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**A B S T R A C T**

In this paper, aqueous two-phase flotation (ATPF) composed of thermo-sensitive ethylene oxide–propylene oxide (EOPO) copolymer and ammonium sulfate was developed for direct recovery of *Burkholderia cepacia* (B. cepacia) strains ST8 lipase from fermentation broth. The effect of varying polymer molar mass, concentration of ammonium sulfate, pH, amount of loaded crude feedstock, initial volume of EOPO phase, concentration of EOPO, initial volume of aqueous phase, nitrogen flow rate and flotation time upon ATPF performance were investigated. Under the optimal conditions of ATPF, the average separation efficiency and purification fold are 76% and 13%, respectively. The recycling of phase components was introduced to minimize the use of organic solvent and salt in ATPF. It was demonstrated EOPO phase in the ATPF system was recovered up to 75%. There was no significant difference in selectivity, purification fold, separation efficiency and recovery yield of lipase obtained between ATPF using fresh and recycled chemicals. *B. cepacia* lipase was successfully purified by using ATPF, which is composed of copolymer EOPO/ammonium sulfate in a single downstream processing step.

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1. Introduction

Aqueous two-phase flotation (ATPF) is a novel separation and purification method, which combines solvent sublation and aqueous two-phase extraction (ATPE) system [1,2]. The main principle of ATPF process is the surface-active compounds have a hydrophobic group (hydroxyl or glucosan) and a hydrophobic group (phenyl or alkyl) in water are adsorbed on the nitrogen bubbles surfaces of an ascending gas stream. Then, the bubbles are dissolved in the polymer layer placed on top of the aqueous solution (Fig. 1). There are many advantages of this technique. This includes high concentration coefficient, soft separation, low dosage of organic solvent, high separation efficiency, simple operation and low environmental impact [3]. ATPF has been widely applied to deal with hydrophobic compound in bioseparation and bioengineering [4] such as using non-recyclable polymer [e.g. polyethylene glycol (PEG)] in separation and concentration of puerarin from *Puerariae* [1], penicillin G [3], lincomycin [5] from fermentation broth and so forth. However, it cannot be denied that one of the limitations of ATPF is that the most phase-forming polymer e.g. PEG could not be effectively recycled [3] (Fig. 1).

Ethylene oxide (EO)–propylene oxide (PO) random copolymers are water-soluble and can be thermoseparated into two phases at temperature that is above a lower critical solution temperature (LCST) [6]. With the use of ethylene oxide–propylene oxide (EOPO) copolymer in ATPF, these systems could be recycled by manipulating temperature [8], thus minimizing the cost [4–7] and environmental impact [9,10]. When applying EOPO in ATPF, the protein purification process is consisted of two steps, namely, primary and secondary ATPF [9]. In primary ATPF, the desired protein is collected on the polymer phase enriched with EOPO copolymer. The secondary ATPF is obtained by the removal of the polymer phase from primary system, which is heated to above LCST. Thus, the desired protein with water was formed in the top phase and concentrated EOPO solution in the bottom phase. The concentrated EOPO copolymer from secondary system could be recycled for the subsequent primary ATPF (Fig. 2). These procedures would give an advantage of reduced cost and minimized environmental pollution. At present, there is no available reports regarding the use of EOPO/(NH4)2SO4 in ATPF for purification of proteins from crude feedstock e.g. fermentation broth.

In this paper, the novel approach of ATPF was applied in the purification of *Burkholderia cepacia* (B. cepacia) strains ST8 lipase...
from fermentation broth. Lipase (EC. 3.1.1.3) is an enzyme that hydrolyzes carboxyl ester bonds that is present in triacylglycerols. It is widely used for industrial and biotechnological applications [11–18]. A detailed study was made with an aim to apply the recycling of both phase-forming components after the purification of B. cepacia strains ST8 from fermentation culture in ATPF. The effect of molecular weight of EOPO, pH, initial volume of EOPO, total volume of (NH₄)₂SO₄ solution, nitrogen flow rate, flotation time, amount of crude feedstock loaded, concentration of EOPO and (NH₄)₂SO₄ solution upon ATPF performance were studied.

2. Materials and methods

2.1. Chemicals and apparatus

Poly (ethylene glycol-ran-propylene glycol), random copolymer (EOPO) with average molecular weight (M_W) of 970, 2500, 3900 and 12000 g/mol, p-nitrophenyl laurate (pNPL), bicinchoninic acid (BCA) solution and standard were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Ammonium sulfate was taken from Merck (Darmstadt, Germany). All of the chemicals were of analytical grade. The ATPF apparatus were the filtration assemblies, obtained from Scott (Scotland, UK) with minor modifications. The G4 porosity was used in sintered glass disk.

2.2. Media and culture condition

B. cepacia strains ST8 was cultivated in a 500 ml shake flask that consisted 100 ml of medium. The fermentation medium comprised of nutrient broth [0.325% (w/v)], CaCl₂ [0.1% (w/v)], gum Arabic [1% (w/v)] and Tween 80 [1% (v/v)]. The pH of medium was 40% (w/w) of crude feedstock.

![Fig. 1. Schematic view of purification in ATPF with non-recyclable polymer (e.g. PEG) and salt solution. The surface active or hydrophobic compounds of the feedstock in aqueous phase are adsorbed on the nitrogen (N₂) bubble surfaces of an ascending gas stream and then collected in non-recyclable polymer phase (e.g. PEG) on top of the aqueous phase.](image)

![Fig. 2. Schematic view of lipase purification in recycling ATPF. The bottom phases of primary and secondary were used to recycle for the subsequent ATPF system. The rest volume was used as samples to conduct the enzyme and protein analysis.](image)
adjusted to 9.0 by addition of NH₃ solution. The fermentation broth was incubated at 37 °C (agitated at 250 rpm) after 16 h of inoculation [5% (v/v)]. After 72 h of incubation, the culture was harvested and immediately used for ATPF studies.

2.3. Lipase activity assay

The lipase activity assay was performed by using the spectrophotometric methods as described elsewhere [19,20], with minor modifications [21–23]. The absorbance of the assay mixture was measured using a microplate reader (Tecan Sunrise) at 405 nm. The lipase assay procedure has been described in a previous publication [22].

2.4. BCA assay

The protein concentrations of samples were determined following the BCA method [24]. The protein standard was bovine serum albumin (BSA). The assay procedure has been described previously [22].

2.5. Determination of selectivity, purification factor, separation efficiency and polymer recovery

Selectivity (S) was calculated as the ratio of the lipase enzyme partition coefficient (Kₑ) to the protein partition coefficient (Kₚ):

\[
S = \frac{Kₑ}{Kₚ} = \frac{Aₚ}{Aₑ} \times \frac{Pₑ}{Pₚ}
\]

where \(Kₑ\) and \(Kₚ\) are the ratios of protein and lipase concentrations recorded in each phase, respectively, \(Aₚ\) and \(Aₑ\) are the lipase activity (in units/ml) seen in the polymer and the aqueous phases, respectively, \(Pₑ\) and \(Pₚ\) are the total protein concentrations (mg/ml) found in polymer and aqueous phases, respectively.

The purification fold (PF) was defined as the ratio of lipase specific activity recorded in polymer phase to the original enzyme specific activity observed in the crude feedstock:

\[
P_F = \frac{\text{Specific activity of polymer phase sample}}{\text{Specific activity of crude feedstock}}
\]

where the enzyme specific activity is the ratio of lipase activity to the total protein concentration of the sample.

Separation efficiency (E) was calculated using Eq. (3):

\[
E = \left(1 - \frac{Cₚ}{Cₑ}ight) \times 100\%
\]

where \(Cₚ\) is the lipase concentration of the aqueous phase at time \(t\), \(Cₑ\) is the original concentration of lipase in aqueous phase.

Polymer recovery (RPₚ) after thermoseparation was defined as Eq. (4):

\[
RPₚ = \left(\frac{mₚ}{mₑ}\right) \times 100\%
\]

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\[
RPₚ = \left(\frac{mₚ}{mₑ}\right) \times 100\%
\]

where \(mₚ\) is the mass of EOPO copolymer captured in the bottom copolymer-rich phase after thermoseparation, \(mₑ\) is the total mass of EOPO copolymer in the polymer phase of ATPF.

Recovery yield (RFₚ) of lipase in the top phase after thermoseparation was determined using Eq. (5):

\[
RFₚ = \frac{1}{1 + \frac{1}{1 + \frac{1}{Vₑ/Vₚ}}} \times 100\%
\]

where \(Vₑ\) is the volume ratio of the top phase to the bottom phase.

2.6. Optimization of separation parameters

The ATPF parameters [molecular weight (\(Mₚ\)) of EOPO, concentration of (NH₄)₂SO₄, concentration of EOPO, pH, initial volume of EOPO phase (\(V₀\)), total volume of ammonium sulfate (\(Vₐ\)), amount of loaded crude feedstock (\(Cₚ\)) flotation time (\(Fₚ\)) and nitrogen flow rate (\(Fₚ\)) were optimized to maximum lipase extraction. The initial total volume of aqueous phase were 250 ml, 50% (w/w) of EOPO, pH 6, 15 ml of V₀, 40% (w/w) of Cₚ, 40 min of \(Fₚ\) and 30 ml/min of \(Fₚ\). All the experiments were carried out at room temperature for three times (triplicates).

2.7. Recycling of phase components in ATPF

The EOPO phase was isolated from the ATPF system and placed in water bath for 15 min (65 °C) [25–28] to induce thermoseparation of the phases. After centrifugation, the top phase water containing the desired enzyme and concentrated EOPO at the bottom phase. The concentrated EOPO solution recovered from the thermoseparation was mixed with the ammonium sulfate solution recovered from first ATPF at the same concentration as used during ATPF (Figs. 1 and 2). The crude feedstock of 40% (w/w) was further introduced to the system. Fresh EOPO and ammonium sulfate were added to obtain the optimized condition of the ATPF if there is necessary. In the experiment, two successive purifications of lipase from fermentation broth were carried out. One of the experiments was carried out by using the recycled EOPO copolymer and ammonium sulfate were recovered in the first ATPF. After purification, the lipase activity and total protein content of all the phases were determined.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out in a SE250 electrophoresis unit (GE Healthcare) as described by Laemmli [29]. The acrylamide gel with 12% of resolving gel and a stacking gel of 4.5% was used in this study. Protein samples were concentrated and precipitated using acetone solution in order to remove polymer compounds and salts presence in the samples as it would affect electrophoresis process. The SDS–PAGE procedure has been described previously [21,22].

3. Results and discussion

3.1. Effect of ammonium sulfate concentration and \(Mₚ\) of EOPO

In ATPF, the concentration of salt (i.e. ammonium sulfate) is the key to sustain an immiscible two-phase system via a salting-out effect [30]. This is due to the volume of the aqueous phase which is much larger than the volume of the EOPO phase if compared with ATPE. The results showed that EOPO phase existed on the top phase when (NH₄)₂SO₄ concentration was 250 g/l or higher. The experiments were conducted by varying the (NH₄)₂SO₄ concentration from 250 to 400 g/l and \(Mₚ\) of EOPO from 970 to 12,000. The influence of (NH₄)₂SO₄ concentration and \(Mₚ\) of EOPO on the separation after thermoseparation is shown in Fig. 3. With the increase of (NH₄)₂SO₄ concentration, the separation efficiency reduced. It is because the interface tension between EOPO phase and aqueous phase were lower enough, thus the mass transfer became easier [3]. Therefore, the lower \(Mₚ\) of EOPO or the lower viscosity mass transfer can be carried out more easily in the separation process. However at high \(Mₚ\) of EOPO, the accumulation of bubbles on the top of flotation system causes controlling of the process more difficult. Therefore, the optimal (NH₄)₂SO₄
concentration of 250 g/l and EOPO 3900 was selected as the extraction phase.

3.2. Effect of pH

The experiments were conducted by varying the pH from 2–8 by adding suitable amounts of hydrochloric acid and sodium hydroxide solution. Fig. 4 shows high purification fold and separation efficiency in the range of pH 2–8. At pH ≥ 8, the separation efficiency and purification fold were decreased because of the decomposition of the NH₄⁺ to NH₃·H₂O. Furthermore, the aqueous two-phase system showed a major change and the characteristic smell of ammonia was found when pH ≥ 8. At pH ≤ 2, the separation efficiency and purification fold are very low because the system exist as uniform which reduces flotation efficiency of the system. The raw fermentation broth was at pH of 6.3 [20]. Since the purification fold and separation efficiency are high at this pH, it can then be chosen as the optimal pH for ATPF operation. It is also to avoid for pretreatment of feedstock with base and acid. This is an advantageous when the entire operation is taken into concern [31].

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Fig. 3. Effect of EOPO molecular weight and ammonium sulfate concentration on ATPF. The effect of four different EOPO molecular weight (970, 2500, 3900 and 12,000) and (NH₄)₂SO₄ concentration in the range of 250–400 g/l were investigated by separation efficiency (%). The results were expressed as the means of triplicate readings with an estimated error of ±5%.

Fig. 4. Effect of pH on ATPF. The ATPF composed of EOPO 3900/(NH₄)₂SO₄ was performed by varying the pH with the range of 2–8. The results were expressed as the means of triplicate readings with an estimated error of ±5%.
3.3. Effect of initial volume and concentration of EOPO

The effect of initial volume (10–30 ml) and concentration [30–60% (w/w)] of EOPO phase upon the ATPF performance was investigated in this part of study. Fig. 5 shows the high separation efficiency obtained in the range of EOPO concentration [30–60% (w/w)]. At concentration of EOPO ≤30% (w/w), the separation efficiency recorded was lower because the systems become homogeneous with the aqueous phase [3]. From our experimental results (Fig. 5), the highest separation efficiency was recorded at 10 ml initial volume and 50% (w/w) concentration of EOPO. In the present study, a 10 ml of initial volume of EOPO phase was the lowest volume that we could apply. This is due to any initial volume of EOPO phase lesser than 10 ml cannot cover the whole surface of aqueous phase. Hence, 10 ml initial volume and 50% (w/w) concentration of EOPO were chosen for further studies.

3.4. Effect of crude feedstock concentration and total volume of aqueous phase

It has been reported that the loaded crude feedstock could change the partition behavior of target protein [32,33] and

Fig. 5. Effect of EOPO concentration and initial volume of EOPO phase on ATPF. The effect of initial volume (10–30 ml) and concentration [30–60% (w/w)] of EOPO phase upon the ATPF performance was investigated. The results were expressed as the means of triplicate readings with an estimated error of ±5%.

Fig. 6. Effect of crude feedstock loaded and total volume of ammonium sulfate solution in the aqueous phase on ATPF. The optimizations of 50% (w/w) EOPO 3900, 250 g/l of (NH₄)₂SO₄ solution and 10 ml volume of EOPO phase at pH 6 were performed by varying crude feedstock loaded [0–50% (w/w)] and volume of aqueous phase (150–400 ml). The results were expressed as the means of triplicate readings with an estimated error of ±5%.
separation efficiency. Fig. 6 illustrates the effect of crude feedstock concentration and total volume of the aqueous phase upon the performance of ATPF. It indicated that crude load of 40% (w/w) is the maximum capacity on the 200 ml of aqueous phase. The separation efficiency for the 40% (w/w) crude feedstock load in ATPF was at 70.8%. Higher amounts of the crude feedstock loaded (>40% (w/w)) into the ATPF will decrease the separation efficiency and subsequently affected the composition of ATPF. It is due to the fact that the components in the feedstock had changed the ATPF properties [34]. These phenomena can be explained by the increase of precipitate accumulation at the interface, causing the loss of targeted enzyme together with other contaminants (e.g. cell debris and other proteins) [21]. Therefore, the 40% (w/w) of feedstock loading in 200 ml aqueous phase system was chosen for further study.

3.5. Effect of nitrogen flow rate in ATPF

Fig. 7 shows the effect of nitrogen flow rate (0–60 ml/min) on the performance of ATPF. A high flow rate was required in order to reduce the flotation time. However, too high gas nitrogen flow rate resulted in the turbulent mixing at the interface of solvent–aqueous solution system [1,35]. The abundance of bubbles accumulated on the top of flotation EOPO phase [36] and such phenomena can result to the re-dissolution of the hydrophobic analytes in the aqueous phase and significantly influence the mass

Fig. 7. Effect of nitrogen flow rate on ATPF. The selectivity (S), purification fold (PF) and separation efficiency (E) were calculated using Eqs. (1)–(3). The results were expressed as the means of triplicate readings with an estimated error of ±5%.

Fig. 8. Effect of flotation time on ATPF. The flotation time upon the ATPF performance was investigated from 0 to 180 min. The results were expressed as the means of triplicate readings with an estimated error of ±5%.
3. Effect of flotation time in ATPF

Fig. 8 shows the effects of varying flotation time on the selectivity, purification fold and separation efficiency of lipase. The selectivity, purification fold and separation efficiency increased with the increasing of the flotation time [37,38]. All the curves achieved separation equilibrium after 60 min of flotation time. The highest flotation time was 50 min or more, the selectivity and separation efficiency were around 12% and 75–78%, respectively. Hence, extending the flotation time into the separation and purification process was not necessary. Thus, a flotation time of 60 min was selected as it is the optimal condition with the values of selectivity and separation efficiency at 13% and 76%, respectively.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>First ATPF</th>
<th>Recycling ATPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feedstock specific activity</td>
<td>9.07</td>
<td>9.07</td>
</tr>
<tr>
<td>Selectivity</td>
<td>12.96</td>
<td>12.79</td>
</tr>
<tr>
<td>Enzyme specific activity (polymer phase)</td>
<td>111.15</td>
<td>97.06</td>
</tr>
<tr>
<td>Purification fold</td>
<td>12.26</td>
<td>17.75</td>
</tr>
<tr>
<td>Separation efficiency (%)</td>
<td>75.87</td>
<td>75.01</td>
</tr>
<tr>
<td>Recovery yield of lipase (%)</td>
<td>98.81</td>
<td>98.22</td>
</tr>
</tbody>
</table>

The table summarizes the selectivity, purification fold, separation efficiency (%), recovery yield of lipase (%), specific activity of enzyme on the polymer phase and crude feedstock by using fresh and recycled chemicals in first and recycling ATPF, respectively. The results were expressed as the means of triplicate readings with an estimated error of ±5%.

3.7. Effect of recycling phase components in ATPF

Fig. 2 illustrated the schematic view of lipase purification in recycling ATPF. It shows that the good surface-activity of lipase can be bound to the surface of the nitrogen bubbles [39,40]. Floating to the top of the aqueous phase consisted of lipase and salt solution where they come across a layer of EOPO copolymer, in which the lipase dissolves [5]. Lipase derived from B. cepacia strain ST8 is suitable for ATPF because it made up of 320 amino acid residues. According to X-ray structure of the B. cepacia strain ST8 lipase shows that its active site can be divided into three domains [41], which are the large hydrophobic pocket, medium-sized pocket and alternate hydrophobic pocket. Under the optimal condition (see Sections 3.1–3.6), ATPF was applied by using the recycling of phase components. The thermoseparation of EOPO copolymer in ATPF gave a copolymer recovery up to 75%. In other words, the high recovery in ATPF can reduce the dosage of organic solvent applied in the study effectively. Hence, recycling ATPF is more beneficial in reducing the operation costs required and protecting environment.

Table 1 indicated that there was just a small variation (<1%) observed in the selectivity, purification fold, separation efficiency and recovery yield of lipase in the ATPF prepared from recovered chemicals. The purity of lipase recovered from the aqueous phase in ATPF was assessed by 12% SDS–PAGES [29] analysis (Fig. 9). The crude feedstock contained multiple bands (Lane 2), representing contaminant proteins present in the fermentation broth. Lane 4 shows the product obtained from ATPF conducted using fresh chemicals. In Lane 5, the sample was obtained from ATPF using recycled phase components. In general, B. cepacia strains ST8 lipases have molecular mass of about 33 kDa [42]. The realization of direct recovery of lipase from unclarified fermentation broth in a partially purified state was confirmed by the SDS–PAGE analyses. In line with the archived lipase specific activities (see Table 1 and Fig. 9), there were

Fig. 9. SDS–PAGE analysis on the purification of lipase. Molecular weight of standard marker ranged from 20 to 100 kDa. SDS–PAGE – Lanes 1 and 3: protein maker; Lane 2: control crude culture; Lane 4: sample of first ATPF conducted using fresh chemicals; Lane 5: sample of recycling ATPF conducted using recovered chemicals from first ATPF.
no gross variation between the products recovered from ATPF composed of fresh and recycled chemicals.

4. Conclusion

The lipase from fermentation broth was successfully purified and separated by using EOPO copolymer in ATPF. Under the optimized condition [3900 molecular mass of EOPO, 250 g/l concentration of ammonium sulfate, pH 6, 10 ml of initial volume of EOPO phase, 50% (w/w) of concentration of EOPO 3900, 200 ml of total volume of aqueous phase system, 40% (w/w) of loaded crude feedstock, 30 ml/min of nitrogen flow rate at 60 min flotation time], a separation efficiency of 76% was achieved in the system. It was demonstrated EOPO phase in the ATPF system was recovered up to 75%. In line with the archived lipase specific activities, there was no gross variation between the products recovered from ATPF composed of fresh and recycled chemicals. The recycling of all the phase-forming components in ATPF is ideally effective in terms of cost (recycling of polymer and salt solution for subsequent ATPF), processing time (reduction of operation time) and environment friendly. This will lead to an extended application of ATPS using alternative technologies.

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References