Production and extraction of Polyhydroxyalkanoates (PHAs) using wastewaters as feedstock: A review

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Abstract

Polyhydroxyalkanoates (PHAs) are biopolyesters accumulated as carbon and energy storage materials under unbalanced growth conditions by various microorganisms. They are one of the most promising potential substitutes for conventional non-biodegradable plastics due to their similar physicochemical properties, but most important, its biodegradability. Production cost of PHAs is still a great barrier to extend its application at industrial scale. In order to reduce that cost, research is focusing on the use of several wastes as feedstock (such as agro-industrial and municipal organic waste and wastewater) in a platform based on mixed microbial cultures.

This review provides a critical illustration of the state of the art of the most likely-to-be-scale-up PHA production processes using mixed microbial cultures platform and waste streams as feedstock, with a particular focus on both, upstream and downstream processes. Current pilot scale studies, future prospects, challenges and developments in the field are also highlighted.

Keywords: Resource recovery, Polyhydroxyalkanoates, Mixed microbial cultures, Wastewater treatment, Biopolymers, Circular economy, scale-up.

1. Introduction

From its origins, the aim of wastewater treatment has always been to remove contaminants in order to protect water quality and thus, human health. However, today scarcity of resources and sustainability are driving major global changes in the wastewater industry. Sustainability demands to look at wastes (from agricultural and industrial sources to those derived from direct human activities) as a renewable resource from which water, materials (e.g., nutrients, metals, bioplastics), and energy can be recovered. In this scenario, wastewater treatment is becoming a key platform to pave the way of a technological development focused on the change from a linear economy model to a circular one. Indeed more than 50% of lost waste resources are contained in wastewater (Guest et al., 2009; Van Loosdrecht and Brdjanovic, 2014; Puyol et al., 2017).

In this sense, Wastewater Treatment Plants (WWTPs) have started to be seen as Water Resource Recovery Facilities (WRRFs) or WasteWater BioRefineries (WWBR) (Regmi et al., 2018; Pott et al., 2018). A WWBR uses mixed microbial culture (MMC) biotechnology, i.e. utilises microorganisms to produce products of value, while treating, at the same time, wastewater for reuse (Verster et al., 2014). Considering the (new) products that can be recovered from wastewater in the context of WWBR, polyhydroxyalkanoates (PHA), completely biodegradable and bio-based biopolymers obtained through bacterial fermentation, are among the most interesting ones due to the role they can have in mitigating the worldwide plastic problem we are facing nowadays.

The amount of non-biodegradable plastic waste entering to the oceans is estimated to be between 4.8 and 12.7 million tons each year and, without the improvement of waste management infrastructures, is predicted to increase by an order of magnitude by 2025 (Jambeck et al., 2015). Furthermore, synthetic polymers are mostly derived from petroleum that is a non-renewable resource and so, with the decrease of its available and the rising of oil prices, the production of plastic from petrochemical resources will also become limited. Moreover, the recently approved European Directive on Single-Use Plastics (European Commission, 2019), entering into force by 2021, will also increase the pressure for a complete ban of conventional polymers. In this scenario, replacing fossil-based non-biodegradable polymers with bio-based biodegradable biopolymers could have meaningful advantages, such as a drastic reduction of the plastic pollution in the environment and the decoupling of plastic production from fossil fuel feedstock.

Polyhydroxyalkanoates are a very interesting class of bio-based biopolymer because they have

similar properties to conventional non-degradable plastics (Singh and Yakhmi, 2017). They can be produced, through bacterial fermentation, from different renewable sources including complex organic substrates such as the ones contained in wastewater, thus integrating resource recovery from wastewater treatment processes into the plastic circular economy (Kržan, 2016). In this review, a critical illustration of the state of the art of the most studied PHA production processes based on MMC is presented, as well as a discussion of the future perspectives and development in the field, with a particular focus on the last developments of the MMC technology and the use of waste streams as feedstock. Special attention is also devoted to the current studies that are being developed at pilot scale using the MMC platform, which now is still under development

In this sense, the paper is organised as follows. First, a description of PHA structure and characteristics is presented. Then, upstream production processes using MMC are discussed. Subsequently, a comprehensive overview of several downstream processes aimed to extract the PHA from MMC is provided, highlighting their application limits. Finally, a summary of the key conclusions and discussion of the future perspectives is presented.

2. Polyhydroxyalkanoates (PHAs) structure and properties

Polyhydroxyalkanoates are a wide class of linear thermoplastic polymers, belonging to bio-based bioplastic category. They can be produced by many microorganisms as intracellular carbon and energy stocks. Depending on the microorganism and the carbon source used, monomers of different length, that will arrange in different polymers, can be obtained.

Structurally, PHA are thermoplastic polyesters of hydroxyacid (HA) monomers that are connected by an ester bond (Akaraonye et al., 2010). The pendant alkyl group of PHA**¡Error! No se encuentra el origen de la referencia.** (R) can vary from methyl (C1) to tridecyl (C13) and it has been isolated over 150 different hydroxyalkanoate units with different R-pendant groups (Akaraonye et al., 2010; Chen et al., 2013). The polymer chain can contain between 100 and 35000 monomer HA units all of which are found with R (-) configuration due to the stereospecificity of the PHA synthase. The number of methylene groups in the monomer's backbone can range from 1 to 4. Commonly, the HA monomers are in the form of 3, 4 or 5 hydroxyalkanoates (3-HA, 4-HA, or 5-HA) (Albuquerque and Malafaia, 2018).

These monomers may be divided into 3 classes depending on the length of their side chain (R), i.e.: short-chain-length PHAs (scl-PHAs), which have a short side chain of only 3 to 5 carbons;

medium-chain-length PHAs (mcl-PHAs) owning a side chain of 6 to 14 carbons and large-chain-length PHAs (lcl-PHAs) that possess a side chain of 15 or more carbons (Zinn et al., 2001). The physical properties of PHA polymers, and thus their final application, are influenced by the length of the side chain and the type of functional group, being the scl-PHAs highly crystalline rigid materials while mcl-PHA are soft, elastic and even sticky materials. The synthesis of either scl- and mcl-PHA homopolymers or PHA copolymers is dependent on the type of microorganism, the process conditions and also, the substrate used as carbon source (Akaraonye et al., 2010; Albuquerque and Malafaia, 2018; Anjum et al., 2016; Chodak, 2008; Kosseva and Rusbandi, 2018). Regarding mechanical properties, the biopolymers are more resistant when its chain length is longer, i.e. when the PHA average molecular weight is high. Molecular weights typically range between 0.2×10^6 and 3×10^6 Dalton (Da) and the mechanical properties of PHA deteriorate when the molecular weight drops below 0.4×10^6 Da (Laycock et al., 2013).

PHA copolyesters of scl- and mcl- monomers such as P(3HBco-3H4MV), P(3HB-co-4HB) and P(3HB-co-3HHx) combine the toughness of scl-PHA and elasticity of mcl-PHA. The studies conducted on these materials show that they have improved mechanical properties similar to elastomers, being more flexible than the P(3HB-co-3HV) copolymers. For these copolymers, as for P(3HB-co-3HV), an increase in comonomer content correspond to an increase in elongation to break and a decrease in Young's modulus and tensile strength. (Laycock et al., 2013; Tanadchangsaeng et al., 2009).

3. PHAs production

Nowadays, the most common way to produce PHA at industrial scale is through the use of pure cultures of natural or engineered microbial strains. One of the biggest drawbacks in pure culture platform is the necessity of a sterile environment that make the process expensive and complicated. In this sense, this section will cover the identified PHA production pathways in pure cultures and, then it will move to MMC to finally make a critical comparison between both platforms.

3.1. Known mechanisms for PHA biosynthesis in pure cultures

Bacterial PHA are synthetized aerobically under conditions of unfavourable growth, like limitation of an essential growth nutrient, and excess of carbon source (Lee, 1996). The precise

composition of the PHAs depends on the type of bacteria and the feedstock from which they are synthesized, in fact eight different pathways have been identified for biosynthesis of PHA up to date (Chen, 2010b). PHAs can also be obtained by chemical synthesis or using genetically modified plants, however the use of bacteria for their biological synthesis is a more attractive process (Chodak, 2008).

One of the most well-known pathways is the one used by *C. necator* (also known as *Ralstonia eutropha*) that is the most used strain at industrial scale nowadays for PHA production (Sudesh et al., 2000). From this pathway scl-PHA are synthesized. The biosynthesis starts with the conversion of an appropriate carbon substrate, such as sugars (through glycolysis) or organic acids (through beta-oxidation), in the form of acetyl coenzyme-A (acetyl-CoA). Two molecules of acetyl-CoA are condensed by the β-ketotiolase (PhaA) enzyme to give acetoacetyl coenzyme-A. This product is then reduced to (R)-3-hydroxybutyril-CoA monomer by acetoacetyl-CoA reductase (PhaB) at the expense of the conversion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. Finally the PHA synthase enzyme (PhaC) catalyses the polymerization of the 3-hydroxybutyril-CoA monomer into P(3HB) (Serafim et al., 2008; Anjum et al., 2016; Sudesh et al., 2000).

. Another pathway described by Sudesh et al. (2000) is commonly used by pseudomonads and occurs by the β-oxidation of fatty acids that are polymerized by the enzyme PHA synthase. The specific enzymes 3-ketoacyl-CoA reductase (FabG) and enoyl-CoA hydratase (PhaJ) are involved in the conversion of fatty acid b-oxidation intermediates, 3-ketoacyl-CoA and trans 2-enoyl-CoA, into (R)-3-hydroxyacyal-CoA that can then be incorporated into PHA polymers by PhaC. It should be noted that the first and second pathways just described use "related" carbon sources, i.e. the synthetized PHA is structurally related to the carbon source employed. . A third pathway was also described by Sudesh et al. (2000), where the carbon source is oxidised into acetyl-CoA and converted into malonyl-CoA and finally, into the intermediate (R)-3-hydroxyacyl of the fatty acid pathway that is converted from their acyl carrier protein (ACP) form to the CoA form by action of the acyl-ACP-CoA transacylase (PhaG). This pathway results attractive as PHA monomers structurally different from the carbon source (such as carbohydrates) can be obtained. According to several authors, this pathway has been observed in some pseudomonads (Anjum et al., 2016; Philip et al., 2007; Kniewel et al., 2017; Sudesh et al., 2000).

3.2. PHA biosynthesis through Mixed Microbial Cultures (MMC)

The PHA production using MMC is based on the natural principles of selection and competition

of the microorganisms with a PHA storage ability against the microorganisms that are not able to accumulate PHA (Kosseva and Rusbandi, 2018). In order to select efficient PHA producers, bacteria should possess several features like high polymer synthesis and cells growth rate, ability to utilize waste streams as feedstock, high substrate to PHA conversion efficiency, maximum polymer accumulation capacity, the possibility of PHA production under cultivation conditions providing high productivities with a minimal energy consumption, such as continuous bioprocesses under open non-sterile conditions. Other characteristics, such as: large cell size and fragile cell wall, that allow to have a simple downstream PHA extraction process, are also highly desirable. These requisites can be indeed covered by the MMC platform and this is why they are gaining more a more attention nowadays.

The PHA production process by MMCs usually takes place in three independent phases (Kourmentza et al., 2017). In the first step, the raw complex organic substrate is fermented to obtain a volatile fatty acids (VFA) rich stream; then in the second step the culture selection of PHA-storing microorganisms by applying transient conditions in sequential batch reactors (SBR) is performed; finally, in the third step, the enriched biomass is fed with the VFA of the first stage, under optimal conditions, to maximize the PHA accumulation. When cells reach the maximum PHA content, they are harvested and sent to downstream extraction processes (Serafim et al., 2008; Kourmentza et al., 2017).

3.2.1. First step for PHA production using MMC: Anaerobic fermentation

In a first step, the raw complex organic substrate is fermented in continuous mode to obtain volatile fatty acids (VFA), precursors of PHA. Acidogenic fermentation is the initial phase of the biogas production by anaerobic digestion of organic matter and, it involves the bioconversion of the monomers produced from hydrolysis into hydrogen (H_2), a mixture of VFA and also CO_2 (Dahiya et al., 2015). When mixed cultures are used for the anaerobic production of VFA from waste substrates, lot of microorganisms are involved and so, several biochemical reactions take place and it can be expected the formation of different intermediates and by-products such as ethanol, butanol, acetate, propionate and butyrate (Ghimire et al., 2015; Zhou et al., 2018).

Despite acetate and butyrate are the most commonly reported by-products of acidogenic fermentation, the production of other kind of VFA, as well as their yields, depends on the type of substrate used, the operational parameters such as loading rate, pH and temperature, the inocula and the environmental conditions. (Ghimire et al., 2015; Silva et al., 2013; Zhou et al., 2018).

In order to achieve high levels of VFA, hydrolysis must be improved in order to produce soluble products for their further fermentation, acidogenesis process must be promoted and acidogens inhibition should be avoided by minimising, for example, methanogens activity (Wang et al., 2014; Zhou et al., 2018). It is important the optimization of process parameters such as temperature, pH, retention time and organic loading rate so that biogas formation is inhibited and most of organic matter is recovered in form of VFAs (Khan et al., 2016).

The VFAs obtained from the first fermentation step can be used as feedstock in the subsequent two steps, both for culture selection and for PHA accumulation. When using MMC it is preferable to use volatile VFAs as carbon source because many other substrates, such as carbohydrates or glycerol tend to form glycogen instead of PHA (Karahan et al., 2006; Kourmentza et al., 2017). Furthermore, this fermentation step makes it possible to use as feedstock for the PHA production process a very various range of complex organic substrates, such as: **food waste** (Zhang et al., 2014; Venkateswar Reddy and Venkata Mohan, 2012b; Jiang et al., 2013; H. Chen et al., 2013; Colombo et al., 2017; Amulya et al., 2015), **sugar cane molasses** (Albuquerque et al., 2011; Albuquerque et al., 2007; Albuquerque, Torres, et al., 2010; Albuquerque, Concas, et al., 2010; Bengtsson, Ana R Pisco, et al., 2010; Bengtsson, Ana R. Pisco, et al., 2010; Duque et al., 2014), **olive mill wastewater** (Yarimtepe et al., 2017; Silva et al., 2013; Beccari et al., 2009; Ntaikou et al., 2014; Campanari et al., 2014; Villano et al., 2010; Dionisi et al., 2005), **waste activated sludge** (Yu et al., 2018; Huang et al., 2015; Jiang et al., 2009; Silva et al., 2013)**, paper mill wastewater** (Chen et al., 2015; Jiang et al., 2012; Bengtsson, Werker, Christensson, et al., 2008; Bengtsson, Werker, and Welander, 2008), **cheese whey** (Gouveia et al., 2017; Valentino, Karabegovic, et al., 2015; Valentino, Riccardi, et al., 2015; Colombo et al., 2016; Silva et al., 2013; Duque et al., 2014)**, palm oil mill effluents** (Lee et al., 2015; Din et al., 2012) as well as some other wastes for instance, **pulp and paper mill effluents, glycerol, soapy residues, winery effluents, leachates, spent coffee grounds, dairy manure, milk whey, and dairy, ice-cream and milk wastewaters** (Queirós et al., 2014; Bosco and Chiampo, 2010; Chakravarty et al., 2010; Silva et al., 2013; Chen et al., 2015; H. Chen et al., 2013; Coats et al., 2016; Cruz et al., 2014).

PHA production from waste feedstocks is very advantageous for two reasons. First, it makes the biopolymer cheaper since waste feedstocks are a free substrate; secondly, it makes the waste management easier, reducing the amount of waste that industries have to treat.

3.2.2. Second step for PHA production using MMC: Culture selection

In the second step, part of the VFAs produced in the first step are used to favour PHA-storing

bacteria following the classical ecological selection principles. Indeed, when using MMC, a desire metabolism can be enforced by varying the operational conditions of the biological system such as applying an intermittent feeding or intermittent availability of electron acceptors, thus causing a stress to the microorganisms. It can be said that, in this way, the ecosystem is engineered instead of the microorganisms (Dias et al., 2006; Koller, 2017). The efficiency of the microbial selection step is pivotal for the overall production of the biopolymer. The aim of this step is to select a culture able to produce a reasonable amount of PHA in stable mode. Furthermore, an high growth rate is important so that volumetric productivity of the process would be as high as possible (Reis et al., 2011).

The key to achieving a successful MMC PHA production process relies on the enrichment of PHA-accumulating bacteria. To that aim two main enrichment strategies are used: i) anaerobic/aerobic enrichment (AN/AE), where the selection of microorganisms is carried out under the alternance of anaerobic and aerobic conditions and ii) aerobic dynamic feeding (ADF), where the selection of microorganisms is carried out alternating availability and unavailability of a carbon source (Kourmentza et al., 2017). Table 1 gives an overview of both strategies.

Table 1. Summary of the most common strategies for MMC selection in PHA-accumulating bacteria

Strategy	Main mechanism applied	Energy requirements	Stability of the PHA-storing capability	PHA Contents (weight %)
Anaerobic/Aerobic Enrichment (AN/AE)	Alternance of anaerobic and aerobic conditions	✓✓✓	✓✓	20 (Kourmentza et al., 2017)
Aerobic Dynamic Feeding (ADF)	Alternance of carbon source availability	✓	✓✓✓	89 (Johnson et al., 2009)

PHA intracellular storage by MMC was first observed during a biological phosphorus removal process in wastewater treatment plants. These systems alternate anaerobic/aerobic cycles and the PHA accumulation occurs during the anaerobic phase where two microorganisms can store carbon source: the polyphosphate-accumulating organisms (PAOs) and the glycogen-accumulating organisms (GAOs). Both microorganisms under anaerobic conditions, since the

electron acceptor becomes limiting, take up the carbon substrate and use it towards PHA synthesis. The energy required for this process is provided by the internally stored polyphosphate (PAOs) or glycogen (GAOs). Then, under aerobic conditions, the stored PHA is consumed for growth, maintenance and glycogen/polyphosphate replenishment using the available oxygen (Koller et al., 2011; Paul et al., 2012; Serafim et al., 2008). From MMC enriched using AN/AE enrichment strategy, stable PHA contents of around 20% had been obtained (Kourmentza et al., 2017). While the aerobic dynamic feeding is more efficient than AN/AE enrichment strategy (maximum PHA contents up to 89% had been reported for MMC enriched with ADF strategy (Johnson et al., 2009), the advantages of AN/AE strategy is a decreased energy consumption due to the lower aeration requirements. With the purpose of enhancing the energy savings in this kind of system, Satoh et al. (1998) proposed to supply a low amount of oxygen in the anaerobic zone of an AN/AE system, so that the enrichment occurs under "microaerophilic-aerobic" conditions, allowing the bacteria to obtain energy from substrate oxidation in the anaerobic zone. Under such conditions maximum PHA content up to 62% has been obtained (Satoh et al., 1998). Furthermore it has been found that the microaerophilic/aerobic process is better than AN/AE process since microbial culture with a more stable PHA storing capacity can be obtained when microaerophilic/aerobic process is used instead of the classic AN/AE process (Takabatake et al., 2000).

Polyhydroxyalkanoates-storing microorganisms with high and stable PHA production can be selected and enriched most commonly under fully aerobic conditions, following the ADF strategy, where the biomass is exposed to cyclic conditions of feast and famine. This mean that under aerobic conditions, short periods of carbon sources availability (feast) are followed by long periods of unavailability (famine). During the feast, the carbon source is taken up and stored as PHA granules. The bacteria capable of storing PHA during this phase, have a competitive advantage over the rest of microbial population because, once the external carbon source is depleted, they will use the PHA accumulated as carbon and energy source to withstand the famine phase. The PHA-storing microorganism’s selection occurs by the competitive advantage of some microorganisms to accumulate PHA over the other microorganisms without this ability. Indeed PHA-storing microorganisms may grow during the famine while non-storing PHA microorganisms will starve (Albuquerque, Torres, et al., 2010; Dias et al., 2006; Salehizadeh and Van Loosdrecht, 2004).

Aerobic dynamic feeding is the most studied and efficient strategy for PHA production by MMCs. The Feast/Famine (F/F) ratio varies greatly from one study to another, especially because of different substrate type and concentrations used in the feed. Nevertheless, the length

of feast phase must be sufficient for complete substrate depletion, and the length of famine phase must be long enough to allow a significant consumption of the previously accumulated PHA (Paul et al., 2012; Hao et al., 2018).

3.2.3. Third step for PHA production using MMC: Maximising the PHA accumulation capacity.

The third stage of the PHA production has the aim to maximize the intracellular accumulation of the biopolymer, in order to ensure a high and stable PHA content in MMC cells. The selected biomass is harvested from the culture selection step and subjected to aerobic conditions of extended feast, in fed-batch mode with pulsed or continuous substrate feeding. In pulsed fed-batch production a high PHA productivity can be obtained, but since after a substrate pulse the microorganisms begin to degrade the accumulated PHA before the addition of the new pulse, PHA productivity eventually decrease. By supplying substrate in a continuous manner PHA accumulation can be maximised, also obtaining a steadier PHA storing activity. Indeed, with this feeding regimen biopolymer losses between pulses would be avoided and a more stable substrate concentration can be maintained through the entire accumulation step (Albuquerque et al., 2011; H. Chen et al., 2013, Montiel-Jarillo et al., 2019).

Generally, nutrient limitation or deficiency is applied in order to maximize PHA accumulation (Johnson et al., 2010; Venkateswar Reddy and Venkata Mohan, 2012a, Montiel-Jarillo et al., 2017; Pokój et al., 2019). Indeed, if under high nitrogen and phosphorous concentrations biomass growth is fostered by protein synthesis, when there is a nutrients limitation the metabolic pathway switch towards PHA accumulation rather than towards protein synthesis (Mohan and Reddy, 2013; Oliveira et al., 2017). Another reason for limiting the nitrogen concentration is to prevent the growth of non PHA-accumulating bacteria (Marang et al., 2014). Nitrogen or phosphorus limitation can increase the polymer content in biomass up to almost 3 time in comparison with the PHA accumulation reached under excess of these nutrients (Montiel-Jarillo et al., 2017; Tu et al., 2019), other studies report that nutrients deficiency rather than limitation lead to higher PHA contents (Johnson et al., 2010; Silva et al., 2017). The requirement for nutrients limitation or deficiency, in order to maximise the PHA cell content, also depends on the type of substrate used and on the composition of the enriched culture. Regarding the overall productivity of the PHA accumulation step is reported that nutrient limitation rather than deficiency lead to higher productivities because while the absence of essential nutrients prevents cell growth causing cellular PHA saturation, limited nutrients concentrations allows bacteria to duplicate without permitting excessive cell growth thus

prolonging PHA accumulation (Kourmentza et al., 2017; Valentino, Karabegovic, et al., 2015).

3.3. Pure cultures vs. MMC

Even though nowadays the most common way to produce PHA is still through pure culture technology of natural or engineered microbial strains (there are still no companies that opt for the use of MMC for the industrial production of PHAs, given the uncertainty of the entire production process), PHA production by MMC is increasingly considered due to the possibility to reduce the costs of the production process and to integrate PHA production in wastewater and organic waste biological treatments (Kourmentza et al., 2017; Morgan-Sagastume, 2016; Frison et al., 2015). In this sense, Table 2 intents to summarise the advantages of MMC compared to pure or engineered microbial cultures.

PHA production by pure cultures is expensive, mostly because of the costs of culture maintenance, substrate formulation and both substrate and reactor sterilization (Villano et al., 2014; Ivanov et al., 2014). Although the amount of PHA that mixed cultures can accumulate is usually lower, as well as cell densities (see below), than that of the single species, there are some mayor advantages that contribute to reduce production costs, making MMC potentially more competitive than other microorganisms from the economic point of view. Such advantages are the following: (i) the ability to grow in non-sterile environments, as opposed to pure culture requiring close sterility conditions, and (ii) the possibility to use a very various range of economical complex organic substrates, including waste/surplus streams, without a previous substrate sterilization while pure cultures mainly use refined carbon substrates, such as glucose or acetate. These simplifications in the production process lead to a minor energy requirement, furthermore less process control is required thus minimizing the equipment cost (Dias et al., 2006; Serafim et al., 2008; Carvalho et al., 2014; Kourmentza et al., 2017).

Another advantage is that MMC can easily produce a wide range of PHA copolymers with different compositions utilizing different feedstock, indeed in P(3HB)-based copolymers synthetized by MMCs various monomer units such as 3-hydroxyhexanoate (3-HHx), 3-hydroxyvalerate (3HV), 3-hydroxy-2-methylvalerate (3H2MV), and 3-hydroxy-2-methylbutyrate (3H2MB), are commonly included in the polymer chains (Dai et al., 2008a; Dai et al., 2008b; Bengtsson, Ana R. Pisco, et al., 2010; Lemos et al., 2006). Pure cultures, on the contrary, often require substantial amounts of co-substrates in order to synthetize polymers with relatively low fractions of monomers other than P(3HB). The cause of this different behaviour could be the heterogeneity of MMCs, since they contain a variety of different microorganisms that can employ various distinct PHA production pathways (Laycock et al., 2013).

In addition to the minor PHA content that mixed cultures can reach compared to pure cultures, also the cell densities reached are lower, usually below 10 g L^{-1}, compared to the cell densities that can surpass 150 g L^{-1} in industrial scale pure culture systems (Kourmentza et al., 2017; Chen, 2009). Nevertheless, comparable productivities, of up to 1.2 gPHA L^{-1} h^{-1}, along with high PHA yields, up to 0.8 Cmol PHA/Cmol S, have already been reached by MMCs (Albuquerque et al., 2011).

Table 2. Mixed microbial cultures versus pure or engineered microbial cultures

Type of Culture	Developed Scale	Operational Costs					Capital Cost	PHA Contents (weight %)	PHA Productivity (g PHA L^{-1} h^{-1})	Versatility of the process for the production of different kinds of PHA	Easiness of the PHA extraction
		Culture maintenance	Substrate formulation	Need of sterility	Ease of operation	Energy requirements					
Pure or engineered microbial culture	Commercial/ Industrial	✓	✓ Very specific	Yes	✓✓	✓	✓✓✓	90 (Chen, 2009)	1.0-3.0 (Kourmentza et al., 2017)	✓	✓✓✓
Mixed microbial culture	Pilot	✓✓✓	✓✓✓ Wastes can be used	No	✓✓✓	✓✓✓	✓✓	75 (Alburquerque et al. 2010)	1.2 (Kourmentza et al., 2017)	✓✓✓	✓

Another major disadvantage in using MMCs is that extracting PHA from MMCs biomass is more difficult than from pure cultures (reader is kindly referred to section 4). Indeed MMCs, compared to pure cultures, are more resistant to cell hydrolysis, have a strong and complex extracellular biomass matrix, a lower starting PHA levels that may result in a lower cell constrains that decrease cellular fragility and furthermore chlorinated compounds seem not to be as effective on it as they are on pure cultures (Patel et al., 2009; Samorì, Basaglia, et al., 2015; Majone et al., 2017).

Nevertheless, MMCs PHA production is promising. It has already been registered a PHA content of 89 wt % (Johnson et al., 2009) and 74% (Montiel-Jarillo et al., 2019) with synthetic substrate and 75 wt % obtained with real substrate, such as fermented molasses (Albuquerque et al., 2010). These results are comparable with the ones obtained using pure cultures, which maximum production reach more than 90 wt % (Chen, 2009). With these results and future improvements in the production and extraction technologies, the advantages of PHA production by MMC would be an improved economy, no requirement of sterile conditions (energy saving), an improved use of wastes, and a simpler process control.

4. PHA extraction from microbial cells: focus on MMC

After the accumulation phase, the biomass is usually separated from the aqueous matrix by centrifugation, filtration or sedimentation, subsequently the PHA is recovered from the non-PHA cell mass (NPCM, which includes polypeptides, phospholipids, DNA, RNA and peptidoglycans). In order to recover the PHA, many methods are described in literature, but all of them basically operate on rupturing the bacterial cell and removing the protein layer that coats the PHA granules, or selectively dissolving the PHA in a suitable solvent that is able to pass through the cell membrane. To the best of our knowledge, there is no PHA production process based on the MMC platform at industrial scale, therefore the extraction methods used up to now are derived or modified from the one already used for pure or engineered cultures.

- In any case, the applicability of the extraction method depends on several factors (Koller et al., 2013):The fragility of the cell wall, which is different for pure cultures and MMC;
- Type of PHA and quantity produced (high intracellular PHAs load can facilitate the release of biopolymer granules due to increased fragility of the cells);
- The type of PHA (scl-PHA or mcl-PHA);
- The required purity (depending on the final application of the biopolymer);
- The impact of the extraction method over the final molecular mass of PHA.

The challenge in the extraction processes for MMC compared to pure cultures is to achieve, in

a cheap and environmentally friendly manner, a high molecular weight with a high degree of purity of the extracted polymer (Kunasundari and Sudesh, 2011). Up to now, the most studied methods for recovering PHAs can be grouped into three categories:

1) Solvent extraction
2) Digestion of the NCPM
3) Mechanical disruption

Often, regardless of the extraction process chosen, a biomass pre-treatment such as heat, mechanical pre-treatment or use of alkali and oxidants, is applied to weaken and break the cell wall in order to facilitate subsequent extraction of the polymer.

Downstream processes for polymer extraction from cells are among the most important factors affecting the overall PHA production cost (Pérez-Rivero et al., 2019). Currently the most used extraction method at industrial scale is the extraction of PHA through the use of solvents, that is very effective on pure cultures although it is not the most economical and environmental friendly method (Chen, 2010a; Pérez-Rivero et al., 2019). The most widely used extraction methods are described below.

4.1. Solvent extraction

Both scl- and mcl-PHAs are insoluble in water but soluble, even at room temperature, in some organic solvents like chlorinated ones such as chloroform, 1,2-dichloromethane and methyl chloride, in some cyclic compounds such as propylene and ethylene and in some azeotropic mixtures such as trichloroethane with water or chloroform either with ethanol, methanol, hexane or acetone. Most mcl-PHA are soluble also in hexane, ether and acetone (Koller et al., 2013). The solvents act in two phases: (i) they modify the cell membrane permeability by dissolving the lipid portion of the NPCM thus (ii) allowing the solubilization and subsequent extraction of PHA. When extracting scl-PHAs, methods involving use of both, PHA non-solvents (acetone and alcohols) for lipid extraction and PHA solvents for the subsequent polymer extraction are commonly applied (Braunegg, 2002; Jacquel et al., 2008).

After the extraction, the dissolved biopolymer is separated by evaporation or precipitating it by the addition of an antisolvent, usually a low molecular weight alcohol (ethanol or methanol) or water in which the PHA is not soluble. For scl-PHA also ether, acetone and hexane can be used as antisolvents. The precipitated biopolymer is then separated from the mixture by centrifugation or filtration (Koller et al., 2013; Madkour et al., 2013). Precipitation can also be triggered by changing temperature or pH to a range where the polymer is not soluble anymore in the specific solvent (Braunegg, 2002).

Solvent extraction presents some advantages in terms of efficiency over others PHA extraction methods such as a higher purity and a negligible degradation of the biopolymer which maintains a high molecular weight and its original composition. Furthermore, with this method bacterial endotoxins can be removed allowing the extracted polymer to be used in medical applications (Lee et al., 1999; Kunasundari and Sudesh, 2011).

Unfortunately, solvent extraction in large-scale applications is generally a not environmentally friendly method. The harmful characteristics of the most used solvents, especially of the chlorinated ones such as chloroform, are the major drawback of this extraction method, since they create hazards for the environment and for the operators. Indeed, the process requires massive amounts of the extraction solvents, up to the 20 time the mass of the PHA-containing biomass. For the biopolymers precipitation, about 10 volumes of the PHA antisolvent must be added per volume of PHA solution (Koller et al., 2013). Also, the purification of the solvent remaining after the precipitation step is a very energy intensive task, usually achieved through distillation. Therefore, the solvent extraction process has high operational and capital costs due to the high quantity of solvents and energy employed and the frequent requirement of drying the biomass before the extraction (Madkour et al., 2013). Another problem is that the viscosity of the PHA solution can interferes with cell debris removal resulting in difficult and expensive separation process (Kunasundari and Sudesh, 2011).

Since the chlorinated compounds are not environmental friendly, the cutting edge technologies in this field are oriented toward the use of recyclable and environmentally friendly green solvents such as dimethyl carbonate (DMC) (Samorì, Basaglia, et al., 2015). Moreover, while chlorinated compounds seem not to be as effective on MMCs as they are on pure culture (is reported that no more than 30% of the PHA produced by MMC can be recovered by chlorinated solvents extraction (Majone et al., 2017; Patel et al., 2009)), Samorì and colleagues proposed a method based on a double extraction with the green solvent DMC that achieved a better recovery (60%) and a polymer purity of 95% (Samorì, et al., 2015).

4.2. NPCM digestion

In light of the fact that PHA producing microorganism can accumulate these polymers in quantities up to 90% of the cell DW, it can be reasonable to remove the small fraction of NPCM surrounding the PHA granules rather than extract the PHA by solubilizing them in a suitable solvent. Therefore, various methods were developed in order to set free PHA granules by solubilizing the surrounding NCPM. Chemical compounds of various kinds can be used for this purpose: sodium hypochlorite, surfactants, acid and alkaline compounds. All these chemicals

can be used in aqueous phase, avoiding the energy consumption needed to dry the biomass when the solvent extraction is applied (Koller et al., 2013; Kunasundari and Sudesh, 2011).
When using oxidants and alkali, if the concentration of the chemical and the other extraction condition used are not well controlled, upon dissolution, not only NPCM are dissolved but also the intracellular polymer, leading to PHA degradation and lower recovery (Kosseva and Rusbandi, 2018). By the way the PHA granules released from the cells are usually surrounded by a membrane that protects PHA to a certain extent from chemical attack; hence under appropriate conditions it can avoid degradation and conserves high molar masses.
Since the applied chemicals convert the NCPM components in water-soluble substances, the released PHA granules can be easily separated by centrifugation, filtration or floatation (Koller et al., 2013).

4.2.1. Digestion by sodium hypochlorite

Sodium hypochlorite degrades most of the NPCM that when oxidised become water soluble, while the PHA that mostly resists to its attack remains solid and can be easily separated. The process is exothermic, therefore when NaClO is used at large scale the temperature of the reaction environment must be controlled. The advantage of using hypochlorite lies in the fact that the cell does not have to be dried before treatment and this saves time and energy and reduces the cost of the downstream process (Madkour et al., 2013). The purity of the polymer at the end of the extraction can reach 96% (Heinrich et al., 2012; Mannina et al., 2019) however the PHA is not completely insoluble in the solution and a reduction of the molecular weight has been observed in some cases (Mannina et al., 2019).
A dispersion of NaClO and chloroform can be used in order to exploit both cell lysis by hypochlorite and solvent extraction. The polymer is recovered by precipitation using an antisolvent. Hahn et al. (1994) recovered 91% of the biomass PHA content with a purity of 97% applying a dispersion of hypochlorite and chloroform for 90 min. The advantage of this method is the high extraction yield associate to a moderate decreased in molecular weight, indeed the PHA released by hypochlorite treatment is immediately dissolved in chloroform and thus protected from degradation. The drawback of this extraction process is that large amount of chloroform required (Hahn et al., 1994).
Another method to reduce PHA degradation is to treat the biomass with a surfactant before the hypochlorite treatment thus reducing the contact time between hypochlorite and PHA granules. In this way, Dong and Sun (2000) reported high purity (> 97%) and minor polymer degradation.

4.2.2. Digestion by surfactants

Thanks to their mode of action surfactants can be used to disrupt the cellular membrane thus allowing the release of PHA granules from bacterial cells. Numerous surfactants, when exceeding the critical micellar concentration, organize into supramolecular aggregates called micelles. The micelles repel each other because of the electrostatic repulsion of their ionized heads, this prevents the particles of oils and fats from re-aggregating again keeping them suspended in the water and allowing their removal.

In the extraction of PHA surfactant disrupt cells by incorporating itself into the lipid bilayer membrane and increasing the volume of the cell envelope until the membrane breaks to produce large micelles of surfactant and membrane phospholipids. This leads PHA to be released into the solution surrounded by the cellular debris. Moreover, surfactants are able to solubilize and denature proteins and other non-PHA cellular materials thus facilitating the disruption of cell membrane (Chen et al., 1999).

Many surfactants, such as sodium dodecylsulphate (SDS) and Triton X-100 have been used with the aim of PHA extraction. When applied on pure cultures of recombinant *E. coli*, the polymer's purity and recovery can reach respectively 97 and 90% without the need for pre-treatment before extraction (Choi and Lee, 1999). The main advantage of this method is that surfactants destroy cells without degrading polymer granules, degradation can still occur if high temperatures or pH are used. The main disadvantages are the high surfactant/biomass ratio needed and the difficulty in the recovery of the surfactant, that then generate a large quantity of wastewater.

The use of small amounts of chelating agents in combination with surfactants increases both purity and recovery of the extracted polymer. Chelating agents act by forming complexes with the divalent cations in the outer cell membrane destabilizing it and thus causing weakness also in the inner membrane (Jacquel et al., 2008). Chen and colleagues demonstrated that by adding a small amount of chelating agent while extracting PHB from *Alcaligenes eutrophus* using surfactants, the recovery rate and the purity of the polymer increased by more than 10%. They obtained a purity of 98.7% and a recovery yield of 93.3% under optimal pH and temperature conditions (Chen et al., 1999).

The main problem when using surfactants in the PHA extraction processes from bacterial biomass, is their recovery. In fact they are very water soluble molecules and are used in large quantities, resulting in high recovery costs and problems in the treatment of wastewater (Kunasundari and Sudesh, 2011). In order to avoid this problem the use of switchable surfactants for the extraction of PHA from biomass was proposed for the first time by Samorì,

Basaglia, et al., 2015. The term “switchable” is used to indicate molecules (solvents, surfactants, additives) that can be reversibly shifted between two stable states when a trigger is applied. For example, they can be reversibly rendered polar/apolar, volatile/non-volatile, protic/aprotic, water miscible/water immiscible and this peculiarity give them the potential to being applied in many specific production fields (Samorì, Basaglia, et al., 2015). Specifically, switchable surfactants, can be directly and reversibly converted between active and inactive forms through the application of an appropriate trigger. So by switching the surfactant to the least soluble form in the reaction medium, they can be removed from the liquid phase and recovered for being reused afterward (Liu et al., 2006).

Based on the work of Samori et al. (Samorì et al., 2015), Mannina and colleagues successfully applied ammonium laurate extraction on MMC biomass with a previous NaClO pre-treatment obtaining a polymer with a purity of 100% (Mannina et al., 2019) and recoveries up to 90%.

4.2.3. Digestion by acids and alkalis

The use of acid or alkaline compounds such as sulfuric acid (H_2SO_4) or sodium hydroxide (NaOH) can be a valid, cost-effective and green alternative to chlorinated compounds in PHA extraction processes.

Hydroxides cause saponification of the lipids present in the cell wall of the microorganism, increasing membrane permeability and helping the release of proteins and non-PHA material (Anis et al., 2012). Mohammadi et al., 2012 used low concentration of NaOH on recombinant *Cupriavidus necator* achieving high purity and recovery yield (> 96%) with only 13% reduction in polymer molecular weight.

Anis et al., 2013 obtained high recovery (86%) and polymer purity (97%) applying the NaOH treatment on recombinant *Cupriavidus necator* biomass pre-treated with NaCl. The combination of NaOH digestion and NaCl pre-treatment increased the purity and recovery of NaOH treatment by almost 10%, but severe degradation of biopolymer was detected. The biopolymer molecular weight was almost halved if compared to polymer extracted using chloroform.

While the intracellular amorphous granules of PHA are quite vulnerable to saponification in alkaline solutions, they are greatly resistant to acidic hydrolysis. Acids act as digester of bacterial cell walls since the non-PHA cell mass and peptidoglycans of cell walls are vulnerable to acidic solutions. High acidity erode the bacterial cell walls by disintegrating the peptidoglycans which lead to the release of protein and other biological macromolecules into the aqueous solution (Yu and Chen, 2006; Anis et al., 2013).

Yu and Chen, (2006) demonstrated that cheap mineral acids, at appropriate concentration are selective agents that solubilizes the non-PHA cell mass into small cellular debris with little decomposition of the polymer. They achieved high purity (>97 wt %) with high recovery yield (>95 wt %) applying the acid treatment followed by final decolorization in a bleaching solution on *Ralstonia eutropha* PHA-containing biomass. Extrapolation on large scale show that this method is much cheaper than conventional extraction, since it can reduce the chemical cost of PHA extraction by 90%.

4.2.4. Enzymatic Cell Digestion

Various types of enzymes such as proteases, phospholipases, lysozyme, and nucleases are highly active in hydrolysis reactions, dissolving proteins and other components of the bacterial cell mass and thus initiating cell lysis, with no or only minor PHA degradation (Yasotha et al., 2006; Kapritchkoff et al., 2006).

An enzymatic method for NCPM digestion was developed by Holmes and Lim, 1990. This method included three phases: (i) a thermal treatment for denaturation of cellular material and initiation of cells lysis, (ii) an enzymatic digestion, and (iii) a surfactant treatment to dissolve the residual biomass. This process was applied to extract PHB granules from *Cupriavidus necato*r biomass by Imperial Chemical Industries (U.K.).

Optimized application of processes involving the use of enzyme cocktails, heat and surfactants, results in polymer purities of about 95% (Koller et al., 2013). Lakshman and Shamala, 2006 employed lytic enzymes from *Microbispora* sp for enzymatic digestion of *Sinorhizobium meliloti* biomass containing 50% PHA, achieving 94% recovery and 92% purity of PHA. A high PHA recovery yield (90%) and purity (93%) was obtained by Yasotha et al., 2006 employing Alcalase for enzymatic digestion of *Pseudomonas putida* biomass combined with surfactant (SDS) and chelate (EDTA) treatment.

With enzymatic processes, extraction of PHA with good yield, purity and quality can be achieved since enzymes are very specific with respect to the reactions they catalyse. However the process is expensive due to the high cost of enzymes and complexity of the extraction process (Kapritchkoff et al., 2006).

4.2.5. Mechanical disruption

The most used mechanical methods for cell disruption are high-pressure homogenization and bead milling. In general, mechanical disruption is favoured because of the little damage it causes to the products and environment since it does not involve the use of any chemicals. The drawbacks of this method are: long processing time, high capital investment cost and difficulty

in scaling up (Kunasundari and Sudesh, 2011). Mechanical cell disruption methods are frequently applied at laboratory scale for the release of PHA granules from bacterial cells. However, these methods are less used for industrial large scale PHA production (Madkour et al., 2013).

During the extraction process when using the bead mill and the homogenizer heat is generated, therefore the process need to be refrigerated. The performances of the homogenizer are better than the mill's ones when cells are highly concentrated (from 45 kg DWm^{-3}) while at low biomass concentration the mill perform better (Jacquel et al., 2008).

Mechanical disruption of cells can also be combined with other treatments using solvents, surfactants or chemicals (Kosseva and Rusbandi, 2018; Kshirsagar et al., 2013). Applying high pressure homogenization to a 5% (w/v) SDS solution containing the biomass, P(3HB) was recovered from *Methylobacterium* sp. V49 achieving a high purity and recovery yield (95% and 98%) (Ghatnekar et al., 2002).

4.3. Other methods

There are other less used methods that has been used at laboratory scale in order to extract PHA from microbial cells.

4.3.1. Supercritical fluid extraction

Supercritical fluid (SCF) extraction is always more implemented for efficient and environmentally friendly extraction of various high-value products from numerous matrices (Darani and Mozafari, 2009). SCF have emerged also as a potential extraction agent in the PHA extraction process due to their suitable physicochemical properties such as low viscosities and high density (Hejazi et al., 2003; Khosravi-Darani et al., 2003).

In this context, the supercritical CO_2 (sCO_2) is considered as a convenient and safe solvent because of its availability, nontoxicity, non-flammability and low reactivity. Furthermore, after the extraction it evaporates completely without letting any residues, thus avoiding a subsequent drying of the product. Since numerous hydrophobic compounds are highly soluble in supercritical CO_2, it was tested as a potential solvent for PHA extraction (Koller et al., 2013).

Supercritical CO_2 has turned out to be unfit to solubilize PHA but it is an efficient solvent for lipids and other hydrophobic components of the bacterial cells. sCO_2 can be used to disrupt the cells and to remove lipid impurities before a solvent extraction (Hampson and Ashby, 1999).

With this method Hejazi et al., 2003 obtained a maximum PHB recovery yield of 89%. They highlight the environmental advantages of the process since, in comparison with other recovery methods, less solvent is consumed.

4.3.2. Floatation

Floatation is an efficient separation method widely used in wastewater treatment. In PHA extraction by flotation follow a previous cell disruption or solvent extraction step and can be used as an alternative to filtration or centrifugation.

van Hee et al., (2006) applied an enzymatic cell lysis, using lysozyme and Novozymes, to release mcl-PHA granules from cells of P. putida, followed by a dissolved air flotation (DAF). After the enzymatic cell disruption, floatation separates PHA granules from NPCM debris according to their different affinities to air/liquid interfaces. While hydrophobic PHA granules are transported to the liquid surfaces by the air bubbles, hydrophilic parts of the cell debris remain in the aqueous phase. A polymer with a purity of 86% was obtained in three consecutive batch flotation steps.

Ibrahim and Steinbüchel, 2009 extracted P(3HB) from cells of *Zobellella denitrificans* by a simple extraction step with chloroform (at 30°C for 72h) followed by self-flotation of cell debris. With this extraction method an excellent P(3HB) purity of 98% with a recovery yield of 85% was achieved.

Flotation methods could be cost effective as additional steps such as centrifugation, and polymer loss during extraction can be avoided. The main limitation of (DAF) is that it can requires several consecutive batch flotation steps (Kunasundari and Sudesh, 2011).

4.3.3. Cell fragility

The accumulation of large amount of PHA in bacterial cells can compromise the cell wall strength. In some bacteria, such as recombinant *E. Coli* and *Azotobacter vinelandii*, cell fragility can also be induced by changing the composition of the growth medium as observed by Lee et al., (1994).

This mechanism was successfully applied to *Bacillus flexus* cells by Divyashree and Shamala, (2010). When the cells were grown in a medium containing inorganic salts, absence of diaminopimelic acid and decreased concentrations of other amino acids were observed compared to cells cultivated on organic nutrients such as yeasts extract or peptone. Appling hot chloroform or a mild alkaline hydrolysis a high PHA recovery of respectively 86 and 100% was obtained for the cells grown in inorganic medium compared to those grown in yeast extract or peptone (32–56%).

Taking advantage of cell fragility simple PHA extraction methods with mild extraction conditions could be successfully used.

4.4. Limits in the application of extraction methods

When selecting an appropriate PHA extraction method a case-based evaluation is always necessary. Indeed, the efficacy and efficiency of extraction methods depend on the microbial strain used (the greater differences being observed between pure and mixed cultures) and its PHA content. Table 3 gives a comparison of all the methods already discussed before.

When comparing solvent extraction with chemical NPCM digestion, higher purities of the extracted polymer are usually obtained with solvent-based methods when the biomass PHA content is moderate. However, if the cells PHA content is high (e.g. 80 % of cell mass) NPCM digestion may give a purity as high or higher than solvent extraction (Gumel et al., 2013). Another important aspect is the PHA degradation provoked by the various extraction methods. Usually solvent extractions cause negligible or only minor degradation compared to NPCM digestion methods but the processes operational conditions, such as temperature, pH and extraction time, have a pivotal role in polymer degradation. PHA macromolecules are likely to be degraded to smaller molecules when exposed to alkaline conditions. Chen and colleagues observed that using a surfactant-chelate method for PHB extraction from *Alcaligenes eutrophus*, the drop in molecular weight was increasing with increasing pH values and the polymer degradation was drastic as the pH exceeded 13. Also temperature was observed to have an important role in polymer degradation, while raising temperature accelerate NPCM disruption also the PHB degradation is speed up (Chen et al., 1999). A very important parameter to be optimized in PHA extraction processes is the contact time, since degradation is a time-dependent process it should be minimized. Samorì end colleagues observed that when performing a Soxhlet extraction of P(3HB-co-3HV) at 90°C, using dimethyl carbonate as solvent changing extraction time from 1 to 24h caused a three time decrease in polymer molecular weight (Samorì et al., 2015). In order to minimize the contact time, Manangan and Shawaphun (2010) suggested that an appropriate agitation could significantly accelerate the NPCM dissolution thus reducing the chance of PHA degradation (Manangan and Shawaphun, 2010).

Table 3. Comparison of PHA extraction methods

Mode of operation	Extraction Method	Applicability at large scale, environmental friendliness	Purity PHA	Preservation of the PHA molecular weight	PHA recovery	Energy requirements	Need of drying biomass before extraction	Tested on MMC?
PHA dissolution inside cells, then recovery.	Solvent	✓	✓✓✓	✓✓✓	✓✓	✓	Yes	Yes, up to lab scale
	Supercritical fluid extraction	✓	✓✓	✓✓	✓✓	✓	Yes	No
Digestion of non-polymer cell mass, then PHA is released	Sodium hypochlorite	✓✓	✓✓✓	✓	✓✓✓	✓✓✓	No	Yes, up to pilot scale
	Surfactants	✓✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	No	Yes, up to pilot scale
	Acids and alkalis	✓✓✓	✓✓✓	✓✓	✓✓✓	✓✓✓	Normally, no	Yes, up to pilot scale
	Enzymatic cell digestion	✓	✓✓✓	✓✓✓	✓✓	✓✓	No	No
	Mechanical cell disruption	✓✓	✓✓	✓✓	✓✓	✓	Yes	No
	Cell disruption +Floatation	✓✓	✓	✓✓	✓✓	✓✓✓	No	No
	Taking advantage of cells' fragility	✓	✓✓	✓✓✓	✓✓	✓✓✓	No	No

Operating with MMCs introduces a further issue in the extraction of polymer from the biomass: MMCs are claimed to be more resistant to cell hydrolysis than pure cultures, in which genetic manipulation and high content of PHA granules increase cellular fragility (Samorì et al., 2015). The reasons for the major difficulty of extracting PHA from MMC than from pure culture could also include factors such as: a strong and complex extracellular biomass matrix that contain the PHA accumulating cells; a stronger NPCM and the lower starting PHA levels that may result in a lower cell constrains that decrease cellular fragility (Patel et al., 2009).

Moreover, chlorinated compounds seem not to be as effective on it as they are on pure culture. In literature is reported that no more than 30% of the PHA produced by MMC could be recovered by chlorinated solvents extraction (Majone et al., 2017; Patel et al., 2009). On the other hand, Samorì and colleagues proposed a method based on a double extraction with the green solvent dimethyl carbonate (DMC) that achieved a better recovery (60%) and a purity of 95% (Samorì et al., 2015).

Chemical dissolution of NPCM is suggested to be a mean to make PHA extraction more competitive for the market also when using MMC, and in this case, it seems to be more performant than solvent extraction. As an example, Jiang and colleagues developed a low-cost process to extract PHB from MMC with a high purity (99%), and more than 90% of recovery. The process consisted of an alkaline pre-treatment (0.2 M NaOH) together with a surfactant (SDS 0.2 w/v%) digestion on fresh biomass samples (Jiang et al., 2015). However, also NPCM digestion is less effective on MMC than on pure culture: the same innovative switchable surfactant extraction used by Samorì, Basaglia, et al., 2015 on a pure culture of *Cupravidus necator* was applied by Mannina and co-workers on a mixed culture enriched trough ADF. The process turned out to be ineffective on MMC without a NaClO pre-treatment that was not necessary on pure culture (Mannina et al., 2019).

Even though the extraction of PHAs from MMCs is more challenging in comparison to single strain cultures, it is possible to achieve good results using green and safe methods avoiding the use of reagents that are harmful to the environment and to human's health such as chlorinated solvents.

5. PHA production at pilot scale using the mixed microbial culture platform

The feasibility of PHA production by MMCs and waste substrate is being demonstrated by several European projects at pilot scale.

Within the framework of the PHARIO project, a project of the Dutch Water Authorities and

industrial partner, a routine kilogram scale PHA production for over 10 months was demonstrated. A commercial quality grades P(3HB-co-3HV) copolymer was produced from surplus activated sludge biomass and fermented volatile fatty acid rich streams, from industry or primary sludge sources, at a competitive cost prize if compared to current PHA market prices. Additionally, life cycle analysis indicated that the PHARIO PHA has a 70% lower environmental impact compared pure culture PHA (Bengtsson et al., 2017; Werker et al., 2018). In response to the European Commission: a) Circular Economy Action Plan and b) European Plastics Strategy, several H2020 aiming at demonstrate PHA production at pilot scale are being funded. For example, RES-URBIS: REsources from URban BIo-waSte in its eighteen-month report states that two pilot plants were continuously operated to produce PHA, using a liquid waste from fruit processing and a liquid slurry after squeezing of the organic fraction of municipal solid waste and excess sludge from urban wastewater treatment. (Res Urbis Project, 2019).

Other H2020 projects are: i) the SMART Plant that aims to demonstrate the feasibility of recovering PHA and struvite from enhanced biological phosphorous removal using the Mainstream SCEPPHAR process (SMARTech2b) developed by GENOCOV Research Group (SMART-Plant, 2019), while ii) the INCOVER (Innovative eco-technologies for resource recovery from wastewater aims to use the microalgae-bacteria platform and wastewater also for recovering PHA and phosphorous.

Finally, the H2020 projects YPACK (2019) and VOLATILE (2019) aims at optimising food packaging applications of PHA and at developing a Volatile Fatty Acid Platform (VFAP) integrated into existing anaerobic digestion plants to be used for PHA production, respectively. In this sense, studies should address to increase cell densities and thus, derived productivities achieved with MMC. It is urgent also to develop mathematical models that can predict the PHA accumulation process. Further improvements are still needed to foster the scale up of the processes and to overall reduce the production costs.

6. Conclusions and perspectives

Today PHAs are not yet widely extended due to the lack of cost-effectiveness in the production process, despite their potentiality. The use of mixed microbial cultures is regarded as the key factor that should lead towards a more cost-effective PHA production process. However, there is an urgent need of feasibility studies, at a pilot-scale level, but most important at pre-commercial scale, in order to really establishing PHA biosynthesis and extraction from waste

as feedstock. These studies should cover all process stages, from feedstock fermentation to PHA extraction and characterisation, with the main challenge of decoupling of the final polymer quality and productivity from variability of waste streams.

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