Cloud-point extraction of green-polymers from Cupriavidus necator lysate using thermoseparating-based aqueous two-phase extraction

Pau Loke Show, The University of Nottingham

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Cloud-point extraction of green-polymers from Cupriavidus necator lysate using thermoseparating-based aqueous two-phase extraction

Yoong Kit Leong, John Chi-Wei Lan, Hwei-San Loh, Tau Chuan Ling, Chien Wei Ooi, and Pau Loke Show

Polyhydroxyalkanoates (PHAs), a class of renewable and biodegradable green polymers, have gained attraction as a potential substitute for the conventional plastics due to the increasing concern towards environmental pollution as well as the rapidly depleting petroleum reserve. Nevertheless, the high cost of downstream processing of PHA has been a bottleneck for the wide adoption of PHAs. Among the options of PHAs recovery techniques, aqueous two-phase extraction (ATPE) outshines the others by having the advantages of providing a mild environment for bioseparation, being green and non-toxic, the capability to handle a large operating volume and easily scaled-up. Utilizing unique properties of thermo-responsive polymer which has decreasing solubility in its aqueous solution as the temperature rises, cloud point extraction (CPE) is an ATPE technique that allows its phase-forming component to be recycled and reused. A thorough literature review has shown that this is the first time isolation and recovery of PHAs from Cupriavidus necator H16 via CPE was reported. The optimum condition for PHAs extraction (recovery yield of 94.8% and purification factor of 1.42 fold) was achieved under the conditions of 20 wt/wt % ethylene oxide-propylene oxide (EOPO) with molecular weight of 3900 g/mol and 10 mM of sodium chloride addition at thermoseparating temperature of 60 °C with crude feedstock limit of 37.5 wt/wt %. Recycling and reutilization of EOPO 3900 can be done at least twice with satisfying yield and PF. CPE has been demonstrated as an effective technique for the extraction of PHAs from microbial crude culture.

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[Key words: Aqueous two-phase system; Bioseparation; Polyhydroxyalkanoate; Purification; Thermoseparating polymer]

Due to increasing concerns towards resource depletion and environmental issues, biodegradable polyesters derived from renewable resources such as polyhydroxyalkanoates (PHAs) have gained much attraction as the potential substitute for conventional plastics (1,2). Accumulated under undesirable growth condition which is deprivation of nutrients (such as oxygen, nitrogen, magnesium and others) in the presence of excess carbon source, PHAs function as intracellular carbon and/or energy storage reserve in more than 300 types of microorganisms (3,4). PHAs are thermoplastics which are biocompatible (5), completely biodegradable (6), non-toxic, water-insoluble, inert, and indefinitely stable in the air (7). Furthermore, PHAs have high structural diversity (8) and good processability on equipment of plastic manufacturing (7). Due to their attractive properties, PHAs have a wide range of applications (9) in packaging, machinery housings, disposable utensils and accessories manufacturing as well as in medical field (10).

However, the production cost of PHAs is much more expensive than that of the conventional petrochemical-based plastics due to the expensive carbon sources and the high cost of downstream processing (11). Thus, a green and cost-effective approach to the downstream processing of PHAs is essential for them to stay competitive among other bio- and synthetic plastics. Solvent extraction is the most conventional and most extensively used method for PHAs recovery due to its rapidity and simplicity (12). However, this method not only consumes a huge amount of volatile toxic solvents such as chloroform, but it also disrupts the native orders of PHA granules’ polymer chains (13). Similarly, other purification techniques such as chemical and enzyme digestion, mechanical disruption and others have their own disadvantages of low recovery and purity, production of a high quantity of wastewater and degradation of PHAs (14).

Aqueous two-phase extraction, ATPE is a purification technique which utilizes two coexisting immiscible phases that formed when two structurally different polymers or an inorganic salt and one
polymer are mixed in water beyond the critical concentration \(15,16\). ATPE serves as a primary step of purification and recovery by partly removing the target product from impurities or substrates, thus reducing the processing volume for subsequent downstream processing \(17\). As compared to the traditional isolation and purification methods, ATPE has many distinctive advantages and unique characteristics that attract the interests of researchers and industries. Consisting of high water content \(80\text{--}90\% \text{wt/wt}\), ATPE provides a mild environment for separation of sensitive biomaterials \(18,19\). Moreover, the phase-forming components of ATPE are generally nontoxic and relatively environmentally-friendly in comparison to the solvents used in the conventional solvent extraction \(20\). Aside from the short processing time \(21\) and the capability to process a high volume of feedstock \(22\), the scale-up phase of purification using ATPE can be easily and reliably predicted from laboratory experiments’ data \(23\). ATPE is a readily available solution to the industry demanding a cost-effective and highly efficient large-scale bioseparation technology with short processing time.

Based on the concept of temperature-induced phase separation, a type of ATPE, also known as cloud point extraction (CPE) has been developed. CPE utilizes thermoseparating polymers (TSPs) which have a decreasing solubility in its aqueous solution as the temperature increases. This unique characteristic not only makes the recycling of TSPs possible but also simplifies the extraction of target product \(17,24\). The common TSPs used include random, diblock, and triblock copolymers of hydrophilic ethylene oxide (EO) and hydrophobic propylene oxide (PO), thus named as EOPO copolymers. The EO/PO ratio and molecular weight of TSPs can be varied to serve a wide range of applications \(25\). EOPO can also be used with potassium phosphate, sodium or ammonium sulfate, dextran and other starch derivatives for ATPE purpose \(26\).

A previous work by Divyashree et al. \(27\) showed that the polyethylene glycol (PEG) 8000/phosphate system was suitable for the recovery of PHAs from the hydrolyzed bacterial cells among conventional ATPEs. However, to date, there is no literature reporting the application of thermoseparation-based ATPE in the extraction of PHAs from the crude feedstock. In view of the advantageous features offered by the thermoseparating ATPE, we report for the first time isolation and purification of PHAs from Cupriavidus necator H16 utilizing CPE technique. In this study, the key ATPE-influencing parameters such as type and concentration of thermoseparating polymer, salt addition, feedstock load, and thermoseparating temperature were optimized to obtain high recovery of PHAs from the crude bacterial lysates. The attempt of recycling and reutilizing the thermoseparating polymer for PHAs extraction has also been done.

### MATERIALS AND METHODS

**Materials**

*C. necator* H16 strain was kindly provided by Yuan Ze University, Taiwan. Poly(ethylene glycol-ran-propylene glycol) monobutyl ether with a molecular weight of 3900 g/mol (EOPO 3900), Ucon HTF 14, benzoic acid and propanol were purchased from Sigma Aldrich (M) Sdn. Bhd. (Selangor, Malaysia). EOPO 3900 and Ucon HTF 14 are both TSPs with EO/PO ratio of 50:50, though EOPO 3900 has a higher molecular weight and is more hydrophobic. All the salts used in this study were obtained from LGC Scientific Sdn. Bhd. (Selangor, Malaysia). All the other chemicals used in this study were of analytical grade.

**Production of PHAs**

The bacterial cells were first cultured on agar plates at 30°C for 24 h. An inoculum was prepared in a 10 ml nutrient broth (8 g/L) by inoculating the medium with a single colony from the cultured agar plate. The culture was then grown aerobically at 30°C for 24 h. Subsequently, the inoculum (5\% v/v) was transferred to a 250 ml shake flask containing 100 ml operating volume of defined medium supplemented with glycerol of 30 g/L and yeast extract of 2 g/L. The inoculated fermentation medium was then incubated aerobically at 30°C for 72 h. The defined medium was composed of \(\text{Na}_{2}\text{HPO}_4\cdot 7\text{H}_2\text{O}, 6.7 \text{ g/L; KH}_2\text{PO}_4, 1.5 \text{ g/L; (NH}_4)_2\text{SO}_4, 2.5 \text{ g/L; MgSO}_4\cdot 7\text{H}_2\text{O}, 0.2 \text{ g/L; and CaCl}_2, 10 \text{ mg/L}}\) and 0.5% v/v of trace mineral solution (Na\(_2\)EDTA, 6.0 g/L; FeCl\(_3\), 6H\(_2\)O, 0.29 g/L; H\(_3\)BO\(_3\), 6.84 g/L; MnCl\(_2\)·4H\(_2\)O, 0.86 g/L; ZnCl\(_2\), 0.06 g/L; CoCl\(_2\), 6H\(_2\)O, 0.026 g/L; and CuSO\(_4\)·5H\(_2\)O, 0.002 g/L). Without undergoing extra clarification or filtration step, the culture broth of *C. necator* H16 was directly subjected to ultrasonic cell disruption with an ultrasonic processor (UP400S, Hielscher) at 30 kHz per cycle for 15 min to obtain intracellular PHA. Disrupted biomass was stored in 500 ml graduated laboratory bottles.

**Partitioning of PHAs in thermoseparating ATPE**

ATPE system was prepared in a 50 ml centrifuge tube by mixing the TSPs (in wt/wt %) into deionized water. The crude PHA feedstock was mixed well with aqueous solution of thermoseparating polymer (agitated in a vortex for 2 min at 2500 rpm) before the mixture was incubated in water bath at 65°C for thermoseparation. After the stages of equilibration and phase separation, the top phase was carefully removed using a pipette, while the bottom phase was then sampled through the interface. The samples from both phases and disrupted biomass were centrifuged and washed with deionized water for three times before left in the oven for overnight at 70°C to be dried. The dried samples from both phases and disrupted biomass were analyzed for PHAs content using gas chromatography (GC). As for the recycling studies, a 40 g system was first prepared at an optimized temperature of thermoseparation. The top phase was removed and the mass of this phase was then determined. Later, the fresh water and feedstock were added to the recycled TSPs and a new stage of ATPE repeats. All experiment works were done at least duplicate.

**Quantification of PHAs by GC analysis**

For the determination of PHAs content, the GC method by Akaraonye et al. \(1\) with minor modification was employed. Chloroform \(2 \text{ ml}\) and 2 ml of propanol with 15\% v/v of sulfuric acid were added to about 2 mg of dried samples. After incubation at 100°C for 2 h, the sample was cooled rapidly and 1 ml of water was then added to the sample to remove the sulfuric acid. The sample was then allowed to settle until separated into organic and aqueous phases. Then, 0.2 \(\mu\)l of the organic phase (bottom phase) was injected into Clarus 500 gas chromatograph (Perkin Elmer, USA) equipped with a DB-WAX capillary column \((0.25 \text{ mm by 30 m; 0.25 } \mu\text{m film thickness})\).**

**Partitioning behaviors of PHAs in ATPE**

The purity, % was defined as ratio between mass of PHAs as quantified by GC and total mass of dried sample used for the GC analysis:

\[
\text{Purity} = \frac{M_{\text{PHA}}}{M_{\text{Sample}}} \times 100
\]

where \(M_{\text{PHA}}\) is the mass of PHAs (g) and \(M_{\text{Sample}}\) is total mass of dried sample used (g).

**Reciprocal yield (Y)**

% of PHA in top phase was calculated as the ratio between PHAs content in the top phase and the initial PHA content in the extract:

\[
Y = \frac{C_{\text{top}}}{C_{\text{extract}}} \times V_{\text{top}}
\]

where \(C_{\text{top}}\) and \(C_{\text{extract}}\) are PHAs concentration of top phase and extract respectively and \(V_{\text{top}}\) is extract volume.

**Purification factor (PF)**

was defined as the ratio of top phase PHA purity to the overall PHA purity:

\[
\text{PF} = \frac{C_{\text{top}}}{C_{\text{extract}} \times V_{\text{top}}}
\]

**RESULTS AND DISCUSSION**

Cloud point extraction of PHAs in thermoseparating aqueous two-phase system CPE is performed by heating TSPs (aqueous) solution above lower critical solution temperature (LCST) or cloud point (CP) temperature which consequently induces thermoseparation \(17\). The solution then becomes cloudy and separates into a top phase which contains mostly water and a bottom phase which consists of concentrated TSPs. The concept of thermoseparation-based CPE is shown in Fig. 1. Polyethylene glycols (PEGs) which commonly used for ATPE is also a TSP, however, it has a LCST above 100°C (around 180°C for those with lower molecular weight) which is undesirable for thermo-based bioseparation as most biocomponents are sensitive to high temperature \(18\). Having a lower CP which as low as 47°C, TSPs are much more suitable for temperature-induced CPE of biomaterials \(17\).

Two types of TSPs, Ucon and EOPO 3900 were used in this thermoseparation-based ATPE of PHAs. These two random copolymers were chosen due to the fact they were widely used for thermoseparating ATPE in literature \(17,28\). Also, some
literature suggested that random copolymer with EO:PO ratio of 50:50 (such as EOPO 3900 and Ucon HTF 14) are more suitable for biomolecule partitioning (18). The study of CPE of PHAs was done over a range of TSP concentrations at 65°C. The systems in CPE normally possess high volume ratio (i.e., large volume of water phase), ranging from 2 to 20. Fig. 2 demonstrates the purification and recovery result of PHAs utilizing Ucon and EOPO 3900 CPE. The recovery yield and PF showed a maximum of 87% and 1.2 fold, respectively at 8 wt/wt % of Ucon concentration. Other than that, the PHAs seem to partition preferably to the Ucon-rich phase in this system as most of the PF obtained were lower than 1. It was also clearly observed that both the recovery yield of PHAs followed a similar trend as PF over a range of Ucon concentrations. Both yield and PF have a decreasing trend from the maximum point at 8 wt/wt % to 13 wt/wt %, which increase afterward with the yield increased at a higher rate than PF. This increasing trend might due to the excluded-volume effect which showed a dominating effect forces both PHAs and contaminants partitioned to the water-rich top phase following the reduction in free-space volume in bottom phase. In this case, PHAs extraction using Ucon-based CPE did not show a promising result as the PHAs recovered in the top phase has a lower purity than that of initial extract, thus, is deemed inefficient and undesirable in the purification process.

On the other hand, for EOPO 3900 system, the recovery yield of PHAs was found to be generally higher than Ucon system, where all yield was greater than 64.5% and reached a maximum of 82.2% at 20 wt/wt % of TSP concentration. The PHAs recovery yield obtained are much higher compared to that of literature which using conventional PEG/phosphate ATPE (51%) (27). The high yield of PHAs compared to that of Ucon is caused by the excluded volume effect due to the stronger hydrophobic effect of EOPO 3900, as the water phase is more entropically favorable for biomolecules, compared to TSP-rich phase (28). The value obtained for PF over a range of TSP concentration was generally higher than 1, with the highest value of 1.3 achieved at 14 wt/wt % of EOPO concentration though it has a significantly lower yield (72.5%) compared to that of 20 wt/wt %. Therefore, thermoseparation-based aqueous two-phase extraction (ATPE) of PHA has been chosen to be done in 20 wt/wt % of EOPO 3900 concentration with the recovery of 82.2% and PF of 1.29.

**Effect of salt addition on PHAs partitioning** Salt addition plays an important role in the partitioning of protein in thermoseparation-based ATPE. Firstly, the addition of cosolutes such as salts which act as counter-ions can promote the partition of biomolecules to the targeted phase due to the diverse affinity of different ions towards the two phases with the anions has a stronger effect than cations. According to the Hofmeister series, the more hydrophobic (chaotropic) ions will partition preferably to the more hydrophobic phase. The addition of salts will cause the cation and anion to partition between the two phases. The

![FIG. 1. Concept of thermoseparation-based aqueous two-phase system (ATPS). Closed circle, bioproducts; closed cloud, contaminants; closed triangle, thermoseparating polymers.](image1.png)

![FIG. 2. Results of PHA partitioning over a range of thermoseparating polymer concentrations (no addition of salt, 33 wt/wt % PHA crude load, thermoseparation temperature of 60°C). Closed circles, EOPO 3900 yield; closed diamonds, Ucon yield; closed squares, EOPO 3900 PF; closed triangles, Ucon PF.](image2.png)
was obtained at 100 mM of salt concentration. It also can be observed that the whole range of 10–100 mM K2HPO4 addition gave higher recovery yield than control, which may due to the alkaline condition helps to release PHA granules more completely (35). On the other hand, further increased concentration of NaCl after 10 mM caused a lower recovery yield and PF in PHAs partitioning compared to that of control. Nevertheless, the addition of 10 mM of NaCl gave both the highest yield (90.5%) and PF (1.34) among all salts across a range of concentrations. Thus, 10 mM of NaCl addition was selected in further optimization of PHAs partitioning.

**Effect of crude load on PHAs partitioning** In the study of PHAs isolation and purification utilizing CPE, PHA crude feedstock was directly added to the thermoseparating system after cell disruption without extra clarification or filtration steps, thus saving extra unnecessary purification cost. For the purpose of scaling-up, it is crucial to understand the limit of PHA crude load that the system can process within a satisfying range of purity and yield. Without considering the mass of crude feedstock, the operating mass of the system throughout the study was maintained at 40 g. Fig. 3 presents the effect of PHAs crude load on the partitioning behavior over a range of crude load. The PF showed an increasing trend from 15 to 37.5 wt/wt %, and it dropped sharply afterwards. It was observed that the optimum crude load for a 40 g system fall in the range of 35–40 wt/wt %. The partitioning of overall biomass to the top phase continued to increase as the crude load increased after 40 wt/wt %, however, the PHAs concentration at top phase increased at a comparatively lower rate (data not shown). This might due to three reasons. Firstly, the huge amount of large descending contaminant particles might have created a downward drag force which is stronger than the hydrophobic affinity between PHAs and the water phase. This directs the partitioning of PHAs molecules towards the bottom phase. Following that, the second possible reason is that the large quantity of PHAs biomolecules may collide and aggregate together during thermoseparation, thus, forming larger and heavier particles which then fall to the bottom phase due to the gravitational force. The last conceivable explanation is that the reduction of free space in the water phase causing it can no longer support the massive amount of bioparticles and contaminants. Hence, the optimized crude load of 37.5 wt/wt % (yield = 92.9%, PF = 1.39 fold) was chosen for further experimental works.

**Effect of thermoseparating temperature on PHAs partitioning** Cloud point (CP) temperature, also known as lower critical solution temperature (LCST) is the temperature where the TSPs started to be “undissolved” from their aqueous solution. However, thermoseparation-based extraction is generally done at a temperature few degrees above the CP to facilitate phase separation, thus reducing the time required (36). Moreover, the electrostatic potential difference caused by uneven partitioning of the ions will generate an electrochemical driving force, thus, influence the partitioning of biomolecules and/or other charged particles in the system (26).

Secondly, the addition of electrolytes such as salts will increase the hydrophobicity difference between the phases by strengthening the hydrophilicity of the water-rich top phase, that in turn causing a decrease in CP. On the other hand, other cosolutes, such as ionic surfactants have opposite effect on CP with an anionic surfactant having a greater impact than cationic surfactant (26). However, literature showed that there is no significant change or a little variation on LCST for the addition of phosphate salts (0–100 mM) (33).

Thirdly, there are also some studies focus on the effect of different salts on the recovery of TSPs (34). These studies concluded a significant increase in TSPs recovery from 69% up to 92% was observed with the addition of 10 mM sulfate, phosphate or chloride salt. It was also pointed out that there is no significant difference in the recovery of the TSPs when utilizing different types of salts (34).

In this study, three different salts have been selected to investigate their influence on PHAs partitioning, which are ammonium sulfate ((NH4)2SO4), dipotassium phosphate (K2HPO4) and sodium chloride (NaCl). The hydrophobicity of the anions studied in this work is ranked in the following order: Cl– > HPO42– > SO42–, while the hydrophobicity of the cations is Na+ > K+ > NH4+. Table 1 presents the results of salt addition on PHAs partitioning. At 10 mM of added salt concentration, every salt gave a higher yield and PF compared to that of control (i.e., without salt addition). However, this phenomenon changed as the salt concentration was further increased. In the case where the concentration of (NH4)2SO4 increased, PHAs partitioned preferably into TSP-rich bottom phase which may due to a fall in pH of the system, causing a decrease in PF and recovery yield. For K2HPO4, the yield reached the highest (87.9%) at 50 mM of salt concentration, while greatest PF (1.26 fold) was observed at 100 mM of salt concentration.

**TABLE 1. Effects of salt addition on PHA partitioning.**

<table>
<thead>
<tr>
<th>Salt type</th>
<th>Concentration (mM)</th>
<th>PF (fold)</th>
<th>Recovery yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH4)2SO4</td>
<td>10</td>
<td>1.21</td>
<td>90.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.20</td>
<td>84.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.17</td>
<td>75.9</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>10</td>
<td>1.23</td>
<td>87.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.03</td>
<td>87.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.26</td>
<td>82.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>10</td>
<td>1.34</td>
<td>90.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.82</td>
<td>54.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.80</td>
<td>53.5</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.17</td>
<td>80.6</td>
</tr>
</tbody>
</table>

* 20 wt/wt % EOPO 3900, 33 wt/wt % PHA crude load, thermoseparation temperature of 60°C.
concentrations of the TSPs in both phases are strongly influenced by thermoseparation temperature. The TSPs-rich phase becomes more saturated as the thermoseparation temperature increases, which reduces the free space for biomolecule per unit volume. This also causes the bioparticles to have an increase tendency to separate into the water phase. Other than that, thermoseparating temperature also has a significant influence on bioseparation as high temperature may cause denaturation of protein, thus affecting the quality of bioproducts (24).

The system composed of 20 wt/wt % EOPO 3900 with the addition of 10 mM NaCl. It was found that the PHAs have partition preferably the water-rich phase at all the investigated temperature. Fig. 4 illustrates the effect of thermoseparating temperatures on the partitioning of PHA. The highest PF was obtained at 60°C (1.42 fold) while thermoseparation at 75°C gave the maximum yield (98.8%). To avoid denaturation of biomolecules due to high temperature and reduce high heat energy consumption, 60°C has been chosen as the most suitable thermoseparation temperature with the yield of 94.8% and PF of 1.42 fold.

Repeated extraction and recycling of thermoseparating polymer in CPE In this thermoseparating-based ATPE, the biomolecules partitioned preferably to the water top phase and the EOPO 3900 can be recycled to form a new stage of ATPE. The recovery and recycling of the EOPO 3900 copolymers are dependent on the geometry of the vessel and heat transfer capacity. The vessel geometry affects the heating rate and sample losses in the interface. A narrow tubing not only promotes the heating rate but also reduces the loss of sample due to the smaller interfacial area (34). Therefore, 50 ml centrifuge tube was used throughout the study for consistency purpose. Moreover, the EOPO 3900-rich bottom phase which contains the contaminants were directly recycled and used in the subsequent stage without going through any extra purification process and additional top-up of fresh TSPs. After the TSP-rich bottom phase was collected and combined with fresh water top phase to form new ATPE, a new batch of feedstock was added to the system.

The results of repeated extraction for PHAs are tabulated in Table 2. The table clearly shown that there is a trade-off between recovery yield and PF as yield drop while PF rise following the repeated extraction. Therefore, there is a need of certain degree of compromise to obtain desired recovery yield and PF.

Table 3 summarizes the results of recycling and reutilization of EOPO 3900 in PHAs recovery. Based on Table 3, it was observed that the purity of PHAs decreased as the EOPO 3900 was being recycled (a reduction rate of approximately 2–3% for each cycle). This was due to the fact that the TSP-rich bottom phase had been increasingly saturated with the contaminants as the thermoseparating polymers being recycled and reused. However, in this case, the EOPO 3900 does not undergo further purification process to remove the contaminants and PHAs in the bottom phase. Moreover, volume exclusion effect occurs due to saturation in bottom phase, promotes partitioning of both PHAs and contaminants to the top phase in the subsequent stage of PHAs extraction. It can also be observed that the overall yield does not follow a specific trend. It can be concluded that the EOPO can be recycled and reutilized for PHA partitioning up to at least twice with insignificant reduction in the purity of PHA. These results are satisfactory where three repeated extraction and purification of PHA was done while retaining similar partitioning behavior. Based on the experimental data, the PF obtained was predicted to reach a constant at 1.356 after the 5th repeated extraction. Taking in the factor there is only a decrease of 6.7% in EOPO 3900 concentration after the first extraction, thus theoretically, the EOPO 3900 can be continuous recycled and reused until the concentration of thermoseparating polymer reach a level too low which it can no longer support a thermoseparation system.

In order to develop a cost-effective and environmental-friendly extraction method for isolation and recovery of PHAs from C. necator H16, the partitioning behavior of PHAs was investigated in the thermoseparation-based ATPE. The purification of PHAs utilizing CPE was satisfactorily achieved in the system of 20 wt/wt % EOPO 3900 and 10 mM NaCl addition at thermoseparation temperature of 60°C with a limit of 37.5 wt/wt % of crude feedstock. PHAs were partitioned preferably to the water-rich top phase with

<table>
<thead>
<tr>
<th>TABLE 2. Repeated extraction of PHAs.4</th>
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<tbody>
<tr>
<td>Extraction</td>
</tr>
<tr>
<td>First</td>
</tr>
<tr>
<td>Second</td>
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<tr>
<td>Third</td>
</tr>
</tbody>
</table>

* 20 wt/wt % EOPO 3900, addition of 10 mM NaCl, 37.5 wt/wt % PHA crude load, thermoseparating temperature of 60°C.

| TABLE 3. Recycling and reutilization of EOPO 3900 in PHA partitioning.5 |
|---------------------------|----------------|----------------|
|                           | PF | Overall yield (%) | Reference |
| First extraction          | 1.45 | 96.7         | Hahn et al. (39) |
| First recycling           | 1.41 | 86.9         |                  |
| Second recycling          | 1.38 | 95.9         | Traussnig et al. (37) |
| Solvent extraction        | 79–90 | 91           | Kim et al. (38) |
| Dispersion by SDS         | >90  | 91           |                  |
| Dispersion of sodium hypochlorite in chloroform | 91 | 91 |                  |

* 20 wt/wt % EOPO 3900, addition of 10 mM NaCl, 37.5 wt/wt % PHA crude load, thermoseparating temperature of 60°C.
a recovery yield of 94.8% and purification factor of 1.42 fold. Recycling and reutilization of EOPO 3900 can be done for at least 2 times without significant loss of yield and purity. The results proved that CPE based aqueous two-phase system (ATPS) is a powerful and potential technique for PHAs purification and recovery. In comparison with literature, the recovery of PHAs by CPE has a much higher recovery yield (approximately 2 times).

In addition, CPE can be coupled with ATP to further improve the quality of PHAs recovered and has a high potential for large-scale application.

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References