ARTICLE; FOOD BIOTECHNOLOGY

Extraction of lipase from Rhizopus microsporus fermentation culture by aqueous two-phase partitioning

Masumeh Anvari*
Department of Biology, Faculty of Sciences, Rasht Branch, Islamic Azad University, Rasht, Iran

(Received 16 December 2014; accepted 15 April 2015)

The purification of a desired protein from the complex mixture of biomolecules in a fermentation broth is one of the main challenges in industrial-scale biotechnological processes. For partial purification of industrial enzymes, one possible approach is to use aqueous two-phase extraction (APTE) as a primary downstream processing step. In the present study, the feasibility of utilizing ATPE for the purification of lipase from Rhizopus microsporus fermentation culture was investigated. The effects of the phase composition and salt type, molecular weight of the polyethylene glycol (PEG), pH of the system, sample loading and addition of neutral salts on lipase partitioning were investigated at 24°C. In most of the examined aqueous two-phase systems (ATPSs), lipase showed affinity to the top phase. Optimum conditions for lipase purification were obtained in 20% PEG 2000/12% (NH₄)₂SO₄, with 5% Na₂CO₃ addition at pH 8 for 30% crude load. For the optimized ATPS, the recovery yield and purification factor in the top phase were determined to be 92.3% and 19.8, respectively.

Keywords: lipase; aqueous two-phase system; Rhizopus microsporus; polyethylene glycol (PEG)

Introduction

Lipases are enzymes that belong to the group of hydro-lases whose main biological function is to catalyze the hydrolysis of insoluble triacylglycerols to free fatty acids, mono- and di-acylglycerols and glycerol. Besides their natural function, lipases can catalyse esterification, inter-esterification and transesterification reactions in non-aqueous media.[1,2]

The use of waste biomaterials for biotechnological products, especially enzymes, has been gaining attention in recent years.[3,4] Guilan province is a large agricultural producer; it is the largest rice producer in Iran. The rice cultivation area in Guilan is over 238,000 ha with an average farm yield of 4.8 tons/ha and an approximate straw production of 2.7 tons/ha.[5] Moreover, there are 450 ha of olive cultivation area and 6000 tons of product yield per year. Thus, a considerable amount of agricultural waste and, more specifically, of olive waste is obtained in large-scale olive oil productions.[4] Such agrochemical wastes could potentially be considered a cheap and valuable source as growth media for different biotechnological processes including microbial enzymes production.[4]

One of the major challenges in the biotechnology industry is the purification of a desired protein from a fermentation broth containing a wide variety of biomolecules.[6] Partitioning using aqueous two-phase systems (ATPSs) has proved to be a valuable tool and a mild technique for separating and purifying mixtures of biomolecules. These systems can form a gentle environment for enzymes and other biologically active proteins. Therefore, the extraction in the systems has the advantages of high capacity and high activity yields and is easy to scale up.[7] These systems have many applications for protein purification in biochemistry and biotechnology.[8,9] The partitioning of biomolecules in ATPS is influenced by pH, temperature, surface properties, size and concentrations of the biomolecules and the types of employed polymers and salts.[10]

Although ATPSs have been applied for recovery of a wide variety of biomolecules, to the best of our knowledge, there is no report on the partitioning behaviour and purification of lipase from R. microsporus fermentation culture in polymer—salt systems. Therefore, in the present study, the partitioning behaviour of lipase in various polyethylene glycol (PEG)—salt ATPS and the feasibility of utilizing these systems for purification of lipase produced from agricultural wastes using R. microsporus was investigated.

Materials and methods

Micro-organisms

The R. microsporus strain used was previously selected as a good lipase producer in solid-state fermentation (SSF).
The cultures were maintained on potato dextrose agar at 30 °C with monthly subculturing.

Inoculum and fermentation conditions
The pre-culture medium was as described previously.[11] Agro-products, including rice straw, rice bran and olive waste, were used as solid substrates for SSF. Ten grams of mixed substrates were moistened with Czapek–Dox medium in 500 mL Erlenmeyer flasks to reach a final moisture content of 70% (w/v). The cultivation conditions were as reported previously.[4] After a 5-day incubation period for fermentation, extraction of the enzyme was carried out and the supernatant was used as a crude enzyme fraction.

Preparation of aqueous two-phase systems
All ATPS were prepared in 15 mL graduated centrifuge tubes at an equal volume of salt and PEG solution in different conditions. To study the effects of PEG molecular weight (MW) and types of salt on the partitioning of lipase from the cell-free fermentation broth, using ATPS, different salts, including Na-citrate, MgSO4, Na2HPO4 and (NH4)2SO4, were mixed with different MWs of PEG (600, 1000, 2000, 4000, 6000 and 8000 Da) in an aqueous system. The glass tube was shaken vigorously by turning upside-down, followed by centrifuged for 10 min at 7000 r/min to assist phase separation. After phase separation and visual estimation of the top and bottom phases, the volumes of the phases were used to estimate the volume ratio. The samples of the top and bottom phases were carefully withdrawn by using a plastic syringe with a short and a long needle, respectively. Aliquots from each phase were analysed to determine the enzyme activity and protein concentration.

Analytical methods
Lipase assay was done spectrophotometrically using p-nitrophenyl palmitate (Sigma) as the substrate.[3]

Protein concentration was measured by the method of Bradford [12], using bovine serum albumin as a standard. The protein partition coefficient (Kp) in the ATPS was defined as [13]

\[ K_p = \frac{C_{p,t}}{C_{p,b}} \]

where \( C_{p,t} \) and \( C_{p,b} \) are the concentrations of protein in the top and bottom phases, respectively.

The partition coefficient for lipase activity (Kc) in the ATPS was calculated to quantify the degree of separation reached in the extraction process:

\[ K_c = \frac{A_t}{A_b} \]

Yield (Y, %) of lipase in the top phase was also calculated based on the partition coefficient to study the efficiency of the system,

\[ Y(\%) = \frac{100}{\left(\frac{V_b K_c}{V_t K_p} + 1\right)} \]

where \( V_R \) is the volume ratio of the top phase to the bottom phase (\( V_t/V_b \)).

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

\[ S = \frac{K_e}{K_p} = \frac{A_t}{C_{p,b}} \frac{C_{p,t}}{A_b} \]

The purification factor in the top phase (PFtop) was defined as

\[ PF_{top} = \frac{SA_t}{SA_i} \]

where SA\(_t\) and SA\(_i\) are the specific activities in the top phase and the crude enzyme, respectively. Also, the specific activity (SA) represents the ratio of the enzyme activity to the protein concentration in a sample,

\[ SA = \frac{A}{C_p} \]

where \( A \) and \( C_p \) are the enzyme activity and the total protein concentration in each phase, respectively.

Data analysis
All experiments were run in duplicate at room temperature (24 ± 1 °C). Statistical analysis was done by Student’s t-test, using Microsoft Excel. A probability level of \( p < 0.05 \) was considered statistically significant.

Results and discussion
Theoretical predictions of biomolecule extraction using ATPS could be difficult. This is because the type of additives and/or impurities and their concentration in the aqueous solution directly affect both the system features and the solute properties.[13] For the efficacy of aqueous two-phase extraction (ATPE), it is very important to select a suitable phase system. In ATPSs, the most significant and difficult step is to choose the appropriate type and composition of the system in terms of achieving efficient extraction of the biomolecule.[14] It is well known that as a result of several factors, such as type of phase-forming
salt, PEG MW, concentration of salt and polymer, tie-line length, volume ratio and ionic composition of the phases, the partition and distribution behaviours of compounds in ATPSs are considered very complex phenomena and therefore, it is not possible to infer a general rule.[15,16] Choosing the phase-forming components types for the distribution of the biomolecule is the second most important step following the selection of the phase system type as polymer—salt.[17] In this study, polymer—salt systems were selected because of some advantages they have, e.g. lower cost and gentler environment, in addition to lower viscosity in comparison to polymer—polymer systems.

**Selection of phase-forming salt**

The choice of a phase-forming salt has a direct effect on the separation and extraction of a given biomolecule in ATPE due to its significant influence on the system environment.[18] The selection of salt employed in the extraction system is also one of the key points of this technique.[19,20] To ensure the efficiency of the extraction, the partition system is also one of the key points of this technique. [21]

The partitions of the target protein and the other molecules in the extract primarily depend on the types and amounts of salts. As shown in the table, the systems containing 10% Na-citrate and 10% MgSO_4 resulted in no phase separation. This could probably be due to the amount of these salts being insufficient for generating the two-phase formation in this partitioning of lipase from *R. microsporus*. The SA and PF decreased the higher the salt concentration was. It has been found that an increase in salt quantity provides a salting-out effect.[22] Consequently, both target and non-target proteins were mostly partitioned to the PEG-rich top phase and resulted in a decrease in SA and PF of the top phase (Table 1), similar to the results reported by Chaiwut et al. [23] for plant protease. Protein hydrophobicity and \( V_R \) reduction are the main reason for lipase to move away to the top phase at increased salt concentration. It has been shown that PF could be decreased with increasing phosphate salt and copolymer concentration because of hydrophobic interactions between copolymer and enzyme molecules.[24]

The performance of salts in promoting phase separation is reflected in the lyotropic series (a classification of ions based upon salting-out or salting-in ability).[25] Salts with multi-charged anions are most effective, e.g. \( \text{SO}_4^{2-} \) > \( \text{HPO}_4^{2-} \) > acetate > \( \text{Cl}^- \), whereas cations are commonly ranked in the following order: \( \text{NH}_4^+ \) > \( \text{K}^+ \) > \( \text{Na}^+ \) > \( \text{Li}^+ \) > \( \text{Mg}^{2+} \) > \( \text{Ca}^{2+} \).[26] Similar to other reports,[23] our results showed that the lipase partitioning was much more strongly affected by anion species than by cations. The highest SA and PF were obtained in the top phase at ATPS of 18% salt containing \( \text{SO}_4^{2-} \) as compared to \( \text{HPO}_4^{2-} \) and citrate, respectively (Table 1). Cations could also be effective in enzyme recovery at the system of 14% salts. In the cation series of \( (\text{NH}_4)^+ > \text{Na}^+ > \text{Mg}^{2+} \), the obtained yields were 31.6%, 25.9% and 23.1%, respectively, which is in accordance with the lyotropic series.

As a result, the highest lipase SA, purification fold and activity recovery were observed in the PEG/(NH_4)_2SO_4 system (Table 1) and this system was, therefore, selected for further studies.

**Selection of polymer molecular weight and system composition**

The partitions of the target protein and the other molecules in the extract primarily depend on the PEG MW and the system composition in ATPSs because these parameters influence the protein partition by changing the number of hydrophobic interactions attributed to interactions between the PEG and the hydrophobic area of protein. [27] Therefore, in order to select a suitable PEG MW for the extraction of lipase from *R. microsporus* fermentation broth, ATPSs were performed with six different MWs of PEG and with different PEG (12%–20%) and (NH_4)_2SO_4 concentrations (12%–20%). The prepared systems are listed in Table 2 together with detailed results. The results showed that the partitioning of lipase in the PEG/(NH_4)_2SO_4 system was strongly dependent on the MW of PEG. In all of the systems, the total percentages of the PEG and salt components were maintained at 30%–32%. However, in the systems prepared with PEG 600, no phase separation was observed in the so-called total percentage.

| Phase composition (% w/w) | \( V_R \) | SA | PF | \( Y (%) \) |
|---------------------------|---------|----|----|----------|
| 12% PEG 4000/10% Na_3HPO_4 | 1.17    | 125.1 | 3.2 | 57.6     |
| 12% PEG 4000/14% Na_3HPO_4 | 0.94    | 124.3 | 3.1 | 23.1     |
| 12% PEG 4000/18% Na_3HPO_4 | 0.42    | 118.7 | 3.0 | 11.8     |
| 12% PEG 4000/10% Na-citrate | ns      | ns   | ns  | ns       |
| 12% PEG 4000/14% Na-citrate | 0.67    | 79.1 | 2.0 | 18.2     |
| 12% PEG 4000/18% Na-citrate | 0.21    | 76.4 | 1.9 | 5.4      |
| 12% PEG 4000/10% (NH_4)_2SO_4 | 1.73    | 241.8 | 11.3 | 58.5     |
| 12% PEG 4000/14% (NH_4)_2SO_4 | 1.27    | 157.7 | 4.7 | 31.6     |
| 12% PEG 4000/18% (NH_4)_2SO_4 | 1.13    | 136.9 | 4.0 | 63.7     |
| 12% PEG 4000/10% MgSO_4 | ns      | ns   | ns  | ns       |
| 12% PEG 4000/14% MgSO_4 | 1.08    | 183.3 | 3.5 | 25.9     |
| 12% PEG 4000/18% MgSO_4 | 0.49    | 130.7 | 3.2 | 9.4      |
Table 2. Effects of molecular weight of PEG in PEG/(NH₄)₂SO₄ on R. microsporus lipase partitioning at 24 °C.

| System              | Kᵞ | Kᵟ | Y (%) |
|---------------------|-----|-----|-------|
| 20%PEG 600/15% (NH₄)₂SO₄ | 9.35 | 11.71 | 73.49 |
| 10%PEG 1000/20% (NH₄)₂SO₄ | 1.38 | 3.09 | 9.46  |
| 13%PEG 1000/17% (NH₄)₂SO₄ | 2.00 | 0.42 | 27.14 |
| 17%PEG 1000/14% (NH₄)₂SO₄ | 2.11 | 0.44 | 37.14 |
| 20%PEG 1000/12% (NH₄)₂SO₄ | 4.05 | 0.24 | 64.56 |
| 10%PEG 2000/20% (NH₄)₂SO₄ | 2.07 | 1.90 | 18.25 |
| 13%PEG 2000/17% (NH₄)₂SO₄ | 3.12 | 1.98 | 38.20 |
| 17%PEG 2000/14% (NH₄)₂SO₄ | 5.86 | 0.68 | 59.04 |
| 20%PEG 2000/12% (NH₄)₂SO₄ | 11.65 | 0.47 | 76.58 |
| 10%PEG 4000/20% (NH₄)₂SO₄ | 3.31 | 3.53 | 29.73 |
| 13%PEG 4000/17% (NH₄)₂SO₄ | 2.49 | 1.27 | 32.65 |
| 17%PEG 4000/14% (NH₄)₂SO₄ | 2.21 | 0.56 | 38.26 |
| 20%PEG 4000/12% (NH₄)₂SO₄ | 1.24 | 0.46 | 42.15 |
| 10%PEG 6000/20% (NH₄)₂SO₄ | 2.79 | 4.65 | 25.51 |
| 13%PEG 6000/17% (NH₄)₂SO₄ | 3.10 | 2.69 | 38.03 |
| 17%PEG 6000/14% (NH₄)₂SO₄ | 2.77 | 1.12 | 43.69 |
| 20%PEG 6000/12% (NH₄)₂SO₄ | 1.88 | 1.05 | 51.25 |
| 10%PEG 8000/20% (NH₄)₂SO₄ | 1.95 | 3.59 | 16.85 |
| 13%PEG 8000/17% (NH₄)₂SO₄ | 1.14 | 2.29 | 13.86 |
| 17%PEG 8000/14% (NH₄)₂SO₄ | 1.02 | 1.29 | 19.27 |
| 20%PEG 8000/12% (NH₄)₂SO₄ | 0.26 | 0.94 | 5.89  |

Certainly, the lower the MW of PEG is, the higher concentration of the polymer or salt will be required for apparent phase formation. In the system prepared with PEG 600 Da, the Kᵞ and Kᵟ values were found to be 9.35 and 11.71, respectively (Table 2). Theoretically, a high Kᵟ value along with a high Kᵞ value indicates that most proteins, including the target one, are more partitioned into the top phase, while a high Kᵞ with a low Kᵟ value implies that it is especially the target enzyme that is more partitioned into the top phase. These results showed that most of the proteins besides lipase in the crude extract were distributed in the top phase non-specifically. This was promising, since one of the most important requirements for effective purification in ATPS is that the contaminating proteins should not be extracted in the same phase with the target enzyme. Therefore, along with a high Kᵞ value, a low Kᵟ value is favourable as well. As Kᵞ > 1 in the PEG 600 Da system, this system could not be considered selective for lipase and was found not suitable for the purification process, although Y was 73.49% (Table 2). Application of PEG with MW that is too low could virtually cause all proteins to run into the PEG phase, resulting in ineffective separation. Therefore, considerable reduction in PEG size would be unacceptable.

As seen from Table 2, the increase in the MW of PEG from 600 to 2000 Da for the same polymer composition led to about 24.9-fold decrease in Kᵞ, while Kᵟ increased slightly. This result showed that the selectiveness of the system increases with the increase in PEG MW. Also, further increase in PEG MW caused a sharp decrease in Kᵞ values from 11.65 to 1.24, 1.88 and 0.26 for the systems prepared with 20% of PEG 2000, 4000, 6000 and 8000 Da, respectively. This result is in agreement with the general rule that the distribution of the target protein in the top phase decreases with higher molecular of PEG. This means that lipase increasingly partitioned to the bottom phase as the PEG MW increased, which could be attributed to two different effects. The most obvious one was the increase in the hydrophobicity of the top phase. In fact, as the PEG chain length increases (with increasing MW) there will be less hydroxyl groups for the same concentration of the polymer and the hydrophobicity of the polymer-rich phase (top) increases. On the other hand, the increase of the chain length will also cause a reduction in the free volume, meaningless space available for the lipase. In other words, the increase of PEG MW had facilitated the partitioning of lipase to the bottom phase. In this system (20% PEG 2000/12% (NH₄)₂SO₄), the best Kᵞ was obtained (11.65) with a Y of 76.58% (Table 2).

Another factor, besides PEG MW, that affects the partitioning behaviour of protein is the PEG/sulphate concentration, which has an effect on the surface hydrophobicity of the enzyme. According to the results, Kᵞ increased with increasing PEG concentrations in the systems prepared with PEG 1000 and 2000 Da. On the other hand, the opposite trend was observed in the PEG 4000, 6000 and 8000 Da systems. Generally, Kᵞ values declined in all of the six different groups considering the MW of PEG with the increasing polymer concentrations (Table 2). It was also observed that with increasing PEG MW and concentration, lipase activity generally decreased in the top phase. For the systems prepared with PEG 4000, 6000 and 8000 Da, the lipase activity generally increased in the bottom phase with increasing polymer concentration (Table 2). These results could be attributed to denaturation due to break-down of lipase into subunits with the hydrophobic interaction between PEG and enzyme or the effect of volume exclusion which became more dominant over salting-out with the increasing MW of PEG. As shown in Table 2, the recovery yield increased with the increase of PEG MW and decrease of salt concentrations, provided that PEG concentration was kept constant. The lowest Y was found to be 5.89% in the system 20% PEG 8000/12% (NH₄)₂SO₄, and the general trend was that higher values of the yield were obtained when the MW of PEG decreased and the concentration of the polymer increased (Table 2).
After selecting the type of salt and MW of PEG, the other process parameters like pH, fermentation broth loading and effect of neutral salts on partitioning of lipase were studied.

**Effect of pH on lipase partitioning**

To study the influence of pH on lipase partitioning for the selected phase system (PEG 2000/(NH₄)₂SO₄), experiments were performed in the pH range of 5.0—9.0 at room temperature (Figure 1). It is well known that biomolecules partitioning in ATPSs is influenced by pH of the system and generally the pH value affects the partitioning behaviour of proteins by changing the charge of the target protein. Also, it affects the ratio of the charged species present in the extract by altering their surface characteristics or the ion composition.[36] The results showed that at system pH of 5 and 6, there was negligible change in protein partitioning ($K_p$) observed (i.e. 0.28 and 0.31, respectively). In the systems with pH > 6 and above, the protein partition coefficients ($K_p$) increased (Figure 1). The change in the partitioning coefficient could be explained by considering the change in the net charge of the enzyme surface compared to its isoelectric point. Lipases are neutral enzymes, with isoelectric points of 6.0—7.0.[37] As the pH of the system increases above the isoelectric point,
Figure 3. Effect of neutral salts in the PEG 2000/(NH₄)₂SO₄ system on \textit{R. microsporus} lipase partitioning: NaCl (A), KCl (B) and Na₂CO₃ (C).

Note: SA, specific activity.
the lipase surface charge becomes negative. As a general rule, negatively charged proteins prefer the top phase in PEG—salt systems, while positively charged ones normally partition in the bottom phase due to electrostatic attraction as a result of charge distribution.[38] As a result, lipase was concentrated in the top PEG-rich phase.

Gautam and Simon [39] have also observed similar effects for total protein partitioning from culture extracts of \( \beta \)-glucosidase in PEG 4000/phosphate salt ATPS. The increase in pH appends the negative charge of the protein surface above the isoelectric point. The negatively charged protein concentrates in the top phase and hence increases the partition coefficient.

Figure 1 also shows the effect of pH on lipase selectivity and recovery yield in the top phase. The maximum recovery of 81.6% with selectivity of 49.4 was observed at pH 8.0. A decrease in the recovery yield of lipase activity and selectivity were observed at pH above 8.0.

Effect of crude load on lipase partitioning

The effect of the loaded crude broth on the lipase partitioning in the PEG 2000/(NH\(_4\))\(_2\)SO\(_4\) ATPS is shown in Figure 2. The amount of added crude broth to the ATPS is important because the loaded feedstock can change the partitioning behaviour of the target protein and the phase \( V_p \).[19] Similar to the results of Amid et al. [40] on pectinase partitioning, the increasing amounts of both lipase and contaminants in the systems could result in a decrease in the ATPS performance. ATPS experiments were carried out by varying the loaded fermentation broth to 40% (w/w). Based on the results, a fermentation broth load of 30% (w/w) was observed to show the maximum capacity. The degree of purification and yield for the 30% (w/w) crude load ATPS was 18.9% and 85.5%, respectively. Higher amount of the sample loading in the ATPS reduced the volume ratio and affected the composition of ATPS. It appeared that the components in the crude fermentation broth had changed the properties of the ATPS; hence, the ATPS was not optimal for purification of lipase. Such behaviour can be described by the increasing accumulation of precipitate at the interface, demonstrating the loss of lipases together with other non-target proteins in the purification process. Therefore, it is the 30% sample loading that would be feasible for the optimal recovery of lipase from the crude extract.

Effect of neutral salts on lipase partitioning

Addition of neutral salts to aqueous solutions provides one of the versatile means by which the selectivity and the yield of target molecules can be manipulated.[41] Changes in the salt type often produce an electrical potential difference between the two phases caused by the preference of one of the ions for one phase relative to the other.[42,43] In order to study the effect of neutral salts on the lipase partitioning, ATPE was carried out with three salts, NaCl, KCl and Na\(_2\)CO\(_3\), at concentrations of 1%, 3%, 5%, 7% and 9% (w/w). The results are shown in Figure 3.

It was observed that the partitioning of lipase in the top phase did not change significantly with the increasing concentration of NaCl and KCl in the 20% PEG 2000/12% (NH\(_4\))\(_2\)SO\(_4\) system (Figure 3(A) and 3(B)). The recovery yield suggests that NaCl and KCl could not improve the partitioning of lipase in the ATPS effectively. However, the results presented in Figure 3(C) show that the partitioning of lipase reached a peak, i.e. highest SA (345.3) and recovery yield (92.3%), in the top phase at 5.0% Na\(_2\)CO\(_3\), in which most lipase shifted from the bottom phase to the top phase. This suggests that Na\(_2\)CO\(_3\) has a strong effect on the partitioning of lipase. A possible explanation of this phenomenon could be sought in two aspects. First, the ranking of CO\(_3^{2-}\) > Cl\(^{-}\) [44] for the salting-out power of ions, indicates that Na\(_2\)CO\(_3\) has a stronger salting-out effect on lipase than other salts, which results in the high partitioning of lipase in the top phase. Second, the salting-out effect and the high surface hydrophobicity of an enzyme can sometimes prove stronger than the effect of the interface electrostatic potential.[45] Thus, at a certain concentration of CO\(_3^{2-}\), the partitioning of lipase could be driven towards the top PEG-rich phase. Compared with Na\(_2\)CO\(_3\), the other two tested salts did not improve the partitioning of lipase effectively.

Thus, taken together, our data showed that the best partitioning was achieved in the system of 20% PEG 2000/12% (NH\(_4\))\(_2\)SO\(_4\)/5% Na\(_2\)CO\(_3\). These results could be considered very promising and further studies would give new insight about the potential application of this system in fermentation product separation at a larger scale.

Conclusions

ATPE was demonstrated to be an efficient primary purification step for \( \textit{R. microsporus} \) lipase. The obtained results showed that, for partitioning of lipase in PEG—sulphate systems, although PEG 600 did not show effective purification, lower MW PEG was more effective than larger ones. Salt type was among the most significant factors affecting the purification factor in the top phase. Addition of non-phase forming salts, NaCl and KCl, could not improve the recovery yield but the partitioning could be modified by pH and sample loading. The highest recovery yield (92.3%) was obtained with 30% (w/w) crude load at pH = 8.0. Hence, the experimental conditions determined here provide an alternative process option for efficient lipase purification, which can be achieved by using PEG 2000/(NH\(_4\))\(_2\)SO\(_4\) ATPS.
Acknowledgements
This study was supported by the Rasht Branch, Islamic Azad University, Rasht, Iran. Also, the author would like to acknowledge Dr Khayatyi for providing the fungal strain.

Disclosure statement
No potential conflict of interest was reported by the author.

References
[1] Houde A, Kademi A, Leblanc D. Lipases and their industrial applications. Appl Biochem Biotech. 2004;118:155–170.
[2] Rigo E, Ninowa JL, Luccio MD, Oliveira JV, Polloni AE, Remonatto D, Arbter F, Vardanega R, de Oliveira D, Treichel H. Lipase production by solid fermentation of soybean meal with different supplements. LWT – Food Sci Technol. 2010;43:1132–1137.
[3] Khayatyi G, Kiani F. A statistical approach for optimization of lipase production by using rice straw: analysis of different inducers and nitrogen sources effect. Minerva Biotecnol. 2012;24:83–89.
[4] Khayatyi G, Gilani HG, Kazemi M. The effect of olive cake types on lipase production by isolated Rhizopus sp. and process statistical optimization. J BioSci Biotech. 2013;2:45–55.
[5] Ministry of Agriculture Jihad Guilan Province. [accessed: 2015 Apr 8]. Persian. Available from: http://amoozesh.gilan.ir/web/guest/agricalture.
[6] Naganagouda K, Mulimani VH. Aqueous two-phase extraction (ATPE): an attractive and economically viable technology for downstream processing of Aspergillus oryzae α-galactosidase. Process Biochem. 2008;43:1293–1299.
[7] Sé RAG, Aznar M. Liquid–liquid equilibrium of the aqueous two-phase system water + PEG 4000 + potassium phosphate at four temperatures: experimental determination and thermodynamic modeling. J Chem Eng Data. 2002;47:1401–1405.
[8] Khayati G, Anvari M. Aqueous two-phase systems composed of different molecular weight of polyethylene glycol and diaminium phosphate for extraction of Bovine Serum Albumin. Ital J Food Sci. 2012;24:279–283.
[9] Khayati G. Optimization of propionic acid extraction by aqueous two-phase system using response surface methodology. Chem Eng Commun. 2013;200:667–677.
[10] Banik RM, Santhiagu A, Kanari B, Sabarinath C, Upadhyay SN. Technological aspects of extractive fermentation using aqueous two-phase systems. World J Microbiol Biotechnol. 2003;19:337–348.
[11] Anvari M, Khayati G. In situ recovery of 2,3-butanediol from fermentation by liquid–liquid extraction. J Ind Microbiol Biotechnol. 2009;36:313–317.
[12] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–254.
[13] Khayati G, Alizadeh S. Extraction of lipase from Rhodotorula glutinis fermentation culture by aqueous two-phase partitioning. Fluid Phase Equilibria. 2013;353:132–134.
[14] Shahbaz Mohammadi H, Omidinia E, Dinarvand R. Evaluation of recombinant phenylalanine dehydrogenase behavior in aqueous two-phase partitioning. Process Biochem. 2007;42:1296–1301.
[15] Raghavarao KSMS, Guinn MR, Todd P. Recent developments in aqueous two-phase extraction in bioprocessing. Sep Purif Rev. 1998;27:1–50.
[16] Rito-Palomares M. Practical application of aqueous two-phase partition to process development for the recovery of biological products. J Chromatogr B. 2004;807:3–11.
[17] Yucekan I, Oral S. Partitioning of invertase from tomato in poly (ethylene glycol)/sodium sulfate aqueous two-phase systems. Process Biochem. 2011;46:226–232.
[18] Randini KE, Rastogi NK. Liquid–liquid extraction of lipase using aqueous two-phase system. Food Bioprocess Technol. 2011;4:295–303.
[19] Pan IH, Yao HJ, Li YK. Effective extraction and purification of β-xylanase from Trichoderma koningii fermentation culture by aqueous two-phase partitioning. Enzyme Microb. Technol. 2001;28:196–201.
[20] Xu Y, He GQ, Li JJ. Effective extraction of elastase from Bacillus sp. fermentation broth using aqueous two-phase system. J Zhejiang Univ Sci. 2005;6B(11):1087–1094.
[21] Wang W, Wan J, Ning B, Xia J, Cao X. Preparation of a novel light-sensitive copolymer and its application in recycling aqueous two-phase systems. J Chromatogr A 2008;1205:171–176.
[22] Babu B, Rastogi NK, Raghavarao KSMS. Liquid–liquid extraction of bromelain and polyphenol oxidase using aqueous two-phase system. Chem Eng Process. 2008;47:83–89.
[23] Chaiwut P, Rawdkuen S, Benjakul S. Extraction of protease from Calostroptis procera latex by polyethylene glycol–salts biphaseic system. Process Biochem. 2010;45:1148–1155.
[24] Rabelo APB, Tambourgi EB, Pessoa A. Bromelain partitioning in two-phase aqueous system containing PO–PPO–PEO block copolymers. J Chromatogr B 2004;807:61–68.
[25] Huddleston J, Veide A, Kohlezi K, Flanagan J, Ensors SO, Lydiatti A. The molecular basis of partitioning in ATPS: review. TrendsBiotech. 1991;9:381–388.
[26] Roe S. Protein purification techniques: a practical approach. 2nd ed. Oxford (UK): Oxford University Press; 2001.
[27] Tanaka H, Kubo R, Komasawa I. Effect of hydrochloric acid on hydrophobicity and partitioning of protein in aqueous two-phase systems. J Chem Eng Jpn. 1991;24:661–664.
[28] Johannson HO, Ishii M, Minaguti M, Feitosa E, Penna TC, Pessoa JA. Separation and partitioning of green fluorescent protein from Escherichia coli homogenate in poly (ethylene glycol)/sodium–poly(acrylate) aqueous two phase systems. Sep Purif Technol. 2008;62:166–174.
[29] Chaiwut P, Pintathong P, Rawdkuen S. Extraction and three-phase partitioning behavior of proteases from papaya peels. Process Biochem. 2010;45:1172–1175.
[30] Cascone O, Andrews BA, Asenjo JA. Partitioning and purification of thiamatin in aqueous two-phase systems. Enzyme Microb Technol. 1991;13:629–635.
[31] Su CK, Chiang BH. Partitioning and purification of lysosome from chicken egg white using aqueous two-phase system. Process Biochem. 2006;41:257–263.
[32] Patil G, Raghavarao KSMS. Aqueous two phase extraction for purification of C-phycocyanin. Biochem Eng J. 2007;34:156–164.
[33] Madhusudhan MC, Raghavarao KSMS. Aqueous two phase extraction of invertase from baker’s yeast: effect of the process parameters on partitioning. Process Biochem. 2011;46:2014–2020.
[34] Porto TS, Medeirose Silva GM, Porto C, Cavalcanti MTH, Neto BB, Lima FJL. Liquid–liquid extraction of proteases from fermented broth by PEG/citrate aqueous two-phase system. Chem Eng Process. 2008;47:716–721.

[35] Nagaraja Viswanatha H, Regupathi I. Fish processing industrial effluent characterization and partitioning of proteins using aqueous two-phase system. In: Sabu A, Augustine A, editors. Prospects in bioscience: addressing the issues. New Delhi: Springer; 2013. p. 1–10.

[36] Karkas T, Onal S. Characteristics of invertase partitioned in poly(ethylene glycol)/magnesium sulfate aqueous two-phase system. Biochem Eng J. 2012;60:142–150.

[37] Ishimoto R, Sugimoto M, Kawai F. Screening and characterization of trehalose-oleate hydrolyzing lipase. FEMS Microbiol Lett. 2001;195:231–235.

[38] Kavakcioglu B, Tarhan L. Initial purification of catalase from Phanerochaete chrysosporium by partitioning in poly (ethylene glycol)/salt aqueous two phase systems. Sep Purif Technol. 2013;105:8–14.

[39] Gautam S, Simon L. Partitioning of β-glucosidase from Trichoderma reesei in poly (ethylene glycol) and potassium phosphate aqueous two-phase systems: influence of pH and temperature. Biochem Eng J. 2006;30:104–108.

[40] Amid M, Murshid FS, Manap MY, Hussin M. A novel aqueous micellar two-phase system composed of surfactant and sorbitol for purification of pectinase enzyme from Psidium guajava and recycling phase components. BioMed Res Int. 2015;2015:815413.

[41] Albertsson PA. Partition of cell particles and macromolecules. New York (NY): Dekker; 1990. p. 287.

[42] Chethana S, Nayak CA, Raghavarao KSMS. Aqueous two phase extraction for purification and concentration of betalains. J Food Eng. 2007;81:679–687.

[43] Shiri S, Khezeli T, Sajjadifar S, Delpisheh A, Avazpour M, Abbasi A. A novel and sensitive method for the determination of vitamin b2 (riboflavin) in urine and pharmaceutical samples using an aqueous two-phase extraction. J Chem. 2013;2013:892380.

[44] Marcus Y. Thermodynamics of solution of ions. Part 6. The standard partial molar volumes of aqueous ions at 298.15 K. J Chem Soc Faraday Trans. 1993;89:713–718.

[45] Yue H, Yuan Q, Wang W. Purification of phenylalanine ammonia-lyase in PEG1000/Na2SO4 aqueous two-phase system by a two-step extraction. Biochem Eng J. 2007;37:231–237.