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Extraction of polyhydroxyalkanoates from mixed microbial cultures: Impact on polymer quality and recovery

Chiara Samorì [a,*], Federica Abbondanzi [a], Paola Galletti [a,b], Loris Giorgini [c,d], Laura Mazzocchetti [d], Cristian Torri [a,b], Emilio Tagliavini [a,b]

[a] Centro Interdipartimentale di Ricerca Industriale Energia Ambiente (CIRI EA), University of Bologna, via S. Alberto 163, 48123 Ravenna, Italy
[b] Dipartimento di Chimica "Giacomo Ciamician", University of Bologna, via Selmi 2, Bologna, Italy
[c] Dipartimento di Chimica Industriale "Toso Montanari", University of Bologna, viale del Risorgimento 4, Bologna, Italy
[d] Centro Interdipartimentale di Ricerca Industriale per la Meccanica Avanzata e i Materiali (CIRI MAM), University of Bologna, Viale Risorgimento 2, Bologna, Italy

HIGHLIGHTS

- PHA-producing MMCs are more resistant than single strain cultures to cell breakage.
- Standard PHA extraction protocols are ineffective on MMCs.
- Total costs of PHA production could be affected by PHA extraction protocols.
- PHA extraction protocols on MMCs are strictly connected to polymer properties.

ABSTRACT

Polyhydroxyalkanoates (PHAs) can be extracted from mixed microbial cultures (MMCs) by means of dimethyl carbonate (DMC) or combination of DMC and sodium hypochlorite (NaClO). The protocol based on DMC, a green solvent never used before for the extraction of PHAs from MMC, allows an overall polymer recovery of 63%; also the purity and the molecular weight of the recovered polymers are good (98% and 1.2 MDa, respectively). The use of NaClO pretreatment before DMC extraction increases the overall PHA recovery (82%) but lowers the mean molecular weight to 0.6–0.2 MDa. A double extraction with DMC results to be the method of choice for the recovery of high quality PHAs from attractive but challenging MMCs.

1. Introduction

Polyhydroxyalkanoates (PHAs) are promising polyesters, produced by bacteria through aerobic fermentation of various carbon sources. These biopolymers are completely biodegradable under aerobic and anaerobic conditions and have elastomeric/thermoplastic properties, which are tunable according to actual co-monomer composition. However, despite the efforts put towards the development of cost-effective fermentative systems, PHA production cost still remains considerably high (~5–6 $/kg), hampering the exploitation of these biopolymers as commodity materials. In recent years, it has been claimed that the use of mixed microbial cultures (MMCs) could represent an alternative and cheap strategy for producing PHAs (Salehizadeh and Van Loosdrecht, 2004). In fact, MMCs do not require sterile conditions and have a wider metabolic potential than single strains (e.g., *Cupriavidus necator* or genetically modified *Escherichia coli*). These two benefits allow to reduce equipment costs (Liu et al., 2013; Moita and Lemos, 2012) and to exploit a large number of cheaper substrates such as wastes (Albuquerque et al., 2010; Carvalho et al., 2014; Jia et al., 2014; Moita et al., 2014a,b), by-passing the use of expensive carbon sources for feeding the bacteria.

Until now, the major research efforts in the field of MMCs have been focused on: (i) the improvement of PHA storage capacity of the bacteria (by adopting multi-steps cultivation strategies), (ii) the use of cheap wastes and feedstock as carbon source (e.g., poultry litter, glycerol, molasses, grass), (iii) the development of continuous process at lab or pilot scale. However, as underlined by Serafim et al. (2008a), little information is available on the characterization of PHAs extracted from MMCs and, above all, on the efficiency of the polymer recovery in the extraction step (Patel et al., 2009). As it is well described in the literature, obtaining PHAs from bacteria through a series of downstream steps (e.g. microbial biomass

Table 1
Summary of the published protocols for the extraction of PHA from MMCs.

Entry	Extraction approach	PHA recovery (%)	PHA $\overline{M}_w$ (MDa)	References
1	Acidic treatment and then acetone (125 °C, 2 h)	n.d.	0.2–0.5	Laycock et al., 2014
2	HCl (3 M, 15 min) and then $CHCl_3$ (3 d, 37 °C)	n.d.	0.2–0.4	Duque et al., 2014
3	HCl (2 M, 10 min) and then $CHCl_3$ (37 °C, 72 h)	n.d.	0.2–0.5	Martínez et al., 2011
4	NaClO (5% Cl_2, 24 h) or NaOH (1 M, 24 h)	80–100	0.3–0.5	Villano et al., 2014
5	Acetone (15 min, reflux) and then $CHCl_3$ (16 h, reflux)	n.d.	0.2–0.4	Hu et al., 2013
6	$CHCl_3$ (100 °C, 2 h)	n.d.	0.4–0.9	Bengtsson et al., 2010
7	Acetone (reflux, 3 h) and then CH_2Cl_2/H_2O (reflux, 30 min)	18–30	2.2	Patel et al., 2009
8	$CHCl_3$ (20 h)	n.d.	2.1–3.4	Serafim et al., 2008
9	$CHCl_3$ (Soxhlet)	n.d.	0.1–0.4	Dai et al., 2007
10	$CHCl_3$ (20 h)	n.d.	0.1–0.9	Lemos et al., 1998

pretreatment, polymer extraction and post-treatment purification) could be challenging and expensive (Jacquel et al., 2008). Specifically, the downstream cost can cover almost 50% of the total production costs, involving an extensive use of non-recyclable (and sometimes highly toxic) chemicals/materials and a high energy consumption (Samorì et al., 2015). Moreover, it is worth mentioning that operating with MMCs introduces a further issue: MMCs are claimed to be more resistant to cell hydrolysis than pure cultures (in which genetic manipulation and/or cell constrains, due to high polymer amount, increase cellular fragility), thus the effort required for PHA extraction/purification can become much more relevant than in the case of single strains. Patel et al. (2009), for example, have hypothesized the existence of a PHA fraction difficult to extract and of a strong and complex Non Polymer Cellular Matrix (NPCM) that surrounds the polymer, preventing the access of the solvent to polymer granules.

The protocols reported in the literature for the extraction of PHA from MMCs (Table 1) can be grouped into two strategies: an optional cell pretreatment under acidic conditions (Entries 1–3, Table 1) followed by solvent extraction (e.g., with acetone or chlorinated compounds, Entries 5–10, Table 1) or a treatment with strong oxidants (e.g., NaClO) or bases (e.g., NaOH) to disrupt the NPCM and release the polymer stored inside the cells (Entry 4, Table 1).

As clearly underlined from the summary reported in Table 1, the use of organic solvents allows a poor PHA recovery (18–30%) but the achieved polymers maintain a high molecular weight (entry 7). On the other hand, the use of strong oxidants such as NaClO provides an excellent recovery (~100%) of a shortened polymer (entry 3), due to alkaline hydrolysis. The range of molecular weights reported in the literature is wide, varying from 0.2 to 0.4 MDa (entry 2) to above 3 MDa (entry 8), but it is not always clear if the low M_w values reported in some cases are related only to some decomposition of the polymer or they are also a specific feature of the polymers produced by MMCs under certain growth conditions.

The aim of the present paper is to develop new and sustainable protocols for the extraction of PHA from MMC, alternative to the current approaches based on the use of environmentally concerned solvents (e.g., chlorinated compounds) or harsh reagents (e.g., NaClO). Thus, novel extraction protocols based on dimethyl carbonate (DMC) and various cell pretreatments were tested, taking into account their impact on polymer quality and recovery. This work demonstrated that the impact of polymer extraction step, in the in the overall analysis of PHA production process from MMCs, should not be overlooked and different extraction strategies were compared for solving this key-issue.

2. Methods

All solvents and chemicals used in this study were obtained from Sigma–Aldrich (purities ⩾ 98%) and used without purification. Standard poly(3-hydroxybutyrate) P(3HB) was purchased from Biomer® (DE).

2.1. Microbial biomass cultivation

2.1.1. Mixed microbial cultures: growth conditions and PHA accumulation

A lab-scale sequencing batch reactor (SBR, 5 L working volume), inoculated with an activated sludge from a municipal wastewater treatment plant (located in Ravenna, Italy), was set up for the selection and PHA-enrichment of MMCs. The following mineral medium composition was prepared per liter of tap water: 600 mg $MgSO_4{\cdot}7H_2O$, 100 mg EDTA, 9 mg K_2HPO_4, 20 mg KH_2PO_4, 70 mg $CaCl_2{\cdot}2H_2O$ and 2 mL of trace element solution. The trace element solution consisted of (per liter of distilled water): 1500 mg $FeCl_3{\cdot}6H_2O$, 150 mg H_3BO_3, 150 mg $CoCl_2{\cdot}6H_2O$, 120 mg $MnCl_2{\cdot}4H_2O$, 120 mg $ZnSO_4{\cdot}7H_2O$, 60 mg $Na_2MoO_4{\cdot}2H_2O$, 30 mg $CuSO_4{\cdot}5H_2O$ and 30 mg of KI. The culture was fed with synthetic organic acids (acetic and propionic acid), to get an overall concentration of 5.5 g COD L^{-1} per day (C/N/P ratio of 100:10:1). The length of the SBR cycle was taken at 12 h (2 cycles per day).Each cycle consisted of an initial feeding phase, an aerobic reaction phase, a sedimentation phase and a withdrawal of depleted water. During the overall cycle, the SBR was stirred and aerated by means of an air stone. For bacteria selection, the reactor was operated at a temperature range of 23–26 °C for about 12 months.

The PHA accumulation tests (accumulation reactor, working volume of 1.5 L) were started after 3 months from the reaching of SBR stable operations and were operated in 6 h batch, as reported by Villano et al. (2014). Simultaneously with the discharge of the biomass from the SBR (1.5 L), the accumulation reactor was fed for 30 min with 100 mL of a more concentrated synthetic mixture of acetic (85%) and propionic (15%) acid (total COD approximately 40 g L^{-1}) and then aerated until the end of the cycle. At the end of the accumulation stage, a biomass slurry was sampled for measuring the biomass concentration and freeze-dried for PHA analyses. In order to obtain a suitable amount of freeze-dried biomass to carry out all extraction tests starting from the same sample, a mix of the best performing batch tests was composed and analyzed in quadruplicate for determining PHA amount and composition. This sample was also analyzed for the microbial community qualitative characterization.

2.1.2. Mixed microbial cultures: qualitative characterization of the microbial community

Microbial community DNA extraction from freeze-dried biomass was carried out using ZR Soil Microbe DNA MiniPrep™, Zymo Research, according to the manufacturer's instructions. In order to assess indicators of biodiversity, a PCR-DGGE analysis with further gene sequencing was carried out in outsourcing (Micro4yoUsrl, Milan, Italy), according to established protocols (Muyzer et al., 1993; Merlino et al., 2013; Piterina and Pembroke, 2013). After sequencing, 16S rRNA fragments were compared with National Center for Biotechnology Information (NCBI) Database, using BLAST Software (http://www.ncbi.nlm.nih.gov/blast).

2.1.3. C. necator DSM 545

C. necator DSM 545 was grown according to the procedure reported by Samorì et al. (2015). The freeze-dried biomass contained 54 ± 2 wt% of a co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-*co*-3HV), 3HB/3HV 80/20).

2.2. Microbial biomass pretreatments

2.2.1. Thermal pretreatment

Freeze-dried MMCs biomass (100 mg) was heated at 150 °C for 24 h in a closed tube, before extracting the polymer with dimethyl carbonate (DMC) (see Section 2.3.1). The experiment was repeated in duplicate.

2.2.2. Mechanical pretreatment

Freeze-dried MMCs biomass (100 mg) was suspended in water (5 mL); glass beads (1 g, ~0.5 mm diameter) were added to the microbial suspension that was sonicated with series of 10 pulses of 2 min each with a Sonicator Ultrasonic Liquid Processor (Heat Systems, model XL2020™).The glass beads were then removed and the microbial suspension was centrifuged at 4000 rpm for 10 min to remove the water phase and achieve a slurry. The polymer was extracted from this slurry with DMC (see Section 2.3.1). The experiment was repeated in duplicate.

2.2.3. Chemical pretreatment

Freeze-dried MMCs biomass (100 mg) was suspended into a sodium hypochlorite (NaClO) solution (10 mL, 5% Cl_2) and stirred at rt or 100 °C for 5, 15 or 60 min. Then the microbial suspension was centrifuged at 4000 rpm for 10 min to remove the water phase and achieve a slurry that was washed twice with water (10 mL). The polymer was extracted from this slurry with DMC (see Section 2.3.1). The experiment was repeated in quadruplicate.

2.3. Extraction of PHA from MMCs

2.3.1. Solvent extraction

Freeze-dried MMCs biomass (100 mg, pretreated or not) was extracted with DMC(4 mL, 1 h at 90 °C), propylene carbonate (PC, 4 mL, 4 h at 140 °C) or dichloromethane (CH_2Cl_2, 4 mL, 4 h at 50 °C) in a closed tube. The solutions were then centrifuged at 4000 rpm for 1 min and filtered with polypropylene membrane filters of 0.45 μm porosity. The polymer was recovered by solvent evaporation and then dried at 60 °C under vacuum overnight. Each extraction was performed in quadruplicate.

The Soxhlet extraction with DMC was performed by placing MMCs biomass (300 mg) into an extraction cellulose thimble that was covered with cotton wool and inserted into the Soxhlet extractor. The extraction was carried out with DMC (150 mL) for 24 h. The polymer was recovered by solvent evaporation and then dried at 60 °C under vacuum overnight.

A series of experiments was also performed by combining a further treatment of the residual biomass after an initial DMC treatment. These experiments were done in two ways:

1) By collecting the residual microbial biomass after DMC extraction and extracting it again with DMC (4 mL, 1 h at 90 °C). This solution was then treated as previously described.
2) By collecting the residual microbial biomass after DMC extraction and treating it with NaClO (15 min, rt or 100 °C); this solution was centrifuged at 4000 rpm for 10 min to remove the water phase and achieve a slurry which was washed twice with water (10 mL). The slurry was submitted again to DMC extraction (4 mL, 1 h at 90 °C) for recovering the polymer.

2.3.2. NPCM disruption with NaClO

Freeze-dried MMCs biomass (100 mg) was suspended into a NaClO solution (10 mL, 5% Cl_2) and stirred at 100 °C for 60 min. The mixture was then centrifuged at 4000 rpm for 10 min. The polymer was recovered on the bottom of the centrifuge tube, washed twice with water (4 mL) and once with ethanol (1 mL), then dried overnight at 60 °C under vacuum. Each NPCM disruption was repeated in quadruplicate.

2.3.3. NPCM disruption with sodium dodecyl sulfate (SDS)

The dissolution of NPCM with SDS was performed by adding freeze-dried microbial biomass (100 mg, form MMCs or *C. necator*) to a solution of sodium dodecyl sulfate (SDS, 200 mg) in water (6 mL). The mixtures were stirred at 90 °C for 3 h and then centrifuged at 4000 rpm for 10 min. The polymer was recovered on the bottom of the centrifuge tube, washed twice with water (4 mL) and once with ethanol (1 mL), then dried overnight at 60 °C under vacuum. Each NPCM disruption was repeated in quadruplicate.

2.4. Analyses

2.4.1. Biomass concentration and PHA amount in microbial cells

Biomass concentration was measured as Volatile Suspended Soils (VSS) as described in Standard Methods (APHA, 1995).

The overall amount of PHA in the cells and the relative abundances of HB and HV monomers were quantified as reported by Torri et al. (2014), using commercial *trans*-2-butenoic acid and *trans*-2-pentenoic acid as standards. After an off-line pyrolysis (CDS 1000 pyroprobe with a resistive heated platinum filament) of freeze-dried biomass (10 mg), the sample was spiked with internal standard solution and eluted with acetonitrile (CH_3CN). The CH_3CN solution was then analyzed using a GC–MS (6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer). Analytes were separated by a polar GC column (Agilent J&W nitroterephthalic-acid-modified polyethylene glycol DB-FFAP 30 m, 0.25 mm, 0.25 μm) with helium as carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200 °C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 12–600 m/z range. The injection port temperature was 280 °C. The thermal program was: 50 °C for 5 min, then 10 °C min^{-1} to 250 °C kept for 5 min.

2.4.2. PHA extraction yield

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

2.4.3. PHA purity (%)

The purity of the extracted PHA (%) was determined by thermogravimetric (TGA) analysis, applying the procedure reported in the literature by Hahn and Chang (1995) on PHA samples of 1–2 mg. Each analysis was performed in duplicate.

2.4.4. PHA recovery

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

$$\frac{\text{PHA extraction yield} \times \text{PHA purity}}{\text{PHA amount in microbial cells}}$$

2.4.5. Molecular weight and polydispersity index

The average molecular weight and polydispersity of the polymers were determined in chloroform ($CHCl_3$) solution by size exclusion chromatography (SEC) using an HPLC Lab Flow 2000 apparatus working with a 1 mL min^{-1} flow, equipped with an

injector Rheodyne 7725i, a Phenomenex Phenogel 5u 10E6A column and a RI detector Knauer RI K-2301. Each sample was filtered with a 0.45 μm porosity Teflon filter and sample injection volume was set to 20 μL. Calibration curves were obtained using several monodisperse polystyrene standards in the range between 0.2 MDa and 3 MDa.

2.4.6. Thermal analysis

Thermogravimetric (TGA) measurements were carried out using a TA Instruments SDT-Q600 instrument. The analyses were performed at 10 °C min^{-1} from rt to 500 °C under nitrogen flow. "T max deg" is defined as the temperature of the maximum weight loss rate.

2.4.7. Infrared spectroscopy

Infrared spectroscopy was carried out in ATR (Attenuated Total Reflectance) mode on a Bruker Alpha spectrophotometer equipped with ATR accessory with a diamond window.

3. Results and discussion

3.1. Mixed microbial cultures: growth conditions and PHA accumulation

The SBR operation period lasted about 150 days and, after about 100 days from the SBR start-up, a clear Feast and Famine regime was obtained (F/F ratio: 0.1, data not shown). This condition allowed the selection of an adapted MMC, specialized on intracellular PHA storage and exploited for the setting up of an accumulation reactor (SBR-Batch).

Fig. 1 shows the comparison between the performance of SBR and SBR-Batch in terms of biomass growth (VSS, g L^{-1}). The average VSS concentrations in the SBR and after accumulation tests were 2.3 and 3.6 g L^{-1}, respectively, according to what has been reported by Villano et al. (2014). An increase of PHA amount in microbial cells (from 19 to 42 wt%) occurred in the accumulation reactor (Fig. 2). At the end of the accumulation phase, a yield of 42 wt% of the co-polymer P(3HB-*co*-3HV) was obtained, with a 3HB/3HV ratio of about 80/20.

In order to obtain a suitable amount of freeze-dried biomass to carry out all extraction tests starting from the same sample, a mix of the best performing batch tests was composed and analyzed in quadruplicate for determining PHA amount and composition. An integrated biomass sample containing 40 ± 5 wt% of PHA, with 3HB and 3HV percentages of 76 and 24, respectively, was obtained.

3.2. Mixed microbial cultures: qualitative characterization of microbial community

PCR-DGGE and gene sequencing allowed to reveal that the microbial community used for the extraction experiments was mainly constituted by Alpha and Beta-Proteobacteria, Gram negative microorganisms. A dominance of genera *Azoarcus* and *Amaricoccus* was observed, together with the presence of genera *Thauera* (Table 2).

All these genera have been already found in activated sludge environments and their PHA storage capacity is well documented (Serafim et al., 2008b; Lemos et al., 2008). In fact, in an active sludge system, the microbial community has to be able to rapidly adapt to different inputs containing xenobiotic organic compounds

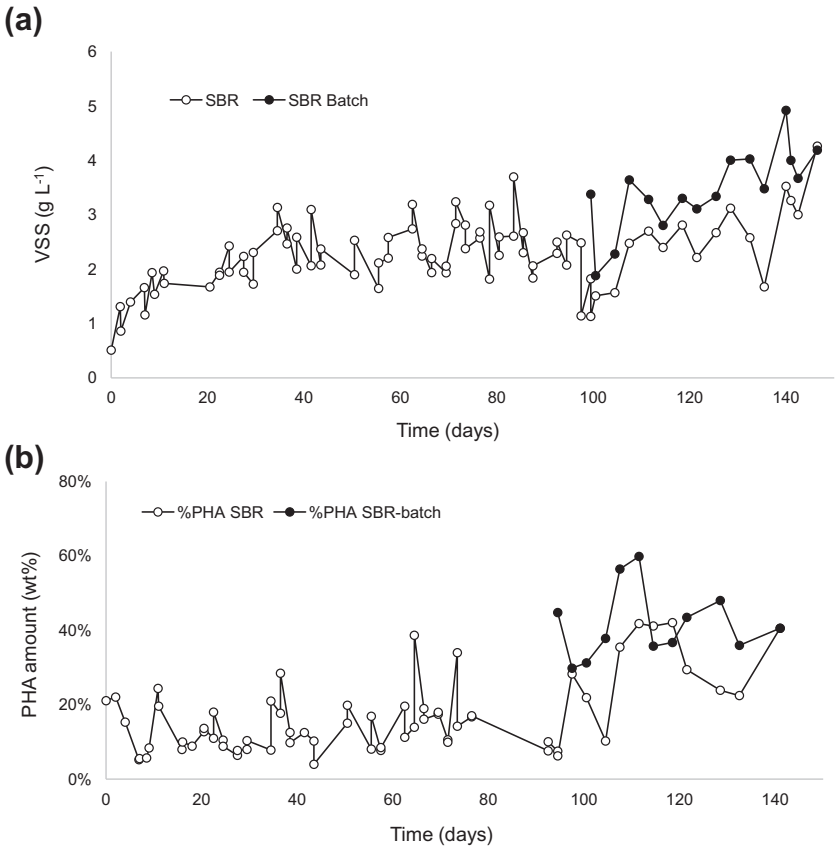

Fig. 1. (a) trend of biomass growth (expressed as VSS, g L^{-1}) in SBR and SBR-Batch over time; (b) trend of PHA amount in microbial cells (wt%) in SBR and SBR-Batch over time.

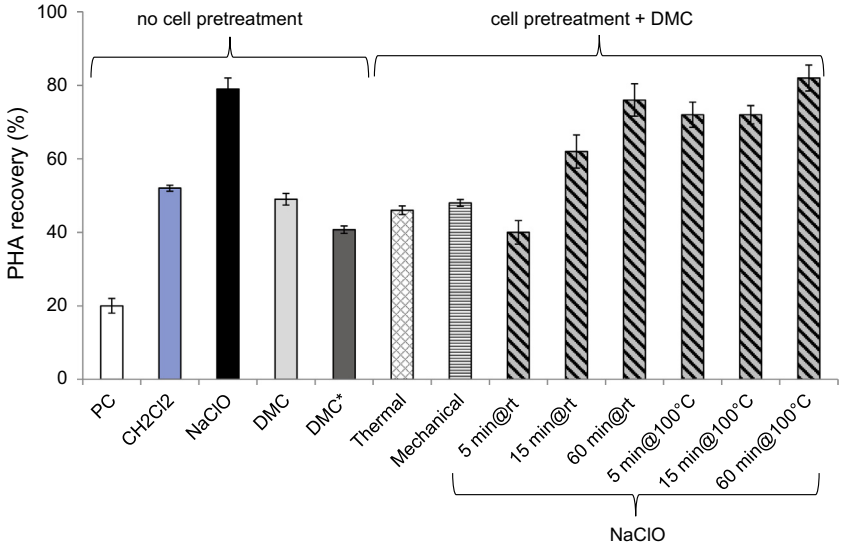

Fig. 2. PHA recovery (%) obtained after various treatments: extraction of non-treated cells with PC (□, 4 h at 140 °C), CH_2Cl_2 (■, 4 h at 50 °C), DMC (□, 1 h at 90 °C) and DMC* (□, 24 h, Soxhlet); disruption of NPCM with NaClO (■, 1 h at 100 °C); extraction with DMC (1 h at 90 °C) after a thermal (▨), mechanical (▤) and NaClO (▨) pre-treatment.

Table 2
Comparison between microbial community analysis of the mixed sample used for PHA recovery and a feast phase sample of the SBR. +: present; –: not present.

	Feast phase sample	Mixed sample for extraction
Azoarcus sp KH32C	+	–
Amaricoccus sp WTFO4	–	+
Uncultured *Azoarcus* sp	+	+
Azoarcus sp BH72 strain BH72	+	+
Azoarcus sp NSC3	+	+
Thauera terpenica 16S rRNA gene, strain 21 Mol	–	+

and, in certain conditions, store intracellular granules as energy and carbon reservoir (Seviour and Blackall, 1999).

Various studies demonstrated that the selection of *Thauera* and *Azoarcusspp* is favored when acetate is used as a carbon source, while propionate seems to favor *Amaricoccus* spp growth (Bergey's Manual, 2005; Serafim et al., 2008b; Albuquerque et al., 2010; Carvalho et al., 2014).

A comparison with microbial community analysis of a *feast* phase sample (Table 2) indicated that *Azoarcus* was the predominant genus, while *Amaricoccus* and *Thauera* were not present. This suggests that process parameters may influence the presence and abundance of different microorganisms, as already reported in other studies (Serafim et al., 2008b; Albuquerque et al., 2010;Carvalho et al., 2014).

3.3. Extraction of PHA from MMCs

3.3.1. Solvent extraction

In the present study various approaches for obtaining purified PHA from MMC biomass have been explored, including three cell pretreatments (thermal, mechanical and chemical) and the use of DMC, a good green alternative to chlorinated solvents for PHA extraction (Samorì et al., 2015).

Until now, chlorinated compounds (Bengtsson et al., 2010; Serafim et al., 2008a; Dai et al., 2007; Lemos et al., 1998) or a mixture of chlorinated and non-chlorinated solvents (Hu et al., 2013; Patel et al., 2009) have been used to extract PHAs from MMCs. Only Patel et al. (2009) reported the actual percentage of the recovered polymer with respect to the amount of PHA stored inside the microbial cells, stressing the fact that no more than 30% of the PHA produced by bacteria could be recovered in this way. This finding result is quite surprising, in particular when compared to the excellent recoveries obtainable with single strain cultures (~100%).

DMC resulted as efficient as CH_2Cl_2in the extraction of PHA, with polymer recoveries of 49 ± 2 and 52 ± 1 %, respectively (corresponding to PHA extraction yields of 20 ± 1 and 22 ± 1 wt%, respectively, calculated on biomass weight basis). PHA purities above 94% were obtained with both solvents (Table 3, entries 1 and 3). Moreover $\overline{M_w}$ measured for the two samples was perfectly comparable, together with PDI, confirming that both solvents were able to extract a very pure polymer without decreasing the molecular weight. These data are in line with the literature on *C. necator* (Samorì et al., 2015) and confirm two relevant aspects:

(1) The suitability of DMC for PHA extraction processes.
(2) The low PHA recoveries when organic solvents are used for extracting MMCs without any cell pretreatment. Although the performances of DMC and CH_2Cl_2 are comparable, it is worth pointing out that the data obtained in the current investigation are slightly better (recovery around 50%) than the literature ones (18–30%, Patel et al., 2009).

The efficiency of the process was not improved when the extraction with DMC was carried out for longer times (24 h *vs.* 1 h) under Soxhlet condition (recovery of 41 ± 1%).On the other hand, the purity of the obtained sample was definitely poorer than what was obtained in 1 h of extraction in closed tubes (Table 3, entry 2). Even the use of propylene carbonate (PC), described in the literature as an excellent solvent for PHA (Fiorese et al., 2009), was largely ineffective (Fig. 2). This finding confirms the scarce performance of PC in PHA extraction process without any cell pretreatment already found for the extraction of *C. necator* (Samorì et al., 2015).

Three pretreatments were also tested in order to improve the recovery percentage. The thermal (temperature >150 °C) and the mechanical (glass beads and sonication) pretreatments did not improve DMC extraction performance. Similar polymer recoveries were obtained in both cases (about 50%, Fig. 2).

On the other hand, a pretreatment of the microbial biomass with NaClO (5 min at 100 °C or 1 h at rt) followed by DMC extraction yielded recoveries of 72 ± 3% and 76 ± 4%, respectively (Table 3, entries 8 and 7). Harsher oxidizing/bleaching conditions (NaClO at 100 °C for 1 h) yielded the highest recovery (82 ± 3%, Table 3, entry 10).

In comparison, a disruption of the NPCM for 1 h at 100 °C with NaClO solely afforded a recovery of 79 ± 3%, thus very similar to what was obtained by combining this treatment followed by DMC extraction. However, the polymer purity was just 77% (Table 3, entry 4), thus definitely lower than what was obtainable if a further extraction step with DMC was applied (purity of 93%, entry 10).

The purity of the polymers extracted with DMC after various pretreatment conditions was overall quite high, ranging from 88% obtained by applying 1 h of NaClO at rt (Table 3, entry 7) to a maximum of 98% with 15 min of NaClO at rt (entry 6).

On the other hand, the time and the temperature of the chemical pretreatment strongly influenced the molecular weight of the recovered polymers. All pretreatments performed at rt (Table 3, entries 5–7) slightly reduced the $\overline{M_w}$ in comparison to the $\overline{M_w}$ of the polymer extracted with DMC (entry 1) or CH_2Cl_2 (entry 3). Furthermore, when the temperature of the NaClO pretreatment was increased to 100 °C, the observed $\overline{M_w}$ reduction was much more relevant, with the lowest value obtained after 1 h (entry 10, the longest treatment duration). It is indeed renown that NaClO has a strong detrimental effect on the molecular weight of PHAs, owing to its oxidizing properties and nucleophilicity. These, in turn, are the same features that make it a good agent for disrupting the NPCM (Kunasundari and Sudesh, 2011). The degrading effect on the polyesters is also emphasized by the increase in the PDI values upon treatment with NaClO, a sign of the polymer chains shortening due to some random chain scission. By comparing the disruption of the NPCM with NaClO solely (Table 3, entry 4) and the cellular pretreatment with NaClO followed by DMC extraction (entry 10), both performed for 1 h at 100 °C, it emerged that the use of DMC had an effect just on the purity of the resulting polymer, being the $\overline{M_w}$ and the PDI value very similar among them. Indeed, equal conditions of NaClO treatments, while helping in the disruption of the NPMC, had the same degrading effect on PHA and the additional DMC extraction step seemed to prevent NPCM residues to come along with the desired PHA.

It is noteworthy that the polymer recovered after DMC-Soxhlet extraction (Table 3, entry 2) had a three-times lower $\overline{M_w}$ (0.5 MDa) than what was recovered from the experiments performed for 1 h in the closed tubes. The poor performance obtained with the Soxhlet method might be attributed to some degradation that affected the polymer in such a long extraction time at 90 °C, as already reported by Dai et al. (2007) in the case of $CHCl_3$-Soxhlet extraction ($\overline{M_w}$ of 0.1–0.4 MDa, Table 1, entry 9). Under these conditions it is possible to hypothesize that hydrolysis promoted by the water still present in the slurry might dramatically reduce the $\overline{M_w}$.

The extracted co-polymer composition was similar for all tested conditions: the weight ratio between the HB/HV monomers was 65/35. This value was significantly different from the monomer ratio of the polymer stored inside the whole cells (76/24 weight ratio). This probably indicates that the tested extractions were slightly more effective on the HV richer PHAs.

A further series of experiments was devoted to the treatment of the microbial biomass residue after a first DMC extraction (named "second treatments") in order to improve the efficiency of the process (Fig. 3). In this way two batches of PHA were obtained: one after the first DMC extraction, named "first PHA" (refer to Table 3, entry 1), and one after the second treatment, named "second PHA". On the basis of the previous results, three second treatments were applied:

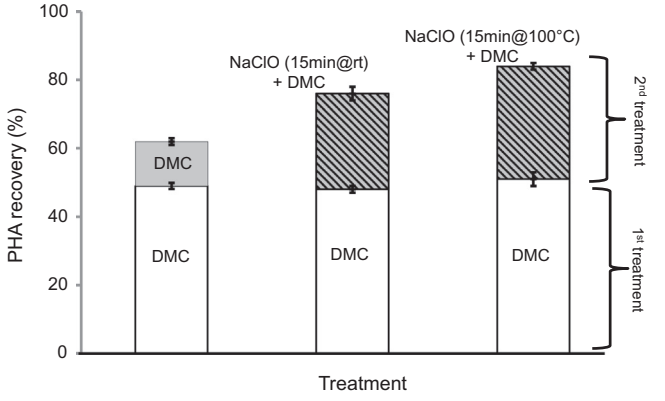

Fig. 3. PHA recovery (%) obtained by applying a first DMC extraction (1 h at 90 °C) followed by a second treatment with DMC (1 h at 90 °C) or NaClO (15 min at rt or 100 °C) and then DMC (1 h at 90 °C).

Table 3
PHA yield, recovery and physical characteristics obtained after various treatments of MMCs biomass: (a) extraction with DMC (1 h at 90 °C), DMC* (24 h, Soxhlet), CH_2Cl_2 (4 h at 50 °C); (b) NPCM disruption with NaClO (1 h at 100 °C); (c) pretreatment with NaClO followed by extraction with DMC (1 h at 90 °C).

Entry	Pretreatment	Treatment	Yield (wt%)[a]	Purity (%)[b]	Recovery (%)[c]	$T_{max\ deg}$ (°C)[d]	$\overline{M_w}$ (MDa)[e]	PDI[f]
1	–	DMC	20 ± 1	98	49 ± 2	254	1.3	1.9
2	–	DMC*	17 ± 1	87	41 ± 1	280	0.5	2.9
3	–	CH_2Cl_2	22 ± 1	94	52 ± 1	246	1.4	2.0
4	–	NaClO	41 ± 2	77	79 ± 3	269	0.3	2.7
5	NaClO (5 min, rt)	DMC	27 ± 3	92	40 ± 3	275	0.6	2.6
6	NaClO (15 min, rt)	DMC	31 ± 5	98	62 ± 4	270	0.8	2.4
7	NaClO (1 h, rt)	DMC	34 ± 4	88	76 ± 4	238	0.6	2.3
8	NaClO (5 min, 100 °C)	DMC	32 ± 3	89	72 ± 3	262	0.5	2.8
9	NaClO (15 min, 100 °C)	DMC	31 ± 1	92	72 ± 2	281	0.5	2.4
10	NaClO (1 h, 100 °C)	DMC	35 ± 2	93	82 ± 3	281	0.2	2.5

[a] Calculated gravimetrically on microbial biomass weight basis (wt%) and expressed as mean ± standard deviation of four independent replicates of each extraction condition.
[b] Evaluated by TGA mass loss intensity.
[c] Calculated as $\frac{\text{PHA extraction yield} \times \text{PHA purity}}{\text{PHA amount in microbial cells}}$.
[d] Maximum decomposition temperature evaluated by TGA.
[e] Mean molecular weight determined by SEC.
[f] Polydispersity index determined by SEC.

Table 4
PHA yield, recovery and physical characteristics of the "second PHAs" obtained by applying a second treatment (DMC or NaClO/DMC) to the residual microbial biomass already extracted with DMC.

Entry	1st treatment	2nd treatment	Yield (wt%)[a]	Purity (%)[b]	Recovery (%)[c]	$T_{max\ deg}$ (°C)[d]	$\overline{M}_w$ (MDa)[e]	PDI[f]
1	DMC	DMC	13 ± 1	95	12 ± 1	n.d.	1.2	2.7
2	DMC	NaClO (15 min, rt) then DMC	28 ± 2	91	25 ± 3	284	0.4	2.4
3	DMC	NaClO (15 min, 100 °C) then DMC	33 ± 1	84	28 ± 2	286	0.2	2.1

[a] Calculated gravimetrically on microbial biomass weight basis (wt%)and expressed as mean ± standard deviation of four independent replicates of each extraction condition.
[b] Evaluated by TGA mass loss intensity.
[c] Calculated as $\frac{\text{PHA extractionyield} \times \text{PHA purity}}{\text{PHA amount in microbial cells}}$.
[d] Maximum decomposition temperature evaluated by TGA.
[e] Mean molecular weight determined by SEC.
[f] Polydispersity index determined by SEC.

- A re-extraction of the residue with DMC.
- A treatment with NaClO for 15 min at rt followed by DMC extraction.
- A treatment with NaClO for 15 min at 100 °C followed by DMC extraction.

The results of the second treatments can be summarized as follow:

- A further DMC extraction increased the overall recovery percentage to 61 ± 1% (a third re-extraction did not improve the recovery, data not shown). Both the first and second PHAs have high purity and very high $\overline{M}_w$, but the PDI value of the "second PHA" was slightly higher than that of the "first PHA"(2.7 *vs*. 1.9, 3 entry 1 and Table 4 entry 1).
- The use of NaClO at rt for 15 min followed by DMC extraction increased the polymer recovery to 76 ± 2%. This cumulative result was higher than the what obtained by applying a single treatment with NaClO at rt for 15 min followed by DMC extraction (recovery of 62%, Table 3, entry 6). In this case the "second PHA" was characterized by a low $\overline{M}_w$ (0.4, entry 2 of Table 4) but it maintained a good purity (91%) and a narrow PDI value (2.4). It is worth noting that the reduction of $\overline{M}_w$ after this second treatment was more intense than what was obtained after a single NaClO pretreatment under the same conditions followed by DMC extraction (0.8, Table 3, entry 6).
- The use of NaClO at 100 °C for 15 min followed by DMC extraction increased the polymer recovery to79 ± 1%. However, this "second PHA" had the lowest $\overline{M}_w$ (0.2) and purity (84%) among all treatments.

The application of a second DMC treatment demonstrates that the unrecovered PHA that remains in the biomass has still a very high potential as a polymer to be recovered, as proved by the very high $\overline{M}_w$ and purity obtained (entry 1, Table 4). On the contrary, the quite stronger degrading effect that a pretreatment with NaClO imparts to biomass residues, might tentatively be attributed to a slightly different condition of the polymer inside the original granules and within the biomass residue. It is renown that the PHA in amorphous phase is easily degradable, while its crystalline phase is more able to resist the alkaline conditions (Yu et al., 2005). It might thus be hypothesized that the pristine polymer in the biomass has some crystalline domain that helps it resisting to the degradation by NaClO during the first extraction (entry 6, Table 3), while the application of DMC could alter this ordered structure.

FT-IR spectra of the MMC biomass before and after DMC extraction show a carbonyl absorption at 1724 cm^{-1}: this signal, typical of a PHA ordered phase (Bloembergen et al., 1986; Xu et al., 2002), is stronger in the biomass before the extraction than in the biomass residue. However, in the latter a contribution to the intensity of the peak might also arise from the overlapping with the 1650 cm^{-1} strong absorption from the amide bond carbonyl stretching of proteins. The band at 1738 cm^{-1}, attributed to amorphous phase (Bloembergen et al., 1986; Xu et al., 2002), is well present in both samples, accounting for the consistent fraction of PHA that easily degrades in the presence of NaClO. This signal is almost absent in the polymer extracted with DMC (the polymer related to entry 1 in Table 3), as expected by a purified PHA that easily crystallizes.

3.3.2. Comparison between MMC and single strain extraction

The same PHA recovery procedures (DMC extraction and disruption of NPCM with SDS) were applied on MMCs and *C. necator* biomass, both containing similar amount (40 ± 5 and 54 ± 2 wt%, respectively) of the co-polymer P(3HB-*co*-3HV) with 20% of HV (Table 5). *C. necator* was chosen since it is described as one of the best performing PHA producer among the single strains tested until now in the literature (Madison and Huisman, 1999.)

Once again these data confirm that the extraction of PHAs from MMCs is much more challenging in comparison to single strain cultures. The recovery obtained with DMC on MMCs was 1.7 times lower than what was obtained from *C. necator* (50% *vs*. 86%, respectively).However, the purity of both polymers was very high (>98%) and comparable.

The treatment with SDS afforded an almost complete polymer recovery with a good purity when applied to single strains. In this case, definitely worse results were obtained on MMCs, both in terms of recovery and purity. This finding confirms what was hypothesized by Patel et al. (2009) who suggested the existence of a strong and complex cellular matrix that creates an impenetrable "barrier" around the polymer granules.

Several authors have claimed that the use of MMCs should bring towards a more cost effective PHA production process, mainly due to lower cost equipment (MMC do not require sterility) and cheaper available substrates for feeding bacteria(Salehizadeh and Van Loosdrecht, 2004). However, the data here presented demonstrate that the extraction step, easy for single strains (e.g., *C. necator*), could represent a non-negligible issue with relevant associated costs in the case of MMCs. Therefore, the research effort in the field of PHA production from MMCs should be directed towards unblocking this bottleneck.

Table 5
PHA recovery and purity obtained with DMC extraction or SDS treatment of MMCs and *C. necator* biomass, both containing around 50 wt% of P(3HB-*co*-3HV).

Treatment	PHA recovery (%)		PHA purity (%)	
	C. necator	MMCs	*C. necator*	MMCs
DMC	86.5 ± 0.01[a]	50.0 ± 1.6	97.5 ± 0.5[a]	98.2 ± 1.0
SDS	92.4 ± 3.1	67.1 ± 4.0	90.8 ± 3.5	56.1 ± 6.6

[a] Data from Samorì et al. (2015).

4. Conclusion

This study demonstrates that the production of PHA from MMCs, claimed to be the most economically attractive option, can be hampered by polymer extraction step. Multiple viable solutions can be found according to the purity, recovery and molecular weight of the target polymer. A pretreatment with NaClO coupled with DMC extraction gives the best recovery result (82%) but the molecular weight is drastically reduced (0.2 MDa). A double extraction with DMC gives acceptable recovery (above 60%) and the highest molecular weight (1.2 MDa), providing a polymer with a high quality comparable to that obtained from single strains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.03.062.

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