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Bioplastic recovery from wastewater: A new protocol for polyhydroxyalkanoates (PHA) extraction from mixed microbial cultures

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ABSTRACT

A new protocol for polyhydroxyalkanoates (PHA) extraction from mixed microbial cultures (MMCs) is proposed. PHA-accumulating capacity of the MMC was selected in a sequencing batch reactor (SBR) fed with a synthetic effluent emulating a fermented oil mill wastewater (OMW). The highest recovery yield and purity (74 ± 8% and 100 ± 5%, respectively) was obtained when using NH_4-Laurate for which operating conditions of the extraction process such as temperature, concentration and contact time were optimized. Best conditions for PHA extraction from MMC turned to be: i) a pre-treatment with NaClO at 85 °C with 1 h of contact time, followed by ii) a treatment with lauric acid in a ratio acid lauric to biomass of 2:1 and 3 h of contact time.

1. Introduction

The ever-increasing presence of plastic in the environment is one of the most relevant environmental issues that nowadays humanity is facing. The mass of plastic waste entering the oceans from land each year is estimated between 4.8 and 12.7 million tons and, without waste management infrastructure improvements, 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 a decrease of its availability and the rising of oil cost, the production of plastic from petrochemical resources will also become limited in the following years. In this scenario replacing synthetic fossil-fuel polymers with bio-based bio-degradable plastics could have significant advantages, such as the drastic reduction of the plastic pollution in the environment and the decoupling of plastic production from fossil feedstock.

Bioplastics are bio-based polymers obtained from organic renewable resources that have a minor impact on the environment since most of them are completely biodegradable. Among them, the Polyhydroxyalkanoates (PHAs) are a very interesting class of bio-based biodegradable polymer because they can be produced, through bacterial fermentation, from a various range of complex organic substrates including the ones contained in waste streams, like agroindustry wastewater to sewage sludge. Therefore, in this context, resource recovery from wastewater treatment processes can have a role in the plastic's circular economy.

Polyhydroxyalkanoates are completely biodegradable and have similar properties to conventional fossil-fuel plastics, making them suitable candidates for fossil-fuel based plastics substitution. These environmentally friendly bio-based bioplastics have a great potential; the main applications range from disposable items and food packaging to chemical synthesis, agricultural, construction, pharmaceutical and biomedical uses (Reddy et al., 2003; Ivanov et al., 2014).

Currently, their production at large scale is still limited by its high production cost compared with conventional fossil-fuel based plastics. In fact, the current PHA price, depending on polymer composition, ranges from 2.2 to 5.0 €/kg that is at least three times higher than the major fossil-fuel based polymers which typically cost less than 1.0 €/kg (Gholami et al., 2016). Despite the high production costs, the convenience in the use of PHA should be evaluated taking into account the missed environmental costs related to their use in substitution of traditional plastics. Indeed the use of PHA avoid plastic pollution (they are biodegradable) and accomplishing the need for the environmentally responsible use of resources.

Among the most important factors in the overall production cost of PHA, the use of pure or genetically modified cultures, the cost of the raw materials used as precursors and the recovery methods employed

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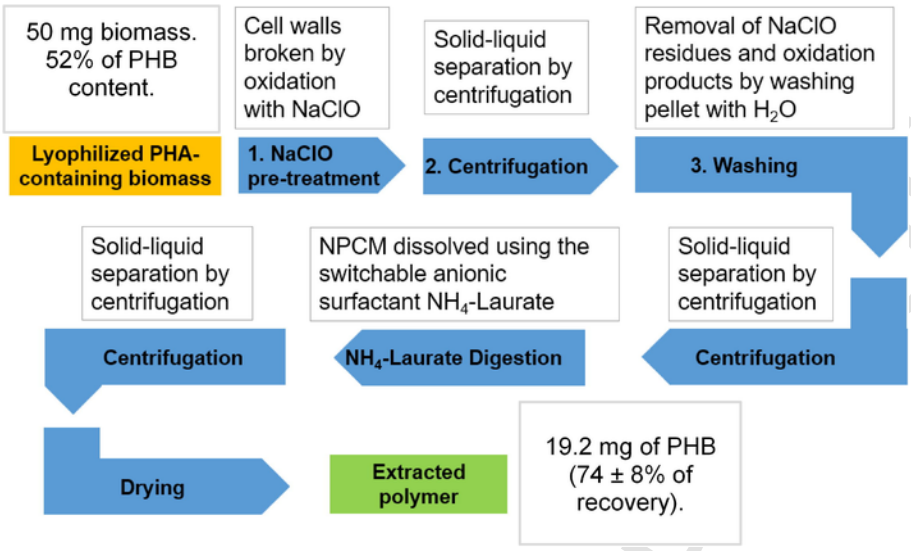

Fig. 1. Extraction protocol schematics taking as example the biomass from the experiment of PHB accumulation.

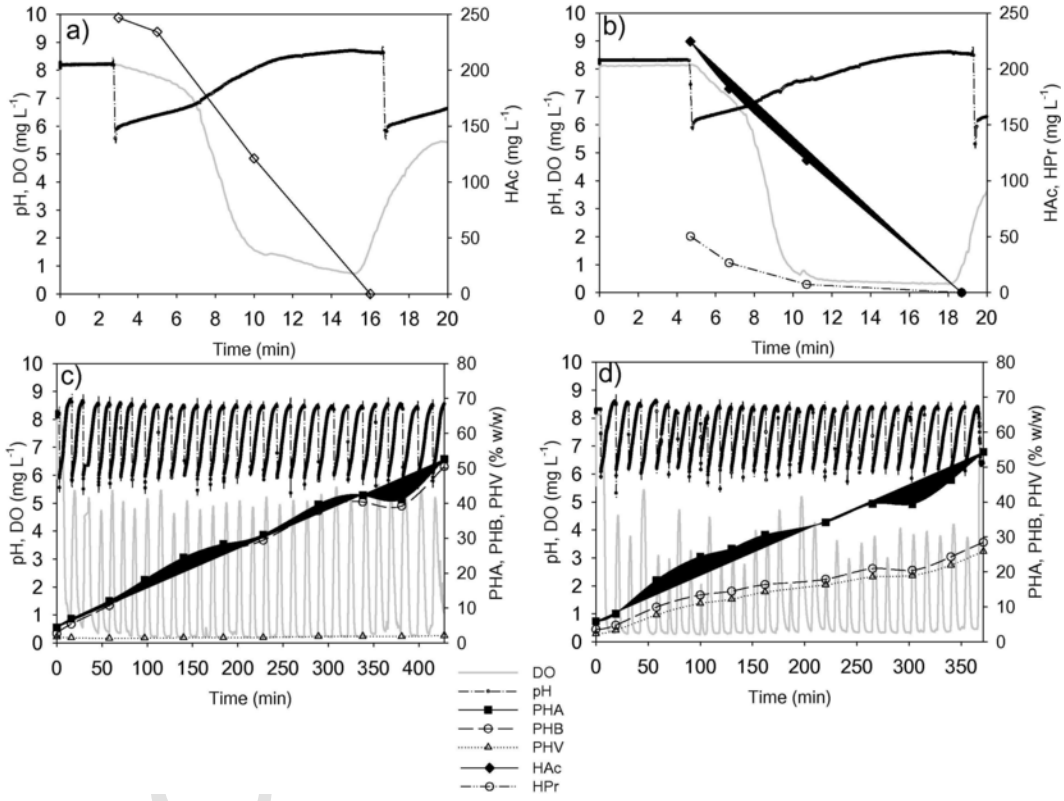

Fig. 2. Trends of pH, DO, acetic acid (HAc) and propionic acid (HPr) concentration and PHA cell content (% w/w) throughout a typical feed event during the accumulation experiments E2 (a), E1 (b) and during the entire accumulation experiments E2 (c) and E1 (d).

are the most important ones (Choi and Lee, 1997; Salehizadeh and Van Loosdrecht, 2004). Industrial processes for PHA production are based on the use of pure or genetically modified cultures of selected strains and of *ad hoc* designed unbalanced growth media (Chen, 2010). Hence, the costs of culture maintenance, substrate formulation and both, substrate and reactor sterilization (Villano et al., 2014; Ivanov et al., 2014) turn to be key factors affecting overall PHA production cost. As alternative, the use of mixed microbial cultures (MMCs) is promising because it will help to reduce the production costs of PHAs, since MMCs do not require sterile conditions and have a wider metabolic potential than single strains. This allow them to utilize a large number of cheaper substrates such as wastes (Carvalho et al., 2014), that avoids the use of expensive carbon sources.

Besides, downstream PHA recovery technologies significantly affects the overall process economics and its environmental sustainability since traditional extraction methods mostly employ halogenated solvents. The challenge in the recovery process is to achieve, in a cheap and environmentally friendly manner, a high recovery efficiency and a high degree of purity of the extracted polymer but, maintaining at the same time its molecular weight and thermal and mechanical properties (Kunasundari and Sudesh, 2011).

Table 1
Operational characteristics and results of accumulation experiments.

Accumulation Experiment	COD supplied	SSV_0	F/M*	PHA content	Storage yields	
	gCOD L^{-1}	gVSS L^{-1}	gCOD g^{-1} VSS	% w/w	$gCOD_{PHA}$ g^{-1} $COD_{substrate}$	$Cmol_{PHA}$ $Cmol^{-1}$ $_{substrate}$
E1	6.5	3.7	1.8	54	0.8	0.8
E3	6.1	6.1	1.0	35	0.8	0.7
E4	6.1	4.0	1.6	43	0.8	0.7

* The F/M ratios were calculated as the total COD provided during the accumulation (expressed as mg L^{-1}) divided by the VSS concentration in the reactor at the start of the accumulation phase (expressed as mg L^{-1}).

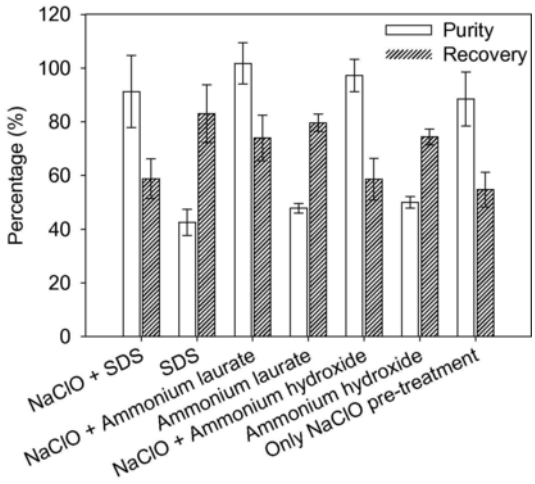

Fig. 3. PHA purity and recovery degree obtained after 3 h of treatment of lyophilized biomass (from accumulation experiment E2) with NH4-laurate (200 wt%), SDS (200 wt%) and NH4OH (0.1 N), with and without 1 h NaClO pre-treatment.

However, operating with MMCs introduces a further issue in the extraction of polymer: 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 behind this could be related to i) a strong and complex extracellular biomass matrix that contain the PHA accumulating cells, ii) a stronger non-polymeric cell material (NPCM) and iii) the lower starting PHA levels that may result in a lower cell constrains that decrease cellular fragility (Patel et al., 2009). Furthermore, chlorinated compounds used as extractants seem not to be as effective on it as they are on pure cultures (Patel et al., 2009; Samorì et al., 2015; Majone et al., 2017; Montiel-Jarillo et al. 2019b). In this sense, the use of surfactants to dissolve the non-PHA cell material is an effective way to extract the polymer with a good purity while maintaining its molecular weight (Jiang et al., 2015). The problems of this methods are the high surfactant to biomass ratio needed and the difficulty of recovering the surfactant, that then generate a large quantity of wastewater if not implemented (Jacquel et al., 2008). For these reasons, the use of surfactant in PHA extraction is still an expensive and not eco-friendly method. In this sense, Switchable Anionic Surfactant (SAS) would emerge as a smart and economically convenient way to easily avoid the unnecessary consumption (and consequent loss) of surfactant when high doses are required for specific processes. In fact, SAS are effectively and simply recoverable by using CO_2 as pH-trigger (Samorì et al., 2015), i.e. they can be directly and reversibly converted to the least soluble form in the reaction medium, so that they can be removed from the liquid phase and recovered for being reused afterward (Liu et al., 2006).

Therefore, a new advanced protocol for PHA recovery from MMCs has been set up in this work, in order to advance towards an even greater environmental and economical sustainability of PHA production. The protocol is based on the destruction of NPCM in aqueous phase using ammonium laurate, a well-known SAS. To the best of our knowledge, this is the first time that SAS has been used with the aim of PHA recovery from MMC biomass. In this sense, the effect of several parameters, such as contact time, extractants concentration, temperature and NaClO pre-treatment efficacy on the PHA recovery from MMC was investigated.

2. Materials and methods

The whole process for PHA production consisted on a first phase in which the biomass is selected in a sequencing batch reactor (SBR) through aerobic dynamic feeding, then the PHA content of the selected biomass is maximized in a feed batch accumulation reactor. The obtained PHA containing biomass is then lyophilised and directed towards the extraction phase where the PHA is recovered applying the advanced extraction protocol described below.

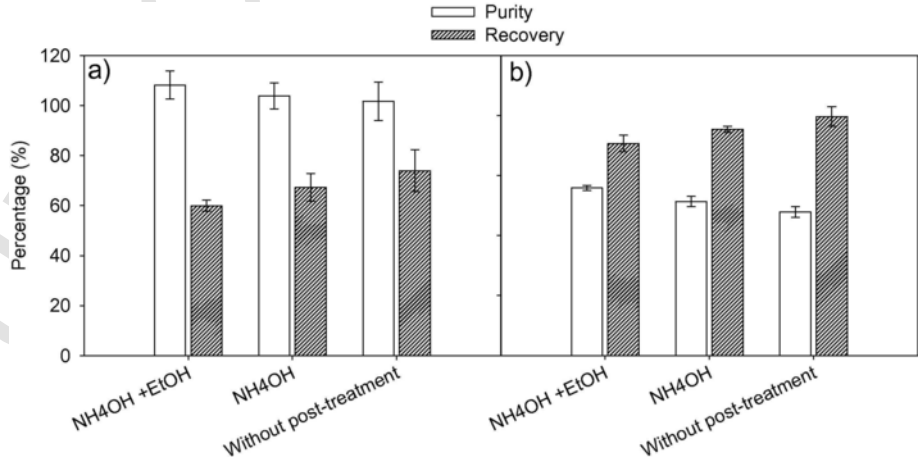

Fig. 4. PHA purity and recovery degree obtained with different post-treatments on the polymer extracted with NH_4-Laurate with (a) and without (b) a previous NaClO pre-treatment.

Table 2
Basic operational conditions used in extraction experiments.

Contact time (h)	Temperature (°C)	Chemicals to biomass ratio
NaClO Pre-treatment	1	85 ± 3	5 mL : 50 mg
NH_4-Laurate digestion	3	75 ± 3	2:1 w/w

2.1. Selection step

For the selection of PHA-storing MMC, a lab-scale SBR was used. It consisted of a 16 L stainless steel tank, stirred by a mechanical mixer and aerated by means of an air diffuser at the bottom of the vessel.

The reactor was run under feast-famine conditions and fed with a synthetic effluent emulating a fermented oil mill wastewater (OMW). The operating scheme was established in cycles of 24 h divided into: an initial idle phase (15 min) at the end of which mineral medium (8 L) was fed; an aerobic reaction phase (1358 min) in which 3 pulses of carbon source (a synthetic mixture of organic acids emulating a fermented OMW) were fed at equal intervals of time, giving an overall organic load of 1.2 g L^{-1} d^{-1} in terms of chemical oxygen demand (COD); a settling phase (60 min); and finally, a discharge phase (7 min) in which 8 L of supernatant were discharged.

The SBR was automatically controlled by a software (LabView, NI), which allowed to manage each phase of the reactor cycle; temperature and pH were let free to vary. The sludge retention time (SRT) was maintained at 15 days and the hydraulic retention time (HRT) was maintained at 2 days.

The carbon source (synthetic effluent emulating a fermented OMW) contained (in COD basis): acetic acid (75.0%), propionic acid (15.0%), benzoic acid (3.3%), cinnamic acid (3.3%) and hydroxyphenylacetic acid (3.3%).

The mineral medium composition was as follows (in mg L^{-1}): KH_2PO_4 (54.4), K_2HPO_4 (43.6), NH_4Cl (129.4), $MgSO_4$ (43.9), $MgCl_2{\cdot}6H_2O$ (160), $CaCl_2{\cdot}2H_2O$ (42), $NaHCO_3$ (30), allylthiourea (ATU, 50) to prevent nitrification and 0.15 mL L^{-1} of a trace elements solution consisting in (in μg L^{-1}): $FeCl_3{\cdot}6H_2O$ (1500), H_3BO_3 (150), $CuSO_4{\cdot}5H_2O$ (30), KI (180), $MnCl_2{\cdot}4H_2O$ (120), $Na_2MoO_4{\cdot}4H_2O$ (60), $ZnSO_4{\cdot}7H_2O$ (120), $CoCl_2{\cdot}6H_2O$ (150) and 68.5 mL L^{-1} EDTA 0.5 M

The reactor performance was monitored through weekly sampling and measurements of total suspended solids (TSS), volatile suspended solids (VSS), COD in the influent and effluent, sludge volumetric index (SVI) and PHA contents.

The reactor was already set up and working from 350 days and it was inoculated with activated sludge from a municipal wastewater treatment plant (Granollers, Catalonia, Spain) (Montiel-Jarillo et al., 2017; Montiel-Jarillo et al., 2019a).

2.2. Accumulation step

The accumulation experiments were carried out in fed-batch mode with pulsed substrate feeding. A 2 L glass reactor was used. It was magnetically stirred and aerated during the whole experiment and it was provided with pH and dissolved oxygen (DO) sensors. Temperature and pH were let free to vary.

The biomass (2 L of mixed liquor) was collected from the selection reactor, washed twice with tap water, left settle down to being able to discard 1 L of supernatant and then, it was mixed with 1 L of mineral medium with the same composition of the one used in the selection step, but without NH_4Cl (the accumulation was promoted under nitrogen limitation). Before the accumulation experiments, the biomass was left stirred and aerated overnight without any substrate supply.

Two different kind of substrate were used. The substrate 1 was a mixture of acetic acid (75% on COD basis) and propionic acid (25% on COD basis) that lead to the accumulation of the copolymer P(3HB-co-3HV); the substrate 2 was only acetic acid and lead to the accumulation of P(3HB).

The accumulation started by the addition of a substrate pulse of 7 mL (60 gCOD L^{-1}). The next pulse was added when the level of DO started to rise after the drop due to the substrate consumption by the biomass. With this operational modality, a total of 29 pulses were added in each accumulation experiment.

During the experiment sludge samples (ca. 10 mL) were collected each 4 pulses in order to follow the PHA accumulation in the biomass; samples were mixed with 0.4 mL of formaldehyde (37 wt% in H_2O) to inhibit biological activity.

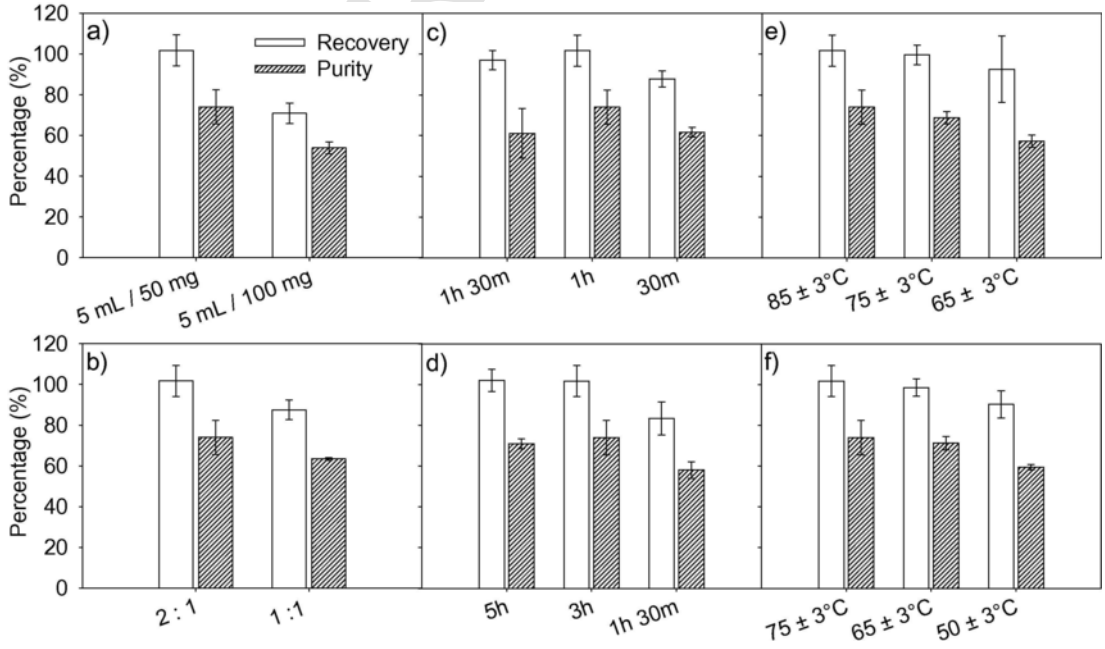

Fig. 5. PHA purity and recovery obtained with different NaClO to biomass ratio (a), lauric acid to biomass weight ratio (b), pre-treatment durations (c), NH_4-laurate digestion durations (d), pre-treatment temperatures (e), NH_4-laurate digestion temperatures (f).

Table 3
Molecular weights for the recovered PHB and P(HB-co-HV).

Biopolymer	M_w ($\times 10^5$ Da)	M_n ($\times 10^4$ Da)	PDI
PHB	0.918	4.18	2.2
P(HB-co-HV)	1.438	4.44	3.2

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

Table 4
Thermal characterisation parameters calculated for the recovered polymers.

PHA	$T_{d\text{-}5\%}$ (°C)	$T_{d\text{-}max}$ (°C)
PHB	185.0	267
P(HB-HV)	205.0	220

$T_{5\%}$ is the degradation temperature corresponding to 5% weight loss in the sample; $T_{d\text{-}max}$ is the range of the maximum degradation rate.

At the end of experiment, 40 mL of formaldehyde (37 wt% in H_2O) was added to the remaining liquor and biomass was separated by centrifugation (40 min, 8500 rpm). The obtained pellet was stored at −80 °C overnight and then lyophilized for 24 h.

2.3. Extraction protocol

In Fig. 1, a schematic representation of the developed protocol is showed. Both, pre-treatment and digestion steps were carried out in 15 mL glass tubes stirred manually every 5 min, the temperature was controlled by using a block heater. The liquid-solid separation was obtained by centrifugation previously transferring the suspension to 15 mL centrifuge tubes. Finally, the obtained polymer was dried at 60 °C overnight. All the experiments were run in triplicate.

In order to enhance the protocol, and to find the optimal condition for the extraction process, key parameters have been tested. Different chemicals were used to destruct the NPCM under variable operational conditions such as contact time, chemicals to biomass ratio and temperature. The efficacy of NH_4-laurate was compared to the performance of sodium dodecyl sulphate (a typical anionic surfactant), and NH_4OH that is the base used to dissolve the lauric acid and obtain the switchable surfactant ammonium laurate.

2.3.1 Sodium hypochlorite pre-treatment

In order to break the cell membrane and make the extraction of the polymer granules easier, a hypochlorite pre-treatment was used. 50 to 100 mg of lyophilized biomass was weighed and then mixed with 5 mL of NaClO (4.7% Cl_2). It was then digested at 85 °C for 1 h. Subsequently, the suspension was centrifuged at 5500 rpm for 10 min. The obtained pellet was washed twice with milliQ grade water (5 mL) and used in the subsequent NPCMs destruction step.

The influence of temperature (85, 75 and 65 °C), contact time (90, 60 and 30 min) and ratio NaClO to biomass (5 mL:50 mg and 5 mL:100 mg) on the process where studied.

2.3.2 Destruction of cellular matrix with surfactants

The dissolution of the NPCM with SDS and NH_4OH were performed with and without pre-treatment by adding of NH_4OH (0.37 mmol, 0.37 mL 1 M NH_3 in H_2O) or SDS (100 mg) in 3 mL of water. Biomass (50 mg) was added to these solutions, the mixtures were stirred at 75 °C for 3 h and then, centrifuged at 5500 rpm for 10 min.

Ammonium laurate was prepared by adding NH_4OH (0.75 mmol, 0.75 mL 1 M NH_3 in H_2O) to a suspension of lauric acid (100 mg, 0.5 mmol) in 3 mL of water. At pH ca. 10 the carboxylic acid switched from the neutral form (insoluble in water) to the ionic form (soluble in water).

The dissolution of NPCM with NH_4-laurate was performed on pre-treated and not pre-treated biomass and different operational conditions were tested. Single effects of three different variables: temperature (75, 65 and 50 °C), contact time (5, 3 and 1.5 h) and lauric acid to biomass ratio (w/w) (2:1 and 1:1) on PHA recovery process were investigated.

Biomass (pre-treated and not) was added to the NH_4-Laurate solution and digested at a fixed temperature for the chosen amount of time. Finally, the suspension was centrifuged at 5500 rpm for 10 min. The extracted polymer, recovered on the bottom of the centrifuge tube, was used in the subsequent post-treatment step or dried at 60 °C overnight.

2.3.3 Purification post-treatment

The polymer, released due to the dissolution of the non-polymeric cellular matrix, was purified by washing it with two different solvents: an NH_4OH solution and ethanol.

Two different kind of post-treatment were used. The post-treatment 1 consisted in washing the recovered polymer once with an NH_4OH solution 0.1 M (1 mL) ant twice with ethanol (1 mL); in the post treatment 2 the polymer was only washed once with an NH_4OH solution.

Actually, the polymer was washed adding the washing agent to the pellet obtained after centrifugation in the precedent step, the tube was vortex mixed until the pellet completely disintegrate. The purified polymer was then, recovered by centrifugation and dried at 60 °C overnight.

2.4. Quantification of PHA amount in microbial cells and extracted biopolymer

For PHA analysis, sludge samples (ca. 10 mL) were mixed with 0.4 mL of formaldehyde to inhibit biological activity. Subsequently, the protocol described for Werker et al. (2008) was followed. Briefly, samples were centrifuged and the obtained pellet was lyophilized. Lyophilized samples were weighted and benzoic acid was added as internal standard. Butanol (1.5 mL) and chlorhydric acid (0.5 mL) were added to each tube and incubated at 100 °C for 8 h. After, 2.5 mL of hexane and 4 mL of MilliQ grade water were added. The tubes were vortex mixed and let stand for 15 min to achieve phase separation. The organic phase was transferred to 15 mL clean falcon tubes and a second 4 mL aliquot of MilliQ grade water was added and the tubes were vortex mixed and centrifuged at 2500 × *g* for 10 min. The organic phase was extracted, filtered through 0.22 μm filters and transferred into gas chromatography (GC) vials. Then, it was analysed following the GC protocol stated by Montiel-Jarillo et al. (2017).

2.5. Biopolymer characterization

The structure of the extracted polymer samples was analysed by nuclear magnetic resonance (NMR). The thermal properties were determined by thermal gravimetric analysis (TGA). The average molecular weight was determined using gel-permeation chromatography (GPC).

The biopolymers chemical structure was determined by quantitative ^1H and ^{13}C NMR spectra using a BRUKER DRX-500 spectrometer. 22.2 mg of sample were dissolved in 0.6 mL of deuterated chloroform ($CDCl_3$). To guarantee the entire sample dissolution, biomass was heated and sonicated for 30 min. The Bruker TopSpin3.5pl7 software was used to process the NMR spectra. The Co-monomer composition distribution (CCD) of the copolymer P(HB-co-HV) was determined by ^{13}C NMR spectra by estimating the degree of randomness on the dyad level (D) and on the triad level (*R*) as proposed by Kamiya et al., 1989 and Laycock et al., 2014.

Thermal gravimetric analysis analyses were performed to assess the degradation temperature (T_d) following the protocol described by Arcos-Hernández et al. (2013).

Finally, the average molecular weights (M_w), number of average molecular weight (M_n) and polydispersity index (PDI) of the extracted biopolymers, were estimated by GPC on a Waters equipment provided with RI and UV detectors. The polymer samples were diluted (0.1% w/

v) and filtered. 100 μL of the sample solution was injected and chromatographed. HHR5E and HR2 Waters linear Styragel columns (7.8 mm × 300 mm, pore size 103–104 Å) packed with crosslinked polystyrene and protected with a pre-column were used. The biopolymers molecular weight was calibrated using as reference Poly (methyl methacrylate) (PMMA).

2.6. Calculations

PHA recovery is defined as percentage with respect to the amount of polymer present in the biomass before the extraction. It was calculated as follows:

$$PHArecovery = \frac{PHAyield * PHApurity}{PHAamountinmicrobialcells} * 100 \quad (1)$$

where:

PHA yield is the fraction of dry mass recovered after extraction on microbial biomass weight basis:

$$PHAyield = \frac{ExtractedPolymerweight}{Biomassweight} \quad (2)$$

PHA purity is the fraction of PHA contained in the extracted polymer:

$$PHApurity = \frac{MassPHA}{MassextractedPolymer} \quad (3)$$

PHA amount in microbial cells is the fraction of PHA contained in the lyophilised biomass:

$$PHAamountinmicrobialcells = \frac{MassPHA}{Masslyophilisedbiomass} \quad (4)$$

Both PHA amount in microbial cells and purity were calculated based on GC results, PHA yield was calculated gravimetrically after drying the extracted polymer. PHA purity was confirmed by NMR spectra.

3. Results and discussion

3.1. Accumulation experiments

With the PHA-storing biomass selected in the SBR (Montiel-Jarillo et al., 2019a), four fed-batch accumulation experiments were performed with the purpose of maximizing the PHA content in the MMCs cells. The accumulation tests were always performed without any nitrogen present in the mineral medium in order to maximize PHA accumulation instead of cell growth.

Fig. 2 represents DO, pH, acetic (HAc) and propionic (HPr) acids profiles in the first minutes of two different accumulation experiment called respectively E1 and E2. In the experiment E1 (Fig. 2b) a mixture of acetic and propionic acid (respectively 75% and 25% on COD basis) was used, while in the experiment E2 (Fig. 2a) only acetic acid was used as substrate.

The accumulation started by the addition of a feed pulse of substrate (7 mL, 60gCOD L^{-1}) that suddenly lowered the pH in the reactor. The consumption process of organics acids is an aerobic process, which means that while the substrates are depleted DO is consumed. The pH values increase along the decrease in the concentration of acetic and propionic acids because of their utilization and decarboxylation (Chua et al., 2003). As the HAc and HPr analysis showed, the moment when the substrate was completely depleted correspond with the moment when the bacteria oxygen uptake rate decreases and consequently the DO concentration starts to increase.

After the first pulse, the following ones were added when the level of DO started to rise following the drop due to the substrate consumption by the biomass. This is the moment when the accumulation has reached the maximum and the consumption of PHA begin.

The DO and pH profiles of the complete fed-batch accumulation tests along with the PHA concentration, expressed as percentage of biomass dry weight, are represented in Fig. 2c (experiment E2) and in Fig. 2d (experiment E1).

The accumulations tests were carried out without oxygen limitation because during the accumulation phase in MMC was observed a higher PHA storage capacity under high DO concentration (Wang et al., 2017; Vargas et al., 2014).

When both HAc and HPr acids are used as precursors, the biomass accumulates a copolymer P(3HB-co-3HV) with a PHV content of ca. 47% in weight. Instead, when HAc is used as the sole carbon source the accumulated polymer mainly consist of polyhydroxybutyrate (PHB) and only a very low amount of polyhydroxyvalerate (PHV) (ca. 4% of polymer weight) is produced. At the end of the accumulation phase, it was reached a maximum PHA content on a dry cell-weight basis of 54% in experiment E1 and 52% in experiment E2, which is a considerably high amount, even though it is still far from the maximum value achieved using MMC reported in literature that is 89% (w/w) (Johnson et al., 2010). The PHA content in the biomass (expressed as % gPHA/gVSS) is a very important parameter for the downstream extraction processes, because the more PHA is accumulated inside bacteria the easier is the PHA recovery (Salehizadeh and Van Loosdrecht, 2004; Lee and Choi, 1998) but, this is not the only way to express the biomass PHA accumulation capacity. PHA storage yield, defined as the amount of PHA produced for unit of substrate provided (both values expressed as C-mol or gCOD) express the biomass efficiency in transforming the substrate in PHA. The PHA storage yields for the experiments E1 and E2 were respectively 0.76 and 0.73 C-mol PHA/C-mol substrate. This storage yields where higher than the 0.6 C-mol PHA/C-mol substrate obtained by Johnson et al. (2009) while reaching the 89% of PHA content (Johnson et al., 2009), therefore the biomass selected in this study is more efficient than the one obtained by Johnson et al. (2009) in accumulating PHA. Probably, the PHA accumulation percentages obtained in this study are lower that the ones of Johnson et al. (2009) due to several facts: i) the length of our experiments were 1 to 2 h shorter, ii) the SRT in the selection step applied in this study was of 2.1 d instead of 1 d applied by Johnson et al. 2009 or iii) Different food to microorganism ratio (F/M) applied.

The percentage of PHA accumulated is influenced by the food to microorganism ratio (F/M) that is the amount of substrate provided divided by the mass of microorganisms in the reactor. In order to elucidate the F/M influence, additional accumulation experiments (E3 and E4) were carried out with different F/M ratio using a mixture of HAc and HPr as substrate, the results are reported in Table 1. As can be seen (Table 1), the biomass reached lower PHA content when lower F/M ratio were applied, despite the PHA storage yield was not significantly affected and remained almost constant.

3.2. Extraction experiments

The biomass used for the extraction experiments is the obtained from accumulation tests E1 and E2 that reached the highest percentage of PHA content. The efficacy of NH_4-laurate was compared to the performance of sodium dodecyl sulphate (SDS) and ammonium hydroxide, and the operational condition were optimized before carrying out two larger scale extractions.

3.2.1. Destruction of NPCM: Chemicals efficacy

The efficacy of ammonium laurate, sodium dodecyl sulphate and ammonium hydroxide, with and without a sodium hypochlorite (NaClO) digestion as pre-treatment was investigated.

Sodium hypochlorite, due to its strong oxidizing properties, is able to digest NPCM by breaking cells membranes, easing PHA recovery before the surfactant digestion. The limit of the hypochlorite treatment is

its non-selectivity, i.e. while it digests NPCM it also could degrades the PHA lowering its molecular weight (Jacquel et al., 2008; Berger et al., 1989).

In all the experiments carried out to investigate the efficacy of the different extraction agents the same operational conditions have been used. The pre-treatment was performed at 85 °C for 1 h using 5 mL of NaClO solution on 50 mg of lyophilized PHB-containing biomass (from accumulation experiment E2) unless otherwise stated. The subsequent extractions were performed at 75 °C for 3 h (after optimization).

Fig. 3 shows the results of different extraction experiment. Values of purity higher than 100% are to be attributed to analytical errors in the GC analysis, the error is reported as standard deviation of triplicate samples.

It is clear that both surfactants (SDS and NH_4-Laurate) and ammonium hydroxide are not effective when used without a previous NaClO treatment. Indeed, the purity of obtained polymer (expressed as weight percentage of PHA on the polymer mass) is the same than in the raw lyophilized biomass. When the biomass is pre-treated, the biopolymer recovery decreases while the purity considerably increases, reaching values around 90–100% for all the extraction agents tested. The extraction agent that exhibited the best performance was the ammonium laurate that lead to a polymer purity of 100% and a recovery of 74 ± 8%.

In order to understand how the surfactant digestion is effective after the pre-treatment, an extraction experiment that consist of only the NaClO pre-treatment has been done. The results show that when NH_4-Laurate digestion is applied after the pre-treatment, a purer polymer is obtained and a greater recovery is reached.

These results confirm the greater difficulty of PHA extraction from MMCs than from pure cultures. Indeed both SAS (Samorì et al., 2015), common surfactants such as SDS and alkalis (Yang et al., 2011; Choi and Lee, 1999) were successfully used under similar conditions, but on pure cultures and without the need of any pre-treatment.

In spite of this, it is possible to develop an environmentally friendly PHA extraction protocol based on the use of surfactants that is effective on MMC, but a pre-treatment of the biomass is needed.

Once the effectiveness of all the tested extraction agents was proven, in order to optimise the operational conditions, ammonium laurate was chosen because it performed slightly better than the others, at least on P(3HB)-containing biomass, and above all because of its low cost and eco-friendly behaviour. Indeed, due to its nature of switchable anionic surfactant, it is possible to recover the NH_4-laurate after having used it in the PHA extraction. When the aqueous phase is separated from the extracted polymer, lowering the pH by simply bubbling CO_2 will cause lauric acid to precipitate, so it can be recovered (with yields around 98% (Samorì et al., 2015)) and reused again to prepare NH_4-laurate. Even the aqueous phase that remain after the precipitation of lauric acid can be recycled, for example as nitrogen source for growing of bacteria in the selection step, since it contains NH_4^+ ions.

3.2.2. Purification post-treatment efficacy

To increase the purity of the recovered polymer, two different purification post-treatments were tested on pre-treated and non-pre-treated biomass. The PHA was washed once with 1 mL of NH_4OH solution (0.1 N), and with 1 mL of ethanol (EtOH) twice or only 1 time with 1 mL of NH_4OH solution. These washing agents should solubilize impurities, and so ease their removal from the polymer washing it away. The results of the experiments are showed in Fig. 4.

Fig. 4a show the results of the post-treatments on the polymer extracted with NH_4-laurate after the NaClO pre-treatment. The extracted PHA has already a purity of 100% so a purification post-treatment is not needed and the only effect is to decrease the recovery degree. The same post-treatment was applied on the PHA extracted using the SDS, this reached a purity of 100% with only being washed once with NH_4OH solution. In both cases, the trend is the same: when the purification treatment is applied the more time the polymer is washed, the more the recovery degree decreases.

In Fig. 4b the results of the post-treatments on the polymer extracted respectively with NH_4-laurate without the NaClO pre-treatment are showed. In this case, the purification treatments are effective because they improve the polymer purity by up to 15% but, at the same time, they lowered the recovery up to the same percentage.

In view of the obtained results, it can be generally stated that a purification post-treatment is not convenient because when the polymer is extracted using the NaClO pre-treatment it already has a high degree of purity, comparable to that of commercial PHA, while when the polymer is extracted without applying a pre-treatment, the PHA does not a high grade of purity and the recovery of the biopolymer decrease.

3.2.3. Extraction optimization

In order to improve recovery, at the end of the extraction phase, the operational conditions such as lauric acid to biomass ratio (w/w), NaClO to biomass ratio (v/w), temperature, and contact time in both the pre-treatment and in the subsequent surfactant digestion were optimized. Moreover, if the amount of chemicals, the time and the temperature needed to dissolve the NPMC are smaller, both the extraction costs and the potential degradation of the polymer would decrease (Rawte and Mavinkurve, 2002; Jiang et al., 2015).

For the optimization experiments, the basic conditions reported in Table 2 were established and each parameter was changed individually while keeping the other constant. All the experiments discussed from now have been carried out on PHB-containing biomass obtained from the accumulation experiment E2. The purity of the extracted polymer was one of the key elements to optimize the protocol, indeed a high grade of purity is one of the most requested features of the PHA for its subsequent industrial use. Therefore, a lack of purity would have hampered a reuse of the material. The results of the optimization experiments are showed in Fig. 5.

In order to optimize the NaClO to biomass ratio, two experiments were carried out, one using 5 mL of NaClO solution on 50 mg of biomass and the other with a ratio of 5 mL to 100 mg of biomass. When the quantity of NaClO is halved, both, the purity and the recovery degree substantially decrease, respectively from 100% to 71% and from 74% to 54%. Another two experiments were run to optimize the surfactant to biomass ratio, where two different amounts of lauric acid were tested: 200 and 100 wt% of the bacterial biomass used. Also, in this case, as showed in Fig. 5b, reducing the amount of lauric acid used lead to a worst purity and recovery.

Regarding the optimal contact time for pre-treatment, with 1 h of NaClO treatment a purity of 100% and the highest level of recovery (74%) is obtained while halving the pre-treatment duration lead to a purity of 87% and a recovery of 61%. In the NH_4-laurate digestion (Fig. 5d) the purity of the polymers recovered after 3 and 5 h of NPCM dissolution was in both case very high (100%) but, a better recovery is obtained with only 3 h of digestion. If the digestion is stopped at one hour and half, both polymer purity and recovery are negatively affected.

With regard to the extraction temperature, as is showed in Fig. 5e and f respectively for pre-treatment and the NH_4-laurate digestion, there is a direct relation between temperature and the process efficiency: in both cases the lower the temperature used for the extraction, the lower the polymer purity and recovery are.

At the end of this series of experiments, it was found that the optimal conditions are those that were fixed as basic condition (Table 2) with the only possible exception of the pre-treatment temperature that when changing from 85 to 75 °C, considering the error, practically it does not affect the overall performances of the extraction process.

3.2.4. Larger scale extractions

With the optimized operational conditions, two larger scale extractions were carried out. One extraction was performed on 5 g of PHB-containing biomass obtained from the accumulation experiment E2,

while the other was performed on 5 g of P(3HB-co-3HV)-containing biomass obtained from the accumulation experiment E1.

The recovered P(3HB-co-3HV) had a lower purity (93%) and recovery rate (73%) compared to the PHB that reach a purity of 100% and a recovery of 77%. These results are comparable with the ones obtained at small scale (mg), suggesting that the extraction protocol developed in this work could be scaled up maintaining similar performances.

3.3. Biopolymers characterization

The composition of the homopolymer PHB and the copolymer P(3HB-co-3HV) can be confirmed by ^{1}H and ^{13}C NMR spectra (Sindhu et al., 2015). The spectra obtained for both biopolymers extracted at larger scale were compared with previous published results and were in agreement with previous literature (Arcos-Hernández et al., 2013; Gobi and Vadivelu, 2015; Liu et al., 2018; Montiel-Jarillo et al., 2019b).

The ^{1}H spectrums for PHB and P(3HB-co-3HV) has been assessed (the reader is kindly referred to the Supporting Information). For the PHB, the typical methyl, methylene and methyne group signals are clearly showed. The first doublet signal at 1.28 ppm corresponds to the methyl group. For the methylene group (B2) the doublet of quadruplet is observed at 2.48–2.60 ppm and finally a multiplet in 5.25 ppm for the methine group (B3) (Sindhu et al., 2015).

The ^{1}H spectrum of the copolymer P(HB-co-HV) and also the typical signals has been assessed, however some unidentified peaks appeared which may correspond to the presence of impurities in the extracted sample, nevertheless, the copolymer peaks can be easily distinguished. The first resonances correspond to the methyl groups of the PHV (V5) and PHB (B4) at 0.90 and 1.28, respectively. Then, at 1.28 ppm, the HB methyl group (B4) signal is observed. Resonance in 1.63 is assigned to the HV methylene protons (V4) and the multiplet observed in the range from 2.50 to 2.60 ppm, corresponds to the methylene protons of HV (V2) and HB methylene group (B2). The methine protons were detected by the typical signals observed at 5.16 (V3) and 5.25 (B3) ppm.

The resonance ^{13}C spectrums for PHB (a) and P(HB-co-HV) (b) has been assessed. The assignment of each carbon peak was done based on data found in the literature (Ivanova et al., 2009; Patel et al., 2009; Montiel-Jarillo et al., 2019b).

The PHB ^{13}C spectrum showed four intense peaks, which corresponds to the carbonyl (169.16 ppm), the methyl carbon in 19.77 ppm, the methylene at 40.78 and the methine in 67.61 ppm. No impurities-related peaks were observed in this spectra. On the other hand, the ^{13}C spectrum for the copolymer P(3HB-co-3HV) revealed that each carbon signal was assigned as previously described in literature (Patel et al., 2009). Unlike the PHB spectrum, and in agreement with the results observed from the ^{1}H spectrum, other peaks are observed in the copolymer ^{13}C spectrum that may be due to the presence of some impurities.

The carbon nuclei sensitivity of the carbonyl and methylene has been previously described and had been used to assign dyads and triads of HB and HV units. The dyad and triad sequence distributions are useful to estimate the degree of randomness of the copolymer (Laycock et al., 2014; Patel et al., 2009; Kamiya et al., 1989). Using the relative peak intensities of each dyad sequence in the carbonyl region and triads in the methylene region of the ^{13}C spectrum, the degree of randomness *D* on the dyad level as proposed by (Kamiya et al., 1989) and *R* on the triad level (Laycock et al., 2014) were calculated. For the copolymer P(3HB-co-3HV) synthesised and extracted in this study, the *D* calculated was of 5.67 and the *R* was of 0.98. The randomness of a copolymer is a key factor to sense their physical properties. A *D* value between 0.99 and 1.5, describes a random copolymer while a *D* value over 1, indicates a "blocky" copolymer. However, if *D* is too much higher than 1 (as in this study), the copolymer structure might be: a) a block copolymer; b) a mixture of random copolymers; or c) a mixture of HB and HV homopolymers (Laycock et al., 2014). In this case, the value of *R* can help to get a better idea of the copolymer structure as it is a more sensitive parameter to the broadness of chemical compositional distribution. A value of $R = 1$, corresponds to a "completely random distribution of HV and HB units" (Arcos-Hernández et al., 2013; Laycock et al., 2014).

Finally, in order to estimate the molar fraction of 3HB and 3HV in the copolymer, the intensity comparison of signals B3 with respect to V3 in the ^{1}H and ^{13}C spectrum were calculated. (Ivanova et al., 2009). The molar fractions were 60.3 %of HB and 39.7% of HV, being results similar to the ones obtained by GC.

3.3.1. Molecular weight

The average molecular weight (M_w), number average molecular weight (M_n) and polydispersity index (PDI) of both polymers were performed by GPC analysis and are shown in Table 3.

The MW obtained in the present study are within the range of the molecular weights reported in the literature (1.0×10^4 Da – 4.0×10^6 Da). However, 10^5 Da are the lowest molecular weights described for PHA biopolymers (Fiorese et al., 2009; Rosengart et al., 2015). Furthermost of the molecular weight, the PDI is of great importance since it is going to be an indicator of the homogeneity of a copolymer. The values reported herein, are in line with previous researches but in general, a value close to 1 is preferred because it reflects a higher polymer homogeneity (Arcos-Hernández et al., 2013; Fiorese et al., 2009).

3.3.2. Thermal properties

The thermal properties of the PHB and P(HB-co-HV) extracted were obtained using TGA analysis and are shown in Table 4. The decomposition temperature (T_d) may give a hint of the range of temperatures in which a polymer can be employed in the manufacture of goods. From the results obtained, it might be concluded that the co-polymer has a high thermal stability (up to 205 °C) with a 5% of weight loss, while the PHB is thermally stable only up to 185 °C. However, the values observed for the biopolymers synthesized in the present study are below the reported range of T_d found in the literature (between 235 and 270 °C) (Arcos-Hernández et al., 2013; López-Abelairas et al., 2015; Xie and Chen, 2008).

4. Conclusions

A PHA extraction method was experimentally assessed and the optimal pre-treatment time was of 1 h using a temperature of 75 °C to destroy the NPCMs, while the best extraction condition was established with a surfactant contact time of 3 h at a temperature of 75 °C. When using NaClO pre-treatment with a ratio of 5 mL NaClO to 50 mg biomass and a biomass to lauric acid mass ratio of 1:2 a very high purity is obtained. Further developments should focus on other types of pre-treatment, the cost analysis and real wastewaters.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.03.037.

References

Arcos-Hernández, M.V., Laycock, B., Donose, B.C., Pratt, S., Halley, P., Al-luaibi, S., Werker, A., Lant, P.A., 2013. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). Eur. Polym. J. 49, 904–913. https://doi.org/10.1016/j.eurpolymj.2012.10.025.

Berger, E., Ramsay, B.A., Ramsay, J.A., Chavarie, C., Braunegg, G., 1989. PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnol. Tech. 3 (4), 227–232.

Carvalho, G., Oehmen, A., Albuquerque, M.G.E., Reis, M.A.M., 2014. The relationship between mixed microbial culture composition and PHA production performance from fermented molasses. New Biotechnol. 31 (4), 257–263.

Chen, G.-Q., 2010. Industrial production of PHA. In: Chen, G.-.Q.G. (Ed.), Plastics from Bacteria: Natural Functions and Applications, Microbiology Monographs. Springer-Verlag, Berlin Heidelberg, pp. 122–131.

Choi, J. Il, Lee, S.Y., 1999. Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals. Biotechnol. Bioeng. 62 (5), 546–553.

Choi, J. Il, Lee, S.Y., 1997. Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess. Eng. 17 (6), 335–342.

Chua, A.S.M., Takabatake, H., Satoh, H., Mino, T., 2003. Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT), and acetate concentration in influent. Water Res. 37 (15), 3602–3611.

Fiorese, M.L., Freitas, F., Pais, J., Ramos, A.M., de Aragao, G.M.F., Reis, M.A.M., 2009. Recovery of polyhydroxybutyrate (PHB) from Cupriavidus necator biomass by solvent extraction with 1, 2-propylene carbonate. Eng. Life Sci. 9, 454–461. https://doi.org/10.1002/elsc.200900034.

Gholami, A., Mohkam, M., Rasoul-Amini, S., Ghasemi, Y., 2016. Industrial production of polyhydroxyalkanoates by bacteria: opportunities and challenges: Minerva Biotecnologica 28 (1), 59–74.

Gobi, K., Vadivelu, V.M., 2015. Bioresource Technology Polyhydroxyalkanoate recovery and effect of in situ extracellular polymeric substances removal from aerobic granules. Bioresour. Technol. 189, 169–176. https://doi.org/10.1016/j.biortech.2015.04.023.

Ivanov, V., Stabnikov, V., Ahmed, Z., Dobrenko, S., Saliuk, A., 2014. Production and applications of crude polyhydroxyalkanoate-containing bioplastic from the organic fraction of municipal solid waste. Int. J. Environ. Sci. Technol. 12 (2), 725–738.

Jacquel, N., Lo, C.W., Wei, Y.H., Wu, H.S., Wang, S.S., 2008. Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem. Eng. J. 39 (1), 15–27.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347, 768–771.

Jiang, Y., Mikova, G., Kleerebezem, R., van der Wielen, L.A.M., Cuellar, M.C., 2015. Feasibility study of an alkaline-based chemical treatment for the purification of polyhydroxybutyrate produced by a mixed enriched culture. AMB Express 5 (1).

Johnson, K., Jiang, Y., Kleerebezem, R., Muyzer, G., Van Loosdrecht, M.C.M., 2009. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromolecules 10 (4), 670–676.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of ammonium on the accumulation of polyhydroxybutyrate (PHB) in aerobic open mixed cultures. J. Biotechnol. 147 (2), 73–79.

Kamiya, N., Yamamoto, Y., Inoue, Y., Chujo, Y., Doi, Y., 1989. Microstructure of bacterially synthesized poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Macromolecules 22, 1676–1682. https://doi.org/10.1021/ma00194a030.

Kunasundari, B., Sudesh, K., 2011. Isolation and recovery of microbial polyhydroxyalkanoates. Express Polymer Lett. 5 (7), 620–634.

Ivanova, G., Serafim, L.S., Lemos, P.C., Ramos, A.M., Reis, M.A.M., Cabrita, E.J., 2009. Influence of feeding strategies of mixed microbial cultures on the chemical composition and microstructure of copolyesters P (3HB-co-3HV) analyzed by NMR and statistical analysis. Magn. Reson. Chem. 47, 497–504. https://doi.org/10.1002/mrc.2423.

Laycock, B., Halley, P., Pratt, S., Werker, A., Lant, P., 2014. The chemomechanical properties of microbial polyhydroxyalkanoates. Prog. Polym. Sci. 39, 397–442. https://doi.org/10.1016/j.progpolymsci.2013.06.008.

Lee, S.Y., Choi, J., 1998. Effect of fermentation performance on the economics of poly(3-hydroxybutyrate) production by Alcaligenes latus. Polym. Degrad. Stab. 59 (1–3), 387–393.

Liu, M.H., Chen, Y.J., Lee, C.Y., 2018. Characterization of medium-chain-length polyhydroxyalkanoate biosynthesis by Pseudomonas mosselii TO7 using crude glycerol. Biosci. Biotechnol. Biochem. 82, 532–539. https://doi.org/10.1080/09168451.2017.1422386.

Liu, Y., Jessop, P.G., Cunningham, M., Eckert, C.A., Liotta, C.L., 2006. Switchable surfactants: Science 313 (5789), 958–960.

Majone, M., Chronopoulou, L., Lorini, L., Martinelli, A., Palocci, C., Rossetti, S., Valentino, F., Villano, M., 2017. PHA copolymers from microbial mixed cultures: synthesis, extraction and related properties (M. Koller, Ed.): Nova Science Publishers.

Montiel-Jarillo, G., Carrera, J., Suárez-Ojeda, M.E., 2017. Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: effect of pH and N and P concentrations. Sci. Total Environ. 583, 300–307.

Montiel-Jarillo, G., Carrera, J., and Suárez-Ojeda, M.E., 2019a. Feasibility of using a simulated pre-fermented olive mil wastewater as precursor for polyhydroxyalkanoates production. In preparation.

Montiel-Jarillo, G., Morales-Urrea, D., Contreras, E., Carrera J., Suárez-Ojeda, M.E., 2019b. Extraction and characterisation of polyhydroxyalkanoates biologically synthesised using mixed microbial cultures and a simulated pre-fermented olive mil wastewater. In preparation.

Patel, M., Gapes, D.J., Newman, R.H., Dare, P.H., 2009. Physico-chemical properties of polyhydroxyalkanoate produced by mixed-culture nitrogen-fixing bacteria. Appl. Microbiol. Biotechnol. 82 (3), 545–555.

Rawte, T., Mavinkurve, S., 2002. A rapid hypochlorite method for extraction of polyhydroxy alkanoates from bacterial cells. Indian J. Exp. Biol. 40 (8), 924–929.

Reddy, C.S.K., Ghai, R., Kalia, V.C., 2003. Polyhydroxyalkanoates : an overview. Bioresour. Technol. 87, 137–146.

Rosengart, A., Cesário, M.T., Almeida, M.C.M.D. De, Raposo, R.S., Espert, A., Díaz, E., Apodaca, D., Manuela, M.R., 2015. Efficient P (3HB) extraction from Burkholderia sacchari cells using non-chlorinated solvents. Biochem. Eng. J. 103, 39–46. https://doi.org/10.1016/j.bej.2015.06.013.

Salehizadeh, H., Van Loosdrecht, M.C.M., 2004. Production of polyhydroxyalkanoates by mixed culture: Recent trends and biotechnological importance. Biotechnol. Adv. 22 (3), 261–279.

Samorì, C., Basaglia, M., Casella, S., Favaro, L., Galletti, P., Giorgini, L., Marchi, D., Mazzocchetti, L., Torri, C., Tagliavini, E., 2015. Dimethyl carbonate and switchable anionic surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass. Green Chem. 17 (2), 1047–1056.

Sindhu, R., Binod, P., Pandey, A., 2015. Industrial Biorefineries and White Biotechnology. Elsevier B.V, pp. 575–605. doi:10.1016/B978-0-444-63453-5.00019-7..

Vargas, A., Montaño, L., Amaya, R., 2014. Enhanced polyhydroxyalkanoate production from organic wastes via process control. Bioresour. Technol. 156, 248–255.

Villano, M., Valentino, F., Barbetta, A., Martino, L., Scandola, M., Majone, M., 2014. Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process. New Biotechnol. 31 (4), 289–296.

Wang, X., Oehmen, A., Freitas, E.B., Carvalho, G., Reis, M.A.M., 2017. The link of feast-phase dissolved oxygen (DO) with substrate competition and microbial selection in PHA production. Water Res. 112, 269–278.

Werker, A., Lind, P., Bengtsson, S., Nordström, F., 2008. Chlorinated-solvent-free gas chromatographic analysis of biomass containing polyhydroxyalkanoates. Water Res. 42 (10–11), 2517–2526.

Yang, Y.H., Brigham, C., Willis, L., Rha, C., Sinskey, A., 2011. Improved detergent-based recovery of polyhydroxyalkanoates (PHAs). Biotechnol. Lett. 33 (5), 937–942.