Optimising Cutinase Enzyme Recovery in Thermo-Induced Phase Separation of LS54/DX ATPS by Enhanced Volume Exclusion Effect

Fariza Akmal Abdul Mutalib
Department of Chemical and Process Engineering, Faculty of Engineering & Built Environment
Universiti Kebangsaan Malaysia (UKM), 43600, Bangi, Selangor, Malaysia

Jamaliah Md Jahim*
Department of Chemical and Process Engineering, Faculty of Engineering & Built Environment
Research Centre for Sustainable Process Technology, Faculty of Engineering & Built Environment
Universiti Kebangsaan Malaysia (UKM), 43600, Bangi, Selangor, Malaysia

Abdul Wahab Mohammad
Department of Chemical and Process Engineering, Faculty of Engineering & Built Environment
Research Centre for Sustainable Process Technology, Faculty of Engineering & Built Environment
Universiti Kebangsaan Malaysia (UKM), 43600, Bangi, Selangor, Malaysia

Qatar Energy and Environment Research Institute, Hamad bin Khalifa University (HBKU), Qatar Foundation, Doha, Qatar

Farah Diba Abu Bakar
Centre of Bioscience & Biotechnology Studies, Faculty of Science & Technology
Universiti Kebangsaan Malaysia (UKM), 43600, Bangi, Selangor, Malaysia

ABSTRACT

Low recovery of cutinase enzyme in water-enriched phase after thermo-induced separation stage of LS54/Dx aqueous two-phase system was improved by enhanced volume exclusion effect in the polymer-water extraction system. It was done by increased the polymer concentration in the polymer-water system. After primary phase separation, more LS54 (polymer) which is the system’s component itself were added into polymer-enriched phase and mixed thoroughly before thermo-induced separation step proceeded. The compositions of LS54 added into the polymer-enriched phase were 0.25, 0.5 and 0.75 g LS54/g top phase. The thermo-induced phase separation was carried out at 37°C. It was found that cutinase recovery in water-enriched phase was increased up to 5-13% with the increment of polymer concentration in the system as compared to a system without polymer addition. The optimum concentration obtained for the polymer added was 0.5 g LS54/g top phase whereby it attained 82% recovery of cutinase enzyme in water-enriched phase after thermoseparation step. Although the increment of enzyme recovery was not exceptionally high as compared to another method such as adding ligand, an affinity tag or neutral salt, still this method is applicable because of its more straightforward work, polymer recycle capability, and enzyme recovery in water phase would definitely give benefit to further downstream processing.

Keywords: Aqueous two phase system (ATPS); thermoseparation; enzyme recovery; volume-exclusion effect

ABSTRAK

Perolehan rendah enzim cutinase dalam fasa diperkaya air selepas peringkat pemisahan teraruh suhu sistem dua fasa berakueus LS54/Dx ditambahbaik dengan meningkatkan kepekatan penyingkiran isipadu dalam sistem pengekstrakan polimer-air. Ia dilakukan dengan meningkatkan kepekatan polimer dalam sistem pengekstrakan tersebut. Selepas pemisahan fasa utama, lebih banyak LS54 (polimer) iaitu komponen sistem ini sendiri, telah ditambah ke dalam fasa diperkaya polimer dan dicampur dengan baik sehingga pemisahan teraruh suhu diteruskan. Komposisi LS54 yang ditambahbaik ke dalam fasa diperkaya polimer adalah 0.25, 0.5 dan 0.75 g LS54/g fasa atas. Pemisahan fasa teraruh suhu dilakukan pada 37°C. Didapati perolehan cutinase dalam fasa diperkaya air meningkat sehingga 5-13% dengan peningkatan kepekatan polimer dalam sistem berbanding dengan sistem tanpa penambahan polimer. Kepekatan optimum yang diperolehi bagi penambahan polimer ialah 0.5 g LS54/g fasa atas dengan mencapai 82% perolehan enzim cutinase dalam fasa diperkaya air selepas penambahan kepekatan teraruh suhu. Walaupun peningkatan perolehan enzim tidak begitu tinggi berbanding dengan kaedah lain seperti penambahan ligan, tag affinity atau garam neutral, namun kaedah ini boleh digunakan untuk perolehan enzim dalam fasa air pasi memberi manfaat untuk pemprosesan hiliran seterusnya.

Kata kunci: Sistem Dua Fasa Berakueus (S2FA); pemisahan teraruh suhu; perolehan enzim; kesan penyingkiran isipadu
INTRODUCTION

Application of thermo-induced polymer in aqueous two-phase system (ATPS) development has earned more attention recently. Unlike the other type of ATPS, the used of the thermo-responsive polymer has made the system capable of extracting product from the polymer-rich phase directly by changing the environment condition which is temperature, and the product-free polymer phase can be recycled for back extraction. Additionally, recovery of target biomolecules/bioproducts in water-rich phase would undoubtedly give benefits to the following steps in downstream processing.

ATPS has been one of the preferred methods in extraction and purification of biomolecules because of its simplicity, selectivity and relatively a low-cost method (Espitia-Saloma et al. 2014; Goja et al. 2013; Leong et al. 2015). The mild condition provided by ATPS has been another factor that makes it keep progressing in bioseparation area. ATPS has been applied not only for the recovery of proteins and enzymes but also had been used for extraction of high-end bioproducst such as recombinant therapeutic proteins, monoclonal antibody-based products (mAb) and nucleic acid-based medicinal products (Rosa et al. 2010).

In order to achieve higher yield, several strategies have been applied in ATPS method. It was either manipulation of system’s parameter (for example type, composition & polymer size, pH), altering the type of ions in the system, adding additional salt, or exploiting the hydrophobic groups) (Asenjo et al. 2012). Yet, most of the strategies were generally used in primary-phase separation. Only several studies had discussed the recovery of proteins/enzymes in a thermo-induced stage. For example, Jo'nsson and Johansson (2003) had modified a hydrophobically thermo-responsive polymer (HM-EO) to a cationic polymer in order to manipulate electronic interaction between protein and the polymer in water/HM-EO system by changing pH, micellar net-charge and salt addition.

The separation of biomolecules in water/thermo-responsive polymer system driven by entropic effect is a well-established concept (Johansson et al. 1998). As the temperature increased, the entropy energy of polymer rich phase would be increased. This situation would drive biomolecules partition exclusively into less chaotic, water-rich phase. This excluded volume concept was similar to the aqueous micellar system. In addition, volume fraction of micelles in micelle-rich phase is vital determinant to the magnitude of the excluded-volume interactions that are driving the partitioning of biomolecules into less-micelle phase (van Roosmalen et al. 2004). With that information, this study was conducted by increasing volume fraction of polymer in polymer-enriched phase, as an attempt to optimise the recovery of cutinase in water-enrich phase. It was done by adding more polymer into polymer-enriched phase at several concentrations before the thermoseparation phase.
recovery in top phase (for primary phase separation) was calculated according to equation (1):

\[ Y(\%) = \frac{C_t V_t}{C_t V_t + C_b V_b} \times 100 = \frac{100}{1 + \left(\frac{1}{k_{cut} \cdot V_b}\right)} \]  

(1)

where \(C_t\) and \(C_b\) represent cutinase activity in top and bottom phase, respectively.

Meanwhile, the yield of cutinase in water-enriched bottom phase, after the thermo-induced separation was determined as below:

\[ \text{Yield}_{\text{ thermo}}(\%) = \frac{\text{To cut act in the water phase}}{\text{To cut act in LS54-enriched phase}} \times 100 = \left(\frac{C_w \cdot V_w}{C_t \cdot V_t}\right) \times 100 \]  

(2)

Where, \(C_w\) and \(C_t\) were cutinase activity in water-enriched phase and LS54-enriched top phase (of primary phase separation), respectively. Whereas \(V_w\) was the volume of water-enriched phase and \(V_t\) was the volume of LS54-enriched top phase (of primary phase separation). Furthermore, as a measure of the concentrating effect of the cutinase enzyme in the water phase, the concentrating factor, \(cf\) was also calculated according to Equation (3):

\[ cf = \frac{V_t + V_b}{V_b} \]  

(3)

where \(V_t\) and \(V_b\): volume of top phase and bottom phase, respectively.

RESULTS AND DISCUSSION

Initially, extraction of cutinase was carried out using LS54/Dx system under condition of 22% LS54/12.5% Dx/0.1M \(Na_2SO_4\) and pH:8.0. This condition was selected as it is the optimum condition for cutinase initial phase separation that obtained in a previous study (Jahim et al. 2012). After primary phase separation, approximately 79.4% of cutinase enzyme was successfully recovered in Dehypon®LS54 (LS54)-enriched top phase. Then, the enzyme extraction was continued with thermo-induced phase separation. At this stage, a new two-phase system consisting of top polymer-enriched phase and bottom water-enriched phase was formed by increasing the LS54-enriched phase temperature up to 35°C in water bath. Ratio top phase to bottom phase volume obtained was 3.4.

After thermoseparation step, approximately 70% of cutinase enzyme in the LS54-enriched top phase (from the primary phase separation) was recovered in water-enriched bottom phase. Hence, due to increase cutinase recovery in the water phase, more polymer (LS54) was added in order to enhance volume excluded effect in the extraction system. According to Ducheyne et al. (2011), as the concentration increases, the swelling of the chain is counteracted by the presence of other chains; thus, leading to a screening effect of the volume excluded interactions between monomers belonging to the same chain. As a result, the polymer chains will pack tightly, which leads to an osmotic penalty; since the protein, volume becomes inaccessible to the polymer...
monomers (Ducheyne et al. 2011). The same self-aggregation will continually occur if the concentration is increased to a certain degree at the same constant temperature. The volume-exclusion effect at high polymer concentration was illustrated in Figure 2.

Furthermore, it was also found that the polymer (Dehypon® LS54) structure had a significant contribution to the accumulation of cutinase in the water phase. Dehypon® LS54 is a non-ionic surfactant that composed of fatty alcohol and two polymers blocks which are ethylene oxide (EO) and propylene oxides (PO) (Figure 3). Besides propylene oxide, fatty alcohol chain (R) that located at the other end of the LS54 structure is also displayed as a hydrophobic character. According to Tani et al. (2001), polymer/surfactant that consists of hydrophobic character at both ends of its structure could perform higher extractability due to micellar network construction that enhances the excluded-volume interaction between the micelles/polymer and protein/enzyme (as shown in Figure 4).

Experimental result for cutinase enzyme recovery in thermoseparation phase was shown in Table 1. At this stage, the top phase of the system was consisted of polymer (LS54), while the bottom phase was enriched with water. As refer to Table 1, it was observed that addition of LS54 in thermo-induced phase had affected phase volume ratio, $v_R$ of the extraction system. The volume of water-enriched phase was decreased significantly with the addition of LS54. Apparently, the addition of the polymer had increased the volume of polymer phase. This initiated exclusion effect which had resulted in a rise of cutinase recovery in water phase. The cutinase recovery had improved by up to 12% (82% recovery) when 0.5 g LS54/g top phase was added. However, enzyme recovery in water-enriched phase had a small increased (84% recovery) with the further addition of polymer (which is a 0.75 g LS54/g top phase) in the system. Meanwhile, a concentration factor of water phase for 0.5 g and 0.75 g LS54/g top phase were 5.5 and 5.8 respectively. The small increment of enzyme recovery in water phase and concentration factor (cf) between the two values of polymer addition (0.5 g and 0.75 g/g top phase) explained that the addition of 0.5 g LS54/g top phase was sufficient to optimize the cutinase recovery in water-enriched phase since more polymer addition, gave a small increment to the enzyme recovery. Furthermore, a system with lower polymer concentration is less viscous. Thus it would be easier to handle compared to a system with 0.75 g LS54/g top phase.

As compared to other strategies, the yield value achieved in this study was slightly lower (Table 2). However, it was expected since other methods were specific in action, and more works were needed to be done. Yet, the polymer addition was managed to increase the cutinase recovery in water phase after thermos-induced step. Besides simple in term of technique, it was also an advantage to be able to recover the enzyme in water-enriched phase.
## TABLE 1. Recovery of cutinase in thermo-induced separation step with and without additional polymer (LS54)

<table>
<thead>
<tr>
<th>Extraction system</th>
<th>Top phase (g)</th>
<th>Bottom phase (g)</th>
<th>Phase volume ratio, $v_R$</th>
<th>Total enzyme act. (U/ml)</th>
<th>Total recovery (%)</th>
<th>cf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary recovery</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>2.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thermo-induced phase separation:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without additional LS54</td>
<td>3.09</td>
<td>0.91</td>
<td>3.4</td>
<td>1.82</td>
<td>70.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Addition of LS54:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 g LS54/g tp</td>
<td>4.08</td>
<td>0.92</td>
<td>4.0</td>
<td>1.95</td>
<td>75.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.50 g LS54/g tp</td>
<td>4.9</td>
<td>1.1</td>
<td>4.5</td>
<td>2.13</td>
<td>81.9</td>
<td>5.5</td>
</tr>
<tr>
<td>0.75 g LS54/g tp</td>
<td>5.8</td>
<td>1.2</td>
<td>4.8</td>
<td>2.17</td>
<td>83.5</td>
<td>5.8</td>
</tr>
</tbody>
</table>

*tp = top phase of primary phase separation

## TABLE 2. Comparison of ATPS studies with different strategies to increase yield value

<table>
<thead>
<tr>
<th>System</th>
<th>Target biomolecule</th>
<th>Strategy</th>
<th>Performance parameter</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCON/dextran</td>
<td>IgG</td>
<td>Add free ligand into system (trietylenglycol-asid diglutarik (TEG-COOH)</td>
<td>$Y = 85%$, $\text{Purity} = 88%$</td>
<td>(Ferreira et al. 2008)</td>
</tr>
<tr>
<td>PEG/dextran</td>
<td>IgG</td>
<td>Attach ligand to a system component (PEG-asid diglutarik)</td>
<td>$Y = 93%$</td>
<td>(Rosa et al. 2007)</td>
</tr>
<tr>
<td>C10G1 micellar aqueous two-phase system</td>
<td>Green flouroescent protein, GFP</td>
<td>Attach affinity tag (CBM9) to target protein (PEG-CBM9)</td>
<td>$k = 3.3$</td>
<td>(Mazzola et al. 2006)</td>
</tr>
<tr>
<td>PEG1000/fosfat</td>
<td>ribonucleose</td>
<td>PEGylation (covalently attached PEG to protein atau drug molecules)</td>
<td>$Y = &gt; 90%$</td>
<td>(González-Valdez et al. 2012)</td>
</tr>
<tr>
<td>PEG300/Na2SO4</td>
<td>Gallic acid</td>
<td>Addition of ionic liquid a. $[\text{C}<em>{4}\text{mpip}]\text{TOS}$ b. $[\text{C}</em>{4}\text{mpip}]\text{SCN}$ c. $[\text{C}_{4}\text{mpip}]\text{(N)CN}<em>2$ d. $[\text{C}</em>{4}\text{mpip}]\text{CH}_3\text{CO}<em>2$ e. $[\text{C}</em>{4}\text{mpip}]\text{Cl}$</td>
<td>$k = 2.3-29.0$, $Y = 78-98%$</td>
<td>(Almeida et al. 2014)</td>
</tr>
<tr>
<td>PEG/K2HPO4</td>
<td>RNA</td>
<td>Addition of neutral salt (NaCl)</td>
<td>$k = 3.5$</td>
<td>(Luechau et al. 2009)</td>
</tr>
<tr>
<td>LS54/dextrin</td>
<td>Cutinase</td>
<td>Addition of more polymers in extraction system at thermo-induced step.</td>
<td>$Y = 82%$</td>
<td>This study</td>
</tr>
</tbody>
</table>

## CONCLUSION

Cutinase recovery in water-enriched phase had been optimized by adding more polymer into LS54-enriched top phase (which is separated from primary phase separation). The optimum concentration obtained for polymer addition was 0.5 g LS54/g top phase. At this concentration, cutinase recovery in water-enriched phase had been increased to 82%. The cutinase recovery in water-enriched phase showed an insignificant increase (84%) with further addition of polymer (0.75 g LS54/g top phase) in the extraction system. It can be concluded that this optimization step is not only able to offer benefit for further enzyme purification stage because the target enzyme accumulated in water-enriched phase, but it also offers minimal use of additives in extraction system since the added polymer was the system component itself.

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Fariza Akmal Abdul Mutalib
Department of Chemical and Process Engineering
Faculty of Engineering & Built Environment
Universiti Kebangsaan Malaysia (UKM)
43600, Bangi, Selangor, Malaysia.

*Jamaliah Md Jahim
Department of Chemical and Process Engineering
Faculty of Engineering & Built Environment
Universiti Kebangsaan Malaysia (UKM)
43600, Bangi, Selangor, Malaysia.

Abdul Wahab Mohammad
Department of Chemical and Process Engineering
Faculty of Engineering & Built Environment
Universiti Kebangsaan Malaysia (UKM)
43600, Bangi, Selangor, Malaysia.

Research Centre for Sustainable Process Technology
Faculty of Engineering & Built Environment
Universiti Kebangsaan Malaysia (UKM)
43600, Bangi, Selangor, Malaysia.

Qatar Energy and Environment Research Instititute
Hamad bin Khalifa University (HBKU)
Qatar Foundation, Doha, Qatar.

Farah Diba Abu Bakar
Centre of Bioscience & Biotechnology Studies
Faculty of Science & Technology
Universiti Kebangsaan Malaysia (UKM)
43600, Bangi, Selangor, Malaysia.

*Corresponding author; email: jamal@ukm.edu.my

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