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Research Article

Chemical Recycling of Polyhydroxybutyrate (PHB) into Bio-Based Solvents and Their Use in a Circular PHB Extraction

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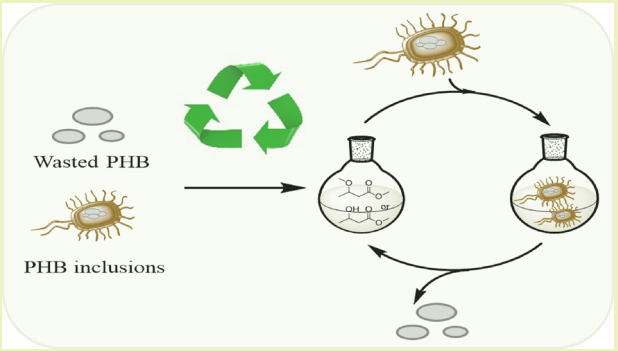
ABSTRACT: Two novel protocols for the chemical valorization of polyhydroxybutyrate (PHB) were developed, aiming at the production of two bio-based molecules: methyl 3-hydroxybutyrate (MHB) and methyl 3-methoxybutyrate (MMB). Optimized reaction conditions were applied to pure PHB and PHB inclusions inside bacterial cells as starting materials. MHB was synthesized through a single-step catalytic methanolysis, while MMB was synthesized through a three-step process: thermolytic distillation to give crotonic acid (CA), esterification to give methyl crotonate (MC), and oxa-Michael addition of MeOH. The obtained MHB and MMB were tested as solvents for the recovery of PHB itself both from freeze-dried single strain cultures (SSC) and mixed microbial cultures (MMC) with low to medium contents of PHB (22−57 wt %). High PHB recovery was achieved: up to 96 ± 1% through MHB and up to 98 ± 1% through MMB. Extraction from MMC slurry (with a PHB content of 39% on dry weight) was also performed, recovering 77 ± 2% using MHB and 92 ± 2% using MMB. High purities and excellent molecular weights and polydispersity indexes of extracted PHB were obtained with both MHB and MMB. Solubility in water, octanol/water partition coefficients (log K_{ow}), and aerobic ready biodegradability of both solvents were also evaluated.



KEYWORDS: *chemical recycle, recycling, polyhydroxybutyrate (PHB), polyhydroxyalkanoates (PHA), extraction, recovery, solvents, bio-based*

INTRODUCTION

The increasing global consumption of plastics demands more sustainable materials, produced from renewable resources, biodegradable, and/or efficiently recyclable, to reuse them or obtain new substances. For this purpose, bioplastics are a good alternative to fossil-based plastics, as they are either bio-based or biodegradable or feature both proprieties.[1] Even if bioplastics are in principle more sustainable for our environment, the importance of recycling applies also to them since, from the point of view of circular economy, the use of bioplastic waste as input for producing new bioplastic materials is more beneficial than the consumption of raw materials (e.g., sugars or polysaccharides) for the same purpose. Among bioplastics, polyhydroxyalkanoates (PHAs) are bio-based and biodegradable polyesters of hydroxy acid monomers bio-synthesized by different kinds of bacteria through the aerobic conversion of various feedstocks[2] and potentially capable of replacing fossil-based plastics thanks to similar mechanical and physical proprieties.[3] At present, the main bottleneck for the production and application of PHA at a large scale is their high production cost (2.2−5.0 €/kg), which is at least three times higher than the main fossil-based polymers that cost less than 1.0 €/kg.[4] This is due to the fact that the most common way to produce PHAs at the industrial scale is through the use of single strain cultures (SSC) of natural or genetically engineered bacteria that need a sterile environment and selected/purified carbon feedstock. These high costs can be overthrown by using mixed microbial cultures (MMC) of PHA producers, capable of producing the polyesters starting from wastes (e.g., food waste, wastewater, molasses, or sludge)[5] under non-sterilized conditions, thus representing a more promising platform than their single strain cousins.[6] For both SSC and MMC, a large part of such costs is due to the PHA recovery process, which is even more challenging for MMC due to the diversity of the microbial cultures and their more complex non-PHA cell material (NPCM).[7] Several alternative methods to the well-known use of toxic chlorinated solvents (e.g., chloroform or dichloromethane)[8] or oxidants (NaClO)[9] are proposed for the recovery of PHAs from SSC (e.g., cyclic carbonates,[10]

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surfactants,[11] and ionic liquids[12]). In the last decade, research studies have focused on the use of more sustainable solvents, and some of them, used for PHA recovery from SSC, can be considered as "super solvents" due to their high affinity for PHAs (e.g., dimethyl carbonate (DMC), γ-valerolactone, and ethyl lactate).[13] On the other hand, very fewer options can be found for PHA recovery from MMC. In fact, to the best of our knowledge, besides NPCM dissolution with surfactants and oxidants[14] and the use of chlorinated compounds, only DMC has been proposed as a true alternative sustainable solvent for PHA recovery from MMC.[15] Evaluations of PHA production costs estimate that solvent extraction methods are quite costly if compared to mechanical disruption or chemical digestion (0.77 vs 0.26 €·kg^{-1} PHA), but in those cases where downstream processing is coupled with other own facility processes and/or products and byproducts (e.g., the utilization of residual heat or process byproducts), solvent extraction methods can be both environmental and economically feasible (0.24 €·kg^{-1} PHA).[16]

Another important aspect for PHA competitive and mature applications is also a convenient and sustainable way to manage the end of life (EoL) and specifically a recycling approach to reuse the polymer or, if not possible or inconvenient, its valorization into high-value molecules that can be inserted in the same industrial production cycle or used for totally different applications. For this purpose, different waste management options have been studied, including mechanical, chemical, or biological recycling.[17] The mechanical route for the recycling of plastic materials is currently quite costly since the municipal plastic waste is a heterogeneous matrix and needs to be separated.[18] The biological way could be applied to produce methane under anaerobic conditions[19] or the monomer 3-hydroxybutyric acid[20] through hydrolysis, even if it has to be considered that the presence of additives can inhibit the microbial activity.[21] Chemical recycling seems to be the most versatile route since it allows the production of several valuable compounds that can be used as building blocks for multiple synthetic applications. Hydrolysis, methanolysis, and pyrolysis are the most explored ways to depolymerize polyesters to produce the corresponding monomers,[22] and some interesting applications on PHAs can be already found in the literature.[23] Hydrothermal depolymerization has been performed on polyhydroxybutyrate (PHB), the homopolymer of hydroxybutyric acid, to obtain hydroxybutyric acid[24] or propylene,[25] useful building blocks for the synthesis of β-hydroxy esters or isopropanol, respectively. α,β-unsaturated acids and oligomers can be produced through pyrolysis[26] or thermolytic distillation[27] and used as convenient feedstock to reobtain PHAs through methanotrophic bacteria.[28] Microwave-assisted PHB degradation in alkaline MeOH has been proposed as a sustainable way to produce PHB monomers,[29] while the use of acidic or basic functionalized ionic liquids in methanol leads to the synthesis of methyl 3-hydroxybutyrate (MHB).[30,31]

Starting from these seminal approaches, the aim of this work is to merge the optimization of the chemical recycling route to the innovation in the extractive step, transforming PHB into valuable C4-building blocks that can be used as solvents for PHB extraction from SSC and MMC (Figure 1). Specifically, methyl 3-hydroxybutyrate (MHB) and methyl 3-methoxybutyrate (MMB) were synthesized following new synthetic thermo-chemical routes starting from PHB of different purities and qualities, like commercial PHB or PHB inclusions inside bacterial cells. MHB and MMB were then tested for the extraction of PHB itself from both *Cupriavidus necator* (*C. necator*) (SSC) and MMC containing low to high contents (from 22 to 57%) of PHB. Moreover, the direct recovery of PHB from MMC slurry was investigated with the aim of avoiding the energy-demanding biomass-drying step. To the best of our knowledge, this is the first example in which the recycling and valorization of PHB to give valuable chemicals are coupled with their use for PHB challenging recovery, thus realizing both an improvement and an effective example of circularity in biopolymer production.

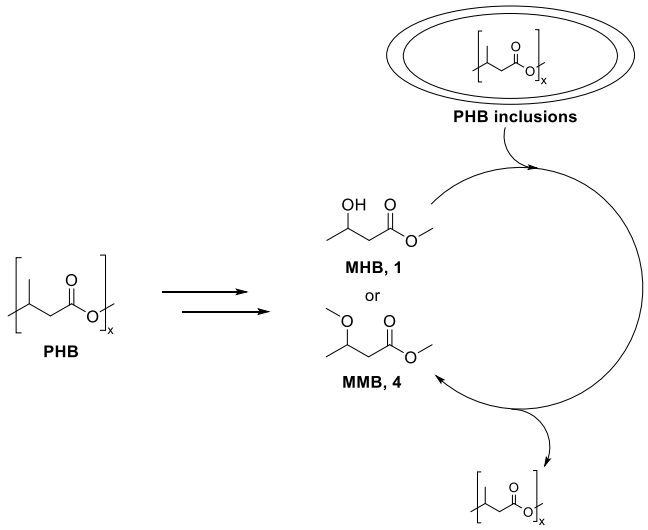

Figure 1. Scope of this work: MHB and MMB synthesis and their use for PHB recovery from SSC or MMC.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich and used without further purification. PHB was purchased from Biomer (DE). SSC (*Cupriavidus necator*) and MMC containing PHB were cultivated following previously reported procedures.[27,32] The types of bacteria used (SSC or MMC) and their PHB content (22, 35, 39, or 57 wt %) are reported in Table 1. A sulfonated acidic heterogeneous catalyst from potato starch (C-SO_3H) was obtained following an already reported procedure.[33]

Table 1. PHB Amounts in SSC and MMC Used Here and the Quantity of Biomass Used for Extraction to Keep Constant the PHB-to-Solvent Ratio (26 mg/mL)

sample	PHB content (%)	biomass extracted (mg)[a]
SSC-35	35 ± 2	75
SSC-57	56.7 ± 2	45
MMC-22	22 ± 2	120
MMC-39	39 ± 1	67

[a]Extraction performed with 1 mL of the solvent.

MHB and MMB Synthesis. *Synthesis of Methyl 3-Hydroxybutyrate (MHB).* PHB (3 g, 34.9 mmol of the HB repeating units) or PHB inclusions (5.3 g of SSC-57, 34.9 mmol of the HB repeating units), MeOH (21.2 mL, 523 mmol, and 15 equiv.), and H_2SO_4 (9.3 μL, 0.17 mmol, and 0.5 mol %; 186 μL, 3.5 mmol, and 10 mol % for SSC-57) were charged in a closed-cap glass reactor. The methanolysis reaction was carried out under autogenous pressure and magnetic stirring at 140 °C for 7 h. After that time, the reaction mixture was cooled to rt, an equimolar amount (with respect to the acid catalyst) of NaOH (6.98 mg, 0.17 mmol, and 0.5 mol %; 140 mg, 3.5 mmol,

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and 10 mol % for SSC-57) was added, and the solution was stirred at rt for 15 min. The resulting solution was distilled to recover the unreacted MeOH (room pressure) and MHB (75−80 °C under a reduced pressure of 10 mbar). MHB was obtained as a colorless liquid with a 95% isolated yield (3.9 g) from pure PHB or an 85% isolated yield (3.47 g) from SSC-57. Tables of optimization of the reaction conditions, catalyst recycle, and NMR characterization of the product are reported in the ESI (Tables S1 and S2).

Synthesis of Methyl 3-Methoxybutyrate (MMB). Synthesis of Crotonic Acid (CA). CA was obtained following a previously reported procedure.[27] Hence, a single-neck round-bottom flask equipped with a distillation apparatus was charged with PHB (20 g) or PHB inclusions (35 g of SSC-57) and then kept under reduced pressure (150 mbar). The flask was inserted in a heating mantle already set at 175 °C and kept at this temperature until no vapors were observed (approximately 1 h). CA was collected in a 100 mL round-bottom flask put at the end of the apparatus, with a yield of 95% from PHB (19 g) or 88% from SSC-57 (15.6 g), and then used without further purification.

Synthesis of Methyl Crotonate (MC). CA (6.5 g, 75.6 mmol, and 1 equiv.), C-SO_3H (163 mg, 2.5 wt %), and MeOH (15.3 mL, 378 mmol, and 5 equiv.) were charged in a closed-cap glass reactor. The esterification reaction was carried out under autogenous pressure and magnetic stirring at 130 °C for 7 h. After that time, the solution was filtered to recover the heterogeneous catalyst. The filtrate was distilled using a Vigreux condenser to recover first unreacted MeOH mixed with H_2O (produced during the reaction) at atmospheric pressure and then MC in a 93% yield (7 g). Tables of optimization of the reaction conditions and NMR characterization of the product are reported in the ESI (Table S3).

Synthesis of Methyl 3-Methoxybutyrate (MMB). MeOH (4.05 mL, 100 mmol, and 2 equiv.) and NaOMe (81 mg, 1.5 mmol, and 3 mol %) were mixed under magnetic stirring in a round-bottom flask at rt for 15 min; then, MC (5 g, 50 mmol, and 1 equiv.) was added dropwise over 30 min, and the solution was stirred for 50 h at rt. After that time, the resulting solution was distilled to recover MeOH, unreacted MC, and MMB (65−70 °C under a reduced pressure of 10 mbar). MMB was obtained in a 92% yield (6.1 g) as a colorless liquid. Tables of optimization of the reaction conditions and NMR characterization of the product are reported in the ESI (Table S4).

The Environmental Factor (*E*-Factor). The *E*-factor[34] was calculated for both MHB and MMB synthetic pathways using the following equation

$$\frac{\text{Mass of wastes (g)}}{\text{Mass of products (g)}} \quad (1)$$

PHB Extraction Procedures. *Extraction of PHB from Freeze-Dried SSC or MMC.* Freeze-dried biomass (SSC or MMC) containing different amounts of PHB (Table 1) was extracted with MHB, MMB (130 °C, 10 min, and 1 mL), or MC (118 °C, 1 h, and 1 mL). The solutions were then centrifuged at 4000 rpm for 30 s, and the supernatant was collected in a round-bottom flask. All the solvents were recovered by distillation under reduced pressure, leaving the extracted PHB in the flask. Recovered MHB and MMB were analyzed by ^{1}H NMR to check the presence of impurities and recycled. Each extraction was performed in triplicate. In comparison, SSC-56 and MMC-22 were extracted with CH_2Cl_2 (1 mL) for 10 min at 60 °C; in this case, the extracted PHB was recovered by solvent evaporation.

The recovery of PHB (%) was calculated as follows

$$\frac{\text{PHB extraction yield (mg)} \times \text{PHB purity (\%)}}{\text{PHB amount in microbial cells (mg)}} \quad (2)$$

where the PHB extraction yield was calculated gravimetrically; PHB purity and the PHB amount in microbial cells were calculated as described in Analyses.

Extraction of PHB from MMC Slurry. MMC slurry (300 mg) with an average content of 20 wt % of dry cell weight and 39 wt % PHB on a dry cell weight basis was extracted with MHB or MMB (130 °C, 10 min, and 1 mL). The solutions were then centrifuged at 4000 rpm for 30 s, and the supernatant was collected in a round-bottom flask. MHB and MMB were recovered by distillation under reduced pressure, leaving the extracted PHB in the flask. Each extraction was performed in duplicate. The recovery of PHB was calculated as described for freeze-dried SSC and MMC.

Analyses. *Estimation of MHB and MMB Solubility in Water.* To determine the solubility in water, 1 mL of MHB or MMB and 1 mL of D_2O were put in a tube and gently stirred at rt for 24 h. The D_2O solution was then recovered using a separation funnel and added in a known amount to a solution of D_2O containing a 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (0.75 wt %) as an internal standard. Concentrations of MHB and MMB in D_2O were determined by ^{1}H NMR using longer relaxation times to ensure quantitative integration.

Estimation of MHB and MMB Octanol/Water Partition Coefficients. Partition coefficient octanol/water (log K_{ow}) determination of MHB and MMB was performed by reverse-phase HPLC according to the OECD guideline 117.[35] A calibration plot was established using 9 reference substances with known log K_{ow}: tetrahydrofuran (0.76), aniline (0.9), phenol (1.5), 2-nitrophenol (1.8), cinnamyl alcohol (1.9), atrazine (2.6), toluene (2.7), 1,4-dichlorobenzene (3.4), and phenanthrene (4.5). The separations were performed on a 4.6 × 150 mm XBridge C8 column with an average pore diameter of 137 Å and a particle size of 3.5 μm at a temperature of 25 °C and the isocratic elution phase composed by 70:30 methanol/water, with a flow rate of 1 mL min^{-1}.

MHB and MMB Biodegradation Tests. Biodegradation tests of MHB and MMB were conducted according to the OECD guideline 301f.[36] The activated sludge was taken from a municipal wastewater treatment plant (Hera) in Ravenna (Italy), aerated at rt for seven days, and then diluted 1:10 in the synthetic medium. The synthetic medium was prepared with 8.5 mg L^{-1} KH_2PO_4, 21.75 mg L^{-1} K_2HPO_4, 22.13 mg L^{-1} $Na_2HPO_4{\cdot}2H_2O$, 1.7 mg L^{-1} NH_4Cl, 36.4 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 22.5 mg L^{-1} $MgSO_4{\cdot}7H_2O$, and 0.25 mg L^{-1} $FeCl_3$ (pH 7.2). Test solutions of MMB and MHB (65 and 84 mg/L, respectively) were inoculated and then continuously stirred with a magnetic bar in the dark. A blank sample (inoculum without any chemicals) was prepared, too. Oxygen consumption was monitored over 28 days. Experiments were performed in duplicate. See the ESI for the biodegradation graph (Figure S1).

Biomass Dry Weight, PHB Amounts in Microbial Cells, and PHB Purity. Biomass dry weight was measured as volatile suspended soils (VSS) as described in Standard Methods.[37] The overall amount of PHB in microbial cells and PHB purity were quantified through low-temperature thermolysis as previously described in the literature.[38] The quantification of the developed crotonic acid was performed by GC−MS, using 2-ethylbutanoic acid as an internal standard.

The Molecular Weight and Polydispersity Index. The average molecular weight and polydispersity of the extracted PHB were determined in $CHCl_3$ solution by size exclusion chromatography (SEC) using an HPLC Lab Flow 2000 apparatus working with a 1 mL min^{-1} flow, equipped with a Rheodyne 7725i injector, a Phenomenex Phenogel 5u 10E6A column, and a Knauer RI K-2301 RI detector. Each sample was filtered with a 0.45 μm porosity Teflon filter, and the sample injection volume was set to 20 μL. Calibration curves were obtained using several monodisperse polystyrene standards in the range 0.2−3 MDa.

GC−MS Analysis. GC−MS analyses of reaction mixtures for MC and MMB synthesis and for PHB quantification (amount in cells and purity) were performed using an Agilent 7820A gas chromatograph connected to an Agilent 5977E quadrupole mass spectrometer. The injection port temperature was 280 °C. Analytes were separated on a DB-FFAP polar column (Agilent J&W nitroterephthalic acid-modified polyethylene glycol DB-FFAP; 30 m, 0.25 mm, and 0.25 μm), with a helium flow of 1 mL min^{-1}. Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 29−450 m/z range. The thermal program was 50 °C for 5 min then 10 °C min^{-1} to 250 °C kept for 5 min.

Nuclear Magnetic Resonance. ^{1}H NMR spectra of extracted PHB and synthesized products and solubility tests of MHB and MMB were

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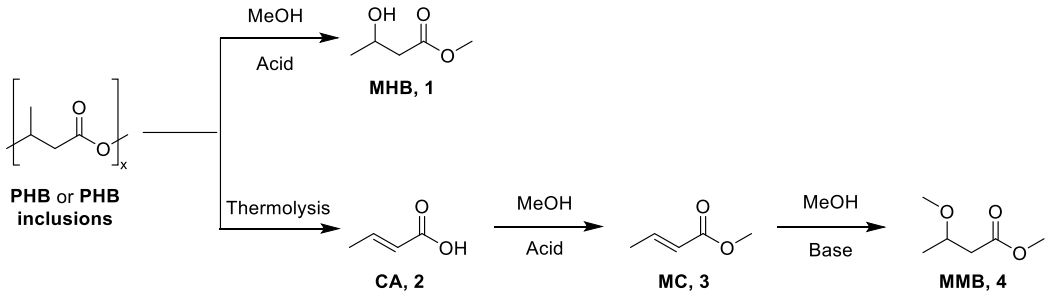
Scheme 1. MHB and MMB Synthetic Pathways from PHB



recorded on a Varian 400 (400 MHz) spectrometer. Chemical shifts were reported in ppm from TMS with the solvent resonance as the internal standard ($CDCl_3$, 7.24 ppm; D_2O, 4.79 ppm).

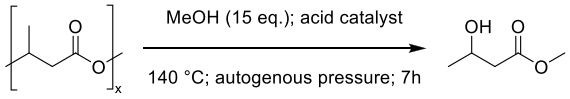
Elemental Analysis. The elemental analysis of MHB, MMB, and extracted PHB was determined using an elemental analyzer (Thermo Scientific, Flash 2000, organic elemental analyzer) through the flash combustion technique.

RESULTS AND DISCUSSION

Solvent Synthesis. MHB and MMB were synthesized starting from commercial PHB or PHB inclusions as reported in Scheme 1.

Methyl 3-Hydroxybutyrate (MHB) from Commercial PHB and PHB Inclusions. Methanolysis of pure PHB was conducted in the presence of acidic or basic catalysts and methanol at 140 °C under autogenous pressure. Various catalysts were tested including $Sn(Bu)_2(OAc)_2$, H_2SO_4, TsOH, $SnCl_2$, CaO, NaOMe, or NaOH, finding that only the first three were effective in totally converting PHB into MHB and oligomers (Table 2, see the ESI for detailed optimization reaction conditions, Table S1). TsOH gave a considerable quantity of oligomers after 7 h of reaction giving a 79% yield of MHB after distillation; $Sn(Bu)_2(OAc)_2$ and H_2SO_4 were the best performing catalysts giving respectively 87 and 95% yields of MHB after isolation (Table 2, entries 2 and 3). Since both $Sn(Bu)_2(OAc)_2$ and H_2SO_4 are frequently used in industrial applications,[39] they can be considered promising catalysts for this transesterification reaction, considering the low loading used (0.5 mol %). H_2SO_4 was selected as the catalyst of choice because of the higher yield and lower cost. A procedure for catalyst recovery and recycle was developed: the recyclability of H_2SO_4 was demonstrated avoiding to quench the acid catalyst with a base and reusing the reaction mixture as it was after distillation of MeOH and MHB (see the ESI for details). With this procedure, the MHB yield decreased from 95 to 82% in the first cycle, and this was probably due to the residual activity of H_2SO_4, which after removal of MeOH catalyzes transesterification between MHB molecules producing non-distillable oligo-esters, thus lowering the initial yield. However, in the second cycle, the residual oligo-esters are converted back to MHB by the addition of MeOH; this allows the second to fifth cycles to reach a higher yield up to 96% (on a PHB input basis) (see ESI, Table S2). H_2SO_4 was thus applied to PHB inclusions inside bacterial cells (SSC-57); in this case, it was necessary to increase the catalyst loading from 0.5 to 10 mol % (Table 2, entry 5) to obtain a good yield, probably due to the alkalinity derived from the bacterial medium and their cellular matrix composed by proteins, phospholipids, and water inside the cytoplasm,[40] which could quench the H_2SO_4 activity. High purity is obtained following the reported procedure both from pure and bacterial PHB, as confirmed by spectroscopic and elemental analyses (reported in the ESI).

Table 2. MHB Synthesis from Pure PHB or Inclusions in Bacterial Cells



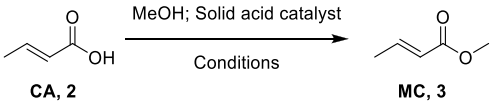
entry	sample	catalyst	cat. loading (mol %)	MHB isolated yield (%)
1[a]	PHB	TsOH	0.5	79
2[a]	PHB	$Sn(Bu)_2(OAc)_2$	0.5	87
3[a]	PHB	H_2SO_4	0.5	95
4[a]	SSC-57	H_2SO_4	0.5	10[b]
5[a]	SSC-57	H_2SO_4	10	85[b]

[a]Reaction conditions: 3 g of PHB, MeOH (21.2 mL), 140 °C, autogenous pressure, and 7 h. [b]The yield is calculated with respect to the PHB content inside bacterial cells.

Methyl 3-Methoxybutyrate (MMB) from Commercial PHB and PHB Inclusions. As depicted in Scheme 1, MMB was obtained via a three-step synthesis:

1. PHB thermolytic distillation affording CA;
2. esterification with MeOH via heterogeneous acid catalysts;

Table 3. Synthesis of MC through the Esterification of CA



entry	catalyst	cat. loading (wt %)	MeOH equiv.	temperature (°C)	time (h)	conversion (%)[a]	isolated yield (%)
1	Amberlyst-15H	10	15	reflux	24	50	
2	Amberlyst-15H	2.5	5	130	7	96	89
3	C-SO_3H	2.5	5	130	7	>99	93

[a]Conversion obtained by GC–MS analysis.

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3. base-catalyzed oxa-Michael addition of MeOH.

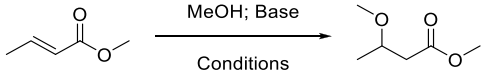
Recently, we reported that PHB thermolytic distillation is a very convenient technique to obtain high-purity CA from PHB in high yields (95%).[27] In this study, we focus on the optimization of the esterification and oxa-Michael addition steps. Even if MC is an already known ester,[41,42] not much is reported in the literature regarding alternative ways for its synthesis from crotonic acid, apart from the use of high loadings of H_2SO_4.[43]

Therefore, to facilitate the workup, two acidic heterogeneous catalysts were tested: Amberlyst 15-H, a commercial acidic resin, and a recyclable catalyst prepared from the char produced through pyrolysis and sulfonation of starch (C-SO_3H) that we already described.[33] Both gave a high conversion of MC after 7 h at 130 °C under autogenous pressure (Table 3, entries 2 and 3, see the ESI for detailed optimization reaction conditions, Table S3). Harsh conditions are thus necessary because of the presence of the double bond in the α,β position that deactivates the carboxylic group; in fact, by using simply reflux conditions, lower conversion was observed (Table 3, entry 1). On the other hand, lower equivalents of MeOH and lower catalyst loadings were enough for an almost quantitative conversion (Table 3, entries 2 and 3).

MMB was obtained through oxa-Michael addition of MeOH to the double bond of MC. Since this kind of reaction is base-catalyzed,[44] various bases were tested to optimize the reaction conditions in terms of MeOH and the catalyst used:

Alkoxides were the most active catalysts (Table 4, entries 3 and 6, see the ESI for detailed optimization reaction conditions, Table S4), providing almost quantitative yields of MMB; both showed the same reactivity due to the fact that NaOtBu forms NaOMe in situ from MeOH, and this is the real catalyst for both reactions.

Table 4. Synthesis of MMB from MC through the Oxa-Michael Reaction



MC, 3 MMB, 4

entry	base	conversion[a]	isolated yield (%)
1[b]	Na_2CO_3	0	/
2[b]	K_2CO_3	82	/
3[b]	NaOMe	98	93
4[b]	NaOH	48	/
5[b]	KOH	42	/
6[b]	NaOtBu	97	94
7[c]	NaOMe	98	92

[a]Conversion obtained by GC−MS analysis. [b]Reaction conditions: MeOH (2.5 equiv.), catalyst (10 mol %), rt, and 24 h. [c]Reaction conditions: MeOH (2 equiv.), catalyst (3 mol %), rt, and 50.

K_2CO_3 behaved similarly to alkoxides, whereas Na_2CO_3 was not active at all (Table 4, entries 1 and 2) probably because of its 10 times lower solubility in MeOH than K_2CO_3 (0.27 vs 3.11 g/100 g).[45,46]

Hydroxides (NaOH and KOH) were not suitable catalysts because of the formation of water during the reaction that reasonably caused the hydrolysis of MC and the quenching of the catalyst (Table 4, entries 4 and 5).

After the optimization of the oxa-Michael reaction using NaOMe, it was possible to obtain a 92% isolated yield of MMB using 2 equiv. of MeOH and 3 mol % of the base (Table 4, entry 7).

MMB was also synthesized from PHB inclusions in bacterial cells. As previously reported,[27] it was possible to produce CA of high purities (up to 97%) from the depolymerization of PHB inside bacterial cells at different concentrations (30 to 60%) through thermolytic distillation. The esterification of CA produced from bacterial inclusions was performed using the same reaction conditions developed for CA produced from pure PHB, with no changes in yields (91%). MC produced from PHB inclusions was analogously converted into MMB with a 90% yield using NaOMe as a catalyst. Under optimized conditions, the percentage of PHB inside bacterial cells needed to produce MMB was at least 30%; lower percentages would have resulted in too low yields of CA making the process unfavorable. High purity is obtained following the reported procedures, both from pure PHB and PHB inclusions, as confirmed by spectroscopic and elemental analyses (ESI). Representative ^{1}H NMR spectra of both products (MHB and MMB) are reported in Figure 2.

The Environmental Factor (*E*-Factor) for MHB and MMB Synthesis. In order to preliminary evaluate the environmental sustainability of the processes for the production of MHB and MMB, the *E*-factor was calculated, taking into consideration MeOH and catalyst recovery and recyclability (Table 5). The *E*-factor was calculated both with and without water as waste for CA esterification since both ways are currently taken into consideration.[34] The processes have been compared in terms of the starting material used (PHB or SSC-57); in all cases, *E*-factors are very promising. When PHB is used, the *E*-factors are lower than 0.1 for MHB and from 0.15 to 0.34 for MMB (depending on water inclusion in the calculation in the second step). When PHB inclusions are used, the *E*-factor increases since NPCM (average 40% of all the initial mass of the starting material) can be considered a waste, and the *E*-factor is around 1 for both processes. On a larger scale, NPCM can be recycled back in the PHB production process, thus diminishing its impact.

Solubility in Water, Octanol/Water Partition Coefficients, and Degradability of MHB and MMB. Both MMB and MHB were investigated in terms of solubility in water, octanol/water partition coefficients (log K_{ow}), and biodegradability (Table 6). As expected, solubility tests confirmed that MHB is totally miscible with water at 20 °C due to the presence of the free hydroxyl group. The strong affinity with H_2O is also confirmed by the low octanol/water partition coefficient obtained. As to less polar MMB, its solubility in water at 20 °C resulted in 145 g/L with a log K_{ow} of 1.43. Biodegradability tests, performed following the OECD guideline 301f, revealed that both MHB and MMB are readily biodegradable since their biodegradation values over 28 days were 72 and 73.5%, respectively (see ESI, Figure S1).

PHB Extraction. *Extraction of PHB from Freeze-Dried SSC or MMC.* A preliminar screeening of PHB extraction conditions was performed on freeze-dried *C. necator* (SSC) containing 56.7 ± 2 wt % PHB, aiming at setting the best extraction temperature, solvent amount, and time. The extraction protocol consisted of a PHB-to-solvent ratio of 2.6% (w/v) (47 mg of biomass with 56.7% PHB corresponding to 26 mg of PHB and 1 mL of the solvent). The biomass and the solvent were loaded in a 4 mL centrifugue tube with a

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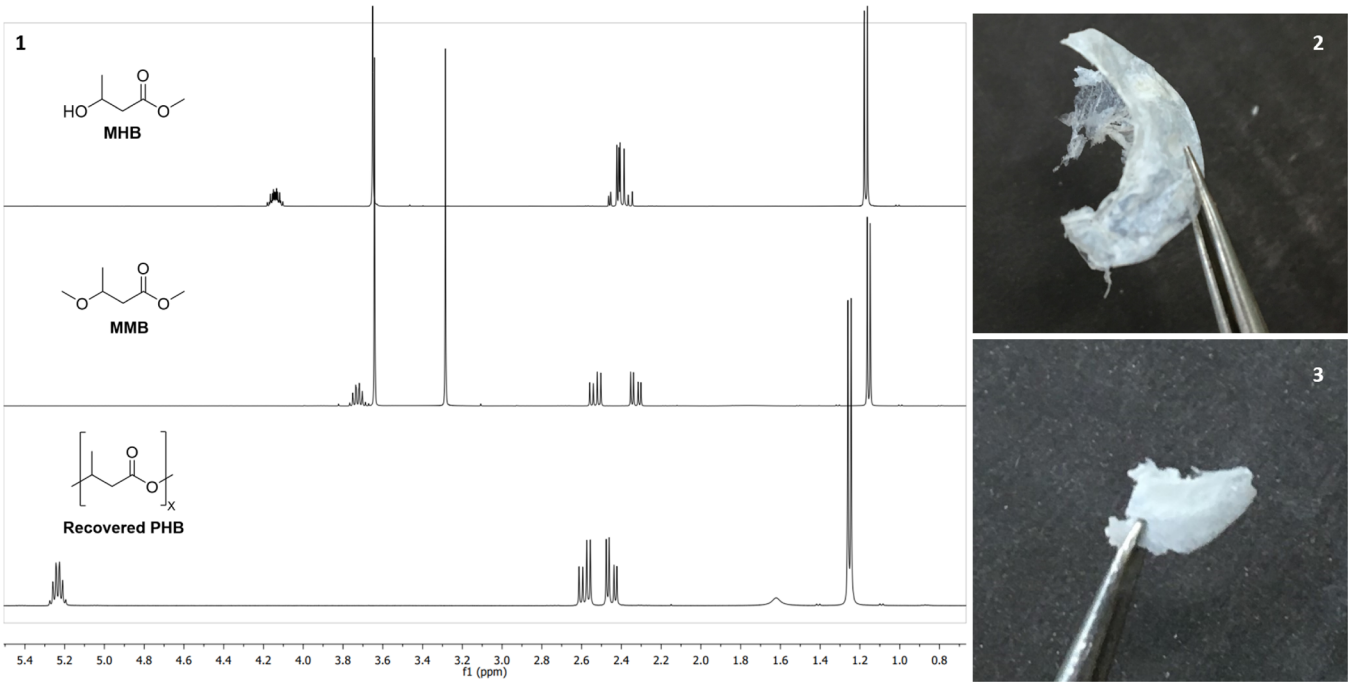

Figure 2. (1) ^{1}H NMR spectra of MHB and MMB from PHB and PHB after extraction. (2) Recovered PHB using MHB. (3) Recovered PHB using MMB.

Table 5. *E*-Factors for MHB and MMB Processes

E-factor				
MHB starting material				total process
PHB[a]				0.072
SSC-57				0.97
MMB starting material	thermolytic distillation	CA esterification (including H_2O)	oxa-Michael	total process (including H_2O)
PHB	0.05	0.045 (0.23)	0.058	0.153 (0.338)
SSC-57	1.24	0.045 (0.23)	0.058	1.343 (1.528)

[a]Calculated following experimental data over 5 cycles for catalyst recycle in MHB synthesis (see ESI, Table S2).

Table 6. Solubility in Water, log K_{ow}, and Biodegradability of MHB and MMB

compound	solubility in H_2O (g/L)	log K_{ow}	biodegradability (%)
MHB	miscible	0.83	72
MMB	145	1.43	73.5

stirring bar and stirred for a specific time and temperature. Both MMB and MHB resulted in effective PHB extraction at temperatures above 115 °C, with 49 and 71% recoveries of PHB, respectively, in 1 h (Table 7, entry 4). The recovery

Table 7. Optimization of PHB Extraction from SSC-57

		PHB recovery (%)	
entry	*T* (°C)	MMB	MHB
1[a]	25	0	0
2[a]	70	0	0
3[a]	100	6	0
4[a]	115	49	71
5[b]	130	98	95

[a]Extraction performed for 60 min. [b]Extraction performed for 10 min.

became even more efficient when the temperature was increased up to 130 °C: in just 10 min, MMB and MHB gave recoveries of 98 ± 1 and 95 ± 1%, respectively (Table 7, entry 5).

Since the boiling temperatures of MMB and MHB (148−149 and 173−174 °C, respectively) are higher than the selected extraction temperature, an autogenous pressure was not necessary to recover quantitatively PHB, contrarily to what is needed with dimethyl carbonate and acetone.[10,47] With MC being an intermediate for the production of MMB, it was tested in the extraction, too. Since its boiling point (120 °C) is lower than the temperature at which both MMB and MHB gave the best results (130 °C), reflux conditions were used; the recovery of PHB was 54 ± 2% in 1 h, much lower than those of MMB and MHB. After setting the best recovery conditions for PHB extraction with MMB and MHB, the efficiency of these solvents was tested on SSC containing a lower amount of PHB (35 ± 2 wt %) since it is known that the extraction of PHB from bacterial cells with a low PHB content is more challenging than the extraction from PHB-rich bacteria. MMB and MHB excellently behaved also in this case, allowing the recoveries of 97 ± 2 and 96 ± 1% PHB in 10 min, respectively (Table 8). The same good results were obtained performing extraction on MMC with a low-medium content of PHB (22−39 wt %): 98 ± 1 (MMB) and 94 ± 2% (MHB) PHB was recovered from MMC-22, while 98 ± 2 (MMB) and 96 ± 3% (HMB) recoveries were obtained extracting MMC-39 (Table 8). Both solvents were recycled more than 10 times after every extraction procedure: while the MMB recovery was almost quantitative at every cycle (98 ± 2%), the MHB recovery was systematically lower (89 ± 2%). No changes in the extracting ability were observed for both MHB and MMB at every cycle (see the ESI for detailed data about solvent recycles, Tables S5 and S6). Dichloromethane (CH_2Cl_2) was used as a benchmark solvent for extracting both SSC-57 and

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Table 8. Extraction of SSC and MMC Containing Different Amounts of PHB with MMB, MHB, MC, and DCM (PHB-to-Solvent Ratio Maintained Constant = 26 mg/mL)

	PHB recovery (%)				
solvent	SSC-57	SSC-35	MMC-22	MMC-39	MMC-39 slurry
MMB (10 min, 130 °C)	98 ± 1	97 ± 2	98 ± 1	98 ± 2	92 ± 2
MHB (10 min, 130 °C)	95 ± 1	96 ± 1	94 ± 2	96 ± 3	77 ± 2
MC (60 min, 118 °C)	54 ± 2				
DCM (10 min, 60 °C)	97 ± 3		95 ± 1		

Table 9. Physical Characteristics of PHB Obtained after 130 °C and 10 min Extraction of Freeze-Dried Biomass or Microbial Slurry with MMB and MHB, in Comparison to Commercial PHB and PHB Extracted with CH_2Cl_2 (10 min, 60 °C)[d]

sample origin	purity (%)[a]			$\overline{M_w}$ (MDa)			PDI		
commercial PHB	98 ± 2			0.8			5.9		
	MMB	MHB	DCM	MMB	MHB	DCM	MMB	MHB	DCM
SSC (freeze-dried)[b]	98 ± 2	95 ± 2	94 ± 3	2.3	1.1	2.1	3.9	2.8	3.3
MMC (freeze-dried)[c]	98 ± 1	96 ± 1	94 ± 2	3.3	1.6	3.5	4.5	4.6	3.1
MMC (slurry)[c]	97 ± 1	94 ± 2	/	3.2	1.4	/	4.7	4.8	/

[a]Evaluated by GC−MS. [b]PHB content in cells: 56.7 ± 2 wt %. [c]PHB content in cells: 39 ± 1 wt %. [d]The data are expressed as the mean ± standard deviation of three independent replicates of each extraction condition ($\overline{M_w}$ average molecular weight; PDI, polydispersity index).

MMC-22: the recovery after 10 min of extraction at 60 °C was 97 ± 3% from SSC and 95 ± 1% from MMC (Table 8).

Extraction of PHB from MMC Slurry. MMB and MHB were tested on microbial slurry, which is known to be more difficult to be extracted than freeze-dried biomass due to the presence of water that can create a barrier between cell membranes and the solvent itself. MMC slurry (containing 39 ± 1 wt % PHB on dry weight and a water content of 79 ± 2%) was extracted at 130 °C for 10 min with the same PHB-to-solvent ratio used for freeze-dried biomass extraction (26 mg of PHB/mL of the solvent). While MMB maintained almost unaltered its activity, recovering 92 ± 2% PHB, MHB had a drop in activity, recovering 77 ± 2% PHB (Table 8). This divergence between MMB and MHB while operating in wet conditions could be explained by their different affinity with water contained in the microbial slurry: as previously discussed, MHB is totally miscible with water, while MMB has a solubility of 145 g/L and its lower affinity with water can explain its better extraction ability.

Characterization of the Extracted PHB. The purity of the PHB extracted from SSC and MMC with both MMB and MHB was determined through low-temperature thermolysis as previously described in the literature,[38] and it was comparable to that of the commercial PHB (94−98 vs 98%, Table 9), confirming the suitability of both solvents in providing high-quality PHB. A representative ^{1}H NMR spectrum of the recovered polymer is reported in Figure 2.

The average molecular weight of the recovered polymers with MMB resulted in between 2.3 and 3.3 MDa, in line with what was obtained with CH_2Cl_2 and almost 3 times higher than the molecular weight of commercial PHB used here. On the other hand, MHB halved the molecular weight, and this evidence was observed independently on the bacterial types (SSC or MMC) and their water content (freeze-dried biomass and microbial slurry). MHB behavior can be explained by the reactivity of its hydroxyl group that at high temperatures could lead to transesterification side reactions on the polymer ester bonds, breaking PHB chains and lowering the molecular weight to half of the original value; this can also explain why the MHB recovery after every cycle is not quantitative. Notwithstanding the transesterification, the average $\overline{M_w}$ attained upon solvent extraction of SSC or MMC biomass is significantly high: this demonstrates the ability of both solvents to extract even the polymers with a very high molecular weight and crystallinity, which usually represent the most difficult fraction to recover. As to the PDI, PHB-derived solvents gave slightly higher values than CH_2Cl_2, while compared to commercial PHB, lower values were obtained. Differences in the M_W and PDI reflect on the aspect of the recovered PHB. In fact, PHB derived from MHB extraction appears as a very thin and bulky film, while a more compact solid was obtained with MMB (Figure 2). No solvent traces were found by ^{1}H NMR in all the extracted polymers (Figure 2).

CONCLUSIONS

In the present study, two new protocols for the chemical recycling and valorization of PHB through bio-polyester depolymerization and functionalization are proposed, producing methyl 3-hydroxybutyrate (MHB) and methyl 3-methoxybutyrate (MMB). Both protocols showed high efficiency in terms of the isolated yield and purity. The reported procedures were then applied to PHB inclusion in bacterial cells, to valorize low-quality batches of PHB, and similar results to pure PHB were obtained. *E*-factors for the synthetic pathways of both MHB and MMB were calculated showing the promising environmental sustainability of the process in terms of waste generated.

The solvents have been tested in PHB extraction from both single strain and mixed microbial cultures. Both MHB and MMB showed a high recovery from freeze-dried microbial biomass, and good efficiency was observed also on microbial slurry. No changes in efficiency were observed with MMB, while a 15% decrease in recovery was observed with MHB, which is explainable by its higher affinity with water. The purity of the recovered PHB was high with both the solvents and comparable to the performance of chlorinated solvents. The molecular weight of the recovered PHB was above 1 MDa with both the solvents.

All these results, together with the excellent recyclability of both solvents and their biodegradability, suggest the possibility

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to generate a new way to chemically recycle PHB to produce C4 molecules that can be applied for the extraction of PHB itself in a circular perspective. Both MMB and MHB can thus be used as "bio-based" key tools for downstream PHB processing, opening the possibility of performing this crucial step in a more environment-friendly way than the use of fossil-based solvents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c03299.

MHB, MMB, and MC synthesis, screening conditions, and characterization; MHB and MMB biodegradation and recyclability tests (PDF)

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Notes

The authors declare no competing financial interest.

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