures, such as whey or lignocellulosic biomass, that requires pre-processing to release its sugars [83,155]. When these effluents are provided as feed in fed-batch PHA accumulations, they provoke substantial increases in the reactor operating volume, thus reducing the productivity.
A promising way to obtain high cell densities and productivities would be the use of cell recycling systems coupled to fed-batch processes [156]. This strategy has been scarcely applied to PHA production until now, with only one report using an MMC [139] and one study in pure strains [157]. The latter obtained cell densities up to 200 g/L with a productivity of 4.6 g PHA/L/h by using external cross-flow membranes to recirculate cells into the fed-batch reactor using C. necator [157]. Thus, the strategy seems to offer good opportunities to increase the cell density and PHA concentration. Likewise, reactor designs preventing the cells from escaping the system, while allowing supernatant removal, could result in high cell densities and reduced reactor volumes.

With the same scope, other research groups have proposed influent concentration. Although evaporation has been suggested [157], other less energy intensive methods such as forward osmosis membranes could more likely be applied [158,159]. A very interesting approach was recently published where forward osmosis Aquaporin® membranes, mimicking biological protein channels, were suggested to concentrate fermentation effluents from glycerol and wheat straw [160]. The novelty lied in the fact that the concentrated feedstock could be used as the water draw solution with the diluted fermentation effluent being the water feed solution. This enabled the recirculation of water from the effluent to the influent in an energy efficient way since the process was based solely on the use of forward osmosis membranes without the need of the costly regeneration of the draw solution (usually performed by applying the energy intensive reverse osmosis). Integration of such systems in the PHA production process could also enhance its productivity.

4. PHA Recovery

The development of new strategies and methodologies for PHA recovery is one of the main factors associated with the feasibility of a PHA production bio-refinery using microbial mixed fermentation. As demonstrated in Figure 2, PHA recovery uses a variation of different techniques. However, PHA purification generally requires five steps: biomass-harvesting, pre-treatment, PHA recovery, PHA accumulation, polishing, and drying. Biomass harvesting is the concentration of biomass using techniques such as filtration or centrifugation. As PHAs are intracellular polymers, it is necessary to concentrate the biomass prior PHA recovery. Nevertheless, some researchers have evaluated PHA recovery without biomass harvesting to facilitate process scale-up and to reduce costs. Pre-treatment’s main objective is to facilitate PHA retrieval from the microbial biomass; these techniques include drying techniques (lyophilization and thermal drying), grinding, chemical and biochemical pre-treatments, etc. The pre-treatment step can combine two or more methods. PHA retrieval phase utilizes two principal methods: PHA solubilization and the disruption of non-PHA cell mass (NPCM). In some cases, NPCM disruption precedes a PHA solubilization step. The PHA accumulation step is dependent on the retrieval technique utilized. In PHA solubilization, the PHA is concentrated by using alcohols precipitation. On the other hand, in NPCM disruption, recovery is performed by collecting the PHA granules. As final steps, recovered PHAs can be polished by removing residues, from the previous steps, or can be dried; depending on the separation steps utilized. As of 2013, two reviews specialized in PHA recovery were published [161,162]. This section primarily focuses in the most recent developments in pre-treatments and PHA retrieval steps; additionally, this section describes a more industrial opinion on the PHA recovery methods.
4.1. Pre-Treatments

Pre-treatments are chemical, physical, or biological methods employed to facilitate the retrieval of PHA. These methods focus on weakening the cell structure that protects and surrounds the PHA granules. After biomass harvesting, drying is the traditional pre-treatment used in PHA recovery, which includes heat drying and lyophilization; the latter is the most employed pre-treatment in PHA recovery processes. This technique removes the majority of water molecules in the biomass facilitating the posterior PHA extraction. Although lyophilization has interesting features, it has economic and technical difficulties that reduce its future application in an industrial PHA recovery process. In recent years, these industrial difficulties increased the interest in PHA recovery from wet biomass instead of dry biomass [163–165]. Lyophilization can be a unique pre-treatment or it can precede a further pre-treatment. Lyophilization has preceded thermal, mechanical, and chemical pre-treatments (see Table 5). All these treatments included a further retrieval technique associated with solvent extraction [166,167]. Samori et al. [166], described chemical pre-treatment with NaClO as the best pre-treatment for PHA recovery compared with thermal and mechanical pre-treatments; however, this pre-treatment generated an important reduction in the molecular weight of the polymer.
### Table 5. Pre-treatment techniques applied for PHA recovery

<table>
<thead>
<tr>
<th>Pre-Treatment</th>
<th>Further PHA Retrieval Treatment</th>
<th>Pre-Treatment Conditions</th>
<th>Purity (%)</th>
<th>Recovery (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>NaOH digestion</td>
<td>NaCl (8 g/L), 30 °C, 3 h</td>
<td>97.7</td>
<td>97.5</td>
<td>[163]</td>
</tr>
<tr>
<td>Ultrasonication</td>
<td>Aqueous two-phase extraction</td>
<td>Ultrasonication at 30 kHz per cycle 15 min</td>
<td>-</td>
<td>-</td>
<td>[164]</td>
</tr>
<tr>
<td>Sodium hypochlorite (NaClO)</td>
<td>Non-halogenated solvent extraction</td>
<td>NaClO (10%), 37 °C, 1 h</td>
<td>-</td>
<td>-</td>
<td>[165]</td>
</tr>
<tr>
<td>Thermal pre-treatment</td>
<td>Enzymatic digestion and chloroform extraction</td>
<td>Autoclave, 15 min 121 °C</td>
<td>94.1</td>
<td></td>
<td>[168]</td>
</tr>
<tr>
<td>Thermal pre-treatment ¹</td>
<td>Non-halogenated solvent extraction</td>
<td>150 °C, 24 h</td>
<td>50</td>
<td></td>
<td>[166]</td>
</tr>
<tr>
<td>Ultrasonication and glass beds ¹</td>
<td>Non-halogenated solvent extraction</td>
<td>Glass beads (0.5 mm) and Ultrasonication (10 pulses of 2 min)</td>
<td>50</td>
<td></td>
<td>[166]</td>
</tr>
<tr>
<td>Sodium hypochlorite ¹</td>
<td>Non-halogenated solvent extraction</td>
<td>NaClO (5%), 100 °C, 15 min</td>
<td>93</td>
<td>82</td>
<td>[166]</td>
</tr>
<tr>
<td>Hot acetone ¹</td>
<td>Non-halogenated solvent extraction</td>
<td>Acetone, 100 °C, 30 min</td>
<td></td>
<td></td>
<td>[167]</td>
</tr>
</tbody>
</table>

¹The pre-treatment included a previous lyophilization step.
Pre-treatments without lyophilization consisted of chemical and physical methods, with chemical methods including sodium chloride (NaCl) and sodium hypochlorite (NaClO). Anis et al. [163], employed NaCl as a pre-treatment for a NaClO digestion. The additional pre-treatment step generated an increment in purity and yield. NaCl pre-treatment modifies the osmotic conditions in the medium leading the cells to dehydrate and shrink, this destabilizes the cell membranes facilitating the PHA granules' liberation. Physical methods include high temperature and ultrasonication methods both methods anteceded NPCM digestion methods. Neves and Muller [168] evaluated three temperatures (121, 100, and 80 °C) during 15 min as pre-treatment conditions. The 121 °C treatment achieved the best results; whereas, 100 and 80 °C treatments recovered significantly lower amounts of PHA. The heat treatment improves the PHA removal by denaturizing proteins, DNA and RNA, destabilizing the microbial cell wall and inactivating the PHB depolymerase [168]. Ultrasonication employs sound waves to create disruption in the cell wall and open the cytosolic material to the aqueous medium. Leong et al. [164] utilized ultrasonication prior an aqueous two-phase extraction. The advantages of this method are the lack of any previous cell harvesting method and the fast pace in which is performed.

Pre-treatments can increase the recovery and purity of the PHA extracted from a fermentation broth. However, their implementation in a PHA industrial recovery process needs to be evaluated using economic and technical analysis. The yield and purity increment should counterbalance with the additional cost associated with the introduction of this step in the purification line. Additionally, it is important to remark that even though several pre-treatments can be used, not all of them are suitable for industrial applications. Industrially suitable pre-treatments require the use of wet biomass or unharvested biomass in order to reduce the number of purification steps and the costs associated with the purification process. Lyophilization use should concentrate on PHA chemical analyses or be replaced with more suitable drying techniques for process scale up. The selection of a pre-treatment is dependent of the bacterial strain, fermentation broth characteristics, and further PHA application; therefore, each PHA process needs to be analyzed individually from an economic, environmental, and technical point of view.

4.2. Retrieval Techniques

4.2.1. Non-PHA Cell Mass (NPCM) Disruption

In recent years, the use of NPCM disruption as a tool for retrieving PHA from bacterial biomass has increased. This increment is associated with the necessity of environmental and safe options to replace the use of halogenated solvents used in the traditional PHA extraction methods. Additionally, some of the NPCM techniques for the PHA extraction have used wet biomass or unharvested biomass, which is an advance through the reduction of purification steps and costs. This review grouped the novel NPCM disruption techniques into chemical, enzymatic, and biological disruption.

Chemical Disruption

NPCM chemical disruption includes methods that utilize chemical compounds to disrupt bacterial cell wall and the denaturalization or degradation of cytosolic material. The three principal methods for the chemical disruption are sodium hydroxide (NaOH), sodium hypochlorite (NaClO), and sodium dodecyl sulphate (SDS). Additional chemical treatments include water and acid treatments. NaOH treatment destabilizes the cell wall by reacting with the lipid layer in a saponification reaction and increases the cell membrane permeability [163]. NaOH treatment has obtained high recovery and purification percentage using pre-treated and unpretreated biomass (see Table 6). However, pre-treated biomass aided the NaOH treatments to achieve improvements in purity and recovery [163,169]. The combination of NaCl pre-treatment and NaOH digestion increased the purity and recovery of NaOH treatment by approximately 10% [163]. Similarly, lyophilization and freezing helped to increase the PHA purity using NaOH; however, these pre-treatments did not increase the recovery percentage [169]. López-Abelairas et al. [170] described
a recovery reduction in treatments with high biomass and NaOH concentrations. Biomass concentration above 2.5% created a constant reduction in recovery and purity; however, biomass concentrations of 7.5% and 10% yielded the greatest recovery reductions. NaOH concentrations over 0.5 N affected recovery, although, the purity percentage at high percentage was constant. Likewise, Villano et al. [171] achieved recovery between 80–87% utilizing high NaOH (1 M), high biomass concentration (6:1 biomass:chemical solution) and extended extraction times (6–24 h). However, the purity achieved by this treatment was below 60%.

Sodium hypochlorite (NaClO) has confirmed its positive aspects as an NCMP disruption treatment [161,162]. Therefore, in recent years, the assessments of this treatment advanced to larger scales or continuous processes [171,172]. A continuous sequential process for PHA production and recovery obtained high polymer recovery (100%) and purity (98%). This continuous process contains three steps: a production step using microbial mixed culture, a PHA accumulation step, and a PHA extraction step using NaClO (5%) disruption for 24 h. This approach produced 1.43 g PHA/L·d and was stable for four months [171].

Another chemical disruption treatment is sodium dodecyl sulphate (SDS). This surfactant is a well-known detergent used in the recovery of genetic material. SDS treatment obtained recovery and purity values comparable with other chemical disruption techniques; moreover, this disruption technique obtained similar retrieval with and without biomass pre-treatment [169,173]. The amount of SDS varied between 0.025% and 0.2%, higher concentrations of SDS generated higher purity as result of SDS micelles formation. High micelles production is also associated with the solubilization of PHA granules generating a reduction in the recovery yield. SDS has complemented NaOH disruption, this combination exhibited superior levels of purity, especially in the removal of hydrophobic impurities [169].

Besides the previous treatments, other authors have described water and acid disruption methods as effective treatments for NPCM disruption. Distilled water achieved high purity (94%) and recovery (98%) percentages; however, the process needed 18h and lyophilized biomass to reach these high percentages. The process duration improved by adding SDS (0.1%) into the mixture [173]. In contrast, distilled water disruption treatment with wet biomass obtained recovery (80%) and purity (58%) percentages lower than lyophilized biomass. Mohammadi et al. [174], described a higher purity and recovery yield using distilled water disruption with recombinant bacteria instead of wild bacteria. Recombinant bacterial cell wall is thinner than in wild type bacteria, which facilitates the cell wall breaking by osmotic pressure. Acid treatments have demonstrated their capability to disrupt NPCM; López-Abelairas et al. [170] described a recovery and purity percentage using a sulphuric acid solution (0.64 M) similar to alkaline treatments (NaOH, NaClO). They selected acid disruption as the best recovery method focused in operational and environmental factors. The authors chose acid disruption because this process had lower cost, environmental impact (greenhouse gas emissions), and polymer degradation than alkaline treatments [170].

Chemical disruption treatments are a significant option in PHA recovery since they present environmental and economic advantages over the traditional PHA extraction using halogenated solvents. Environmentally, chemical disruption avoids the use of toxic solvents such as chloroform. Economic advantages include liquid current recycling, the use of wet biomass, and the reagent cost. The principal drawback for chemical disruption has been polymer degradation; however, the use of mixtures of chemicals and process optimization has reduced polymer degradation. The selection of a chemical disruption method for PHA recovery from mixed microbial cultures needs an all-around evaluation of technical, economic, and environmental factors that consider the positive and negative effects and how they can affect the feasibility of a PHA bio-refinery plant.

Enzymatic NPCM Disruption

Enzymatic NPCM disruption utilizes purified enzymes or crude extracts to disrupt the bacterial cell wall. Proteases are the principal enzymatic activities employed in enzymatic disruption; however, other types of enzymes or enzymatic cocktail have effectively degraded NCMP. Enzymatic disruption advantages include their low energy requirements, aqueous recovery, and low capital
investment; in contrast, the enzymes production cost is the principal disadvantage for industrial implementation [175]. Gutt et al. [176], evaluated the recovery of P3HBHV from *Cupriavidus necator* by several methods including enzymatic disruption. Simple enzymatic treatment (lysozyme) obtained low recovery and purity (Table 6). The authors attributed these low percentages to the absence of additional chemical or mechanical treatments, which have proved necessary for achieving high recovery and purity. Martino et al. [177] evaluated enzymatic disruption using simultaneously enzymatic (Alcalase) and chemical treatments (SDS and EDTA). SDS and EDTA contributed to cell wall and membrane lysis whereas Alcalase solubilized the cytosolic material. This enzymatic/chemical digestion treatment eliminated the requirement of heat pre-treatment used in previous enzymatic disruption researchers [161,162]. Kachrimanidou et al. [175], developed a novel enzymatic disruption method by using crude enzymes from solid-state fermentation of *Aspergillus awamori*. This method achieved good recovery (98%) and purity (97%) without using additional chemicals; however, it required heat and lyophilization as pre-treatments. Approaches focused in the reduction of enzyme costs are necessary to facilitate the industrial application of enzymatic disruption including the use of immobilized enzymes, integration of enzymes production as part of a PHA biorefinery, and genetically engineering enzymes.

Biological NPCM Disruption

Biological disruption utilizes biological agents (virus) or organisms to liberate PHA from bacterial cells. The first biological disruption technique used viral particles to break bacterial cells. Bacteriophages were included in bacterial lines to utilize the viruses’ lytic cycle to liberate PHA granules. When the lytic cycle is completed, the virus escapes from the host cell by breaking down the cell wall; this breaking down also liberates the PHA particles allowing their recovery [162]. In recent years, biological disruption methods included bacteria predators, rats, and mealworms [178–180].

Martinez et al. [181], proposed the use of obligate predatory bacteria *Bdellovibrio bacteriovorus* as an innovative cell lytic agent suitable to recover intracellular bioproducts such as PHA. *B. bacteriovorus* achieved a PHA recovery of 60%, the recovery percentage obtained was attributed to PhaZ depolymerase activity which hydrolyses PHA and expresses during all the stages of *B. bacteriovorus*’s life cycle [181]. To improve the use of *B. bacteriovorus* in PHA recovery, Martinez et al. [180], developed *B. bacteriovorus* mutant strains, one with an inactive medium-chain-length PHA depolymerase (*B. bacteriovorus* Bd3709) and another with inactive short-chain-length-PHA depolymerase (*B. bacteriovorus* Bd2637). *B. bacteriovorus* Bd3709 increased PHA recovery from 60 to 80% when predating *Pseudomonas putida*, whereas, *B. bacteriovorus* Bd2637 increased PHB recovery from 48% to 63% when predating PHB-accumulating *E. coli* ML35 [180]. Biological recovery using *B. bacteriovorus* has advantages such as avoiding the use of cell harvesting and pre-treatments. However, it has disadvantages such as the use of organic solvent steps, processing time, and low recovery. In the future, this biological method can be complemented with other recovery treatments to avoid solvents’ usage and to increase PHA recovery.

In recent years, authors have used complex organisms’ digestive system as a NPCM disruption technique. In these treatments, different organisms were fed with PHA-rich bacteria; afterwards, PHA granules were recovered from these organism’s feces. This process selectively digested the NPCM without reducing the PHA molecular weight. Kunasundari et al. [182], fed Sprague Dawley rats with lyophilized cells of *Cupriavidus necator* in a single cell protein diet during several days. The fecal pellets were whitish and rich in PHA (82–97%). The authors also demonstrated the safety and tolerability of the Sprague Dawley rats to a *Cupriavidus necator* diet. Kunasundari et al. [179], studied the purification of the Sprague Dawley PHA-rich fecal pellets using water and surfactants. The use of SDS 2% as a further purification step increased the purity of the PHA biological recovered at levels similar to solvent extraction. Similar to Sprague Dawley rats, Murugan et al. [180] fed lyophilized bacteria to mealworms and recovered PHA granules from their fecal pellets. The PHA granules had an 89% purity when washed with water and reached almost 100% purity when treated with SDS. The authors reported higher protein content in mealworms fed with *C. necator* cells than
mealworms fed with oats. Mealworms fed with *C. necator* can be an alternative protein source in aquaculture and poultry diets. Biological NPCM disruption is an alternative to other disruption methods; it does not require expensive instrumentation, solvents, or strong chemicals, and the organisms doing the PHA recovery can be a marketable product too. However, the biological recovery process takes longer than any other recovery process and needs biomass pre-treatment. Depending on the final use, PHA biological recovery can be an integrated process for renewable production of feed, food, and materials.
Table 6. NPCM chemical disruption treatments.

<table>
<thead>
<tr>
<th>NPCM Digestion Type</th>
<th>NPCM Disruption Method</th>
<th>Pre-Treatment</th>
<th>PHA Accumulation Method</th>
<th>Disruption Conditions</th>
<th>Microbial Strain</th>
<th>PHA Content in Biomass (%)</th>
<th>Purity (%)</th>
<th>Recovery (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>NaOH</td>
<td>Chem Pre-treatment</td>
<td>Centrifugation</td>
<td>NaOH (0.1 M), 30 °C, 1 h, 350 rpm</td>
<td>C. necator</td>
<td>68</td>
<td>90.8</td>
<td>95.3</td>
<td>[163]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaOH</td>
<td>Chem Pre-treatment</td>
<td>Centrifugation</td>
<td>NaOH (0.1 M), 30 °C, 1 h, 350 rpm</td>
<td>C. necator</td>
<td>68</td>
<td>82.7</td>
<td>94.4</td>
<td>[163]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaOH</td>
<td>Lyophilization</td>
<td>Centrifugation</td>
<td>NaOH (0.1 M), 30 °C, 1 h, 350 rpm</td>
<td>C. necator</td>
<td>68</td>
<td>80–90</td>
<td>80–90</td>
<td>[183]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaOH</td>
<td>Lyophilization</td>
<td>Centrifugation</td>
<td>NaOH 0.05 M, 3 h, 0 rpm, 4 °C</td>
<td>C. necator</td>
<td>30</td>
<td>98.6</td>
<td>96.9</td>
<td>[174]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaOH</td>
<td>Lyophilization and milling</td>
<td>Centrifugation</td>
<td>NaOH (0.5 N), 4 h, 37 °C, 500 rpm</td>
<td>C. necator</td>
<td>65</td>
<td>93</td>
<td>80</td>
<td>[170]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaOH</td>
<td></td>
<td>Centrifugation</td>
<td>NaOH (0.2 M), 200 rpm, 30 °C, 1 h</td>
<td>Partial Culture</td>
<td>62–72</td>
<td>87</td>
<td>97</td>
<td>[169]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaClO</td>
<td></td>
<td>Centrifugation</td>
<td>NaClO (5%) 24 h</td>
<td>Mixed Culture</td>
<td>46</td>
<td>90</td>
<td>–100</td>
<td>[171]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaClO</td>
<td>Mechanical pre-treatment</td>
<td>Precipitation</td>
<td>NaClO 13% (v/v), room temperature, 1 h</td>
<td>Ralstonia eutropha</td>
<td>65.2</td>
<td>95.6</td>
<td>91.3</td>
<td>[172]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaClO</td>
<td>Lyophilization and milling</td>
<td>Centrifugation</td>
<td>NaClO (13%), 37 °C, 500 rpm, 4 h</td>
<td>C. necator</td>
<td>65</td>
<td>97</td>
<td>82</td>
<td>[170]</td>
</tr>
<tr>
<td>Chemical</td>
<td>NaOH and SDS</td>
<td></td>
<td>Centrifugation</td>
<td>NaOH (0.2 M) and SDS (0.2 %), 200 rpm, 30° C, 1 h</td>
<td>Mixed Culture</td>
<td>62–72</td>
<td>99</td>
<td>91</td>
<td>[169]</td>
</tr>
<tr>
<td>Chemical</td>
<td>SDS</td>
<td></td>
<td>NaClO and Centrifugation</td>
<td>SDS (0.1%), 24 h</td>
<td>H. mediterranei</td>
<td>70</td>
<td>–100</td>
<td>97</td>
<td>[55]</td>
</tr>
<tr>
<td>Chemical</td>
<td>SDS</td>
<td></td>
<td>Centrifugation</td>
<td>SDS (0.1%), 24 h</td>
<td>H. mediterranei</td>
<td>71.2</td>
<td>–100</td>
<td>97</td>
<td>[56]</td>
</tr>
<tr>
<td>Chemical</td>
<td>SDS</td>
<td></td>
<td>Centrifugation</td>
<td>SDS (0.1%), 60 °C, 2 h</td>
<td>Halomonas sp. SK5</td>
<td>48</td>
<td>94</td>
<td>98</td>
<td>[173]</td>
</tr>
<tr>
<td>Chemical</td>
<td>H2SO4</td>
<td>Lyophilization and milling</td>
<td>Chemical treatment and Centrifugation</td>
<td>H2SO4 (0.64 M), 6 h, 80 °C</td>
<td>C. necator</td>
<td>65</td>
<td>98</td>
<td>79</td>
<td>[170]</td>
</tr>
<tr>
<td>Chemical</td>
<td>Water</td>
<td>Lyophilization</td>
<td>Centrifugation</td>
<td>dH2O, 30 °C, 1 h</td>
<td>Comamonas sp. Halomonas sp.</td>
<td>30</td>
<td>80.6</td>
<td>96</td>
<td>[174]</td>
</tr>
<tr>
<td>Chemical</td>
<td>Water</td>
<td>Lyophilization</td>
<td>Centrifugation</td>
<td>dH2O, 30 °C, 18 h</td>
<td>Comamonas sp. Halomonas sp.</td>
<td>48</td>
<td>94</td>
<td>98</td>
<td>[173]</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>Alcalase, SDS and EDTA</td>
<td></td>
<td>Centrifugation</td>
<td>Alcalase (0.3 U g−1), SDS (0.3 g g−1), EDTA (0.01 g g−1), NaH2PO4 buffer, 150 rpm, 55 °C, 1 h</td>
<td>C. necator</td>
<td>37</td>
<td>94</td>
<td>97</td>
<td>[177]</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>Crude extract</td>
<td>Heat treatment and lyophilization</td>
<td>Centrifugation</td>
<td>NaH2PO4-citric acid buffer and 47 °C</td>
<td>C. necator</td>
<td>78.9</td>
<td>98</td>
<td>97</td>
<td>[175]</td>
</tr>
</tbody>
</table>

104
<table>
<thead>
<tr>
<th>Type</th>
<th>Organism</th>
<th>Preparatory Method</th>
<th>Treatment Details</th>
<th>Recovery (%)</th>
<th>Growth Rate (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic</td>
<td>Lysozyme</td>
<td></td>
<td>Lysozyme solution (2 mg/mL). 1 h, 3 °C</td>
<td>C. necator</td>
<td>41</td>
<td>100</td>
</tr>
<tr>
<td>Biological</td>
<td>Mealworm (Tenebrio molitor)</td>
<td>Lyophilization</td>
<td>50 g of mealworms fed 5% of their body weight per day for 16 days.</td>
<td>C. necator</td>
<td>37</td>
<td>89%</td>
</tr>
<tr>
<td>Biological</td>
<td>Sprague Dawley rats</td>
<td>Lyophilization and grinding</td>
<td>150–200 g rats were fed 15 g/day/animal, 28 days 25 °C</td>
<td>C. necator</td>
<td>37</td>
<td>89.3</td>
</tr>
<tr>
<td>Biological</td>
<td>Sprague Dawley rats</td>
<td>Lyophilization</td>
<td>150–200 g rats were fed 15 g/day/animal, 28 days 25 °C</td>
<td>C. necator</td>
<td>54</td>
<td>82–97</td>
</tr>
<tr>
<td>Biological</td>
<td>Bdellovibrio bacteriovorus HD100</td>
<td>Lyophilization</td>
<td>Water</td>
<td>P. putida</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>Biological</td>
<td>Bdellovibrio bacteriovorus HD100 and Bd3709</td>
<td>Lyophilization</td>
<td>Centrifugation P. putida was inoculated with B. bacteriovorus strains 48 h, 30 °C</td>
<td>P. putida</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. putida and E. coli cultures were inoculated with B. bacteriovorus strains 48 h, 30 °C</td>
<td></td>
<td></td>
<td>[180]</td>
</tr>
</tbody>
</table>
4.2.2. PHA Extraction

As PHA are produced inside the cellular biomass, in order to be retrieved they have to be separated from the non-PHA cell mass (NPCM). The simplest, least destructive to biopolymer and most direct way for PHA is to be extracted from the biomass; significant quantities of hazardous solvents and energy input are required for this, creating a potential counterbalance to sustainability and economics towards commercialization [184].

Several studies have proposed various solvent extraction methods for PHA recovery that improve parameters such as yield, purity, and cost of extraction, while at the same time maintaining the physicochemical properties of the biopolymer [185].

Non-Halogenated Solvents

Although several types of extraction systems exist for the production of biopolymers, the majority of extraction methods for PHA still involve the use of organic solvents in which the polymer is soluble. Fei et al. [186], aimed to develop an effective and environmentally-friendly solvent system so as to extract PHB from bacterial biomass. In order to accomplish that, they used a solvent mixture of acetone/ethanol/propylene carbonate (A/E/P, 1:1:1 v/v/v) for extracting PHB from Cupriavidus necator. When the A/E/P mixture was used at high temperature, it could recover 92% pure PHB with 85% yield from dry biomass, and 90% purity with 83% PHB yield from wet biomass. Additionally, if hexane was added, it could further enhance the purity and recovery quantities of PHB.

Bacterial PHA could be used for medical applications due to its biocompatibility. However, using inappropriate solvents or techniques during extraction of PHA from bacterial biomass could result in contamination by pyrogenic compounds (e.g., lipopolysaccharides), which eventually leads to rejection of the material for medical use. This problem could be overcome by using a temperature-controlled method for the recovery of poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) from Pseudomonas putida GPo1. Non-chlorinated solvents were found to be the optimal solvents for such tests and, specifically, n-hexane and 2-propanol. The purity reached more than 97% (w/w) and the endotoxicity between 10–15 EU/g PHA. Further re-dissolution in 2-propanol at 45 °C and precipitation at 10 °C resulted in a purity of nearly 100% (w/w) and endotoxicity equal to 2 EU/g PHA [187].

Another approach, however, is that the use of aqueous solvents could benefit the integration into a biorefinery scheme. A study aimed at connecting the exploitation of a raw biowaste, such as used cooking oil (UCO), and producing a desired final product (i.e., amorphous granules of PHA) based on aqueous solvents in a way that will prove the effective reliability of an overall biotechnological approach. Used cooking oil was utilized as the only carbon source for the production of PHB by cultivating Cupriavidus necator DSM 428 in batch reactors. The PHB granules were extracted from the biomass using sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and the enzyme Alcalase in an aqueous medium. The PHB granule recovery reached more than 90% and highly pure amorphous polymer was finally obtained [177].

A different alternative to the use of halogen-free and environmental-friendly methods implemented the use of water and ethanol for the recovery of PHA from recombinant Cupriavidus necator, in comparison to the well-established chloroform extraction method. Comparing the results obtained from experiments under different incubation times (1, 3, and 5 h) and temperatures (4 and 30 °C) showed that the optimized halogen-free method produced a PHA with 81% purity and 96% recovery yield, whereas the chloroform extraction system resulted in a highly pure PHA with 95% recovery yield. This method could potentially be developed as an alternative and more environmentally-friendly method for industrial application [188].

Aqueous Two-Phase Extraction Systems (ATPS)

In addition to the conventional isolation and purification methods, such as solvent extraction, aqueous two-phase systems (ATPS) have many advantages and important characteristics that attract
the attention of researchers and industries. The main advantages are that because ATPS comprise of high water content (70–90% w/w), thus they provide a beneficial environment for separation of sensitive biomaterials. Also, the materials that form the different phases/layers of ATPS are, in principle, safe and environmentally-friendly compared to conventional solvent extraction methods; additionally, an intricate ability for high capacity processing which leads to reduced purification steps; finally, large-scale purification using ATPS can be easily and reliably predicted from laboratory experimental data. ATPS is a feasible solution for industrial demand of cost-effective and highly efficient large-scale bioseparation technologies with short processing times [189],[190].

Regarding PHA retrieval methods, one interesting approach is the use of aqueous two-phase systems with the aim of enhancing the accumulation of PHA in one phase using environmentally-friendly layer-forming constituents. This method offers advantages of supporting a beneficial environment for bioseparation, capability of handling high operating capacity, and reducing downstream processing volume, thus proving extractive bioconversion via ATPS can be an optimum solution. Leong et al. [189], examined the effect of pH and salts’ addition in Ralstonia eutropha H16 cultures, using an ATPS system as a mechanism for PHA extraction. The optimum result obtained in this study was a PHA concentration of 0.139 g/L (purification factor: 1.2–1.63) and recovery yield 55–65% using ATPS of polyethylene glycol (PEG) 8000/sodium sulphate adjusted to pH 6 and the addition of 0.5 M NaCl.

In another study using ATPS, the thermal separation of the phases and how it affected the PHA extraction was studied. The most important ATPS parameters (type and concentration of thermoseparating polymer, salt addition, feedstock load, and thermoseparating temperature) were optimized in order to achieve high PHA retrieval from the bacterial lysate. By taking advantage of the properties of the thermo-responsive polymer (whose solubility decreases in its aqueous solution as the temperature increases), cloud point extraction (CPE) is an ATPS technique that offers the capability to its phase-forming component to be reused. Extraction of PHA from Cupriavidus necator H16 via CPE was investigated. The best conditions for PHA extraction (recovery yield of 94.8% and purification factor 1.42) were reached under the following conditions: 20 w/w% ethylene oxide-propylene oxide (EOPO), 10 mM NaCl addition, and a thermoseparating temperature of 60 °C with crude feedstock limit of 37.5 w/w%. Another benefit of this process is the ability to recycle and reuse EOPO 3900 at least twice, achieving a satisfying yield and purification factor [164].

5. Conclusions

Wide production, commercialization, and thus application of PHAs as a biodegradable alternative to conventional plastics is still limited due to high production cost. Bioprocess technologies are still being developed while bacterial resources are still being explored.

Pure cultures are constantly investigated for their potential to valorize waste byproducts as a low-cost feedstock. The ability of certain bacteria to directly utilize lignocellulosic biomass as the carbon source for PHA production is a huge bioprocess advantage. In this case, the need for chemicals, energy, and labor is minimized. PHA production can be also used as a tool for the bioremediation of oil contaminated sites, as bacterial strains can degrade environmental pollutants, minimizing their toxicity and environmental impact. Moreover, halophilic PHA producers combine a series of advantages, such as growth on seawater and possibility of continuous processes under non-sterile conditions. Those features will significantly contribute to the PHA cost reduction and minimize the requirements for fresh water. Synthetic biology tools are expected to aid in the enhancement of PHA production efficiency, simplify downstream process, and regulate PHA composition providing customized materials for specific applications. However, robust strains are yet to be developed.

Efforts have been also made towards the PHA production using mixed microbial cultures (MMC). MMC-based processes, apart from offering a reduction in the operational costs and the possibility to adapt to a wider range of waste substrates, they could be integrated in current wastewater treatment plants. Recent developments regarding different enrichment strategies and
the PHA production step offer new opportunities to make the PHA production more feasible. Cell densities and derived productivities attained with MMC are the main current bottleneck.

The development of economic and simple downstream processes is crucial for the recovery of PHAs. Methods based on the utilization of environmentally friendlier techniques are constantly being investigated, with enzymatic methods advancing the bio-based profile of the process. Reduction of large amounts of chemicals, used per cell dry mass, is going to benefit the economics of the process as well as society and the environment.

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Polyhydroxyalkanoates (PHA) production from fermented crude glycerol: Study on the conversion of 1,3-propanediol to PHA in mixed microbial consortia

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Abstract
Crude glycerol, a by-product from the biodiesel industry, can be converted by mixed microbial consortia into 1,3-propanediol (1,3-PDO) and volatile fatty acids. In this study, further conversion of these main products into polyhydroxyalkanoates (PHA) was investigated with the focus on 1,3-PDO. Two different approaches for the enrichment of PHA accumulating microbial consortia using an aerobic dynamic feeding strategy were applied. With the first approach, where nitrogen was present during the whole cycle, no net production of PHA from 1,3-PDO was observed in the fermented effluent, not even in a nitrogen-limited PHA accumulation assay. Nevertheless, experiments in synthetic substrates revealed that the conversion of 1,3-PDO to PHA was possible under nitrogen limiting conditions. Thus, a different enrichment strategy was formulated where nitrogen was limited during the feast phase to stimulate the storage response. Nitrogen was still supplied during the famine phase. With the latter strategy, a net production of PHA from 1,3-PDO was observed at a yield of 0.24 Cmol PHA/Cmol 1,3-PDO. The overall yield from the fermented effluent was 0.42 Cmol PHA/Cmol substrate. Overall, the PHA yield from 1,3-PDO seemed to be limited, similarly to when using glycerol as a substrate, by a decarboxylation step and accumulation of other storage polymers such as glycogen, and possibly, lipid inclusions.

1. Introduction

When exposed to unbalanced growth conditions, some bacteria have the ability to store carbon and energy in the form of intracellular compounds such as lipids (e.g. triacylglycerols and wax esters), polyphosphates, carbohydrates (e.g. glycogen) or polyhydroxyalkanoates (PHA) (Dias et al., 2006; Thomson et al., 2010). PHA are polyesters described in more than 300 microorganisms with a diverse monomer composition, although the most commonly reported polymers are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) (Dias et al., 2006). PHA present similar properties to polyethylene and polypropylene, and therefore they have been proposed as a possible renewable and biodegradable bio-based alternative to these petroleum-derived plastics (Laycock et al., 2014). Nevertheless, the market size is still limited, mostly due to the elevated costs of PHA production. The use of waste substrates and/or mixed microbial consortia are of increasing interest as strategies to reduce production cost (Laycock et al., 2014; Serafin et al., 2008).

Crude glycerol is among the substrates recently tested for PHA production. This by-product of the biodiesel industry represents 10% of the biodiesel output and contains on average 70% glycerol, among other impurities such as methanol, salts or fats (Zhu et al., 2013). Limited research has been conducted so far in respect to the production of PHA in mixed microbial consortia using crude glycerol as a substrate (Dobroth et al., 2011; Freches and Lemos,
2017; Moita et al., 2014). In synthetic glycerol, Moralejo and colleagues (Moralejo-Garate et al., 2011) obtained a PHB content of 67% of the cell dry weight and surpassed the results attained with pure strains in terms of specific productivity and yield, reaching these parameters values of 0.34 g PHA/g biomass.h and 0.57 Cmol PHA/Cmol S, respectively.

Nevertheless, the production of PHA from glycerol has three main drawbacks: 1) the side production of glycozyme (Moita et al., 2014; Moralejo-Garate et al., 2013), 2) the reduced molar mass of the polymer due to glycerol chain termination (Zhu et al., 2013) and 3) the production of HB as only monomer, which has less desirable thermal properties than PHB copolymers (Laycock et al., 2014; Zhu et al., 2013). For these reasons a three step process was chosen in the present work, where a fermentation of glycerol into volatile fatty acids (VFA), recognized as preferred substrates for PHA production, was performed prior to the culture selection and PHA accumulation (Serafim et al., 2008). To our knowledge, this strategy has only been tested once using crude glycerol as a substitute (Shen et al., 2014). Nonetheless, it has never been tested in fermentation effluents containing 1,3-propanediol (1,3-PDO), a major metabolite of glycerol fermentation. As a matter of fact, production of PHA from 1,3-PDO has rarely been studied and never described in mixed consortia. Such conversion has only been reported in recombinant bacteria (e.g. Meng et al., 2012) and in two defined strains producing a mixture of medium and long chain PHA and poly(3-hydroxybutyrate-co-3-hydroxypropionate) (Kimura et al., 2002; Lee et al., 1995), but no conversion yields have been reported.

The most efficient enrichment strategy so far developed for the PHA production in mixed consortia is the aerobic dynamic feeding (ADF), based on the transient limitation of substrate in feast/famine cycles performed in sequential batch reactors (SBR) (Dias et al., 2006; Serafim et al., 2008). The availability of nitrogen during the famine phase is crucial for PHA-accumulating microorganisms to proliferate and overgrow non-accumulating bacteria (Albuquerque et al., 2007). Conversely, PHA yields are generally higher in the absence of nitrogen during the PHA accumulation phase (Johnson et al., 2010a; Marang et al., 2014; Serafim et al., 2004). This discrepancy, together with the incapacity of certain bacteria to accumulate PHA in the presence of nitrogen (Lee, 1996), set the ground for an alternative enrichment strategy. Nitrogen limitation was applied during the feast phase to promote PHA accumulation, while nitrogen was supplied for growth during the famine phase. The same strategy was very recently reported to increase the PHA yield and productivity using cheese whey as a substrate (Oliveira et al., 2016).

This study focuses on the production of PHA from the two main substrates of the glycerol fermentation: VFA and 1,3-PDO, and evaluates the results obtained from a conventional ADF enrichment strategy and an alternative enrichment strategy based on the nitrogen limitation during the feast phase.

2. Materials and methods

2.1. Crude glycerol fermentation

Crude glycerol fermentation run in a 2.5 L Minifors reactor (INFORS HT) with a working volume of 1.7 L operated under continuous mode (CSTR). The reactor ran at a hydraulic retention time (HRT) of 12 h at 37 °C and an agitation of 150 rpm under non-sterile anaerobic conditions. The pH was maintained at 5.5 with 4 M KOH. The feed medium consisted of BA (Basic Anaerobic) growth medium supplemented with crude glycerol to a final glycerol concentration of 10 g/L (14.74 g crude glycerol/L). Both the medium and the crude glycerol characteristics are reported elsewhere (Varrone et al., 2015). The reactor was inoculated (10% v/v) with anaerobic sludge previously submitted to an enrichment process (Varrone et al., 2015), and later cultivated under the abovementioned CSTR conditions for 116 HRT (Varrone et al., 2017). Nitrogen in the feed was progressively lowered from a C:N:P ratio of 100:5.7:0.5 to a ratio of 100:1:3:0.5 to obtain an effluent with less than 10 mg/l N-NH_3 (2.56 ± 1.74). Distribution of products in the steady state was as follows (g/L): 1,3-PDO: 4.15 ± 0.52, butyrate: 1.66 ± 0.2, propionate: 0.78 ± 0.26, acetate: 0.19 ± 0.07, glycerol: 0.18 ± 0.21, TSS: 0.89 ± 0.21. Measured metabolites accounted for an average of 11.6 ± 0.4 g COD/L, while the total COD was around 14 g COD/L. The effluent was kept at 4 °C and at ~20 °C for short and long term storage, respectively. Before PHA production, it was centrifuged (15 min at 4000 g) and filtered (1 μm).

2.2. Sequential batch reactors (enrichment of PHA accumulating microorganisms)

Enrichment of PHA accumulating microorganisms was performed in the same reactors as the fermentation, but operated in a Sequential Batch Reactor (SBR) mode under a feast-famine regime and non-sterile conditions. Two types of enrichments were evaluated, one with nitrogen availability during the whole cycle (SBR_N) (Fig. 1A), and another one with nitrogen limitation during the feast phase (SBR_noN) (Fig. 1B).

2.2.1. Enrichment under nitrogen availability (SBR_N)

The reactor was initially inoculated with 30% inoculum consisting in equal parts of anaerobic sludge from Lundtofte wastewater plant (Lyngby, Denmark) and aerobic secondary sludge from Daka EcoMotion Biodiesel wastewater treatment plant (Løsning, Denmark). Every 12 h, half of the volume of the reactor (850 mL) was replaced with fresh medium using timer-controlled external pumps. No settling phase was allowed, acquiring a HRT and solids retention time (SRT) of 1 day. Aeration was supplied at a rate of 1 L/min with an agitation of 500 rpm. pH was maintained at 8 with 1 M NaOH and 0.5 M HCl. Temperature was set at 30 °C. The reactor and the tubing material were emptied and cleaned once a week to avoid excessive biofilm formation.

The medium was prepared with clarified fermentation effluent diluted with distilled water (320 mL of effluent per L of medium on average) to obtain a concentration of 90 C_mmol (mM of carbon) (45 C_mmol at the beginning of the cycle), which resulted in an organic loading rate (OLR) of around 4.4 g COD/m^3d (day). Nitrogen and phosphorous were initially adjusted to a C:N:P molar ratio of 100:8:1 with NH_4Cl and K_2HPO_4 and later on to a molar ratio of 100:12:1.5. Allylthiourea (5 mg/L) (Marang et al., 2014) and Anti-foam 204 (Sigma Aldrich) (85 μL/L) were supplied to inhibit nitrification and foaming, respectively.

The reactor was controlled with the Iris Software (INFORS HT) monitoring online the dissolved oxygen concentration (pO_2). Periodically, a whole cycle was fully characterized by harvesting offline samples. The stability of the reactor was assessed based on the duration of the feast and famine phases (determined by the pO2 profile) and PHA content at the end of the feast phase.

2.2.2. Enrichment under nitrogen limitation during the feast phase (SBR_noN)

SBR_noN was operated under nitrogen limitation during the feast phase and nitrogen availability during the famine phase (Fig. 1B). The reactor was inoculated from the culture from SBR_N and was initially operated with a SRT and HRT of 4 days, where one fourth of the volume of the reactor (425 mL) was replaced with new medium every 24 h. Nevertheless, after cycle 24, the cycle length was adjusted back to 12 h as in SBR_N, given that 1,3-PDO consumption time decreased, resulting in a HRT and SRT of 2 days and
the same OLR as SBR_N. Aeration, agitation, temperature, and pH were maintained as described for SBR_N, as well as a substrate concentration at 45 mM at the beginning of the cycle.

In the medium, the C:N:P ratio was 100:0:1.5. Nitrogen was supplied in the famine phase by means of a control algorithm based on the slope of the pO2 saturation curve, given that a sharp increase was observed after substrate consumption. Addition of nitrogen in the form of NH4Cl started at 30 mg/L NH3-N. However, in order to provide the precise amount of nitrogen needed, the residual nitrogen at the end of the cycle was monitored and the concentration of NH3-N was progressively corrected to 40 mg/L.

2.3. Batch PHA accumulation assays

PHA accumulation assays were performed in batch mode using the effluent from the respective SBR at the end of the cycle as inoculum.

When the performance on fermented effluent was evaluated (section 3.1.2), the same medium as for the SBR_N was prepared (45 mM at time 0), but without addition of nitrogen. As in the SBR, 850 mL of culture and 850 mL of medium were used. Alternatively, when individual substrates were evaluated (sections 3.1.1 and 3.2.1), the BA growth medium supplemented with butyrate or 1,3-PDO to the same concentrations as those in the fermentation effluent were used. In these experiments, the culture from the SBR was centrifuged and resuspended in medium to avoid any interference from the effluent matrix. In order to keep the same ratio of [culture]:[total volume] as in the SBR, 850 mL of culture were centrifuged and re-suspended in 1700 mL total volume for SBR_N (section 3.1.1), while 425 mL of culture were centrifuged and re-suspended in 567 mL total volume for SBR_noN (section 3.2.1).

Phosphorous, allylthioureia and antifoam were supplied to the concentrations described for the SBR. Temperature, pH, agitation and aeration were also controlled as in the SBR.

2.4. PHA extraction

PHA was extracted from 1 g of lyophilized cells with 50 mL of chloroform at 65 °C for 4 h with frequent manual mixing. The suspensions were then filtered through 0.2 µm PTFE membranes and PHA was precipitated from the solution by dropwise addition of methanol under stirring (ratio methanol:chloroform 10:1) and finally recovered in 0.7 µm glass fiber filters.

2.5. Analytical methods

Offline samples of the reactors were analyzed in the following manner. OD600, total soluble solids (TSS) and Nile Blue fluorescence were assessed in duplicates in fresh samples. Nile Blue was measured according to the protocol of Oshiki et al. (2011), as an indirect indicative measurement of the PHA concentration. TSS were measured according to standard protocols using Whatman filters GF/F (APHA et al., 2005).

A sample of approximately 50 mL was then centrifuged for 10 min at 4000 g and the supernatant was filtered through 0.2 µm filters. VFA (acetic, propionic, lactate, iso and n-butyrate and iso and n-valerate) and alcohols (1,3-PDO, ethanol, butanol and glycerol) in the supernatant were analyzed by HPLC in a Shimadzu HPLC system equipped with an RI detector and an Aminex HPX-87H column (BioRad). Soluble nitrogen in the form of ammonia (N-NH3) and soluble COD were determined using Hach-Lange kits LCK 305/303 and LCK 914/514, respectively. Soluble inorganic reactive phosphorous was determined photometrically by the ascorbic acid method (APHA et al., 2005) in a plate reader.

Cell pellets after centrifugation were washed with 10 mL of PBS buffer and freeze-dried overnight. Lyophilized cell pellets were used for the determination of PHA and glycogen, both performed in duplicates. The glycogen content in the cell pellets was assessed using the protocol optimized for mixed cultures in the study of Lanham et al. (2012): 2 mL of 0.9 M HCl were added to 2 mg of freeze-dried cells and incubated at 100 °C for 3 h in a block heater. The glucose content was then analyzed by HPLC as mentioned above.

The PHA content of the cells was assessed according to the protocol described in Johnson et al. (2009) with minor modifications: 10 mg of freeze dried cell pellets were dissolved in 1.5 mL of 1,2-dichloroethane (DCE) and 1.5 mL of HCl:1-propanol solution (1:4 v/v). 25 µL of a solution of 40 mg/mL of benzoic acid in 1-propanol were added as internal standard. The mixture was incubated for 2 h at 100 °C in a block heater. After incubation, 3 mL of an 80 g/L NaHCO3 solution were added to induce phase separation and neutralize the acidity. 1.7 mL of the organic phase were transferred into a microcentrifuge tube with 0.2 g of sodium sulfate to absorb residual water and filtered through 0.2 µm PTFE filters. Samples were then analyzed in a GC-FID system (Agilent 6890) equipped with a SGE BP21 column. Quantification was done with commercial poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) and 3-hydroxypropionic acid (Sigma Aldrich) submitted to the same assay.

Nuclear magnetic resonance spectroscopy (NMR) on the chloroform extracted polymer was performed on a 300 MHz Cryo magnet from Spectrospin & Bruker (1H NMR at 300 MHz, 13C NMR at 75 MHz), at room temperature. Fourier Transform Infrared spectroscopy (FT-IR) was performed on a Thermo-Fischer is50 FT-IR with a universal attenuated total reflection (ATR) sampling accessory on a diamond crystal.
2.6. Calculations of yields and rates

The concentration of active biomass (cells excl. PHA) was obtained by subtracting the PHA and the glycogen from the TSS concentration. A formula of C5H7NO2 was considered for the cells (Serfim et al., 2004). Substrate consumption rates (\( -r_s \) \( \text{Cmol S/h} \)) and product formation rates (\( r_p \) \( \text{Cmol PHA/h} \)) were calculated from the slope of the function of concentration vs. time. Likewise, specific substrate consumption rates (\( -q_s \) \( \text{Cmol S/Cmol X.h} \)) and specific product formation rates (\( q_p \) \( \text{Cmol PHA/Cmol X.h} \)) were calculated with the function of ratio of the substrate or product concentration to the respective biomass concentration at each time point. The value of the rates was the slope of the function of the ratio vs. time.

The yields of PHA (\( Y_{\text{PHA}} \) \( \text{Cmol PHA/Cmol substrate} \)) and glycogen (\( Y_{\text{glycogen}} \) \( \text{Cmol Glycogen/Cmol substrate} \)) were calculated by dividing the amount of polymer formed by the amount of substrate consumed. Biomass yield (\( Y_X \) \( \text{Cmol X/Cmol substrate} \)), where calculated in the same way, where X refers to the active biomass (TSS excluding PHA and glycogen). Yields and rates reported in terms of \( \text{COD}_{\text{total}} \) were obtained with HPLC, while yields reported in terms of \( \text{COD}_{\text{total}} \) were obtained with the measurements of the soluble COD in the supernatants.

2.7. Microbial community analysis

At certain time points, 10 mL of culture were withdrawn from the reactors and frozen at \(-20^\circ\text{C}\). Cultures were thawed at \(4^\circ\text{C}\) for 5 h, centrifuged at 4000 \(g\) for 10 min prior to freeze-drying the pellet. DNA extraction was performed using the ZR Soil Microbe DNA MiniPrep™ (ZymoResearch) and DNA concentrations were determined using the Qubit™ dsDNA BR Assay Kit (ThermoFisher Scientific). 16S rRNA gene amplicon sequencing was basically performed according to the procedures described previously (Varrone et al., 2015), except that version v.2.5.1 of the MiSeq Reporter Software was used. Comparisons between samples were done at the genus level. Sequencing reads have been deposited to the Sequence Read Archive of NCBI under the BioProject ID PRJNA389518.

3. Results

3.1. Enrichment with nitrogen availability (SBR_N)

Using the effluent from the fermentation as substrate, an SBR was established (SBR_N) to enrich a population able to produce PHA from VFA and 1,3-PDO (Fig. 1A). Initially, a molar ratio C:N:P of 100:8:1 was applied. With this ratio, nitrogen and phosphorous were depleted before the end of the cycle, which could have compromised the fate of the enrichment, given that the nutrients were not available to enable full growth from the PHA during the famine phase (Albuquerque et al., 2007). Consequently, the molar ratio of C:N:P of the enrichment was increased to 100:12:1.5 from cycle 19.

After the C:N:P ratio was increased, the feast phase for 1,3-PDO decreased considerably (Fig. 2A), and after a few cycles of fluctuations, it was stabilized at around 4 h. Likewise, the time needed to consume VFA settled at around 1.5 h. These would represent a feast/famine ratio of 0.1 and 0.4 for butyrate and 1,3-PDO, respectively, which are in the range of values allowing PHA accumulation reported in literature (Albuquerque et al., 2010).

A cycle of the SBR_N after stabilization of the feast phase duration (cycle 109) is presented in Fig. 3 (first column of graphs). PHA production was observed from the VFA (up to 20% of the TSS) (Fig. 3D), leading to HB (3-hydroxybutyrate) and HV (3-hydroxyvalerate) monomers, most probably coming from butyrate and propionate, respectively. Glycogen did not vary significantly during the cycle and accounted for up to 5% of the cell dry weight. No net production of PHA from 1,3-PDO was observed when only this substrate was present in the medium (after 2 h) and only cell growth was observed (Fig. 3C). The depletion of VFA and 1,3-PDO was clearly distinguished by a sharp increase in the oxygen saturation of the broth (Fig. 3A).

Besides VFA and 1,3-PDO, the substrate also accounted for around 300 mg/L of undefined COD, meaning the difference between the total COD measured in the sample and the sum of the metabolites identified in the supernatant by HPLC. Part of this undefined COD was consumed simultaneously to the known substrates (Fig. 3B). No further consumption occurred during the famine phase, thus not affecting the feast-famine regime. The presence of undefined COD was most probably derived from the presence of residual fats and oils in crude glycerol. Methanol was not expected to contribute significantly to the undefined COD fraction, as its initial concentration was estimated to be around 25 mg/L based on methanol concentration in the crude glycerol (Varrone et al., 2015).

Nitrogen was consumed mostly during the feast phase (Fig. 3B), coinciding in time with cell growth. In regard to the Nile Blue fluorescence (Fig. 3D), used as a rapid indication of the PHA concentration, a similar tendency to the PHA measured by GC chromatography was observed.

3.1.1. PHA accumulation with synthetic substrates

PHA production from 1,3-PDO was not observed in the enrichment cycles. Nevertheless, it could be that this transformation was masked by a simultaneous consumption of the PHA produced from VFA. In order to rule that out, a series of batch experiments with synthetic substrates were performed.

PHA accumulation from butyrate and 1,3-PDO was tested in the concentrations present in the fermented effluent, using the culture of the enrichment (SBR_N). Both substrates were tested in the presence and absence of nitrogen. In the presence of nitrogen, only butyrate was transformed into PHA (entirely PHB), while 1,3-PDO mainly led to biomass growth (Fig. 4B and E). The yield obtained on butyrate was 0.51 Cmol PHA/Cmol butyrate (Table 1).

In the absence of nitrogen, both substrates produced PHA to a final polymer percentage of around 10% of the cell dry weight (Fig. 4C).
Butyrate produced exclusively PHB, while 1,3-PDO led to trace amounts of HV and HP (3-hydroxypropionate) (less than 0.5%) besides HB. The PHA yield was higher for butyrate than for 1,3-PDO, with the respective values being 0.28 and 0.15 PHA Cmol PHA/Cmol substrate (Table 1). Noteworthy, a slight glycogen production was also observed when 1,3-PDO was used as substrate (Fig. 4I). The PHA yield on butyrate was lower in the absence of nitrogen than in the presence of nitrogen (0.51 vs. 0.28 Cmol PHA/Cmol butyrate, respectively) (Table 1). In all cases, the Nile Blue fluorescence presented a similar tendency to the PHA detected by GC.

For both substrates, the specific substrate uptake (-qs) was faster when nitrogen was available. In the absence of nitrogen, the rate in 1,3-PDO was approximately the same compared to that with butyrate (0.12 vs. 0.11 Cmol S/Cmol X.h) (Table 1), despite that in the experiment with 1,3-PDO the substrate was consumed in 8 h.

3.1.2. PHA accumulation with fermentation effluent under nitrogen limiting conditions

Results obtained with synthetic substrates implied that in the SBR_N, where nitrogen was available, no PHA from 1,3-PDO was being produced. Nevertheless, the culture had the capacity to produce PHA from both substrates in the absence of nitrogen (see above). Therefore, a batch PHA accumulation experiment was conducted in nitrogen limiting conditions with the culture enriched in the SBR_N and fermentation effluent as substrate. Results are presented in Fig. 3 (E–G) and Table 1 together with the data obtained during a cycle of the SBR_N for comparison.

Despite the limitation of nitrogen, still no PHA production was observed when 1,3-PDO was the only substrate in the fermentation (Fig. 3G). However, differently to what had been observed in all previous experiment, an important discrepancy between the measured PHA and the Nile Blue fluorescence was observed (Fig. 3G). Lower substrate consumption rates were observed compared to the SBR cycle (Table 1). Nonetheless, as in SBR_N, both substrates presented similar specific substrate consumption rates.

3.1.3. Confirmation of the polymer structure (enrichment SBR_N)

To further confirm that no other PHA than the ones detected by the GC analysis was being produced, a chloroform extraction was
performed with the cells at the end of the batch experiments with synthetic substrates (1,3-PDO and butyrate without nitrogen) and at the end of the PHA accumulation with fermentation effluent. The NMR and IR analyses of the extracted material confirmed in all cases the structure of the PHA, showing the presence of HB and HV in the NMR, as well as clear ester stretches in the IR. Trace amounts of HP units were also confirmed in the experiment with 1,3-PDO without nitrogen (see spectra in the Appendix A).

3.2. Enrichment with nitrogen limitation during the feast phase (SBR_noN)

Experiments performed so far proved the capacity of the culture to produce PHA from 1,3-PDO only in absence of nitrogen. Nevertheless, during the enrichment in SBR_N, nitrogen was available. This would mean that the selection was being performed in conditions not promoting PHA production from 1,3-PDO. Thus, a new

---

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phase</th>
<th>Nitrogen</th>
<th>( Y_{PHA} )</th>
<th>( Y_{X/S} )</th>
<th>( r_s )</th>
<th>( r_p )</th>
<th>( q_s )</th>
<th>( q_p )</th>
<th>( Y_{PHA}/S_{COD} )</th>
<th>( Y_{X/S} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-PDO</td>
<td>Yes b</td>
<td>0.68</td>
<td>0.55</td>
<td>0.30</td>
<td>11.54</td>
<td>7.84</td>
<td>0.08</td>
<td>0.11</td>
<td>0.48</td>
<td>0.22</td>
</tr>
<tr>
<td>Butyrate</td>
<td>No</td>
<td>0.68</td>
<td>0.55</td>
<td>0.30</td>
<td>11.54</td>
<td>7.84</td>
<td>0.08</td>
<td>0.11</td>
<td>0.48</td>
<td>0.22</td>
</tr>
<tr>
<td>1,3-PDO</td>
<td>No</td>
<td>0.01</td>
<td>0.43</td>
<td>5.70</td>
<td>0.00</td>
<td>0.24</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Butyrate</td>
<td>Yes</td>
<td>0.51</td>
<td>0.18</td>
<td>8.85</td>
<td>4.55</td>
<td>0.32</td>
<td>0.32</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Butyrate</td>
<td>No</td>
<td>0.28</td>
<td>-0.32</td>
<td>2.41</td>
<td>0.66</td>
<td>0.11</td>
<td>0.11</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

---

*The values were reported for the feast phase of the batch. For the fermentation effluent, VFA and 1,3-PDO phase refer to the interval where VFA were consumed and the interval where only 1,3-PDO was left, respectively.*

*The values for this condition were obtained from cycle 109 of SBR_N.*

*The values corresponding to this phase were calculated accounting for an initial concentration of 1,3-PDO of 27.6 \( C_{mol} \). Determination of 1,3-PDO at time 0 in Fig. 3 E was considered to be a sampling error.*
strategy was formulated consisting of an SBR where nitrogen was limited in the feast phase. Nitrogen was provided during the famine phase to enable growth of PHA accumulators out of the stored PHA (SBR_noN Fig. 1B).

Given that in the absence of nitrogen 1,3-PDO was consumed within 10 h (Fig. 3E), the reactor was operated at higher biomass concentrations, with cycles of 24 h but a SRT and HRT of 96 h, where only 1/4 of the volume of the reactor was replaced at the end of each cycle. After around 15 cycles though, the 1,3-PDO consumption time started decreasing, and it was finally settled around 4 h, very similar to that obtained in SBR_N (Fig. 5). For this reason, the cycle length was lowered again to 12 h after cycle 24, which provided practically the same feast/famine ratios as for SBR_N: 0.1 and 0.4 for VFA and 1,3-PDO, respectively.

A cycle of the SBR_noN reactor after stabilization is presented in Fig. 3 (last column of graphs). The most important observation was that, in this case, PHA concentration increased when 1,3-PDO was the only substrate left after VFA consumption, with HB being the main product. HV was only produced during the VFA consumption phase, and trace amounts of HP were observed from both substrates (less than 1% of the cell dry weight). Also, a slight increase in the glycogen content associated with 1,3-PDO consumption was observed in agreement with the experiments with synthetic substrates. PHA reached approximately 20% of the cell dry weight within 10 h (Fig. 3E), the reactor was operated at higher biomass concentration, the PHA concentration in the reactor broth was higher than that obtained in the previous enrichment (Fig. 3J).

As in a typical feast-famine cycle, the nitrogen supplied at the beginning of the famine phase was consumed to generate biomass from the PHA stored during the feast phase; this was also reflected in the pO2 saturation curve (Fig. 3H). As in SBR_N, part of the unknown COD was consumed along with the depletion of VFA and 1,3-PDO, but approximately half of the undefined COD remained unconsumed after the feast phase (Fig. 3I).

Although 1,3-PDO was consumed simultaneously with the VFA and at similar rates (Table 2), the PHA yields and rates largely differed between the VFA and the 1,3-PDO consumption phases. When both substrates were present the PHA yield was 0.74 Cmol PHA/Cmol S, this value decreased to 0.24 when 1,3-PDO was the only substrate.

In the supernatant of the samples from this experiment, increasing areas of a new peak were detected with the 1,3-PDO disappearance (Fig. 3I). The retention time of this peak coincided with the consumption of 1,3-PDO.

3.2.1. PHA accumulation in synthetic 1,3-PDO

In order to determine the net PHA yield from 1,3-PDO and rule out if PHA from VFA was masking the PHA production from 1,3-PDO, a new experiment using synthetic 1,3-PDO under nitrogen limiting conditions was established, using the culture enriched in SBR_noN. The PHA yield obtained in this experiment was 0.15 Cmol PHA/Cmol S, thus lower than the one obtained when 1,3-PDO was the only substrate available in the real effluent, which was 0.24 (Table 2). As in the enrichment cycle, increasing concentrations of 3-hydroxypropionate were detected in the supernatant coinciding with the consumption of 1,3-PDO.

3.2.2. Confirmation of the polymer structure (enrichment SBR_noN)

Like in the previous enrichment, PHA was extracted from the enriched biomass collected just after 1,3-PDO consumption. NMR and IR of the isolated polymer confirmed the expected structure with repeated units of HB, HV and traces of HP, bonded by ester linkages (see spectrum in Appendix A). Signals corresponding to a possible 1,3-propanediol hydroxytermination of the polymer as described before for glycerol (Tanadchangsaeng and Yu, 2012) were not observed.

3.3. Microbial community analysis

Samples from both enrichment reactors were analyzed by 16S rRNA gene amplicon sequencing, which showed a very diverse population in both cases (Table 3) (see analysis at class and genus level in Appendix B). The community in SBR_N was dominated by bacteria from the genera Xanthobacter, Rhodococcus, Paracoccus and Acinetobacter, which together accounted for around 50% of the OTUs (Operational Taxonomic Units). SBR_noN presented a similar percentage of the genera Paracoccus and Xanthobacter, but Rhodococcus and Acinetobacter practically disappeared from the culture. Instead, two more genera appeared in high percentages: Gordonia (14.9%) and Segetibacter (9.2%).

Also a sample at the end of the PHA accumulation with synthetic 1,3-PDO in the presence of nitrogen (correspondent to the experiments presented in section 3.1.2) was analyzed, to obtain indications of the bacteria responsible for growth on 1,3-PDO in the SBR_N. Rhodococcus was the genus presenting a higher increase (from 14.1% to 24.4%) compared to the mother culture (SBR_N cycle 109). The genera Tepidimonas and Hylemonella also presented a high relative increase (108% and 77%, respectively), although their abundance remained below 10%.

4. Discussion

4.1. The requirement of nitrogen limitation for the PHA production from 1,3-PDO

PHA is produced in the cells under conditions of unbalanced growth. This can mean a limitation in external factors such as nutrients (nitrogen or phosphorous) or electron acceptors (oxygen), as it is the case for the aerobic/anaerobic enrichments. Nevertheless, the limitation can also be internal, such as insufficient RNA or enzymes required for growth after a starvation period, with this being the most accepted mechanism for the PHA storage under aerobic
dynamic feeding (Dias et al., 2006; Ren et al., 2009). The latter has proven to be effective in selecting PHA accumulators in mixed microbial consortia fed with many different substrates (Serafim et al., 2008). However, there are some strains able to produce PHA only under nutrient limiting conditions, such as Cupriavidus necator, the most well-known PHA producer (Lee, 1996). There is no evidence that the feast-famine strategy, which provides a competitive advantage for PHA accumulators, rather than imposing a limitation, is enough for the enrichment of PHA accumulators from all types of substrates.

A possible example of this can be observed in the first of the strategies evaluated in the present study (SBR_N), a classical feast and famine enrichment, where nitrogen was available during the whole cycle. In this enrichment, 1,3-PDO contained in the fermentation effluent was not converted to PHA but mostly contributed to cell biomass increase (section 3.1). Moreover experiments performed in the presence of only 1,3-PDO or butyrate showed that only butyrate, but not 1,3-PDO, was converted to PHA when nitrogen was present in the medium (Section 3.1.2). On the other hand, when nitrogen was absent, both butyrate and 1,3-PDO were converted to PHA (from synthetic substrates). Thus, the results implied that, under the conditions of the enrichment, only the internal factor limitation was not enough to obtain PHA production from 1,3-PDO, and nitrogen limitation was also required.

Nonetheless, when nitrogen limitation was applied to this culture using effluent as a substrate, no net production of PHA from

### Table 2
Yields and rates obtained with the culture SBR_noN (enrichment without nitrogen), both using fermentation effluent (enrichment cycle 44) and in synthetic 1,3-PDO medium as substrates. Both experiments were performed under nitrogen limitation. Y_P/S: yield product/substrate Y_X/S: Yield biomass/substrate. 

<table>
<thead>
<tr>
<th>Substrate Phase</th>
<th>Overall</th>
<th>Overall</th>
<th>VFA</th>
<th>VFA</th>
<th>Overall</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y_P/S</td>
<td>g COD PHA/g COD_total</td>
<td>Cmol PHA/ Cmol S</td>
<td>Cmol Glycogen/ Cmol S</td>
<td>Cmol X/ Cmol S</td>
<td>Cmol S/h</td>
<td>Cmol S/h</td>
</tr>
<tr>
<td>1,3-PDO</td>
<td>0.42</td>
<td>0.24</td>
<td>0.08</td>
<td>0.10</td>
<td>9.82</td>
<td>3.49</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.35</td>
<td>0.21</td>
<td>0.14</td>
<td>0.17</td>
<td>4.12</td>
<td>1.99</td>
</tr>
</tbody>
</table>

* The values were reported for the feast phase of the batch. For the fermentation effluent, VFA and 1,3-PDO phase refers to the interval were VFA were consumed and the interval where only 1,3-PDO was left, respectively. The term overall refers to the entire feast phase.

** Product rates for the overall feast phase could not be calculated from the slope of the experimental data because of the distinct behavior of both phases, thus these parameters were calculated dividing the increment in Cmol PHA or Cmol PHA/Cmol X by the time interval.

### Table 3
Relative abundance of the predominant bacterial genera identified by 16S rRNA amplicon sequencing (>1% in at least one of the samples). Grey scale intensity corresponds to relative abundance.

<table>
<thead>
<tr>
<th>Genera</th>
<th>SBR_N Cycle 109 (time 0 of the cycle)</th>
<th>SBR_noN Cycle 44 (time 0 of the cycle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU %</td>
<td>1,3-PDO Nitrogen available (End of batch)</td>
<td>1,3-PDO Nitrogen available (End of batch)</td>
</tr>
<tr>
<td>Xanthobacter</td>
<td>15.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Rhodococcus</td>
<td>14.1</td>
<td>24.4</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>11.0</td>
<td>9.1</td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>9.3</td>
<td>12.8</td>
</tr>
<tr>
<td>Unclassified</td>
<td>8.1</td>
<td>10.3</td>
</tr>
<tr>
<td>Gordonia</td>
<td>7.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>5.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Teptidimonas</td>
<td>4.1</td>
<td>8.6</td>
</tr>
<tr>
<td>Hylemonella</td>
<td>2.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Leuconostrix</td>
<td>2.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Flexibacter</td>
<td>2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Rhodobacter</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Agrobacter</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Sargassum</td>
<td>1.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Komamonas</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Aquamonas</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Shigella</td>
<td>0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Aminobacter</td>
<td>0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Segelbacter</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Sphingobacter</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Methylobacter</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Others</td>
<td>10.1</td>
<td>9.1</td>
</tr>
</tbody>
</table>

1,3-PDO was observed when the latter was the only remaining substrate (just after the VFAs were up-taken) in the fermentation (section 3.1.3). As a matter of fact, the PHA degradation yield was the same as in the presence of nitrogen (−0.22 vs. −0.21 Cmol PHA/Cmol 1,3-PDO) (Table 1). Consequently, it could not be concluded that PHA production from 1,3-PDO was masked by the degradation of the PHA produced from VFA. However, the PHA yield during the VFA consumption phase was higher than obtained in the presence of nitrogen (0.68 vs 0.56 Cmol PHA/Cmol S) (Table 1), leading to almost 30% PHA in the cells at the moment of VFA depletion. This would not be justified with a higher PHA production from butyrate (which was observed to have a lower yield without nitrogen; see Table 1). Thus, it could be possible that certain PHA production from 1,3-PDO did occur. Yet, also other compounds present in the fermentation effluent could have been used as a substrate.

4.2. PHA production from 1,3-PDO in the enrichment without nitrogen during the feast phase (SBR_noN)

The presence of nitrogen during the SBR cycles is generally accepted to favour enrichment of PHA producers given that in this condition, bacteria can grow during the famine phase from the accumulated PHA, which otherwise is just used for maintenance (Albuquerque et al., 2007). On the other hand, PHA yields in the accumulation phase are higher in absence of nitrogen (Johnson et al., 2010b; Marang et al., 2014; Serafin et al., 2004). This fact, together with the above described production of PHA from 1,3-PDO exclusively under nitrogen limiting conditions, justified a strategy where nitrogen was limited during the feast phase and supplied in the famine phase for the growth of PHA accumulating bacteria (SBR_noN; Fig. 1B).

This enrichment strategy enabled a net production of PHA once VFA had depleted and only 1,3-PDO was present in the medium. The conversion had a low yield (0.24 Cmol PHA/Cmol S or 0.21 g CODPHA/g CODtotal), which could not be attributed to the simultaneous degradation of the PHA produced from VFA since the experiment with only 1,3-PDO as a substrate gave an ever lower yield of 0.15 Cmol PHA/Cmol S or 0.13 g CODPHA/g COD1,3-PDO (Table 2). The higher yield in fermented effluent could be partially explained by the contribution of residual COD to the PHA production. As a matter of fact, certain undefined COD was also consumed during this phase (Fig. 3I). Remarkably though, this COD alone (70 mg/L) would not explain an increment of 213 mg COD/L in PHA. Thus, 1,3-PDO was certainly contributing to the PHA production.

4.2.1. 3-Hydroxypropionate (3-HP) as possible intermediate

The culture enriched without nitrogen during the feast phase (SBR_noN) showed 3-HP increase along with the degradation of 1,3-PDO, both when using fermentation effluent and with synthetic 1,3-PDO. 3-HP-CoA was suggested as an intermediate of 1,3-PDO conversion to PHA in a Chromobacterium strain producing a mixture of medium and long chain PHA (Kimura et al., 2002) (Fig. 6). Interestingly, the accumulation of intermediates is believed to be a key regulation mechanism for PHA production in certain species that require nitrogen limitation (Kojima et al., 2004).

The fact of 3-HP being detected in the supernatant suggested that 3-HP may be excreted, and allows for hypothesizing a cross-feeding phenomenon, where 3-HP could be transformed to PHA by other species than the ones degrading the 1,3-PDO. Moreover, co-metabolism effects could also be occurring in the culture, which could contribute to explain the lower PHA yield obtained in pure synthetic 1,3-PDO compared to the one obtained in the fermentation effluent (section 3.2.2). More explicitly, it could be proposed that in real effluent, 3-HP excreted into the medium was converted into PHA by other bacteria, producing as well PHA from VFA. In the absence of VFA, the PHA production enzymes might not be induced, thus lowering the yield of PHA.

Differences in the behavior of cultures when submitted to mixed substrates or single substrates have been previously described in studies combining the labelling of substrates and microbial strains with MAR and FISH (Albuquerque et al., 2013), where co-metabolism effect were also suggested.

Is it worth emphasizing the fact that in both enrichments, HB was the main monomer produced from 1,3-PDO, while only traces of HP and HV were detected. On the contrary, whenever 1,3-PDO conversion had been reported, HP contributed significantly to the polymer fraction (Kimura et al., 2002), following the pattern that odd carbon substrates lead to odd-carbon monomers (Dias et al., 2006) (Fig. 6). This observation might support the suggestion that in the present study 1,3-PDO was not directly converted to PHA in the same microorganism.

4.2.2. Further advantages of the enrichment without nitrogen

Apart from enabling the conversion of 1,3-PDO to PHA, this type of enrichment could also imply that a separate PHA accumulation phase would no longer be needed, given that the enrichment itself would be performed at the conditions maximizing the PHA production. If part of the biomass was removed from the reactor at the end of the feast phase, cells with maximum PHA content could be obtained without the need of an extra reactor (Fig. 1B). Needless to say, the viability of this configuration would be conditioned to an increment of the PHA content of the cells at this stage by incrementing the substrate load as discussed in section 4.4.

4.3. Insights into the microbial community

4.3.1. The presence of lipid accumulating bacteria

The most predominant genera identified in the reactors were Xanthobacter, Rhodococcus, Paramicrococcus, Acinetobacter, Gordonia, and Segetibacter. All of them except Segetibacter (a relatively undescribed genus) have been previously detected, in higher or lower frequency, in SBR working under feast-famine regimes for PHA production (Morgan-Sagastume, 2016). Moreover, representatives of all the genera, except Segetibacter, have been directly linked to PHA accumulation (Alvarez, 2003; Kojima et al., 2004; Schembri et al., 1995; Wiegel, 2006).

It is important to notice though, that Rhodococcus, Acinetobacter and Gordonia, are especially well known for their capacity to accumulate internal lipid inclusions, such as wax esters and triacylglycerols. This ability has been reported from various carbon sources such as organic acids and alcohols, particularly under nitrogen limiting conditions (Alvarez and Steinbüchel, 2002). In contrast to PHA synthesis, which is widespread among prokaryotes, the capacity to synthesize lipid inclusions is limited to a very narrow spectrum of bacterial genera. Consequently, their presence should not be disregarded. Moreover, the metabolism of lipid storage compounds and PHA is largely interconnected, given that both are produced as storage compounds under unbalanced growth conditions and use acyl-CoA molecules as intermediates (Alvarez and Steinbüchel, 2002; Thomson et al., 2010) (Fig. 6). As a matter of fact, Kimura and colleagues suggested that malonyl-CoA, the first metabolite in the fatty acid biosynthesis, could be directly formed from propionyl-CoA in the oxidation of 1,3-PDO, suggesting a direct link to the fatty acid biosynthesis. Thus, the accumulation of lipids instead of PHA could be a possible explanation of the low PHA yields obtained from 1,3-PDO.

Further evidence of the accumulation of lipids could be found in the results obtained from the Nile Blue analysis. This compound has typically been used for screening of PHA-producing bacteria and even suggested as a rapid method of quantification of PHA (Oshiki
et al., 2011). Nevertheless, it can also bind to lipid inclusions, given that the interaction is due to hydrophobicity (Spiekermann et al., 1999). In the experiments with pure substrates (Fig. 4), PHA and Nile blue followed similar trends, as well as in the fermentation effluent in the presence of nitrogen (Fig. 3D). Nonetheless, in the experiment with fermented effluent in the absence of nitrogen (Fig. 3G), a significant difference was observed in the curves of PHA and Nile Blue, which could be attributed to Nile Blue binding to lipid inclusions.

Given the known interactions of Nile Blue to lipid inclusions, its use in PHA quantification should be carefully considered.

4.3.2. Genera related to 1,3-PDO consumption

Most of the main genera described in the reactors (Xanthobacter, Rhodococcus, Paracoccus, Acinetobacter and Gordonia) are known to be able to use various alcohols as carbon sources (De Carvalho and Da Fonseca, 2005; Eberly et al., 2013; Kojima et al., 2004; Singer and Finnerty, 1985; Wiegel, 2006). Thus, the characteristics of the communities itself did not give further insights into the substrate consumption preferences.

The main changes occurring with the different enrichment strategies corresponded to the reduction of OTUs corresponding to the genera Rhodococcus and Acinetobacter, together with the appearance of OTUs classified as Gordonia and Segetibacter when nitrogen was limited (Table 3). As the most important change in the behavior of the culture occurred in terms of 1,3-PDO conversion, it could be suggested that those genera were the ones involved in the 1,3-PDO consumption. A further indication of this could be the fact that Rhodococcus was the genus increasing more in abundance with respect to the mother culture when only 1,3-PDO was supplied in the presence of nitrogen. As no PHA was detected in that case, the assumption could be that 1,3-PDO was being used mostly for growth in the members of this genus, whose presence was dramatically reduced when nitrogen was limited in SBR_noN.

Furthermore, also Tepidimonas and Hylemonella, increased considerably their relative abundance when only 1,3-PDO was present in the medium. Thus, these genera could also have been contributed to the utilization of 1,3-PDO for growth. Like Rhodococcus, their presence was reduced in the reactor operating under nitrogen limitation (SBR_noN).

4.4. Overall discussion on utilization of 1,3-PDO to produce PHA

A moderate PHA yield was obtained from 1,3-PDO in the fermented effluent (0.24 Cmol PHA/Cmol S). As discussed above, this yield could be partially explained by the carboxylation step occurring in its conversion to acetyl-CoA (Fig. 6), but was also limited by a flow of carbon to glycogen, cell biomass and possibly lipid inclusions. Thus, even though this study represents an important step in demonstrating the possibility of the transformation of 1,3-PDO into PHA, further investigation is needed to enhance the feasibility of the process. Along this line, it would be important to elucidate the competition between PHA and other storage polymers, and study the operational parameters that maximize PHA production. Such parameters have been observed to have a very important influence in the carbon distribution between glycogen and PHA in other studies (Freches and Lemos, 2017). Likewise, higher substrate feedings should be tested in order to achieve higher productivities and PHA content. Only low OLRs were tested in this study in order to limit inhibition phenomena in the process of studying a novel substrate. Possible inhibition phenomena resulting from the presence of long-chain fatty acids and/or methanol should be addressed when testing higher substrate concentrations. Moreover, it would be interesting to study the fate of 1,3-PDO within the culture by means of combined MAR and FISH to elucidate possible cross-feeding phenomena and the implications of 3-hydroxypropionate excretion.

In terms of overall yields, the PHA yield obtained from the fermentation effluent in the present study was 0.42 Cmol PHA/Cmol S, which, taking into account the stoichiometry of the fermentation step, corresponded to 0.36 Cmol PHA/Cmol glycerol (0.31 g COD_{PHA}/g COD_{total}). This yield is slightly lower than the maximum obtained from crude glycerol in mixed cultures (0.36–0.51 Cmol PHB/Cmol S) (Freches and Lemos, 2017), but similar to reported yields in terms of COD (0.32 g COD/g COD_{total} in Moita et al., 2014). Thus, this study proved the possibility of an alternative strategy with similar perspectives in terms of yields. Moreover, the use of glycerol as a substrate leads to only PHB, while the polymer obtained in this study contained both HB and HV monomers. Such copolymers have been reported to have more desirable properties as a biopolymer than PHB, such as higher flexibility and tensile strength (Laycock et al., 2014). Furthermore, the PHA composition and yield can be further modulated by studying the distribution of metabolites.
depending on the fermentation operational conditions.

5. Conclusions

The present study investigated the possibility of producing PHA from a crude glycerol fermentation effluent containing volatile fatty acids and 1,3-propanediol as main substrates, focusing on the potential of 1,3 PDO to be up-taken for PHA production in mixed microbial consortia. The following results could be extracted:

- The conventional aerobic dynamic feeding strategy with nitrogen availability during the whole cycle failed to produce PHA from 1,3-PDO in the fermentation effluent, under the tested conditions.
- Experiments with individual synthetic substrates (butyrate and 1,3-PDO) showed the capability of the culture to produce PHA from 1,3-PDO only under nitrogen limiting conditions, proving the relevance of nitrogen limitation for this conversion.
- PHB was the main polymer produced from 1,3-PDO under nitrogen limiting conditions.
- 1,3-PDO conversion to PHA in the fermentation effluent was attained by means of an enrichment strategy that limited nitrogen during the fast phase and provided nitrogen during the famine phase, with an overall yield of 0.42 Cmol PHA/Cmol Substrate (including both VFA and 1,3-PDO). The yield when 1,3-PDO was the only substrate was 0.24 Cmol PHA/Cmol substrate. Noteworthy, this is to our best knowledge, the first time that conversion yields from 1,3-PDO are reported.
- The two enrichment strategies led to important differences in the microbial community composition.
- Besides PHA, glycogen accumulation was also detected. Moreover, indications of lipid accumulation were also found. Thus, multiple storage responses seemed to be competing in the culture.

Acknowledgements

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jwatech.2017.10.046.

References

Appendix A

1) $^1$H-NMR spectra of the extracted PHA

Fig. A 1: $^1$H-NMR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation experiment with 1,3-propanediol without nitrogen using the culture from the enrichment with nitrogen (SBR_N).

Fig. A 2: $^1$H-NMR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation experiment with butyrate without nitrogen using the culture from the enrichment with nitrogen (SBR_N).
Fig. A 3: $^1$H-NMR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation experiment with fermentation effluent without nitrogen using the culture from the enrichment with nitrogen (SBR_N).

Fig. A 4: $^1$H-NMR spectrum of the chloroform extracted PHA at the end of the feast phase (after 1,3-propanediol depletion) in the enrichment without nitrogen (SBR_noN).
2) **IR spectra of the extracted PHA**

Fig. A 5: IR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation experiment with 1,3-propanediol without nitrogen using the culture from the enrichment with nitrogen (SBR_N).

Fig. A 6: IR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation experiment with butyrate without nitrogen using the culture from the enrichment with nitrogen (SBR_N).
Fig. A 7: IR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation experiment with fermentation effluent without nitrogen using the culture from the enrichment with nitrogen (SBR_N).

Fig. A 8: IR spectrum of the chloroform extracted PHA at the end of the feast phase (after 1,3-propanediol depletion) in the enrichment without nitrogen (SBR_noN).
### Appendix B

Table B1: Relative abundance of the predominant bacterial OTUs at the class level (>10%) and genera level (> 1 %) identified by 16S rRNA gene amplicon sequencing. Grey scale intensity corresponds to relative abundance.

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<th>Reactor</th>
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<th>Genus</th>
<th>OTU %</th>
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Combined polyhydroxyalkanoates (PHA) and 1,3-propanediol production from crude glycerol: Selective conversion of volatile fatty acids into PHA by mixed microbial consortia

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ABSTRACT

Crude glycerol is an important by-product of the biodiesel industry, which can be converted into volatile fatty acids (VFA) and/or 1,3-propanediol (1,3-PDO) by fermentation. In this study, a selective conversion of VFA to polyhydroxyalkanoates (PHA) was attained while leaving 1,3-PDO in the supernatant by means of mixed microbial consortia selection strategies. The process showed highly reproducible results in terms of PHA yield, 0.99 ± 0.07 Cmol PHA/Cmol S (0.84 g COD PHA/g COD S), PHA content (76 ± 3.1 g PHA/100 g TSS) and 1,3-PDO recovery (99 ± 2.1%). The combined process had an ultimate yield from crude glycerol of 0.19 g COD PHA and 0.42 g COD 1,3-PDO per g of input COD. The novel enrichment strategy applied for selectively transforming fermentation by-products into a high value product (PHA) demonstrates the significance of the enrichment process for targeting specific bio-transformations and could potentially prove valuable for other biotechnological applications as well.

1. Introduction

Crude glycerol is a by-product of the transesterification of fats and oils in the biodiesel industry, with an average glycerol content of 70% (Zhu et al., 2013). Its world annual production is in the magnitude of millions of tons, which has stimulated the interest for finding high-value applications such as the production of polyhydroxyalkanoates (PHA) (da Silva et al., 2009). PHA are biopolymers produced in microbial cells under conditions of unbalanced growth (Anderson and Dawes, 1990). Their properties largely resemble some commonly used petroleum derived plastics such as polyethylene and polypropylene, for what PHA have been explored as possible renewable and biodegradable alternative during the last decades (Bugnicourt et al., 2014). Nevertheless, the success of PHA in the market is still limited by their high production costs. In this regard, the use of mixed microbial consortia (MMC), which do not require sterilization and generally present a higher tolerance and adaptability to waste substrates, is being studied as a possible alternative (Kourmentza et al., 2017; Valentino et al., 2016).

Only a few studies have attempted the conversion of glycerol into PHA using MMC (Dobroth et al., 2011; Freches and Lemos, 2017; Moita et al., 2014; Moralejo-Garate et al., 2013), with a common observation of co-accumulation of glycogen, which limits the PHA yield. In order to circumvent that, the substrate can first be fermented to volatile fatty acids (VFA), which can then be converted into PHA at high yields and without glycogen production. The resulting three-step process consists of: 1) Fermentation of the substrate into VFA; 2) Enrichment of PHA accumulating MMC; 3) PHA accumulation (Albuquerque et al., 2007; Serafim et al., 2008).

However, glycerol fermentation presents another challenge, which is the production of 1,3-propanediol (1,3-PDO) besides VFA. PHA production from 1,3-PDO has recently been demonstrated in MMC subjected to nitrogen limitation during the feast phase of the enrichment step (Burniol-Figols et al., 2018). Conversion of 1,3 PDO to PHA still needs optimization, as the conversion yields obtained so far were lower than the ones generally obtained from VFA and...
certain glycogen accumulation also occurred.

Given that 1,3-PDO is a high value product with a similar market price as PHA (1.8 €/kg for 1,3-PDO (Gargalo et al., 2016) and 1.5–5 €/kg for PHA (Chanprateep, 2010)), an alternative approach could be to avoid the consumption of 1,3-PDO while converting the VFA into PHA. The presence of substrates which favor the channeling of carbon towards microbial growth instead of PHA accumulation is a general obstacle, as it limits the PHA content of the cells (Marang et al., 2014; Tamis et al., 2014). In a recent study, the selective consumption of VFA over methanol was achieved in an MMC enriched from aerobic sludge using a strategy based on the selective settling capacity of PHA-containing cells (Korkakaki et al., 2016). By reducing the presence of bacteria utilizing methanol, the maximum PHA storage capacity of the culture increased from 48 to 70 wt% PHA. Along the same line, a selective consumption of VFA over 1,3-PDO has recently been reported using defined co-cultured strains. In the latter case, the outcome was achieved by testing PHA producing strains for their inability to consume 1,3-PDO (Pan et al., 2016).

In the present study, the selective consumption of VFA over 1,3-PDO in the accumulation phase was attained in MMC by not supplying 1,3-PDO during the enrichment phase. More precisely, the enrichment culture was fed with a synthetic medium supplemented with VFA and crude glycerol (Fig. 1). Different substrate concentrations in the accumulation phase were tested in order to evaluate possible inhibition phenomena. Furthermore, the study assessed the stability of the culture and the reproducibility of the process, which are of vital interest and sometimes put under concern in MMC based open-culture processes.

2. Materials and methods

2.1. Crude glycerol fermentation effluent

Crude glycerol continuous fermentation was performed with an influent concentration of 10 g/L of glycerol (14.7 g/L of crude glycerol) as described elsewhere (Burniol-Figols et al., 2018), based on the results obtained in Varrone et al. (2017). The distribution of metabolites in the effluent utilized during the PHA accumulation experiments was the following (g/L): 1,3-PDO: 4.43 ± 0.16, butyrate: 1.59 ± 0.15, propionate: 0.80 ± 0.04, acetate: 0.19 ± 0.04, glycerol: < 0.01, TSS: 0.89 ± 0.21. Measured soluble metabolites accounted for an average of 11.6 ± 0.4 g COD/L, while the total soluble COD was around 14 g CODtotal/L. Soluble nitrogen in the form of NH4+ was 2.56 ± 1.74 mg/L. The effluent was kept at 4 °C and at –20 °C for short and long term storage, respectively, and was centrifuged (15 min at 4000 g) and filtered (1 μm) before being used in the PHA accumulation phase.

2.2. Enrichment (SBR)

The enrichment in PHA producers was carried out in a 2.5 L Minifors reactor (INFORS HT) operating as Sequential Batch Reactor (SBR) with a working volume of 1.7 L. The reactor was operated in cycles of 12 h. At the end of each cycle, half of the volume of the reactor was replaced with fresh medium resulting in a Hydraulic Retention Time (HRT) and Solids Retention Time (SRT) of 1 day. The inoculum (30% v/v in the first cycle) was a mixture of equal parts of anaerobic sludge from the Lundtofte wastewater treatment plant and aerobic sludge from the Daka Biodiesel wastewater treatment plant (Denmark). Air was supplied at a rate of 1 L/min and agitation was set at 500 rpm. pH and temperature were maintained at 8 and 30 °C, respectively. The reactor was emptied and cleaned along with the tubing once a week to avoid excessive biofilm formation.

The medium consisted of BA Medium (Varrone et al., 2015) supplemented with VFA and crude glycerol to a carbon concentration of 90 mM of Carbon (CmM) (45 CmM at the beginning of the cycle). Half of the carbon corresponded to glycerol (45 CmM), which was added in the form of crude glycerol (1.88 g/L). The crude glycerol source and characteristics can be found elsewhere (Varrone et al., 2015). The other half of the carbon was in the form of VFA (45 CmM), which were added to the same proportions as in

**Fig. 1.** Strategy for production of PHA from volatile fatty acids and recovery of 1,3-propanediol from fermented crude glycerol. (CSTR: Continuous Stirred Tank Reactor, SBR: Sequential Batch Reactor).
the crude glycerol fermentation effluent: (acetate 2.38 C_m, propionate 12.10 C_m, butyrate 30.52 C_m). C:N:P molar ratio in the medium was fixed at of 100:12:1.5 with NH4Cl and K2HPO4. Allylthiourea (5 mg/L) and Antifoam 204 (Sigma) were supplied to inhibit nitrification and foaming, respectively. The resulting organic loading rate of the SBR was 3.7 g COD_{total}/L^{1}.day^{1}.

The oxygen saturation (pO2) was monitored online and used as an indicator of the duration of the feast phase. This parameter was used, together with the PHA content at the end of the feast phase, to assess the stability of the reactor. Moreover, a full cycle was periodically characterized by offline samples (see analytical methods).

2.3. PHA accumulation

Batch tests were performed using the culture from the enrichment at the end of the cycle (12 h). 0.85 L of cell culture were allowed to settle in a volumetric cylinder for 30 min, followed by removal of 0.65 L of supernatant. At time 0 of the batch experiment, the remaining 0.2 L of settled cells were added to 1.5 L of medium consisting of clarified crude glycerol fermentation effluent diluted with distilled water to reach different VFA concentrations (45 C_m, 60 C_m, 75 C_m and 90 C_m) at the beginning of the batch, corresponding to 5.6, 7.5, 9.5 and 12 g COD_{total}/L. The correspondent cycle of the SBR for each batch is indicated in Table 1. Allylthiourea and antifoam were supplied to the concentrations described above for the SBR. Aeration, agitation and pH control were also carried out and antifoam were supplied to inhibit nitri-

2.4. Analytical methods

Offline samples of the reactors were characterized as follows. Total suspended solids (TSS) were measured in fresh samples using Whatman® filters GF/F and according to standard protocols (APHA et al., 2005). Samples were thereafter centrifuged (at 4000 g for 10 min) and the pellets were washed with PBS solution (phosphate-buffered saline) and freeze dried. PHA and glycogen in lyophilized cell pellets were measured in duplicate according to the protocols described in Burniol-Figols et al. (2018), and expressed as a weight percentage of the TSS. The PHA and glycogen content was subtracted from the TSS to obtain the concentration of active biomass (cells excl. polymers or X). The supernatant was filtered (0.45 µm) and kept at -20 °C prior to analysis of soluble nitrogen (N-NH3) (Hach-Lange kits LCK 305/303), COD (Hach-Lange kits LCK 914/514), as well as VFA and alcohols concentrations. VFA (acetate, propionate, iso and n-butyrate, lactate, iso and n-valerate and hexanoate) and alcohols (1,3-PDO, butanol and glycerol) in the supernatant were analyzed by HPLC in a Shimadzu HPLC system equipped with an RI detector and an Aminex HPX-87H column (BioRad). Yields and rates were calculated as previously reported (Burniol-Figols et al., 2018). Parameters reported in terms of C_{mol} were based on the compounds measured by HPLC, while parameters reported in terms of COD_{total} were based on the measurements of the soluble COD in the supernatants. The dilution effect of acid and base addition for the pH control, as well as the effect of the sampling, were taken into account for all calculations.

PHA were extracted in chloroform and analyzed by Nuclear Magnetic Resonance spectroscopy (1H NMR and 13C NMR) as described in Burniol-Figols et al. (2018). The molar mass of the polymer was determined by Size-Exclusion Liquid Chromatography (SEC) in a Shimadzu HPLC system using two columns in series (SDV 5 µm 8 × 300 mm 1000 Å and 10000 Å, PSS Polymer Standards Service GmbH). The oven temperature was set at 31 °C. Chloroform was used as eluent at a flow rate of 1 mL/min. The system was calibrated with low polydispersity polystyrene standards (Polymer Standards Service GmbH).

Microbial analyses were performed in samples corresponding to time 0 of the SBR cycles indicated in Table 1. Sampling, DNA extraction, 16S rRNA gene amplicon sequencing and data processing were performed as described in Burniol-Figols et al. (2018).

Table 1

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<th>Cycle SBR</th>
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3. Results and discussion

3.1. Enrichment of PHA-accumulating MMC in absence of 1,3-PDO

The present study was based on the hypothesis that an MMC subjected to a feast and famine regime using medium supplied with VFA, but not 1,3-PDO, would be enriched in bacteria adapted to accumulate PHA predominantly from VFA. Consequently, a culture selected in this manner would not consume 1,3-PDO when supplied with real fermentation effluent containing both substrates in the following PHA accumulation phase (Fig. 1). Therefore, the PHA enrichment was performed in an SBR fed with a synthetic VFA medium. Nevertheless, in order to avoid any inhibition phenomena when the culture faced the real waste effluent in the PHA accumulation, the VFA synthetic medium was supplemented with crude (non-fermented) glycerol.

During the first cycles, VFA were consumed faster than glycerol (Fig. 2 A). Nonetheless, from cycle 50 onwards, the depletion of either substrate was not distinguishable any more from the pO2 profile, as exemplified by a typical cycle of the stabilized reactor in Fig. 3A and B. Complete substrate consumption occurred within 65 min, representing a feast/famine ratio of 0.1 (Fig. 3 B). Substrate consumption coincided with a PHA production up to 37.3 wt%, detecting an increment in both HB (3-hydroxybutyrate) and HV (3-hydroxyvalerate) monomers (Fig. 3 C and D). Glycerogen was also formed during the feast phase, probably from glycerol, given the tendency of the latter to lead to co-accumulation of glycogen and PHA (Moralejo-Gárate et al., 2011). The PHA yield was 0.68 $\text{Cmol PHA/Cmol substrate}$ and the glycerogen yield represented 0.12 $\text{Cmol glycogen/Cmol substrate}$ (Table 2). During the famine phase, these polymers were transformed into biomass (Fig. 3 C) at a yield of 0.62 $\text{Cmol biomass/Cmol polymers}$ using the nitrogen present in the medium (Table 2).

The SBR proved to be very stable both in terms of duration of the feast phase and the PHA content of the cells at the end of the feast phase (Fig. 2A and B). Table 2 displays the yields and rates obtained in two different cycles separated for more than 3 months, which presented practically the same values.

3.2. Microbial community analysis

The culture in the SBR was dominated by OTUs (Operational Taxonomical Units) classified within the genera *Amaricoccus* (class Alphaproteobacteria) and *Thauera* (class Betaproteobacteria), which together accounted for 56.3–72.4% of the sequences at different cycles of the SBR (Fig. 4). The other identified OTUs rarely presented abundances higher than 5%, suggesting a highly diverse flanking population.

Both dominant genera, *Thauera* and *Amaricoccus* have largely been reported to be prevalent genera in PHA enrichment reactors operated under feast/famine aerobic regime, especially in the case of *Thauera* (Morgan-Sagastume, 2016). Moreover, both genera have been directly correlated with PHA accumulation by means of Nile Blue staining (Lemos et al., 2008).

In regard to substrate utilization, different substrate preferences have been reported for these genera. Members of the *Amaricoccus*...
genus were described to become dominant over *Thauera* when the substrate of the SBR changed from acetate to propionate (Lemos et al., 2008). However, Yang et al. (2013) identified *Thauera* to be the main genus in a propionate-fed SBR. On the other hand, bacteria from the genus *Thauera* were described to primarily use butyrate as a carbon source when submitted to a mixture of VFA (Albuquerque et al., 2013). To our knowledge, neither of the two genera has been previously described in glycerol-fed systems.

Fluctuations of the relative abundance of the main genera, as well of the sum of the two, were observed in samples taken at different points, despite a steady behavior of the SBR (Fig. 4). This is very well exemplified in the two SBR cycles presented in Table 2 (cycle 298 and 76). The yields and rates of these cycles were very similar despite substantial variations in the microbial population identified at the beginning of the cycles (Fig. 4). The percentage of OTUs classified within the genus *Thauera* was much higher in cycle 298 (29.1 vs. 18.9), and concurrent with a clear drop in the genus *Amaricoccus* (37.9 vs 27.2). Nonetheless, the sum of the two main genera was virtually the same. Stable operation of SBRs with variations in the relative composition of the microbial population has been previously reported (Bengtsson, 2009; Serafin et al., 2006; Valentino et al., 2014). Albuquerque et al. (2013) demonstrated that microbial strains with PHA production capacity can compete for the same substrate. Thus, small variations in the microbial composition do not necessarily imply a change in the overall performance. From the results obtained here, a relative abundance of the two main genera over 50% seemed to be a common denominator for a stable operation of the SBR.

### 3.3. PHA accumulation with 1,3-PDO recovery

Using the enriched inoculum from the SBR, a set of experiments of PHA accumulation with crude glycerol fermentation effluent was performed at different initial VFA concentrations: 45 mM, 60 mM, 75 mM and 90 mM, with the last corresponding to undiluted effluent (Fig. 5 and Table 3).

For all the concentrations tested, butyrate was used up before acetate and propionate, even though the initial concentrations of

### Table 2

<table>
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<tr>
<th>SBR Cycle</th>
<th>Phase</th>
<th>Yields</th>
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<td>$Y_{Gly/S}$</td>
<td>$Y_{X/S}$</td>
<td>$Y_{PHA}$</td>
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<tr>
<td></td>
<td></td>
<td>$C_{PHA}/C_{total}$</td>
<td>$C_{Gly}/C_{total}$</td>
<td>$C_{X}/C_{total}$</td>
<td>$C_{PHA}$</td>
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<td>39.01</td>
</tr>
<tr>
<td></td>
<td>famine</td>
<td>0.62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 298</td>
<td>feast</td>
<td>0.78</td>
<td>0.14</td>
<td>0.05</td>
<td>34.37</td>
</tr>
<tr>
<td></td>
<td>famine</td>
<td>0.88</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Y P/S*: Yield PHA/substrate; *Y Gly/S*: Yield glycogen/substrate; *Y X/S*: Yield biomass/substrate; *Y X/polymers*: Yield biomass/polymers (PHA + glycogen). - $r_s$: Substrate consumption rate. $r_P$: PHA formation rate. $q_P$: Specific PHA formation rate. $q_s$: Specific substrate uptake rate. $m$: Specific growth rate.

* TSS concentration and PHA contents (wt%) correspond to the end of the feast phase.

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Fig. 4. Relative abundance of the predominant bacterial genera identified by 16S rRNA gene sequencing at different cycles of the SBR (>1% in at least one of the samples). Numbers within the bars indicate the relative abundance of the correspondent genus. Numbers on top of the bars indicate the summed abundance of OTUs classified as the genera *Thauera* and *Amaricoccus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
these VFA were lower (Fig. 5C). Acetate and propionate concentrations decreased right from the beginning, but their consumption rates increased once butyrate was depleted (Table 3, Fig. 5 C). This fact was translated into two different phases of PHA accumulation, which could be clearly distinguished by the \( pO_2 \) saturation profile (Fig. 5 A).

During the first phase, coinciding with butyrate consumption, a steep increase in the PHA concentration and polymer content was observed. HB was the main monomer produced, but a notable production of HV also occurred (Fig. 5D and E). A small increment in 3-hydroxypropionate (HP) was detected as well, but never reaching values over 1 wt%. PHA yields during this phase (hereafter referred to as butyrate phase) were higher than 0.9 \( C_{\text{mol}} \) PHA/\( C_{\text{mol}} \) S in all experiments (Table 3). Around 80% of the substrate was consumed during this phase.

During the second phase, when only acetate and propionate were available, only a slight increase in PHA concentration was observed (Fig. 5 D). The PHA yields were much lower than during the butyrate phase and a considerable increase in the carbon derived to growth was observed for all batches (Table 3). As a result, the overall PHA yields for the entire batch presented always a lower value than during the butyrate phase (Table 3).

Similar observations regarding the preference of butyrate over acetate and propionate have been reported previously in cultures.
can be converted to propionyl-CoA and incorporated into HV. The formation of other substrates (such as butyrate), all the propionate will be transformed into propionyl-CoA. When acetyl-coA is available from the trans-acyl-CoA pool, it can be decarboxylated to acetyl-coA, thus lowering the maximum theoretical PHA yield to 0.67 Cmol PHA/Cmol S. In the present case, propionyl-coA from propionate can be combined with acetyl-coA originated from the other VFA during the butyrate phase, and as a matter of fact, over 70% of propionate consumed was recovered as HV (mol basis). During the second phase, the little acetate present in the medium could not provide enough acetyl-coA molecules, so decarboxylation of propionyl-coA to acetyl-coA was needed. During this phase, under 30% of the propionate consumed was recovered as HV (mol basis). This behavior, which might partially explain the lower yields, is very well illustrated in Fig. 5, where despite the propionate consumption rate increased during the second phase of the batch (Fig. 5 C), the HV monomers continued increasing at the same rate as earlier (Fig. 5 D). Overall, the lower PHA yields combined with lower substrate uptake rates (in Cmol) led to a reduction of the PHA production rate during the second phase. Moreover, a reduction of the apparent PHA production rate could have occurred due to a simultaneous degradation of the PHB produced during the butyrate phase. Nonetheless, the last hypothesis could not be confirmed from the data obtained here.

The lower PHA yields obtained during the second phase could be related to various factors. Higher PHA yields have been associated to butyrate compared to acetate, and attributed to the lower ATP needs of the transformation to PHB, thus a reducing carbon flux to the TCA cycle (Marang et al., 2013). Propionate has also usually been reported to present lower PHA yields and higher growth yields compared to acetate (Dias et al., 2008; Jiang et al., 2011). In this case, a lower PHA yield would not be justified by higher energy requirements, but partially because of the loss of carbon during HV production (Pardelha et al., 2012), which justifies in the following argument. HV arises from the condensation of acetyl-CoA and propionyl-CoA. When acetyl-coA is available from the transformation of other substrates (such as butyrate), all the propionate can be converted to propionyl-CoA and incorporated into HV.

dominated by different microbial genera (Albuquerque et al., 2013; Jiang et al., 2012; Marang et al., 2013). As a matter of fact, this phenomenon has been observed even in cultures that were not exposed to butyrate during the enrichment (Dionisi et al., 2005). In a study with only butyrate and acetate as a substrates (Marang et al., 2013), butyrate depletion before acetate led to the same phases observed here, where specific substrate carbon consumption rates (in Cmol S Cmol X−1 h−1) decreased during the second phase. Nonetheless, the specific substrate consumption rate in terms of mol S Cmol X−1 h−1 remained constant throughout the two phases. This fact indicated that the substrate uptake and activation was the rate-limiting process, and that butyrate and acetate shared the same transporter, but it had preference for butyrate. The same phenomenon was observed in the present study: a decrease in the specific substrate carbon uptake rate (in Cmol S Cmol X−1 h−1) was observed after butyrate exhaustion, but the specific substrate rate in terms of substrate mols was maintained (except for the batch at 75 Cmol. Table 3). Nevertheless, this condition was only met when propionate was considered, which, following the same reasoning as in Marang et al. (2013), would indicate that propionate was also uptaken through the same regulation system.

The lower PHA yields obtained during the second phase could be related to various factors. Higher PHA yields have been associated to butyrate compared to acetate, and attributed to the lower ATP needs of the transformation to PHB, thus a reducing carbon flux to the TCA cycle (Marang et al., 2013). Propionate has also usually been reported to present lower PHA yields and higher growth yields compared to acetate (Dias et al., 2008; Jiang et al., 2011). In this case, a lower PHA yield would not be justified by higher energy requirements, but partially because of the loss of carbon during HV production (Pardelha et al., 2012), which justifies in the following argument. HV arises from the condensation of acetyl-CoA and propionyl-CoA. When acetyl-coA is available from the transformation of other substrates (such as butyrate), all the propionate can be converted to propionyl-CoA and incorporated into HV. Nonetheless, when acetyl-coA is not available, propionyl-coA has to be decarboxylated to acetyl-coA, thus lowering the maximum theoretical PHA yield to 0.67 Cmol PHA/Cmol S. In the present case, propionyl-coA from propionate could be combined with acetyl-coA originated from the other VFA during the butyrate phase, and as a matter of fact, over 70% of propionate consumed was recovered as HV (mol basis). During the second phase, the little acetate present in the medium could not provide enough acetyl-coA molecules, so decarboxylation of propionyl-coA to acetyl-coA was needed. During this phase, under 30% of the propionate consumed was recovered as HV (mol basis). This behavior, which might partially explain the lower yields, is very well illustrated in Fig. 5, where despite the propionate consumption rate increased during the second phase of the batch (Fig. 5 C), the HV monomers continued increasing at the same rate as earlier (Fig. 5 D). Overall, the lower PHA yields combined with lower substrate uptake rates (in Cmol) led to a reduction of the PHA production rate during the second phase. Moreover, a reduction of the apparent PHA production rate could have occurred due to a simultaneous degradation of the PHB produced during the butyrate phase. Nonetheless, the last hypothesis could not be confirmed from the data obtained here.

Measurements of the COD in the supernatant always resulted in higher values than calculated from the sum of identified compounds in the HPLC. Partial consumption of this undefined COD, probably crude glycerol impurities, was observed (Fig. 5 B). Whether these compounds contributed to the observed PHA production could not be ruled out with the analyses performed here. Thus, the PHA yields calculated from the overall COD consumed are also reported in Table 3. It is noteworthy to mention that, despite these yields were lower, they presented the same tendencies as the ones calculated on VFA basis.

Another very important general observation in all experiments was the negligible consumption of 1,3-PDO, which was recovered to a minimum of 95% (Fig. 5 B and Table 3). This value was much lower than the theoretical 50%.
higher than the recovery obtained when a similar strategy was tested by selecting defined PHA strains for their incapacity to consume 1,3-PDO (Pan et al., 2016). That strategy resulted in a 1,3-PDO recovery of 80% and a PHA content of around 20 wt% after 28 h of incubation. The higher selectivity for VFA obtained in the present study using MMC might be due to the high responsiveness to substrate addition developed by cultures submitted to feast/famine regimes.

The presence of VFA as by-products is common in biological processes such as lactic acid, ethanol or butanol fermentations. The strategy presented here could be tested in other fermentation effluents in order to increase the purity of the desired product while producing PHA as an additional high value product.

### 3.4. Effect of increasing substrate concentration on the PHA accumulation

The PHA accumulation experiments performed at different VFA concentrations also exhibited some differences in the followed parameters, which are summarized in Fig. 6. Given the different phases observed (described in the previous section) the results are presented both for the overall VFA consumption and the butyrate consumption phase. The polymer percentage inside the cells increased linearly with the initial substrate concentration until 75 Cmol, where 70 wt% PHA was reached (Fig. 6 A). No further increase was observed at 90 Cmol initial concentration. Nevertheless, a higher PHA concentration was obtained at 90 Cmol derived from the higher TSS obtained at this concentration. As a matter of fact, there was a linear increase in the maximum PHA concentration obtained at the end of the feast phase (Fig. 6 B), reaching up to 1.79 g/L of PHA at the highest concentration.

The overall PHA yield and the one obtained during the butyrate phase decreased from 45 to 60 Cmol (Fig. 6 C), but remained approximately constant after that. From these data points, one could suggest a decreasing trend of the PHA yield (considering the data points at 60 Cmol as outliers). Alternatively, no trends would be perceived if the batch at 45 Cmol was considered an outlier. Looking at the specific substrate uptake rates (qs) and the specific product formation rates (qp) (Fig. 6D and E), no important differences could be observed. Both parameters seemed to be independent of the substrate concentration. Thus, substrate inhibition could not be concluded from the data. Noteworthy, the lack of inhibition occurred despite increasing concentrations of 1,3-PDO.

Overall, increasing the VFA initial concentration up to 90 Cmol did not have an apparent effect on the main outcome of the PHA accumulation, the specific substrate concentration rate (qp). At this concentration, a higher PHA concentration was obtained, which would benefit the downstream costs of the process. Given the different phases observed, it would be reasonable to stop the process after butyrate depletion in order to maintain a high productivity. At this point, the PHA would be produced at a yield of 0.91 Cmol PHA/Cmol 5 with a specific rate of 0.87 Cmol-Cmol X^-1.h^-1 (Table 3). The 1,3-PDO would be recovered at 97% and would represent 80% of the COD of the supernatant. This figure would increase up to 85% if the rest of the VFA were allowed to be depleted. Nevertheless, the yield would decrease to an overall yield of 0.81 Cmol PHA/Cmol 5 and the productivity would drop to 0.5 Cmol PHA/Cmol X^-1.h^-1 (Table 3). Consequently, different strategies could be formulated based on a general economic evaluation, taking into account the cost of the further purification of 1,3-PDO depending on the purity of the effluent and the repercussion of the decrease of productivity.

### 3.5. Reproducibility of the PHA accumulation

The PHA batch accumulation at the highest initial VFA concentration (90 Cmol) was repeated 11 times over a period of 2 months to study the stability of the culture (Table 1). The batches were stopped after butyrate depletion, indicated by a sudden increase in the oxygen saturation (Fig. 5 A). The average PHA content obtained at the end of the 11 batches was 76 ± 3.1 wt% (Table 4) implying a relative error of just 4%. The HB: HV ratio was also reproducible, with the average value being 84:16 (±2.0:1.8). In the same line, 1,3-PDO was always completely recovered from the fermentation broth, with an average recovery of 99 ± 2.1%.

In 5 of those batches, the yields and rates of the process were also evaluated (Table 5). Highly reproducible values were obtained in terms of PHA yield and maximum PHA concentration, with average values of 0.99 ± 0.07 Cmol PHA/Cmol 5 (0.84 g COD PHA/g COD 5) and 1.48 ± 0.14 g PHA/L, respectively.

A slightly higher degree of variability was observed in terms of rates (Table 5). The substrate uptake rate (rs) and PHA production rate (rp) presented relative standard deviations of 15% and 12%, respectively. These standard deviations were mainly caused by a decline of the rates in batches 9 and 10, in which the duration of the batches (defined by the depletion of butyrate and thus by the rate of butyrate consumption (rs but)) increased from 3 h to 5 h and 4 h respectively. In those batches, the rs but presented values of around 13 Cmol/h, much lower than obtained in the initial test (21.6 Cmol/h). Noteworthy, the decrease in rs but was not accompanied with a decrease in the specific substrate consumption for butyrate (qs but), which, on the contrary, increased considerably in all the repeats compared to the initial PHA accumulation test. Nonetheless, an important decrease of the initial biomass concentration (TSSini) was observed (from 0.77 g/L in the initial test to an average of 0.46 g/L in the repetitions). A linear correlation was observed between the

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**Fig. 6.** Comparison of calculated parameters from the PHA accumulation experiments with crude glycerol fermentation effluent at increasing initial concentration of VFA. A: Maximum PHA content inside the cells (g polymer/100 g TSS). B: Maximum PHA (g/L). C: Yield PHA/substrate (YP/S). D: Specific substrate consumption rate. E: Specific PHA formation rate. For all graphs, values for the overall batch (black circle ●) and for the butyrate phase (green triangle▲) are presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
correlations were not established with the biomass composition of the culture. It is important to notice though, that these seemed to be governed by the initial cells concentration (TSS), and giving further evidence that the performance of the systems in later cycles of the SBR (Table 5), but was due to a loss of biomass biomass at the end of the SBR cycle, which even slightly increased and \( r_p \) with the sum of \( \text{initial TSS concentration and } \text{rs,rs but and } \text{rp} \) (significant correlation was obtained between \( \text{TSS end} \) and \( \text{rs but} \)).

Remarkably, the lower initial TSS was not caused by a decreased biomass at the end of the SBR cycle, which even slightly increased in later cycles of the SBR (Table 5), but was due to a loss of biomass in the settling step between the SBR and the PHA accumulation. The absolute TSS lost in the clarified fraction was quantified to be 8% of the inoculum in the initial test (B90), while around 50% in the batches 9, 10 and 12 (Table 5).

Correlations were also investigated between the observed parameters and the composition of the microbial population used as inoculum (Fig. 4). No significant correlations \((\alpha < 0.05)\) were obtained between any of the rates \( r_b \), \( r_p \), \( \text{rs but} \), \( \text{qs} \) and \( \text{qp} \) and the relative abundance of \( \text{Thauera, Amaricoccus} \) or the sum of the two. However, in terms of concentration of those genera \((\text{g/L instead of relative abundance})\), a significant correlation was obtained between \( r_b \) and \( r_p \) with the sum of \( \text{Amaricoccus and Thauera} \) \((\alpha < 0.05)\). Thus, giving further evidence that the performance of the systems seemed to be governed by the initial cells concentration (TSS), and consequently of the main genera, rather than the relative composition of the culture. It is important to notice though, that these correlations were not established with the biomass composition present at the beginning of the PHA accumulation but with the biomass used as inoculum, which was the main interest of the study and the most likely source of variations. Consequently, possible changes in relative percentages occurring during the sedimentation step, which could have hampered the correlations, cannot be excluded.

Insufficient settling of the biomass can be caused by overgrowth of filamentous bacteria (or filamentous bulking) \((\text{Martins et al., 2004; Morgan-Sagastume et al., 2015})\). Reported filamentous genera \((\text{Nielsen et al., 2009})\) were found in very minor quantities in the analyzed samples, generally below 0.2%. Only OTUs classified in the genera \( \text{Acidovorax (0.3–1.1%), Acinetobacter (0.05–3.4%) and Dyadobacter (1.2–9.6%)}, \) of which some members are described to have filamentous growth \((\text{Chellius and Tripplett, 2000; Nielsen et al., 2009})\), reached higher abundances. Nevertheless, no correlations were observed between the TSS and the relative abundance of filamentous genera, neither individually nor as a sum. On the other hand, bulking effect can also be caused by the overproduction of exopolysaccharides, typically attributed to the genus \( \text{Zooglea} \) \((\text{Janarthanan et al., 2016; Jenkins, 1992})\). Members of the genus \( \text{Thauera} \), closely related to \( \text{Zooglea} \), have also been reported to be related to such episodes in activated sludge \((\text{Allen et al., 2004; Lajoie et al., 2000})\). In the present study, the relative abundance

### Table 4

Yields and rates from the PHA accumulation repeats with fermentation effluent at 90 \( \text{C} \text{initial concentration of VFA.} \)

<table>
<thead>
<tr>
<th>Batch</th>
<th>Total PHA (wt%)</th>
<th>HB (wt%)</th>
<th>HV (wt%)</th>
<th>HB:HV ratio</th>
<th>1,3-PDO recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B90_2</td>
<td>70.7</td>
<td>57.1</td>
<td>13.5</td>
<td>80.9: 19.1</td>
<td>100.0</td>
</tr>
<tr>
<td>B90_3</td>
<td>74.8</td>
<td>61.8</td>
<td>13.0</td>
<td>82.6: 17.4</td>
<td>94.4</td>
</tr>
<tr>
<td>B90_4</td>
<td>73.1</td>
<td>60.5</td>
<td>12.6</td>
<td>82.8: 17.2</td>
<td>100.0</td>
</tr>
<tr>
<td>B90_5</td>
<td>75.9</td>
<td>63.7</td>
<td>12.1</td>
<td>84.1: 15.7</td>
<td>97.8</td>
</tr>
<tr>
<td>B90_6</td>
<td>74.6</td>
<td>65.8</td>
<td>8.9</td>
<td>88.1: 11.9</td>
<td>99.1</td>
</tr>
<tr>
<td>B90_7</td>
<td>79.4</td>
<td>66.8</td>
<td>12.5</td>
<td>84.1: 15.7</td>
<td>97.8</td>
</tr>
<tr>
<td>B90_8</td>
<td>78.7</td>
<td>66.0</td>
<td>12.6</td>
<td>83.8: 16</td>
<td>98.7</td>
</tr>
<tr>
<td>B90_9</td>
<td>81.8</td>
<td>67.1</td>
<td>14.6</td>
<td>82.7: 17.8</td>
<td>97.2</td>
</tr>
<tr>
<td>B90_10</td>
<td>78.2</td>
<td>65.4</td>
<td>12.7</td>
<td>83.5: 16.3</td>
<td>99.2</td>
</tr>
<tr>
<td>B90_11</td>
<td>78.2</td>
<td>67.3</td>
<td>12.3</td>
<td>86.5: 15.7</td>
<td>107.9*</td>
</tr>
<tr>
<td>B90_12</td>
<td>75.7</td>
<td>64.2</td>
<td>11.4</td>
<td>84.8: 15.1</td>
<td>102.4</td>
</tr>
</tbody>
</table>

### Table 5

Yields and rates from the PHA accumulation repeats with fermentation effluent at 90 \( \text{C} \text{initial concentration of VFA.} \)

<table>
<thead>
<tr>
<th>Batch</th>
<th>Duration (h)</th>
<th>YPH</th>
<th>YP/S</th>
<th>( r_s )</th>
<th>( r_p )</th>
<th>( q_s )</th>
<th>( q_p )</th>
<th>PHA\text{ formation rate}</th>
<th>( \text{PHA end} )</th>
<th>( \text{TSS inv} )</th>
<th>( \text{TSS end} )</th>
<th>( \text{TSS lost} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>B90_initial test</td>
<td>3</td>
<td>0.91</td>
<td>0.75</td>
<td>29.7</td>
<td>0.86</td>
<td>4.2</td>
<td>21.6</td>
<td>26.8</td>
<td>0.91</td>
<td>0.69</td>
<td>0.87</td>
<td>4.4</td>
</tr>
<tr>
<td>B90_4</td>
<td>3</td>
<td>0.99</td>
<td>0.78</td>
<td>21.7</td>
<td>0.11</td>
<td>2.8</td>
<td>18.5</td>
<td>21.4</td>
<td>0.95</td>
<td>0.83</td>
<td>1.03</td>
<td>2.6</td>
</tr>
<tr>
<td>B90_5</td>
<td>3</td>
<td>0.99</td>
<td>0.88</td>
<td>21.8</td>
<td>0.08</td>
<td>2.7</td>
<td>18.8</td>
<td>21.2</td>
<td>1.00</td>
<td>0.92</td>
<td>1.18</td>
<td>2.3</td>
</tr>
<tr>
<td>B90_9</td>
<td>5</td>
<td>1.02</td>
<td>0.88</td>
<td>16.2</td>
<td>0.35</td>
<td>2.3</td>
<td>13.0</td>
<td>16.3</td>
<td>0.89</td>
<td>0.75</td>
<td>1.08</td>
<td>1.8</td>
</tr>
<tr>
<td>B90_10</td>
<td>4</td>
<td>1.08</td>
<td>0.82</td>
<td>16.6</td>
<td>0.01</td>
<td>2.0</td>
<td>14.0</td>
<td>17.5</td>
<td>0.85</td>
<td>0.83</td>
<td>1.13</td>
<td>1.6</td>
</tr>
<tr>
<td>B90_12</td>
<td>3.5</td>
<td>0.89</td>
<td>0.84</td>
<td>22.5</td>
<td>0.73</td>
<td>3.4</td>
<td>17.4</td>
<td>20.5</td>
<td>1.23</td>
<td>0.94</td>
<td>1.27</td>
<td>1.8</td>
</tr>
</tbody>
</table>

\( Y_{PH} \): Yield PHA/substrate. \( r_s \): Substrate consumption rate. \( r_p \): PHA formation rate. \( q_s \): Specific PHA formation rate. \( q_p \): Specific substrate uptake rate.


\( \alpha < 0.05 \). Thus, giving further evidence that the performance of the systems seemed to be governed by the initial cells concentration (TSS), and consequently of the main genera, rather than the relative composition of the culture. It is important to notice though, that these correlations were not established with the biomass composition present at the beginning of the PHA accumulation but with the biomass used as inoculum, which was the main interest of the study and the most likely source of variations. Consequently, possible changes in relative percentages occurring during the sedimentation step, which could have hampered the correlations, cannot be excluded.

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of OTUs classified as *Thauera* increased in all PHA accumulation repeats compared to the initial test (Fig. 4). Thus, exopolysaccharides production, as well as its potential causes, such as nutrient availability and toxicity factors (Sheng et al., 2010), should be further investigated as possible elements causing differences in the settlement behavior.

Interestingly, the TSS at the beginning of the batch had a linear correlation with the initial PHA content of the cells (Table 5). High PHA contents are known to favor cell settlement, due to increased density (Korkakaki et al., 2016). Nonetheless, differences observed here (from 1.6 to 4.4 wt%) could not justify important differences in the cell density. Alternatively, the PHA content at the end of the famine phase could be read as an indication of the physiological state of the cells, affecting their settling behavior. Similar observations were reported in the study of Valentino et al. (2015).

All in all, the causes of insufficient settling should be further investigated and avoided in order to consolidate a robust process and to maximize the transfer of biomass from the enrichment to the PHA accumulation. Alternatively, the implementation of flocculation agents or a centrifugation step could be considered.

### 3.6. Analysis of the polymer

In order to rule out the presence of any other PHA unit besides the ones measured by GC chromatography (HB, HV, HP), NMR analysis of the polymer was performed. This analysis was especially important given the observed partial consumption of undefined COD during the batches, which could have given unexpected PHA monomers. However, NMR results of the chloroform extracted PHA at the end of the butyrate phase confirmed the presence of only HB and HV monomers. Moreover, HB-HV bonds could be identified in the $^{13}$C NMR, revealing that the HB and HV units were in the form of a copolymer $\text{P(HB-co-HV)}$ (Fig. A1 and A.2 Supplementary materials).

SEC analysis displayed a unimodal distribution, corroborating that a copolymer was formed from HB and HV monomers during PHA accumulation (Fig. A3 Supplementary materials). The weight-average molar mass ($M_w$) was 529 kg/mol and the polydispersity index 2.37, which are values in the range of those previously reported for MMC, and a priori acceptable for thermoplastic applications (Laycock et al., 2014).

### 3.7. Overview of the developed process

The average PHA yield (0.99 $\text{Cmol PHA}/\text{Cmol S}$; 0.84 g COD/g COD), PHA content (76 wt%) and rate (1.13 $\text{Cmol PHA}/\text{Cmol X}\times 1^{-1}\times h^{-1}$ or 0.41 g PHA/L$\times h^{-1}$) obtained in the present study during the PHA accumulation were in the very high range of results previously reported in the literature (Valentino et al., 2016). The highest yield obtained from crude glycerol in MMC is of 0.51 $\text{Cmol PHB}/\text{Cmol glycerol}$ (Freches and Lemos, 2017), which led to 59 wt% PHA. Considering as well the stoichiometry of the fermentation, 1 $\text{Cmol}$ of glycerol would produce 0.50 $\text{Cmol}$ of 1,3-PDO and 0.23 $\text{Cmol}$ PHA, resulting in an overall carbon recovery of 0.73 $\text{Cmol}/\text{Cmol}$. This value would be higher than producing PHA directly from glycerol, which has a theoretical maximum of 0.67 $\text{Cmol PHB}/\text{Cmol glycerol}$ due to the CO$_2$ release occurring during the production of acetyl-coA as intermediate (Moraileo-Garate et al., 2011).

In terms of total COD, and considering the total COD input of the three steps of the process (consumed or not), the overall COD recovered in the products would be 0.61 g COD/g COD$_{\text{in}}$ (0.19 g COD PHA/g COD$_{\text{in}}$ and 0.42 g COD 1,3-PDO/g COD$_{\text{in}}$) (Fig. 7 B). Remarkably, this value would not increase any further by leaving the substrate to be totally consumed. Moreover, it is important to note that the COD provided in the enrichment represented only 12% of the total input, due to the low organic loading rate of this reactor. The total COD recovered as products was much higher than the maximum overall yield reported from PHA production directly from crude glycerol (0.32 g COD PHA/g COD$_{\text{in}}$ in Moita et al., 2014). It was also higher than preliminary results obtained through crude glycerol fermentation and conversion of both VFA and 1,3-PDO to PHA (0.31 g COD PHA/g COD$_{\text{total}}$ in Burniol-Figols et al., 2018).

Besides the advantages in terms of COD recovery, the process may also present a value in terms of the type of PHA produced. No other monomers besides HB have been described in PHA produced from direct transformation of glycerol (Zhu et al., 2013). In contrast, the PHA produced here from fermented crude glycerol included both HB and HV units. This could represent a further advantage of the suggested three step process, as P(HB-co-HV) copolymers are described to have better thermoplastics properties than PHB (Laycock et al., 2014).

A future development of the process should include the investigation of higher substrate concentrations, observing possible inhibition phenomena from 1,3-PDO or other inhibitory components in crude glycerol. Moreover, avoiding the use of synthetic substrates in the SBR could be evaluated. Despite the input being minor compared to the total COD (Fig. 7), its substitution would probably benefit the economics of the process. The use of other fermentation effluents containing only VFA or crude glycerol alone could be tested.

### 4. Conclusions

Using fermented crude glycerol and mixed microbial consortia (MMC), the present study assessed the possibility to selectively convert volatile fatty acids (VFA) into polyhydroxyalkanoates (PHA) while leaving 1,3-propanediol (1,3-PDO) in the supernatant. The following conclusions could be extracted:

- Selective consumption of VFA over 1,3-PDO was attained by using microbial biomass enriched in the absence of 1,3-PDO.
- The PHA accumulation led, on average, to 76±3.1 wt% PHA in the form of a copolymer of P(HB-co-HV), with a complete recovery of 1,3-PDO (99±2.1%).
- The SBR reactor presented a steady behavior despite changes in the relative percentage of the main genera of the MMC: *Thauera* and *Amaricoccus*.
- Highly reproducible values were obtained in terms of PHA yield and maximum PHA concentration, with average values of 0.99±0.07 $\text{Cmol PHA}/\text{Cmol S}$ (0.84 g COD PHA/g COD S) and 1.48±0.14 g PHA/L, respectively.
- Small variations in the substrate uptake rates and PHA formation rates during the PHA accumulation were attributed to variations in the biomass settling behavior prior to the PHA accumulation.
- The MMC presented preference for butyrate over acetate and propionate.
- The microbial culture was not inhibited when exposed to up to 90% COD of VFA and 150% COD$_{\text{in}}$ of 1,3-PDO, and presented a high PHA production rate of 0.41 g PHA/L$\times h^{-1}$.
- Taking into account the three steps of the process (crude glycerol fermentation, enrichment and PHA accumulation), the overall COD recovered as products was 0.61 g COD/g COD$_{\text{in}}$, where 0.19 g were in the form of PHA and 0.42 g in the form of 1,3-PDO.
Fig. 7. COD Balance of the process with influents indicated in red and effluents in green. Values next to the compounds indicate the g COD consumed or produced for one PHA batch accumulation (90 h CHZ) in Fig. A and the mass fraction of each component referring to the total COD input (g COD/g COD input) in Fig. B. For the PHA and 1,3-PDO streams, two sets of values are indicated separated by a bar, corresponding to values obtained after the butyrate phase or after depletion of all VFA. The term “Others soluble” refers to soluble COD excluding glycerol, 1,3-PDO, butyrate, acetate and propionate, thus contemplating mostly undetermined COD present in the crude glycerol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data
Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.02.029.

References
Appendix A: Supplementary figures of the polymer analysis

**Fig. A.1.** $^1$H-NMR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation in crude glycerol fermentation effluent (90 C$_{\text{mm}}$ initial VFA concentration).

**Fig. A.2.** A: $^{13}$C-NMR spectrum of the chloroform extracted PHA at the end of the batch PHA accumulation in crude glycerol fermentation effluent (90 C$_{\text{mm}}$ initial VFA concentration). B: Expanded spectrum for carbonyl carbon resonance, where peak e indicates the presence of HB-HV bonds.
Fig. A.3: SEC (Size-Exclusion Liquid Chromatography) signal of the chloroform extracted PHA at the end of the batch PHA accumulation in crude glycerol fermentation effluent (90 CmM initial VFA concentration).
To be submitted for publication
High polyhydroxyalkanoate productivity using cell-retention membrane bioreactors

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Abstract

One of the factors limiting the economic viability of polyhydroxyalkanoates (PHA) is the low volumetric productivity obtained with second-generation feedstocks, resulting from their low carbon concentration. In the present study, the use of membrane bioreactors (MBRs) was evaluated as a strategy to retain the microbial cells in the reactor and to enable a repeated supply of substrate without increasing the reactor volume. Immersed pressure-driven and diffusion-based MBR configurations were tested in a fed-batch PHA accumulation process using mixed microbial consortia. In the diffusion-based configuration, the rate of substrate diffusion (volatile fatty acids) was found to be lower than the substrate consumption rate, and thus, not suitable to attain high productivities. On the other hand, pressure-driven configurations (hollow fibers and ceramic filters) led to high values of productivity (0.87-1.44 g PHA/L/h). No flux reduction was observed in a 24 h fed-batch process, which allowed for a reduction of up to 82 % of the reactor volume, demonstrating the potential of this strategy. Advantages and limitations identified with hollow fibers and ceramic filters are discussed.
1 INTRODUCTION

The interest in biodegradable and biobased plastics has increased intensely during the last decades, due to their potential to mitigate some of the environmental impacts caused by petroleum-derived plastics. Among them, polyhydroxyalkanoates (PHA) have received significant attention given their high biodegradability, wide range of applications, and possible raw materials (Kourmentza et al., 2017). Yet, PHA represent only a minor percentage of the bioplastics market, what is to a big extend, due to their high production costs (Kourmentza et al., 2017).

Microbial cultivation techniques allowing for high values of PHA productivity (frequently in the range of 2-3 g PHA/L/h) were reported already during the 90s (Blunt et al., 2018; Ienczak et al., 2013). These strategies implied the use of axenic cultures, highly purified and concentrated feedstocks, and pure O₂ supply. All of which are factors that translate into high operational costs. Over the years, the focus has shifted to organic waste streams which, besides reducing costs, would bring improvements in terms of sustainability (Blunt et al., 2018; Koller et al., 2017; Kourmentza et al., 2017). For similar reasons, the use of mixed microbial consortia (MMC) is emerging, owing to their low operational demands (Kourmentza et al., 2017; Valentino et al., 2016).

Values of volumetric PHA productivity obtained with second-generation feedstocks have rarely surpassed values of 1 g PHA/L/h, neither in pure cultures or MMC (Blunt et al., 2018; Koller et al., 2017; Valentino et al., 2016). Hence, there is a need for developing efficient cultivation techniques to produce PHA from these substrates. One of the main challenges is the low carbon concentration in such feedstocks, which causes volume increase during fed-batch cultivations (Ienczak et al., 2013; Koller et al., 2017; Kourmentza et al., 2017). A way to circumvent this issue is the use of membrane bioreactors (MBRs) with cell-retention systems, which enable removal and addition of feed while keeping the cells in the bioreactor.

MBRs have been extensively applied in wastewater treatment (Judd, 2010) and increasingly in other biotechnological applications (Carstensen et al., 2012), but little research has been performed on the frame of PHA production. A few studies have explored the use of external loop MBRs in PHA production bioreactors with pure cultures (Ahn et al., 2001; Haas et al., 2017; Ienczak et al., 2016; Schmidt et al., 2016). In such systems, the fermentation broth is recirculated through an external cross-flow filtration module, where part of the supernatant is removed (Figure 1). High PHA productivities were reported, up to 4.6 g PHA/L/h in Ahn et al. (2001). However, external loop MBRs rely on high cross-flow velocities to control membrane fouling, which result in high operational costs (Gander et al., 2000; Judd, 2010). Moreover, cell recirculation can lead to shear stress or oxygen limitation, resulting in cell death or reduced cell growth (Carstensen et al., 2012; de Andrade et al., 2014; Judd, 2010).

Operational costs are generally lower in immersed MBR configurations (iMBR), in which the filtration modules are submerged in the cell broth and aeration can provide fouling control (Carstensen et al., 2012; Gander et al., 2000) (Figure 1). To the extent of our knowledge, iMBRs have only been tested for PHA production in the study of Kumar et al., (2018), resulting in a 2 fold increase in the cell concentration. Yet, the productivity of the system was low (< 0.1 g PHA/L/h) due to the low PHA yield of the strain.
Both immersed and external loop MBRs are based on the application of a pressure differential as the driving force to achieve liquid permeation through a membrane. More recently, an alternative MBR strategy has been suggested, using concentration gradients as the driving force (Judd, 2010; Mahboubi et al., 2016) (Figure 1). In such systems, the microorganisms are separated from the feed medium by means of a membrane, which allows diffusion of the substrate to the cell compartment due to the difference of concentration. Experience on these systems is very limited, and so far only focused on soluble products and anaerobic processes (Mahboubi et al., 2016). Membrane fouling - the main challenge of MBRs - can still occur, but it is less likely, as the magnitude of the force bringing the cells and other foulants to the surface of the membrane is smaller.

In the present study, immersed pressure-driven and diffusion-based MBR configurations were tested for the first time in the frame of PHA production using MMC. The main objective was to provide a proof of concept of their utilisation to overcome the volume limitations imposed by diluted feed solutions. Despite they are both immersed systems, the abbreviation iMBR is kept for conventional immersed pressure-driven MBRs, while dMBR is used to refer to diffusion-based MBR configurations.

Figure 1: Schematic representation of different MBR configurations. Alternative reactor designs are possible for each configuration. Modified from Mahboubi et al. (2016).

2 MATERIALS AND METHODS

2.1 PHA-producing culture

PHA producing culture was enriched and maintained in a Sequential Batch Reactor (SBR) run under aerobic feast-famine regime as described in Burniol-Figols et al. (2018b). Briefly, the reactor followed a cyclic operation, where every 12 h, half of the volume of the reactor was replaced with fresh medium containing crude glycerol, butyrate, propionate, and acetate, to a substrate concentration of 45 C_mM at the beginning of each cycle. The effluent at the end the cycle (850 mL) was used as inoculum for the PHA accumulation without any prior sedimentation.
2.2 Feed solution

The feed for the PHA accumulation was a synthetic mixture of VFA, simulating the concentration of VFA obtained experimentally after optimisation of crude glycerol fermentation in continuous mode (operated at pH 6.4, HRT of 10 h and 50 g/L of glycerol) (Varrone et al., 2018). The fermentation effluent contained butyrate (6.4 g/L), propionate (1.8 g/L), acetate (1.1 g/L) and lactate (0.75 g/L). Soluble ammonical nitrogen (N-NH₃) and phosphate (PO₄³⁻) were 10.8 and 501 mg/L, respectively, corresponding to a nitrogen/carbon ratio (N/C) of 1.8 Nmmol/Cmol and a phosphorous/carbon ratio (P/C) of 11.9 Pmmol/Cmol.

For the experiments described in section 2.3 (effect of N/C ratio) and due to volume limitations (see below), the VFA concentration was 10 times higher than in the model effluent. The proportion of each VFA and the P/C ratio was the same as in the model effluent. Different N/C ratios were tested (explained in section 2.3). For the experiments described in section 2.4 and 2.7 the VFA concentration and the P/C ratio was the same as in the model effluent and the N/C ratio was adjusted to 15 Nmmol/Cmol.

For all experiments, the VFA solution was prepared in BA Medium (Varrone et al., 2018) and adjusted to pH 6.4 with NaOH. N/C and P/C ratios were adjusted with NH₄Cl and K₂HPO₄. Antifoam 204 (Sigma Aldrich) and allylthiourea were added to a final concentration of 85 µL/L and 5 mg/L.

2.3 Fed-batch PHA accumulation: effect of the nitrogen/carbon ratio

PHA accumulation experiments were performed in a Multifors reactor (1.4 L) (Infors HT, Switzerland) with a starting volume of 650 mL of inoculum. The culture was fed with a VFA medium (section 2.2), in which the VFA concentration was 10 times higher than in the model effluent to circumvent the volume limitation of the reactors. Four experiments were performed at different N/C ratios (N mmol/Cmol): 1.8 (as in the model effluent), 15, 30 and 50. The experiments with N/C ratios of 1.8 and 15 ran simultaneously in parallel reactors using inoculum from the same SBR cycle. The experiments at N/C ratios of 30 and 50 also ran simultaneously on another SBR cycle.

Experiments were performed in a fed-batch mode for 24 h. Medium was initially added to a VFA concentration of 90 C mmol (14 mL of medium). Thereafter, the same feeding volume was repeated whenever butyrate was exhausted, manifested by an increase of more than 8 % in the dissolved oxygen saturation (pO₂) with respect to the pO₂ immediately after substrate addition. Aeration, agitation, temperature and pH were kept at 1 L/min, 750 rpm, 30 °C and pH 8, respectively.

2.4 Diffusion-based MBR configuration (dMBR)

VFA diffusion was tested in two membrane materials: PSf (Polysulphone) and PVDF (Polyvinylidene fluoride), both with a polypropylene as a support layer. The PSf membrane had a pore size of 0.2 µm (GRM0.2PP, Alfa Laval, Sweden - 270 µm thickness). Two pore sizes (0.15 and 0.45 µm) were tested for the PVDF membrane (FSM0.15PP and FSM0.45PP, Alfa Laval, Sweden - 285 µm thickness). Before use, the membranes were
cleaned with distilled water (dH₂O) and dilute NaOH (pH 10) at 45 °C according to the manufacturer instructions.

Figure 2: Diffusion-based MBR (dMBR). A: Membrane support. B: Schematic representation of the diffusion membrane inside the bioreactor. Blue arrows indicate diffusion across the membrane.

The membranes were placed in an aluminium support inside the bioreactor, separating the bioreactor in two concentric compartments (Figure 2). The internal compartment contained the microbial culture. Aeration (1 L/min), agitation (750 rpm) and pH control (pH 8) were performed in this compartment. The feed was supplied in the external compartment, which was cell free. The membranes were placed with the active membrane surface in contact with the external compartment. The total active surface of the membrane was 312 cm².

To test VFA diffusion across the membranes, the external compartment was filled with 160 mL of medium at pH 6.4 (section 2.2). The internal compartment was loaded with 650 mL of autoclaved inoculum (to avoid VFA consumption). The VFA diffusion rate (in Cmmols/h) was calculated from the slope of the VFA amount detected in the inner compartment vs. time during the first 30 min, and thus corresponded to the initial and maximum VFA diffusion rate.

2.5 Immersed pressure-driven MBR configurations (iMBR)

2.5.1 Hollow fibers

Hollow fiber modules were purchased from Zena Membranes (Czech Republic). They consisted of polypropylene fibers with an average pore size of 0.1 µm and an external and internal diameter of 310 and 240 µm, respectively. Two types of membrane configurations were tested: a rod configuration (Figure 3 A) and a loop configuration (Figure 3 B). The rod consisted of 800 fibers of 10 cm length, and the loop was made of 400 fibers of 20 cm. Both configurations had a filtration surface of 779 cm², and were connected to a PVC (polyvinyl chloride) pipe placed in a 12 mm port in the bioreactor. In order to minimise the internal volume of the PVC pipe, a tube of 2 mm diameter was placed inside, directly connecting the outlet of the pipe to base of the pipe. For the loop configuration, a plastic net was fixed between the agitation shaft and the filter module to avoid damage by the impellers (Figure 3 B).
Before the first use, the fibers were soaked in 2-propanol for 30 min and rinsed with dH2O. The fibers were always kept in dH2O to prevent drying. In between experiments, the fibers were cleaned with NaOH 0.1 wt%, citric acid 2 wt% and dH2O. For each reagent, the cleaning consisted of three steps: soaking, filtering and backflushing (30 min each). dH2O permeability was measured before each experiment.

Figure 3: Hollow fiber configurations: A: Rod configuration, B: Loop configuration placement in the bioreactor. C: Schematic representation of the hollow fiber rod module inside the bioreactor.

2.5.2 Ceramic filters

The ceramic filtration module was built with 8 ceramic cylinders (silicone carbide) of 10 mm external diameter, 6 mm internal diameter and 12 cm length (LiqTech International, Denmark). The active filtration layer was on the external part of the cylinders (0.5 µm pore size) (Figure 4). The filters were fit in a stainless steel support secured with threaded rods and sealed with silicone. The upper part of the support connected the internal volume of all filters to a single steel outlet tube fixed to one of the ports of the bioreactor. The active surface area of the module was 276 cm². No pretreatment was applied before the first use. In between experiments, the filters were cleaned by soaking, filtering and backflushing for 30 min with NaOH 2 wt% (30 °C), followed by 30 min backflushing air at 1.5 bar. The cleaning procedure was then repeated with dH2O. dH2O permeability was measured before each experiment.

Figure 4: A: Ceramic filtration module. B: Schematic representation of the ceramic filtration module inside the bioreactor
2.6 Evaluation of fluxes in iMBR configurations

Filtrations were carried out using a peristaltic pump (Masterflex L/S 7518-00), in which the relation between rpm and flow (mL/min) was calibrated with dH₂O at room temperature without being coupled to any filtration device. Critical and limiting fluxes were assessed by the flux stepping method (Bacchin et al., 2006). The setpoint of the pump was increased step-wise from 8-25 mL/min, recording the permeate flow and the transmembrane pressure (TMP) (measured between the peristaltic pump and the filter with a gauge pressure manometer (GMS20, Professional Instruments – measuring range ± 350 mbar). For each setpoint, the filtration proceeded until 160 mL of permeate had been removed (target volume exchange in fed-batch as explained in section 3.2.2). Thereafter, the permeate was returned to the bioreactor through the filter in reverse flow. The reverse flow setpoint was the same as the filtration flow for setpoints higher than 16 mL/min. For lower filtration flows, the reverse flow was constant at 16 mL/min.

This cycle of filtration and backflushing was repeated 5 times for each setpoint. Flow-TMP graphs were constructed using the TMP read at the end of the 5th filtration of each setpoint flow.

During these tests, aeration, agitation, temperature and pH were maintained as during cultivation (section 2.3). The experiments were performed with dH₂O and cell cultures at different concentrations (1.7 and 16 g/L), corresponding to the ones observed at time 0 h and 20 h during the fed-batch with an N/C ratio of 15 Nmmol/Cmol (section 3.1).

2.7 Fed-batch PHA accumulation in iMBRs

PHA accumulation was performed similarly to the description in section 2.3. The key difference was that when butyrate depletion was detected, 160 mL of cell-free liquid were removed though the filtration modules (hollow fibers loop or ceramic filters). Thereafter, 160 mL of fresh feed (at the concentration of the model effluent, section 2.2) were added through the filter in reverse flow, keeping the volume constant at 750 mL throughout the 24 h fed-batch (except for sampling and acid/base addition). The batch started with 850 mL of inoculum from the SBR and thus the first filtration removed slightly more permeate (260 mL) to reach the 750 mL after the first feeding.

Based on the results of section 3.2.2, the filtration flow was set at 16 mL/min for the hollow fiber module (loop configuration). For the ceramic module, the filtration flow was set at 16 mL/min for the first filtration and at 13 mL/min for the subsequent ones. Feed addition occurred at 16 mL/min in reverse flow. In order to avoid accumulation of feed in the inner space of the filters after feed addition, about 7 mL of permeate were returned to the reactor in reverse flow.

2.8 Analytical methods

Samples taken from the bioreactors were centrifuged at 8000 g for 5 min. The supernatant was filtered through 0.45 µm Nylon filters and analysed for VFA and N-NH₃ concentration as described in Burniol-Figols et al. (2018a). The pellet was washed with distilled water and centrifuged again twice before freeze-drying.
The PHA content of the biomass pellet was determined by GC chromatography as described in (Burniol-Figols et al., 2018a), using methyl-3-hydroxybutyrate (99 %, J&K Scientific®) and methyl-3-hydroxyvalerate (≥ 98 %, Sigma-Aldrich®) as calibration standards (Burniol-Figols et al., 2020). Total biomass concentration was estimated by measurement of the total suspended solids (TSS) in fresh samples, performed according to standard protocols (APHA et al., 2005) using glass fiber filters of 0.7 µm pore size (Merk Millipore). PHA concentration (g/L) was obtained by multiplying the TSS (g/L) by the PHA content (wt%). PHA and active biomass (cells excl. PHA or X) concentrations were then estimated by subtracting the calculated PHA (g/L) from the TSS (g/L).

In the membranes used in the diffusion set-up, water contact angle (θ°) was measured by the sessile drop method (Hebbar et al., 2017) in a goniometer (OCA20, Dataphysics Instruments, Germany). For each membrane, the reported values correspond to the average θ° measured in five random locations, recorded immediately after dropping 1 µL of MiliQ® water at room temperature onto the membrane. Membrane surface charge was assessed by the zeta potential, measured in a SurPASS electrokinetic analyzer (Anton Parr, Austria) as described in Zhang et al. (2017).

3 Results

In order to maximise productivity in a MBR configuration, two aspects have to be met. The first is that the culture is able to sustain PHA accumulation and reach high cell densities. This aspect is evaluated in section 3.1. The second aspect is that the MBR configuration is able to operate for the whole PHA accumulation and meet the requirements of the culture. This is presented in section 3.2.

3.1 Effect of the nitrogen/carbon ratio on culture saturation

The main advantage of an MBR is the possibility of operating at a constant volume. Extended PHA accumulation batches (maximising the g PHA/g initial biomass) would be desirable to fully exploit this advantage and minimise the volume of the reactor producing inoculum (SBR). However, it is very common that PHA production ceases when cells reach maximum PHA accumulation capacity. This is a result of operation under severe nitrogen limiting conditions, which maximise PHA accumulation and prevent cell growth (Kourmentza et al., 2017; Valentino et al., 2015). Recent studies have reported the possibility of extending the PHA accumulation and increase the PHA productivity by supplying limiting rather than starving nutrient conditions, which allow cell growth and PHA accumulation to occur simultaneously (Valentino et al., 2015).

In here, four nitrogen to carbon (N/C) ratios were tested during fed-batch PHA accumulation. Feed addition occurred whenever butyrate was exhausted, as previous studies on this culture evidenced that net PHA production drastically reduced during consumption of the remaining VFA (acetate and propionate) (Burniol-Figols et al., 2018b). The culture produced a copolymer (poly (3-hydroxybutyrate-co-3-hydroxyvalerate)) referred simply as PHA in the text and figures.
The four N/C ratios tested (expressed in terms of $N_{\text{mmol}}/C_{\text{mol}}$) resulted in 4 different scenarios in terms of nitrogen availability (Figure 5 A). In the two lowest ratios (1.8 and 15) all the nitrogen provided was consumed and both cultures were nitrogen limited for the whole batch. However, the degree of limitation was more severe in the first case, which could be considered nitrogen starved. For the N/C ratio of 30, the culture had nitrogen in excess for the first 12 h, but thereafter became nitrogen limited. At an N/C ratio of 50, the culture had excess of nitrogen throughout the cultivation.

Figure 5: Fed-batch PHA accumulation experiments at different nitrogen/carbon ratios.*Volumetric productivity was calculated considering a constant volume equal to the initial volume, to better reflect the obtainable productivity in an MBR configuration with constant volume.
With the N/C relation present in the model effluent (1.8 N\text{mmol}/C\text{mol}), substrate consumption and PHA production considerably slowed down after 6 h (Figure 5 B and C). Cell growth was almost negligible (Figure 5 E), what probably led to a saturation of the cells once they had reached its maximum PHA content of about 70 % (Figure 5 G).

For the other three N/C ratios tested, the culture had a sustained substrate consumption throughout the 24 h (Figure 5 B). For all parameters evaluated, these three experiments behaved almost identically for the first 12 hours (Figure 5). At this point, about 8 g of PHA had been produced (Figure 5 C) at a yield of 0.7-0.8 C\text{mol} PHA/C\text{mol} S (Figure 5 H). The productivity observed for these three experiments during the first 12 h more than doubled the one observed for the N/C ratio of 1.8 (1.0 vs 0.43 g PHA/L/h) (Figure 5 D).

From 12 h to 24 h, the behaviour of the culture differed slightly according to the nitrogen loading. Higher nitrogen concentrations enabled a higher production of active biomass (Figure 5 E), which translated into a slightly higher substrate consumption (Figure 5 B). For the nitrogen limited case (N/C ratio of 15), this did not have negative effects on the PHA yield or PHA content. At the end of the batch, these parameters presented values of 0.72 C\text{mol} PHA/C\text{mol} S and 78 %, respectively, very close to the obtained in nitrogen starving conditions (Figure 5 H and G). On the other hand, for the N/C ratios of 30 and 50, the higher biomass yield translated in a decrease in the PHA yield and PHA content, especially remarkable for the N/C ratio of 50 (nitrogen excess throughout the batch). The latter presented a PHA yield of 0.45 C\text{mol} PHA/C\text{mol} S and a decrease of the PHA content from 70 % at 12 h to 60 % at 24 h (Figure 5 H and G). This decrease in the PHA content would indicate that the growth response had taken over the PHA accumulation of the culture.

The N/C ratio of 30 presented values only slightly lower than the N/C ratio of 15 for all parameters. The differences were usually not significant when considering the measurements’ standard deviation, but could already indicate that the growth response was starting to take over at this nitrogen loading. Therefore, an N/C ratio of 15 was chosen for further experiments in MBR configurations.

In terms of productivity, the values calculated for the whole 24 h batch were considerably lower than obtained during the first 12 h (Figure 5 D). The reason behind that was an important decrease in the specific substrate consumption rate (q_s) with time (Figure 5 F). Hence, even though the productivity could be greatly enhanced by supplying extra nitrogen and allowing cell division to occur, PHA accumulation rates decreased for all the N/C ratios tested. For the N/C ratio of 15, the productivity decreased from 1.00 g PHA/L/h at 12 h to 0.78 g PHA/L/h after 24 h, being this the scenario with the least drop in productivity (Figure 5 D).

### 3.2 Evaluation of MBR configurations

#### 3.2.1 Evaluation of the dMBR configuration

For this configuration, microfiltration membranes were used to create a cell-free compartment, from where clarified broth could be withdrawn without removing cells from the bioreactor (Figure 2). The same compartment would be used to supply fresh medium containing VFA, which would diffuse across the membrane to the cell-containing compartment to support PHA accumulation.
In order to maximise PHA productivity with this configuration, VFA diffusion across the membrane had to be at least equal to the substrate consumption rate of the culture ($r_s$). Therefore, VFA diffusion was measured in different membrane materials (PSf and PVDF) and compared to $r_s$ (36.7 Cmmols VFA/h in the experiments in section 3.1). This minimum threshold of VFA diffusion was not reached for any of the membranes (Figure 6 A), and thus the configuration would not be able to sustain the PHA accumulation process.

Figure 6: Results of the diffusion-based MBR with PSf and PVDF membranes at different pore size. A: VFA diffusion across the membranes (Cmmols/h). The dashed line across the graph indicates the substrate consumption rate of the culture ($r_s$) (Cmmols/h). B: Water contact angle. C: Zeta Potential.

For all experiments, the diffusion rate of each of the VFA (butyrate, propionate, acetate and lactate) was proportional to their concentration, suggesting no preferential membrane selectivity. At a similar pore size, the PSf membrane (0.2 µm) presented a much higher diffusion rate than the PVDF membrane (0.15 µm) (19 vs. 5 Cmmols VFA/h). With the first, the diffusion was comparable to the achieved with the PVDF membrane at 0.45 µm (18 Cmmols VFA/h). Hence, PSf resulted in a higher diffusion rate.

The hydrophilicity of the materials was measured by contact angle measurements (Figure 6 B), were values below 90° are generally accepted to correspond to hydrophilic surfaces, while hydrophobic for higher values (Hebbar et al., 2017). According to this, PSf was hydrophilic, but PVDF was slightly hydrophobic, with values around 95° and no significant differences between pore sizes ($p = 0.1$).

All membranes presented a negative surface charge at the pH of the feed solution (pH 6.4) (Figure 6 C). At this pH, the VFA also have a negative charge. Thus, charge repulsion could have hindered diffusion across the membrane. For the PVDF membranes, the charge was more negative for the 0.15 µm pore membrane compared to the 0.45 µm, which could be a possible explanation for the reduced VFA diffusion obtained at the lower pore size.

Based on these results, higher VFA diffusion rates obtained with PSf compared to PVDF at a similar pore size could be related to the higher hydrophilicity of this membrane combined with the lower negative charge density.

### 3.2.2 Evaluation of fluxes iMBR configurations

The main limitation of iMBRs is the occurrence of fouling and the consequent reduction of the membrane performance. This phenomenon has led to the definition of two flux concepts: the critical flux and the limiting flux (Bacchin et al., 2006). The critical flux is the one at which irreversible membrane fouling starts to occur.
Assuming weak form behaviour, this can be defined as the point at which the relation between the transmembrane pressure (TMP) and flux starts to deviate from linearity, or the point at which the TMP increases at a constant flux (Bacchin et al., 2006). The limiting flux is the highest flux that can be maintained in a given system. Below this flux, fouling might be forming, but it is still possible to maintain the flux by increasing the TMP. Above the limiting flux, the fouling saturates the filtration capacity of the membrane. Here, the discussion is done in terms of flow (instead of flux) for the ease of comparison between setups with different filtration areas.

The ultimate objective of the fed-batch cultivation using MBRs was to remove supernatant every time that butyrate was exhausted and replace it with fresh VFA medium. In order to minimise the time that the culture was in absence of butyrate, the filtration time interval had to be as short as possible, but without surpassing the limiting flow, or in the ideal case, the critical flow. The target was to remove 160 mL of supernatant (about 1/5th of the working volume) in 10 min (16 mL/min). By replacing this volume, the initial concentration of substrate after each feeding would be about 90 mM of VFA, which was found not inhibitory for the culture in previous studies (Burniol-Figols et al., 2018b). The two submerged membrane types (hollow fibers and ceramic filters) were tested at flows between 8-25 mL/min, to determine the critical and limiting fluxes. Two cell concentrations were evaluated (1.7 and 16 g/L), corresponding to the cell concentration at the beginning of the fed-batch and after 20 h, respectively (section 3.1).

**Hollow fibers**

Two type of hollow fiber configurations were tested: a rod configuration and a loop configuration (described in Figure 3). With the rod configuration, the target flow of 16 mL/min (12.3 L/h/m²) could not be attained, even with the initial cell concentration of the fed-batch (1.7 g/L) (Figure 7 A). The pump could not maintain flows over 10 mL/min, which was identified as the limiting flow of the system.

The loop configuration was much less prone to fouling. The peristaltic pump could always maintain the setpoint flows at the tested range, even with a concentrated cell culture (16 g/L) (Figure 7 B). In other words, the limiting flow was not reached. At the cell concentration of the beginning of the batch (1.7 g/L), the system did not show significant increases in the TMP in consecutive filtrations at the same setpoint (Figure 7 D). Moreover, the relation between TMP and flow was linear for the whole range (Figure 7 C). Hence, the results did not show signs of having reached a critical flow.

With the cell culture of 16 g/L, the critical flow could be identified at 19 mL/min (14.6 L/h/m²). At this point, the TMP vs. flow curve started to deviate from linearity (Figure 7 C), and a slight increase in the TMP in consecutive filtrations could be observed (Figure 7 D). Based on these results, operation of the hollow fiber loop module at 16 mL/min (12.3 L/h/m²) was considered for the fed-batch PHA accumulation experiments presented in section 3.3.
Ceramic filters

At the initial cell concentration (1.7 g/L), the ceramic filtration module presented a very similar behaviour to the hollow fiber loop. Neither a limiting flow nor a clear critical flow could be identified (Figure 8). The pump could always maintain the setpoint flow (Figure 8 A) and there were no clear deviations from linearity in the curve TMP vs. flow (Figure 8 B). No significant increase in the TMP was observed in successive filtrations at the same setpoint (Figure 8 C).

At the high cell concentration (16 g/L) though, important fouling was observed. Already at 13 mL/min, the TMP increased considerably from the first to the fifth filtration (Figure 8 C), and the TMP increased exponentially in relation to the flow (Figure 8 C). The critical flow was identified at about 13 mL/min (28.2 L/h/m²), although lower flows should be tested to better identify the linear region of the function TMP vs. flow in Figure 8 B. At 13 mL/min irreversible fouling started to occur, but it was not until much higher flows (25 mL/min) where flow reduction was observed (Figure 8 A) (limiting flow).

Given that the system presented clear fouling at the target flow (16 mL/min), an additional anti-fouling strategy was considered, consisting of backflushing air through the filter. This is a common cleaning strategy (Judd, 2010), which can be applied in non-filtration periods for fed-batch processes (Suzuki, 1994). The filters presented a very high resistance to air and very little air flow could be obtained at pressures lower than 1.5 bar. At this pressure, 250 mL/min of air could be transferred. However, when this strategy was tested with the cell culture, it led to intense foam formation, which could not be controlled even with addition of antifoam.
at concentrations above the recommended by the supplier (Antifoam 204 Sigma-Aldrich at 0.2%). Hence, air backflushing could not be applied as additional antifouling strategy.

In order to avoid fouling and eventual saturation of the filters, operation below the critical flux is preferred. For the present case, a sub-critical flux would represent a decrease in the productivity of the system, as it would extend substrate-limiting periods. Based on these results the fed-batch PHA accumulation with ceramic filters operated at 13 mL/min (28.2 L/h/m²), which was already at the critical flow, but far below the limiting flow. A flow of 16 mL/min was applied to the first filtration of the batch, as this was below the critical flow observed for the cell concentration at the beginning of the batch.

Figure 8: Evaluation of critical and limiting flux for the ceramic filtration module. In graphs A and B, the values of flow and transmembrane pressure (TMP) are the ones recorded at the end of the 5th filtration at each flow setpoint.

3.3 Fed-batch PHA accumulation in iMBRs

Hollow fibers (loop configuration) and ceramic filters were tested in a fed-batch PHA accumulation regime using synthetic VFA medium. The two systems presented very similar results (Figure 9).

The fed-batch proceeded using the filters to remove permeate (1/5th of the working volume) whenever butyrate was consumed. Fresh feed solution was then provided through the filter in reverse flow. During a 24 h experiment, this procedure occurred over 20 times, without observing any reduction on the filtration flow (Figure 9 A and D). Both iMBRs allowed to feed over 3.5 L of substrate through the reactor, while keeping the working volume at 0.75 L. As a result, they enabled an 82 % reduction on the working volume, compared to a scenario where these experiments would be performed without a cell-retention system. The concentration of PHA at this point was 18.5 g/L (Figure 9 B and E).
The use of submerged filtration during PHA accumulation resulted in high values of productivity (0.87-1.44 g PHA/L/h) (Figure 9 C and F). However, a decrease of PHA productivity was observed during the fed-batch, especially after 12 h, as already observed in the experiments without cell-retention systems presented in section 3.1. After 12 h, the productivity was 1.20 and 1.27 g PHA/L/h, for hollow fibers and ceramic filters, respectively. At 24 h, the values decreased to 0.89 and 0.87 g PHA/L/h, respectively. If stopped after 12 h, the bioreactors would present a total TSS concentration of about 20 g/L with a PHA content of 70 % PHA (14 g PHA/L) (Figure 9 B and D). At this point, the use of iMBRs would represent a 75 % reduction of the working volume with respect to a system without cell recycling (about 2 L of feed had been circulated).

Overall, despite the iMBRs were capable of sustainable operation for 24 h, such long cultivations would not appear as a clear choice looking exclusively at the values of productivity during PHA accumulation (Figure 9 C and F). However, the perspective might change when taking into consideration the volume and duration of the cycle of the SBR (inoculum reactor), and the overall productivity of the process; as extended PHA accumulation batches would bring a reduction of the inoculum need and thus a reduction of the SBR reactor.

It is important to mention that the results presented for the hollow fibers module were obtained with a periodical rotation of the hollow fiber module during the fed-batch to release the cell cake. When this procedure was not done, considerable cell deposition was observed on the hollow fibers, and in the zones in

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**Figure 9: Fed-batch PHA accumulation in iMBR: loop hollow fibers (left) and ceramic filters (right).**
contact with the wall of the reactor. The cake did not affect the performance of the membrane, which could still maintain the flow during the whole 24 h fed-batch, but it compromised the performance of the culture and the determination of microbial biomass. By slightly rotating the filter, cell deposits were released by the turbulence created by the agitation and aeration. Immediately after rotating, the concentration of suspended cells would increase by 13%, and a drop in the oxygen saturation was observed, indicating an increase in the metabolism. It is a known fact that oxygen and substrate can become limiting in the inner layers of the cake, in which the cells are at risk of death (Iorhemen et al., 2016). In the present setup, cake formation led to a decrease of 46% in the substrate consumed and PHA produced (when considering the whole 24 h batch). Such an important effect could be a result of the limited space between the fiber loop and the wall of the reactor (Figure 3 B), which allowed little movement of the fibers.

4 Discussion

4.1 Perspectives on the use of dMBRs for PHA production

With the reactor design and membranes studied in this work, diffusion of VFA across the membrane did not meet the substrate requirements of the culture (section 3.2.1). At the highest porosity tested, PSf (0.2 µm) and PVDF (0.45 µm) allowed for a diffusion rate between 18-19 mmol VFA/h (corresponding to a flux of 576-610 mmol/h/m²) (Figure 6). This value should have been double in order to meet the substrate requirements of the present culture (36 mmol VFA/h).

Diffusion rate of VFA across the membranes is proportional to the membrane surface and the concentration difference between the two sides of the membrane (Mahboubi et al., 2016). Thus, the strategy could still be valuable in a reactor design with double surface of contact. In the same line, substrate requirements could be met with a feed solution of approximately double VFA concentration (assuming a proportional concentration polarisation effect). This would correspond to a concentration of about 20 g/L of VFA, which is common in many fermentation effluents (Valentino et al., 2016). However, even in that scenario, the concentration of VFA in the feed side would decrease as acids diffuse to the cell compartment, decreasing the transmembrane gradient. Hence, the VFA diffusion rate would decrease with time, eventually leading again to a situation where substrate requirements would not be met. A possible way to achieve a constant VFA flux would be a continuous recirculation of broth from the fermentation bioreactor to the feed compartment of the PHA accumulating reactor, similar to what was suggested by Du and Yu, (2002).

In regards to membrane materials, the results showed higher diffusion rates with PSf compared to PVDF, with the first being more hydrophilic. Higher diffusion for hydrophilic materials was also reported by Du and Yu (2002) and Youngsupkasem et al. (2013), which are, to our knowledge, the only previous reports evaluating VFA diffusion studies in a similar context. Moreover, the present study showed that VFA diffusion was highly correlated with the negative charge density on the membrane surface (Figure 6), possibly justified by repulsive forces with the ionised carboxyl groups of VFA at pH 6.4. To reverse these interactions, the pH should be lower than the isoelectric point of the membranes (about pH 4 in Figure 6 C), or lower than the pKa of the acids (3.8 - 4.9). Given that these are rather low values of pH to carry out a biological process, it would
be interesting to study other hydrophilic membrane materials with positive or neutral surface charge at a higher pH range.

Small pore sizes are generally preferred in MBR applications, as they minimise pore clogging by microbial cells (Ilorhemen et al., 2016). Yet, our results showed that smaller pore size had an important effect on the charge density and led to lower diffusion rates. Therefore, bigger pore sizes might be beneficial for diffusion-based applications.

4.2 Perspectives on the use of iMBRs for PHA production

This section discusses the main challenges and opportunities identified with the use of iMBR configurations (hollow fibers and ceramic filters) for PHA production, summarised in Table 1.

Two types of hollow fiber configurations were tested in this study: a rod configuration and a loop configuration (Figure 3). As described in section 3.2.2, the rod configuration was more prone to fouling than the loop. In the rod, the fibers were very compact, allowing little circulation of liquid in between. Conversely, the fiber loop allowed higher movement of air and liquid in between the fibers, which provided membrane scouring and alleviated fouling. However, this looser configuration also led to higher fiber surface, creating a cell-retention net. Cells would get trapped in this fiber net, and between the fibers and the reactor wall, resulting in cell deposits. As explained in section 3.3, this did not lead to problems of membrane operation, but it led to an important decrease on the substrate consumption rate (46 %), most likely caused by substrate and oxygen limitation in the inner layers of the cell cake. In the current setup, this could be solved by rotating the fibers, what changed the flow patterns between the fibers and released the cell cake. This aspect would be of critical relevance when considering a scale-up of the process, especially considering that the product (PHA) would also be trapped.

Table 1: Main opportunities and challenges of hollow fibers and ceramic filters applied as submerged filtration systems during PHA accumulation.

<table>
<thead>
<tr>
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<th>Hollow fibers</th>
<th>Ceramic filters</th>
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<tbody>
<tr>
<td><strong>Advantages/opportunities</strong></td>
<td>• Stable permeate flux during 24 h fed-batch process.</td>
<td>• Stable permeate flux during 24 h fed-batch process.</td>
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<td></td>
<td>• Low relation footprint/filtration area.</td>
<td>• Not prone to cell cake formation.</td>
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<tr>
<td></td>
<td></td>
<td>• More resistant to chemical cleaning. Possibility to couple to PHA purification through chemical digestion methods.*</td>
</tr>
<tr>
<td><strong>Disadvantages/challenges</strong></td>
<td>• Prone to cake formation. The fiber net traps cells leading to reduced cell activity and lower PHA production.</td>
<td>• High relation footprint/filtration area.</td>
</tr>
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</table>

*According to literature information. Not tested in this study.

Cake formation was not an issue in ceramic filters. Their tubular smooth surface was not prone to accumulate cells, aided by the turbulence of the bioreactor. However, the ceramic filters presented a lower critical flow
compared to the hollow fiber loop (13 vs. 19 mL/min) (section 3.2.2). This was a result of the higher filtration surface provided by the hollow fibers, which was almost three times bigger (779 cm² vs. 276 cm²). In terms of flux, the critical point was higher for the ceramic filters (28.2 vs. 14.6 L/h/m²). Space limitations in the bioreactor impeded the possibility of increasing the surface of filtration. Given the lesser propensity of ceramic filters to cell cake formation, it would be interesting to explore filter configurations that could increase the filtration area without increasing the footprint.

A successful fed-batch operation with effluent removal could be attained with both the fiber loop and the ceramic filters (section 3.3), meaning that no reduction of the flow was observed. Both systems allowed a rapid exchange of exhausted supernatant for fresh medium (aprox. 10 min) below their limiting flow. However, in order to minimise periods without substrate, the ceramic module operated above its critical flow towards the end of the batch, when high cell concentrations were reached (according to the results in Figure 8 section 3.2.2). At this point of the discussion, it is interesting to introduce the concept of the sustainable flux (Bacchin et al., 2006). Operation below the critical flux, where no fouling occurs, is a good strategy to prolong membrane performance and to minimise the costs of membrane cleaning. However, operation at such fluxes might not be feasible for certain processes. Instead, it might be feasible to operate at fluxes with a low fouling rate, in which the cost and downtime from membrane cleaning are reasonable. In this respect, it would be interesting to consider the integration of membrane cleaning and PHA purification. In recent years, PHA purification methods based on the use of chemicals to digest and solubilise non-PHA material have gained interest. Such processes involve the same type of chemicals used for membrane cleaning (alkalis, acids or oxidizing agents) (Burniol-Figols et al., 2020; Judd, 2010; Kourmentza et al., 2017), and thus integration of both operations could be envisioned. In this regard, ceramic membranes would offer an additional advantage, given their higher resistance to chemicals and overall robustness (Carstensen et al., 2012).

The main objective of this study was not to define a sustainable flux, but to perform a proof of concept of the utilisation of MBRs for PHA accumulation. In this sense, both iMBR configurations proved valuable to overcome the limitations of dilute feeding solutions, reaching high PHA productivities (0.87-1.44 g PHA/L/h). These values are among the highest reported for PHA production processes in MMC - generally below 0.5 g/L/h (Valentino et al., 2016) - showing the great potential of this cultivation strategy. Besides being valuable for fed-batch cultivations, iMBR could also be an asset in PHA production in continuous mode, as they could offer the possibility of uncoupling the hydraulic retention time from the cell retention time and cell growth.

5 CONCLUSIONS

One of the main limitations to obtain high PHA productivities with second-generation substrates is the dilute nature of the feed solutions. The present study evaluated for the first time the use of membrane bioreactors (MBRs) in the frame of PHA production in mixed microbial consortia. The main conclusions of the work were:

- In pressure-driven immersed MBR configurations (iMBR), both hollow fibers and ceramic filters allowed to feed over 3.5 L of substrate in a fed-batch period of 24 h, while keeping a constant reactor volume of 0.75 L, representing an 82 % reduction of the reactor volume.
• A high volumetric productivity could be attained in iMBR configurations (0.87-1.44 g PHA/L/h) despite the use of a diluted substrate feed (approximately 10 g/L of volatile fatty acids).
• Hollow fibers allowed to operate at higher flows, due to their lower ratio of surface/footprint. However, they presented a risk of cell deposition, resulting in a decreased metabolic activity and PHA production rate.
• Ceramic filters were less prone to cell deposition and fouling, but they had a higher footprint, leading to a lower critical flow with the surface available in the bioreactor.
• VFA diffusion across PSf and PDVF microfiltration membranes was not enough to satisfy the substrate requirements of the culture in a diffusion-based MBR configuration.

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References


Polyhydroxyalkanoate (PHA) purification through dilute aqueous ammonia digestion at elevated temperatures

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Abstract

BACKGROUND: Purification of polyhydroxyalkanoates (PHA) is a challenging step, given the difficulty of achieving high PHA purity, while maintaining polymer integrity, in a sustainable and cost-efficient manner. This study evaluated the potential of dilute ammonia digestion as a method to purify PHA from mixed microbial consortia.

RESULTS: Digestion temperatures were critical to the obtainable purity and the amount of recovered PHA. At temperatures below 75 °C (regardless of the incubation time and ammonia concentration), a low PHA recovery (down to 65 %) and no increase in purity was observed. By increasing the temperature above 75 °C, a significantly higher PHA purity and higher recovery (above 90 %) could be achieved. Temperatures maximizing the purity (140 °C) led to a detrimental reduction in the molar mass of the isolated PHA, but the use of a sonication pre-treatment enabled to increase the purity at temperatures leading to limited molar mass loss (75-115 °C). The impurities still present in the recovered PHA did not compromise its thermal stability, and no significant degradation occurred during melting of PHA with 86 % purity (comparably to pure chloroform-extracted PHA). Conversely, PHA recovered through H₂SO₄ digestion underwent severe degradation during melting, despite presenting higher purity (98 %).

CONCLUSIONS: High PHA purity, recovery and thermal stability can be obtained with dilute ammonia digestion. These observations, combined with the possibility of reusing ammonia within the process, make this method a promising approach for a more sustainable purification of PHA.
1 | INTRODUCTION

Polyhydroxyalkanoates (PHA) are bio-based and biodegradable polyesters produced in prokaryotic microbes as storage granules in the cytoplasm.\(^1\) They have similar properties to polyethylene and polypropylene, and could replace these plastics in many of their applications.\(^2\) Several companies have brought PHA to industrial scale, but high production costs are still a major obstacle for a wider presence in the market.\(^3\)

In the production stage, several strategies are being tested in order to reduce the costs, such as the use of waste substrates or mixed microbial consortia (MMC) instead of pure strains.\(^3\) After PHA production, the granules have to be extracted from the cells and purified from non-PHA cell material (NPCM), a step with significant impact on the overall costs and sustainability.\(^3,5\) The methods studied so far for PHA recovery and purification can be divided into two main categories based on their basic approach, described in the following paragraphs.

The first approach relies on the use of solvents to break the cell membranes and ultimately solubilise the PHA granules, which is typically attained with carcinogenic solvents like dichloromethane or chloroform. Subsequently, PHA is precipitated by the addition of a non-solvent (e.g. methanol). Although lower efficiencies have been described for MMC compared to pure strains,\(^6,7\) these processes usually result in high PHA recovery and purity, while maintaining the integrity of the polymer. Nonetheless, these methods present obvious concerns in terms of safety and sustainability, and imply significant costs due to the large amounts of solvent and non-solvent utilised. Substantial efforts are therefore being directed towards finding non-hazardous and easily recyclable organic solvents (such as ionic liquids)\(^3,5\), as well as towards identification of other more environmentally friendly processes.

The second approach for PHA purification aims at the digestion and solubilisation of the NPCM, leaving PHA as an insoluble solid. Such digestion processes can be biological (e.g. enzymes) or chemical.\(^2\) The major advantage of the latter is reduction of the processed volumes (compared to the use of solvents), as well as lower reagent costs. Several chemicals have been tested including alkalis (e.g. NaOH, KOH or NH\(_3\)), acids (e.g. H\(_2\)SO\(_4\)) surfactants (e.g. sodium dodecyl sulfate (SDS)) and oxidising agents (e.g. NaOCl). High PHA purity and recovery (> 90 %) have been attained with some of these reagents (or combinations of them) both in pure strains and MMC.\(^8,13\) Nevertheless, the ester bonds of PHA are susceptible to hydrolysis and PHA degradation (reduction of molar mass) is usually reported as a significant drawback of these processes.\(^8,10,11,13\) In this sense, one of the most promising treatments so far is the use of dilute H\(_2\)SO\(_4\) followed by NaOCl. Limited PHA degradation (around 50 % molar mass reduction) has been reported for this treatment, while achieving high PHA purity (up to 99 %) and a high recovery (up to 79 %).\(^9,12,14\)

Besides PHA degradation, another disadvantage of the chemical digestion methods is the generation of significant volumes of wastewater containing chemicals. In this regard particularly, ammonia could bring important benefits to the process, given that NH\(_3\) (and other nutrients derived from NPCM digestion) could be used as a nitrogen source during the PHA production steps (and as such would not be considered a waste stream). Moreover, due to its high volatility, residual ammonia can more easily be separated from the...
extracted PHA, which could prevent negative effects of residual chemicals on the thermal stability of the polymer.

Ammonia digestion was first suggested in 1993,\textsuperscript{15} when a PHA purity up to 94 % was reported with no decrease in the molar mass by using relatively high ammonia concentrations (1 N) at 45 °C. In addition, the authors proved that NH\textsubscript{3} could be successfully recycled as a nitrogen source for the PHA production. However, the strategy was only successful in strains with compromised cell membranes. Later on, a patent was published in which ammonia digestion at high concentrations (5-10 N) followed by NaOCl (4-6 %) washing reached 92 % PHA purity. No values of PHA recovery or polymer degradation were reported.\textsuperscript{16} Despite these encouraging results, little research has been dedicated to this digestion method. Some studies included ammonia in initial screenings, which generally resulted in lower PHA recoveries and purities compared to other alkalis (such as NaOH and KOH), after which NH\textsubscript{3} was excluded from further experiments.\textsuperscript{13,17-19} To the extent of our knowledge, only two studies have reported the molar mass of PHA after NH\textsubscript{3} digestion.\textsuperscript{15,20}

Up to date, there has not been a comprehensive evaluation of the potential of NH\textsubscript{3} digestion as a PHA purification method. There is lack of knowledge about the effect of digestion conditions on the PHA purity and polymer degradation. Likewise, there are no studies on the impact of NH\textsubscript{3} digestion on the thermal stability under different conditions. The objective of the present study was therefore to investigate ammonia digestion with a focus, not only on PHA purity and recovery, but also on the effects on the polymer integrity (reduction of molar mass and thermal stability), which are commonly overlooked in PHA recovery studies.

2 MATERIALS AND METHODS

2.1 Biomass for PHA purification experiments (crude PHA)

Cells containing PHA were a MMC dominated by the genera Thauera and Ammaricoccus, obtained according to the process developed by Burniol-Figols et al.\textsuperscript{21} Briefly, the process consisted of three main steps: 1) Fermentation of crude glycerol into volatile fatty acids (butyrate, propionate and acetate) and 1,3-propanediol; 2) Enrichment of PHA-accumulating bacteria using synthetic volatile fatty acids and crude glycerol; 3) PHA accumulation using the biomass from step 2 as inoculum and the effluent of step 1 as substrate. In the last step volatile fatty acids were converted into P(3HB-co-3HV) (poly(3-hydroxybutyrate-co-3-hydroxyvalerate)). After PHA accumulation, cells were centrifuged (4000 g 15 min), washed with distilled water and freeze-dried. In order to exclude sources of variation, the starting material for the PHA recovery assays was the end-product of 11 different PHA batch accumulations pooled together after freeze drying. The P(3HB-co-3HV) content of the joined biomass was 64 ± 1.3 wt%, where 3-HV monomers represented a 13 wt% of the polymer. This starting material is referred in the text as crude PHA, and was kept in a desiccator in the dark.
2.2 Outline of experiments

The first experiments evaluated the effect of the incubation time, the ammonia concentration and the temperature on the performance of the digestion of NPCM on crude PHA (detailed in section 2.3). The outcome of these experiments was mainly evaluated in terms of PHA purity and PHA recovery. Moreover, the latter parameter was complemented with the determination of the molar mass ($M_w$) of the recovered polymers and the quantification of PHA monomers lost in the supernatant to identify predominant hydrolysis patterns (leading to PHA loss or to reduction of $M_w$). These analyses are described in section 2.6.

The trends in the hydrolysis observed at different temperatures were further investigated by two means. First, by evaluating how the equilibrium constants were affecting the hydrolysis rate at different temperatures. This was done by performing experiments with a simple ester (ethyl 3-hydroxybutyrate (E3HB)) instead of PHA, in order to avoid possible bias from the polymer conformation (section 2.7). Secondly, by studying possible conformational changes occurring on PHA with different treatment conditions. This was attained by Differential Scanning Calorimetry (DSC) analysis on the polymers recovered with NH$_3$ digestion at different temperatures (section 2.6).

Next, sonication was evaluated as a pre-treatment to increase the PHA purity at elevated temperatures (75 and 115 °C) (section 2.4). Lastly, the polymer recovered with the best conditions from this study was compared in terms of thermal stability (section 2.8) with PHA obtained through other suggested methods for PHA purification (section 2.5).

2.3 PHA purification through NH$_3$ digestion

PHA extraction experiments were performed by incubating 100 mg of freeze-dried crude PHA with 4 mL of aqueous ammonia solution in pre-weighted 10 mL glass centrifuge tubes in a heating block. The tubes were vortexed every 5 min. Different incubation times (10-60 min), temperatures (30-140 °C) and ammonia concentration solutions (0-1 M NH$_3$) were tested during the experiments. When one parameter was changed, the others remained at a central value: 30 min, 0.2 M and 45 °C. All experiments were performed in triplicate. Ammonia solutions were prepared from concentrated ammonia solution 32 wt%.

Unless otherwise stated, after incubation the tubes were allowed to cool at room temperature for 5 min and centrifuged for 5 min at 2500 g at room temperature. The time of centrifugation was increased to 15 min in samples incubated at 140 °C due to the observation of high turbidity. The supernatant was removed and the pellets containing the PHA were washed twice with 8 mL of distilled water and freeze-dried. The recovered material was analysed as described in section 2.6. All experiments were performed in triplicates.

2.4 Sonication as a pre-treatment

Sonication was performed in glass centrifuge tubes containing 100 mg of freeze dried crude PHA suspended in 5 mL of distilled water and using a UP400S ultrasonic processor (400 W, 24 kHz, Heilscher) coupled to an H3 sonotrode (Heilscher) set to 100 % amplitude and a pulse of 0.5 for 10 min. Due to the energy delivered
to the samples, the temperature increased to 70 °C. After sonication, the tubes were centrifuged for 15 min at 2500 g and the pellets washed twice with distilled water. Thereafter, cell pellets were freeze-dried again to not introduce a dilution effect on the posterior NH₃ digestion, which was performed as described above (15 min centrifugation time).

### 2.5 PHA purification through reference methods

NaOH digestion, as well as combined NaOH and SDS digestion, were performed according to the protocols suggested by Jiang et al. Incubations were done using 100 mg of crude PHA and 4 mL of digestion solution, containing NaOH 0.2 M or NaOH 0.2 M with SDS 0.2 % (w/v). Assay tubes were incubated at 30 °C for 60 min, centrifuged (2500 g 15 min) and washed twice with distilled water before freeze-drying the solids.

Acid mediated digestion was done as proposed in Lopez-Abelairas et al. 100 mg of biomass was incubated with 2 mL of H₂SO₄ 3.5 % (v/v) for 6 h at 80 °C. Afterwards, the solution was adjusted to pH 10 with NaOH 0.5 M, centrifuged 15 min at 2500 g, and washed twice with distilled water. The recovered solids were then treated with 5.5 mL of NaOCl 3 % (w/v) for 1 h at room temperature, and again centrifuged and washed twice with distilled water before freeze-drying. The recovered material was analysed as described in section 2.6. All experiments were performed in triplicates. Chloroform extraction (65 °C 4 h) followed by methanol precipitation was performed as previously described.

### 2.6 Analyses on the material recovered after digestion experiments

For all digestion methods, the total solids recovered were assessed by weighing pre-tared tubes before and after the digestion and freeze-drying. PHA purity (expressed as g PHA/100 g solids) was measured by Gas Chromatography (GC) after HCl-mediated propanolysis in 1,2-dichloroethane as previously described. Methyl-3-hydroxybutyrate (99 %, J&K Scientific®) and methyl-3-hydroxyvalerate (≥98 %, Sigma-Aldrich®) were used as standards for the calibration of 3-HB and 3-HV monomers, given that the standard described in the referenced work (P(3HB-co-3HV) with 12 mol% 3-HV from Sigma-Aldrich®) led to an average overestimation of 10 % of the PHA purity (Supporting information -Appendix F). A secondary calibration curve was built with methyl-esters not submitted to the transesterification reaction dissolved in 1,2-dichloroethane, in order to take into account the extent of incomplete transesterification in the standard samples.

This value was then used to calculate the PHA recovery according to the following formula:

\[
\text{PHA recovery} (\%) = \frac{\text{PHA recovered (g)}}{\text{PHA initial (g)}} \times 100
\]

where:

\[
\text{PHA recovered (g)} = \text{solids after extraction (g)} \times \text{PHA Purity after extraction (g PHA/g solids)}
\]

\[
\text{PHA initial (g)} = \text{solids before extraction (g)} \times \text{PHA Purity before extraction (g PHA/g solids)}
\]

The percentage of non-PHA cell material (NPCM) removed was assessed with the formula:
NPCM removal (%) = \( \frac{\text{NPCM removed (g)}}{\text{NPCM initial (g)}} \times 100 \)

where

\[ \text{NPCM removed (g)} = \text{Solids removed (g)} - \text{PHA lost (g)} \]

\[ \text{Solids removed (g)} = \text{solids before extraction (g)} - \text{solids after extraction (g)} \]

\[ \text{PHA lost (g)} = \text{PHA initial (g)} - \text{PHA recovered (g)} \]

\[ \text{NPCM initial (g)} = \text{Solids before extraction (g)} \times (1 - \text{Purity PHA before extraction ((g PHA/g solids))}) \]

Molar mass distribution of the recovered material was assessed by Size Exclusion Chromatography (SEC). The analyses were conducted in a Shimadzu HPLC (High Pressure Liquid Chromatography) system using two columns in series (SDV 5μm 8x300mm 1000Å and 10000Å, PSS Polymer Standards Service GmbH). Chloroform was used as eluent at a flow rate of 1 mL/min with an oven temperature of 31 °C. Before analysis, 5 mg of the recovered PHA was dissolved in 1 mL of chloroform overnight and filtered through 0.45 µm PTFE filters. The system was calibrated with low dispersity polystyrene standards (Polymer Standards Service GmbH). The copolymer P(3HB-co-3HV) with 12 mol% 3-HV from Sigma-Aldrich® was used as a reference for comparisons.

For selected digestion conditions, the thermal behaviour of the PHA after digestion was assessed by Differential Scanning Calorimetry (DSC) (TA Instruments - Discovery series DSC) using hermetic aluminium pans. The first heating ramp (in order to erase thermal history) proceeded at a heating rate of 10 °C/min from -90 to 185 °C (run I), and was followed by a cooling ramp (10 °C/min), and a second heating step (run II) identical to run I. The glass transition (\( T_g \)) was determined at the inflection point, while crystallisation (\( T_c \)) and melting (\( T_m \)) temperatures were determined at the peak temperature on the second run.

In experiments evaluating the temperature effect, additional analyses were performed in the supernatant of the digestion experiments. The concentration of 3-hydroxybutyrate (3-HB) and crotonic acid were determined in an HPLC system (Shimadzu) equipped with an Aminex HPX-87H column (BioRad) with 12 mM H₂SO₄ (0.6 mL/min) at 40°C. 3-HB was quantified through the refractive index detector signal, while crotonic acid was determined by the UV signal at 210 nm. Calibration curves were done with 3-hydroxybutyrate (HPC standards®) and crotonic acid 98 % (Sigma-Aldrich®).

2.7 Experiments on the hydrolysis rate using ethyl 3-hydroxybutyrate (E3HB)

E3HB (100 mg) was incubated with 4 mL of NH₃ solution 0.2 M for different time intervals (0, 10, 20 and 30 min) in duplicate, using the same materials and equipment used for the experiments in crude PHA. After incubation, tubes were cooled on ice for 5 min and immediately neutralised to pH 7 with H₃PO₄. 3-HB and crotonic acid were determined by HPLC as described above (section 2.6). E3HB and ethanol were determined in a GC-FID (Agilent 6890) with a SGE BP21 column using nitrogen as a carrier gas. Samples were acidified with H₃PO₄ to pH 3, and diluted 1:1 with acetone to enable 0.5 µL split injections. Acetone used for dilution was spiked with 3-methyl-1-butanol (in order to verify that ester hydrolysis was not occurring before the
analysis) and with 1-hexanol (internal standard to correct for injection variance). Ethyl-3-hydroxybutyrate (>99 %, Acros Organics®) and ethanol (99.9 %, Merck) were used as calibration standards.

### 2.8 Thermal stability

Samples obtained with the different methods were analysed by Thermogravimetric Analysis (TGA) (TA Instruments - Discovery series TGA). The analyses were run under nitrogen at a rate of 20 °C/min and in a temperature range from 25 to 700 °C coupled with gas analysis on a FT-IR (Thermoscientific iS10) equipped with a gas-cell. Moreover, thermal stability during melt was assessed by determining the $M_w$ change after melting the recovered polymers in a hot press (170 °C for 5 min) in duplicate.

### 3 Results and Discussion

#### 3.1 Effect of time and NH$_3$ concentration on ammonia digestion

Effects of different digestion times (10-60 min) and ammonia concentrations (0-1 M NH$_3$) were investigated at a constant temperature (45 °C). The main results in terms of PHA recovery, PHA purity and elimination of NPCM (non-PHA cell material) are presented in Figure 1.

Long incubation times had a detrimental effect for the PHA recovery, while little effect on the NPCM removal (Figure 1 A). As PHA and impurities were lost in a similar degree, the samples maintained a PHA purity similar to the material before digestion. In short, longer incubation times seemed to contribute more to the loss of PHA than to the increase of PHA purity.

Similar trends were observed when increasing the ammonia concentration (Figure 1 B). PHA recovery decreased progressively from 80 to 66 %, while the percentage of impurities removed was independent of the ammonia concentration (approximately 25 % of NPCM removal). PHA purity presented a slight decline with increasing ammonia concentrations, as a result of a combination of decreased PHA recovery and steady NPCM removal. At the highest concentration of ammonia, PHA purity was even lower than in crude PHA (61 % vs 64 %). A certain loss of PHA (around 12 %) and NPCM removal (6.2 %) was also observed in the control experiment without ammonia (distilled water) (Figure 1 B).

Despite the fact that rather low PHA recoveries were obtained with these experiments (65 - 82 %), no indications of significant PHA degradation were observed. The recovered polymers presented very similar values of average molar mass ($M_w$) and dispersity (Đ) to the crude PHA in all the conditions assayed (Table S1 - Supporting information).
3.2 Effect of temperature on ammonia digestion

Besides ammonia concentration and time, temperature was also evaluated as a parameter influencing the effectiveness of the PHA recovery through ammonia digestion (Figure 2).

An important decrease in the PHA recovery (from 82 to 72 %) was observed when increasing the temperature from 30 to 45 °C, without being accompanied by an increase in the purity (Figure 2 A). Nonetheless, the opposite effect was observed when the temperature was further increased to 75 °C. At this temperature, a higher PHA purity and recovery were obtained (68 and 88 %, respectively), and more NPCM was solubilised. Consequently, incubation at 75 °C seemed to affect the impurities more than the PHA itself. No significant changes were observed in molar mass ($M_w$) or $\bar{D}$ for any of the temperatures with respect to the original material at this temperature range (Figure 2 B).

At 115 °C and 140 °C, PHA purity reached even higher values (75 and 83 %, respectively), while maintaining high PHA recovery (almost 90 %) (Figure 2 A). For all temperatures, 3-HB and 3-HV monomers were recovered to a similar extent. However, incubations at elevated temperatures resulted in a considerable decrease in the molar mass of the recovered polymer (Figure 2 B). At 115 °C, extraction led to a molar mass similar to that of commercially available PHA ($M_w$ of around 200 kg/mol), while extraction at 140 °C led to a lower molar mass ($M_w$ of 85 kg/mol). Differences in $\bar{D}$ were statistically non-significant ($p > 0.05$).
Mechanical properties of polymers are affected by their molar mass, usually following an asymptotical trend. For example, the tensile strength of polymers is generally compromised at low molar mass, but does not increase further once a molar mass threshold is surpassed. Hence, loss of molar mass does not necessarily imply detrimental effects in a polymer’s properties. The molar mass threshold varies among polymer properties, and it is also affected by the monomer distribution and the polymer purity. Thus, a limit of acceptable molar mass cannot be established without a characterisation of a range of mechanical parameters, as well as taking its life-span degradation mechanism into account. Having said that, other studies have reported deteriorated properties for P(3HB-co-3HV) with \( M_w \) below 112 kg/mol. Hence, digestion at 140 °C would not be recommended, due to the loss in molar mass. However, the treatment at 115 °C would still lead to an a priori acceptable value (200 kg/mol).

![Figure 2](image)

**Figure 2**: A: Effect of temperature during ammonia digestion on the PHA purity, PHA recovery and Non-PHA Cell Material (NPCM) removal (t: 30 min, NH\(_3\): 0.2 M). The line across bars indicates the PHA purity of the material before digestion (crude PHA). B: Weight-average molar mass (\( M_w \)) and dispersity (D) in the range of 30 to 140 °C.

It is important to note that only the temperature was studied as a parameter in these experiments, whereas the ammonia concentration and the incubation time were maintained at constant values (0.2 M, 30 min). Nonetheless, negative effects of long incubation times and high ammonia concentrations upon the PHA recovery were described in the previous section. To observe the effect of the incubation time at these temperatures, an experiment at 115 °C for only 15 min (vs. 30 min in Figure 2) was performed. With only 15 min difference, the polymer degradation was reduced from 67 % to 38 %, while PHA purity was only reduced from 75 to 72 %. Thus, a high sensitivity to digestion parameters was observed at high temperature, contrasting the minor changes observed at low temperatures (Figure 1). Consequently, a fine-tuning of variables in a multivariate approach, as well as a consideration of the importance of a high purity product versus molar mass, would be needed in order to identify specific conditions.
3.3 Why higher PHA recovery at elevated temperatures?

In general, the use of elevated temperatures led to a high degree of PHA purity, which can be attributed to the tendency of most compounds to increase their solubility in water at increasing temperatures. Moreover, the rate of hydrolysis reactions increases with temperature, resulting in faster digestion of NPCM. Similarly, a higher degree of PHA degradation would also be expected, as the rate of ester bond cleavage reactions - hydrolysis and/or β-elimination - increase with temperature. Counterintuitively, PHA recovery improved with increasing temperature (Figure 2). The following sections (3.3.1-3.3.3) elaborate further on this phenomenon, and its possible causes.

3.3.1 PHA loss vs. reduction in the PHA molar mass

In the digestion experiments, ester cleavage phenomena are reflected in two different measured responses: PHA recovery and reduction of molar mass. The reduced PHA recovery would account for the ester bonds breakage occurring towards the end of the polymer chains, leading to the formation of soluble oligomers and monomers that would be lost in the supernatant, resulting in PHA loss. Conversely, ester cleavage occurring at the middle of the chains would produce shorter chains (reduction of molar mass), but still result in water-insoluble polymers that would be recovered in the solid fraction. The occurrence of these two degradation phenomena is summarised in Figure 3 A, showing opposite trends with temperature. At low temperatures PHA loss predominated, whereas at high temperatures reduction in $M_w$ was more pronounced. The inflection point was between 45 and 75 °C.

Figure 3: A: Reduction of weight-average molar mass ($M_w$) (%) and PHA loss (%) in the experiments performed with NH$_3$ 0.2 M during 30 min at different temperatures. B: 3-hydroxybutyrate (3-HB) and crotonic acid detected in the supernatant of the experiments in A.

PHA recovery could not be significantly increased by raising the centrifugation speed (from 2500 to 4300 g), nor by solid-liquid separation through filtration (Figure S1 Supporting information – Appendix B). Similarly, no significant increment in the PHA recovery was obtained by decreasing the centrifugation temperature to 4 °C or with a previous cooling of the solution (Figure S1), which has been reported to result in enhanced precipitation and increased PHA recovery in previous studies employing NaOH.25
In order to further confirm that PHA loss was indeed due to the formation of soluble monomers and oligomers and not due to the loss of long PHA chains during the solid-liquid separation - 3-hydroxybutyrate (3-HB) and crotonic acid were analysed in the supernatants of the NH₃ digestions at different temperatures (Figure 3 B).

Crotonic acid was only detected in trace amounts, representing less than 1 % of the 3-HB monomers lost. Its concentration increased exponentially with temperature, similarly to the trend observed for the reduction of $M_w$ (Figure 3 A). This was in good agreement with previous findings, which attributed the generation of crotonic acid to $\beta$-elimination reactions occurring in the P3HB backbone. This reaction is the predominant mechanism of chain scission and $M_w$ reduction at elevated temperatures.\(^{14,26}\)

On the other hand, the amount of 3-HB in the supernatants (resultant from P3HB hydrolysis) presented the same trend as the PHA loss, with a maximum at 45 °C. The quantified 3-HB represented more than 60 % of the 3-HB monomers lost at temperatures up to 75 °C. This result confirmed a higher degree of hydrolysis leading to monomer formation occurring at temperatures below 75 °C, which resulted in PHA loss. Given that this trend was unexpected, further experiments were performed to elucidate if the phenomena could be related to changes in the hydrolysis rate, or to changes in the conformational structure of PHA. These experiments are presented and discussed in the following two sections (3.3.2 and 3.3.3).

### 3.3.2 Changes in the hydrolysis rates with digestion temperature

The rate of basic-catalysed hydrolysis of esters is generally dependent on the concentration of hydroxide ions (OH\(^-\)), which act as nucleophiles in the reaction. However, in aqueous ammonia solutions, unionised ammonia (NH₃) can also act as a nucleophile and cleave ester bonds (producing primary amides). The overall rate of the reaction can be expressed as a function of the reaction constants ($k$) and the concentration of OH\(^-\) and NH₃ (eq.1).

$$\frac{d[\text{ester}]}{dt} = (k_{\text{OH}}[\text{OH}^-] + k_{\text{NH}_3}[\text{NH}_3])[\text{ester}]$$  \hspace{1cm} (eq.1)

$$\frac{d[\text{ester}]}{dt} = k_{\text{obs}}[\text{ester}]$$  \hspace{1cm} (eq.2)

By increasing the temperature, the reaction constants ($k_{\text{OH}}$ and $k_{\text{NH}_3}$) increase exponentially (according to the Arrhenius equation). However, the temperature also affects the dissociation constants of water and ammonia, as well as their vapour pressures. This translates into changes in the effective concentration of OH\(^-\) and unionised NH₃, which could affect the overall reaction rate. To simulate the changes in the OH\(^-\) and NH₃ concentrations occurring due to temperature increase, a theoretical model was constructed, taking into account the equilibrium reactions of NH₃ and water, and the vapour-liquid equilibrium of NH₃ (Figure 4 and Supporting information Appendix C).

The most interesting result of this model was the trend of pOH with the temperature (Figure 5). Maximum OH\(^-\) concentration was identified at 45 °C, coinciding with the maximum PHA loss presented in Figure 3. This trend was a result of the ammonia ionisation reaction, which is endothermic under 50 °C, but exothermic at higher temperatures (Figure 4).\(^{27}\) Consequently, and according to the Van Hoff Equation, the equilibrium shifts to NH₄\(^+\) with increasing temperatures up to 50 °C, resulting in higher concentration of OH\(^-\), but does the opposite for higher temperatures (Figure 4 and 5).
The model showed only small changes in the concentration of OH\(^-\) and unionised NH\(_3\) at different temperatures. These changes would not seem enough to overrule the effect of temperature in increasing the rate constants and decrease the overall hydrolysis rate (as expressed in eq.1). Nonetheless, given the correlation between the concentration of OH\(^-\) and the PHA loss (attributed to PHA hydrolysis in the previous section), the overall hydrolysis rate at different temperatures was assessed experimentally. The experiments were performed by submitting ethyl-3-hydroxybutyrate (E3HB) to NH\(_3\) digestion at different temperatures, as previously done with crude PHA (Figure 2 and 3). By replacing PHA for E3HB as a model ester, bias in the hydrolysis rate dues to conformational changes of the polymer was excluded.

Figure 4: Shift of the equilibrium reactions with the increase of temperature from 0 to 50 °C (A) and from 50 to 140 °C (B). The direction of the shift is indicated by the bold arrows.

Figure 5: pH, pOH and concentration of unionised NH\(_3\) obtained with the model at varying temperatures (25 - 140 °C) and constant initial concentration of ammonia (0.2 M).

For each temperature, the concentration of OH\(^-\) and NH\(_3\) were assumed to be constant during the reaction, and the overall reaction constant (\(k_{\text{obs}}\)) was calculated based on a pseudo-first order reaction kinetics (eq.2). The results showed an increase in \(k_{\text{obs}}\) at increasing temperature (Figure 6), which enabled two important inferences: 1) the decrease in OH\(^-\) at elevated temperatures was not enough to overrule the increase in the reaction constants; and 2) the decrease of hydrolysis at elevated temperatures exposed in Figure 3 could not
be explained by a decrease in the hydrolysis rate. Further investigations were centred on the conformation of PHA and presented in the next section (3.3.3).

In regards to the products identified from E3HB hydrolysis, only trace amounts of crotonic acid were detected, falling below the quantification limit (0.9 mg). Ethanol and 3-HB were the prevailing products, and were determined practically in equimolar concentrations (as exemplified in Figure 6 B corresponding to the experiment at 140 °C). This enabled another important conclusion from these experiments, which was that OH catalysed hydrolysis of the ester bond (leading ethanol and 3-HB) was clearly predominant over ammonolysis (resulting in ethanol and 3-hydroxybutyramide) at the conditions studied. Consequently, a high presence of amide groups would not be expected in the recovered polymer.

**Figure 6**: A: Hydrolysis constant observed with incubations of ethyl-3-hydroxybutyrate with 0.2 M NH₃ at different temperatures. B: Ethyl-3-hydroxybutyrate (E3HB), 3-hydroxybutyrate (3-HB) and ethanol detected during the hydrolysis of E3HB with NH₃ 0.2 M at 140 °C.

### 3.3.3 Changes in the PHA conformation with digestion temperature

PHA decomposition is known to be affected by the polymer conformation, as highly crystalline polymers are more resistant to alkali attack. Therefore, thermal transitions occurring in the crude PHA submitted to different digestion temperatures were examined by DSC analysis (Figure 7).

In crude PHA, the glass transition (T_g) was identified at -6.8 °C, the melting temperature (T_m) at 156 °C, and a small cold crystallisation (T_c) at around 50 °C (Figure 7 B). The samples purified through NH₃ digestion at different temperatures presented the same thermal profile, although the enthalpies of the phenomena were much more pronounced (Figure 7 B). This indicated that the samples had a higher degree of crystallinity than
the crude PHA, most likely due to the removal of impurities that compromised the formation of crystalline structures in the crude PHA.

Cold crystallisation was only observed in the second run of the DSC (after cooling from the molten state), and not in the first heating curve (Figure 7 A). Instead, the first run exhibited a “shoulder” in the DSC baseline at around 60 °C. In reference to Figure 7 A, it should be noted that the curve presented as well some sharp endotherms at temperatures above 120 °C. These peaks were attributed to the evaporation of impurities resulting in leakages from the capsules, as presented in the Supporting information (Appendix D).

![DSC curves recorded for crude PHA and NH₃-digested samples (0.2 M NH₃ for 30 min at different temperatures). A: Run I (first heating ramp) of crude PHA. B: Run II (second heating ramp) of crude PHA and NH₃-digested samples.

The thermal transition observed in the first heating curve at 60 °C (Figure 7 A) - hereafter Tₑ₂ - has previously been reported for samples stored at room temperature,²⁸⁻³⁰ and has been attributed to the glass transition temperature of the rigid amorphous fraction (RAF).³⁰ The RAF is formed even after very short periods (< 24h) of storage at temperatures above the glass transition temperature of the mobile amorphous fraction.³¹ During this process, the amorphous regions in contact with the crystals undergo a restriction of mobility. Moreover, small imperfect inter-lamellar crystallites are formed (secondary crystallisation) which further constrain the mobility of the amorphous regions.²⁹,³₁⁻³³ Above the Tₑ of the RAF, melt and recrystallisation start occurring, which translates into a negative slope in the DSC curve until the melting of the primary lamellae occur at the Tₑ.³⁰

The secondary crystallisation of PHA has been widely studied, given its effect on the mechanical properties of the polymer, and it has been found to be reversible after annealing polymers at temperatures above...
The heating step enables the melting of the imperfect crystallites developed during secondary crystallisation, as well as mobilisation of the rigid amorphous fraction, which allows a reorganisation of the amorphous fraction and a thickening of the stable crystallites. The abovementioned phenomenon could be a potential explanation for the decrease in PHA loss observed above 75 °C (Figure 3). By incubating PHA above the T_g (60 °C), there is a mobilisation of the RAF, which could enable the formation of thicker crystals. Moreover, digestion above 75 °C led to a higher release of impurities possibly compromising crystallisation. Both factors would contribute to a higher degree of crystallinity, and thus, possibly explain a lower degree of hydrolysis leading to PHA loss.

On the other hand, the start of the endotherm observed above 100 °C (Figure 7 B) would indicate a progressive melting of the crystalline structure, thus making the chains more accessible to hydrolysis in the middle of the chains. This fact, together with the onset of the exponential increase in β-elimination reactions at elevated temperatures (resulting in crotonic acid formation - Figure 3) could be a reasonable hypothesis to explain the higher degree of reduction of molar mass observed from 115 °C.

### 3.4 Sonication pre-treatment to increase the PHA purity

Ammonia digestion at elevated temperatures proved to be a valuable method to increase the PHA purity while maintaining high recovery yields. Nonetheless, severe reduction of the molar mass was observed at conditions favouring high purity (140 °C) (Figure 2). Thus, a sonication pre-treatment was investigated as a strategy to enhance ammonia digestion at lower temperatures. Sonication was chosen based on its reported efficiency in disaggregating the exopolysaccharide structures of microbial cultures, which have been suggested as the main reason behind the recalcitrance of MMC to PHA extraction. The results of these experiments are displayed in Figure 8.

Through sonication as the only means of purification (control without posterior NH₃ digestion), a PHA purity of almost 70 % was reached (Figure 8 A). 33 % of the impurities were removed with this physical treatment, resulting in only 11 % PHA loss. However, a certain reduction of the molar mass was observed compared to the starting material (Figure 8 B).

When sonication was followed by ammonia digestion at 75 or 115 °C, PHA purity reached a value of 79 and 86 %, respectively (Figure 8 A). These values represented a 15 % relative increase in the PHA purity compared to the non-pretreated ammonia digestion at the same temperatures (Figure 2), without affecting PHA recovery. The average molar mass did not decrease significantly with the treatment at 75 °C after sonication (Figure 8 B), in agreement with observations for samples without sonication at this temperature (Figure 2 B). The treatment at 115 °C led to a similar value of M_\text{w} as without pretreatment (around 200 kg/mol), and thus, still within a priori acceptable ranges according to the discussion in section 3.2. A distinctly whiter polymer was obtained after this treatment.
Figure 8: Combined sonication and ammonia digestion at high temperatures, performed with incubations of 30 min with 0.2 M NH₃. Only sonication but no ammonia digestion was performed on the control samples. A: PHA purity, PHA recovery and Non-PHA cell material (NPCM) removal. The line across bars indicates the PHA purity of the material before digestion (crude PHA). B: Weight-average molar mass ($\bar{M}_w$) and dispersity (Đ).

3.5 Thermal stability of NH₃ purified samples (comparison with reference methods)

The thermal stability of the PHA purified through NH₃ digestion (NH₃ 0.2 M at 115 °C with sonication) was evaluated and compared to other previously suggested methods aiming at the solubilisation of the NPCM (summarised in Table 1). Namely, the methods included digestion with NaOH, combined NaOH and SDS, and H₂SO₄ treatment. Moreover, chloroform extraction was used as a reference method, for being known as a method not leading to PHA degradation.

Previous studies on alkali extraction have reported up to 96 % PHA purity by using NaOH, and up to 99 % when combined with SDS. When applied to the crude PHA from the present study, these methods led to 72 and 76 % purity, respectively (Table 1), giving further indication of the recalcitrance of the mixed microbial culture used for the PHA production. No important decrease in molar mass was observed in the recovered PHA with either method (Figure 9). PHA recovery was approximately 90 % for both treatments.

Sulphuric acid digestion followed by mild NaOCl treatment has so far been identified as the most promising method for solubilising NPCM, due to the notable outcomes in terms of PHA purity and the limited extent of the PHA degradation. Moreover, it presented less CO₂ emissions and costs compared to methods employing NaOCl or solvents. In the present study, H₂SO₄ treatment gave indeed practically pure PHA and a
97 % PHA recovery (Table 1). The \( M_w \) after digestion was in the range of the one obtained after ammonia extraction at 115 °C (about 200 kg/mol) (Figure 9).

Table 1: PHA purity, type of impurities and melting temperature \((T_m)\) of samples purified through ammonia digestion or other comparison methods.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Purification method</th>
<th>PHA recovery (%)</th>
<th>PHA purity (%) GC*</th>
<th>PHA purity (%) TGA**</th>
<th>Organic impurities (%)†</th>
<th>Inorganic impurities (%)†</th>
<th>( T_m ) (°C) ††</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude PHA</td>
<td>--</td>
<td>--</td>
<td>64 ± 1.2</td>
<td>69</td>
<td>16.8</td>
<td>9.9</td>
<td>156</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Chloroform 65 °C 4h + methanol precipitation</td>
<td>n.d.</td>
<td>99 ± 0.2</td>
<td>99</td>
<td>0.03</td>
<td>0.8</td>
<td>162</td>
</tr>
<tr>
<td>NaOH</td>
<td>NaOH 0.2 M 30 °C 60 min</td>
<td>90 ± 0.9</td>
<td>72 ± 0.8</td>
<td>76</td>
<td>13.4</td>
<td>6.8</td>
<td>152</td>
</tr>
<tr>
<td>NaOH SDS</td>
<td>(NaOH 0.2 M + SDS 0.2 %) 30 °C 60 min</td>
<td>91 ± 0.9</td>
<td>76 ± 0.9</td>
<td>76</td>
<td>13.4</td>
<td>6.2</td>
<td>154</td>
</tr>
<tr>
<td>H(_2)SO(_4)</td>
<td>H(_2)SO(_4) 3.5 % 80 °C 6h + NaOCl 3 % 1h room T</td>
<td>97 ± 2.5</td>
<td>98 ± 2.6</td>
<td>97</td>
<td>1.3</td>
<td>0.8</td>
<td>147</td>
</tr>
<tr>
<td>NH(_3)</td>
<td>sonication + NH(_3) 0.2 M 115 °C 30 min</td>
<td>92 ± 0.2</td>
<td>86 ± 0.8</td>
<td>89</td>
<td>5.1</td>
<td>2.8</td>
<td>154</td>
</tr>
</tbody>
</table>

* PHA purity (g PHA/100 g Total solids) measured by Gas Chromatography  
** PHA purity (g PHA/100 g Total solids) measured from the slope of the TGA curve  
† Determined from the TGA curve  
†† Determined from the DSC curves in Figure S 7 (Appendix E)  
n.d. not determined  

The samples purified through the different methods were submitted to TGA analysis to observe their thermal stability (Figure 10 A). All samples presented a major drop in mass at temperatures between 200-330 °C, corresponding to the thermal decomposition of PHA. Infrared spectra obtained in-line confirmed the presence of crotonic acid in the off-gas during this temperature range. Only the samples purified with chloroform and \( H_2SO_4 \) presented an almost complete loss of mass during PHA degradation, indicating the high purity of the samples. The rest of the analysed samples presented further weight loss until 550 °C, representing the presence of other organic compounds. Furthermore, total weight loss was not observed at the end of the heating ramp (700 °C), which pointed out the presence of residual inorganics. The content of organic and inorganic impurities calculated from these mass loses, as well as the PHA percentage (calculated from the mass loss in the main slope) are presented in Table 1. The PHA purity calculated from the TGA gave similar results to that obtained by GC.

Figure 10 B shows the derivative curve of the TGA, where the position of the peaks indicates the temperature of maximum PHA decomposition \( (T_d) \). PHA purified through ammonia digestion presented a \( T_d \) above 300 °C, very close to the sample purified with chloroform. On the other hand, the samples purified through NaOH or \( H_2SO_4 \) presented much lower values. More specifically, the \( T_d \) for the acid purified sample was in the range of the crude PHA (without any purification), while the alkali methods led to an even lower \( T_d \) than the starting material. Thus, the thermal stability of the purified material did not seem to be related to the degree of PHA purity obtained with different methods. Likewise, no correlation was identified between the \( T_d \) and the quantities of organic or inorganic impurities determined from the TGA curve.
An absence of correlation between the $T_d$ and the purity was also observed when comparing the samples obtained through ammonia digestion at different conditions (Figure 10 C). The $T_d$ of all these samples was in line with the one of the chloroform purification, although the purity of these samples was much lower. Especially noteworthy is the case of the sample purified at 75 °C compared to the crude PHA, where just an increase of 4 % in the PHA purity (from 64 to 68 %) led to an increase of the $T_d$ of 36 °C (from 265 to 301 °C).

The $T_d$ is an important indication of the thermal stability of PHA, but it only accounts for PHA degradation resulting in absolute mass loss, occurring generally at temperatures above 250 °C. Chain scission reactions are known to occur at much lower temperatures, close to the melting temperature, and lead to reduction of the molar mass without absolute mass loss (and thus not observed in the TGA). This degradation has important implications during polymer processing.

Crude PHA, as well as samples purified through the different comparison methods were melted at 170 °C in a hot press in order to observe changes in molar mass (Figure 9). Severe reduction of molar mass (86 %) was observed in samples treated with NaOH or H$_2$SO$_4$, all leading to $M_w$'s below 100 kg/mol. Interestingly, these samples presented almost twice the reduction of molar mass compared to the crude PHA. Conversely, NH$_3$-purified PHA (115 °C with previous sonication) showed only a 10 % reduction of molar mass, comparable to the degradation observed for chloroform-extracted PHA.

While no important differences were observed in the TGA with regards to the $T_d$ of samples obtained with NH$_3$ digestion under different conditions (Figure 10 C), significant differences were observed in the melt-stability (Figure 9). Higher purities correlated with an increase in the thermal stability, although samples with higher than 70 % PHA purity presented already a low reduction in the molar mass.

Overall, the results suggested that biomass and fermentation residues had a limited effect on catalysing thermal degradation, whereas the method of purification had a drastic impact. A potential explanation could be the nature of the chemicals used. H$_2$SO$_4$ and NaOH are difficult to remove completely from the recovered polymer, and traces of such chemicals can catalyse degradation reactions during melting. On the other hand, NH$_3$ is highly volatile, and can easily be removed.
Several studies have led to similar observations in regards to the effect of the biomass residues and the purification methods on the thermal stability. In addition, it has been reported that some impurities can behave as plasticisers, enhancing the mechanical properties of PHA, which emphasise the importance of testing other properties besides the PHA purity when evaluating purification methods. The presence of impurities should only be considered detrimental when affecting negatively the PHA properties and applicability.

*Figure 10:* TGA curves. A: Weight % vs. temperature in samples purified through NH₃ digestion at 115 °C (0.2 M) with previous sonication compared to other reference methods and the crude PHA. B: Derivate of the weight % vs. temperature of the samples in A in the temperature range of PHA degradation. C: Derivate of the weight % vs. temperature of samples obtained through different NH₃ digestion conditions: 75 °C 0.2 M NH₃, 115 °C 0.2 M NH₃ and sonication without NH₃ digestion. Values in parenthesis in the legends indicate PHA purity.
3.6 Comparison of results obtained with ammonia digestion and perspectives

Ammonia-assisted extraction of PHA has not been extensively reported in scientific literature. Discrepancies have been reported in regards to the outcomes observed at similar conditions in different cultures (Table 2). As an example, by applying the same digestion conditions, Anis et al.17 observed no increase of the PHA purity accompanied with very low PHA recoveries, whereas Choi and Lee18 observed an important increase in the PHA purity and high PHA recovery. Likewise, no increase in the PHA purity was observed when increasing the temperature from 45 °C to 100 °C in the study of Page and Cornish15, whereas significant improvements in the efficiency of the extraction were observed in the present study with a similar temperature increase (Figure 2). Higher PHA purity and recovery were also observed at elevated temperatures using NH₃-laurate digestion in MMC.19 These differences might imply that the efficiency of dilute aqueous ammonia digestion depends on the PHA accumulating culture.

Most of the studies up to now, have only tested NH₃ digestion in a screening phase, and discarded the method due to lower initial PHA purity and recovery compared to other alkalis (such as NaOH, KOH or NH₃-laurate).13,17–19 Nonetheless, the whole picture of the results obtained here and reported previously, suggests that NH₃ digestion efficiency largely depends on the digestion conditions (and possibly on the PHA accumulating culture), and that adjustment of the parameters can lead to relatively pure PHA and high PHA recovery.

Additionally, our study shows for the first time that NH₃ digestion can lead to a higher thermal stability than achieved using other digestion methods, even with PHA of lower purity. The maximum degradation temperature (Tₜ) was comparable to solvent-extracted PHA (around 300 °C), and much higher than that obtained with NaOH or H₂SO₄ digestions (lower than 270 °C) (Figure 10). Similarly, a much lower Tₜ has been reported for NH₃ digestions complemented with lauric acid as surfactant (Table 2). Moreover, PHA recovered through NH₃ digestion presented almost no reduction of the molar mass during melting, while almost complete degradation was observed for H₂SO₄ or NaOH mediated extractions. These observations are of crucial importance for polymer applications.

Our results showed that a high degree of thermal stability was obtained with PHA purities above 70 % (obtained at temperatures between 75 °C and 115 °C). Higher purities and thermal stability could be achieved at treatments above 75 °C and including a sonication pre-treatment, but as a trade-off, they led to higher loss of molar mass and would be expected to present higher energy costs. Thus, further optimisation should take into consideration the required purity level and molar mass, which were seen to be very sensitive to operational conditions at elevated temperatures.

The results obtained in this study are very promising also from a sustainability point of view. Besides avoiding the use of a toxic solvent, the digestion solution could be re-used in PHA production process. Previous studies have observed that the use of the digestion solution as a nitrogen source led to even better results than the use of ammonium salts15. Moreover, the high pH of the digestion solution (9-12) could represent a further advantage in processes requiring base addition. In such processes (like the fermentation and enrichment step of three-stage PHA production in MMC21), direct addition of this solution could substitute external alkali, and thus lead to additional savings. For each process, a balance between the needs for nitrogen and alkali should
be performed, taking into account as well the possibility of reusing the NH₃ solution in the following digestion batch.

4 CONCLUSIONS

Dilute ammonia digestion of the non-PHA cell material was studied as a method for purifying PHA produced by mixed microbial consortia; a method which could bring important advantages in terms of costs and sustainability due to the possibility of recycling ammonia within the process.

The results showed that the efficiency of the method largely depends on the digestion conditions - especially in regards to the temperature. Low PHA purities (around 64 %) and PHA recoveries (from 65 to 80 %) were obtained at mild temperature conditions, with little changes upon increased incubation time or ammonia concentration. On the other hand, treatments performed between 75 °C and 115 °C led to high recovery of PHA (above 90 %) and increased PHA purity, while maintaining the molar mass of PHA at reasonable values. Higher PHA recovery at this temperature range was a result of a lower degree of hydrolysis leading to monomers and soluble oligomers, which was hypothesised to occur as a result of PHA conformational changes at elevated temperatures. A pre-treatment by sonication also proved valuable to further increase the PHA purity without severely impacting the molar mass.

High thermal stability was observed in PHA recovered with NH₃ digestion at different conditions when its purity was above 70 %. Conversely, almost complete degradation during melting was observed in PHA recovered with other chemicals aiming at digestion of non-PHA cell material (e.g. H₂SO₄), despite presenting higher purity values (98 %). Stability during melt after NH₃-assisted recovery at 115 °C with previous sonication was comparable to pure chloroform-extracted PHA. This fact further underlined the potential of NH₃ digestion as a method for PHA purification.

The study provided the grounds for further optimisation of the method, which would be dependent on the PHA-accumulating culture and the requirements in terms of PHA purity and molar mass. Both parameters were highly sensitive to operational conditions at elevated temperatures.

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Supporting information

Appendix A: Effect of time and NH₃ concentration on the molar mass.
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Appendix D: The sharp endotherm peaks in DSC curves.
Appendix E: DSC curve of samples purified with reference methods.
Appendix F: Comparison of values of PHA purity with different PHA Standards.
Table 2: Comparison of results obtained with ammonia digestion published in the scientific literature.

<table>
<thead>
<tr>
<th>Ref</th>
<th>Polymer</th>
<th>Culture</th>
<th>Biomass state</th>
<th>Pre-treatment</th>
<th>Conc. NH₃</th>
<th>T (°C)</th>
<th>t (min)</th>
<th>Post-treatment</th>
<th>PHA ini (wt %)*</th>
<th>PHA Purity (wt %)**</th>
<th>PHA Recovery (%)</th>
<th>M_w ref (kg/mol)*</th>
<th>M_w final (kg/mol)**</th>
<th>Td (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>P3HB</td>
<td>Azobacter vienlandii</td>
<td>wet biomass</td>
<td>--</td>
<td>1 N</td>
<td>45</td>
<td>10</td>
<td></td>
<td>84</td>
<td>94</td>
<td>n.r.</td>
<td>1700-2700</td>
<td>1700-2700</td>
<td>n.r.</td>
</tr>
<tr>
<td>13</td>
<td>P3HB</td>
<td>MMC</td>
<td>wet biomass</td>
<td>--</td>
<td>0.2 M</td>
<td>30</td>
<td>60</td>
<td></td>
<td>68.6</td>
<td>62.6</td>
<td>63.3</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>freeze dried</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>69.9</td>
<td>87.4</td>
<td>95</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>16</td>
<td>P3HB</td>
<td>Alcaligenes latus</td>
<td>wet biomass</td>
<td>--</td>
<td>5-10 N</td>
<td>60-100</td>
<td>60-120</td>
<td>NaOCl 4-6 %</td>
<td>n.r.</td>
<td>92</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>18</td>
<td>P3HB</td>
<td>Escherichia coli</td>
<td>wet biomass</td>
<td>--</td>
<td>0.1 N</td>
<td>30</td>
<td>60</td>
<td></td>
<td>77</td>
<td>85.4</td>
<td>94.7</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>17</td>
<td>P3HB(co-3HHx)</td>
<td>Cupriavidus necator</td>
<td>freeze dried</td>
<td>--</td>
<td>0.1 M</td>
<td>30</td>
<td>60</td>
<td></td>
<td>60</td>
<td>≈ 60</td>
<td>≈ 62</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>20</td>
<td>P3HB</td>
<td>Cupravirus necator</td>
<td>freeze dried</td>
<td>--</td>
<td>0.1 M</td>
<td>90</td>
<td>180</td>
<td></td>
<td>≈ 70</td>
<td>≈ 78</td>
<td>700</td>
<td>272</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>--</td>
<td>0.25 M NH₃-laurate</td>
<td>90</td>
<td>180</td>
<td></td>
<td>74</td>
<td>98</td>
<td>100</td>
<td>1200</td>
<td>600</td>
<td>264</td>
</tr>
<tr>
<td>19</td>
<td>P3HB(co-3HV)</td>
<td>MMC</td>
<td>freeze dried</td>
<td>--</td>
<td>0.1 N</td>
<td>90</td>
<td>180</td>
<td></td>
<td>52</td>
<td>50†</td>
<td>75†</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td></td>
<td>P3HB(co-3HV)</td>
<td>MMC</td>
<td></td>
<td>--</td>
<td>NaOCl 4.7%</td>
<td>0.2 M NH₃-0.13 M laurate</td>
<td>75</td>
<td>180</td>
<td></td>
<td>52-54</td>
<td>93</td>
<td>73</td>
<td>n.r.</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>This study</td>
<td>MMC</td>
<td>freeze dried</td>
<td>--</td>
<td>0.2 M</td>
<td>115</td>
<td>30</td>
<td></td>
<td>64</td>
<td>75</td>
<td>88</td>
<td>586</td>
<td>195</td>
<td>302</td>
</tr>
</tbody>
</table>

n.r.: not reported; *Before purification; **After purification; †value extrapolated from a figure; MMC: Mixed Microbial Consortia; M_w: weight-average molar mass; Td: temperature of maximum decomposition
References


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Appendix A – Effect of time and NH₃ concentration on the molar mass

Table S1: Weight-average molar mass ($\tilde{M}_w$) and dispersity (Đ) of the material obtained after ammonia digestion at different incubation times and NH₃ concentrations (incubations at 45 °C).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>NH₃ (M)</th>
<th>$\tilde{M}_w$ (kg/mol)</th>
<th>Đ</th>
</tr>
</thead>
<tbody>
<tr>
<td>--*</td>
<td>--*</td>
<td>586 ± 16</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.2</td>
<td>540 ± 34</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>0.2</td>
<td>560 ± 4.7</td>
<td>2.2 ± 0</td>
</tr>
<tr>
<td>60</td>
<td>0.2</td>
<td>588 ± 27</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>539 ± 27</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>0.05</td>
<td>583 ± 13</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>0.1</td>
<td>628 ± 41</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>0.2</td>
<td>587 ± 25</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
<td>589 ± 22</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>621 ± 16</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>

*crude PHA (before digestion).
Appendix B – PHA solid-liquid separation

In order to evaluate the efficiency of the solid-liquid separation after ammonia digestion, several modifications of this step were carried out after digesting crude PHA with 0.2 M NH₃ at 45 °C during 30 min. A higher centrifugation force was tested (4300 g vs. 2500 g), as well as a separation through filtration (glass fiber filters 0.7µm (Whatman® GF/F)). Lastly, the effect of the temperature of the centrifugation was evaluated, by allowing the digestion tubes to cool down in ice and/or centrifuging at 4 °C (2500 g) instead of room temperature. The results are presented in Figure S 1.

Figure S 1: PHA purity, PHA recovery, and Non-PHA cell material (NPCM) removal in the experiments with different separation methods (A) and centrifugation temperatures (B). For all experiments, the extraction as performed at 45 °C for 30 min with an NH₃ concentration of 0.2 M. The line across bars indicates the PHA purity of the material before digestion (crude PHA).
Appendix C – Model of aqueous ammonia species and pH at varying temperatures

The model considered the following equilibria:

![Diagram of Headspace and Aqueous phase]

**Figure S 2: Equilibrium reactions considered for the model of aqueous ammonia species and pH**

The following system of equations was stated, where water and ammonia were assumed to behave as ideal gases and as an ideal mixture of liquids.

1) Ammonia equilibrium

\[ k_b = \frac{[NH_3^+][OH^-]}{[NH_3]} \]

2) Water equilibrium

\[ k_w = [OH^-][H^+] \]

3) Electroneutrality aqueous solution

\[ [NH_3^+] + [H^+] = [OH^-] \]

4) Vapour-liquid equilibrium ammonia

\[ [NH_3]H_{NH_3,cp} = P_{NH_3} \]

5) Gas law

\[ P_{NH_3}V_H = n_{NH_3(g)}R.T \]

6) Total NH3 balance

\[ n_{NH_3(total)} = [NH_3]V_L + [NH_3^+]V_L + n_{NH_3(g)} \]

The temperature dependence of the equilibrium constants \((K_b\) and \(K_w\)) as well as the Henry’s constant of ammonia \((H_{NH_3})\) were calculated based on equations S1-3, which account for a temperature range from 273-475 K.

\[ \ln (k_b) = -686.514 + 33110.5/T(K) + 110.7182\ln (T(K)) - 0.145962.T (K) - 2033789/(T(K))^2 \]  
(\text{eq.S1})

\[ \ln (k_w) = 140.932 - 13445.9/T(K) - 22.4773\ln (T(K)) \]  
(\text{eq.S2})

\[ \ln (H_{NH_3} \text{ (MPa.mol/kg)}) = 3.932 - 1879.0/T(K) - 355100/(T(K))^2 \]  
(\text{eq.S3})

According to eq. S1 and S2, the equilibrium constants would follow the trends represented in Figure S 3.
The system of equations was solved using the solver tool in Excel and the outputs of the model were verified with the software ProII version 9.4.

Figure S 3: $K_b$ and $K_w$ as a function of temperature
Appendix D – The sharp endotherm peaks in DSC curves

Sharp endothermic peaks were typically observed during the first run of the DSC at temperatures over 120 °C, especially in samples with low degree of purity. As an example, Figure S 4 displays the first heating curve of the crude PHA sample, the PHA recovered after purification at 75 °C (NH₃ 0.2 M) and the chloroform purified sample.

![Figure S 4: DSC curves recorded during the first heating scan (run I) of the material before digestion (crude PHA), digestion with NH₃ 0.2 M at 75 °C and chloroform-extracted PHA. The run was performed using sealed hermetic lids.](image)

The peaks could not be attributed to PHA degradation, as the correspondent endotherm peak was observed at much higher temperatures in STA analyses (Simultaneous Thermal Analysis) (Figure S 5). Noteworthy to mention, the sharp endotherms were not observed in the heat flow curves of the STA analyses, what suggested that the peaks observed in the DSC did not correspond to true melting events. Instead, pressure build-up in the DSC cups was identified as a possible cause. This is a common experimental phenomena in thermal analysis, attributed to the rapid volatilisation of gases trapped in partially sealed hermetic cups, which would not occur in STA as it was performed in open alumina cups. This hypothesis was supported by the small losses of weight occurring in the TGA at early temperatures (below 120 °C) in samples containing impurities (e.g. 75 °C purification Figure S 5 A).

The later argumentation was further endorsed by performing DSC analysis in cups with a perforation in the lid, which would allow the evaporation of gases. These runs did not show anymore the sharp peaks at high temperatures (Figure S 6), but displayed a wide endotherm starting at 50 °C, which would be in agreement to the evaporation losses observed in the STA analysis. Based on these results, volatilisation of impurities was considered to be the most plausible explanation for the sharp endotherms observed in the first heating curves of samples containing impurities.
Figure S 5: Heat flow curves (dashed lines) and weight % (continuous lines) recorded during STA (Simultaneous Thermal Analysis) of the PHA recovered after digestion with NH$_3$ 0.2 M at 75 °C (A) and chloroform-extracted PHA (B).

Figure S 6: DSC curves recorded during the first heating scan (run I) of the material before digestion (crude PHA), digestion with NH$_3$ 0.2 M at 75 °C and chloroform-extracted PHA. The run was performed with cups with a perforation in the lids.
Appendix E- DSC curve of samples purified with reference methods

Figure S 7: DSC curves recorded during the second heating scan (run II) of the crude PHA purified according to the methods in Table 2 of the manuscript.
Appendix F- Comparison of values of PHA purity with different PHA Standards

The use of Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) P(3HB-co-3HV) 12 mol% P3HV from Sigma-Aldrich® as a standard for PHA determination by Gas Chromatography (GC) analysis has been widely reported (e.g.3,4,13,14,5–12), although its purity is not specified. Using this standard, some of the samples in this study were calculated to have purities over 100 % (around 110 % for H2SO4 and chloroform purified samples) (Table S 2).

The commercial polymer P(3HB-co-3HV) was analysed by 1H-NMR, showing peaks of important intensity not corresponding to the P(3HB-co-3HV) structure (Figure S 10). The polymer appeared as a semi-opaque white solution when dissolved in chloroform-d, also suggesting the presence of other components hampering the solubility (Figure S 8). Such impurities could be partially removed by filtration (PTFE filters 0.2 µm) and methanol precipitation, leading to a transparent solution. However, the polymer still presented some non-assigned peaks on the 1H-NMR spectrum (Figure S 11). The purity of the purified polymer was quantified to be 94 % (by referencing the intensity to known chloroform spikes).

On the other hand, PHA purified with the same method from the initial material used in this study (crude PHA - described in section 2.1 of the manuscript), resulted in an 1H-NMR spectrum without detectable impurities Figure S 12. The quantification gave a value of 104.9 % purity.

Two different sets of standards were set for the GC determination of 3-HB and 3-HV monomers. The first one was made with known amounts of the P(3HB-co-3HV) purified from crude PHA, considering a 100 % purity and the 3-HV molar ratio determined by 1H-NMR. The second set was done with methyl-3-hydroxybutyrate (99 %, J&K Scientific®) and methyl-3-hydroxyvalerate (≥ 98 %, Sigma-Aldrich®). Both sets of standards were submitted to the same procedure as samples to be analysed (described in the analytical methods of the manuscript). Moreover, for the standards build with methyl-esters, a secondary calibration curve was built with methyl-esters not submitted to the transesterification reaction dissolved in 1,2-dichloroethane, in order to take into account the extent of incomplete transesterification.

Very similar results were obtained with the two calibration curves when determining the PHA content of several samples (Table S 2). All standard curves presented R² higher than 0.999 and the relative error between the two calibrations in the determination of total PHA was in the range of 1.5 %. Likewise, both standard sets predicted a purity close to 100 % in the samples where calibration with P(3HB-co-3HV) from Sigma-Aldrich lead to values over 110%. Thus, the results suggested that both standard sets could determine with acceptable accuracy the content of 3-HB and 3-HV monomers, and total PHA of the samples. For practical reasons, the study proceeded using the standard set produced using methyl-esters, as the 3-HB to 3-HV ratio can be more flexibly adjusted to fit the needs of the analyses. The methyl-esters not converted to propyl-esters in this standard samples was in the range of 0.4 % of the initial amount of methyl-esters in the reaction.

The purity of Sigma Aldrich P(3HB-co-3HV) was determined to be 89.5 % (±0.9) by GC analysis, based on average quantification of both calibration sets.
Figure S8: Samples of P(3HB-co-3HV) dissolved in chloroform-d (30mg/mL). From left to right: 1) P(3HB-co-3HV) purified from crude PHA in this study; 2) purified P(3HB-co-3HV) 12 mol% from Sigma Aldrich; 3) raw P(3HB-co-3HV) 12 mol% from Sigma Aldrich.

Table S2: PHA and monomer determination in several samples using different standard sets.

<table>
<thead>
<tr>
<th>Standard set</th>
<th>Sample</th>
<th>3-HB</th>
<th>3-HV</th>
<th>3-HB</th>
<th>3-HV</th>
<th>3-HB</th>
<th>3-HV</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(3HB-co-3HV) 12 mol% Sigma Aldrich</td>
<td>Crude PHA</td>
<td>59.6 ± 0.4</td>
<td>11.9 ± 0.13</td>
<td>71.5 ± 0.42</td>
<td>54.6 ± 0.37</td>
<td>3-HB</td>
<td>59.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Crude PHA after sonication and NH3 digestion at 115 °C</td>
<td>81 ± 0.69</td>
<td>16.2 ± 0.24</td>
<td>97.2 ± 0.73</td>
<td>74.2 ± 0.63</td>
<td>3-HV</td>
<td>16.2 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>Crude PHA after H2SO4 purification</td>
<td>92.9 ± 2.45</td>
<td>18.7 ± 0.46</td>
<td>111.6 ± 2.5</td>
<td>85.1 ± 2.25</td>
<td>Total PHA</td>
<td>92.9 ± 2.45</td>
</tr>
<tr>
<td></td>
<td>Crude PHA after chloroform purification</td>
<td>98.7 ± 0.15</td>
<td>119.0 ± 0.04</td>
<td>110.6 ± 0.16</td>
<td>90.4 ± 0.14</td>
<td>Total PHA</td>
<td>98.7 ± 0.15</td>
</tr>
<tr>
<td>Methyl-3-HB and Methyl-3-HV</td>
<td>Total PHA</td>
<td>63 ± 0.38</td>
<td>85.7 ± 0.65</td>
<td>98.4 ± 2.27</td>
<td>98.8 ± 0.14</td>
<td>3-HB</td>
<td>54.6 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>Total PHA</td>
<td>63 ± 0.38</td>
<td>85.7 ± 0.65</td>
<td>98.4 ± 2.27</td>
<td>98.8 ± 0.14</td>
<td>3-HV</td>
<td>8.4 ± 0.09</td>
</tr>
<tr>
<td>P(3HB-co-3HV) purified from crude PHA</td>
<td>Total PHA</td>
<td>63 ± 0.38</td>
<td>85.7 ± 0.65</td>
<td>98.4 ± 2.27</td>
<td>98.8 ± 0.14</td>
<td>3-HV</td>
<td>9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Total PHA</td>
<td>63.9 ± 0.38</td>
<td>87 ± 0.66</td>
<td>99.9 ± 2.29</td>
<td>100 ± 0.15</td>
<td>Total PHA</td>
<td>63.9 ± 0.38</td>
</tr>
</tbody>
</table>

Figure S9: TGA curves (weight % vs. temperature) of Sigma Aldrich P(3HB-co-3HV) polymer (2 batch lots), Sigma Aldrich P(3HB-co-3HV) purified through filtration in chloroform and methanol precipitation and P(3HB-co-3HV) purified from crude PHA.
Figure S10: $^1$H-NMR spectrum of P(3HB-co-3HV) 12 mol% from Sigma Aldrich® (CDCl$_3$, 300 MHz).
Figure S11: $^1$H-NMR spectrum of purified P(3HB-co-3HV) 12 mol% from Sigma Aldrich$^\dagger$ (CDCl$_3$, 300 MHz). Purification was done by dissolving the polymer in chloroform, filtering (0.2µm PTFE) and precipitating with methanol.
Figure S 12: $^1$H-NMR spectrum of P(3HB-co-3HV) purified from crude PHA in this study (CDCl$_3$, 300 MHz). Purification was done by dissolving the polymer in chloroform, filtering (0.2µm PTFE) and precipitating with methanol.
References in supporting information


