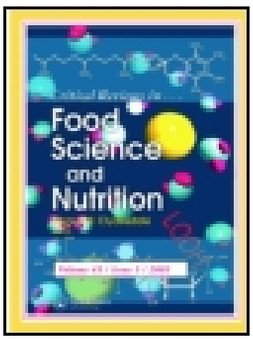

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Three-phase partitioning as an elegant and versatile platform applied to non-chromatographic bioseparation processes

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Abstract

In recent years, a big trend has been the development of rapid, green, efficient, economical, and scalable approaches for the separation and purification of bioactive molecules from natural sources, which can be used in food, cosmetics, and medicine. As a new non-chromatographic bioseparation technology, three-phase partitioning (TPP) is attracting the attention of a growing number of scientists and engineers. Although a number of studies have been published in the last 40 years regarding the extraction, separation, and purification of numerous bioactive molecules

using TPP systems, a background review on TPP partitioning fundamentals and its applications is much needed. Therefore, the present review focuses in detail on the TPP separation process, including the definition of TPP, partitioning mechanisms, parameters for establishing the suitable condition to form precipitate such as concentration of ammonium sulfate, content of tert-butanol, pH and temperature, and the application for separation and purification of protein, enzyme, plant oil, polysaccharide and other small molecule organic compounds. In addition, the possible directions of future developments in TPP technology are discussed. The review presents a good opportunity, as well as a challenge for scientists, to understand the detailed partitioning rule and to take better use of TPP for the production and separation of various bioactive molecules, which have been intensively applied in the food and medical fields.

Keywords

Three-phase partitioning; Bioseparation; Purification; Protein; Enzyme

INTRODUCTION

Currently, there is a pronounced tendency in the food industry toward the development and manufacture of functional products (Biesalski et al., 2009). This new class of food products has been a large success in current markets owing to growing customer interest for healthy foods (Gil-Chávez et al., 2013). Thus, pharmaceutical and food domains share a similar interest to obtain and characterize new bioactive compounds from natural sources, which can be used as drugs, functional food ingredients, or nutraceuticals. To date, a number of methods have been developed for the isolation and production of bioactive compounds from different natural matrices that are involved in various extraction techniques (Gil-Chávez et al., 2013; Grilo, Aires-Barros & Azevedo, 2016), including soxhlet or solvent extraction (SE), pressurized liquid extraction (PLE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), enzyme-assisted extraction (EAE), and aqueous two-phase systems (ATPS). Moreover, numerous advanced purification techniques, including ultrafiltration (UF), nanofiltration (NF), ion exchange chromatography (IEC), gel filtration chromatography (GFC), and affinity chromatography (AC) have been subsequently applied to further purify the desired molecules (Akin, Temelli & Köseoğlu, 2012; Bucar, Wube & Schmid, 2013). However, these reported techniques are usually expensive and time consuming, requiring enormous amounts of organic solvents and the exposure of the extract to the risk of

degradation or modification of some of the constituents with multistep protocols. In addition, many current techniques require equipment with high investment and maintenance costs. In light of these aspects, there is a growing need in the research field for a fast, green, efficient, versatile, and inexpensive technique for the recovery and purification of large quantities of bioactive molecules.

In 1972, Tan and Lovrein, for the first time, proposed the use of three-phase partitioning (TPP) as a promising alternative strategy to traditional extraction and separation techniques, which commonly required large amounts of volatile organic solvents, energy, and time. In TPP process firstly an inorganic salt (generally ammonium sulfate) is added to the crude extract containing proteins then mixted with *tert*-butanol in an appropriate amount. When *t*-butanol (co-solvent) is added in the presence of ammonium sulfate, it pushes the protein out of the solution. In this process *t*-butanol binds to hydrophobic part of the proteins to reduce the density of the proteins and leading to float above the denser aqueous salt phase. Within an hour, it forms an interfacial precipitate between the lower aqueous and upper organic phase that usually contains *t*-butanol (Dennison & Lovrein, 1997; Pike & Dennison, 1989).. Since then, TPP has been considerably exploited for the efficient recovery and purification of a wide variety of bioactive molecules, including proteins (Dennison & Lovrein, 1997; Pike & Dennison, 1989; Szamos & Kiss, 1995), enzymes (Dennison & Lovrien, 1997; Gagaoua & Hafid, 2016; Pol, Deutsch & Visser, 1990; Rachana & Jose, 2014; Sharma, Sharma & Gupta, 2000), enzyme inhibitors (Roy & Gupta, 2002;

Saxena, Iyer & Ananthanarayan, 2007; Wang, Chen, Jeng & Sung, 2011; Wati et al., 2009), edible oils or lipids (Sharma et al., 2002; Vidhate & Singhal, 2013), Carbohydrates (Coimbra, Lopes & Calazans, 2010; Mondal, Sharma & Gupta, 2004; Tan et al., 2015), and small-molecule organic compounds (Rao & Rathod, 2015; Vetal, Shirpurkar & Rathod, 2015), from various natural sources such as plants, animals, and microorganisms, as well as fermentation broths. In this regard, the TPP process is easily scalable and has been used for both upstream and downstream biomolecule purification processes and has simultaneously been used as a single-step purification protocol. To date, there are more than 100 research studies that have been published on the use of TPP separation technology for the extraction and separation of a variety of bioactive molecules. Among them, approximately 70% have focused on the recovery and purification of proteins and enzymes, while the other 30% have focused on the recovery and purification of oils, lipids, small-molecule organic compounds, and carbohydrates (Fig. 1). Although TPP has been extensively employed by many researchers for the extraction and purification of a wide range of bioactive molecules, a comprehensive review of the literature on the use of TPP and its potential applications and future developments is lacking.

This review attempts to summarize the current state of knowledge on the development of TPP as a simple, green, and efficient bioseparation technique with a focus on the individual aspects of the TPP separation process, including partitioning mechanisms and factors that may influence the TPP process, as well as their potential applications to green separation processes for different

bioactive molecules. Future trends and challenges are also discussed in order to provide in the near future an innovative TPP bioseparation technology for the improved recovery and purification of specific bioactive molecules for use as nutraceuticals or food ingredients in the design of functional foods.

THE TPP PROCESS AND PARTITIONING MECHANISMS

As an alternative strategy, TPP is a simple, efficient, and green bioseparation technology to separate and enrich various proteins in comparison to conventional separation techniques. This method, which is also used to isolate enzymes, involves the sequential mixing of an organic solvent (primarily *t*-butanol) with a sufficient amount of aqueous antichaotropic salt {typically ammonium sulfate [$(NH_4)_2SO_4$]} in the crude extracts or suspensions. After agitation and centrifugation, the mixture separates into three different phases, namely, an upper *t*-butanol phase, a lower aqueous phase, and a protein-enriched intermediate phase (Fig. 2) (Dennison & Lovrien, 1997; Roy & Gupta, 2002). Pigments, lipids, enzyme inhibitors, and other non-polar compounds contained in the crude extracts are usually enriched in the upper *t*-butanol phase, while some polar compounds such as saccharides are concentrated in the lower aqueous phase (Dennison & Lovrein, 1997; Özer et al., 2010). The third phase is formed through a protein-enriched intermediate phase (in the form of a precipitate) and, generally, an increase in enzymatic activity in this intermediate phase can be attained (Dennison & Lovrein, 1997). In addition to enhanced concentration, purification with TPP has been found to be comparable to chromatographic

techniques (Saxena, Iyer & Ananthanarayan, 2007). Although the cost is only slightly higher than the simple "salting out" approach, the fold purification is generally found to be much higher (Lovrein et al., 1987). Consequently, TPP can be interpreted as a special combination of "salting out" and alcoholic precipitation approaches, with several advantages. TPP usually can be performed at room temperature in less than one hour, and the chemicals used in the process can be recycled. In addition, the process does not use polymers that have to be removed later. Moreover, the method is easily scalable and can be used directly with large volumes of crude extracts or suspensions. Indeed, TPP was first developed as an upstream technique for the liter scale precipitation of crude cellulases and other enzymes (Odegaard, Anderson & Lovrein, 1984). However, to date, TPP remains primarily useful downstream for isolation and purification on a semi-micro, milliliter volume scale.

Although the exact mechanism of TPP is not well reported or defined in the literature, it is generally believed that the separation process involves a combination of different operating principles including kosmotropy, "salting out," co-solvent precipitation, isoionic precipitation, osmolytic electrostatic forces, conformation tightening, and protein hydration shifts, which all contribute to protein precipitation at the interface during the TPP process (Dennison & Lovrein, 1997; Lovrein et al., 1987). Proteins exhibit various behaviors under these conditions, primarily depending upon their source, hydrophobicity, molecular weight, charge, isoelectric point (*pI*), and temperature (Dennison, 2011; Pike & Dennison, 1989). At the same time, because ammonium

sulfate and *t*-butanol are widely used in TPP, the dehydration properties of ammonium sulfate salt and the protein buoyancy enhancement properties of *t*-butanol are also believed to account for protein concentration in the middle phase (Paule et al, 2004). Moreover, according to a recent report (Dennison & Lovrein, 1997), TPP-precipitated proteins are actually protein-*t*-butanol coprecipitates whose buoyancies are increased by increasing *t*-butanol.

Based on previous reports (Dennison & Lovrein, 1997; Rachana & Lyju, 2014), the basis of the TPP process to enrich proteins is mainly attributed to the abilities of the organic solvent *t*-butanol and sulfate anions to push on the left and pull from the right in two principal reactions. It is generally believed that pushing and pulling is meant in both the thermodynamic sense and in a measurable physical sense. Pushing and pulling are both related to macromolecule hydrate water. Water molecules are compelled to move out of the macromolecule domain for the sake of squeezing a macromolecule shut, thus forcing conformation tightening. How different agents push or pull depends on how much, and even whether, such agents interact or bind directly with the macromolecule. Pushing via a large concentration of inorganic salts such as ammonium sulfate is usually regarded as a thermodynamic consequence of an exclusion crowding mechanism (Jacobsen, Wills & Winzor, 1996; Timasheff, 1992). Based on the results of viscosity changes and related physical methods, pushing generates conformation tightening together with protein hydration changes. Meanwhile, pulling also often leads to conformation tightening of protein molecules, whereas different reasons are involved and much lower concentrations of sulfate

anions are needed to push. In pulling, sulfate anions can bind to the cation groups of protein molecules by electrostatic interactions, which draws protein conformation inward and further decreases protein net hydration. In this case, protein molecules will become sulfate salts. The bound sulfate salt form is usually believed to be more stable. Nonetheless, while lacking salt, protein molecules are relatively fragile and easily denatured. Several physicochemical approaches and spectroscopic means are used to investigate the conformational changes fostered by sulfate anions, and related agents, when proteins are still in solution (Fink, 1995; Matulis & Lovrein, 1996). Pushing means raising the chemical potential of the protein in solution at the left of reaction 1, in a positive direction. Pulling means lowering the chemical potential of the product of reactions 1 and 2, on the right, in a negative direction. It is likely that some versions of TPP are hybrids; they both push and pull. Analogous to many conventional chemical reactions that can be pushed, pulled, or both (LeChatelier's principle), the forward driving reactions 1 and 2 produce a net negative change in chemical potentials in the overall conversion, with Protein (aqueous solution) $\Leftrightarrow$ Protein (TPP precipitate or coprecipitate), and, hence, a net negative Gibbs free energy change. Other detailed explanations on the partitioning mechanisms of the TPP process are likewise found in the reported study (Dennison & Lovrein, 1997).

There is a reciprocal relationship between the amounts of ammonium sulfate and *t*-butanol required to precipitate proteins during TPP separation processes (Pike & Dennison, 1989). The interrelated effects of ammonium sulfate and *t*-butanol are investigated in a quantitative way using

the phase behavior of a three-component (protein-free) system (Kiss et al., 1998). The obtained phase diagram (in weight ratio) of the water/ammonium sulfate/*t*-butanol three-component system at 25°C (Fig. 3) represents the one liquid-phase area at the water corner and the composition area with tie-lines, where the two immiscible liquid phases are formed by ammonium sulfate and *t*-butanol. Above that, the upper part represents excess ammonium sulfate present in solid form. Phase separation can be induced by increasing the amounts of ammonium sulfate and *t*-butanol. The area within the triangle (with the tie-lines) corresponds to the composition range that could be used for the partitioning experiment. The compositions of the upper and lower liquid phases (the end points of the tie-lines) indicate that both phases contain all three components, albeit with different ratios. The longer the tie-line, the greater the difference between the compositions of the equilibrium liquid phases. Based on this, partitioning systems with different initial compositions can be compared by investigating the behavior of single model proteins, including bovine serum albumin (BSA), ovalbumin (OVA), lysozyme (LYS), and gelatin (GE). From these comparisons, the dependence of precipitation on protein concentration casts light on the character of the process.

FACTORS INFLUENCING THE TPP PROCESS

Ammonium sulfate

Ammonium sulfate, $(NH_4)_2SO_4$, is the most popular inorganic salt used for protein "salting out" due to its high solubility (about 3.6 M) and ionic strength [e.g., 1 M $(NH_4)_2SO_4$ has three

times the ionic strength of 1 M NaCl]. Apart from that, NH_4^+ and SO_4^{2-} are usually at the ends of their respective Hofmeister series and have been shown to stabilize protein structure. Ammonium sulfate concentration or saturation plays a critical role in the TPP system because it is responsible for protein-protein interactions and precipitation. In this regard, it must be optimized to obtain the maximum recovery or yield of the desired proteins or enzymes. The efficiency of "salting out" first relies on $(NH_4)_2SO_4$ and, second, on net charge. In general, researchers start at a minimum salt concentration of 20% (w/v) to optimize the partitioning parameters of the TPP process (Dennison & Lovrein, 1997). As the $(NH_4)_2SO_4$ concentration is increased, the dissipation of water molecules from the salvation layer around the protein molecules is promoted. As a result, hydrophobic patches of the protein surface are exposed, which then interact with hydrophobic patches on other protein surfaces. At higher salt precipitation, water molecules are adsorbed by salt ions, which causes stronger protein-protein interactions and leads the protein molecules to precipitate via hydrophobic interactions (Choonia & Lele, 2013; Gagaoua et al., 2014). Generally, the mass fraction of ammonium sulfate in TPP varied from 20% (w/v) to 60% (w/v). Pike and Dennison (1989) and Lovrein et al. (1987) have reported that 30% (w/v) is the best salt concentration for carrying out TPP efficiently.

As a traditional kosmotropic salt, $(NH_4)_2SO_4$ is responsible for the "salting out" of proteins from aqueous solutions. There are five different principles for "salting out" proteins with SO_4^{2-} ions: (1) ionic strength effects; (2) cavity surface tension enhancement osmotic stressors

(dehydration); (3) kosmotropy; (4) exclusion crowding agents; and (5) the binding of SO_4^{2-} to cationic protein sites (Dennison, 2011). These five possible factors, and the potential interactions between them, operate in different proportions primarily depending on sulfate concentration and protein molecule charge (denoted as Z_H^+) in response to pH. In addition, there is a sixth factor that has slowly emerged, but has equal weight now. Namely, the divalent sulfate anion SO_4^{2-} tends to bind into a few cationic sites of many proteins when these proteins have a net positive charge Z_H^+. Consequently, this behavior promotes macromolecular contraction and conformational shrinkage, which is the main underlying cause of the strong pH dependency of the above-mentioned five principles or mechanisms of sulfate "salting out." (Dennison & Loverian, 1997)

tert-butanol

Solvent *t*-butanol is almost always used in the TPP process as it has a higher boiling point (84°C) and is much less flammable compared to *n*-hexane, acetone, methanol, or ethanol, all of which are used in conventional SE (Vetal, Shirpurkar & Rathod, 2014). In addition, a number of organic solvents, including *n*-propanol, isopropanol, *n*-butanol, *n*-pentanol, isoamyl alcohol (isopentanol), and *n*-hexane, have been studied as differentiating co-solvents in the TPP process (Dennison & Loverian, 1997). However, compared with *t*-butanol, these organic solvents displayed higher deactivation and lower interfacial precipitation via hydrophobic interactions with proteins (Dennison & Loverian, 1997; Lovrein et al., 1987). Meanwhile, other co-solvents such as methanol and ethanol are not kosmotropic or considered crowding agents except at temperatures

near or below zero. It is generally accepted that, due to its size and branched structure, *t*-butanol does not easily permeate inside folded protein molecules and, thus, does not lead to denaturation (Dennison et al., 2000). Additionally, at 20-30°C, *t*-butanol displays significant kosmotropic and crowding effects and increases TPP (Dennison et al., 2011). At lower concentrations of *t*-butanol, it may not adequately synergize with ammonium sulfate (Sharma & Gupta, 2001a); however, if the amount of *t*-butanol is high, it is more likely to cause protein denaturation and hinder protein precipitation (Chaiwut et al., 2010). In addition, it is noteworthy that increased amounts of *t*-butanol do not increase the yield of the target product during the TPP process because as the amount of *t*-butanol increases, the viscosity of the solution also increases. Indeed, as the viscosity increases, molecular mobility decreases, making it relatively difficult for the molecules to interact. Hence, an appropriate amount of *t*-butanol should be chosen to set up an optimal TPP separation process. Generally, *t*-butanol was added to the crude extract at the volume ratios of crude to *t*-butanol of 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0 and 1.0:2.5 (v/v) with a constant ammonium sulfate concentration of 30% (w/v). In fact, the appropriate amount of *t*-butanol could be selected in various TPP systems according to the practical requirements.

pH

During the TPP separation process, pH has a positive influence on the efficiency of protein enrichment and purification (Dennison & Lovrein, 1997). This influence is usually associated with resultant changes in amino acid residues at the surface of proteins due to pH changes. In addition,

electrostatic interactions between charged proteins and phases also affect partitioning behavior to some extent. In general, partitioning of the target protein to the interface, or the aqueous phase in the TPP system, mainly relies on its pI. Moreover, there are four or five different pH values ranging between 3.0 and 7.0 that could be suitable for screening the distribution of proteins in TPP systems (Çalci et al., 2009; Dennison & Lovrein, 1997; Özer et al., 2010). When pH values are above the pI, surface-exposed amino acid residues on the protein surface carry a net negative charge and make the protein negatively charged. Consequently, the protein will be left in the aqueous phase. On the contrary, the protein will be precipitated and enriched in the middle phase if the pH of the TPP process is below its pI (Dennison & Lovrein, 1997; Wang et al., 2011). Therefore, in light of the above reasons, it is necessary to investigate the effects of pH on the TPP process and to find the optimal pH condition for the partitioning of target products, particularly proteins and enzymes.

Temperature

Because TPP is carried out by multiple effects that involve conformational tightening and changes in protein hydration, the effect of varying the process temperature has been evaluated. The majority of concentration processes are often operated at low temperatures and the use of low temperatures in solvent or salt precipitation can be beneficial for dissipating the heat produced, thus, ensuring minimal protein denaturation (Dogan & Tari, 2008; Garg & Thorat, 2014). Although the requirement of a low temperature in TPP separation processes has not been reported

in the literature, temperature appears to have an important influence on the performance of TPP processes consisting of ammonium sulfate and *t*-butanol. In this regard, temperature can affect the rate of mass transfer, and most of the TPPs were usually carried out around normal temperatures. Many studies have demonstrated that a temperature range of 20-40°C is optimal using the TPP approach; however, this is difficult to confirm in light of the complexity of the process parameters involved (Garg & Thorat, 2014; Sharma & Gupta, 2001a). Moreover, as temperature increases above 40°C, the volatility of *t*-butanol slowly increases and a lesser volume of the solvent will be available for extraction. The availability of lesser amounts of *t*-butanol results in a reduced synergistic effect with ammonium sulfate, thus producing a lower extraction yield. Additionally, a higher temperature during the TPP process will lead to the greater consumption of energy.

ASSISTED OR MODIFIED TPP SYSTEM

Ultrasound- or microwave-assisted TPP

Ultrasound is process intensification tool that has been widely applied in extraction, fermentation, adsorption, aqueous two-phase extraction, bioremediation, and biocatalysis processes as it enhances mass transfer and yield (Gogate & Kabadi, 2009; Santos, Lodeiro & Capelo-Martienz, 2009). TPP is also a mass transfer-dependent phenomenon. Thus, the applicability of ultrasound on TPP processes has been evaluated in order to overcome the limitations of mass transfer in TPP due to the cavitational phenomenon. To date, TPP combined

with ultrasound is often used for the extraction of bioactive molecules, such as mangiferin (Kulkarni & Rathod, 2014), ursolic acid and oleanolic acid (Vetal, Shirpurkar & Rathod, 2014), astaxanthin and fibrinolytic enzyme (Avhad, Niphadkar & Rathod, 2014), and serratiopeptidase (Pakhale & Bhagwat, 2016), with promising results compared to conventional TPP.

Unlike conventional extraction methods, MAE requires less solvent, time, and energy. As microwave irradiation time increases, microwaves cause the polar molecules to rotate at the same frequency for a longer time, creating molecular friction. This friction releases heat, which further raises the temperature. Microwave-assisted TPP has been occasionally applied for the effective extraction of lipase (Saifuddin & Raziah, 2008) and mangiferin from the leaves of *Mangifera indica* (Kulkarni & Rathod, 2015).

In addition, the effect of various pretreatments, such as enzymes and ultrasound, have also been explored to further improve the TPP process (Gaur et al., 2007; Kurmudle et al., 2011; Shah, Sharma & Gupta, 2004; Sharma & Gupta, 2004; Tan et al., 2016; Varakumar, Umesh & Singhal, 2017).

Two-step TPP

It has been observed that, under certain circumstances, upon subjecting crude protein solutions to TPP, three phases are formed; however, the desired protein remains predominantly in the aqueous layer. In this case, it was expected that the target proteins or enzymes would display

greater partitioning to the interphase with maximum yield and purity. Therefore, in such cases, the three layers are separated and the lower aqueous layer is further subjected to a second TPP by adding more $(NH_4)_2SO_4$ and *t*-butanol. Three phases are formed again and most of the desired protein is found to be partitioned in the interfacial precipitate (Fig. 4) (Raghava et al., 2008). As shown in Fig. 4, TPP was carried out twice. In the first step, the solubilized inclusion body was saturated with 5% (w/v) ammonium sulfate and vortexed gently, and then the *t*-butanol was added in 1:1 (v/v) ratio of solubilized sample to *t*-butanol. After incubation for 1 h at 25°C, the mixture was centrifuged and the three phases were formed. With solubilized inclusion bodies, the first step was useful in removing host cell proteins as the interfacial precipitate. The lower aqueous was subjected to a second round of TPP. The aqueous phase was saturated with 35% (w/v) ammonium sulfate followed by addition of *t*-butanol (in 1:1 v/v ratio of aqueous phase to *t*-butanol). Again three phases were formed and collected. The interfacial precipitate was dissolved and dialyzed for activity measurements. Compared with TPP-one step, the overall fold purity of the separated proteins by two-step TPP is improved about 2~4 times (Jain, Singh & Gupta, 2004; Rajeeva & Lele, 2011; Saxena et al., 2007). For example, Saxena et al. (2007) reported that the aqueous phase contained the majority of the desired protein, and when subjected to a two-step TPP, this process results in considerable purification of the target protein. Meanwhile, such two-step TPP processes have also been utilized to recover and purify enzymes with substantial yields compared to traditional TPP techniques. These include laccase from submerged cultures of *Ganoderma* sp.

WR-1 (Rajeeva & Lele, 2011), protease from *Calotropis procera* latex (Rawdkuen et al., 2010), pectinases (Sharma & Gupta, 2001b), phospholipase D from *Dacus carota* (Sharma & Gupta, 2001c), and alkaline phosphatase from chicken intestine (Sharma, Sharma & Gupta, 2000). It should be pointed out that only one-step TPP was sufficient when considering enzyme recovery. However, in terms of enzyme purity, two-step TPP appears necessary.

Macroaffinity ligand-facilitated three-phase partitioning

In recent years, Gupta's research group has developed an extension of the TPP system called macroaffinity ligand-facilitated three-phase partitioning (MLFTPP) for the separation and purification of enzymes (Sharma & Gupta, 2002a). Compared with simple TPP, MLFTPP displays much higher selectivity under the same or similar partitioning conditions, and it can easily be designed in a more predictable fashion by choosing an appropriate and smart polymer. In the MLFTPP system, smart polymers chosen for this application are reversible soluble-insoluble polymers that precipitate upon the application of an appropriate stimulus, which may involve a change in pH or temperature or the addition of a chemical species, depending on the individual smart polymer (Roy & Gupta, 2003). In general, both naturally occurring polymers (*e.g*., alginate, chitosan, and κ-carrageenan) and synthetic polymers (*e.g*., eudragit S-100, L-100) have been employed for the direct precipitation of proteins in MLFTPP. To date, MLFTPP has been utilized to directly extract and purify xylanase, pectinase, cellulase, α-amylases, glucoamylase, and pullulanse, as well as to refold recombinant proteins (Gautam et al., 2012; Mondal, Sharma &

Gupta, 2003a,b; Sharma & Gupta, 2002a; Sharma, Mondal & Gupta, 2003a; Sharma, Roy & Gupta, 2004). Hence, it may be worthwhile to note that the strategy of sequential MLFTPP as a programmable bioseparation technology can be used to separate important biological activities like proteins and enzymes directly from a crude broth for potential applications in food processing. Additionally, a metal-affinity based TPP has been developed as a highly selective technique to recover and purify proteins with surface histidine residues using a soybean trypsin inhibitor (Roy & Gupta, 2002).

Ionic liquid-based three-phase partitioning

Although TPP is a quick, easy, and scalable way for the enrichment of proteins and enzymes that can be used directly with crude suspensions, the main drawback of TPP is the use of conventional organic solvents (*t*-butanol) with a flash point and a volatility similar to that of ethanol which may limit the large-scale use of this technique (Przybycien et al., 2004). As a result, a novel technique called (ILTPP), which combines the advantages of ionic liquid-based aqueous two-phase systems (ILATPS) and TPP, has been developed in recent years to concentrate and recover lactoferrin and BSA (Alvarez-Guerra & Irabien, 2014, 2015; Alvarez-Guerra et al., 2014a, b). Regarding ILATPS, they are usually formed by a hydrophilic ionic liquid and a salt, which are mutually incompatible though both miscible in water so that two aqueous phases are obtained (an ionic liquid-rich phase and a salt-rich phase) (Freire et al., 2012). Thus, an appropriate hydrophilic ionic liquid should be selected in ILTPP. Consequently, most of the target protein should be

accumulated at the liquid-liquid interface between the aqueous ionic liquid-rich phase and the aqueous salt-rich phase. At present, several feasible ternary systems, including 1-butyl-3-methylimidazolium tetrafluoroborate ($BmimBF_4$)/NaH_2PO_4/H_2O and 1-butyl-3-methylimidazolium trifluoromethanesulfonate (BmimTfO)/(NaH_2PO_4/Na_2HPO_4)/ H_2O, for the development of ILTPP have been selected and studied. Even though ILTPP is regarded as a promising alternative to the traditional TPP method, the recyclability of the ionic liquid used in the process must also be studied to minimize the economic and environmental influences associated with these compounds. For this purpose, Alvarez-Guerra et al. (2014a) has studied two alternatives to enhance ionic liquid recyclability. These include: (a) the addition of salt to increase the concentration of the ionic liquid in the ionic liquid-rich phase; and (b) the concentration of the salt-rich phase by vacuum evaporation. Furthermore, the characterization of the thermodynamic equilibrium of the principal components of ILTPP (ionic liquid, salt, and water) has also been evaluated by Alvarez-Guerra et al. (2014b) results obtained from thermodynamic data and mass balances demonstrated that ILTPP could potentially reuse more than 99% of the ionic liquid involved in the process.

Others

Most of the previous work on the TPP process was customarily pointed toward the use of *t*-butanol-$(NH_4)_2SO_4$-water as the necessary components of the process. Nonetheless, Torres et al. (2015) recently developed a pseudo-heterogeneous mathematical modeling method of

bioconversion in a three-phase (air-water-ionic liquid) partitioning bioreactor (TPPB) for estimating and describing the mass transfer mechanism. The proposed model promised to be a useful platform for understanding and evaluating the design and optimization of bioconversions in a TPPB during further scale-up or scale-down operations.

Recently, a new strategy was suggested by Sui et al. (2014) to remove Fe, Al, and Si impurities from rare-earth leach solutions by controlling the partitioning and selective enrichment of those impurities and rare earths in established three-liquid-phase systems (TLPS) such as Bis(2,4,4-trimethylpentyl) phosphinic acid (Cyanex 272)/PEG 2000/$(NH_4)_2SO_4$-H_2O and Cyanex 272/PEG 600/$(NH_4)_2SO_4$-H_2O. Results showed that TLPS could be a potential separation media for the efficient removal of Fe, Al, and Si from complex rare-earth leach solutions and the group separation of different target metals into different liquid phases.

APPLICATIONS IN SEPARATION AND PURIFICATION OF BIOMOLECULES

Proteins

TPP was originally explored as an upstream technique for the isolation of proteins. In a successful TPP protocol, the desired protein is recovered in the concentrated form as the interfacial layer, while some contaminating material (e.g., pigments, oils, lipids) is recovered in the organic solvent phase. TPP has been proven to be a good strategy for protein purification in several cases, leading it to be regarded as a potential non-chromatographic approach for protein purification in

industrial enzymology. Pike and Dennison (1989) selected a group of standard proteins with known physico-chemical properties (molecular weight, pH, concentration, solubility), including BSA, cytochrome C, γ-globulin, hemoglobin, LYS, myoglobin, and OVA, to investigate and evaluate their behavior in the TPP process under different conditions. The results obtained indicate that the TPP method, with its best advantages, may subsequently be used in a more systematic manner for the fractionation and concentration of various proteins. So far, several specific target proteins have also been extracted and purified from different natural sources using the TPP system, including immunoreactive-secreted proteins from *Corynebacterium pseudotuberculosis* (Paule et al., 2004), recombinant green fluorescent protein (GFP) from *Escherichia coli* DH5α (Jain, Singh & Gupta, 2004), and the proteins (e.g., GFP, lipase, penicillin G acylase, alcohol dehydrogenase) released from permeabilized microbial cells such as *Thermus thermophilus*, *Saccharomyces cerevisiae*, and *E. coli* (Raghava & Gupta, 2008).

In order to gain insight into the detailed role of factors affecting the TPP process for the separation of proteins, Kiss et al. (1998) investigated the phase diagrams and behaviors of a ternary (protein-free) system and the interfacial tensions between the equilibrium upper and lower liquid phases formed in a system containing water, *t*-butanol, and $(NH_4)_2SO_4$. In this TPP system, the partitioning experiments of four model proteins (BSA, OVA, LYS, and GE) were determined. The amount of protein precipitated in the middle layer casted light on the character of the partitioning system as well as the initial protein concentration. Moreover, the interfacial adsorption of a single

protein showed a good correlation with the accumulation of the same protein in the TPP system as a middle layer for the various proteins investigated.

Previous findings have demonstrated that protein interfacial adsorption, and the rheological properties of such films, may have important influences on the separation process of proteins by emulsification in TPP. The amount of protein concentrated in the middle layer is dependent on the extracted total amount, which is characteristic of partitioning rather than precipitation (Kiss et al., 1998). At the same time, the partitioning of proteins usually occurs between the lower phase and the interfacial layer, with a large surface area in the case of an emulsion with small drop size. Moreover, the middle phase formed in the TPP system is believed to be a coherent layer showing bulk viscoelastic behavior. Hence, the interfacial viscoelasticity with a high value in the TPP process was widely believed to play a crucial role in the stability of dispersed systems stabilized by proteins (Pezennec et al., 2000). For instance, Borbás et al. (2001) studied the mechanical properties of the middle phase in TPP model systems using BSA and OVA. The results demonstrated that the middle phase became harder upon compression, and the BSA gels showed higher elastic and viscous deformation and lower viscosity than the OVA gels, thereby indicating that the middle phase formed in the TPP system is an emulsion gel. In addition, Borbás et al. (2003) further investigated the interfacial rheological properties of four various proteins (BSA, OVA, β-lactoglobulin, and LYS) in model systems of TPP. The achieved interfacial rheological results, and the microscopic observation of the middle layer, supported the crucial role of

emulsification and emulsion stability related to the viscoelastic character of the interfacial film in protein separation during TPP.

Although TPP has previously been used for protein concentration and purification (Dennison & Lovrein, 1997; Jain, Singh & Gupta, 2004), Raghava et al. (2008), for the first time, successfully applied the TPP method coupled with dialysis to refold and purify a variety of recombinant proteins from inclusion bodies (Fig. 4). Moreover, a strategy called MLFTPP has also been used to refold a diverse set of recombinant proteins from solubilized inclusion bodies in approximately 10 min (Fig. 5) (Gautam et al., 2012). In addition, Rather and Gupta (2013a) has reported that urea-denatured OVA in the TPP system under various conditions produced many refolded soluble conformational variants. Indeed, both ammonium sulfate and *t*-butanol have been reported to be useful additives during protein refolding. In particular, *t*-butanol acts as an "aggregation suppressor" (Wang & Engel, 2009). However, the results of current studies indicate that the structures of proteins subjected to TPP differ from the native structures of the proteins. In addition, conformation changes presumably occur in the proteins, but with little differences in the rate and extent of the partitioning process in spite of the rigorously applied conditions. For example, when pure proteinase K was subjected to TPP treatment and its structure analyzed by X-ray diffraction at 1.5 Å resolution, a higher overall temperature factor was observed. This was determined to be the result of which side chains of several amino acid residues in the binding site were found to adopt more than one conformation (Singh et al., 2001). In order to confirm the safety of the TPP

technique for protein purification and refolding, Rather and Gupta (2013b) further examined the structural changes in several proteins by comparing their biophysical parameters, such as thermal stabilities, secondary structure contents, surface hydrophobicities, hydrodynamic radii, and solubilities in the presence of ammonium sulfate. The obtained results revealed that, while the nature or extent of structural changes may vary, in all the cases the changes were rather subtle and not drastic in nature, indicating that the use of TPP for protein purification and refolding is an acceptable method.

Enzymes

Enzymes, or biocatalysts, are important biomolecules that act on substrates specifically and ultimately give rise to the transformation/conversion of substrates to desired products. The advantages of enzymes as biocatalysts over inorganic catalysts include their high specificity, high rate of reaction, non-toxicity, biodegradability, and reproducibility under mild conditions of temperature, pH, and pressure. To date, many techniques have been used for purifying enzymes from different plant, animal, and microbial sources, such as "salting out," precipitation, membrane filtration, dialysis, IEC, hydrophobic interaction, GFC and AC (Azmir et al., 2013; Kula et al., 1981, 1982). However, these techniques are usually multistep, expensive, time consuming, and difficult for scale enlargement.

TPP has been widely reported and successfully used in the highly efficient purification and recovery of more than 50 industrially important enzymes (Dennison & Lovrien, 1997; Gagaoua & Hafid, 2016; Pol, Deutsch & Visser, 1990; Rachana & Jose, 2014), as well as several enzyme inhibitors, including α-amylase inhibitor (αAI) (Wang, Chen, Jeng & Sung, 2011), bifunctional amylase/protease inhibitors (Saxena, Iyer & Ananthanarayan, 2007; Sharma & Gupta, 2001a), and trypsin inhibitor (Roy & Gupta, 2002; Wati et al., 2009) from crude suspensions or extracts. TPP process conditions may vary from enzyme to enzyme, thus optimization is desirable in each individual case. In many cases, TPP enhances the activity of various enzymes, resulting in an obvious higher yield in excess of 100% (Singh et al., 2001). As a result, the purified enzymes after the TPP procedure are generally characterized with respect to their biochemical properties such as catalytic activity, stability, and structure at different temperature and pH ranges using SDS-PAGE analysis, X-ray diffraction analysis, and determination of kinetic parameters (K_m and V_{max}). In addition to the enhanced concentration, purification with TPP has been found to be comparable to chromatographic techniques. Furthermore, TPP has been reported to recover different enzyme activities (*e.g.*, xylanase, cellulase, cellobiase, β-glucosidase, and α-chymotrypsin) from their inactivated/denatured forms (Roy, Sharma & Gupta, 2004, 2005; Sardar, Sharma & Gupta, 2007). These findings suggest that TPP may be a valuable technique for the simultaneous renaturation/purification of the multiple enzymes present in a protein mixture. Table 1 summarizes

some important industrial enzymes and their respective optimum partitioning parameters for recovery and purification by TPP.

Proteases

Proteases, or proteolytic enzymes, play an important role in food biotechnology (Walsh, 2002). In food industries, proteases are widely utilized in various productions, such as cheese-making, milk-clotting, and meat tenderizing. The bulk of these enzymes come from microbial sources, but vegetable proteases, extracted from higher plant organs, have been extensively investigated in recent years as potential proteolytic enzymes in the food industry. Considering their potential and actual uses in the food industry, the development of simple and effective methods for their recovery and purification are important. The TPP system is an attractive process that has been frequently used to purify numerous proteases. The purification and recovery of these proteases from crude extract suspensions during partitioning has been performed under various conditions. Hence, the effects of partitioning at different $(NH_4)_2SO_4$ concentrations, crude extract to *t*-butanol ratios, times, temperatures, and pHs on TPP (purification fold, activity recovery) have been investigated and evaluated. Recently, Gagaoua and Hafid (2016) gave a rapid overview on some important protease recovery and purification experiments with plant proteases using the TPP system, including papain from papaya peels (Chaiwuta, Pintathonga & Rawdkuen, 2010), zingibain from *Zingiber officinale* rhizomes (Gagaoua et al., 2015, 2016), ficin from *Ficus carica* latex (Gagaoua et al., 2014), and calotropain from *C. procera* latex (Rawdkuen et al., 2010).

Recently, the use of alkaline proteases from marine digestive organs, particularly trypsin, has significantly increased because they are both stable and active under harsh environments. Important proteases in the viscera of fish and aquatic invertebrates primarily include aspartic protease (pepsin) and serine proteases (*e.g.*, trypsin, chymotrypsin, collagenase, and elastase). These proteases, recovered from fish viscera, have been successfully used as seafood processing aids, such as the acceleration of fish sauce fermentation, the extraction of carotenoprotein, and the production of protein hydrolysates (Klomklao, Benjakul & Simpson, 2012). The TPP technique has also been used to separate and purify these proteases from fish viscera. For example, Rawdkuen et al. (2012) determined the best conditions for separating alkaline proteases from farmed giant catfish viscera, which consisted of a crude enzyme extract to *t*-butanol ratio of 1.0:0.5 in the presence of 50% (w/v) $(NH_4)_2SO_4$. Ketnawa et al. (2014) further optimized the partitioning parameters for the recovery of alkaline proteases from farmed giant catfish viscera using TPP. The optimized system consisted of a crude enzyme extract to *t*-butanol ratio of 1:0.5 (v/v), with 50% (w/v) $(NH_4)_2SO_4$, a pH of 8.0, and an incubation temperature of 25°C. This system gave the highest enzyme recovery (220%) and demonstrated a 6-fold purification.

Amylase

Amylases are other important industrial enzymes, and represent about 25-33% of the world enzyme market. They have been used worldwide in starch liquefaction to produce glucose, fructose, and maltose, and in brewing, baking, textile, paper, detergent, and sugar industries as

well (Nguyen et al., 2002; Noman et al., 2006). Although amylases are frequently applied in the food and chemical industries, the production of these enzymes on a large scale is restricted to certain microorganisms, underlying the necessity to find alternative sources (Amid & Abd Manap, 2014). In recent years, TPP has been successfully used in single procedures for the concentration and purification of several amylases, including α-amylase from *Bacillus amyloliquifaciens* (MTCC-610) (Mondal, Sharma & Gupta, 2003a), β-amylase from stems of *Abrus precatorius* (Sagu et al., 2015), and glucoamylase from *Aspergillus niger* (Mondal, Sharma & Gupta, 2003b).

α-Galactosidase

α-Galactosidase (α-D-galactoside galacto-hydrolase, EC 3.2.1.22) is widely distributed in nature and found in plants, mammals, and microorganisms, where it mainly catalyzes the hydrolysis of terminal α-1,6-linked galactose residues found in galacto-oligosaccharides or sugars of the raffinose family and galactomannan polysaccharides (Gote et al., 2006; Guimarães et al., 2001; Pessela et al., 2008). They have been reported worldwide to be potentially applied in the fields of biotechnology and medicine, such as food processing, animal feed processing, the sugar producing industry, the pulp and paper industry, enzymatic synthesis, structural analysis, conversion of blood type, and treatment of Fabry's disease (Anisha et al., 2008; Clarke et al., 2000; Guimarães et al., 2001; Pessela et al., 2008; Schiffmann, 2009). To date, there are several studies available in the literature regarding the purification and recovery of α-galactosidases from different sources using the TPP system, such as *Aspergillus oryzae* (Dhananjay & Mulimani, 2008, 2009), tomato

(*Lycopersicon esculentum*) (Çalci et al., 2009), pepino (*Solanum muricatum*) (Şen et al., 2011), *Rhizopus sp*. A01 (Wang et al., 2012), and watermelon (*Citrullus vulgaris*) (Bayraktar & Önal, 2013). In addition, Duman and Kaya (2013a) has purified and recovered β-galactosidase (EC 3.2.1.23) from chick pea (*Cicer arietinum*) in a single step using TPP. The best combination for providing the highest β-galactosidase recovery (10.1-fold purification and 133% recovery) under optimal purification conditions, TPP also improved the substrate affinity of the purified enzyme by enhancing its flexibility during the partitioning process based on the results of SDS-PAGE analysis and kinetic properties. At the same time, *Lactobacillus acidophilus* β-galactosidase, with 78% recovery and 7.5-fold purity, was also purified through TPP in single- and two- step procedures (Choonia & Lele, 2013).

Oils, fats, and lipids

SE with *n*-hexane is a conventional approach that has been widely used to extract edible oils (or fats) from plant materials (Mattil et al., 1964). However, *n*-hexane is flammable and non-biorenewable, and emitted from oilseed extraction is a source of volatile organic compounds. In addition, *n*-hexane has been commonly regarded as an air pollutant because it can react with other pollutants to produce ozone and photochemical oxidants (Gandhi et al., 2003). *t*-Butanol has a higher boiling point (84°C) than *n*-hexane (69°C). *t*-butanol also has a freezing point of 11°C, which is comparable to *n*-hexane (-95°C). In addition, it is more economical and facilitates separation by chilling rather than heating (Sharma et al., 2002). Therefore, TPP with *t*-butanol

promises to be a good feasible and eco-friendly strategy for the extraction and separation of oils, fats, and lipids. Sharma et al. (2002), for the first time, reported that TPP was used for the extraction of oil from soybean, and 82% oil was extracted with the simultaneous addition of *t*-butanol and 30% ammonium sulfate (1:1, v/v) to the soybean slurry at 25°C for 1 h. In general, oils in oilseeds were present in lipid bodies that are enmeshed in a protein network and, thus, pretreatment with proteases before extraction has contributed to considerable enhancement in oil recovery (Rosenthal et al., 1996). Gaur et al. (2007) has demonstrated that pretreatment with proteases (ProtizymeTM), followed by the TPP procedure, was used for the extraction of oils from mango kernel, soybean, and rice bran, with maximum oil yields of 98%, 86%, and 79% (w/w), respectively, under optimized conditions. This method was preferable to conventional *n*-hexane extraction, with the additional merit of its operation at room temperature, along with the ease in reusing the solvent phases. Subsequently, Vidhate and Singhal (2013) reported a maximum recovery of 95% (w/w) fat obtained from *kokum* (*Garcinia indica*) kernels with an optimized TPP system consisting of a 50% (w/v) salt concentration, 1:1 ratio of slurry to *t*-butanol, and a pH of 2.0 within 2 h. Recently, Tan et al. (2016) extracted oil from Flaxseed (*Linum usitatissimum* L.) using the enzyme-assisted TPP (EATPP) method. The optimized enzymolysis conditions were as follows: a mixed enzyme concentration of 600 U/g, pH of 5.0, and temperature of 40°C. The maximum extraction yield of 71.68% was obtained under optimized conditions of a 49.29% *t*-butanol concentration, 30.43% $(NH_4)_2SO_4$ concentration, and a 35°C extraction temperature.

Additionally, TPP or TPP coupled with assisted methods (e.g., ultrasound, enzyme, high-pressure homogenization) is also used for the extraction of oils and lipids from various plants such as almond, apricot, and rice bran (Sharma & Gupta, 2004), *Jatropha curcas* L. seed kernels (Shah, Sharma & Gupta, 2004), *Cassia sophera* Linn. (Caesalpiniaceae) (Mondal, 2015), *Crotalaria juncea* (Dutta, Sarkar & Mukherjee, 2015), microalgae *Chlorella* spp. (Li, Li, Zhang & Tan, 2015), and *Chlorella saccharophila* (Mulchandani, Kar & Singhal, 2015). Consequently, based on previous reports, TPP or TPP combined with assisted methods has a higher extraction yield and better oil quality compared with traditionally used methods, and it as a green, simple, and inexpensive technique with significant potential to be utilized for both pilot- and large-scale extractions of oils, fats, and lipids from naturally available sources.

Carbohydrates

In the last few years, TPP has been successfully described in the separation and purification of carbohydrates. Sharma and Gupta (2002b) reported that TPP was initially used for the precipitation and purification of three different commercial preparations of alginates under different TPP conditions. Subsequently, Sharma et al. (2003b) described the use of TPP to chitosan and investigated the changes in some of the properties (e.g., solubility, structure, and biodegradability) of this carbohydrate polymer. It is worth noting that TPP treatment may be a novel and simple method to produce desirable changes in the properties of chitosan for some specific applications. At the same time, TPP has been extended to precipitate and recover starch

from potato and tapioca and some chemically modified starches. It has been found that starch and its modified forms could be recovered at more than 90% yield, and TPP treatment results in considerable changes in the susceptibility of potato starch to amylolytic hydrolysis (Mondal, Sharma & Gupta, 2004). Subsequently, a saccharide TPP technique was tested to precipitate and separate levan and fructooligosaccharide (FOS) from aqueous solutions with success (Coimbra, Lopes & Calazans, 2010). According to these reported studies, more than 90% carbohydrate polymers were usually concentrated in the interfacial precipitate during the TPP process. Alternatively, Tan et al. (2015) has recently reported on the application of TPP for simultaneously purifying aloe polysaccharide (APS) and protein from the crude slurry of aloe powder using a single-step extraction. APS was extracted in the lower phase, and the protein was extracted in the middle phase, under optimized conditions of 26.35% (w/w) ammonium sulfate and 20.82% (w/w) *t*-butanol at 30°C and pH 6.5. Based on these findings, our research group has successfully extracted and separated antioxidative polysaccharides (CFPS) from *Corbicula fluminea* in the lower aqueous phase using TPP (Yan et al., 2017). The highest extraction yield of CFPS (9.32%) was obtained under optimized process conditions of a 20.0% (w/v) mass fraction of $(NH_4)_2SO_4$, 9.8 mL of *t*-butanol, 35.3°C reaction temperature, extraction time of 30 min, and pH 6.0. The purified CFPS after TPP coupled with dialysis was a proteoglycan with high purity (86.5%) and various molecular weights, which showed obvious free radical scavenging capacities and antioxidant activities *in vitro*. Therefore, TPP as a novel, simple, and green bioseparation method

that is a promising technique for separating active PS, PS-protein, or -peptide complexes from natural resources for various food, cosmetic, and pharmaceutical industries.

Small-molecule organic compounds

Recently, the extraction and purification of bioactive small-molecule organic compounds from natural sources have attracted increasing attention because phytochemicals are widely applied in foods, perfumes, cosmetics, paints, and pharmaceuticals. Thus, the isolation of natural products and the identification of new natural sources of bioactive small-molecule organic compounds have gained scientific and industrial importance. The extraction and purification of these phytochemicals are the first steps for achieving these growing demands. As reported in previous studies (Wang & Weller, 2006), different conventional (*e.g*., SE and batch SE) and advanced extraction (*e.g*., UAE, MAE, SFE and TPP) techniques have been currently employed for the extraction of small-molecule organic compounds as natural drugs from plants, animals, and microorganisms.. Although TPP has been extensively evaluated for the simultaneous separation and purification of proteins and enzymes from crude extracts, it has also been widely used to extract small-molecule organic compounds from the upper *t*-butanol phase in recent years such as antioxidative andrographolide (AP) from the leaves of *Andrographis paniculata* (Rao & Rathod, 2015) and ursolic acid and oleanolic acid from *Ocimum sanctum* leaves (Vetal, Shirpurkar & Rathod, 2015) (Table 2).

In order to enhance the yield in the TPP process, Kulkarni and Rathod (2014, 2015) reported that treatments with microwave and ultrasound, combined with TPP, have been carried out for the extraction of mangiferin from *M. indica*. The results showed that the treatments of microwave-assisted three-phase partitioning (MATPP) and ultrasound-assisted three-phase partitioning (UATPP) for the extraction of mangiferin from *M. indica* leaves significantly increased the yield and reduced the time of extraction compared with TPP, further indicating that the combination of microwave (or ultrasound) and TPP enhances the benefits of both technologies and makes this process an interesting alternative to downstream purification procedures. Chougle et al. (2014) has reported that a combination of ultrasound pretreatment and TPP was found to be a very effective approach for the extraction of astaxanthin (AX) from the bacterial biomass of *Paracoccus* NBRC 101723. Additionally, enzyme-assisted TPP was used to efficiently extract oleoresin from turmeric (*Curcuma longa* L.) within approximately 4 h (Kurmudle et al., 2011).

CONCLUSION AND PERSPECTIVES

As an emerging bioseparation technique, TPP has been widely used by many researchers for the recovery and purification of different bioactive molecules, including proteins, enzymes, inhibitors, oils, carbohydrates, and small-molecule organic compounds from natural sources such as drugs, functional food ingredients, or nutraceuticals, which are potentially applied in medicine, food, and cosmetics. Although TPP has received increasing attention and showed many advantages in comparison to traditional extraction and separation techniques, there is still a lot to learn about

TPP systems. First, the precise partitioning mechanism and phase behavior, which rule the formation of TPP, should be paid extra attention in order to gain a more in-depth understanding. Second, quicker and more efficient assisted or modified TPP systems based on the traditional TPP should be developed for the "green" and effective extraction and separation of different bioactive molecules. The use of more benign and biocompatible ionic liquids instead of the organic solvent *t*-butanol in TPP formation is still in demand. Third, the effect of the TPP separation process on physicochemical properties, molecular structure, and bioactivity of the separated biomolecules should not be ignored. Finally, in order to highlight the potential of the "novel" TPP system for practical purposes, large-scale and industrial extraction and separation of bioactive molecules using the TPP system needs to be addressed in the near future. Overall, this presents a good opportunity, as well as a challenge for scientists, to understand the detailed partitioning rule and to take better use of TPP for the production and separation of various bioactive molecules, which have been intensively applied in the food and medical fields.

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Table 1. TPP for the recovery and purification of different enzymes from different sources

Name	Source	TPP process parameters	Adopted method	Purification fold	Activity recovery	Reference
AAO	*Pleurotus ostreatus*	$(NH_4)_2SO_4$ 30% (w/v), *t*-butanol in a ratio of 1:1 (v/v), 30 °C	TPP	10.19	10.95%	Kumar & Rapheal, 2011
alkaline proteases	farmed giant catfish viscera	$(NH_4)_2SO_4$ concentration 50% (w/v), crude extract to *t*-butanol ratio 1.0:0.5 (v/v), pH 8.0 and 25 °C	TPP	6	220%	Rawdkuen et al., 2012; Ketnawa et al., 2014
α-amylases	wheat germ, porcine pancreas, *Bacillus amyloliquefaciens*	alginate ester 0.5% (w/v), $(NH_4)_2SO_4$ 20% (w/v), *t*-butanol in a ratio of 1:1 (v/v), 37 °C, 1 h, and pH 5.6 (or 6.8)	MLFTPP		77%, 92%, 74%	Mondal, Sharma & Gupta, 2003a
cholesterol oxidase	COX4-8 (*Rhodococcuse sp*.)	$(NH_4)_2SO_4$ saturation 70% (w/v), ratio of broth to *t*-butanol 1:0.8 (v/v), 20 °C, 30 min and pH 9.0	TPP	4.5	95%	Wang et al., 2014
α-chymotrypsin		$(NH_4)_2SO_4$ saturation 50% (w/v), *t*-butanol 6 mL, 25 °C, 60 min and pH 7.8	TPP	3.58	119%	Roy & Gupta, 2004
α-galactosidase	*Aspergillus oryzae*	$(NH_4)_2SO_4$ saturation 30% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), 40 °C, 60 min and pH 4.3	TPP	15	50%	Dhananjay & Mulimani, 2008
α-galactosidase	fermented media *(Aspergillus oryzae)*	$(NH_4)_2SO_4$ concentration 50% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), 37°C, and	TPP	12	92%	Dhananjay & Mulimani, 2009

		60 min				
α-galactosidase	tomato (*Lycopersicon esculentum*)	$(NH_4)_2SO_4$ saturation 50% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), 25 °C, 60 min and pH 4.5	TPP	4.3	80%	Çalci et al., 2009
α-galactosidase	pepino (*Solanum muricatum*)	$(NH_4)_2SO_4$ saturation 50% (w/v), ratio of crude extract to *t*-butanol 1.0:1.5 (v/v), 25 °C, 60 min and pH 5.25	TPP	6.2	127%	Şen et al., 2011
α-galactosidase	Watermelon (*Citrullus vulgaris*)	$(NH_4)_2SO_4$ 50% (w/v), crude extract to *t*-butanol ratio 1.0:1.0 (v/v), 25 °C, 60 min and pH 5.5	TPP	2.7	76.7%	Bayraktar & Önal, 2013
β-galactosidase	chick pea (*Cicer arietinum*)	$(NH_4)_2SO_4$ saturation 60% (w/v), ratio of crude extract to *t*-butanol 1.0:0.5 (v/v), 37 °C, 30 min and pH 6.8	TPP	10.1	133%	Duman & Kaya, 2013b
β-galactosidase	*Lactobacillus acidophilus*	$(NH_4)_2SO_4$ concentration 30% (w/v), crude extract to *t*-butanol ratio 1.0:0.5 (v/v), 25 °C, and 60 min	TPP	7.5	78%	Choonia & Lele, 2013
β-amylase	Stems of *Abrus precatorius*	$(NH_4)_2SO_4$ saturation 49.46% (w/v), a ratio of crude extract/ *t*-butanol 0.87 (v/v), and pH 5.2	TPP	10.17	156.2%	Sagu et al., 2015
catalase	sweet potato tubers (*Solanum tuberosum*)	$(NH_4)_2SO_4$ saturation 40% (w/v), crude extract/*t*-butanol ratio 1.0:1.0 (v/v), 35 °C, 60 min and pH 7.0	TPP	14.1	262%	Duman & Kaya, 2013a
cellulase	Pectinex Ultra SP-L	chitosan 0.2% (w/v), $(NH_4)_2SO_4$ 45% (w/v), *t*-butanol in a ratio of	MLFTPP	16	92%	Sharma, Mondal & Gupta,

		1:1 (v/v), 37 °C, and 60 min				2003a
exo-polygalacturonase	*Aspergillus sojae*	$(NH_4)_2SO_4$ saturation 30% (w/v), crude extract to *t*-butanol ratio 1.0:1.0 (v/v), 25 °C and pH 6.6	TPP	6.7	25%	Dogan & Tari, 2008
fibrinolytic enzyme	*Bacillus sphaericus* MTCC 3672	$(NH_4)_2SO_4$ saturation 80% (w/v), broth to *t*-butanol ratio 1.0:0.5 (v/v), frequency 25 kHz, power 150 W, duty cycle 40%, pH 9.0, 30 °C and 5 min	UATPP	16.15	65%	Avhad, Niphadkar & Rathod, 2014
ficin	Mediterranean fig (*Ficus carica* L.) latex	$(NH_4)_2SO_4$ saturation 40% (w/v), ratio of crude extract: *t*-butanol 1.0:0.75 (v/v), 25 °C, 45 min and pH 7.0	TPP	6.04	167%	Gagaoua et al., 2014
glucoamylase	*Aspergillus niger*	esterified alginate 0.5% (w/v), $(NH_4)_2SO_4$ 30% (w/v), *t*-butanol in a ratio of 1:2 (v/v), 37 °C, 60 min, and pH 4.5	MLFTPP	20	83%	Mondal, Sharma & Gupta, 2003b
β-glucosidase	bitter almond	$(NH_4)_2SO_4$ saturation 50% (w/v), crude extract to *t*-butanol ratio 1.0:1.5 (v/v), 25 °C, 60 min and pH 5.0	TPP	5.97	85.7%	Wei et al., 2016
invertase	*Aspergillus oryzae*	$(NH_4)_2SO_4$ saturation 30% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), 40 °C, 60 min and pH 5.0	TPP	12	54%	Dhananjay & Mulimani, 2008
invertase	tomato *(Lycopersicon esculentum)*	$(NH_4)_2SO_4$ saturation 50% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), 25 °C, 60 min and pH 4.5	TPP	8.6	190%	Özer et al., 2010

invertase	*Saccharomyces cerevisiae*	$(NH_4)_2SO_4$ saturation 50% (w/v), crude extract to *t*-butanol ratio 1.0:0.5 (v/v), 25 °C, 60 min and pH 4.0	TPP	15	363%	Akardere et al., 2010
inulinase	*Aspergillus niger*	$(NH_4)_2SO_4$ saturation 30% (w/v), ratio of *t*-butanol to crude extract 1.0:0.5 (v/v), 25 °C and pH 4.0	TPP	10.2	88%	Kumar et al., 2011a
laccase	*Pleurotus ostreatus*	$(NH_4)_2SO_4$ saturation 50-60% (w/v), ratio of crude extract to *t*-butanol 1.0:1.8 (v/v), and 42-45 °C	TPP	7.22	184%	Kumar et al., 2011b
laccase	Submerged cultures of *Ganoderma sp. WR-1*	Step 1: $(NH_4)_2SO_4$ 20% (w/v), aqueous phase-to-*t*-butanol ratio 1.0:0.5 (v/v), 35 °C, 60 min and pH 7.0; Step 2: $(NH_4)_2SO_4$ 90% (w/v)	Two-step TPP	13.2	60%	Rajeeva & Lele, 2011
lipase	*Candida rugosa*	$(NH_4)_2SO_4$ 50% (w/v), ratio of the enzyme solution to *t*-butanol 1.0:2.0 (v/v), 25 °C and pH 7.8; microwave time 10 s, frequency 2.45 GHz, power 100 W, 100 °C	MTPP	5.4	123%	Saifuddin & Raziah, 2008
lipase	*Chromobacterium viscosum*	$(NH_4)_2SO_4$ saturation 50% (w/v), *t*-butanol 6 mL, 25 °C, 60 min and pH 7.8	TPP		320%	Roy & Gupta, 2005
nattokinase	Fermentation broth of *Bacillus natto* NRRL-3666	$(NH_4)_2SO_4$ 30% (w/v), ratio of crude extract to *t*-butanol 1.0:1.5 (v/v), 37 °C, 60 min and pH 8.0	TPP	5.6	129.5%	Garg & Thorat, 2014
papain	papaya peels	$(NH_4)_2SO_4$ concentration 20% (w/v), crude extract to *t*-butanol	TPP	15.8	253.5%	Chaiwut, Pintathong & Rawdkuen, 2010

		ratio 1.0:0.5 (v/v)				
pectinase	Pectinex Ultra SP-L	alginate 1% (w/v), $(NH_4)_2SO_4$ 45% (w/v), *t*-butanol in a ratio of 1:1 (v/v), 37 °C, and 60 min	MLFTPP	13	96%	Sharma, Mondal & Gupta, 2003a
pectinase	Pectinex Ultra SP-L	esterified alginate 0.5% (w/v), $(NH_4)_2SO_4$ 30% (w/v), *t*-butanol 4 mL, 37 °C, 60 min and pH 3.8	MLFTPP	4	100%	Sharma, Roy & Gupta, 2004
peroxidase	leaves of *Ipomoea palmata*	$(NH_4)_2SO_4$ 30% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), 37 °C, 30 min and pH 9.0	TPP	18	81%	Narayan, Madhusudhan & Raghavarao, 2008
peroxidase	Orange peels (*Citrus sinenses*)	$(NH_4)_2SO_4$ saturation 50% (w/v), broth to *t*-butanol ratio 1.0:1.5 (v/v), 30 °C, 200 rpm, 80 min and pH 6.0	TPP	18.20	93.96%	Vetal & Rathod, 2015
phospholipase D	*Dacus carota*	$(NH_4)_2SO_4$ 30% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), 25 °C, 30 min and pH 7.0	Two-step TPP	13	72%	Sharma & Gupta, 2001c
PPO	potato peel *(Solanum tuberosum)*	$(NH_4)_2SO_4$ 0-40% (w/v), crude extract to *t*-butanol ratio1.0:1.0 (v/v), frequency 25 kHz, power 150 W, duty cycle 40%, 25 °C, 5 min and pH 7.0	UATPP	19.7	98.3%	Niphadkar & Rathod, 2015
PPO	*Trachystemon orientalis L.*	$(NH_4)_2SO_4$ 15% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v), temperature 25 °C, 60 min and pH 6.5	TPP	3.59	68.75%	Alici & Arabaci, 2016
proteinase	*Calotropis procera* latex	Step 1: $(NH_4)_2SO_4$ 50% (w/v), ratio of crude extract to *t*-butanol 1.0:0.5 (v/v), 60 min; Step 2:	Two-step TPP	6.92	132%	Rawdkuen et al., 2010

		$(NH_4)_2SO_4$ 65% (w/v)				
proteinase K		$(NH_4)_2SO_4$ saturation 30% (w/v), *t*-butanol 6 mL, temperature 25 °C, 60 min and pH 6.0	TPP		210%	Singh et al., 2001
pullulanase	*Bacillus acidopullulyticus*	esterified alginate 0.5% (w/v), $(NH_4)_2SO_4$ 20% (w/v), *t*-butanol in a ratio of 1:1 (v/v), 37 °C, 60 min and pH 5.0	MLFTPP	38	89%	Mondal, Sharma & Gupta, 2003b
serratiopeptidase	*Serratia marcescens* NRRL B 23112	$(NH_4)_2SO_4$ saturation 30% (w/v), *t*-butanol to crude extract ratio 1.0:1.5 (v/v), pH 7.0, frequency 25 kHz, intensity 0.05 W/cm^2, dute cycle 20%, 5 min	UATPP	9.4	96%	Pakhale & Bhagwat, 2016
SOD	*Kluyveromyces marxianus*	$(NH_4)_2SO_4$ 20% (w/v), ratio of crude extract to *t*-butanol 1.0:1.0 (v/v)	TPP	9.8	80%	Simental-Martínez et al., 2014
xylanase	Pectinex 3XL	eudragit S-100 1% (w/v), $(NH_4)_2SO_4$ 30% (w/v), *t*-butanol in a ratio of 1:1 (v/v), 40°C, and 60 min	MLFTPP	95	60%	Sharma & Gupta, 2002a
xylanase	urea-denatured xylanase	$(NH_4)_2SO_4$ saturation 30% (w/v), ratio of denatured enzyme solution to *t*-butanol 1.0:1.0 (v/v), 40°C, 60 min and pH 5.6	TPP	21	93%	Roy, Sharma & Gupta, 2004
zingibain	*Zingiber officinale* Roscoe rhizomes	$(NH_4)_2SO_4$ saturation 50% (w/v), crude extract to *t*-butanol ratio 1.0:1.0 (v/v), 25 °C, 60 min and pH 7.0	TPP	14.91	215%	Gagaoua et al., 2015, 2016

Table 2. TPP for separation and purification of small molecular organic compounds from natural resources

Name	Source	TPP process parameters	Adopted method	Extraction Yield	Reference
andrographolide	*Andrographis paniculata*	$(NH_4)_2SO_4$ 40% (w/v), solute to solvent 1:40, slurry to *t*-butanol ratio 1:1 (v/v), 120 min, 40 °C, and pH 7.0	TPP	26.55 mg/g	Rao & Rathod, 2015
forskolin	*Coleus forskohlii* roots	$(NH_4)_2SO_4$ 30% (w/v), slurry to *t*-butanol ratio 1:1 (v/v), pH 5.5; (1): stargen® 002 (64 U/g of substrate), Accellerase® 1500 (90 FPU/g of substrate), pH 4.5,50 °C; (2)UATPP: duty cycle 50%, power 50 W	(1)EATPP, (2)UATPP, (3)UATPP+EATPP	0.53%; 0.51%; 0.55%	Harde & Singhal, 2012
mangiferin	leaves of *Mangifera indica*	(1) $(NH_4)_2SO_4$ 40% (w/v), solute to solvent ratio 1:20, slurry to *t*-butanol ratio 1:1, soaking time 5 min, microwave time 5 min, power 272 W, duty cycle 50%; (2) solute to solvent ratio 1:40, frequency 25 kHz, power 180 W, duty cycle 50%, 30 °C	(1)MATPP; (2)UATPP	54 mg/g; 41 mg/g	Kulkarni & Rathod, 2014, 2015
mangiferin	leaves of *Mangifera indica*	$(NH_4)_2SO_4$ concentration 40% (w/v), solute to solvent 1:40, slurry to *t*-butanol ratio 1:1 (v/v), 120 min, 30 °C, and pH 6.0	TPP	28 mg/g	Kulkarni & Rathod, 2016
Oleanolic acid	*Ocimum sanctum* leaves	(1) $(NH_4)_2SO_4$ saturation 50% (w/v), crude extract to *t*-butanol ratio 1:1, feed loading 7.5% (w/v), 120 min and pH 7.0; (2) frequency 40 kHz, power 180 W, 14 min.	(1)TPP, (2) UATPP	80.67%,85.58%	Vetal et al., 2014
oleoresins	Ginger (*Zingiber officinale*) rhizome	(1) $(NH_4)_2SO_4$ concentration 10% (w/v), slurry to *t*-butanol ratio 1:0.5 (v/v), ~32 °C and pH 5.0; (2) stargen or accellerase 0.5% (v/v) at 50 °C; (3) duty	(1)TPP, (2)EATPP, (3)UATPP	61.6, 69, 67, 64 mg/g	Varakumar et al., 2017

		cycle 40%, power 36 W			
turmeric oleoresins	*Curcuma longa* L.	$(NH_4)_2SO_4$ 30% (w/v), slurry to *t*-butanol ratio 1.0:1.0 (v/v), α-amylase & glucoamylase 1.5% (w/w), 240 min and pH 4.5	EATPP	14.21% (w/w)	Kurmudle et al., 2011
ursolic acid	*Ocimum sanctum* leaves	(1) $(NH_4)_2SO_4$ saturation 50% (w/v), crude extract to *t*-butanol ratio 1:1, feed loading 7.5% (w/v), 120 min and pH 7.0; (2) frequency 40 kHz, power 180 W, 14 min.	(1)TPP, (2) UATPP	79.48%, 83.36%	Vetal et al., 2014

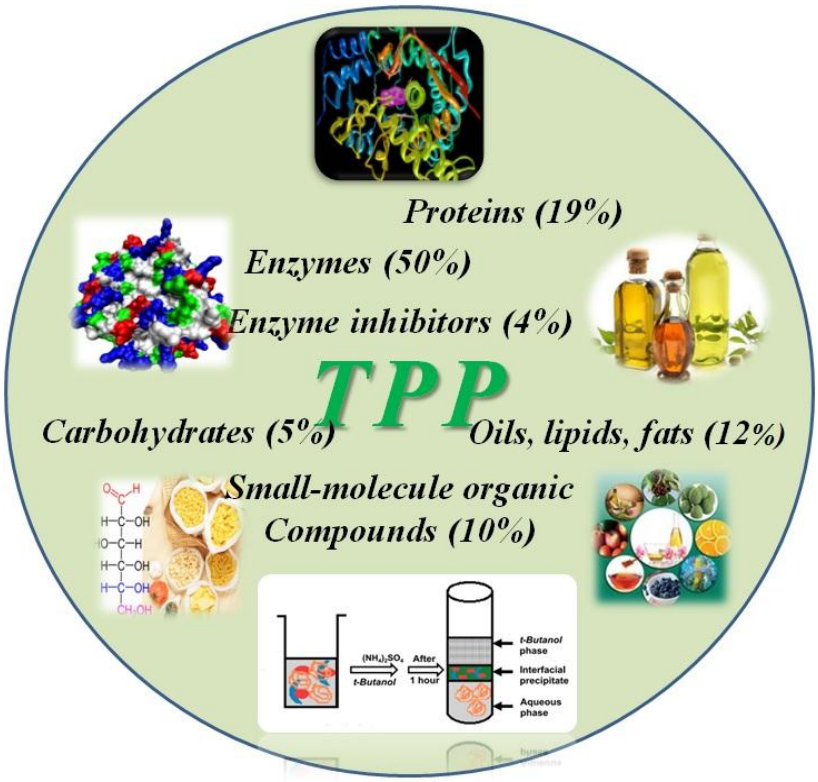

Fig. 1. TPP separation technology for the extraction and separation of a variety of bioactive molecules from different natural sources.

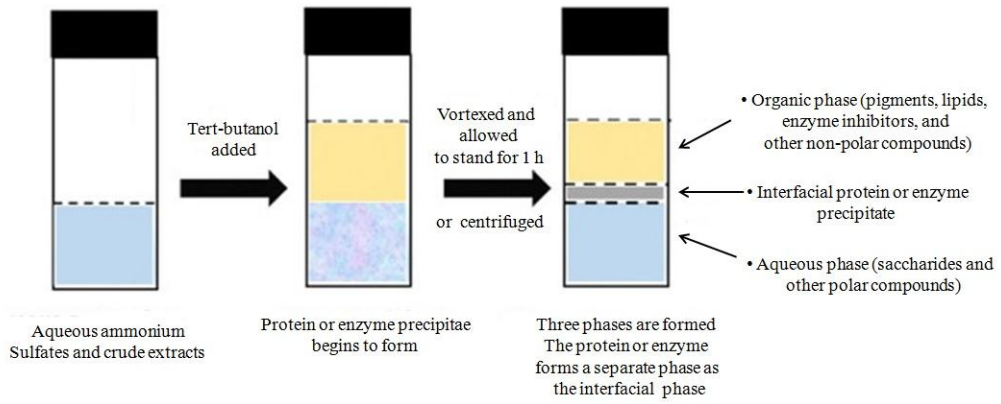

Fig. 2. Schematic representation of the TPP System.

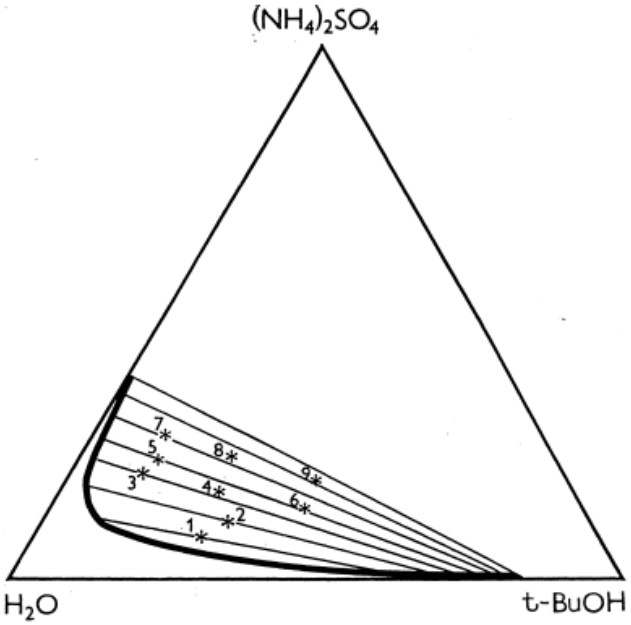

Fig. 3. Phase diagram (in weight ratio) of the water/$(NH_4)_2SO_4$/*t*-butanol three-component system at 25 °C. Number from 1 to 9 represented different components (weight ratios) of the three component partitioning systems with the corresponding length of the tieline and interfacial tensions between the equilibrium upper and lower phases (Kiss et al., 1998)

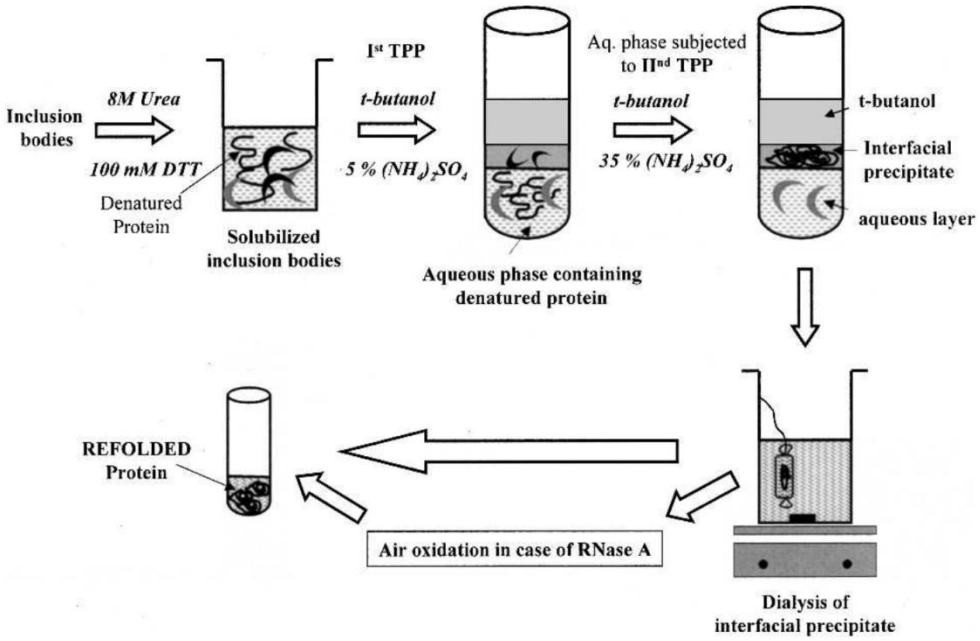

Fig. 4. Schematic representation for refolding of proteins from inclusion bodies by two-step TPP.

(Raghava et al., 2008)

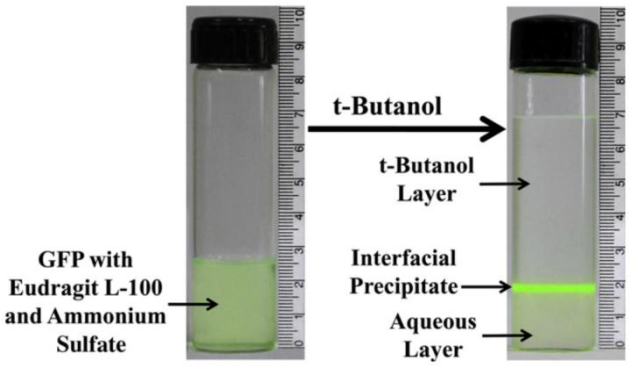

Fig. 5. MLFTPP of recombinant GFP with Eudragit L-100. (Gautam et al., 2012)