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Citation: Yang, Yung-Hun, Jong-Min Jeon, Da Hye Yi, Jung-Ho Kim, Hyung-Min Seo, ChoKyun Rha, Anthony J. Sinskey, and Christopher J. Brigham. "Application of a Non-Halogenated Solvent, Methyl Ethyl Ketone (MEK) for Recovery of Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) [P(HB-Co-HV)] from Bacterial Cells." Biotechnol Bioproc E 20, no. 2 (April 2015): 291–297.

As Published: http://dx.doi.org/10.1007/s12257-014-0546-y

Publisher: The Korean Society for Biotechnology and Bioengineering

Persistent URL: http://hdl.handle.net/1721.1/105530

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Massachusetts Institute of Technology

Biotechnology and Bioprocess Engineering 20: 291-297 (2015)
DOI 10.1007/s12257-014-0546-y

RESEARCH PAPER

Application of a Non-halogenated Solvent, Methyl Ethyl Ketone (MEK) for Recovery of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(HB-*co*-HV)] from Bacterial Cells

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Received: 11 August 2014 / Revised: 3 November 2014 / Accepted: 8 November 2014
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Abstract Conventional solvent-based methods are still the most practical approaches for recovery of polyhydroxyalkanoate (PHA) polymer from cellular biomass, even though potential alternatives exist, including chemical, mechanical, and enzymatic methods. It is still necessary, however, to avoid dangerous and environmentally unfriendly solvents (*e.g.*, chloroform and dichloromethane) in the polymer recovery process. In the work presented here, we applied various solvent systems to recover PHA from *Ralstonia eutropha* and recombinant *Escherichia coli* cells. It was demonstrated that methyl ethyl ketone (MEK) is a promising solvent for PHA recovery from bacterial cells, particularly for the copolymer poly(hydroxybutyrate-*co*-hydroxyvalerate) [P(HB-*co*-HV)], exhibiting > 90% polymer recovery. Even though MEK did not solubilize PHAs to the same extent as chloroform, it can recover a comparable amount of polymer because of its processing advantages, such as the low viscosity of the MEK/PHA solution, and the lower density of MEK as compared to cellular components. MEK was found to be the best alternative, non-halogenated solvent among examined candidates for recovery of P(HB-*co*-HV) from cells. The MEK treatment of PHA-containing cells further allowed us to eliminate several costly and lengthy steps in the extraction process, such as cell lysis, centrifugation, and filtration.

Keywords: polyhydroxyalkanoate, recovery, solvent extraction, poly(hydroxybutyrate-*co*-hydroxyvalerate), methylethylketone

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1. Introduction

Naturally occurring polyesters, polyhydroxyalkanoates (PHAs), have been studied for several decades. Their importance has been emphasized as an alternative for petroleum-based plastics by virtue of their physicochemical properties and biodegradability [1,2]. PHAs exhibit superior biocompatibility and have been used to develop materials for medical applications including sutures, artificial organ construction scaffolds, and therapeutic composites [3]. For most bacteria, including the model organism of PHA biosynthesis, *Ralstonia eutropha*, polymer production is induced when carbon is plentiful and nutrients such as nitrogen, sulfur, oxygen, or phosphate are in short supply [4]. However, biosynthesis of polymer has also been shown to occur concomitantly with growth in certain organisms [5]. As storage materials for excess carbon and reducing potential, PHAs can comprise over 80% of the cell's dry

weight in some bacterial species [6]. Molecular weight and monomer composition can be controlled through substrate feeding and PHA synthase activity [4,7,8], and the resulting polymers demonstrate different mechanical properties, depending on both the type of monomers incorporated and the polymer chain length [9,10]. PHAs can be degraded by many species of bacteria and other microorganisms in nature in a relatively short period of time [11], and, as a result, they are often considered to be environmentally friendly plastics. Despite the advantages of PHAs, the current price of these bioplastics is relatively high when compared with conventional plastics. Efforts must be made to lower production costs in order for PHAs to compete with petroleum-based plastics in the global marketplace.

To date, most research has been focused on development of new bacterial strains and discovery of cost-effective starting materials for polyhydroxyalkanoate (PHA) production, while relatively few breakthroughs in the area of recovery of PHAs from cellular biomass have been discussed in the scientific literature. A key component of lowering bioplastic production costs is the formulation of improved processes for recovery of bioplastic from microbial cells. Currently, the method of solvent-based recovery of PHAs from cells, which is seemingly most effective in terms of polymer yield and purity [8], has been largely dependent on extraction using halogenated hydrocarbon solvents, such as chloroform and dichloromethane [8,12]. However, these extraction methods often require large quantities of toxic solvents to avoid the occurrence of highly viscous polymer solutions (containing > 5% (w/v) PHA). Other non-solvent-based polymer recovery methods have been attempted, including chemical-based recoveries using sodium hydroxide or sodium hypochlorite [13], yet both of these chemical methods result in severe reduction of the molecular weight of recovered PHA [14]. Chemical recovery of intracellular polymer using detergents, such as SDS [12] or LAS-99 [15], has been attempted previously. While these detergent-based recovery methods show promise, there are still questions regarding excess water usage and the presence of salts in the waste streams that could potentially lead to contamination. Enzymatic digestion in aid of PHA recovery from cells has been attempted at laboratory scale [14], but it is questionable as to whether this technique would be cost-effective at the pilot or industrial scale. Thus, the use of non-halogenated solvents could represent the best alternative for PHA recovery from microbial cells, especially if solvent recycling is implemented in a designed process.

Considerations for improvement of a solvent-based recovery system include the choice of solvent, the environmental implications of operations, the quantity of waste products, the overall toxicities of substances used, and the practical design of facilities [16]. In light of these considerations, our efforts have focused on the discovery and application of alternative solvent candidates for PHA recovery.

Here, we report the use of a non-halogenated solvent system to substitute for chloroform, taking safety, cost-effectiveness, and other processing parameters into account. Furthermore, we demonstrate the effectiveness of the solvent system when applied without any requisite cell pretreatment steps such as grinding or spray drying. We show that methyl ethyl ketone (MEK) is a strong candidate solvent for PHA recovery, particularly for the copolymer P(HB-*co*-10 wt% HV). Although MEK did not solubilize PHAs to the same extent as the halogenated solvent, chloroform, it has favorable processing characteristics, such as the lower viscosity of an MEK/PHA solution as compared to a chloroform/PHA solution with same solvent: polymer ratio, and the lower density of MEK compared to the cellular components. These characteristics represent a processing advantage over chloroform extraction by streamlining the polymer recovery process.

2. Materials and Methods

2.1. Bacterial strains and media

To prepare P(4HB), P(HB-*co*-12 wt% HHx), and P(HB-*co*-10 wt% HV), different strains of *R. eutropha* and *Escherichia coli* were cultured (Table 1). The copolymer P(HB-*co*-10 wt% HV) was prepared by mixed acid growth using *R. eutropha* H16 (WT) in minimal medium culture using 0.3% (v/v) short-chain organic acids (1:1:1 ratio of acetate:propionate:butyrate) as the carbon source and incubated at 30°C for 72 h, as described previously [17]. In order to obtain cell-based P(HB-*co*-HHx), Re2000 cells were grown in minimal medium [18] containing 0.5% (w/v) sodium hexanoate (Sigma-Aldrich, St. Louis, MO) for 72 h at 30°C [19] (Table 1). Under these conditions, Re2000 synthesizes PHA with 12 wt% 3HHx monomer. For the preparation of PHA containing a higher concentration (32 wt%) of 3HHx, Re2135/pCB81 cells were grown in minimal medium with 1% palm oil (Wilderness Family Naturals, Finland, MN) for 72 h at 30°C [19]. To prepare P(4HB), *E. coli* YH091 containing pASK-IBA7 (gift from T. Selmer, Philipps-Universität Marburg, Germany), pLW487 [15], and pRARE were grown in M9 minimal media with 0.1% yeast extract and 0.1% sodium 4-hydroxybutyrate at 30°C for 48 h.

2.2. Materials for PHA extraction and analysis

Acetone, isobutanol, butyl acetate (BA), methyl isobutyl ketone (MIBK, 4-methyl-pentanone), methyl ethyl ketone

Table 1. Bacterial strains and plasmids used in this study

Strains, plasmids, primers	Genotype/Relevant information	Source or reference
Bacterial strains		
E. coli strains		
DH5α	F^- φ80*lacZ* M15 *endA recA hsdR*($r_k^- m_k^-$) *supE thi gyrA relA* Δ(*lacZYA-argF*)U169	[27]
BL21(DE3)	F^- *ompT hsdS_B*($r_B^- m_B^-$) *gal dcm*	Novagen
S17-1	recA pro hsdR RP4-2-Tc::Mu-Km::Tn7	[28]
YH091	BW25113 (Δ(*araD-araB*)567 Δ*lacZ*4787 Δ(*rhaD-rhaB*)568) + DE3 containing pLW487, pASK-IBA7 and pRARE	This study
R. eutropha strains		
H16	Wild-type; Gm resistant	[18]
Re2000	H16Δ*phaC* with *phaC*(C09) from *Rhodococcus aetherivorans* inserted into *phaC* deletion allele.	[29]
Re2135	H16Δ*phaB*1, Δ*phaB*2, Δ*phaB*3 Δ*phaC*	[29]
Plasmids		
pEP2	Km^r, a 1.85-kb derivative of pNG2	[30,31]
pRARE	A compatible chloramphenicol-resistant plasmid, pRARE supplies tRNAs for the codons AUA, AGG, AGA, CUA, CCC, and GGA	Novagen
pASK-IBA7	Vector carrying PCR product of propionyl-CoA from *Clostridium propionicum* [32]	[32]
pCB81	pBBR1MCS2 carrying PCR products *phaC* (D12) from *Rhodococcus aetherivorans* I24, and *phaJ1* from *Pseudomonas aeruginosa*	[29]
pLW487	Spectinomycin-resistant pEP2-based plasmid carrying PCR products of *bktB*, *phaB* and *phaC* under *trc* promoter from *R. eutropha*	[15]

(MEK), dichloromethane, chloroform, dimethyl sulfoxide, isoamyl alchohol (3-methyl-1-butanol), 4-hydroxybutyrate and P(HB-*co*-12 wt% HV), methanol and polystyrene standards were all purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Solvent-based PHA extraction

Before applying each solvent, cell lysis was achieved by incubating 1 mL of acetone with 10 mg of dried cells, containing 65 wt% PHA, for 30 min at 100°C. The resulting biomass was dried in a fume hood at room temperature [20]. Aliquots (2 mL) of each solvent were then mixed with dried cell mass (1 mL solvent: 10 mg cells), and incubated for 15 h at 60°C. Following centrifugation or filtration to remove cell debris, 1 mL of PHA solution was transferred to a new glass vial and precipitated using an equal volume of MeOH:Water (7:3). Solubility of commercially available P(HB-*co*-12 wt% HV) (Sigma-Aldrich, St. Louis, MO, USA) was examined with an attempt to dissolve this reference polymer using different solvents (1 wt%) at different operational temperatures from 50 to 110°C.

2.4. Molecular weight measurement

The molecular weights of P(HB-*co*-HV) were determined by gel permeation chromatography (GPC) with a refractive index detector (RID) using an ultrastyragel column with an Agilent 1100 high-performance liquid chromatography (Santa Clara, CA). Chloroform was used as the mobile phase at a flow rate of 1.0 mL/min. A calibration curve was determined using low polydispersity polystyrene standards from Sigma-Aldrich (St. Louis, MO). The GPC data were calculated by the use of standard analysis software (Agilent Technologies, Santa Clara, CA), using an integrator for number average molecular weight (M_N), weight average molecular weight (M_W) and polydispersity (M_W/M_N).

2.5. Analytical methods

PHA composition was analyzed by gas chromatography using a slight modification of the method described previously [21]. Approximately 10 mg of freeze-dried cells or recovered PHAs were placed in Teflon-stoppered glass vials. Methanolysis of PHAs was performed as described previously [17]. Volatile acids present in growth media were monitored by high-pressure liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-RAD, CA, USA) at 50°C with a flow rate of 0.6 mL/min of 5 mM sulfuric acid as the mobile phase. Samples were detected using a diode array detector at a wavelength of 210 nm [18].

3. Results and Discussions

3.1 Evaluation of solvents with emphasis on Methyl Ethyl Ketone (MEK)

To formulate a more cost-effective and environmentally

friendly PHA extraction process, we systematically compiled data on the use of different solvents used to date, and compared different properties, such as boiling point, water solubility, safety, price and others (Supplementary Table 1). Considering a large number of applications for PHA involving food, the US FDA classification for food was specially considered because halogenated solvents were classified as limited solvents (class 2). Initially, we examined commercially available (cell-free) P(HB-*co*-12 wt% HV), and attempted to dissolve this reference polymer using different solvents (1 wt% polymer, final concentration) at different operational temperatures, from 50 to 110°C. Dichloromethane and chloroform have been shown to dissolve P(HB-*co*-HV) in this range of temperatures [22]. Acetone and dimethyl sulfoxide (DMSO) could dissolve P(HB-*co*-HV) only at the high temperature of 110°C (data not shown). However, other candidate solvents such as BA, isobutanol, MIBK, and MEK could not dissolve the polymer at all, even at high temperatures. These findings were likely a result of the crystallinity of the commercial P(HB-*co*-HV) polymer, resulting in poor solubility in many of the solvents tested. Moving forward, we decided to examine the effectiveness of solvents using bacterial cells containing different types of PHA. Since polymer present in cells is completely amorphous [9,23], the solubility of the intracellular polymer in the candidate solvents is likely to be quite different. Candidate solvents were applied directly to dried cell samples containing polymer. In these experiments, prior to application of solvent for polymer extraction, 1 mL of acetone per 10 mg of dry cells containing P(HB-*co*-12 wt% HHX), P(100 wt% 4HB) and P(HB-*co*-10 wt% HV) was used to facilitate cell lysis. In order to screen solvents, cells containing PHA with different concentrations of 3HB, 3HHx, 4HB, or 3HV monomers were used (Figs. 1A, 1B, and 1C). These types of polymer are generally more soluble in a wider variety of different solvents than PHB homopolymer [20]. Among the examined solvents, BA, MIBK, MEK were each able to dissolve P(HB-*co*-HHx) (50% CDW polymer, 12 wt% 3HHx).

Of all the non-halogenated solvents tested in this work, MEK emerged as the most promising for general PHA recovery, because it showed about 50% of chloroform's solubility for P(HB-*co*-HHx) polymer from recombinant *R. eutropha*, without any optimization, under the conditions tested (Fig. 1A). Approximately 62% of chloroform's solubility was demonstrated for P4HB (15% CDW polymer, 100% 4HB) recovered from engineered *E. coli* (Fig. 1B), also without any optimization. MEK showed the highest solubility towards P(HB-*co*-HV) with 10 wt% 3HV from *R. eutropha* (Fig. 1C). Among those tested, MEK was the only non-halogenated solvent that could solubilize P(HB-*co*-10 wt% HV) to any extent. Previous studies indicated

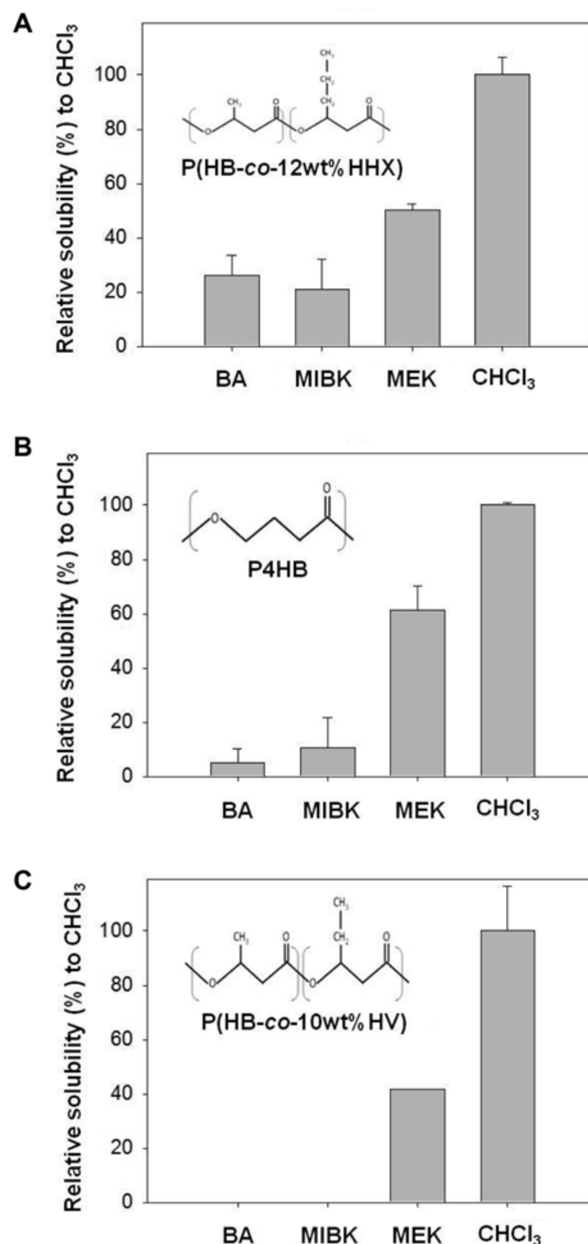

Fig. 1. Solubility of PHAs in different solvents relative to chloroform (100%) with different types of bacterial cells (see Materials and Methods) containing: 50 wt% of P(HB-*co*-12 wt% HHx) (A), 15 wt% of P4HB (B), and 65 wt% of P(HB-*co*-10 wt% HV) (C). Abbreviations: Butyl acetate (BA), Methyl isobutyl ketone (MIBK), Methyl ethyl ketone (MEK), Chloroform ($CHCl_3$).

MIBK and MEK as strong candidates for P(HB-*co*-HHx) recovery [24]. However, in our studies, MIBK was shown not to be an applicable solvent for P(HB-*co*-10 wt% HV). In addition to MEK, it was also shown that acetone could dissolve PHAs (data not shown), as reported previously [25]. However, MEK (b.p.: 79.6ºC) has a notably higher boiling point than acetone (b.p.: 56.5ºC), thus avoiding a potential explosion risk due to high pressure as a result of

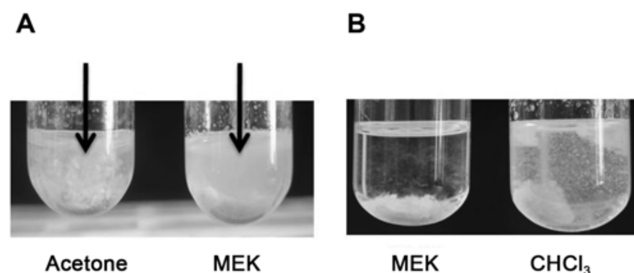

Fig. 2. Properties of MEK as a solvent for polymer extraction from cells. Reaction and lysis of dry cells containing 65 wt% of P(HB-*co*-10 wt% HV) with acetone (A, left) and MEK (A, right), sedimentation of cells with MEK and chloroform with gravity (B). In (A), arrows indicate gel-like substances, observed upon cell lysis.

vapor buildup during heating to temperatures of 60ºC and above.

3.2. Properties of MEK as a solvent for PHA

MEK was shown to lyse cells and dissolve P(HB-*co*-10 wt% HV) copolymer without acetone pre-treatment. After treatment with MEK for 5 min at 100°C, the presence of gel-like polymers were observed, similar to results observed during acetone pretreatment (Fig. 2A), which was suggestive of cell lysis. The amounts of recovered polymer were quite similar, regardless of whether acetone pretreatment was used, suggesting a pretreatment (*i.e.* cell lysis) step could be skipped in a polymer recovery process using MEK.

In addition to the processing properties discussed above, MEK exhibits a lower density (0.8 g/cm^3) as compared to cellular components (1.1 ~ 1.3 g/cm^3) [26], which presents additional recovery process simplifications. Simple centrifugation (2,000 × *g* for 30 sec) or sedimentation for 30 min can separate the PHA solution from cell debris, and this separation allows for easier filtration of the PHA solution. Furthermore, filtration is likely unnecessary as the separation of the supernatant from cell debris is notably clear (Fig. 2B). For chloroform, with its density of 1.5 g/cm^3, there is not as distinct a separation of PHA solution from cell debris, and therefore filtration is required, which could potentially lead to the persistent clogging of the apparatus during filtration due to buoyant cell debris (Fig. 2B).

Gel permeation chromatography shows that the molecular weights (M_n) of P(HB-*co*-10 wt% HV) recovered by MEK or chloroform are similar (414.4 ± 41.0 kDa, compared to 468.5 ± 71.0 kDa, respectively). However, the polydispersity index (PDI, M_w/M_n) of polymer recovered by MEK (2.84 ± 0.29) is much lower than that of polymer recovered by chloroform (3.81 ± 0.19), suggesting that the polymer recovered by MEK is more homogeneous in chain length. This implies that MEK can extract polymers of a specific

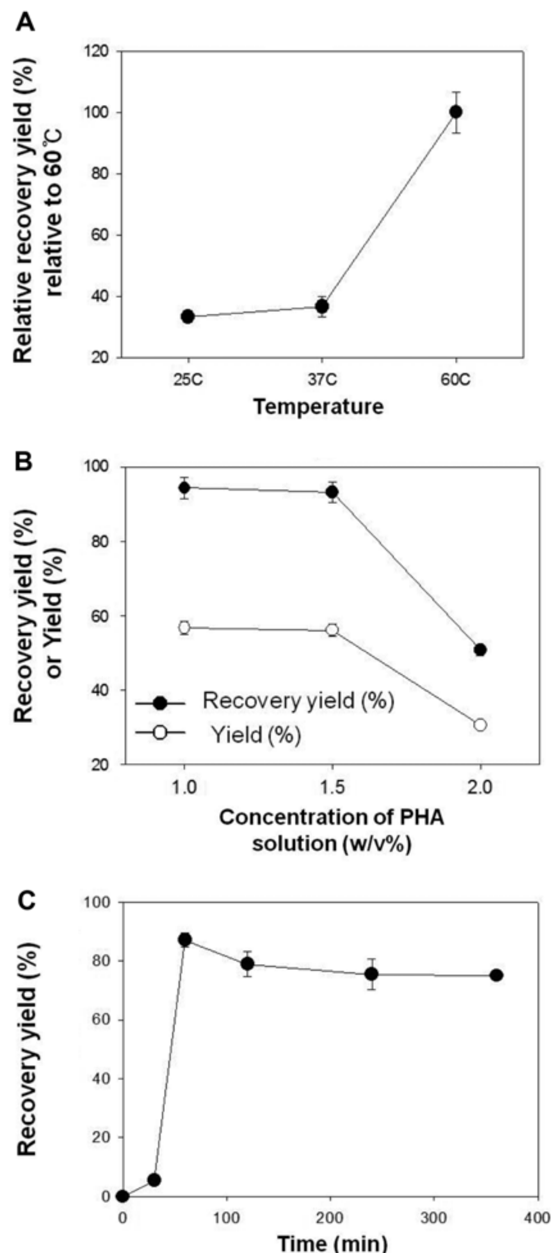

Fig. 3. Effect of temperature (25, 37, or 60°C) on relative recovery yield of PHA (A); effect of ratio of dry cell weight and MEK on yield and recovery yield, using 0.3 g of dried cells containing 65 wt% of P(HB-*co*-10 wt% HV) (B); and effect of different incubation times of extraction on yield and recovery yield with 1:66 ratio (P(HB-*co*-10 wt% HV) and MEK with 2 g of dried cells) (C). Relative recovery yield (%) in (A) was calculated on relative amount to recovery at 60°C (100%).

molecular weight range out of a distribution of polymers in the cell.

3.3. Investigation of MEK processing variables

To optimize processing variables for efficient polymer recovery, we examined several factors, such as incubation

time, temperature and ratio of cell dry weight to volume of MEK used. Using MEK (10 mg cells/ 1 mL MEK), the solubility of P(HB-*co*-10 wt% HV) increased with increasing temperature. When MEK was applied to cells containing P(HB-*co*-10 wt% HV), recovery at 25°C was only 30% of the amount recovered at 60°C (Fig. 3A).

To find an optimal PHA:solvent ratio, different ratios of dry cell weight (g) to MEK (mL) were examined. At a ratio of 1:50 (0.3 g dry cells: 15 mL of MEK or 2 w/v%), a recovery yield of 50.7% and a yield of 30.5% were observed. The recovery yield is defined as the mass percentage of polymer recovered from the cells (mass PHA recovered/mass PHA present in cells), while the yield is the mass percentage of total biomass that is recovered as PHA (mass PHA recovered/initial (dry cell mass). With 1.5 w/v% and 2.0 w/v% PHA solutions, a recovery yield of 93.8 ± 0.6% with purity of 91.4 ± 0.3% and a yield of 56.5 ± 0.4%, respectively, were seen (Fig. 3B) using 0.3 g dried cells. In this work, the optimal reaction time to extract P(HB-*co*-10 wt% HV) polymer from cells was shown to be 60 min at 60°C (Fig. 3C).

4. Conclusion

Applying an MEK solvent system to polymer-containing cells was found to be the best alternative solvent tested for recovery of P(HB-*co*-HV), and demonstrated promise in recovering other polymers examined in this study (P(HB-*co*-HHx) and P4HB). Although MEK exhibited lower solubility towards PHAs than chloroform, it delivered comparable recovery yield with chloroform due to its processing advantages, such as low viscosity and easy separation by sedimentation. MEK-based recovery allowed for potential elimination of several costly and lengthy steps in the extraction process, such as cell lysis and filtration. As a result, MEK-based extraction represents a simplified PHA extraction process, potentially reducing capital, operational, and material costs. This is especially true if solvent recycling is employed in a recovery process, although care must be employed in the selection of a precipitant, in order to ensure recovery of usable solvent in the absence of a solvent/precipitant azeotrope. Furthermore, MEK-based extractions have the potential to significantly reduce cycle times, greatly improving the overall environmental safety of the polymer extraction.

Acknowledgements

The authors thank Mr. Jacob Palmer and Mr. John Quimby for their contributions to this manuscript. The work was partially supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A2A10004690), KOPRI (PE14030), the Energy Efficiency & Resources of the Korea Institute of Energy Technology Evaluation and Planning (KETEP) grant funded by the Korea Government Ministry of Trade, Industry and Energy (20133030000300), R&D Program of MOTIE/KEIT [10047910], and National Research Foundation of Korea Grant (NRF-2011-619-E0002).

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