Improved detergent-based recovery of polyhydroxyalkanoates (PHAs)

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Abstract Extracting polyhydroxyalkanoate (PHA) polymer from bacterial cells often involves harsh conditions, including use of environmentally harmful solvents. We evaluated different detergents under various conditions to extract PHA from Ralstonia eutropha and Escherichia coli cells. Most detergents tested recovered highly pure PHA polymer from cells in amounts that depended on the percentage of polymer present in the cell. Detergents such as linear alkylbenzene sulfonic acid (LAS-99) produced a high yield of high purity polymer, and less detergent was needed compared to the amount of SDS to produce comparable yields. LAS-99 also has the advantage of being biodegradable and environmentally safe. Chemical extraction of PHA with detergents could potentially minimize or eliminate the need to use harsh organic solvents, thus making industrial PHA production a cleaner technology process.

Keywords Detergent · Polyhydroxyalkanoate · Polymer recovery · Ralstonia eutropha

Introduction

Polyhydroxyalkanoates (PHAs) are naturally-occurring biopolymers, produced by a diverse group of Gram-negative and Gram-positive bacteria (Madison and Huismann 1999; Sudesh et al. 2000). These PHAs have characteristics similar to those of chemically-synthesized polymers (Steinbüchel and Fuchtenbusch 1998) and can be broken down in natural environments such as soil, sea water, and lake water (Mergaert et al. 1992; Khanna and Srivastava 2005). In general, microbial biosynthesis of PHA results from the limitation of nutrients, such as nitrogen, oxygen, or phosphate (Anderson and Dawes 1990), in the presence of abundant carbon. These polymers serve as storage for carbon or reducing equivalents and, in some strains of...
bacteria, PHA accounts for more than 80% of the cell dry weight (cdw) (Khanna and Srivastava 2005). The characteristics of PHAs differ, depending on the type of monomer incorporated and particularly on the monomer chain length (Doi et al. 1995; Jendrossek and Handrick 2002).

Most processes developed to recover PHAs from microbial cells are based on extraction with organic solvents, including halogenated hydrocarbon solvents such as chloroform and dichloromethane (Choi and Lee 1999). A major drawback of these methods is the requirement of large quantities of these solvents, which in turn requires solvent recovery/recycling processes. Using too little solvent could result in a highly viscous polymer solution, if the solution concentration is more than 5% (w/v) PHA.

Several alternative, non-solvent based recovery methods were explored, including differential digestion with sodium hypochlorite, thermal treatment of biomass followed by enzymatic digestion, and chemical disruption by SDS and NaOH (Choi and Lee 1999; Thakor et al. 2005). The detergent method of PHA recovery differs from solvent-based extraction in that detergents disrupt various cell components while leaving the PHA intact, which is clearly the main goal of an extraction process (Choi and Lee 1999). A previous study suggested that a detergent-based method has drawbacks, including low purity of the extracted polymer and high cost of detergents. In addition, this study suggested that the detergent-based method requires large amounts of detergent per gram of PHA to recover polymer and large quantities of water to wash out cell debris (Jiang et al. 2006). However, another study has discussed advantages of detergent-based extraction, including that the method can be applied to wet cells directly after fermentation, eliminating the need for drying prior to polymer extraction (Thakor et al. 2005). One obvious advantage to a detergent-based recovery scheme is that toxic solvents can be avoided. Furthermore, a detergent-based method, if optimized, could give high recovery yield and be applied to any PHA regardless of monomer composition (Choi and Lee 1999).

In this work, we examined the effectiveness of several detergents in recovering PHA from whole cells of Ralstonia eutropha and an Escherichia coli strain expressing PHA production genes. Under the conditions described in this paper, detergents can be effective PHA recovery agents and are clearly safer for the environment than halogenated solvents.

Materials and methods

Materials

Detergents linear alkylbenzene sulfonate (LAS-99), sodium alphaolefin sulfonate (AOS-40), and sodium polyoxyethylene sulfate (ES 702) were purchased from Pilot Chemical Company (Cincinnati, OH, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Growth of bacteria

For preparation of PHA-containing cells in the following experiments, R. eutropha H16 was precultured in 3 ml dextrose-free tryptic soy broth (TSB) for 24 h at 30°C. One ml of cells taken from this preculture was used to inoculate 50 ml of TSB in a 250 ml flask, which was incubated for 24 h at 30°C. The cells were harvested, washed twice with sterile water, used to inoculate 1 l of high phosphate minimal medium containing mixed organic acids as a carbon source (acetate/propionate/butyrate 3:1:1 by wt), and then grown for 72 h at 30°C before harvesting. The cells grown in the aforementioned manner produce polyhydroxybutyrate-co-polyhydroxyvalerate [P(HB-co-HV)] with 6% (w/w) 3-hydroxyvalerate monomer (6 wt% 3HV) (Yang et al. 2010). The cells were harvested, washed twice with cold water, and lyophilized for 48 h prior to polymer recovery.

Recovery of PHA and analytical procedures

The PHA recovery procedure is outlined in Fig. 1. Quantitation of cellular and purified PHA was performed as described previously (Yang et al. 2010).

Results

Use of SDS for recovery of PHA from cells

Various compounds, such as laboratory and industrial detergents, were examined in order to determine their
relative effectiveness in the extraction of PHA. In addition to the type of detergents used, we studied incubation time, temperature, and solution pH to determine whether changing these parameters would enhance yield and decrease the amount of detergents required for optimal recovery.

Initially, SDS was used to optimize PHA recovery. For polymer recovery with 5% (w/v) SDS, incubation for 3–6 h typically gave reasonable recovery yield (~95%, Fig. 2a), while purity was best following 6–24 h of incubation (~87%, Fig. 2a). Temperature was also an important variable for the separation of cellular components from PHA in SDS (Fig. 2b). Increasing temperature up to 90°C enhanced recovery of PHA from cells (Thakor et al. 2005), and in the present study increasing temperature enhanced the purity of the recovered polymer. Also, the concentration of SDS was less important than temperature. When SDS was adjusted from 0.625 to 5% (w/v) and the extraction performed at 37°C, there was no direct correlation between the amount of SDS and the purity or recovery yield. However, incubation of cells with 0.625% (w/v) SDS at 60°C recovered PHA with a 20% (or larger) increase in purity compared to recovery using 0.625% (w/v) SDS at 37°C (data not shown).

Compared to solvent extraction with chloroform, the effectiveness of polymer recovery using SDS was influenced by PHA content of cells, because the detergent is able to dissolve non-PHA materials as well as PHA. With cells containing a larger amount of PHAs (82% cdw PHA in Fig. 3), both solvent extraction and chemical disruption methods produced results of similar purity. However, the chemical disruption of cells containing much lower amounts of PHA (45% cdw PHA and 33% cdw PHA, from R. eutropha and E. coli, respectively) resulted in extracted polymer of lower purity compared to those obtained by solvent extraction (Fig. 3). Although the addition of a sonication step after cellular disruption by detergent produced PHA with higher purity, overall polymer content of the cell influenced recovery (Fig. 3). Therefore, the recovery system may necessitate adjustment, depending on the particular PHA-producing strain. In the case of industrial PHA production, a higher concentration of PHA per cell is desirable, suggesting that detergent-based methods could be suitable for polymer recovery at the industrial scale, provided the polymer production strain accumulated high intracellular concentrations of PHA.

Screening of detergents for polymer recovery

We also explored various detergents, including commercial laundry detergents; industrial detergents such as SDS, IGEPAL CA 630 (Nonidet P40) and Brij 58; (see Table 1), and one non-detergent chemical, NaOH (see Table 1). The protocol for our chemical recovery method is shown in Fig. 1. As discussed earlier, SDS recovered polymer of 85–98% purity depending on the PHA content of the cells (Fig. 2). Although SDS is expensive, it effectively extracted polymer, as reported by a previous study (Thakor et al. 2005). NaOH showed lower recovery yield and purity than reported previously (Choi and Lee 1999) because we used a higher pH and higher incubation temperature than previously described (Table 1). Treatment with all detergents listed in Table 1 recovered PHA to a relatively high yield and with high purity, especially when compared to polymer recovery by NaOH. This suggests that, while PHA recovery with NaOH is an inexpensive, non-solvent-based option, only a low yield and low purity polymer is obtained compared to the detergent recovery method.
Among detergents studied here, AOS-40 is promising for both purity and recovery yield, while Brij58 showed the highest recovery yield (Table 1). However, with LAS-99, using one-fifth the amount of detergent, gave similar PHA extraction results, as compared with other promising detergents (Fig. 4a). Interestingly, ES-702, AOS-40 and LAS-99 effectively recovered PHAs from R. eutropha cells. Components of many of these detergents are biodegradable (Mieure et al. 1990). For example, LAS-99 was broken down readily in aerobic environments (Scott and Jones 2000). The half life of LAS-99 in soil was 1–3 weeks, and does not significantly affect biota (Jensen 1999), thus suggesting that this detergent is also environmentally safe. Also, there is no indication that LAS-99 is toxic to plant and animal life.

We next examined LAS-99 more closely for recovery of PHAs from R. eutropha cells grown on a mixed acid substrate. LAS-99 is biodegradable, pourable, and pumpable at ambient working temperatures (http://www.pilotchemical.com/productgroups/index/7). A small amount of LAS-99 could dissolve non-PHA materials better than the other detergents tested, allowing PHA to be easily separated from non-PHA materials (see comparisons in Fig. 4a). We then optimized the pH of LAS-99 treatment (Fig. 4b) using an incubation time of 3 h and temperature of 60 °C, conditions which were found to increase recovery yields for SDS treatment. Figure 4b shows that a pH of 3.77 is optimal for LAS-99 mediated recovery of PHA from cells containing 65.7 ± 2.2% cdw PHA and 3–9% (w/w) 3HV, resulting in a yield of ∼86% and purity of ∼88%.

These results were achieved by using a mass ratio of 1:2.5 (g cell dry weight:g sodium dodecyl sulfate) was used. In b, the asterisk denotes cellular PHA content (56.4 ± 0.1% cell dry weight polyhydroxybutyrate-co-hydroxyvalerate with 9–10% 3-hydroxyvalerate monomer).

Fig. 2 Effect of incubation time (a) on purity and recovery yield of polyhydroxyalkanoates (PHA) with 5% sodium dodecyl sulfate at 60°C. Effect of temperature (b) on purity of recovered PHA with 5% sodium dodecyl sulfate at 60°C.

Fig. 3 Purity of PHA recovered using sodium dodecyl sulfate (SDS) treatments. SDS treatment (SDS), SDS treatment with sonication (SDS*), and chloroform extraction (CHCl₃) on various microbial strains containing different PHA content. R. eutropha containing 82 wt% PHA (Ralstonia 82%), R. eutropha containing 45 wt% PHA (Ralstonia 45%) and E. coli containing 33 wt% of PHA (E. coli 33%) were examined with 5% SDS. The E. coli YH091 strain used in this study was constructed by adding plasmid pLW487 (pEP2-based plasmid (Zhang et al. 1994) containing PHA synthesis genes bktB, phaB, and phaC from R. eutropha under the control of a trc promoter) into E. coli strain BL21. YH091 was grown in M9 minimal media with 1% glucose. For R. eutropha, low nitrogen concentration (0.01% NH₄Cl, for Ralstonia 82%) or high nitrogen concentration (0.1% NH₄Cl, for Ralstonia 33%) were used with high phosphate minimal medium at 30°C for 72 h (Yang et al. 2010). See Supplementary Fig. S1 for a map of pLW487

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easier and safer, despite the relatively lower purity of the PHAs obtained (88–92%) compared to that obtained with chloroform (94–98%) (Ibrahim and Steinbüchel 2009). To examine the source of impurities present in the polymer recovered by LAS-99, we performed methanolation and examined the gas chromatography traces of purified P(HB-co-HV) and various control substances (Supplementary Fig. S2).

Table 1  PHA purity and recovery yields with various detergents

<table>
<thead>
<tr>
<th>Structure</th>
<th>Purity (%)</th>
<th>Recovery yield (%)</th>
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<tbody>
<tr>
<td>SDS C_{12}H_{25}-OSO_3^-Na^+</td>
<td>90 ± 1</td>
<td>81 ± 2</td>
</tr>
<tr>
<td>ES-702a C_{12}H_{25} - [OCH_3CH_2]_2 - OSO_3^-Na^+</td>
<td>90 ± 1</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>AOS-40b C_{12}H_{25} - CH=CH - CH_2 - OSO_3^-Na^+</td>
<td>91 ± 2</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>Brij®58 C_6H_{13} - [OCH_2CH_2]_{20} - OH</td>
<td>83 ± 1</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>LAS-99c</td>
<td>86 ± 0</td>
<td>87 ± 2</td>
</tr>
<tr>
<td>IGEPAL®CA 630</td>
<td>86 ± 1</td>
<td>91 ± 9</td>
</tr>
</tbody>
</table>

Detergent 2d N.A. 84 ± 4 90 ± 5
Detergent 3 N.A. 86 ± 3 81 ± 0
NaOH NaOH 78 ± 10 45 ± 20

PHAs (70% CDW, 2% 3HV) were treated with various detergents [1:2.5 (CDW: detergent)] for 3 h at 60°C. Purity is defined as: g of PHA/total g sample. Recovery yield is defined as g of PHA/total cellular PHA (prior to recovery).

a ES702: Sodium polyoxoethylene sulfate
b AOS-40: Sodium alpha olefin sulfonate
c LAS-99: Linear alkylbenzene sulfonic acid
d Detergent 2 and 3: Proprietary formulas

Fig. 4  Linear alkyl sulfonate (LAS-99) as a detergent for harvesting PHA from cells: separating the PHA from the cellular material/detergent solution (a), and the effect of pH on purity and recovery yield with LAS-99 (b). Comparatively small amounts of LAS-99 were able to disrupt cells. Solution concentrations of 1% (g cell dry weight:g detergent = 1:0.5) were used to recover PHA. In the middle tube in a, the precipitant on the bottom is recovered PHA. Cells used in b contained 66 ± 2% cell dry weight polyhydroxybutyrate-co-hydroxyvalerate with 7 ± 1% 3-hydroxyvalerate monomer.

According to Supplementary Fig. S2, the polymer recovered by LAS-99 contains small amounts of non-PHA cellular material and trace amounts of detergent. There is also a contaminant peak of unknown origin with a retention time of ~4.5 min present in the detergent-purified polymer sample. A similar peak is seen in the commercial P(HB-co-HV) sample, and its presence may be a result of the recovery process.
Discussion

To the best of our knowledge, this is the first example of a multiple detergent screen for PHA recovery from whole cells of *R.* *eutropha*. The detergent-based processes effectively recovered high yields of polymer from cells containing large amounts of PHA. We also showed specifically that LAS-99 is a promising detergent for use in the chemical recovery of PHA due to the ability of a relatively small quantity of detergent to recover a high yield of high purity polymer. Yields and purities of recovered PHA obtained by the detergent disruption method were affected by the choice of recovery system and also by the intracellular PHA content, and were therefore quite dependent on the PHA-producing strains and production conditions used. Our results suggest that a greater accumulation of PHA as a percentage of cell dry weight may be necessary for effective purification and recovery. However, the use of bacterial strains that accumulate a large amount of cellular PHA is in line with most industrial PHA production processes, indicating that detergent-based polymer recovery is a potential alternative recovery process for PHA.

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