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Mini Review

Phase partitioning for enzyme separation: An overview and recent applications

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Abstract

Conventional purification techniques those were used to recover and purify enzymes are composed of several steps. Besides, procedures have proven laborious, costly, time consuming and yielding low recovery. Hence, an alternative recovery technique such as an aqueous two phase system (ATPS), thermoseparation (TMP) and three phase partitioning (TPP) is created to solve these disadvantages. Multiple factors affecting recovery and purity are investigated including the polymer molecular weight and concentration, the salt type and concentration, the system pH, the NaCl addition and the total number of cycles. The alternative recovery technique has been widely applied for separating and purifying enzymes from various sources such as plants (bromelain, papain, invertase, polyphenol oxidase and trypsin inhibitors), animals (trypsin, α-chymotrypsin, chymosin, pepsin and luciferase) and microbial (lipase, α-amylase, α-galactosidase and cyclodextrin glycosyltransferase). In this article, the backgrounds and phase forming materials are presented. The parameters that affect the enzymes partitioning behavior as well as recent applications of these methods are reviewed and summarized.

Introduction

There are many conventional purification methods used for recovering and purifying enzymes. They include ammonium sulfate precipitation followed by size-exclusion and ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, or some combination of these methods. All of these are time consuming, burdensome and costly. Furthermore, at each step of these conventional protocols, some quantity of the target enzyme is lost and thus yields a low recovery. Other extraction methods such as liquid-liquid extraction like an aqueous two phase system (ATPS), thermoseparation (TMP) and three phase partitioning (TPP) are promising alternatives for separating biomolecules, especially proteins and enzymes. The procedural steps are less, the processing time is shorter and scaling up operation is easier. Because of these improvements, there can also be a higher recovery with acceptable purity than results obtained from conventional purification techniques (Dreyer, 2010).

The separation of components in a liquid mixture by means of direct contact of the solution with a solvent (in which one of the compounds is preferentially soluble) is known as liquid-liquid extraction (Da Rós *et al*., 2010). It involves the use of organic solvents that are not suitable for protein recovery as most proteins are either insoluble in organic solvents or are irreversibly denatured. An appropriate alternative to traditional bio-separation processes is partitioning in ATPS, which has been successfully used for isolating proteins and other biological organic materials (Zugina *et al.*, 2010).

ATPS is a very attractive method for separating biomolecules. It makes use of two aqueous phases, which consist of two water-soluble polymers (i.e. polyethylene glycol; PEG), or a polymer and a salt (i.e. dextran and ammonium sulfate), or alcohol and a salt (i.e. 1-propanol and ammonium sulfate) (Amid *et al*., 2012). It is a technique that has high potential for applications in biotechnology for separating and purifying biological materials such as proteins, enzymes, nucleic acids, viruses, antibodies and cell organelles. The rather simple process and lower cost of the phase forming materials allows for larger-scale purification. There are many advantages to consider such as the simplicity of the technique, the rapid separation with minimal enzyme denaturation, the low interfacial tension and the selective separation (Albertsson, 1986).

In TMP, thermoseparating copolymer i.e. ethylene oxide (EO), propylene oxide (PO) a co-

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polymer, poly(ethylene glycol-ran-propylene glycol), monobutyl ether (EOPO) was used instead of a conventional polymer (PEG) and a salt (i.e. sodium citrate and potassium hydrogen phosphate) was also used in the system. In this system, enzyme purification is a two-step process. In a primary TMP, the target protein should preferentially enter the EOPO phase at the start. The secondary system is formed during the next step by a temperature-induced phase separation of the EOPO solution. Consequently, a water solution of the target protein and a concentrated EOPO solution are obtained (Johansson *et al.*, 1998). It has been previously suggested that the EOPO copolymer can be recovered and reused to prepare the next primary ATPS (Persson *et al*., 1999).

TPP is another relatively recent bio-separation technique, which employs collective operation of principles involved in numerous techniques for protein precipitation. Ammonium sulfate is used with a certain saturation to precipitate the protein and t-butanol is added to make three-phase layers and to remove some low molecular weight compounds such as lipids, phenolics and some detergents (Dennisson *et al.*, 1997). Pigments, lipids and enzyme inhibitors are concentrated in the upper solvent phase, which is separated from the lower aqueous phase that is enriched with polar components like saccharides by an intermediate protein precipitated layer (Kiss *et al.*, 1998). In general, biomolecules are recovered in a purified form at the interphase, while the contaminants mostly partition to t-butanol (top phase) and to the aqueous phase (bottom phase) (Roy and Gupta, 2002).

Phase partitioning has been a more effective technique for recovering biological materials over other conventional methods because it requires more gentle environmental conditions with high water content of up to 70-90% in both liquid phases. The interfacial tension between the two phases is low, resulting in high mass transfer. Many polymers used in the system have stabilizing effects on the biological activity and on the structure of the proteins and enzymes (Albertsson and Tjerneld, 1994), so the denaturing of labile biomolecules possibly decreases. This technique is straightforward and also requires relatively simple equipment that is easy to operate (Berlo *et al*., 1998). Moreover, the results of large scale production are not considerably different in quality from small scale production, making the up-scaling reliable (Ratanapongleka, 2010). Because of these advantages, phase partitioning has been used in many kinds of applications such as in the recovery of biopharmaceuticals, environmental remediation, enzyme and protein purification and extractive bioconversion.

However, the drawback is that there is still little known about the mechanisms involved in the partitioning process and there is only a rudimentary understanding of the proper technique (Rito-Palomares and Hernandez, 1998), so it is difficult to predict the phase equilibrium and the extent of the product partitioning. The high consumption of phase-forming components requires costly phase forming polymers. Another important issue for the application of these systems on a large scale is their potential impact on wastewater treatment (Ratanapongleka, 2010).

Despite some limitations of these three methods, the advantages are far greater than those of the conventional enzyme separation techniques. ATPS, TMP and TPP techniques have been widely applied for the partitioning and recovery of various proteases from assorted sources. In this review, these three major phase partitioning systems are reviewed in depth. The factors affecting the partitioning behavior of enzymes, specifically the molecular weight and concentration of the polymers, the ionic strength of the salt phase, the pH, the addition of NaCl, the temperature and the number of cycles are all reviewed. Recent applications of these methods are also described and reviewed.

Phase partitioning

Aqueous two phase system (ATPS)

Liquid-liquid binary systems can also be formed using two polymers or polymer/salt solutions. These so-called aqueous two-phase systems (ATPS) were first discovered in 1896 by the Dutch microbiologist, M. Beijernick. He noticed the separation of the two phases while solubilizing gelatin and agar or starch in water (Dreyer, 2010). However, this first report remained unnoticed until 1956 when a Swedish biochemist, Albertsson rediscovered the phenomenon and further developed the phase separation technique. ATPS has been widely used in various applications. It has proven to be highly suitable for the gentle separation of cell membranes and organelles from crude cell lysates. It has also been effective for the selective purification of proteins and enzymes from protein mixtures, nucleic acids, viruses, antibodies and cell organelles (Hatti-Kaul, 2000; Raja *et al*., 2011).

Phase forming materials and formation

ATPS is generally composed of either two water soluble polymers (e.g. dextran and polyethylene glycol; PEG) with different molecular weights (MW),

or one polymer and one salt (e.g. phosphate, sulphate, or citrate) at appropriate concentration. Other chemicals like poly(acrylic acid) (PAA), solvents (e.g. ethanol and methanol) or some surfactants (e.g. Triton X-114, Pluronic L31, 61, 81 and 121) and salt have been also used to invent ATPS (De Lencastre Novaes *et al.,* 2013; Amid *et al*., 2013). Formation of the PEG/salt ATPS is produced by the salting out of the polymer whereas the PEG/dextran ATPS is formed due to the incompatibility of both polymers. Due to some limitation of PEG/dextran ATPS such as viscosity increasing with raising concentration of dextran, dextran is too expensive to scale-up extraction process and difficulties to carry out cyclic re-utilization, PEG/salts combination is the most commonly used in this technique (Azevedo *et al.*, 2009; Liu *et al.*, 2011). As PEGs are nontoxic, non-flammable, nonvolatile and economical price, PEG-based ATPS causes less environmental problems compared to conventional solvent extraction systems that utilize water-immiscible organic solvents (Lladosa *et al*., 2012).

Phase diagrams of aqueous two phase systems

The ATPS is formed when two polymers, or one polymer and one salt, are mixed above their critical thermodynamic conditions. It is composed of two immiscible phases that promote the separation of components in a proper environment and preserve the principle characteristics of the products being separated (Pereira *et al*., 2003; Zuniga *et al*., 2010). The phase diagram (Figure 1) provides information about:

1) The concentration of the phase-forming components necessary for forming a system with two phases at equilibrium.

2) The subsequent concentration of phase components in the top and bottom phase.

3) The ratio of each phase volume along with the tie-line.

This information can be drawn from a solubility curve and the tie-lines, which are characteristic for a phase diagram. The final concentrations of phase components in the top and bottom phases of a generated ATPS can be drawn from the tie-line that connects two nodes on the solubility curve (C-D in Figure 1.). Tie-line refers to the line that joining points representing the top and bottom phase composition in the phase diagram. The nod represent the total compositions of the three systems (1,2,3) on the same tie-line that have the same phase composition but different volume ratios. In a phase diagram, the binodal curve represents the concentration boundary separating the monophasic from the biphasic region

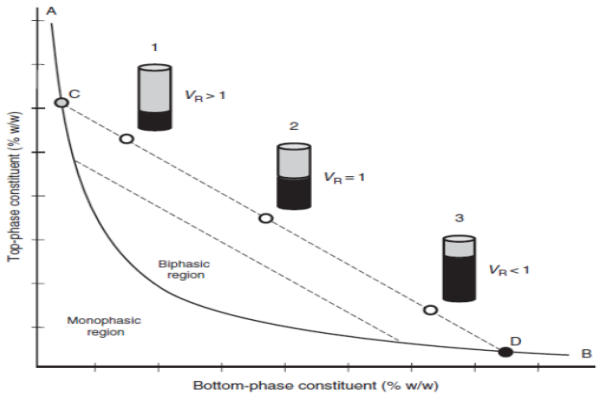

Figure 1. Schematic illustration of a phase diagram for an aqueous two-phase system (Dreyer, 2009)

in an appropriate phase diagram. A composition (% w/w) over the binodal curve (Figure 1, line A-B) should be selected in order to form a biphasic aqueous system. Commonly, in the biphasic diagram, the axis of the ordinate is used for the top-phase-rich constituent, while the axis of the abscissa is used for bottom-phase rich constituent (Benavides *et al*., 2011).

The tie-line length (TLL) is a numerical indicator of the composition difference between the two phases and is generally used to correlate trends in the partitioning of solutes between both phases. After mixing, phase separation is accomplished either by settling under gravity or by centrifugation. The phases are separated and analyzed or used to recover the separated components of the initial mixture as shown in Figure 2A, the target product (i.e. biomolecules) should be concentrated in one of the phases and the contaminants in the others. In many cases, recovery and concentration of products with yields exceeding 90% can be achieved using a single extraction step (Raja *et al*., 2011). When single-stage extraction does not give sufficient recovery, repeated extractions can be carried out in series (Raja *et al*., 2011; Ketnawa *et al*., 2014a).

Advantages of ATPS

Compared to other separation techniques, ATPS has been proposed as an ideal purification technique for the separation, extraction and concentration of biomolecules. It has been applied widely because of its interfacial tension, non-toxicity and inflammable properties, which provides for mild conditions suitable for large scale purification processes (Persson *et al*., 1999; Becker *et al*., 2009). It is also economical, has a shorter processing time, requires low energy and is biocompatible making it relatively easy to scale up production (Show *et al*., 2012a, b). ATPS has been applied for separating and purifying various enzymes such as those derived from plants, animals and microbial (Table 3) of which more than

100% recovery (104-335%) of enzymes are generally observed in a single step separation due to migration of other proteins and impurities to the bottom phase when appropriate concentration of polymer and salt that being tested.

Thermoseparation (TMP)

Some limitations of conventional ATPS partitioning (e.g. PEG/salts and PEG/dextran) such as Phase-forming chemicals cannot be recycled effectively, large consumption of chemicals, denaturation of biomolecules when high salt concentrations, difficulty in polymer withdrawal and environmental problem of salt discard (Zuniga *et al.*, 2010; Ng *et al.*, 2012). Besides, large amounts of polymers/chemicals are required, driving up the cost of operation (Alred *et al.*, 1994). Therefore, a more economical and environmentally friendly TMP with the ability to retain the biological activity of enzymes is preferred to others. The recycling of the phase components by temperature induced phase separation has caught the attention of researchers as it can partition and recover various enzymes. Apart of polymer recycling, salts phase can also be reused leading to cost effectiveness and environmental benefits. In this type of partitioning, the aim is to partition the target protein to the top polymer (EOPO) phase and leave contaminants collected in the bottom polymer or salt phase. Moreover, EOPO hydrophobically modified with meristic groups ($C_{14}H_{29}$) (HM-EOPO) has also been introduced to overcome the limitations of the traditional ATPS (Johansson *et al.*, 1997).

Phase forming materials and formation

A random copolymer, poly(ethylene glycol-ran-propylene glycol) mono-butyl ether (EOPO) has been shown to form two-phase systems with dextran, starch derivatives, potassium phosphate, magnesium sulfate and ammonium or sodium sulfate. This polymer can be used to replace PEG that is commonly used in traditional ATPS (Li *et al.*, 1998; Xu *et al.*, 2004).

A schematic diagram (Figure 2B) depicts the recycling of phase components when using EOPO as the thermoseparating polymer and salt in TMP. In TMP, a primary two-phase system is first formed and the target protein is partitioned to the EOPO top phase. Next, the EOPO phase is withdrawn and heated above the cloud point of the polymers to induce thermoseparation and subsequently a new two-phase system consisting of a water top phase and a polymer bottom phase is observed. The target enzyme is harvested from the water phase and the EOPO polymer can be recovered from the EOPO

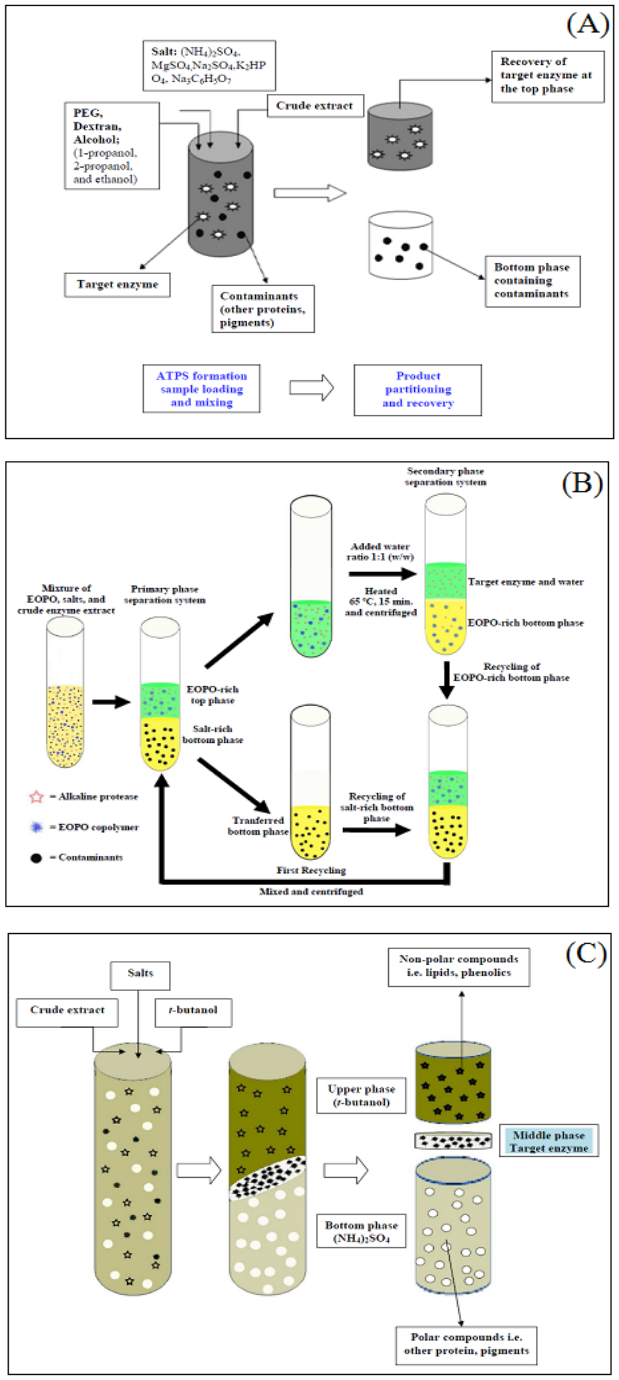

Figure 2. Schematic diagram of aqueous two phase partitioning system (ATPS) (A), thethermoseparating system (TMP) (B) and three phase partitioning system (TPP) (C) system of biomolecule

bottom phase of the system (Johansson *et al.*, 1999; Ng *et al.*, 2012). EOPO random copolymers (linear and non-ionic) can be separated from aqueous solution by heating the solution above the cloud point (also known as the lower critical solution temperature or LCST) at a low temperature of around 50ºC (Show *et al.*, 2011). In this case, the polymers can be recycled and the salt component can also be reused in subsequent ATPS extractions (Johansson *et al.*, 1999; Li *et al.*, 2002). During the thermoseparation, (in

accordance with a model based on the Flory-Huggins theory of polymer solution), it was shown that the separation of enzymes from polymers by temperature-induced phase separation results from an excluded volume effect because the thermoseparated polymer rich phase is entropically unfavorable for proteins, compared to a water phase (Johansson *et al.*, 1996). The thermo-responsive polymer chains show coil-to-globule-to-aggregate transition exhibiting LCST behavior when temperature of an aqueous solution is increased. There is loss of ordered water molecule arrangement around the hydrophobic polymer chain, which leads to chain collapse showed interruption in polymer water hydrogen bonding interactions during phase transition. Besides, phase transition relies on both hydrogen bonding and hydrophobic interactions in polymer solvent system resulting in the change of hydrated random coil to hydrophobic globule transition above critical solution temperatures (Teotia *et al*., 2015)

Advantages of TMP

In addition to short processing time and low energy consumption, another noticeable advantage of TMP is that the EOPO copolymers can be easily recovered after use (due to its thermoseparating properties) and then reused in the following system. The salt concentration required to form two phases is lower than that in other phase partitioning techniques, so the overall cost and waste can be minimized. Furthermore, the process of separating the target enzyme from phase solutions is simplified (Persson *et al*., 2000; Dembczynski *et al*., 2010a, b). Moreover, the target protein/enzymes can be recovered in a water phase. Because of this advantage, it can minimize the purification step of the procedure by no requirement for desalinating or exterminating the polymer. Thus, the obtained enzymes are well-prepared for further application.

Three-phase partitioning (TPP)

TPP is an upcoming bio-separation technique developed for the extraction of proteins, especially enzymes from multi-component systems. TPP employs collective operations of principles involved in numerous techniques such as salting out, isoionic precipitation, co-solvent precipitation, osmolytic and kosmotropic precipitation of proteins (Dennison and Lovrein, 1997; Saxena *et al*., 2010). It was developed as an 'upstream' technique, but frequently it is also useful for the downstream method for isolation on a milliliter volume scale. Besides, it is used as a one-step purification protocol (Saxena *et al*., 2010; Narayan *et al.*, 2008). In many cases, TPP enhances the activity of various enzymes, resulting in apparent higher yields (>100%) due to the target enzymes or protein precipitates out in the middle layer between the organic and aqueous phases. Removal of polymers is not necessary (Dennison and Lovrein, 1997; Singh *et al.,* 2001). Furthermore, TPP has been extensively evaluated for the simultaneous separation and purification of proteins, enzymes and inhibitors from crude suspensions (Vidhate and Singhal, 2013; Rawdkuen *et al*., 2010). Thus, the novelty of TPP lies in its ability to concentrate proteins from crude broths with higher purification than conventional concentration methods (Rajeeva and Lele, 2011).

Phase forming materials and formation

A schematic diagram of phase forming materials and target enzyme partitioning by TPP is depicted in Figure 2C. TPP is a technique in which a salt (generally ammonium sulphate) and an organic solvent (generally t-butanol) are added to an aqueous solution of protein(s) to remove some small molecular weight compounds such as lipids, phenolics and some detergents (Dennison and Lovrein, 1997). Sulphate ions increase the dehydration action when it is added in the water phase. Thus the proteins are segregated after being precipitated out of the water phase due to the large ions crowd together. Kulkarni and Rathod (2015) found that the solvent like ethanol or methanol were not able to form a three phase and recommend to use t-butanol. Thus t-butanol is used as the solvent as it is capable of forming three phases. Besides, t-butanol increases the buoyancy of the precipitated protein by binding to it, so it then floats above the denser aqueous salt layer (Rajeeva and Lele, 2011). Within an hour, three phases are formed. The upper t-butanol-rich phase (containing non-polar compounds) is separated from the lower aqueous phase (containing polar compounds) by an interfacial protein precipitate (Roy *et al.*, 2005).

Advantages of TPP

TPP is an efficient, economical and eco-friendly technique, which is promising because of its utilization of agro-processing waste (Saxena *et al.*, 2010; Vidhate and Singhal *et al*., 2013). In addition, TPP requires less time, enables working at room temperature, permits recycling of chemicals, is easily scalable and shows rapid recovery. Moreover, its requirement for only minimal pretreatment is an additional benefit (Kiss *et al.*, 2003; Shah *et al*., 2004). For crude extract containing high lipids, TPP is also very useful because it is easy to remove undesirable substances from the system such as impure lipids that dissolve in the t-butanol phase.

Table 1. Critical properties used to achieve and factor affecting phase separation of particular protein/enzymes

Partitioning properties	Description	Factor
Hydrophobicity	-to separate according to hydrophobicity of proteins	-Molecular weight/size of polymer -Concentration of polymer - Ionic strength of salt
Electrochemical	-to separate according to molecules or particles according to their charge	-Ionic strength of salt -pH system adjustment -NaCl addition
Size-dependent	-molecular size of the proteins/enzymes or surface area of proteins/enzyme	-Molecular weight/size of polymer
Biospecific affinity	-affinity between sites on the proteins and ligands attached to one of the phase polymers	-Affinity ligands
Conformation-dependent	-Conformation of the proteins	-Molecular weight/size of polymer -Concentration of polymer -pH system adjustment -NaCl addition

Adaped from Asenjo and Andrews (2011)

However, denaturation of biomolecules by excess amount t-butanol and discard of solvent and salt can be a limited the potentiality this partitioning method.

Parameters affecting partitioning behavior of enzymes

Critical properties used to achieve phase separation for particular proteins/enzymes are summarized and shown in Table 1. Such critical enzyme properties like hydrophobicity, charge, molecular weight, bio-specific affinity and conformation are used to separate target enzymes from the system. In addition, multiple factors in the phase partitioning system should be considered such as the polymer molecular weight and concentration, salt types and concentration, system pH, NaCl addition, temperature and the number of cycles in partitioning. These may all affect the partitioning parameters, including the enzyme volume ratio (VR), the partition coefficient (KE), the protein partition coefficient (KP), the purification fold (PF) and the enzyme recovery (RE). VR is defined as the volume ratio in the top phase to that in the bottom phase. KE or Kp is determined as the ratio of the enzyme activity or protein content in the upper fraction to that in the lower fraction. PF is defined as the ratio of the specific activity of each fraction to the initial specific activity of the initial extract. RE is identified by total enzyme activity in the upper fraction compared to the initial total activity of the sample. Parameters affecting the partitioning behavior of enzymes are summarized in Table 2.

Effect of polymer molecular weight (MW) on partitioning

In ATPS and TMP, phase separation will occur if the concentrations of polymers or salts are above a certain critical value of the phase diagrams (Figure 1). Raghavarao *et al.* (1995) reported that two phases were formed when the polymer concentration was in the range of 8-16% (w/w) and the salt concentration had to be as high as 10% (w/w). In addition, the lower the MW, the higher the polymer concentration required for phase formation (Rawdkuen *et al*., 2011). The target enzymes were partitioned prominently in the polymer phase, principally those with hydrophobic characteristics. In polymer-salt systems, the target enzyme partitioning depends on a "volume exclusion effect" of the polymer and a "salting-out effect" (Hatti-Kaul, 2001; Babu *et al*., 2008). The distribution of target enzymes and proteins in ATPS are reported as KE and KP, respectively. High KP values indicate that most of the proteins from the extract partitioned more to the polymer rich-top phase, while the high KE was the only target enzyme to favor the polymer rich-top phase (Rawdkuen *et al*., 2011).

Because the proteins/enzymes to be partitioned have a defined size (MW and hydrodynamic diameter) as well as geometry (tridimensional conformation), they are subjected to the steric effects imposed by the constituents of the system (Asenjo and Andrews, 2011). According to partitioning theory, increasing the PEG concentration will result in the increase of viscosity and the interfacial tension between the top phases of the ATPS/TMP (Berlo *et al*., 1998; Hatti-Kaul, 2001). The MW of the polymer influences the protein partition by changing the number of polymer-protein interactions. This is usually attributed to hydrophobic interactions between the chains of PEG and the hydrophobic area of the proteins.

In ATPS, most reports have indicated that at the same PEG concentration, KE decreases with increased MW of PEG. Furthermore, better partitioning of protein is achieved with lower MW

Table 2. Parameters affecting partitioning behavior of enzymes

Parameters	Effect		Partitioning behavior			
			K_E	K_P	RE	PF
Polymer molecular weight	-Volume/steric exclusion -Interfacial tension -Hyrophobicity	↑	↓	↑	↓	↓
Polymer concentration	-Volume/steric exclusion -Alter the phase volume ratio	↑	↓	↑	↓	↓
Salt types	-Salting-out or salting-in ability (lyotropic series) -Anions; SO_4^{2-}>HPO_4^{2-}>CH_3COO^- >Cl^- -Cations; NH_4^+>K^+>Na^+>Li^+ >Mg^{2+}>Ca^{2+}		Depend on salt types and enzymes			
Salt concentration	-Salting out	↑	↑	↓	↑	↑
Crude load volume	-Alter the phase volume ratio and the partition behaviour		Depend on enzymes			
System pH	-Electrochemical interactions -Alter charge of polymer to loses/gains charge	>pI; the enzyme was negatively charged and prefers polymer rich top phase	↑	↓	↑	↑
		<pI; the enzyme was positively charged and prefers the salt-rich phase	↓	↑	↓	↓
NaCl addition	-Electrical potential change	↑	↑	↓	↑	↑
Number of cycle	-The first TPP cycle could not remove the contaminant proteins efficiently -Considerable purification of the target protein in second cycle	1 or 2 cycle	↑	↓	↑	↑

↑ refers to that subject was increased
↓ refers to that subject was decreased
KE: partition coefficient
KP: protein partition coefficient
RE: enzyme recovery
PF: purification fold

of PEG as opposed to that with higher MW (Tubio *et al*., 2009; Nalinanon *et al*., 2009; Klomklao *et al.*, 2005; Ketnawa *et al.*, 2010; Rawdkuen *et al*., 2010; Ketnawa *et al.*, 2011; Chaiwut *et al*., 2010; Yucekan and Onal, 2011). A preferential interaction between PEG molecule and protein domains decrease when the MW of PEG was increased due to its exclusion from the protein domain (Reh *et al*., 2002; Klomklao *et al*., 2005). The influence of the MW of PEG on protein partitioning can also be explained on the basis of Flory Huggins theory for polymers in solution (Reh *et al*., 2002; Klomklao *et al*., 2005; Nalinanon *et al.*, 2009).

From previous results, the partitioning of enzymes was strongly dependent on the MW of the PEG. The highest partition parameters (recovery and PF) were obtained by PEG with low MWs (i.e. 1000, 1500, 3000, 2000 and 4000) (Babu *et al*., 2008; Nalinanon *et al.*, 2009; Chaiwut *et al*., 2010; Yucekan and Onal, 2011). PEG-1000 was a suitable polymer for partitioning various enzymes such as stomach protease of albacore tuna (Nalinanon *et al*., 2009), and spleen protease of yellowfin tuna (Klomklao *et al*., 2005) . It also provided higher PF and yield than PEG with higher MWs (2000, 3350, 4000 and 8000). PEG-2000, 3000 and 4000 provided higher enzyme recovery in partitioning bromelain, porcelain and invertase, respectively (Ketnawa *et al*., 2010; Chaiwut *et al.*, 2010; Yucekan and Onal, 2011). PEG with higher MW (8000) excluded the protein from the top phase driven by an entropically unfavored term in the ATPS of bovine trypsin (Tubio *et al*., 2009). The enzyme activity recovery, specific activity and degree of purification increased with a decrease in MW of PEG from 8000 to 1500 in the partitioning of luciferase (Priyanka *et al*., 2012). In this case, an increase in MW of PEG results in an increase in the chain length of the polymer, which leads to a reduction in the free volume in the top phase (Porto *et al*., 2005; Babu *et al.*, 2008; Yucekan and Onal, 2011; Madhusudhan and Raghavarao, 2011).

In TMP, EOPO polymers exhibit different degrees of hydrophobicity with varying PO content. As the PO content increases, the hydrophobicity of EOPO increases as a result of the longer hydrocarbon chain (Ng *et al*., 2012). It has been reported that hydrophobicity increases with increasing the MW of polymers as the ratio of hydrophilic groups to hydrophobic area decreases. There was a significant decrease in lipase production recorded when the culture medium contained EOPO with a MW of 12,000. Show *et al*. (2011) also reported that the lower MW of EOPO or the lower viscosity mass transfer can be carried out more easily in the separation process. However, at a high MW of EOPO, the accumulation of bubbles on the top of the flotation system makes

the process more difficult to control.

According to the results obtained from the partitioning of Bacillus cereus cyclodextrin glycosyltransferase (CGTase), a higher KE value was achieved in ATPSs comprising of EOPO polymer with 50% of PO content (i.e. EOPO 970 and EOPO 3900) (Ng *et al*., 2012). The CGTase partitioning in EOPO 3900/phosphate ATPSs was believed to be enhanced by increasing the hydrophobicity difference between the polymer-rich top phase and the salt rich bottom phase. Persson *et al.* (2000) reported that 50% of PO content (i.e. EOPO 970 and EOPO 3900) was more suitable for protein (CGTase) partitioning than PO content of 80% (i.e. EOPO 2500 and EOPO 12,000).

Effect of polymer concentration on partitioning

The polymer concentration was generally varied over the range of 8-18% (w/w) in ATPS to form two phases with polymers that have a certain critical value of the phase diagrams (Figure 1). In general, an increase in polymer concentrations relates to high density, refractive index and the viscosity of the phase properties. Thus, high concentration of polymer provides large differences in properties between the phases. The role of MW is also concerned with the concentration used in phase forming. The viscosity of the phase is affected by the MW of the polymers because the viscosity of a polymer solution mainly depends on the concentration. The higher the MW of the polymer is used, the lower concentration is required for phase forming because high viscosity might impact further process. In a polymer-salt system, when using a higher concentration of polymer, lower concentration of salt is required for ATPS preparation. In general, low polymer concentration is required for phase separation when the salt concentration is increased. Furthermore, the interfacial tension depends on the polymer and salt composition. When the polymer concentration is increased, the composition of the phase system moved from the critical point and then the interfacial tension increased, as a result, the biomolecules favor to move more to the top or bottom phase (Walter and Johansson, 1994).

Several studies report that increasing the PEG concentration results in a higher recovery. However, a decrease of KE and KP in bromelain partitioning was observed due to volume exclusion, which increases with an increase in polymer concentration (Babu *et al.*, 2008; Ketnawa *et al*., 2011). The highest bromelain recovery at each MW of PEG was found when the highest concentration (18%, w/w) of PEG was applied. In one investigation of the effect of PEG at the concentration of 1500, the polymer concentration varied over the range of 12-16% (w/w), but the KE of luciferase increased. The KP of the total protein decreased with an increase in PEG concentration (Priyanka *et al.*, 2012). Babu *et al*. (2008) found that increasing the concentration of PEG1500 from 12 to 18% (w/w) resulted in increased partitioning of polyphenol oxidase to the bottom phase (KE decreased). The decrease in KP of total proteins could be attributed to the influence of volume exclusion, which increases with an increased concentration of polymer (Babu *et al.*, 2008).

In TMP, it revealed that the phase diagrams of all EOPO polymers with the MWs of 970, 2500, 3900 and 12,000 Daltons are shifted towards lower polymer concentrations and almost reach the salt axis, indicating that the copolymers are probably not included in the salt-rich phase (Show *et al*., 2012a). According to the results from Ng *et al.* (2011), higher KE values were achieved in TMP, comprising of EOPO polymer with 50% of PO content (i.e. EOPO 970 and EOPO 3900).This indicates optimum partitioning of the target enzyme to the top phase without signify cant loss of enzyme activity. It has been reported that EOPO with 50% of PO content (i.e. EOPO 970 and EOPO 3900) were more suitable for most enzymes partitioning as compared to EOPO with a PO content of 80% (i.e. EOPO 2500 and EOPO 12,000) (Persson *et al*., 2000). Lower PO content polymers (i.e. EOPO 970 and EOPO 3900) produced better enzyme partitioning because it enabled maximal solubility of enzymes in the polymer phase and subsequently, enzyme precipitation in inter-phase could be avoided (Huang and Forciniti, 2002). The result indicates that a relatively higher polymer concentration is required in the formation when using a low MW of EOPO (i.e. EOPO 970). The concentrations of EOPO 3900 at around 20%-50% (w/w) were generally applied to the system to form two phases based on phase diagrams (Dembczynski *et al*., 2010a ; Ng *et al*., 2011; Show *et al*., 2012b). The high polymer concentration might lead to volume exclusion and the formation of inter-phase precipitation, which then causes a decrease in KE and PF (Ng *et al*., 2011). It has been also reported that increasing the MW or concentration of the phase-forming polymer increases the medium viscosity (Yang *et al*., 2008).

Effect of salt types and concentration on partitioning

It is well known that the addition of salts alter the partition behavior of biological materials (Hatti-Kaul, 2000). Zaslavsky (1994) reported that salts can change the structure of water, which acts with hydrophilic enzymes to modify the interaction between the water structures. The addition of salts to

the aqueous solution separation led to an arrangement of ordered water around the polymer molecules. This was due to their effect of breaking the water structure (Farruggia *et al.*, 2004; Nalinanon *et al.*, 2009). The formation of a water layer around the cation resulted in a more compact structure with a minor volume of PEG molecules. Electrical interaction and repulsion between charged aqueous phase systems and the proteins affects the partitioning of the system. The presence of salt may affect the partitioning in two ways: 1) the weakening or strengthening of the interactions, or 2) the interaction between ionized groups with the opposite net charge of the proteins. Huddleston *et al.* (1991) concluded that the effectiveness of various salts in promoting phase separation reflects the lyotropic series (a classification of ions based upon the salting-in or salting-out ability). The effectiveness of the salt is mainly determined by the nature of the anion. Multi-charged anions are most effective in the order of SO_4^{2-}> HPO_4^{2-}> CH_3COO^-> Cl^-. The order of cations is usually given as NH_4^+> K^+> Na^+> Li^+> Mg^{2+}> Ca^{2+} (Roe, 2000). When increased salt concentration results in less activity recovery, this might be due to the denaturation of enzymes caused by a salting out effect (Babu *et al*., 2008; Ooi *et al.*, 2009). In salt solutions protein solubility initially increases (salting in) and after a maximum of solubility it starts to decrease (salting out). Increase in salt concentration greater than 1.0 M caused a decrease in protein solubility. Water molecules are strongly bound to the salt and there is competition between the salt ions and the protein molecules for the water molecules. This could segregate the protein to the bottom phase (Babu *et al*., 2008; Ooi *et al.*, 2009).

Due to the effectiveness of salt to promote phase separation, multi-charged anions like SO_4^{2-}, HPO_4^{2-} from sulfate ($(NH_4)_2SO_4$, Na_2SO_4, $MgSO_4$) and phosphate (K_2HPO_4, NaH_2PO_4) salts are widely used in ATPS, TMP and TPP. The sulphate salts, especially ammonium sulphate, have an ability to promote hydrophobic interactions between the target and contaminant proteins (Yue *et al*., 2007). Many researchers have reported the suitability of PEG/$(NH_4)_2SO_4$ ATPS for extracting various oxidoreductase enzymes, namely peroxidase (Srinivas *et al*., 1999), phenylalanine dehydrogenase (PheDH) (Mohamadi *et al*., 2007) and lipoxygenase (Lakshmi *et al*., 2012). It can be observed that the PEG 1500/$(NH_4)_2SO_4$ system had the highest enzyme activity recovery (97.30%) and highest degree of purification (3.61 fold) of luciferase (Priyanka *et al*., 2012). Other sulfate salts like magnesium sulfate are also prevalently used in ATPS. A phase system containing 20% PEG1000 and 20% $MgSO_4$ gave the highest recovery (85.70%) and PF (7.2-fold) (Nalinanon *et al.*, 2009). K_2HPO_4 was reported as a widely used salt for enzyme recovery (Porto *et al.*, 2005; Ooi *et al*., 2009; Vidhate and Singhal, 2012). In order to study the effect of the salt concentration on partitioning enzymes, the concentration of salt was varied in the range of 12-18% (w/w) while keeping the polymer at a controlled concentration. Increasing salt concentrations resulted in less activity recovery. Reduced activity may be due to the denaturation of enzymes caused by the salting out effect (Babu *et al.*, 2008). Enhancement of salt quantity provided a higher proportion of a salt-rich bottom phase, leading to a practically reduced VR. It also can be observed that at an increased salt concentration, the partition coefficient of luciferase (KE) increased rapidly as compared to that of total proteins (KP) (Priyanka *et al.*, 2012).

Ammonium sulfate and potassium phosphate have been widely used in the range of 10-35% (w/w) with EOPO in TMP. Both salts are keys to sustaining an immiscible two-phase system through a salting-out effect (Wan *et al*., 2010). This is due to the volume of the aqueous phase, which is much larger than the volume of the EOPO phase if compared with ATPS. The influence of the ammonium sulfate concentration on the separation of lipase was optimal at a concentration of 250 g/L (Show *et al*., 2011). The increase of $(NH_4)_2SO_4$ concentration led to a reduced separation efficiency because the interface tension between the EOPO phase and aqueous phase were low enough, so the mass transfer became easier (Bi *et al*., 2009).

Effect of pH on partitioning

Electrochemical interactions can influence the partitioning behavior of proteins. As opposite charges attract, the presence of charged constituents may generate a selective fractionation of oppositely charged solutes and proteins towards a specific phase. It has been reported that the system pH will modify the partitioning of the targeted protein and alter the concentration of the phase-forming polymer because the polymer loses/gains charge from functional groups, which causes repulsion/attraction and material expansion/contraction (Saravanan *et al*., 2008). If the pH tested is above the isoelectric point (pI) of that enzyme, the enzyme is negatively charged and repelled from the salt rich bottom phase to the polymer rich top phase (Hatti-Kaul, 2000). Its interaction with PEG becomes stronger and the partition coefficient increases. Negatively charged proteins prefer the upper polymer-rich phase and

positively charged proteins partition to the lower salt phase (Andrews and Asenjo, 1996). However, some results show that a major increase in KE is observed when the protein charge changes from positive to neutral (Marcos *et al.*, 1998). When proteins are in a solution at the same pH of their pI, they will have no net molecular charge. Electrostatic repulsion between protein molecules will then be at a minimum and interactions via hydrophobic groups on the surface of the protein are more likely to occur. For this reason, the protein/enzyme favors more to the polymer-rich top phase (Chaiwut *et al*., 2010). Therefore, the pH of the system may be manipulated in order to promote selective separation. The use of pH values above the isoelectric point (pI) of proteins may induce an additional affinity towards the polymer-rich phase (Benavides *et al*., 2000). Generally, the experiments were conducted by varying pH in the range of 3-11, which was accomplished by adding appropriate amounts of hydrochloric acid and sodium hydroxide solution (Yucekan and Onal, 2011; Amid *et al.*, 2012).

In ATPS, the tested pH range was determined based on the pI of the target enzymes. For example, the majority of the lipase derived from *Burkholderia cenocepacia* strain ST8 (pI about 6.3) migrated to the top polymer phase when the pH was above 7.0. At a pH below 7.0, lipase migrated to the bottom salt phase. At a pH above 7, the lipase enzyme was negatively charged and repelled from the phosphate-rich bottom phase (Mohamadi *et al*., 2007; Ooi *et al*., 2009; Show *et al.*, 2012a, b). Acidic enzymes (pI < 7) such as that which come from the bromelain in pineapple (pI = 4.6) (Babu *et al*., 2008), recombinant PheDH from *Bacillus badius* (pI = 5.3) (Mohamadi *et al.*, 2007) and invertase from tomato (Yucekan and Onal, 2011) all moved to the top phase when the pH was increased above the pI of those respective enzymes. For example, invertase from tomato preferred to partition to polymer rich top phase when a lower pH (at 4.5) was applied; hence, the KE, PF and recovery values increased compared to those obtained at pH 7.5 (Yucekan and Onal, 2011). With regards to an alkaline enzyme (pI > 7), an adjustment of pH to be lower than the pI resulted in proteins/enzymes to shift to the salt-rich bottom phase. For example, the recovery of *Calotropis porcera* protease was decreased when the system (pH system 5.7) was adjusted to pH 4.0 when compared to that set at a neutral pH. At pH 4.0, the target protease is positively charged and prefers the salt-rich phase (Chaiwut *et al*., 2010).

In TMP, it has been reported that EOPO tends to be positively charged at a pH above 7, which allows it to interact with some enzymes (Li and Peeples, 2004). Furthermore, if enzymes represent a large hydrophobic region on the surface, this strongly interacts with the hydrophobic tails of EOPO (Show *et al*., 2010a). Dembczynski *et al.* (2010a) reported that at pH 9.0, the lysozyme (pI = 10.7) was positively charged, while all major egg white proteins such as ovalbumin, ovotransferrin, ovomucoid and ovomucin had pI values lower than 6.5 and were negatively charged (Ghosh *et al.*, 2004). As mentioned above, the target enzyme behaved as a negatively charged protein that preferably migrated into the polymer rich top phase whereas positively charged proteins would partition into the phosphate salt-rich bottom phase (Ng *et al*., 2012).

Effect of NaCl on partitioning

The effect of NaCl concentration on the partition has been analyzed because it is well known that salt is able to modify the enzyme partition to one of the phases (Reh *et al*., 2002). The non-toxicity of NaCl is useful in modifying the partition process without affecting the use of enzymes in any biological process (Amid *et al.*, 2012). Generally, the addition of neutral salts speeds up phase separation by influencing the phase potential or by decreasing protein hydrophobicity, in which the interaction between hydrophobic surface area of the protein and hydrophobic chain (ethylene group) of PEG will be facilitated. The addition of neutral salts is known to generate an electrical potential difference between the two phases that were able to drive proteins from one phase to another phase depending on the charge. Generally, the addition of sodium chloride affects the partitioning in aqueous two phase system by speeding up phase separation, by influencing the phase potential or by protein hydrophobicity (Hatti-Kaul, 2000).The addition of sodium chloride salts in ATPS can affect water structure and hydrophobic interactions differently in which the interaction between hydrophobic chain (ethylene group) of PEG and hydrophobic surface area of the protein will be facilitated (Marcos *et al*., 1998). Asenjo *et al.* (2011) reported that the resolution of the ATPS is increased with NaCl concentration. Therefore, adding NaCl in ATPS can be done to separate enzymes more efficiently. Abbott and Hatton (1998) reported that NaCl affects recovery yields, causing the separation of phases or changing the electrical potential. At high NaCl concentrations, KE increases as a function of positive charge. This suggests that the polymer-rich top phase becomes more negative as the concentration of NaCl in the systems increases and, therefore, attracts the positively charged proteins. The addition of salts to the aqueous polymer solution

leads to an arrangement of ordered water molecules around PEG molecules due to their water structure breaking effects (Farruggia *et al.*, 2004). The formation of a water layer around the cation results in a more compact structure with a minor volume of polymer molecules (Nalinanon *et al.*, 2009). However, higher concentrations of NaCl have shown to have a significantly negative effect on partitioning and yields of the enzyme, probably due to protein denaturation and precipitation at high concentrations of this salt (Amid *et al.,* 2012). There are some reports that indicate that in PEG-salt systems with NaCl addition, the salt increased the difference in the hydrophobicity of the phases, promoting the partition of the more hydrophobic proteins to the upper phase.

In ATPS, NaCl concentrations in the range of 1 to 6 % (w/v) present in the system are observed to increase KE and recovery of enzymes and the concentration can be increased in the system until reaching the saturation point. Most researchers have reported that KE can be dramatically increased when high concentrations of NaCl are added to the system (Marcos *et al*., 1998). In addition, increased NaCl enhances interactions between 2-propanol's hydrophobic chains and the serine protease's hydrophobic surface area and the enzyme could be attracted to the propanol-rich top phase (Amid *et al*., 2012). These results are consistent with others; the addition of NaCl can increase KE, PF and recovery by more than double as opposed to unmodified systems of trypsin (Tubio *et al.*, 2009) and lipase derived from *Burkholderia pseudomallei* (Ooi *et al*., 2009). In Ooi *et al*. (2009), an addition of NaCl of 4.5% (w/v) to 16% 2-propanol-16% K_2HPO_4 ATPS improved the recovery from 76 to 99% and purity from 6.4 to 13.5-fold. The same trend of improved recovery and purity was observed in Chaiwut *et al.* (2010), Yucekan *et al.* (2011) and Amid *et al*. (2012). The recovery of protease from *Calotropis procera* was significantly increased from 23.58 to 107% when NaCl was added by 6% (w/w) to 12% PEG 4000-17% $MgSO_4$ (Chaiwut *et al.*, 2010). Yucekan and Onal (2011) reported that when KCl 5% (w/w) was added to 15% PEG3000-12% Na_2SO_4, the recovery and purity greatly increased from 68 to 90% (recovery) and 3.3 to 5.5-fold (PF). In addition, the recovery and purity of serine protease from mango peel increased when the pH was adjusted to 7.5 and NaCl was added by 5% (w/w) to a system of 16% 2-propanol-19% K_2HPO_4 (Amid *et al*., 2012).

According to the TMP accounts described in the literature, the addition of NaCl was intended to create an electrical potential difference between phases since Cl^- preferentially partitions to the EOPO-rich phase. It has previously been shown that the partition of proteins can be affected by various salts, where positively charged proteins partition to the phase at which negative ions have an affinity (Johansson *et al*., 1996). Hence, the positively charged proteins also preferentially partition to the EOPO copolymer phase. NaCl in crystalline form has been added to a final concentration of 0.85 M in each system to improve partitioning of lysozyme from hen egg whites (Dembczynski *et al*., 2010a, b). The addition of 4% (w/w) NaCl in partitioning of CGTase by TMP exhibited a highest KE of 11.7 and PF of 16-fold as compared to that without NaCl addition (KE of 1.8 and PF of 8.5-fold) (Ng *et al*., 2011). The recovery of alkaline protease from farmed giant catfish viscera was increased by triple when 17% (w/w) of NaCl was added to the system (Ketnawa *et al*., 2014a). From the above previous works, it can be concluded that NaCl improves the partitioning of target enzymes to the top phase due to the chemical potential of solutes altered by the salt, resulting in increased protein/ enzyme recovery and purification.

Effect of crude extract/protein concentrations on partitioning

In ATPS, TMP and TPP, the amounts of crude load volume or protein concentration are important factors for enzyme partitioning. It can alter the phase volume ratio and the partition behavior of the target molecules (Abbott and Hatton, 1998). The partition coefficient (KP) could be satisfactorily predicted, as a function of the overall protein concentration (Andrews and Asenjo, 1996). In the top phase, maximum protein concentration is determined mainly by the steric exclusion effect of PEG and also by the hydrophobic interaction between PEG and proteins. In the bottom phase, maximum protein concentration is determined mainly by a salting-out effect. As the ionic strength is increased in the systems, the concentration in the top phase increased for all proteins. In the bottom phase, an increase in ionic strength increased the salting-out effect. The concentration limit depends on the properties of the protein (Asenjo and Andrews, 2011). When the concentration of a protein exceeds relatively low values, precipitation at the interface can be observed (Amid *et al*., 2012; Show *et al*., 2012a; Ng *et al*., 2012). It has been found that only in rare cases will the proteins be completely in solution as the concentration is increased until a solubility limit is reached and then the protein precipitates fully out of the solution (Andrews and Asenjo, 1996).

ATPS experiments are normally performed by varying the crude load volume from 10 up to 70% (w/w) in order to investigate the effect of crude

load volume on enzyme partitioning (Dreyer, 2010; Ketnawa *et al.*, 2014a; Show *et al*., 2012a; Ng *et al.*, 2011). A crude load of 20% (w/w) provided the maximum yield (95.8%) of serine protease from mango peel (Amid *et al.*, 2012). The same result was observed by Ng *et al.* (2011) and Show *et al.* (2012a); 20% (w/w) of crude load provided the highest recovery (70.53%) and PF (13.1-fold) in partitioning cyclodextrin glycosyltransferase derived from *Bacillus cereus* from rotten potato by ATPS and 95% and 6.7-fold in *Burkholderia cenocepacia (B. cenocepacia)* strain ST8 lipase by TMP, respectively. Larger amounts [>20% (w/w)] of crude load volume in the both ATPS and TMP affect the composition/ properties of the systems and decrease the VR. There is a decrease in ATPS/TMP performance when the amounts of crude load volume are increased in the system (Amid *et al.*, 2012; Show *et al.*, 2012a; Ng *et al*., 2012; Ketnawa *et al*., 2014a). Therefore, the partitioning and volume ratio are decreased when a higher amount of the sample is loaded and thus the system is not at optimum condition for various enzyme purification at higher concentrations of the crude extract (Amid *et al*., 2012; Show *et al*., 2012a; Ng *et al*., 2012; Ketnawa *et al*., 2014a). However, the crude concentration of up to 70% (w/w) in the system provided the best recovery (157.13%) and purity (3.94) of alkaline protease from giant catfish viscera by ATPS (Ketnawa *et al*., 2014a). This depended on the higher levels of solubility of the protein in each phase. Hence, the partitioning observed at low protein concentrations can be very different to that observed at high concentrations (Asenjo and Andrews, 2011).

For other partitioning techniques like TPP, the ratio of the crude sample to t-butanol content is also critical since the denaturation of the protein occurs when t-butanol to crude enzyme extract is greater than one (Dhananjay and Mulimani, 2008). This optimum ratio is presumably a result of two factors. If the amount of t-butanol is less, it does not adequately synergize with ammonium sulphate. If the t-butanol ratio is high (>1.0:1.0), the denaturation of the protein is more likely occur (Sharma and Gupta, 2001; Barros *et al*., 2014). The ratio between crude enzyme extract to t-butanol was varied from 1.0:0.5 to 1:2.0 in various studies. The system ratio crude extract to t-butanol of 1.0:0.5 provided the highest protease recovery (67.3%) and PF (2.4) for papaya peel protease partitioning (Chaiwut *et al*., 2010) and 132% and 6.29 in *C. procera* protease partitioning (Rawdkuen *et al*., 2010). The same ratio offered the highest purity of 15-fold and 363% activity recovery for invertase from baker's yeast (Akardere *et al.*, 2010). The ratio crude extracts to t-butanol of 1.0:1.0 provided the highest recovery and PF of certain enzymes such as wheat germ amylase inhibitor (Sharma and Gupta, 2001), amylase/protease inhibitor (Saxena *et al.*, 2010), α-galactosidase from fermented media of Aspergillus oryza (Dhananjay and Mulimani, 2008), α-galactosidase from tomato (Çalci *et al.*, 2009) and investase from tomato (Özer *et al.*, 2010). It was observed that an increase of t-butanol volume of more than 1.0 resulted in a decrease of purification fold and activity recovery.

The amount of crude extract in TMP was studied and showed that only a crude load of 20% (w/w) was suitable for TMP of lipase from fermentation broth of *Burkholderia cenocepacia* strain ST8 and Cyclodextrin glycosyltransferase (CGTase) from fermentation broth of *Bacillus cereus*. They found that increasing crude extract volume caused precipitate accumulation at the interface was increased and that there was a loss of enzyme activity (Show *et al.* 2012a; Ng *et al.*, 2012). However, it indicated that a crude load of 40% (w/w) is the maximum capacity for 200 ml of the aqueous phase in partitioning of *Burkholderia cepacia* strains ST8 lipase by TMP. The separation efficiency for 40% (w/w) crude feedstock load was 70.8%. Higher amounts of the crude feedstock loaded [>40% (w/w)] into the TMP decreased the separation efficiency and subsequently affect the composition of TMP (Show *et al*., 2011).

Effect of temperature on partitioning

The effect of temperature is very different for each phase system that relies on the type of polymer used. For example in ATPS at high temperature, two phases are formed easily with small concentrations of PEG/ salt whereas in a PEG/dextran system, two phases will easily form at lower temperatures (Walter and Johansson, 1994). An increase in temperature results in increased differences in the phase composition. With the increase in temperature, the phase diagram (Figure 1) moves towards the higher-phase-forming concentration and decrease in the tie-line length (Albertsson, 1986). Under this influence, the partition coefficient of a few proteins has been found to increase with the rise in temperature. Consequently, the number of water molecules available for solute salvation in the bottom phase decrease due to an increase in salt concentration. This also reduces the solubility of biomolecules in the phase. The partition coefficient of the biomolecules is probably influenced by this variation in the phase compositions. Naganagouda *et al*. (2008) indicated that the KE of α-galactosidase in a PEG-salt system increased at a temperature from 25 to 55ºC. Furthermore, increasing temperature can destroy the bonds of the biomolecules. As these

bonds are weakened and broken, the structure of the biomolecules becomes more flexible. Water in two phase systems can interact and form new hydrogen bonds with the functional group of the biomolecules. The presence of water further weakens nearby hydrogen bonds by causing an increase in the effective dielectric constant near them. As the structure is broken, hydrophobic groups are exposed to the solution. As a consequence, losses in molecule solubility have been observed (Ratanapongleka, 2010).

For TMP, it is important to determine these effects judiciously as the thermoseparation parameters will improve the copolymer recovery and the separation of protein from copolymer. Yet, temperature can be a limiting factor in thermoseparation systems and much depends on copolymers with different EO/PO compositions. The copolymers with different EO/PO compositions have been investigated at EO50PO50 [50% EO and 50% PO (w/w)], EO30PO70 and EO20PO80. The temperature required for thermoseparation decreases when the PO content of the copolymer is increased (Persson *et al.*, 1999). EO20PO80 has the lowest cloud point (LCST) at 30ºC. EO30PO70 and EO50PO50 have LCSTs at 40 and 50ºC, respectively. Low temperature could not cause phase separation since the random copolymers of EOPO are not separated from aqueous solution above a critical temperature or cloud point. Conversely, at extremely high temperatures, enzymes were usually thermally denatured (Show *et al*., 2011). Temperatures above 50ºC will cause thermoseparation because of the cloud point (LCST) for EOPO3900 is 50ºC at 10% (w/w) (Persson *et al*., 1999). In Ketnawa *et al.* (2014b), no phase separation was observed below 55ºC and increasing the temperature in the thermoseparation step provided a lower recovery. The maximum KE value (24.09) and recovery (77.98%) of alkaline proteases were obtained at a system temperature of 55ºCwhen compared to 60ºC and 65ºC. The results indicated that the KE value, PF and lipase production using a 10% (w/w) solution of EOPO with a MW of 3900 g/mol were increased when increasing the system temperature from 20 to 50ºC (Show *et al.*, 2011).

Effect of number of cycles on partitioning

The reason for carrying out the second cycle of each partitioning is that sometimes the first partitioning cycle cannot remove the contaminant proteins efficiently (Chaiwut *et al*., 2010; Rawdkuen *et al*., 2011). It is expected that the target protease will be more partitioned to the upper phase (ATPS/TMP) or interphase (TPP) with the highest yield and purity. Saxena *et al.* (2010) reported that the aqueous phase containing most of the desired protein when subjected to a second cycle of TPP results in considerable purification of the target protein. Rajeewa and Lele (2011) investigated a two-step TPP. In the first step, proteins and polysaccharides that precipitated in the interfacial layer at low levels of ammonium sulfate were separated. In addition, hydrophobic components such as pigments, lipids and enzyme inhibitors were also removed along with the upper organic phase. In the second step, the pre-purified aqueous layer was further subjected to higher levels of ammonium sulfate.

In ATPS, performing the second cycle could increase the recovery of alkaline protease from farmed giant catfish by triple (157 to 360%) (Ketnawa *et al.,* 2014a). Furthermore, Chaiwut *et al*. (2010) partitioned papain by TPP with 55% (w/v) $(NH4)_2SO_4$ in the second TPP cycle, which provided 89.4% protease recovery. In another study, the second TPP of *C. procera* with 60% saturated $(NH_4)_2SO_4$ resulted in a 132% recovery with higher purity (6.92-fold) (Rawdkuen *et al.*, 2010). It was observed that 60% ammonium sulphate saturation and a ratio of aqueous phase to t-butanol of 1:2 gave a maximum 20.1 and 16-fold purification with 39.5% and 32% yield of amylase inhibitor and trypsin inhibitor, respectively (Saxena *et al.*, 2010). The high recovery of protease was probably because the TPP led to simultaneous activation of the enzyme, resulting in a clearly observed value of more than 100% (Rawdkuen *et al.*, 2011). However, the research reported that there was lower recovery gained from the second cycle of TPP, yet a higher purity was obtained. Thus, a single step liquid-liquid phase partitioning method was insufficient to achieve the desired purification; an alternative two-step partitioning procedure was used to increase the purification fold.

Recent applications of phase partitioning on enzyme separation

Aqueous two phase partitioning for enzyme separation

Recent applications of ATPS partitioning on various enzymes from different sources are shown in Table 3. ATPS has been widely used for the partitioning and recovery of enzymes from various sources such as plants, animals and microbial. The best conditions that provided the maximum recovery and purity are also demonstrated in Table 3. The reasonably high recovery (nearly or more than 100%) and purity (>1.5-fold) are derived by ATPS partitioning. Specifically, the recovery more than

Table 3. Recent applications of aqueous two phase, thermoseparation and three phase partitioning on enzyme separation

Enzyme	Source	The best condition	Recovery (%)	Purity (fold)	References
Aqueous two phase partitioning					
Bromelain	Pineapple fruits (*Ananus comosus* L. Merryl)	14% PEG-17.66%K_2HPO_4+KH_2PO_4, 1 mM NaCl, pH 7.5.	89.65	2.80	Navapara *et al*., 2011
Polyphenol oxidase	Patato (*Solanum tuberosum*) peel	17.62 % PEG1500-15.11% KH_2PO_4+K_2HPO_4, 2.08mM NaCl, pH 7	77.8	4.5	Niphadkar *et al*., 2014
Fibrinolytic enzymes	*Auricularia auricular*-judae, mushroom	20%PEG 8000-11.6%K_2HPO_4, system pH 7.0, 25%Crude extract	78.0	7.01	Mohamed Ali *et al*., 2014
Alkaline protease	Farmed giant catfish viscera	Step1:15% PEG2000-15% $Na_3C_6H_5O_7$, pH8.0	157.13	3.9	Ketnawa
		Step2: 10% $Na_3C_6H_5O_7$	365.53	11.60	*et al*., 2014a
Luciferase	Firefly	4.0%PEG1500-20.5%$(NH_4)_2SO_4$	118.34	13.69	Priyanka *et al*., 2012
Trypsin	Bovine pancreas	40%PEG3350-25% $Na_3C_6H_5O_7$, pH 5.20, crude load 9.30%, 3.34% NaCl	99.7	2.55	Pérez *et al*., 2015
Protease	Hepatopancreas of Pacific white shrimp	Step1:15%PEG1000 -25%$MgSO_4$	65.5	8.6	Senphan
		Step2:25%PEG8000-10%$MgSO_4$	46.2	9.9	*et al*., 2014
Pectinase	*Aspergillus oryzae* in a passion fruit peel	8% Triton X-114, 20% Crude extract, 25°C	261.6	1.34	Duque Jaramillo *et al*., 2013
Proteases	*Penicillium restrictum* from Brazilian Savanna	4%PEG2000-20 %sodium polyacrylate (NaPA), 45% fermented broth, 25°C, pH 6.5, 1.05%NaCl	93.47	1.98	Barros *et al*., 2014
Lipase	Cell free fermentation broth of *Rhodotorula glutinis*	17.5%PEG4000-12.5% oxalate potassium, 12.5 (%, v/w) fermentation broth, pH 6.6, 24°C	71.2	13.9	Khayati *et al*., 2013
Lysozyme	Hen egg white	40% EOPO3900-20% K_2HPO_4, 0.85 M NaCl, pH 9.0	99.4	29.4	Dembczynski *et al*., 2010a
Lipase	Fermentation broth of *Burkholderia cepacia* strains ST8	50% EOPO3900, 25% $(NH_4)_2SO_4$, crude load 40% (w/w)	76	13	Show *et al*., 2012a
Lipase	Fermentation broth of *Burkholderia cenocepacia* strain ST8	EOPO3900- K_2HPO_4 comprising TLL of 48.5% (w/w),crude load 20% (w/w), pH 7.0	99	14	Show *et al*., 2012b
Cyclodextringlycosyltransferase	Fermentation broth of *Bacillus cereus*	EOPO 3900- K_2HPO_4 comprising TLL of 41.2% (w/w),crude load 20% (w/w), pH 7.0	87	13.1	Ng *et al*., 2012
Three phase partitioning					
Peroxidase	Orange peels (*Citrus sinenses*)	50% (w/v) ammonium sulfate with with 1.0:1.5 (v/v) ratio of crude extract: *t*-butanol at pH 6, 30°C with shaking 200 rpm for 80 min .	93.96	18.29	Vetal *et al*., 2015
Ficins	*Fig (Ficus carica)* latex	40% (w/v) ammonium sulfate saturation with 1.0:0.75 (v/v) ratio of crude extract: *t*-butanol at pH 7.0	167	6.04	Gagaoua *et al*., 2015
Mangiferin	Mango (*Mangifera indica) leaves*	40% (w/v) ammonium sulfate with solute to solvent ratio 1:20 (w/v) slurry to *t*-butanol ratio 1:1 (v/v) microwave power 272 W, 5 min irradiation time and duty cycle 50%	54	-	Kulkarni *et al*., 2015
Alkaline proteases	Farmed giant catfish viscera	50% sodium citrate and enzyme extract:*t*-butanol 1:0.5 (w/v), pH 8.0 with incubation temperature 25 °C	220	5.04	Ketnawa *et al*., 2014c
Protease	Hepatopancreas of Pacific white shrimp	30%ammonium sulfate saturation with 1:1 (v/v) ratio of crude extract to *t* -butanol	76	2.6	Senphan *et al*., 2014
Fibrinolytic enzyme	*Bacillus sphaericus* MTCC 3672	80% ammonium sulfate saturation with 1:0.5 ratio of crude extract to *t*-butanol, pH 9, 30°C, at 25 kHz &150 W ultrasonication power with 40% duty cycle for 5 min irradiation time	65	16.15	Avhad *et al*., 2014

200% was derived from optimum condition ATPS of several enzymes such as bromelain 335.27% (De Lencastre Novaes *et al*., 2013), alkaline protease 365.53% (Ketnawa *et al.*, 2014a) and Pectinase 261.6% (Duque Jaramillo *et al.*, 2013). In addition, partitioning by ATPS provided enzymes with the high purity of more than 1.5 to 15-fold. The highest purification fold of 25.78 and 13.69 was derived from ATPS of 8%PEG2000 -6%PAA15000, 6%Na_2SO_4 at 30°C and 5%PEG8000-28.5%K_2HPO_4 in the partitioning of in the partitioning of bromelain (De Lencastre Novaes *et al.*, 2013) and luciferase from firefly (Priyanka *et al*., 2012), respectively. All studies have been focused on finding the best condition for gaining the highest purity and recovery for target enzyme. Hence, researchers pay a lot of effort with optimization condition of ATPS in recent years. Extraction optimization using a conventional one factor-at-a-time method involves changing one independent variable (polymer molecular weight, polymer concentration, salts concentration, pH, NaCl concentration, crude load volume, temperature, number of cycle etc.) while fixing all others at a certain level (Barros *et al*., 2014). However, optimization of a large number of independent variables for maximum response by the one factor-at-a-time approach is a tedious and time consuming task and often not capable of signifying the interactions between the different variables. So, response surface methodology (RSM) mainly involves the collection of mathematical and statistical techniques that are useful for modeling and analyzing systems where a response of interest is simultaneously influenced by several variables to achieve maximum response in extraction of target enzymes by ATPS (De Lencastre Novaes *et al.*, 2013; Duque Jaramillo *et al*., 2013; Barros *et al*., 2014; Niphadkar *et al*., 2014). It reduces the number of experiments and also can identify the independent variables with statistical significance for the process, as well as their significant interactions (Pérez *et al*., 2015). A central composite design (CCD) was carried out to optimize the extraction factors and maximize purification factor and the yield in experiments simultaneously. RSM is a collection of mathematical and statistical techniques that are useful for modeling and analyzing systems where a response of interest is simultaneously influenced by several variables. Navarapa *et al*. (2011) studied bromelain extraction

by using Central Composite Rotatable Design (CCRD) of RSM. A PEG1500 concentration 12, 15 and 18 (%, w/w), potassium phosphate concentration 14, 17 and 20 (%, w/w), system pH 6, 7.5 and 9 and NaCl concentration 0, 0.5 and 1 (mM) were evaluated to identify the best bromelain extraction conditions. They found that an optimized to purify bromelain by ATPS was the system composed of 14% (w/w) PEG 1500, 17.66% (w/w) potassium phosphate and 1 mM sodium chloride at pH 7.5 A maximum enzyme partition coefficient of 12.62 and % yield of 90.33 in the top PEG-rich phase with a purification factor of 2.4 was predicted. However, from the experiment, enzyme partition coefficient, % yield and purification factor as 12.22, 89.65 and 2.8, respectively. From the model, they can report that the partition coefficient and yield decreased with an increase in molecular weight of PEG, in an ATPS formed by PEG and potassium phosphate. In another study, the independent variables analyzed were PEG and PAA concentrations, PEG and PAA molar mass, salt (Na_2SO_4) concentration and temperature, was applied to the model to achieve the highest purity and recover (De Lencastre Novaes *et al.*, 2013). The results from the RSM model showed that PEG and PAA concentrations and temperatures showed a significant positive effect, suggesting that an increase in these parameters would improve bromelain extraction by ATPS (PEG/PAA). PEG molar mass had a negative effect, which means that work with lower PEG molar mass could improve both responses (yield and purification factor). Pérez *et al*. (2015) also confirmed about the robustness of the statistic model used and suggested that the experimental design using RSM is a suitable tool for a quick and efficient study on the optimization of experimental conditions for bovine pancreas trypsin extraction. A purification factor of 2.55 and a yield of 99.7% were obtained experimentally while the optimized conditions, predicted by the software was NaCl concentration, crude volume were 3.34% (w/w) and 9.30%, respectively giving theoretical purification factor of 2.3 and a yield of 97% (Pérez *et al*., 2015).

From the above studies, it can be concluded that ATPS can be used for partitioning various enzymes very effectively with high recovery and purity. Moreover, the efficacy in partitioning can be further enhanced by simple modification of the ATPS with pH adjustment or addition of NaCl. Furthermore, statistical techniques like RSM can be applied to study factors more than 2 factors at a time. It describes also the effect of each factor on response value finally lead to obtain the optimum condition for each interesting enzymes.

Thermoseparation for enzyme recovery

For polymers that should be used in TMP for the purification of biomolecules, it is important to observe that the cloud point temperature or critical micellization temperature (CMT) cannot be too high as this could lead to protein denaturation. At concentrations below critical micelle concentration (CMC), polymer chains exist as individual unimer coils, whereas on further increasing the polymer concentration in aqueous solution above CMC or when solution temperature is raised above CMT, there is formation of stable micelles. Micelle formation at room temperature was not observed in solutions of less hydrophobic polymers. It has also been shown that the CMC or CMT for a polymer depends on its molecular weight (EO/PO block length) and EO (hydrophilic)-PO (hydrophobic) content ratio (Deng *et al*., 1992; Teotia *et al.*, 2015). Rabelo *et al.* (2004) found that when the copolymer concentration varied from 5 to 30% (m/m), the cloud point temperature changed from 32 to 18°C, respectively, for solutions containing copolymer with 10% EO (EOPO 1100). The high number of copolymer molecules increases the interactions and easily aggregate, resulting in phase separation at lower temperatures. However, the cloud point temperature of the solution containing copolymer with 50% EO (EOPO 3900) was not influenced significantly by the copolymer concentration, unlike what was observed in the solution containing copolymer with 10% EO. The solutions with 50% EO have presented a cloud point temperature of 65ºC (Rabelo *et al*., 2004).

A lot of recent reports have been launched to date about ATPS plus with special material that can be separating enzyme out easily and economically. For example, thermo responsive polymer, pH which they show responsive behavior, such as pH, temperature, variation in ionic strength, light, redox conditions, charged moieties in vicinity, electrical changes, magnetic field and biological and chemical stimuli (Teotia *et al*., 2015). Thermo-responsive polymers are the most comprehensively studied responsive polymers because of their unique property of sol-gel transition above certain temperature. This chapter focuses on polymer materials with thermo-responsive behavior in enzyme recovery. Poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) copolymer (EOPO) is one of synthetic thermo-responsive polymers that have been pervasively used in separation of biomolecules in thermoseparation (TMP).

EOPO can be modified to show sol-gel transition at desired temperature and becomes insoluble above a critical temperature called the lower critical solution

temperature (LCST). For a polymer in aqueous solution, LCST is the point in the phase diagram at which entropy of the water in the system increases due to less ordered arrangement of water molecules and becomes more than enthalpy of water hydrogen bonded to the polymer (Kumar *et al*., 2007), therefore entropy of system governs LCST (Southall *et al*., 2002). When temperature of an aqueous solution of thermo-responsive polymer exhibiting LCST behavior is increased, the polymer chains show coil-to-globule-to-aggregate transition. Structurally, these are block copolymers with a poly(propylene oxide) (PO) middle block flanked by poly(ethylene oxide) (EO) block on either side. At concentrations below critical micelle concentration (CMC), polymer chains exist as individual unimer coils, whereas on further increasing the polymer concentration in aqueous solution above CMC or when solution temperature is raised above critical micellization temperature (CMT), there is formation of stable micelles. Micelle formation at room temperature was not observed in solutions of less hydrophobic polymers. It has also been shown that the CMC or CMT for a polymer depends on its molecular weight (EO/PO block length) and EO (hydrophilic)-PO (hydrophobic) content ratio (Deng *et al*., 1992; Teotia *et al*., 2015). In fact, the thermo-separating copolymer showed different degrees of hydrophobicity with different between EO and PO content. Polymers in this class have been designed to have phase separation temperatures ranging from 10°C (polymers with low EO contents) to 100°C (polymers with high EO contents).

The recent applications of TMP on various enzymes from different sources are summarized and shown in Table 3. TMP has been used for recovering and purifying various enzymes including plant, animal and microbial sources specifically bromelain in pineapple (De Lencastre Novaes *et al*., 2013), amylase in red pitaya peel (Amid *et al*., 2014), lysozyme from hen egg (Dembczynski *et al*., 2010a, b), lipase from fermentation broth (Show *et al*., 2011; Show *et al*., 2012b) and CGTase from Bacillus cereus (Ng *et al*., 2012). The best conditions that provided the highest recovery and purity are also noted. Most studied showed that EOPO3900 showed the optimum result in partitioning differenced enzymes and provides the best purity and recovery (Table 3). Some preliminary studies have showed EOPO copolymer with higher PO content (above 50%) would have an efficient thermoseparation and yield at lower temperature. EOPO with low PO content (25%) (i.e., 2,500) was more suitable for the certain enzymes than high i.e. EOPO 3900 with low PO content (50%) depends on target enzymes. It has been reported that the EOPO with 50% of PO content (i.e. EOPO 970 and EOPO 3900) were more suitable for protein CGTase partitioning as compared to EOPO with PO content of 80% (i.e. EOPO 2500 and EOPO 12000) (Persson *et al.*, 2000). Ng *et al*. (2012) also reported that EOPO 3900 showed higher purity and recovery of *Bacillus cereus* CGTase than those by using EOPO 970, EOPO 2500 and EOPO 12000. CGTase showed optimum partitioning with purity up to 13.1-fold and 87% yields in EOPO 3900/phosphate ATPS comprising TLL of 41.2% (w/w), VR of 1.25 and crude load of 20% (w/w) at pH of 7.0. Lower PO content polymers (i.e. EOPO 970 and EOPO 3900) gave better CGTase partitioning because it enables maximal solubility of CGTase in the polymer phase and subsequently the CGTase precipitation in inter-phase could be avoided (Ng *et al.*, 2012). Lipase from *B. cenocepacia* strain ST8 has also been satisfactorily partitioned by a system composed of EOPO 3900 and potassium phosphate, with a tie-line length of 48.5% (w/w), a volume ratio (VR) of 2.3 and a crude load of 20% (w/w) at pH 7 (Show *et al.*, 2012a). However, another study, the highest amylase activity from red pitaya peel was achieved in the presence of 30% (w/w) of EOPO 2500, while EOPO with higher molecular weight significantly ($p<0.05$) decreased the amylase activity (Amid *et al.*, 2014). It could be considered that EOPO 2500 with low PO content has better effect on the enzyme partitioning because it enables maximal solubility of the amylase polymer phase and subsequently the amylase precipitation in the interphase could be avoided. Apart from type of polymer hydrophobic group (depends on proportion between PO and EO), concentration of polymer, type of salts/solvents, temperature for separating were investigated for optimum the highest purity and recovery. Dembczyński *et al.* (2010a) have incorporated 10% K_2HPO_4 in a system containing 40%EOPO 4000 for partition of lysozyme and this system provided a recovery of 85% of protein and PF of 16.9. One interesting factor to promote the recovery and purity is additional of NaCl. Demdczynski *et al.* (2010b) observed that the addition of 0.85 M NaCl to the systems EOPO 3900 (17.40%, w/w) and K_2HPO_4 (22.67%, w/w) in lysozyme extraction. The same trends from Ketnawa *et al*. (2014c) that studied partitioning of alkaline protease from fish viscera by TMP using EOPO 3900 and varying type of salts, temperature for separation and NaCl addition. The additional of NaCl 17% to the optimum system contained 40% EOPO 3900-10% $MgSO_4$, induced phase separation at 55ºC, increased the recovery from 45 to 77.98% and purity from 4 to 21.50-

fold. Another study, 1-propanol, 2-propanol and ethanol were used instead of salt to separate amylase enzyme from red pitaya *(Hylocereus polyrhizus)* peel (Amid *et al*., 2014). In this study, the enzyme was satisfactorily partitioned into the polymer-rich top phase in the system composed of 30% (w/w) EOPO 2500 and 15% (w/w) 2-propanol, at a volume ratio of 1.94 and with a crude load scale of 25% (w/w) at pH 5.0 with a high purification factor of 14.3 and yield of 96.6% and copolymer was also recovered and recycled at a rate above 97%, making this TMP was more economical than the traditional ATPS method.

Even though this method does not provide recovery as high as ATPS and TPP, purity in some experiments was very high up to 21-29% (Demdczynski *et al*., 2010b; Ketnawa *et al.*, 2014b). The above studies demonstrated that very high purity was obtained from TMP. Moreover, direct recovery using thermoseparating polymers is far less expensive than most polymer-polymer and polymer-salt ATPS. The recycling of all the phase-forming components in TMP is ideal in terms of cost (recycling of polymer and salt solution for subsequent TMP), processing time (reduction of operation time) and environmental benefits. Thus, TMP could be a valuable alternative to conventional methods for enzyme recovery and purification.

Three phase partitioning for enzyme separation

Three phase partitioning is one of such integrated approaches for purification of protein and enzymes from different sources such as plants, animals and microbial. Besides, TPP has also been widely used for the extraction and purification of other small biomolecules and natural products. TPP increases the partitioning of the target enzyme by enhancing the mass transfer process which successively improves the purification fold and activity recovery (Vetal and Rathod, 2015). Table 3 shows the number of recent works in which the best conditions that supplied the maximum recovery and purity are also depicted. TPP was used successfully for the isolation of some enzymes and achieved very high enzyme recovery and purity levels (Rawdkuen *et al*., 2010; Vetal and Rathod, 2015; Duque Jaramillo *et al*., 2013; Barros *et al.*, 2014; Amid *et al.*, 2014). The recovery ranged from 100 to >300% and purity ranged from 1 to >10-fold in the partitioning of any enzymes by TPP. Ammonium sulfate salt at the range of 30 to 50% (w/w) saturation and crude extract: t-butanol in ratio of 1:1 (v/v) was normally used to gain the maximum recovery and purity of enzymes.

Several studies pay more attention in these following factors, for example, $(NH_4)_2SO_4$ saturation, crude extract to t-butanol ratio and pH. Apart of aforementioned factors, time in incubation, temperature, speed in agitation and number of cycle were also investigated to enhance purity and recovery. Generally, most research study started the investigation by concentration of ammonium sulfate or crude extract: t-butanol ratio in the first step. For example, the effect of salt concentrations (30, 40, 50, 60, 70 and 80%) (w/v) on the crude enzyme extract for the TPP at the constant crude extract: t-butanol ratio (1.0:1.0) was investigated first. After that, various t-butanol ratios (crude extract: t-butanol; 1.0: 0.5, 1.0: 0.75, 1.0: 1.0, 1.0: 1.25, 1.0: 1.5 and 1.0: 2.0) were employed with a constant ammonium sulfate saturation (Gagaoua *et al*., 2015). From Table 3, ammonium sulfate salt at the range of 30 to 50% (w/w) saturation was normally used to gain the maximum recovery and purity of enzymes. The best crude extract to t-butanol ratio seems to be 1:1 (v/v). After, the optimum t-butanol and ammonium sulfate was known, effects with different pH values of were tested. Crude extract was saturated with selected amount of ammonium sulfate and pH was adjusted form pH 3- pH 11, then selected ratio of crude extract: t-butanol was added and the best pH value on the partitioning behavior of enzymes was obtained. pH of the system depends on enzymes types based on their isoelctric point (pI). When the pH value scale of the system is above isoelectric point (pI) of protein, hydrophilic amino acid residues are charged negatively and the protein will acquire net negative charge and will be propelled to interphase or bottom aqueous media (Ketnawa *et al*., 2014b; Gagaoua *et al*., 2015). The enzyme was found to be exclusively partitioned in the aqueous phase. Most of enzyme as precipitant in the middle-phase as *Calotropis procera*, papain and giant catfish (Rawdkuen *et al.*, 2011; Chaiwut *et al.*, 2012; Rawdkuen *et al*., 2012) and some enzymes in the aqueous phase like zingibain and mangiferin (Gagaoua *et al*., 2015; Kulkarni and Rathod, 2015).

After optimization of the parameters, three repetitions were conducted to confirm the overall results using amount of ammonium sulfate, ratio crude extract to t-butanol and a pH based on the best results from previous steps. Furthermore, some groups focused more factors like time and temperature in incubation, speed in agitation, also and type of solvent. Vetal and Rathod (2015) studied partitioning of peroxidase from orange peel by TPP. This group studied the time in incubation for the first factor, because the reason that time is one of the key factors required for completion of any industrial reaction or process should be minimized to make it economically

feasible. They started by varying it from 20 to 120 min and keeping other experimental parameters constant. The study showed that purification fold and activity recovery of peroxidase was no significant increase after 80 min. Temperature is an important parameter that affects the enzyme configuration and overall stability. Many researchers have found higher purity at a temperature range of 20-30°C such as fish viscera alkaline protease at 25°C , Fibrinolytic enzyme at 30°C, galactosidase from pepino at 25°C (Şen *et al.*, 2011; Ketnawa *et al.*, 2014b; Avhad *et al*., 2014). At higher temperature (above 30°C), there may not be significant kosmotropic and crowding effects which resulted in decreased purity and activity recovery (Dennisson and Lovrein, 1997). So, around room temperature (25-30°C) can be applied with TPP due to considering economic and preventing enzyme from denaturation. Speed of agitation is one of the factors that have been paid attention. Speed of agitation is associated with the turbulence created in the batch reactor. It is clear that high turbulence means high speed which subsequently offers high mass transfer rate. Vetal and Rathod (2015) investigated to boost purity and recovery of fibrinolytic enzyme by varying the shaking speed from 100 to 500 rpm. The purity and recovery were the lowest at 100 rpm and lower than others around 1.5 times. However, it has been clearly observed that with an increase in the speed of agitation from 100 rpm to 300 rpm, there is an increase in purification fold and activity recovery but there is no significant improvement in the results after 300 rpm. Therefore, considering energy required for mixing and economy of the process, lower speed like 200 rpm enough to improve the purity and recovery. For effect of type of solvent, most researchers reported t-butanol was the most popular using in TPP. However, among the same group solvent, t-butanol is the most expensive. In the view of industry or mass production, the cheaper one can be selected if no significance different in purity or recovery. However, safety, toxicity and odour of that solvent are the limitation in case application of enzyme in food. Ketnawa *et al.* (2014c) revealed that t-butanol provided the purity (4.2-fold) and recovery (142%) of alkaline protease from giant catfish viscera. However, 2-butanol gave 4.5-fold purification with 116% recovery and 2-propanol gave higher purification (6-fold), the recovery activity was much lower (86%). Meanwhile, comparing to t-butanol and 1-butanol, 1-propanol provided the same proteases purity but lower recovery (95%). Eventhough, ethanol and methanol are cheaper than t-butanol, they are not normally used in TPP. They were not able to form a three phase so t-butanol is used as the solvent as it is capable of forming three phases (Kulkarni and Rathod, 2015). Besides, t-butanol can be practically used either at room temperature, or even at higher temperature (Ketnawa *et al.*, 2014c). The two-step TPP was done to improve the recovery and purity of enzymes (Rawdkuen *et al*., 2010; Navarapa *et al.*, 2011; Niphadkar *et al*., 2014; Pérez *et al*., 2015). Specifically, the recovery of 441% and PF of 14-fold of trypsin inhibitors were gained from TPP consisting of 30%ammonium sulfate and a ratio of 1:1 crude extract to t-butanol (95). This ratio was observed by many researchers to provide the best enzyme recovery and purity (Çalci *et al*., 2004; Özer *et al*., 2010; Şen *et al*., 2011; Harde *et al.,* 2012; Ketnawa *et al.*, 2014c; Avhad *et al*., 2014; Vetal and Rathod, 2015; Gagaoua *et al*., 2015; Chaiwut *et al*., 2010; Rawdkuen *et al*., 2012; Kulkarni and Rathod, 2015).

Some studies used assisted method to augment extraction efficiency, such as microwave and ultrasonication, especially shorten tome of separation and increase the yield Harde and Singhal (2012) reported that the yield of forskolin from *Coleus forskohlii* roots by TPP using ultrasound and enzyme treatment. The enzymatic treatment followed by ultrasonication and TPP gave 79.95% and 83.85% recovery in 4 h. While the time required for Soxhlet extraction was 12 h. Avhad *et al.* (2014) studied about ultrasound assisted three phase partitioning (UATPP) to enhance recovery of a fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672. They studied by varying different process parameters such as ammonium sulfate saturation concentration, pH, broth to t-butanol ratio, temperature, ultrasound frequency, ultrasonication power and duty cycle. The optimized parameters yielding maximum purity of 16.15-fold with 65% recovery comprised of 80% ammonium sulfate saturation, pH 9, temperature 30°C, broth to t-butanol ratio 0.5 (v/v), at 25 kHz frequency and 150 W ultrasonication power with 40% duty cycle for 5 min irradiation time. The similar study using microwave irradiation assisted TPP to improve extraction of mangiferin form leaves of Mango *(Mangifera indica)* was studied by Kulkarni and Rathod (2015). The mixture of mango leave powder, water, ammonium sulphate and t-butanol was further exposed to microwave for a desired time in a microwave for the formation of three layers. The results showed high mangiferin recovery of 54 mg/g by ammonium sulphate concentration 40% w/v, leaves powder to water ratio 1:20, slurry to t-butanol ratio 1:1, microwave power 272 W at irradiation time 5 min and duty cycle 50% (one minute off and one minute on the machine), while convention Soxhlet

extraction provided 57 mg/g yielded in 5 h (Kulkarni and Rathod, 2015).

Hence, TPP is one of the best partitioning methods that provide dramatically high recovery and purity of biomolecules. Using this method also reduces cost because it requires cheaper organic solvents instead of expensive polymers. Alternative novel method such as ultrasound or microwave irradiation assisted TPP can enhance recovery of interested compounds and consume considerably less time than conventional methods.

Conclusion

The phase partitioning techniques ATPS, TMP and TPP are vastly superior to the conventional processes for enzymes separation. They have higher capacity and yield higher recovery. The processing time is shorter, the methods are simpler and they are more biocompatible. Moreover, these techniques are more economical making higher scale production more feasible and environmentally friendly. The possibility up-scaled operation drives these three alternative means to be distinctive techniques to purify enzymes instead of other conventional separation methods. Parameters including molecular weight and concentration of polymers, type and concentration of salts, pH, NaCl addition and number of cycles could enhance or alter the recovery and purity of enzymes. From this extensive review, it is strongly suggested that all technique is very attractive and has clear potential to provide very high recovery and purity and has highly desirable effectiveness and efficiency for enzyme purification.

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