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## ARTICLE

# Dimethyl carbonate and Switchable Anionic Surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass

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The availability of green and cheap technologies to recover polyhydroxyalkanoates (PHAs) from microbial biomass is crucial for the development of a reliable and sustainable production chain. Here, two novel protocols are proposed to extract PHAs from *Cupravidus necator*. The first method is based on PHA-extraction with dimethyl carbonate (DMC), a green solvent that is completely biodegradable and less harmful to humans and the environment than most solvents. The procedure can be applied directly to concentrated microbial slurries or to dry biomass, affording very high polymer recovery (>85%) and excellent purity (>95%). No degradation/decomposition of the polymer is observed in both cases. The second protocol uses fatty acid carboxylates as surfactants, which disrupt cell membranes, providing excellent polymer recovery (>99%) and high purity (>90%). Ammonium laurate can be successfully used and easily recycled (98%) by lowering the pH through CO<sub>2</sub> addition. Therefore, both protocols reported here are effective and sustainable: the recovery and purity of the obtained PHAs are very high, the use of toxic chemicals is avoided, and the recycling of various solvents/surfactants used in the processes is optimal.

## Introduction

The replacement of petroleum-based non-biodegradable plastics with alternative biopolymers that are biodegradable, compostable and often biocompatible, has become an industrial, social and environmental priority. The demand for more sustainable solutions to the “plastic issue” has been reflected in recent years in a growing interest towards biopolymers: market forecasts predict an increase in the global production from 1.4 Mt in 2012 to more than 6 Mt in the next five years.<sup>1</sup> Among the various bio-based plastics, polyhydroxyalkanoates (PHAs), which are linear polyesters produced by bacteria through aerobic conversion of various carbon sources, are very promising. PHAs are biosynthetic, biocompatible and biodegradable into harmless organic waste. Moreover, PHAs display thermoplastic properties that can be tuned according to their co-monomer composition. However, on the basis of the existing technologies and current high production costs, the share of PHAs on the bioplastic market is limited to around 2% of all available bioplastics.<sup>1</sup> In fact, PHAs are currently around 5 times more expensive than standard petrochemical plastics (~5-6 €/kg vs 1 €/kg)<sup>2</sup> and even more costly than the most widespread commercially available bioplastics (polylactic acid or starch-based polymers) already produced at large manufacturing scale. Thus, the price of PHAs is a strong limit to their potential application as commodity materials rather than niche-market high-value products.

Downstream processes may represent up to 50% of the total polymer production costs.<sup>3</sup> Specifically, the current downstream strategies include several steps (e.g. microbial biomass pre-

treatment, polymer extraction, and post-treatment purification) characterized by undeniable drawbacks: energy consumption, high costs, toxicity, extensive use of non-recyclable material/chemicals, and side reactions that can detrimentally affect polymer properties.

The polymer recovery phase can be accomplished in two main ways: i) PHA solubilization/recovery with organic solvents or ii) selective dissolution of Non-PHA Cell Mass (NPCM) with chemicals (e.g. surfactants or chelating agents) or enzymes.<sup>4</sup> However, there are concerns about both approaches:

- chloroform and dichloromethane are the best performing organic solvents, but they are problematic both for humans and the environment, rendering them as undesirable for industrial applications. Many “green” and more sustainable alternatives to chlorinated solvents have been investigated in the past years (e.g. propylene carbonate,<sup>5,6</sup> ethyl acetate,<sup>7</sup> methyl isobutyl ketone,<sup>7</sup> ionic liquids<sup>8</sup> and supercritical fluids<sup>9</sup>). However, in spite of giving excellent results in terms of purity and recovery yields, some of the proposed “green” approaches come with certain disadvantages. For example, cyclic carbonates display good PHA-solubility behavior, but the required high extraction temperature could be detrimental to the final polymer quality by diminishing its molecular weight. Moreover, some of the explored green alternatives, such as ionic liquid and supercritical fluids, are still too costly to represent a valid alternative.
- in the case of NPCM dissolution with surfactants, a wide range of compounds has been explored, including anionic (e.g. sodium dodecyl sulfate, SDS), cationic (e.g.

hexadecyltrimethylammonium bromide, CTAB) or non-ionic surfactants (e.g. polysorbate surfactant, Tween 20 and Triton X-100).<sup>10</sup> These surfactants can be directly applied to high cell density cultures (50-300 g L<sup>-1</sup>), by-passing the biomass dewatering step that can increase process costs. In addition they allow a polymer recovery without any degradation. However, high detergent doses are often required. This implies a great amount of wastewaters that has to be recovered, purified and recycled to meet wastewater treatment regulatory standards and to keep the process costs low. Moreover, this approach is affected by lower PHA purity; thus an additional purification step is often required.

The aim of the present research is to explore novel green solutions to overcome some of the main issues related to the above described approaches. Hence, we propose two new protocols for the extraction of PHAs from *Cupravidus necator* containing a high content (from 50 to 70 wt%) of the homopolymer poly(3-hydroxybutyrate) P(3HB) or the co-polymers poly(3-hydroxybutyrate-co-4-hydroxybutyrate) P(3HB-co-4HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV). The first method is based on the solubilization of PHAs with dimethyl carbonate (DMC), whereas the second is based on the dissolution of NPCM with fatty acid carboxylates used as Switchable Anionic Surfactants (SAS). To the best of our knowledge, this is the first time that both SAS and DMC have been applied as “green” tools for the treatment of microbial biomass with the aim of PHA recovery.

DMC is an acyclic alkyl carbonate, industrially produced by catalytic oxidative carbonylation of methanol through a green process developed by Enichem (IT) and UBE Industries (JP). In the past decades, DMC has become increasingly important in the chemical industry mainly because of its versatility as reagent and solvent, and its relatively low toxicity for human health and for the environment (DMC is fully biodegradable, is non-irritating and has no mutagenic effects either by contact or inhalation).<sup>11</sup> Moreover, in 2009, US-EPA has excluded DMC from the list of the volatile organic compounds (VOC), since scientific studies have shown that DMC has “negligible reactivity” in the formation of photochemical smog.<sup>12</sup>

SAS represent a peculiar class of surfactants that can be reversibly converted from water-insoluble neutral forms into anionic water-soluble compounds through pH shift. The pH change can be achieved in many ways, but the addition and removal of CO<sub>2</sub> is the simplest and most effective method.<sup>13</sup> The concept of “switchable compounds” was proposed for the first time by Jessop et al.<sup>14</sup> This idea is based on the possibility of reversibly switching some properties of a substance when a “trigger”, such as CO<sub>2</sub>, is applied. Switchable solvents or surfactants can be reversibly rendered polar/apolar, volatile/non-volatile, protic/aprotic, water miscible/water immiscible, exploiting this peculiarity for specific applications.<sup>15</sup> The cheapest available SAS are long chain carboxylates. For this work, we chose to use ammonium dodecanoate (laurate).

## Results and Discussion

In this study, *C. necator* DSM 545, an efficient producer of PHAs, was selected to obtain microbial cells with high content of the homopolymer P(3HB) or more “valuable” co-polymers P(3HB-co-3HV) and P(3HB-co-4HB). In fact, depending on the co-monomers composition, co-polymers might exhibit a wide range of physical properties, ranging from crystalline plastic to elastic. It is known that

the incorporation of 4HB or 3HV co-units into PHB improves the material toughness, acting as intra-chain plasticizers.

The strain produced up to 12 g L<sup>-1</sup> dry cell biomass using glucose as the only carbon source. After an appropriate accumulation phase, cell biomass was harvested and freeze-dried to obtain a unique batch of powder to be used for all PHAs extraction experiments, avoiding misleading results due to the variability that occurs between cultures. The amount of P(3HB) was found to be 74±2 wt% of the dry cell mass as determined by the conventional methanolysis method.<sup>16</sup> The use of valeric acid as carbon source resulted in the production of P(3HB-co-3HV) (54±2 wt%, 3HB/3HV 80/20) while the addition of  $\gamma$ -butyrolactone effectively promoted the accumulation of the co-polymer P(3HB-co-4HB) (64±1 wt%, 3HB/4HB 90/10).

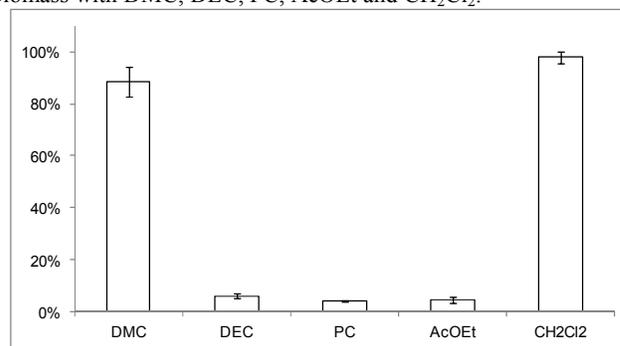
## Extraction of PHAs with DMC

### Extraction from freeze-dried biomass

In a first set of experiments, *C. necator* freeze-dried biomass containing 74±2 wt% of P(3HB) was extracted with DMC and various “green” solvents already proposed in the literature for the recovery of PHAs from microbial biomass, such as polypropylene carbonate (PC), diethyl carbonate (DEC) and ethyl acetate (AcOEt). Dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) was used as reference solvent. The extraction protocol required a biomass to solvent ratio of 2.5% (w/v) (50 mg of biomass were extracted with 2 mL of solvent). The biomass and the solvent were loaded in a centrifuge tube that was closed with a cap and kept at a specific temperature (90°C for DMC, PC and DEC, 80°C for AcOEt and 50°C for CH<sub>2</sub>Cl<sub>2</sub>) for 4 h. The residual biomass was then centrifuged and the polymer was recovered after i) filtering and evaporating the solvent or ii) the addition of ethanol (EtOH) and precipitation. These two recovering strategies were tested because the way in which the dissolved polymer is separated from the solvent can influence its chemical and physical properties as well as its purity.

The results of the various extractions are compared in Fig. 1.

**Figure 1.** P(3HB) recovery (expressed as percentage of the polymer content in the cells) obtained after 4 h-extraction of freeze-dried biomass with DMC, DEC, PC, AcOEt and CH<sub>2</sub>Cl<sub>2</sub>.



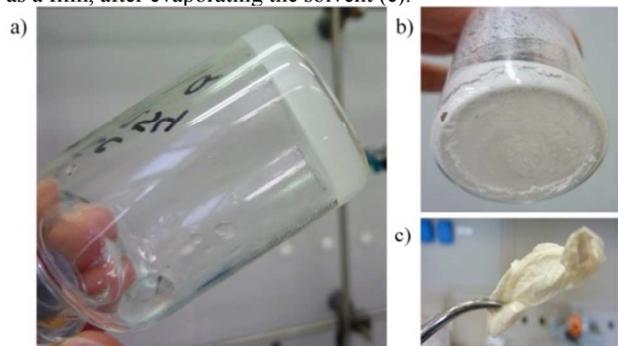
The recovery of P(3HB) extracted with CH<sub>2</sub>Cl<sub>2</sub> was 98±3%, corresponding to a polymer yield of 73±1 wt% (expressed as amount of polymer with respect to the extracted biomass).

Among the alkyl carbonates, DMC yielded a high P(3HB) recovery (88±6%), while PC and DEC were ineffective, with 4±1 and 6±1%, respectively. The limited performance of PC was unexpected as the excellent ability of this solvent (and other cyclic carbonates such as ethylene carbonate) in the solubilization and extraction of PHAs from microbial cells has been widely described.<sup>5,6</sup> The behavior reported here could be explained by two factors: i) the absence of biomass pre-treatment before the extraction, and, above all, ii) the lower temperature (90°C) at which

the extraction was performed in comparison to published results (above 130°C). Reis et al. described an improved recovery from 40 to 90% by increasing the temperature from 100 to 130°C, thus demonstrating how this parameter is crucial to extraction efficiency.<sup>5</sup> This trend was confirmed by the poor performance of DMC at 50°C, where the recovery was only 11±1% instead of 88±6% at 90°C (see ESI). Also the poor performance of DEC compared to DMC could be ascribed to a temperature-effect: we hypothesize that the mass transfer of the solvent can be facilitated at 90°C in the case of DMC compared to DEC, since the former is operating close to its boiling point (b.p. of DMC is 90°C; b.p. of DEC is 127°C). In these conditions DMC is expected to show higher mobility.

After centrifugation, filtration and cooling to room temperature, the P(3HB)/DMC solution formed a gel phase, analogously to what was reported in the literature with other non-halogenated solvents (e.g. methyl isobutyl ketone).<sup>7</sup> The polymer can then be recovered as a film, in the case of solvent evaporation, or as granules, in the case of precipitation with EtOH. (Fig. 2).

**Figure 2.** Formation of P(3HB)/DMC gel phase after leaving the solution of extracted P(3HB) in DMC (30 mg in 2 mL) at r.t. for 5 min (a); the polymer was recovered as granules by adding EtOH (b), or as a film, after evaporating the solvent (c).



AcOEt performed similarly to DEC and PC, with a polymer recovery of 5±1%. This result contrasted with the excellent ability of AcOEt to extract poly(hydroxybutyrate-co-hydroxyhexanoate) copolymer (> 15 mol% of 3-hydroxyhexanoate) from an engineered *C. necator* strain.<sup>7</sup> Sinskey et al. reported a PHA recovery of 95% after a 4 h extraction at 75°C using AcOEt, largely higher than our results. This discrepancy could be explained by two factors: i) the microbial biomass used for our extraction experiments (a wild-type *C. necator* strain) in comparison to the strain used in the literature (engineered *C. necator*); ii) the presumably lower solubility of P(3HB) in AcOEt than long chain monomer-based PHAs.

By evaluating the kinetics of the extraction with DMC, we found no significant differences between the recovery of P(3HB) obtained after 1 h of extraction (87±2%) and 4 h (88±3%); therefore further experiments were set up and optimized at 1 h.

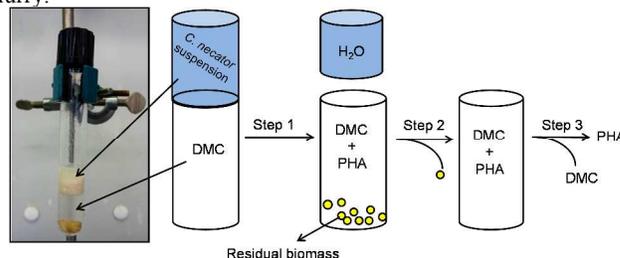
#### Extraction from microbial slurry

A series of extraction experiments were performed directly on microbial slurry to verify the possibility of bypassing the biomass drying step. If successful, this will reduce the energy consumption of the process while maintaining suitable extraction performances.

A first set of experiments was performed on freeze-dried *C. necator* biomass. The samples were re-suspended in water (1 mL) to get biomass concentrations of 50, 100 and 250 g L<sup>-1</sup>. This approach was applied to avoid misleading results due to the variability that occurs between cultures. In parallel we performed another set of experiments by using “fresh” microbial slurry (1 mL) with a biomass

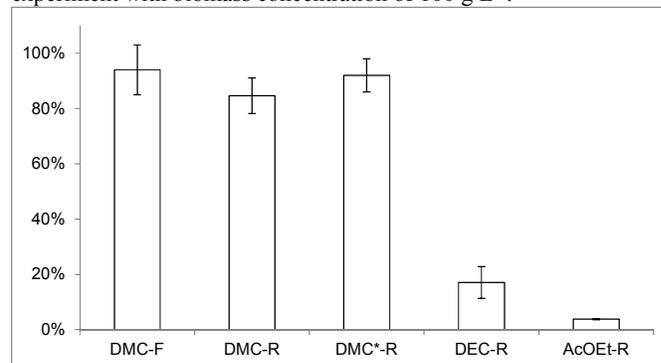
concentration of 50 g L<sup>-1</sup>, achieved by centrifuging and concentrating the microbial culture after the accumulation phase. The slurries were extracted with DMC (2 mL) for 1 h at 90°C (Step 1, Fig. 3). The water phase and the residual biomass were then centrifuged and separated (Step 2), and the extracted polymer was recovered after filtration and evaporation of the solvent or precipitation with EtOH (Step 3). DEC and AcOEt (2 mL) were used for comparison.

**Figure 3.** DMC-based protocol for the extraction of *C. necator* slurry.



The results of the various extractions are shown in Fig. 4.

**Figure 4.** P(3HB) recovery (expressed as percentage of the polymer content in the cells) obtained after 1 h-extraction of “fresh” (labelled as “F”) or “re-suspended” (labelled as “R”) microbial slurry (biomass concentration of 50 g L<sup>-1</sup>). The label “DMC\*” indicates the experiment with biomass concentration of 100 g L<sup>-1</sup>.



After 1 h of extraction at 90°C the recovery of P(3HB) with DMC was 85±6% and 92±6% for the re-suspended biomass concentrations of 50 and 100 g L<sup>-1</sup>, respectively. Similar results were obtained by extracting “fresh” microbial slurry at the same biomass concentration (94±9%). This findings demonstrated two points: i) the same efficiency was achieved by applying the protocol on both fresh or re-suspended microbial slurry. This fact underlined that the re-suspended microbial slurry (chosen for having a “standard” biomass on which performing all extractions) was a valid substitute for the “fresh” one; ii) the procedure developed could be efficiently applied to highly concentrated microbial slurries without decreasing its performance. However, when applied to slurries containing 250 g L<sup>-1</sup> biomass (data not shown), a gel of DMC/P(3HB) formed immediately when the test tube was removed from the hot silicon bath. In this case the separation of the DMC phase from cellular debris was impossible.

The polymer recovery rates obtained with DEC and AcOEt were 17±5 and 4±0.1%, respectively, much lower than that of DMC. In the case of DEC, a slight improvement was achieved in comparison to the results obtained on freeze-dried biomass (Fig. 1).

Most of DMC used can be recovered from the extraction phase by distillation (Fig. 3). However, due to its water solubility ( $139 \text{ g L}^{-1}$ ,  $\sim 14 \text{ wt}\%$ ), the losses of DMC in 1 mL of microbial slurry were 140 mg, around 6 wt% of the DMC used for the extraction. For this reason, the recycling of the upper water phase would be desirable, particularly when it will reach a threshold DMC saturation.

Considering that the existing separation technologies (e.g. pervaporation) only deal with removal of dilute organic compounds (usually less than 10 wt%) from aqueous streams,<sup>17</sup> we investigated if it was possible to use this residual DMC as a carbon source for industrially relevant microbiological processes. Therefore, several bacterial, yeast and fungal strains, selected for their possible industrial applications (*i.e.* PHA-, enzymes-, ethanol-, hydrogen-producers) were screened for their ability to grow using DMC dissolved into the cultivation medium as the sole carbon source (see ESI). DMC concentrations from 0.125 to 4 wt% have been tested. Among all screened microorganisms, only the yeast *Saccharomyces ludwigii* (an ethanol producer) was able to grow and consume up to 60% of the DMC present in the medium, even at the concentration of 1 wt%. Further research is in progress to improve this preliminary result.

#### Characterization of the extracted polymers

With the aim of evaluating the efficacy of the DMC-based protocols also as a function of the quality of the extracted P(3HB), a detailed characterization of the polymers was performed. The elemental analysis was carried out to assess the presence of cellular impurities on the samples, the temperature of maximum degradation rate together with the purity analysis (as described in the literature<sup>18</sup>) were both determined by thermogravimetric analysis (TGA). The average molecular weight ( $\overline{M}_w$ ) and the polydispersity index (PDI) were both determined by gel permeation chromatography (GPC). For the sake of comparison a commercial sample of P(3HB) (purchased from Biomer) was also analyzed (Table 1).

The purity of the recovered PHAs was comparable to that of the commercial polymer, suggesting that our protocol was appropriate to achieve highly pure materials from both freeze-dried biomass and microbial slurry. The amount of nitrogen, determined through CHN analysis, is a good indicator of protein content, one of the major residual impurities in the polymer.<sup>19</sup> In all analyzed samples, N was below the detection level, indicating that the amount of residual proteins was negligible. These data were also confirmed by the Fourier Transform-Infrared Spectroscopy (FT-IR) spectra of the samples, in which no significant absorption bands related to amide I ( $1630\text{-}1660 \text{ cm}^{-1}$ ) and amide II ( $1510$  and  $1580 \text{ cm}^{-1}$ ) vibrations were detected (see ESI).

**Table 1.** Physical characteristics of P(3HB) obtained after 1-h extraction of freeze-dried biomass or microbial slurry with DMC, in comparison with commercial P(3HB) (purchased from Biomer) and P(3HB) extracted with  $\text{CH}_2\text{Cl}_2$  (4 h). The data are expressed as mean  $\pm$  standard deviation of four independent replicates of each extraction condition ( $T_{\text{deg}1\%}$ : temperature at which 1% weight loss occurs;  $T_{\text{max deg}}$ : maximum decomposition temperature,  $\overline{M}_w$ : mean molecular weight; PDI: polydispersity index)

Treatment	Purity (%) <sup>a</sup>	N	C	H	$T_{\text{deg}1\%}$ (°C)	$T_{\text{max deg}}$ (°C)	$\overline{M}_w$ (MDa)	PDI
Commercial P(3HB)	98 $\pm$ 2	-	55.6 $\pm$ 0.8	7.0 $\pm$ 0.1	258	284	0.8	5.9
$\text{CH}_2\text{Cl}_2$ (freeze-dried biomass) <sup>b</sup>	94 $\pm$ 2	-	55.2 $\pm$ 1.8	7.0 $\pm$ 0.3	263	290	1.1	2.7
DMC (freeze-dried biomass) <sup>b</sup>	95 $\pm$ 2	-	55.8 $\pm$ 0.6	7.0 $\pm$ 0.1	236	280	1.0	2.7
DMC (microbial slurry) <sup>b</sup>	93 $\pm$ 1	-	55.6 $\pm$ 0.3	6.9 $\pm$ 0.1	266	289	1.1	2.7
DMC (microbial slurry) <sup>c</sup>	96 $\pm$ 3	-	55.1 $\pm$ 0.2	7.1 $\pm$ 0.2	242	273	0.9	1.8

<sup>a</sup> evaluated by TGA mass loss intensity; <sup>b</sup> polymer obtained after solvent evaporation; <sup>c</sup> polymer obtained after precipitation with EtOH

TGA measurements also provide information regarding thermal stability of all analyzed samples: the polymers were thermally stable up to at least 230°C and they all displayed a single degradation step at a comparable temperature (273-290°C). It is worth noting that published studies reported the highest temperature of maximum degradation rate for natural P(3HB) at 290°C.<sup>20</sup> This temperature is comparable to that of the sample extracted with DMC from the microbial slurry and obtained upon solvent evaporation.

Concerning the molecular weight of the obtained polyesters, GPC analyses of all extracted samples displayed quite similar  $\overline{M}_w$ , slightly higher than commercial P(3HB), while PDI was definitely lower. These results show that DMC-based protocols did not affect the polymer properties because they were comparable to those of the commercial reference sample and to the reference extraction protocol with  $\text{CH}_2\text{Cl}_2$ .

Moreover, it is clear that the method used for recovering P(3HB) after the extraction did not influence the characteristics of the materials obtained, even though the precipitation with EtOH led to some fractionation of the low molecular weight macromolecules that remained soluble in the solvent mixture, thus narrowing PDI (1.8 vs 2.7). These results are significant since it has been widely reported that the molecular weight of PHAs is strongly influenced by the temperature and the length of the thermal treatment applied during the extraction procedure.<sup>5</sup>

#### Extraction of the co-polymers P(3HB-co-3HV) and P(3HB-co-4HB).

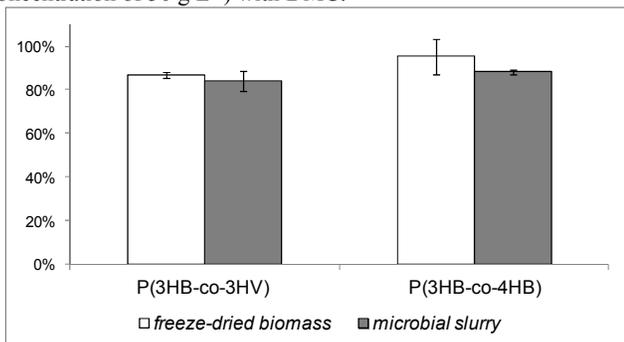
To deepen the versatility of DMC-based protocols, we evaluated the efficiency in the extraction of *C. necator* biomass containing the co-polymers P(3HB-co-3HV) (54 wt%) or P(3HB-co-4HB) (64 wt%) from both freeze-dried samples and microbial slurries (Fig. 5).

After 1 h of extraction of the freeze-dried biomass at 90°C, the recovery of the co-polymers P(3HB-co-3HV) and P(3HB-co-4HB) was 84 $\pm$ 5 and 88 $\pm$ 1%, respectively. The same conditions applied to microbial slurries yielded a polymer recovery of 87 $\pm$ 1 and 95 $\pm$ 8%, respectively.

Thus, even in these cases, the performance of DMC was good, confirming the validity of these protocols for the extraction of various PHAs, independently from polymer composition and quantity.

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**Figure 5.** P(3HB-co-3HV) and P(3HB-co-4HB) recovery (expressed as percentage of the PHA content in the cells) obtained after 1 h-extraction of freeze-dried biomass and microbial slurry (biomass concentration of 50 g L<sup>-1</sup>) with DMC.



### Dissolution of NPCM with Switchable Anionic Surfactants

The use of SAS represents 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, as recently demonstrated for the remediation of oil-contaminated sands,<sup>13</sup> SAS are effectively and simply recoverable by using CO<sub>2</sub> as pH-trigger.

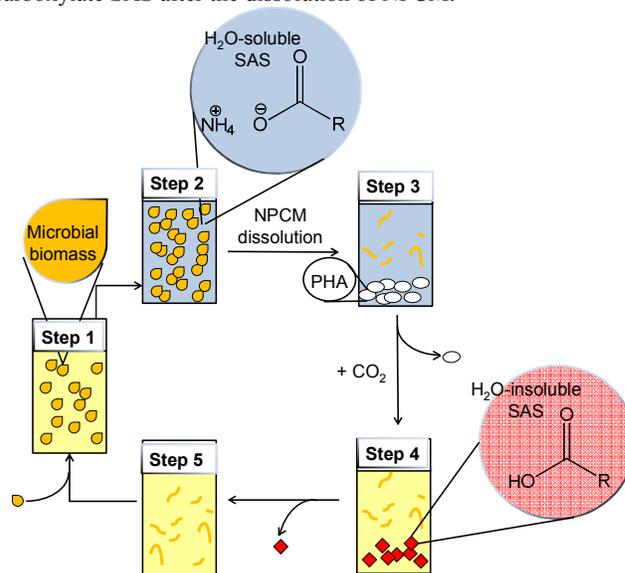
Here we developed a new protocol to disrupt NPCM from *C. necator* biomass and then recover PHAs, based on cheap, biocompatible and easily available long-chain carboxylates. Fatty acid carboxylates of different lengths have been investigated: sodium and ammonium laurate, myristate, palmitate and stearate. All of them were prepared by mixing the corresponding carboxylic acid (1 eq) and the necessary base (1.5 eq of NH<sub>4</sub>OH or 1 eq of NaOH) in water.<sup>13</sup> Only sodium and ammonium laurates were completely water soluble even at concentrations of 150 mM. All other longer chain sodium or ammonium carboxylates were insoluble even at very low concentrations (5 mM). Among the two laurate salts, we decided to focus on the ammonium one, because the water phase deriving from the process of dissolution would contain nitrogen (as NH<sub>4</sub><sup>+</sup> ions) and could be recycled as N source for re-growing bacteria (Fig. 6).

The process described in Fig. 6 starts with a microbial slurry (Step 1) that is treated with a NH<sub>4</sub>-laurate solution (pH 10, Step 2), providing the dissolution of NPCM. The released water insoluble polymer precipitates and can be recovered by centrifugation (Step 3). Upon CO<sub>2</sub> addition, the pH of the remaining suspension decreases to about 7, so that the surfactant switches from a water soluble salt into neutral lauric acid plus ammonium hydrogen carbonate. Lauric acid can be easily recovered by centrifugation (Step 4), whereas the aqueous phase, containing cellular residues and (NH<sub>4</sub>)HCO<sub>3</sub> (Step 5) can be recycled as nitrogen source to grow microorganisms. A NH<sub>4</sub>OH-solution can be added to the carboxylic acid, to re-obtain the ammonium laurate for another reuse.

For these experiments, *C. necator* freeze-dried biomass (50 mg) was re-suspended in water containing NH<sub>4</sub>-laurate. The surfactant to biomass ratio was 2:1 w/w (200 wt% of NH<sub>4</sub>-laurate), 1:1 (100 wt%), 0.5:1 (50 wt%) and 0.2:1 (20 wt%). Water was added to keep

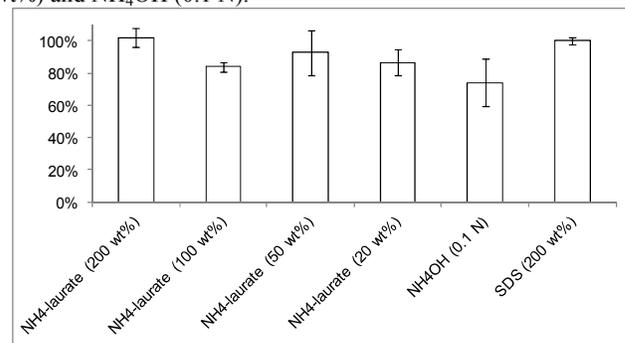
a SAS concentration of 150 mM. The activity of NH<sub>4</sub>-laurate was compared to the performance of sodium dodecyl sulfate (SDS, 200 wt%), a typical anionic surfactant, and NH<sub>4</sub>OH (0.1 N solution), to verify the effect of free bases. All experiments were carried out under stirring for 3 h at 90°C.

**Figure 6.** Use of CO<sub>2</sub> as a pH-trigger for recovering ammonium carboxylate SAS after the dissolution of NPCM.



The polymer recovery achieved with the various treatments is reported in Fig. 7, the purity and the elemental analysis are reported in Table 2, together with the mean molecular weight ( $\overline{M}_w$ ), the polydispersity index (PDI) and the thermal properties.

**Figure 7.** P(3HB) recovery (expressed as percentage of the polymer content in the cells) obtained after 3 h of treatment of freeze-dried biomass with NH<sub>4</sub>-laurate (200, 100, 50 and 20 wt%), SDS (200 wt%) and NH<sub>4</sub>OH (0.1 N).



The polymer stored inside the cells was completely recovered with both NH<sub>4</sub>-laurate and SDS used at 200 wt% (102±6 and

100±2%, respectively). NH<sub>4</sub>OH or lower amount of NH<sub>4</sub>-laurate led to less satisfactory results (recoveries from 70 to 90 % in all these cases).

The purity of the polymers recovered after NPCM dissolution with NH<sub>4</sub>-laurate and SDS, both used at 200 wt%, was high and compared well to that of commercial P(3HB) (Table 1). On the other hand, by decreasing the amount of NH<sub>4</sub>-laurate, the level of purity decreased down to around 80%. The treatments with NH<sub>4</sub>OH yielded a largely impure material (70%), similarly to what was previously reported in the literature.<sup>21</sup> The large standard deviation associated to the purity value of the polymers recovered with NH<sub>4</sub>OH and with 50 or 20 wt% of NH<sub>4</sub>-laurate testified the scarce homogeneity of the materials.

The evaluation of the N content in the samples through elemental analysis specified that, when using both NH<sub>4</sub>-laurate (200 wt%) and SDS, the possible residual protein contaminations were quite low. On the other hand, the treatments with lower amount of NH<sub>4</sub>-laurate or with NH<sub>4</sub>OH were less satisfactory: nitrogen amount progressively increased from two to five times by decreasing the NH<sub>4</sub>-laurate amount from 200 to 20 wt%, while in the case of NH<sub>4</sub>OH the protein impurities were almost 10 times more than what found with NH<sub>4</sub>-laurate 200 wt%. This trend was also confirmed by the FT-IR spectra of the samples: the absorption bands related to amide I (1630-1660 cm<sup>-1</sup>) and amide II (1510 and 1580 cm<sup>-1</sup>) vibrations were clearly visible in the polymer recovered with NH<sub>4</sub>OH. Only a very weak shoulder was observed in the polymer obtained with NH<sub>4</sub>-laurate 200 wt% (see ESI).

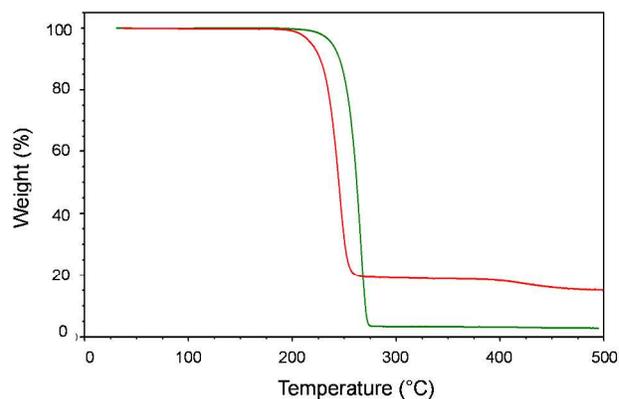
The thermal stability of the obtained polymers ( $T_{\text{deg } 1\%}$  and  $T_{\text{max deg}}$ ) was generally lower than what was obtained with DMC extraction (TGA results). Moreover, as clearly shown in Fig. 8, an earlier degradation was observed when lower amounts of SAS or when simple ammonia solutions were used. This behavior is comparable to published data for impure samples, which might display temperature of maximum degradation rate down to 235°C when the polymer is still embedded in the proteinaceous cellular residue.<sup>20</sup>

According to GPC analysis it results that the samples treated with SDS at neutral pH displayed, as expected,  $\overline{M}_w$  values closer to those of the polymer obtained with the DMC-based method. On the other hand, all treatments with NH<sub>4</sub>-laurate resulted in a reduction of the  $\overline{M}_w$  of the recovered polymer, suggesting that the high pH at

which the treatment was performed (pH 10), was detrimental, eventually providing a partial hydrolysis of ester bonds. Specifically, a slight decrease of  $\overline{M}_w$  was observed when SAS concentration was diminished. However, NH<sub>4</sub>-laurate solution is a buffered system and the pH value is relatively independent from the concentration of the buffering salt. If we assume that the nucleophilic reagent causing ester bond cleavage is HO<sup>-</sup>, then its concentration is about the same in all experiments with the SAS system and a similar rate of saponification is to be expected.

These findings suggest that there is a complex interaction between polymer, surfactant and extraction environment which has to be elucidated for a further optimization. However, it seems clear from all of the chemico-physical characterizations that a reduction in the loading of the surfactant seriously compromises the properties of the polymer.

**Figure 8.** Comparison of TGA profiles for pure and impure P(3HB) obtained with SAS: i) P(3HB) recovered with 200 wt% NH<sub>4</sub>-laurate (— green line) and ii) P(3HB) recovered with 100 wt% NH<sub>4</sub>-laurate (— red line).



**Table 2.** Physical characteristics of P(3HB) obtained after 3-h treatment of *C. necator* slurry. The data are expressed as mean ± standard deviation of four independent replicates of each extraction condition ( $T_{\text{deg } 1\%}$ : temperature at which 1% weight loss occurs;  $T_{\text{max deg}}$ : maximum decomposition temperature;  $\overline{M}_w$ : mean molecular weight; PDI: polydispersity index).

Treatment	Purity (%) <sup>a</sup>	N	C	H	$T_{\text{deg } 1\%}$ (°C)	$T_{\text{max deg}}$ (°C)	$\overline{M}_w$ (MDa)	PDI
NH <sub>4</sub> -laurate (200 wt%)	98±2	0.06±0.01	55.6±0.9	7.0±0.2	204	264	0.6	1.9
NH <sub>4</sub> -laurate (100 wt%)	80±1	0.16±0.03	54.7±0.9	7.2±0.2	191	245	0.4	2.6
NH <sub>4</sub> -laurate (50 wt%)	90±10	0.13±0.03	52.9±1.4	6.9±0.1	192	244	0.4	2.4
NH <sub>4</sub> -laurate (20 wt%)	80±7	0.27±0.04	53.9±1.4	7.1±0.3	196	239	0.3	2.7
NH <sub>4</sub> OH (0.1 N)	70±11	0.8±0.1	51.3±1.2	6.7±0.4	143	272	0.7	2.2
SDS (200 wt%)	99±1	0.06±0.01	53.9±0.5	6.9±0.1	225	271	1.2	2.2

<sup>a</sup>evaluated by TGA mass loss intensity

To recycle the SAS after having removed the polymer by centrifugation, CO<sub>2</sub> was bubbled for 20 min in the aqueous solution to decrease the pH from 10 to 7.2. Lauric acid precipitated and it was then recovered by centrifugation with a yield of 98.3% (Phase 3, Fig. 6). These results clearly confirm the improvement in terms of recyclability of such SAS-based protocols, in comparison with the use of other surfactants (anionic, cationic and non-ionic) proposed so far, which are highly water soluble and thus very difficult to be recycled. Thus, in spite of the need for high surfactant doses to achieve a suitable polymer extraction yield and purity, the excellent surfactant recovery achieved here by means of CO<sub>2</sub> provides a cheap and eco-friendly strategy to keep the overall cost process low.

### Comparison between the two protocols

To summarize the *pros* and *cons* of the proposed protocols and suggest their application under specific operative conditions, we identified four parameters for comparison: i) polymer purity, ii) polymer  $\overline{M}_w$ , iii) chemical/solvent recyclability, and iv) costs.

The main advantages of the DMC-based approach are:

- the excellent polymer purity
- the high  $\overline{M}_w$  of the recovered polymer

On the other hand, the most relevant drawback is that the recovery of DMC after polymer extraction or from water phase requires considerable energy consumption with undeniable economic costs.

The major advantages of SAS rely on the following points:

- the treatment does not require to dry the microbial biomass, reducing the overall energy consumption of the process;
- the surfactant recyclability is excellent;
- SAS are cheaper than the majority of the existing surfactants (e.g. SDS).

The major drawbacks are:

- the treatment with SAS (as well as with other surfactants) does not guarantee the abatement of endotoxins that can be detrimental for some specific applications (e.g. biomedical);
- SAS significantly reduce the  $\overline{M}_w$  of the recovered polymer.

From this preliminary comparison we deduce that the choice between the two methods strictly depends on the desired polymer application. For high-added value applications (e.g. biomedical), the polymer features (endotoxin abatement, purity and  $\overline{M}_w$ ) are fundamental, thus the acceptable process costs would be higher. In this case the use of DMC would be preferable.

On the other hand, for large scale applications such as (non-food) packaging, for which keeping the costs low is fundamental, the use of SAS would provide the cheapest scenario.

However, a more detailed evaluation of the entire process is needed, taking into account both the energy/economic costs and the relationship between physico-chemical properties and the subsequent use of the extracted polymer.

### Conclusions

In this study two new protocols were developed and evaluated as green alternative to well-established approaches (e.g. use of chlorinated solvents and typical anionic surfactants) for the recovery of PHAs from *C. necator* biomass.

The DMC-based method was applied successfully to both freeze-dried biomass and highly concentrated microbial slurry without any pre-treatment before the extraction, bypassing an energy intensive and costly step. The recovery and the purity of the polymer were very high, for both homopolymer P(3HB) or co-polymers P(3HB-co-3HV) and P(3HB-co-4HB); also the thermophysical

characteristics of the recovered polymers were excellent, due to the relatively low extraction temperature applied.

All of these findings, together with its low toxicity and high biodegradability, confirm that DMC is an effective solvent for this process, representing a valid alternative to the use of environmentally concerning chlorinated solvents.

The NH<sub>4</sub>-laurate-based method was also applied successfully to highly-concentrated microbial slurry without any pre-treatment of the biomass before the extraction. Recovery and purity of the polymer were very high when using an amount of surfactant of 200 wt%, in the range of what is obtained with typical anionic surfactants such as SDS. The recyclability of SAS system, achievable by simply adding CO<sub>2</sub>, allowed a cheap and successful recovery of more than 98% of the surfactant, underlying the great green potential of these compounds in comparison to typical anionic surfactants. Since the pH-change has resulted to have an effect on the final properties of the polymer, further work will be directed to the exploration of other switchable surfactant systems for which a less severe pH change is required.

## Experimental

### Chemicals

All solvents and chemicals used in this study were obtained from Sigma-Aldrich (purities  $\geq 98\%$ ) and were used without purification. Standard P(3HB) was purchased from Biomer (DE).

### *Cupriavidus necator* DSM 545: growth conditions and PHA accumulation.

*C. necator* DSM 545 cells containing high amounts of P(3HB) were obtained after two-step aerobic batch incubation according to the literature.<sup>22</sup> Briefly, in the first step, aimed at biomass production, *C. necator* was grown at 30°C in 1 L shaking flask containing 300 mL of DSMZ81 medium, using glucose (30 g L<sup>-1</sup>) as carbon source. An overnight culture (OD<sub>600</sub> nm=1.5) at 0.25% (v/v) was used as pre-inoculum. After 48 h, cells were harvested by centrifugation at 4000 x g at 4°C for 15 min and, to promote PHA synthesis, transferred into 1 L shaking flasks containing 300 mL nitrogen-free DSMZ81 medium with 30 g L<sup>-1</sup> glucose.

To produce the co-polymers P(3HB-co-3HV) and P(3HB-co-4HB), *C. necator* DSM 545 was grown, during the first step, in DSMZ81 medium supplemented with glucose (3 wt%) and valeric acid (0.1 wt%) or  $\gamma$ -butyrolactone (0.11 wt%), respectively. In the second phase, the cells, harvested as described above, were transferred into nitrogen-free DSMZ81 medium with 3 wt% glucose and 0.15 wt% of valeric acid or 1.3 wt% of  $\gamma$ -butyrolactone, respectively.

After incubation at 30°C for 72 h, cells were harvested by centrifugation and lyophilized for further analyses. To measure microbial dry biomass, 10 mL of culture broth were centrifuged, washed twice in distilled water, and the pellet dried to constant mass at 80°C before weighting.

### Extraction of PHB with organic solvents

*Freeze-dried biomass extraction.* *C. necator* freeze-dried samples (50 mg) were extracted with organic solvents (2 mL) for 1-4 h. The tested solvents and the corresponding temperatures of extraction were: DMC (90°C and 50°C), PC (90°C), DEC (90°C), ethyl acetate (80°C) and CH<sub>2</sub>Cl<sub>2</sub> (50°C). At the end of the extraction, the solutions were centrifuged at 4000 rpm for 1 min and then filtered with polypropylene membrane filters of 0.45  $\mu$ m porosity. The polymer was recovered by solvent evaporation or by precipitation with EtOH, then dried at 60°C under vacuum overnight.

Each extraction was performed in quadruplicate.

**Microbial slurries extraction.** *C. necator* freeze-dried samples (50, 100 and 250 mg) were re-suspended in water (1 mL) and then extracted with DMC, DEC or AcOEt (2 mL) for 1-4 h at 90°C (for DMC and DEC) or 70°C (for AcOEt). At the end of the extraction, the solutions were centrifuged at 4000 rpm for 1 min; in the case of DMC, the aqueous layer (on the top) was withdrawn and the organic layer was filtered with polypropylene membrane filters of 0.45 µm porosity. The polymer was then recovered by solvent evaporation or by precipitation with EtOH, then dried at 60°C under vacuum overnight.

Each extraction was performed in quadruplicate.

To evaluate the perspective of recycling DMC resulting in the water phase during the cell extractions, twenty-two microbial, yeast and fungal strains, capable of producing valuable compounds<sup>23,24,25,26</sup> were screened for their ability to use DMC as single carbon source (for details see ESI). The strains, pre-inoculated in the appropriate defined broth, were grown in the presence of increasing concentrations of DMC (0.125, 0.25, 0.5, 1, 2 and 4 wt%). For comparison, each broth was supplemented with the equivalent amount of glucose. Cell growth was monitored at regular intervals by measuring absorbance ( $A_{600\text{nm}}$ ). The experiment was carried out in triplicate.

#### Dissolution of NPMC with SAS

Ammonium carboxylate of lauric acid ( $\text{NH}_4$ -laurate) was prepared according to the procedure reported in the literature, using a stoichiometry of 1:1.5 for the ratio lauric acid to base.<sup>13</sup> Briefly,  $\text{NH}_4\text{OH}$  (0.75 mmol) was added to a suspension of lauric acid (100 mg, 0.5 mmol) in water (3 mL): at pH 10, the carboxylic acid switched from an insoluble form to a soluble ionic liquid. *C. necator* biomass (50 mg) was added to this solution, the mixture was stirred at 90°C for 3 h and then centrifuged. The polymer was recovered on the bottom of the centrifuge tube, washed once with a  $\text{NH}_4\text{OH}$  solution (0.1 N), water (1 mL) and twice with EtOH (1 mL), then dried overnight at 60°C under vacuum.  $\text{CO}_2$  was bubbled for 20 min into the aqueous phase until pH 7.2 was reached (by increasing the time of bubbling to 1 h the pH did not decrease to less than 7). Lauric acid, precipitated from this neutral aqueous phase, was recovered by centrifugation, washed once with  $\text{H}_2\text{O}$  and then dried overnight at 60°C under vacuum.

The experiments with the ratio surfactant to biomass 1:1, 0.5:1 and 0.2:1 were performed with the following amount of lauric acid in  $\text{H}_2\text{O}$ : 50 mg (0.25 mmol) in 2 mL of  $\text{H}_2\text{O}$ , 25 mg (0.12 mmol) in 1 mL of  $\text{H}_2\text{O}$ , and 10 mg (0.05 mmol) in 0.5 mL of  $\text{H}_2\text{O}$ .  $\text{NH}_4\text{OH}$  (1.5 eq) was then added to these suspensions.

The dissolutions of NPCM with  $\text{NH}_4\text{OH}$  and SDS were performed by adding of  $\text{NH}_4\text{OH}$  (0.37 mmol) or SDS (100 mg) in 3 mL of  $\text{H}_2\text{O}$ . *C. necator* biomass (50 mg) was added to these solutions, the mixtures were stirred at 90°C for 3 h and then centrifuged.

Each extraction was performed in quadruplicate.

#### Analysis

**PHA amount in microbial cells** (wt%). The polymer amount in *C. necator* biomass was determined by gas chromatography following the procedure reported in the literature with slight modifications:<sup>14</sup> microbial biomass samples (5 mg) were incubated for 3.5 h at 100°C with a 20% acidic MeOH solution (1 mL containing 0.2 mL of  $\text{H}_2\text{SO}_4$ ) and  $\text{CH}_2\text{Cl}_2$  (1 mL); after that, brine (0.5 mL) and the internal standard (*n*-nonane, 0.05 mL of 1 wt% solution in DMC) were added to the mixture. The solutions were centrifuged at 4000 rpm for 10 min and water phase was withdrawn.

In order to remove any trace of  $\text{H}_2\text{SO}_4$ ,  $\text{CaCO}_3$  was added to the  $\text{CH}_2\text{Cl}_2$  phase before analyzing the organic phase by GC-MS.

Each analysis was performed in triplicate.

**PHA yield** (wt%). The yield of the extracted PHA was calculated gravimetrically (after evaporating the solvent under vacuum or after adding EtOH) on microbial biomass weight basis (wt%).

**PHA purity** (%). The purity of the extracted PHA was determined by TGA analysis, applying the procedure reported in the literature<sup>18</sup> on PHA samples of 1-2 mg.

Each analysis was performed in duplicate.

**PHA recovery** (%). The recovery of PHA was calculated as follows:

$$\frac{\text{PHA yield} * \text{PHA purity}}{\text{PHA amount in the microbial cells}}$$

**GC-MS analysis.** Quantitative analyses were performed with a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 mm film thickness), with helium as carrier gas (at constant pressure, 33  $\text{cm s}^{-1}$  linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan  $\text{s}^{-1}$  within the 12-600  $\text{m/z}$  range.

For the quantitative analysis, the temperature of the column started from 40°C hold for 6 min, and then increased up to 120°C at 10°C  $\text{min}^{-1}$ , followed by a post-run at 325°C hold for 1 min. Calibration solutions for GC-MS analysis were prepared by applying the previously described procedure on the commercial P(3HB) standard and used to determine the GC-MS response factor of methyl 3-hydroxybutanoate relative to nonane.

DMC concentration of spent broths was determined by 10 min SPME (PDMS 100 µm) performed on headspace of the solution and GC-MS analysis. GC-MS was performed with the same parameters described before with isothermal GC-program at 40°C. Quantitation was assessed by external calibration performed in the culture broth samples spiked with known quantity of DMC.

**Elemental analysis.** The elemental composition of the extracted polymer was determined by using an elemental analyzer (Thermo Scientific, Flash2000, Organic Elemental Analyzer) by means of the flash combustion technique.

**Molecular weight and polydispersity index.** Number average molecular weight and polydispersity were determined in  $\text{CHCl}_3$  solution by size exclusion chromatography (SEC) using an HPLC Lab Flow 2000 apparatus working with a 1 mL  $\text{min}^{-1}$  flow, equipped with an injector Rheodyne 7725i, a Phenomenex Phenogel 5u 10E6A column and a RI detector Knauer RI K-2301. Calibration curves were obtained using several monodisperse polystyrene standards.

**Thermal analysis.** Thermogravimetric (TGA) measurements were carried out using a TA Instruments SDT-Q600 instrument. The analyses were performed at 10°C  $\text{min}^{-1}$  from room temperature to 600°C under nitrogen flow. T deg 1% is defined as the temperature at which the sample weight loss is 1%, while T max deg is defined as the temperature of the maximum weight loss rate.

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## Notes and references

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† Electronic supplementary information (ESI) available: detailed PHA yield and recovery, FT-IR spectra of some extracted PHAs; bacterial, yeast and fungal strains screened for their ability to use DMC as carbon source.

[1] <http://en.european-bioplastics.org/>

[2] S. Chanprateep, *J. Biosci. Bioeng.*, 2010, **110**, 621-632

[3] N. Jacquet, C. Lo, Y. Wei, H. Wu, S. Wang, *Biochem. Eng. J.*, 2008, **39**, 15-27

[4] Koller, H. Niebelschütz, G. Braunnegg, *Eng. Life Sci.*, 2013, **13**, 549-562

[5] M. Fiorese, F. Freitas, J. Pais, A. Ramos, G. De Aragao, M. Reis, *Eng. Life Sci.*, 2009, **9**, 454-461

[6] C. Mc Chalicher, F. Srienc, D. Rouse, *AIChE J.*, 2010, **56**, 1616-1625

[7] S. Riedel, C. Brigham, C. Budde, J. Bader, C. Rha, U. Stahl, A. Sinskey, *Biotechnol. Bioeng.*, 2013, **110**, 461

[8] US Pat., 7 763 715 B2, 2010

[9] J. W. Hampson, R. D. Ashby, *J. Amer. Oil Chem. Soc.*, 1999, **76**, 1371-1374

[10] J. Choi, S. Y. Lee, *Biotechnol. Bioeng.*, 1999, **62**, 546-553

[11] D. Delledonne, F. Rivetti, U. Romano, *Appl. Catal. A-Gen*, 2001, **221**, 241-251

[12] [www.epa.gov/ttn/caaa/t1/fr\\_notices/vocexclusion\\_prop092507.pdf](http://www.epa.gov/ttn/caaa/t1/fr_notices/vocexclusion_prop092507.pdf)

[13] E. Ceschia, J. Harjani, C. Liang, Z. Ghoshouni, T. Andrea, R. Brown, P. Jessop, *RSC Adv.*, 2014, **4**, 4638-4645

[14] P. G. Jessop, D. J. Heldebrant, X. Li, C. A. Eckert, C. L. Liotta, *Nature*, 2005, **436**, 1102

[15] C. Samori, D. López Barreiro, R. Vet, L. Pezzolesi, D. W. F. Brillman, P. Galletti, E. Tagliavini, *Green Chem.*, 2013, **15**, 353-356

[16] L. Serafim, P. Lemos, R. Oliveira, M. Reis, *Biotechnol. Bioeng.*, 2004, **87**, 145-160

[17] P. Shao, R.Y.M. Huang, *J. Membrane Sci.*, 2007, **287**, 162-179

[18] S. K. Hahn, Y. K. Chang, *Biotechnol. Tech.*, 1995, **9**, 873-878.

[19] M. Kim, K. Cho, H. Ryu, E. Lee, Y. Chang, *Biotechnol. Lett.*, 2003, **25**, 55-59

[20] F. D. Kopinke, M. Remmler, K. Mackenzie, *Polym. Deg. Stab.* 1996, **52**, 25-38

[21] Y. Yang, C. Brigham, L. Willis, C. Rha, A. Sinskey, *Biotechnol. Lett.*, 2011, **33**, 937-942

[22] S. Povolo, P. Toffano, M. Basaglia, S. Casella, *Bioresour. Technol.*, 2010, **101**, 7902-7907

[23] L. Favaro, M. Basaglia, S. Casella, I. Hue, X. Dousset, B.D. Gombossy de Melo Franco, S.D. Todorov, *Food Microbiol.*, 2014, **38**, 228-239

[24] L. Favaro, T. Jooste, M. Basaglia, S.H. Rose, M. Saayman, J.F. Görgens, S. Casella, W.H. van Zyl, *Appl. Microbiol. Biotechnol.*, 2012, **95**, 957-968

[25] L. Favaro, M. Basaglia, A. Trento, E. van Rensburg, M. Garcia-Aparicio, W.H. van Zyl, S. Casella, *Biotechnol. Biofuels*, 2013, **6**, 168

[26] A. Alibardi, L. Favaro, M.C. Lavagnolo, M. Basaglia, S. Casella, *Water Sci. Technol.*, 2012, **66**, 1483-1490