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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 sodium dodecyl sulfate (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.

Response to Reviewers: December 13, 2010

Dr. Colin Ratledge, Editor
Biotechnology Letters

Dear Dr. Ratledge,
Thank you for your helpful editorial suggestions with our recent submission to Biotechnology Letters, "Improved detergent-based recovery of polyhydroxyalkanoates (PHAs)" by Yang, et al. We are very pleased to hear that this manuscript has been accepted for publication in the journal. As you have also listed some items that require correction before the final version is to be published, we have addressed these points, and the responses are listed below.
1. Please give full names of the detergents in Materials, page 5 line 10. SDS can, however, be used without definition.
Response to point 1: As suggested, we have added the full names of detergents listed in Materials, page 5, lines 10-11.
2. Is it legitimate to quote purities and recoveries (Table 1) to three significant figures bearing in mind the analytical methods being used?

Response to point 2: We have trimmed back the significant figures on the values listed in Table 1, page 18.

3. There are a lot of phrases, such as "...has been shown to..." etc. that can be drained to make the text more concise and the Results section seems rather wordy and some pruning would be appreciated.

Response to point 3: We have addressed this concern. A few examples of text trimming are shown below. Page 4, line 14: changed "a previous study also suggested" to "another study discussed." Page 4, line 2: the phrase "which normally affects PHA solubility" was eliminated. Page 7, line 6: the phrase "has been shown to enhance" was changed to "enhanced." Page 7, line 12: the phrase "could recover" was changed to "recovered." Page 7, line 15: the word "greatly" was eliminated. Page 8, line 4: the phrase "helped to recover" was changed to "produced." Page 9, line 14: the phrase "has been shown to biodegrade" was changed to "was broken down." Page 9, line 15: the phrase "has been shown to be" was changed to "was." Page 10, line 4: the phrase "we found that" was eliminated.

4. Supplementary Figure 1 should be used as a normal figure as it provides useful information. However, please state whether the ratio of cells to detergent is w/v, w/w or whatever.

Response to point 4: We have made Supplementary Figure 1 into Figure 1 and adjusted the other Figures accordingly. The new Figure 1 is found on page 19, with the legend at the top of page 16. We have also added "(w/w)" to indicate how the ratio of cells to detergent was calculated.

Thank you again for your most helpful comments with our manuscript. If anything else is needed, please let us know. We look forward to seeing the printed copy of the paper.

Sincerely,

Anthony J. Sinskey

Improved detergent-based recovery of polyhydroxyalkanoates (PHAs)

Selection for consideration: Bioprocessing and Bioengineering

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Keywords: detergent, polyhydroxyalkanoate, polymer recovery, *Ralstonia eutropha*

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 sodium dodecyl sulfate (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 Huisman 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. In nature, 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 polyoxoethylene 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 bacterial cells. For preparation of PHA-containing cells in the following experiments, *R. eutropha* H16 was precultured in 3 mL of dextrose-free Tryptic Soy Broth (TSB) for 24 h at 30°C. A 1-mL aliquot 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 (a 3:1:1 ratio of acetate: propionate: butyrate), 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, the common detergent sodium dodecyl sulfate (SDS) was used to optimize PHA recovery parameters. For polymer recovery with 5% (w/v) SDS, an incubation time of 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 the concentration of SDS was adjusted from 0.625% (w/v) to 5% (w/v) and the extraction performed at 37ºC, 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, low-yield and low purity polymer is obtained as compared to the detergent recovery method. Among detergents studied here, AOS-40 is promising for both purity and recovery yield, while Brij®58 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 (Figure 4A).

Interestingly, the detergents 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 wt% 3HV, resulting in a yield of ~86% and purity of ~88%. These results were achieved by using a mass ratio of 1: 0.5 (dried cell mass: LAS), 5X less detergent than the ratios used in Table 1. Compared to recoveries with solvents such as chloroform (less than 70% yield) (Ibrahim and Steinbüchel 2009), LAS gave a higher yield (~86%), and the process used was 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 methanolysis and examined the gas chromatography traces of purified P(HB-*co*-HV) and various control substances

(Supplemental Figure 2). According to Supplemental Figure 2, 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

We examined the use of laboratory and industrial detergents for recovery of PHA from *R. eutropha* cells. To the best of our knowledge, this is the first example of a multiple detergent screen for PHA recovery from whole cells that has been described. The detergent-based processes reported here 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.

Acknowledgements

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Figure legends

Fig. 1 General protocol for detergent-based polymer recovery experiments discussed in this work. For LAS-99 polymer recovery, the cell dry weight to detergent ratio was 1:0.5.

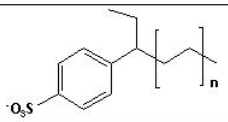
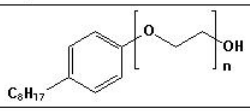
Fig. 2 Effect of incubation time (a) on purity and recovery yield of polyhydroxyalkanoates (PHA) with 5% sodium dodecyl sulfate at 60°C. Effect of temperature (b) on purity of recovered PHA with 5% sodium dodecyl sulfate at 60°C. The ratio of 1:2.5 (g cell dry weight:g sodium dodecyl sulfate) was used. 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. 3 Purity of PHA recovered using sodium dodecyl sulfate (SDS) treatments. SDS treatment (SDS), SDS treatment with sonication (SDS^{*}), and chloroform extraction ($CHCl_3$) 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% NH_4Cl, for Ralstonia 82%) or high nitrogen concentration (0.1% NH_4Cl, for Ralstonia 33%) were used with high phosphate minimal medium at 30°C for 72 hr (Yang et al. 2010). See Supplemental Figure 1 for a map of pLW487.

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.

Table 1: PHA purity and recovery yields with various detergents.

	Structure	Purity (%)	Recovery yield (%)
SDS	$C_{12}H_{25}$-$OSO_3^-Na^+$	90±1	81±2
ES-702 [a)]	$C_{12}H_{25}(OCH_2CH_2)_2$-$OSO_3^-Na^+$	90±1	85±2
AOS-40 [b)]	$C_{11}H_{23}$-CH=CH-CH_2-$OSO_3^-Na^+$	91±2	87±3
Brij ® 58	$C_{16}H_{33}(OCH_2CH_2)_{20}$-OH	83±1	99±1
LAS-99 [c)]		86±0	87±2
IGEPAL® CA 630		86±1	91±9
Detergent 2 [d)]	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

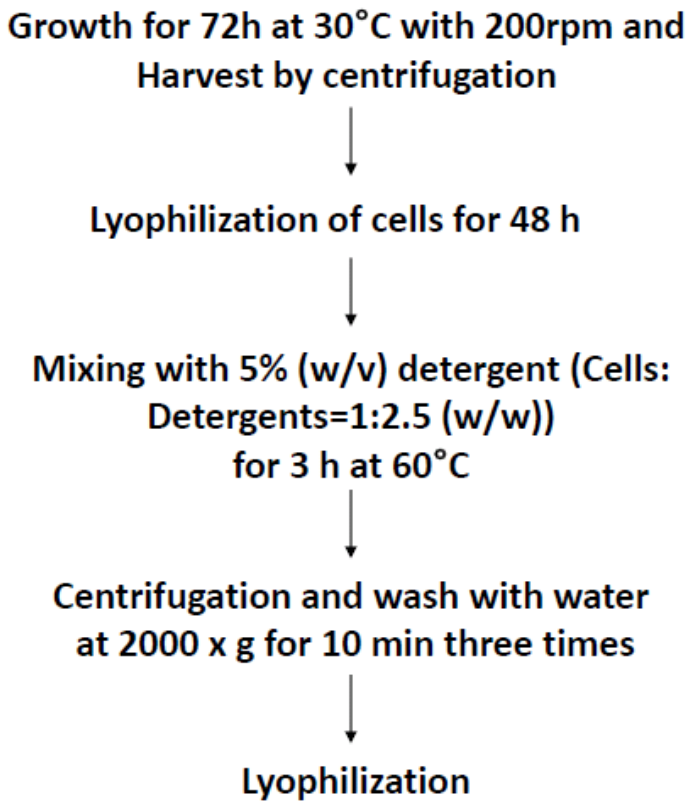
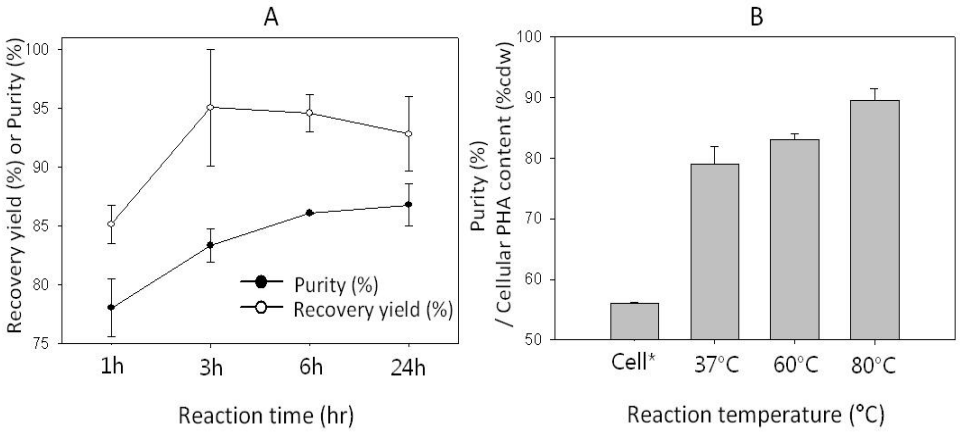

Growth for 72h at 30°C with 200rpm and
Harvest by centrifugation
Lyophilization of cells for 48 h
Mixing with 5% (w/v) detergent (Cells:
Detergents=1:2.5 (w/w))
for 3 h at 60°C
Centrifugation and wash with water
at 2000 x g for 10 min three times
Lyophilization

Figure 1.

1



2

3 **Figure 2.**

4

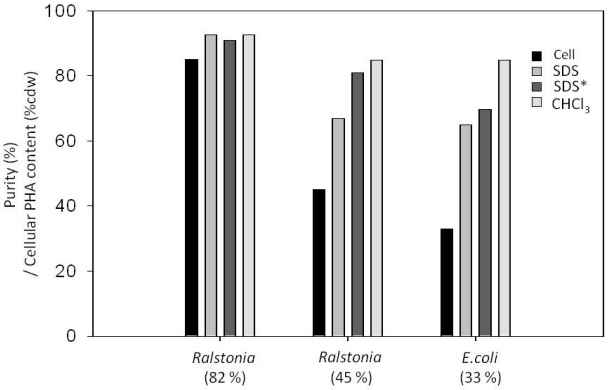

100
80
60
40
20
0
Purity (%)
/ Cellular PHA content (%cdw)
Cell
SDS
SDS*
CHCl3
Ralstonia
(82 %)
Ralstonia
(45 %)
E.coli
(33 %)

Figure 3.

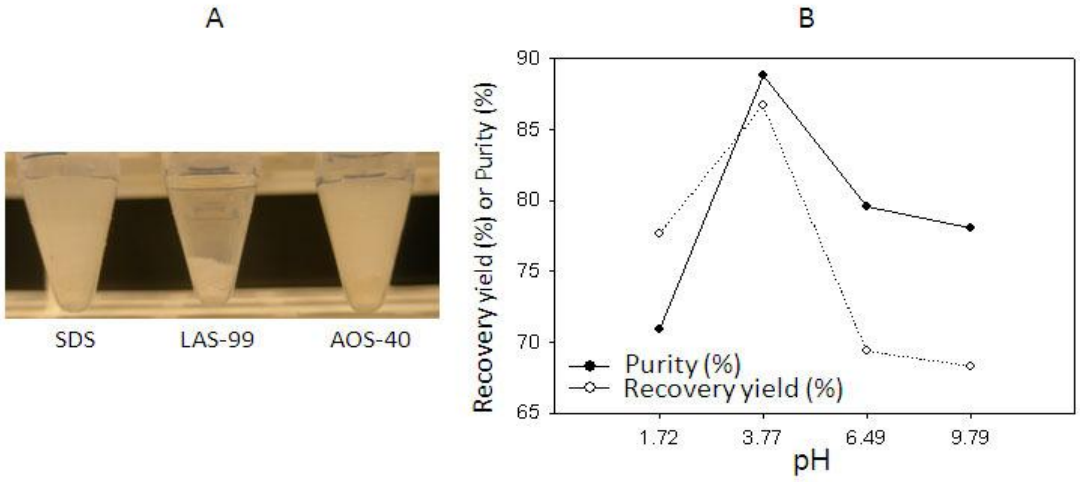

A
B
SDS
LAS-99
AOS-40
Recovery yield (%) or Purity (%)
90
85
80
75
70
65
Purity (%)
Recovery yield (%)
1.72
3.77
6.49
9.79
pH

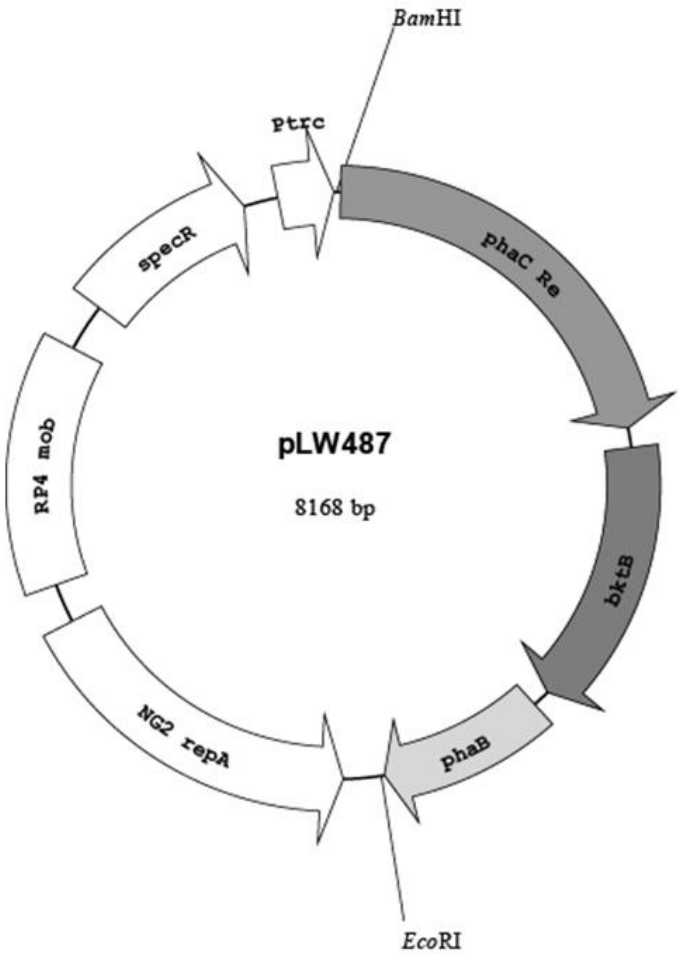
Figure 4.

Supplementary Material

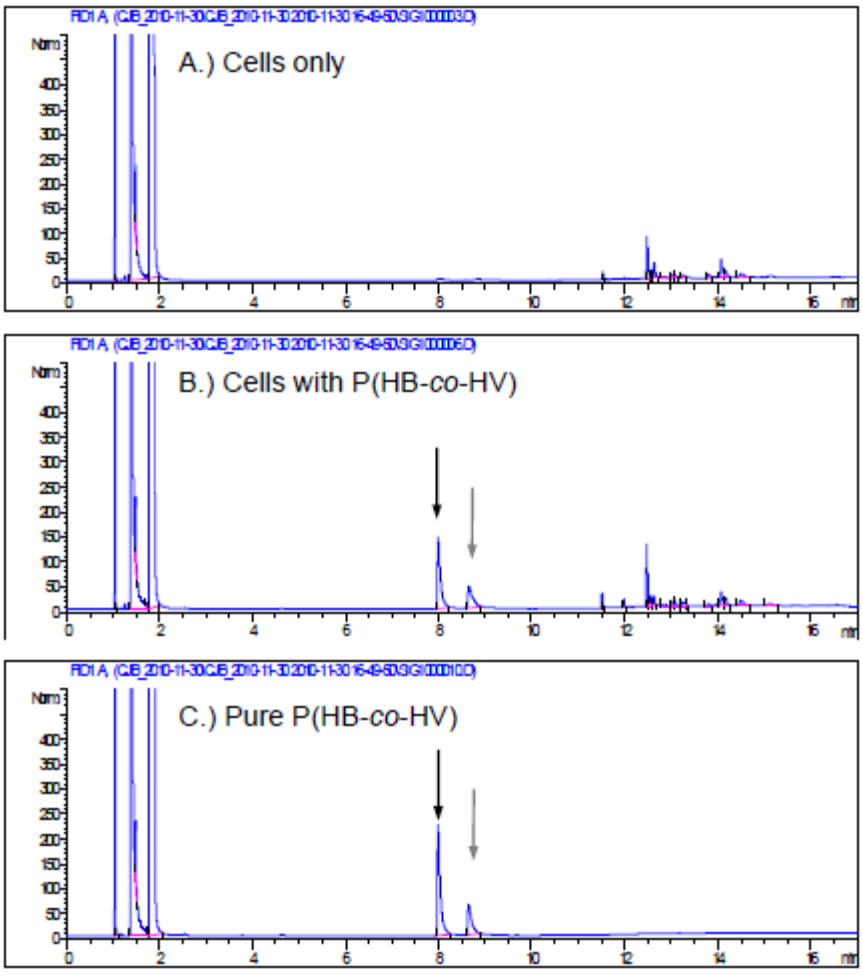
Figure 1. Vector map of pLW487.

Figure 2. Contaminant profile of P(HB-*co*-HV) purified by LAS-99. Solution concentrations of 1% (g cell dry weight:/g detergent = 1:0.5) were used to recover PHA. Samples were subjected to methanolysis and methyl esters were detected by gas chromatography using an Agilent 6850 Series gas chromatograph. (A) *R. eutropha* H16 cells grown in the presence of 0.1% mixed acids (limited carbon); (B) *R. eutropha* H16 cells grown in the presence of 0.5% mixed acids, containing P(HB-*co*-HV); (C) Pure P(HB-*co*-HV) containing 14 mol% 3HV monomer (Aldrich); (D) 5 mg of LAS-99 detergent; (E) P(HB-*co*-HV) polymer purified from *R. eutropha* H16 mixed acid cultures by LAS-99. In (B), (C), and (E), the solid black arrow points to peak corresponding to 3HB methyl ester and the solid grey arrow points to the peak corresponding to 3HV methyl ester. In (E), the dashed boxes correspond to the presence of small amounts of impurities in the purified sample. The impurities with retention times of 10-12 min are likely cellular material and LAS-99 detergent. The impurity with a ~ 4.5 min elution time is

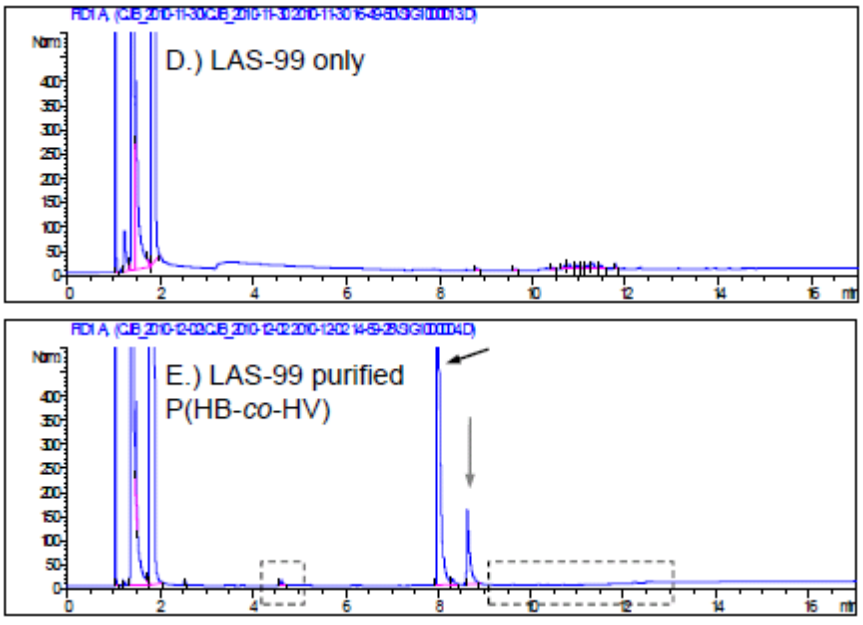
unknown, but small amounts of an impurity with a similar retention time are seen in the commercially available P(HB-*co*-HV).



Supplementary Figure 1.



1



2

3 **Supplementary Figure 2.**