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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.

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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

* Corresponding author. Tel.: +39 (0)544 937353; fax: +39 (0)544 937411.
E-mail address: chiara.samori3@unibo.it (C. Samorì).

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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).