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Aqueous Methods for Extraction/Recovery of Macromolecules From Microorganisms of Atypical Environments: A Focus on Three Phase Partitioning

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1 INTRODUCTION

Owing to developments in molecular biology over recent decades, there is a growing demand for the separation of macromolecules including proteins and enzymes from numerous sources. This has resulted in the innovation and optimization of a myriad of separation and purification processes (Johansson, 1994; Mondal & Gupta, 2006; Nadar, Pawar, & Rathod, 2017; Przybycien, Pujar, & Steele, 2004). The interest in the development of extraction and purification/recovery techniques of macromolecules from various sources, including atypical microorganisms, has increased due to their beneficial aspects for human life such as industry, laboratory facilities and other research aspects including therapeutic needs. Biological separation operations have long been dominated by packed-bed chromatography (Anspach, Curbelo, Hartmann, Garke, & Deckwer, 1999; Chavez Crocker, Sako, & Uchida, 1999; Hakamada, Koike, Kobayashi, & Ito, 1997; Przybycien et al., 2004; Ronimus, Koning, & Morgan, 1999). These chromatographic techniques, which are mainly based on ion exchange, hydrophobic interaction and membrane affinity (Andronopoulou & Vorgias, 2003; Bouanane-Darenfed et al., 2016; Chavez Crocker et al., 1999; Kim, Kim, Lee, Park, & Oh, 2000; Li, Xiang, Liu, Zhou, & Tan, 2003), give more accurate resolution, however, they are generally costlier, time consuming and complicated to scale up compared to nonchromatographic techniques (Przybycien et al., 2004). Recently, there have been novel approaches using nonchromatographic techniques to aid the growing demand for large-scale production of macromolecules, mainly

proteins and enzymes. These techniques were developed because in some industries it is not necessary to use macromolecules of high purity. In such cases, nonchromatographic methods constitute an attractive alternative to protocols involving packed-bed chromatography. The several alternatives were classified under bulk, field-based and adsorptive separation techniques that aim to increase the throughput and overcome the drawbacks of the chromatographical tools (Przybycien et al., 2004).

Among the bulk separations, aqueous systems have the potential for high-throughput purifications (Alvarez-Guerra & Irabien, 2014a; Puthirasigamany et al., 2014). Even though the resolution is not comparable to chromatographical techniques, aqueous systems represent a highly biocompatible and continuously operating purification process, which is easy to scale up for macromolecules from animal, plant or microbial sources using fluids, tissues or cells (Boland, 2002; Chew, Ling, & Show, 2018; Johansson, 1994; Nadar et al., 2017; Nadar, Rao, & Rathod, 2018; Nathan, Rogers, Matthew, Lynn, & John, 2010). These recovery systems are largely based on fractionation (precipitation) separations where two main systems can be distinguished: aqueous two-phase systems (ATPS) (Asenjo & Andrews, 2011; Dutra, Araújo, Pessoa, Gava, & Viccari, 2013; Lee, Khoiroh, Ooi, Ling, & Show, 2017) and Three Phase Partitioning (TPP) (Dennison, 2011; Gagaoua & Hafid, 2016; Yan et al., 2017).

The former, ATPS, known also as aqueous biphasic systems are formed when either two polymers, one polymer and one kosmotropic salt, or two salts (one chaotropic and the other a kosmotropic salt) are mixed at appropriate concentrations or at a particular temperature (Asenjo & Andrews, 2011; Pereira et al., 2013). The two phases are mostly composed of water and nonvolatile components, hence eliminating volatile organic compounds. ATPS can be used as a separation process based on the partition of a target compound between the two immiscible aqueous-rich phases formed by polymer–polymer, polymer–salt and salt–salt mixtures as detailed in the comprehensive review by Freire et al. (2012). Previous studies demonstrated that ATPS can preserve enzymatic activity and satisfactory stability results were obtained for different proteins (Li, Kim, & Peeples, 2002; Silvério, Rodríguez, Tavares, Teixeira, & Macedo, 2013; Wei, Zhu, & Cao, 2002).

The later, TPP is a one-step purification approach which consists of biomolecule recovery due to the sequential mixing of an organic solvent (mainly *tert*-Butanol = *t*-BuOH) with a sufficient amount of salt (typically ammonium sulphate [(NH₄)₂SO₄]) in the crude extracts or suspensions to form a two-phase system in which organic macromolecules precipitate at the interface, hence forming the third phase (Chew et al., 2018; Dennison, 2011; Dennison & Lovrien, 1997; Gagaoua & Hafid, 2016; Nadar et al., 2017). TPP involves collective operation of principles such as salting out, isoionic precipitation, cosolvent precipitation, osmolytic and kosmotropic precipitation (Gagaoua & Hafid, 2016).

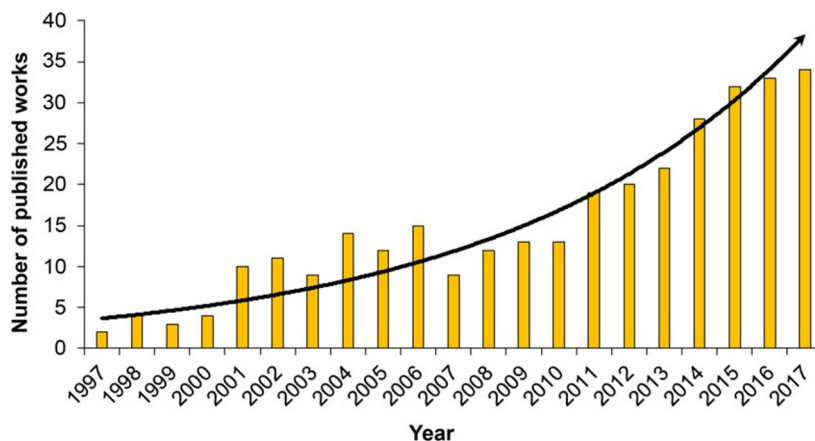
Both ATPS and TPP have been exploited for the separation/purification of macromolecules from atypical microorganisms (Ait Kaki El-Hadef El-Okki, Gagaoua, Bourekoua, et al., 2017; Baweja et al., 2017; Boucherba et al., 2017; Cavalcanti et al., 2006; Gupta, Beg, Khan, & Chauhan, 2002; Kammoun, Chouayekh, Abid,

Naili, & Bejar, 2009; Lario, Malpiedi, Pereira, Sette, & Pessoa-Junior, 2016; Li & Peeples, 2004; Periyasamy et al., 2017; Porto et al., 2008; Sadaf, Sinha, & Khare, 2018; Show et al., 2012; Wu, Liu, Zhao, Wang, & Sun, 2006; Yadav, Gupta, & Khare, 2017; Yan et al., 2017; Zhi, Song, Bi, & Ouyang, 2004). Therefore, this chapter focuses on TPP separation process only and intends to describe its potential as an emerging alternative to extract and purify macromolecules (proteins, enzymes, carbohydrate polymers, plasmid, DNA, oils and pigments) from microorganisms of atypical environments since chromatographic techniques are too complicated to be used as simple, versatile and effective industrial methods. Thus, in the following, TPP will be highlighted by giving its uses and advantages for the recovery of macromolecules, namely proteins and enzymes. The chapter will further summarize the partitioning mechanisms of TPP, the main factors influencing the recovery process such as ammonium sulphate amount, *t*-BuOH to slurry ratio, pH and temperature and also the variants and unique characteristics of TPP as an emerging elegant nonchromatographical tool for the separation and recovery of biomolecules from different sources, including fermentation broths and extremophile microorganisms.

2 TPP AS AN EMERGING AND ATTRACTIVE TECHNIQUE FOR MACROMOLECULES RECOVERY

TPP, a technique first described by Lovrien's group in 1972 (Tan & Lovrien, 1972), was intensively used to purify several target macromolecules and has become a versatile, early-exploratory tool and a common bioseparation tool with wide applications (Chew et al., 2018; Dennison, 2011; Gagaoua & Hafid, 2016; Mondal, Jain, Teotia, & Gupta, 2006; Nadar et al., 2017; Yan et al., 2017). As stated earlier and due to the drawbacks of chromatographic techniques, this elegant nonchromatographic bioseparation approach, has gained the attention of numerous scientists and laboratories as shown in Fig. 1, which contains the number of published works related to TPP during the last 2 decades. To date, about 70% of the studies on TPP have focused on the recovery and purification of proteins and enzymes, while the other 30% concerned oils, lipids, small-molecule organic compounds, DNA and carbohydrates (Dennison, 2011; Ward, 2009; Yan et al., 2017). TPP has been successfully used for the recovery of a wide variety of macromolecules from various natural sources (fluids, tissues and cells) such as plants, animals and microorganisms (including extremophiles), as well as fermentation broths.

Table 1 summarizes some examples of the recovered macromolecules, most of which are from microorganisms of atypical environments. From the large literature base, the targeted macromolecules were proteins (Alvarez-Guerra & Irabien, 2014a; Chew et al., 2018; Kiss, Szamos, Tamas, & Borbas, 1998; Paule et al., 2004; Szamos & Kiss, 1995) including enzymes/proteases (Ait Kaki El-Hadef El-Okki, Gagaoua, Bourekoua, et al., 2017; Akardere et al., 2010; Dennison, 2011; Gagaoua et al., 2014; Gagaoua & Hafid, 2016; Gagaoua, Hafid, Ziane, & Nait Rabah, 2015; Gagaoua, Hoggas, & Hafid, 2015; Gagaoua et al., 2017;

**FIG. 1**

Bar plot representation of the number of the related works on Three Phase Partitioning published over the past 20 years.

Mondal, Sharma, & Gupta, 2003; Rawdkuen, Chaiwut, Pintathong, & Benjakul, 2010; Rawdkuen, Vanabun, & Benjakul, 2012) and their inhibitors (Kansal, Sharma, & Gupta, 2006; Roy & Gupta, 2002; Saxena, Iyer, & Ananthanarayan, 2007; Sharma & Gupta, 2001b; Wang, Chen, Jeng, & Sung, 2011; Wati, Theppakorn, Benjakul, & Rawdkuen, 2009). It was further used to extract DNA (Szamos, Aubrecht, & Gelencser, 1998; Ward, 2008), to separate edible oils (Gaur et al., 2007; Mondal, 2015; Mulchandani, Kar, & Singhal, 2015; Panadare & Rathod, 2017; Tan et al., 2016), carbohydrates (Coimbra, Lopes, & Calazans, 2010; Mondal, Sharma, & Gupta, 2004; Sharma & Gupta, 2002b; Sharma, Mondal, & Gupta, 2003b; Tan et al., 2015) and small-molecule compounds including pigments, antibodies and polyphenols (Chougale et al., 2014; Rao & Rathod, 2015; Varakumar et al., 2017; Vetal, Shirpurkar, & Rathod, 2014; Ward, 2008, 2009).

3 ADVANTAGES OF TPP

The increasing interest in TPP may be attributed to the advantages that it offers over conventional techniques which include (i) high throughput of desired product, (ii) easily scalable, (iii) adaptability for both upstream and downstream purification processes, (iv) cost effective and (v) simultaneous use as a single-step or a combined purification protocol (for rapid screening of natural or expressed proteins). Furthermore, TPP can be defined as a simple, efficient, economical and green (clean) bioseparation method for the extraction and purification of target macromolecules (bioactives), with respect to the conventional techniques (Chew et al., 2018; Dennison, 2011; Gagaoua & Hafid, 2016). TPP stands out because

Table 1 Examples of Three Phase Partitioning (TPP) and Its Variants for the Recovery and Purification of Macromolecules and Small Molecules From Different Sources Including Fermentation Broth and Microorganisms From Atypical Environments

| Target Biomolecules | Sources | Applied TPP System ^a | Recovery Yield | Purification Fold | References |
|-------------------------|---|---------------------------------|--------------------|--|--|
| α -Galactosidase | <i>Aspergillus oryzae</i> | TPP | 50% | 15 | Dhananjay and Mulimani (2008) |
| Alkaline protease | <i>Bacillus pumilus</i> MP27 | | 186% | 16.65 | Baweja et al. (2017) |
| Inulinase | <i>Aspergillus niger</i> | | 88% | 10.2 | Kumar et al. (2011) |
| α -Galactosidase | | | 97.2% ^b | 6.27 | Gu et al. (2012) |
| exo-Inulinase | | | 88% | 10.2 | Kumar, Premkumar, et al. (2011) |
| Invertase | <i>Saccharomyces cerevisiae</i> | | 363% | 15 | Akardere, Ozer, Celem, and Onal (2010) |
| Xylanase | <i>Trichoderma citrinoviride</i> | | 99.8% | 5.7 | Periyasamy et al. (2017) |
| Carboxymethyl cellulase | AUKAR04 | | 96.5% | 5.5 | |
| β -1,3-Glucanase | | | 98.4% | 5.6 | |
| β -Galactosidase | <i>Lactobacillus acidophilus</i> | | 78% | 7.5 | Choonia and Lele (2013) |
| α -Amylase | <i>Rhizopus oryzae</i> FSIS4 | | 168.8% | 14.94 | Ait Kaki El-Hadef El-Okki, Gagaoua, Bourekoua, et al. (2017) |
| Xylanase | <i>Bacillus oceanisediminis</i> SJ3 | | 107% | 3.48 | Boucherba et al. (2017) |
| Lipase | <i>Chromobacterium viscosum</i> | | 320% | 3.24 | Roy and Gupta (2005) |
| Nattokinase | <i>Bacillus natto</i> NRRL-3666 | 129.5% | 5.6 | Garg and Thorat (2014) | |
| Laccase | <i>Coriolopsis trogii</i> | 75% | 20 | Liu, Yan, and Huang (2015) | |
| Naringinase | <i>Aspergillus brasiliensis</i> MTCC 1344 | 21.41% | 4.2 | Shanmugapakash, Vinothkumar, Ragupathy, and Reddy (2015) | |
| Superoxide dismutase | <i>Kluyveromyces marxianus</i> | 80% | 9.8 | Simental-Martinez, Rito-Palomares, and Benavides (2014) | |
| β -Lactamase | <i>Bacillus cereus</i> EMB20 | 15% ^c | 30 | Sadaf et al. (2018) | |
| exo-Polygalacturonase | <i>Aspergillus sojae</i> ATCC 20235 | 25% | 6.7 | Dogan and Tari (2008) | |
| Asparaginase | <i>Escherichia coli</i> k12 | 36.21% | 11 | Biswas, Kumari, Singh, and Jha (2014) | |
| Aryl alcohol oxidase | <i>Pleurotus ostreatus</i> | 10.95% | 10.19 | Kumar and Rapheal (2011) | |
| Mannitol dehydrogenase | <i>Aspergillus parasiticus</i> | 77% | 4.8 | Niehaus and Dilts (1982) | |

Continued

Table 1 Examples of Three Phase Partitioning (TPP) and Its Variants for the Recovery and Purification of Macromolecules and Small Molecules From Different Sources Including Fermentation Broth and Microorganisms From Atypical Environments—cont'd

| Target Biomolecules | Sources | Applied TPP System ^a | Recovery Yield | Purification Fold | References |
|---------------------------|---|---------------------------------|-----------------------|-------------------|--|
| Immunoreactive protein | <i>Corynebacterium pseudotuberculosis</i> | | — | — | Paule et al. (2004) |
| Bioactive constituent | <i>Bacillus subtilis</i> EMB M15 | | — | — | Yadav et al. (2017) |
| DNA | <i>Escherichia coli</i> | | — | — | Shabani (2009); Ward (2009) |
| Green fluorescent protein | | | 78% | 20 | Jain, Singh, and Gupta (2004) |
| Surfactin | <i>Bacillus subtilis</i> ATCC 21332 | | 81% | — | Chen, Chen, and Juang (2008) |
| Fibrinolytic enzyme | <i>Bacillus sphaericus</i> MTCC 3672 | UA-TPP | 65% | 16.15 | Avhad, Niphadkar, and Rathod (2014) |
| Serratiopeptidase | <i>Serratia marcescens</i> | | 96% | 9.4 | Pakhale and Bhagwat (2016) |
| Astaxanthin | <i>Paracoccus</i> NBRC 101723 | | 428 µg/g ^d | 1.2 | Chougale, Singhal, and Baik (2014) |
| Peroxidase | <i>Citrus sinensis</i> | | 91.84% | 24.28 | Vetal and Rathod (2016) |
| Lipase | <i>Candida rugosa</i> | MA-TPP | 123% | 5.4 | Saifuddin and Raziah (2008) |
| Mangiferin | <i>Mangifera indica</i> | | 54 mg/g ^d | — | Kulkarni and Rathod (2015) |
| Laccase | <i>Trametes hirsuta</i> | | 59.8% | 13.9 | Patil and Yadav (2018) |
| Glucoamylase | <i>Aspergillus niger</i> | MLF-TPP | 83% | 20 | Mondal, Sharma, and Gupta (2003) |
| Pullulanase | <i>Bacillus acidopullulyticus</i> | | 89% | 38 | Mondal, Sharma, and Gupta (2003) |
| Xylanase | <i>Aspergillus niger</i> | | 60% | 95 | Sharma and Gupta (2002a) |
| α-Amylase | <i>Bacillus amyloliquefaciens</i> | | 74% | 5.5 | Mondal, Sharma, Lata, and Gupta (2003) |
| Pectinase | <i>Aspergillus niger</i> | | 96% | 55 | Sharma, Mondal, and Gupta (2003a) |
| Cellulase | | | 92% | 13 | |
| Edible oils | <i>Mangifera indica</i> | EA-TPP | 72% | — | Gaur, Sharma, Khare, and Gupta (2007) |
| Oleoresin | <i>Zingiber officinale</i> | | 69 g/kg ^d | — | Varakumar, Umesh, and Singhal (2017) |

^aUA-TPP: ultrasound-assisted TPP; MA-TPP: microwave-assisted TPP; MLF-TPP: macroaffinity ligand-facilitated TPP; EA-TPP: enzyme-assisted TPP.

^bAqueous two-phase system (ATPS) was further used to compare the recovery efficiency, which purified the enzyme with a purification factor of 3.25% and 84.41% yield.

^cA two-step TPP was combined with gel filtration chromatography.

^dThe yield is given as the highest recovery compared to the conventional methods (TPP-based technique was more efficient than conventional approach).

it has short processing time that can be used directly with crude suspensions, works at room temperature, and the chemicals required can be easily recycled (Saxena et al., 2007).

According to Mondal et al. (2006) and Ward (2009) and our experience in the field (Gagaoua & Hafid, 2016), further technical advantages can be attributed to TPP such as:

- (i) purification as well as concentration of macromolecules (e.g. proteins), unlike chromatography which dilutes them;
- (ii) lowering of viscosity by removing DNA, polysaccharides and lipid micelles;
- (iii) higher fold purification than achieved in simple ‘salting out’ procedures;
- (iv) can be used with direct crude cultures containing cell debris without any preclarification;
- (v) use of mild operational conditions and structural stability of proteins in their native form;
- (vi) the process does not use polymers, unlike ATPS, which have to be removed later.

For these reasons, TPP seems to be an attractive prepurification, early-stage exploratory research tool and a preparation/recovery method of macromolecules (Lovrien & Matulis, 2001) including those from microorganisms of atypical environments (Champdoré, Staiano, Rossi, & D’Auria, 2007; Elleuche, Schröder, Sahm, & Antranikian, 2014; Saggiu & Mishra, 2017; Sarmiento, Peralta, & Blamey, 2015).

4 TPP PROCESS

As stated earlier, the TPP bioseparation technique is a three-stage recovery batch method, which is a hybrid of ‘salting out’ and alcohol precipitations for extracting, dewatering, purifying and concentrating macromolecules, especially proteins for use in small or large manufacturing operations (Chew et al., 2018; Dennison, 2011; Gagaoua & Hafid, 2016; Yan et al., 2017). The principle of this fast-emerging tool consists in mixing the crude extract or suspension of cells with solid salt (ammonium sulphate, potassium phosphate and sodium citrate) but mostly ammonium sulphate [(NH₄)₂SO₄] and an organic solvent (*t*-BuOH, 2-butanol, 1-propanol and 2-propanol), but usually *t*-BuOH in order to obtain three phases (Fig. 2A).

Most general procedures followed for TPP include an incubation step and an extraction step. The former consists of mixing the crude slurry with salt solution for a fixed short time, and the later includes slow addition of *t*-BuOH to initiate the separation. Thus, the process is based on the ability of the salt to separate a *t*-BuOH–water mixture, an otherwise miscible solution, into two phases, an upper organic phase and a lower aqueous phase (Gagaoua & Hafid, 2016). When this is carried out in the presence of an organic molecule (e.g. proteins), an intermediate protein layer is formed from which the target macromolecule can be extracted (Fig. 2A). Subsequently, the desired macromolecules are selectively partitioned

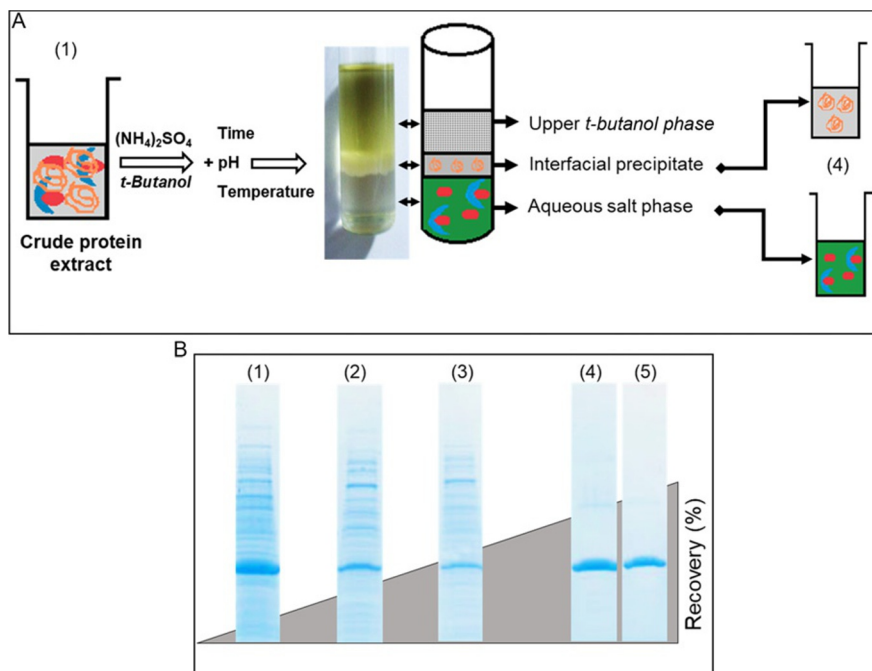


FIG. 2

Three Phase Partitioning (TPP) recovery system. (A) Example of schematic representation of three phases after TPP separation highlighting the main steps from crude protein extract to macromolecule recovery (Dennison, 2011; Gagaoua et al., 2014; Gagaoua & Hafid, 2016; Gagaoua, Hafid, & Hoggas, 2016). In this example, the desired macromolecule (orange) is selectively partitioned in the interfacial precipitate phase (based on the principle detailed in Fig. 3) while contaminants such as carbohydrates and other proteins (red and blue) or pigments are all partitioned in the lower aqueous or within *t*-butanol phases, respectively. A TPP result depicted by a picture of the glass test tube highlights the aspect of the partitioning of the three distinct separated phases, showing the partitioned organic layer. (B) Example of *Coomassie blue* stained gel lanes of the protein samples from the interfacial precipitate phase assessed by SDS-PAGE to highlight the purity of the target protein after each step of TPP optimization as well as the corresponding recovery percentage (grey triangle). Lanes (1) crude and dialyzed protein extract (also shown in A); (2) a protein sample after crude protein extract to *t*-butanol ratio after optimization at 30% $(\text{NH}_4)_2\text{SO}_4$; (3) a protein sample after ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ concentration optimization at a constant *t*-butanol ratio; (4) a protein sample (also shown in A) after pH optimization on the degree of purification and recovery and finally (5) the recovered protein of interest after dialysis against the appropriate buffer before assaying or storage.

after a short separation period (mostly less than 1 h), agitation and slow-speed centrifugation at a given temperature (mostly 4°C or at room temperature) (Gagaoua et al., 2016), to one phase while contaminants such as pigments, enzyme inhibitors and lipids to the other one. As detailed by Dennison (2011) and Ward (2009), if proteins (and cell wall polysaccharides and DNA) are additionally present in the slurry solution, then they may precipitate into a third layer between the aqueous and *t*-BuOH layers. The upper *t*-BuOH layer contains dissolved membrane lipids and low molecular weight pigments. This may efficiently separate proteins from DNA and cell wall components (Ward, 2008, 2009). As depicted in Fig. 2A, the upper *t*-BuOH phase which contains nonpolar compounds is separated from the lower aqueous phase that contains the polar compounds such as carbohydrates by an interfacial protein precipitate (Chew et al., 2018; Gagaoua et al., 2014; Gagaoua, Hoggas, & Hafid, 2015; Li, Jiang, Li, Zhang, & Tan, 2013). The third phase is formed through an organic (protein)-enriched intermediate phase in the form of a precipitate (Fig. 2A).

Most studies with proteins, especially enzymes, have reported enhancement of both concentration and activities that are similar or better than those of chromatographic techniques (Chew et al., 2018; Gagaoua et al., 2016; Gagaoua & Hafid, 2016; Saxena et al., 2007). The well-accepted explanation behind this efficiency in protein isolation is that *t*-BuOH (organic solvent) binds to the precipitated proteins, thereby increasing their buoyancy and causing them to float above the denser aqueous salt layer forming the third phase (interphase).

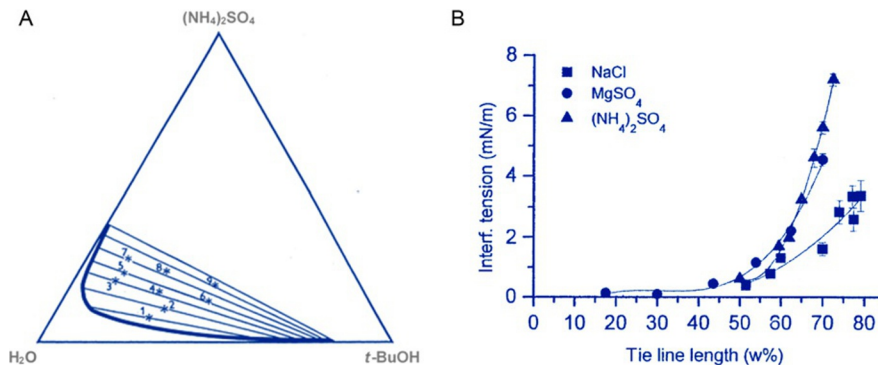
5 MECHANISMS OF TPP

Despite the high number of studies concerning TPP, the exact mechanism behind this elegant nonchromatographical system is not well reported. However, it is generally recognized that the separation process involves collective operation of principles involved in numerous techniques like (i) ‘salting out’, (ii) isoionic precipitation, (iii) cosolvent precipitation, (iv) osmolytic, (v) kosmotropic precipitation of proteins, (vi) protein hydration shifts and (vii) conformation tightening, which all may contribute positively to the best recovery of the target macromolecules (Dennison, 2011; Gagaoua et al., 2017; Gagaoua & Hafid, 2016; Yan et al., 2017). In the TPP process, *t*-BuOH may be first added to the aqueous solution of protein to about a 1–2 ratio. It is hypothesized that this results in the protein equilibrating with the solvent (water) and the cosolvent. The protein thus becomes partially hydrated and partially *t*-butanolated, in proportion to the relative abundance of the solvents in the mixture. Upon addition of (NH₄)₂SO₄ (initially at an amount of 20%–30% before optimization), water is abstracted by the salt ions (especially SO₄²⁻ ions) as these become hydrated. The salt apparently has a higher affinity for water than for *t*-BuOH, and thus preferentially sequesters the water. In the absence of protein, this results in the solution dividing into two phases, as some of the water is made unavailable to the *t*-BuOH. If protein is present, the protein equilibrates with the new proportions of solvent

and cosolvent available. The dehydration properties of $(\text{NH}_4)_2\text{SO}_4$ and the protein buoyancy enhancement properties of *t*-BuOH are believed to contribute considerably to the recovery of the protein in the interfacial phase (Paule et al., 2004). Furthermore, pushing and pulling are both known to be related to macromolecule hydrate water. Thus the partitioning process is affected by the hydrophobicity, the molecular weight, and in an important manner by the charge and isoelectric point (*pI*) of the macromolecule and also by the physical conditions of the phase system (Chew et al., 2018; Dennison, 2011; Dennison & Lovrien, 1997; Mondal et al., 2006). Accordingly, an optimization of the process concerning the main factors affecting TPP is necessary to achieve the desirable yield and purity of the targeted macromolecule as shown in Fig. 2B.

To determine the best TPP system for the purification of macromolecules, various process parameters including the amount of $(\text{NH}_4)_2\text{SO}_4$ for the precipitation, crude extract to *t*-BuOH ratio, pH and temperature are optimized to get the highest purification fold and recovery yield (Fig. 2B). The impact of these parameters is discussed in Section 6. An example of SDS-PAGE results is given in Fig. 2B showing protein samples from the interfacial phase at each step of TPP workflow, i.e., from protein extracts to the recovered target macromolecule. In this case, the sampled interfacial phases (based on the principle described in Section 6.3), were subjected to Coomassie blue gel electrophoresis to visualize the purity of the target protein after each step of TPP optimization as well as the corresponding recovery percentage (yield).

The example given in Fig. 2B shows that a good optimization of the main factors led to the best recovery of the target macromolecule. Indeed, there is a reciprocal relationship between the amounts of $(\text{NH}_4)_2\text{SO}_4$ and *t*-BuOH required to precipitate proteins in a solution during TPP separation processes (Dennison, 2011; Pike & Dennison, 1989). For example, the interrelated effects are investigated in a quantitative way using the phase behaviour of a three-component system (Kiss et al., 1998). This is highlighted in this chapter by a phase diagram (in weight ratio) of the water/ $(\text{NH}_4)_2\text{SO}_4$ /*t*-BuOH three-component system at 25°C (Fig. 3A). It 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 $(\text{NH}_4)_2\text{SO}_4$ and *t*-BuOH. Above that, at higher salt concentrations, the system contains the excess salt in a solid form. According to the literature and especially to the studies by Kiss' research group (Kiss et al., 1998; Szamos, Janosi, et al., 1998; Szamos & Kiss, 1995), the phase separation can be induced both by increasing the amount of salt and by the amount of the *t*-BuOH. Keeping the *t*-BuOH content of the system at 10%, the maximum amount of salt that can be dissolved in the aqueous solution is 10% $(\text{NH}_4)_2\text{SO}_4$. In the presence of larger quantities of salt or *t*-BuOH, the system forms two liquid phases, the compositions of which are determined by the end points of the tie lines in the phase diagram (Fig. 3A). The area of the triangle covered by tie lines represents the range of compositions that can be used for partitioning experiments. The extension of this range is determined by the solubility of the electrolyte applied as shown in Fig. 3B. The length of each tie line reflects the difference in composition of the

**FIG. 3**

Interfacial behaviour of proteins in Three Phase Partitioning using salt-containing water/*t*-butanol (*t*-BuOH) systems. (A) Phase diagram (in weight ratio) of the water/ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$)/*t*-butanol three-component system at 25°C according to Kiss et al. (1998). This *phase diagram* represents the liquid phase area at the water corner, the composition area with *tie lines*, where two immiscible liquid phases are formed, and *above the upper part* where the excess salt is present in solid form. The area within the *triangle* with the *tie lines* corresponds to the composition range, which could be used for the partitioning experiment (Szamos, Janosi, Tamas, & Kiss, 1998). Owing to the considerable solubility of $(\text{NH}_4)_2\text{SO}_4$ in water, the partitioning area in the phase diagram is large. The numbers from 1 to 9 highlight the different weight ratios of water/ammonium sulphate/*t*-butanol partitioning systems with the corresponding length of the tie line and interfacial tensions between the equilibrium upper and lower phases. (B) Interfacial tension between the upper and lower phases of the three-component systems as a function of tie line length under three different salts (Szamos, Janosi, et al., 1998).

The figures were reprinted with permission from Szamos, J., Janosi, A., Tamas, B., & Kiss E. (1998).

A novel partitioning method as a possible tool for investigating meat. I. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung A—Food Research and Technology, 206, 208–212.

equilibrated liquid phases (Kiss et al., 1998; Szamos, Janosi, et al., 1998; Szamos & Kiss, 1995). Overall, the upper phase is composed of more than 60% *t*-BuOH, some water, and less than 1% salt, while the lower phase is an aqueous salt solution with small amounts of *t*-BuOH.

6 FACTORS AFFECTING TPP PROCESS

As stated earlier, the TPP bioseparation process is affected by numerous critical parameters like salt and cosolvent types as well as the concentration, temperature and pH of the medium. Best results can be obtained by streaming these parameters as detailed in the following subsections. Therefore, the optimization of these mentioned parameters is already conducted in most TPP processes, however further

factors may be also involved. For example, earlier studies have reported that isoelectric point (pI), hydrophobicity, interfacial tension and density differences in the protein-free phase, influences the amount of the macromolecules (e.g. proteins), partitioned from the crude slurry (Dennison, 2011; Dennison & Lovrien, 1997; Gagaoua et al., 2014; Szamos & Kiss, 1995; Wati et al., 2009). It is worthwhile noting that the relative measure of accumulation in the interfacial precipitate does not depend on the initial protein concentration of the sample (Kiss et al., 1998).

For the purpose of optimization of TPP systems, different partitioning experiments at various saturations of $(\text{NH}_4)_2\text{SO}_4$ (20%, 30%, 40%, 50%, 60%, 70% and 80%, w/v), crude extract to *t*-BuOH ratios (1.0:0.25, 1.0:0.5, 1.0:0.75, 1.0:1.0, 1.0:1.25, 1.0:1.5, 1.0:1.75 and 1.0:2.0, v/v), pH and temperature are all performed in a stepwise manner. The limits of the two later parameters depend on the targeted macromolecules.

6.1 IMPACT OF AMMONIUM SULPHATE

Prior to the TPP process, different salts ($(\text{NH}_4)_2\text{SO}_4$, potassium sulphate, sodium sulphate, magnesium sulphate, sodium chloride and potassium chloride) that are classified as kosmotropic, chaotropic or neutral can be investigated to achieve high macromolecule recovery (Gagaoua, Hoggas, & Hafid, 2015). Among these, $(\text{NH}_4)_2\text{SO}_4$ was found as the best salt in the TPP process (Chew et al., 2018) that provided the most effective effect on the macromolecule partitioning. The main principle behind the use of salt is 'salting out' which is usually performed to separate proteins based on their solubility in the presence of high salt concentration (Burgess, 2009). $(\text{NH}_4)_2\text{SO}_4$, is the most popular salt used for 'salting out' as it is cheap, readily available, gentle on proteins, stabilizes some proteins, and it has a high solubility (about 3.6M). Additionally, NH_4^+ and SO_4^{2-} are at the ends of their respective Hofmeister series and have been shown to stabilize intermolecular interactions in macromolecules such as protein structures, namely enzymes. Apart from kosmotropy, the effect of the ionic strength of SO_4^{2-} , cavity surface tension enhancement, osmotic stressing (dehydration), exclusion crowding and its binding to cationic sites of positively charged proteins (Fink, 1995) are the additional advantages that prevent protein aggregation/denaturation. Hence, protein precipitation in TPP occurs as a result of two types of sulphate ions: the bound ions which help in cocrystallization of proteins (Conroy & Lovrien, 1992) and the free ions in the bulk solvent that act as kosmotrope.

The peculiarity of $(\text{NH}_4)_2\text{SO}_4$ compared to the other salts can be also viewed in another way. Indeed, it is known that Hofmeister anions like phosphate are not as successful as the sulphate ion because the later remains as an active kosmotrope and a protein cationic site binding anion even at pH less than 6.5, while phosphate does not. Moreover, sulphate binding to cationic sites of protein entities is a function of their isoelectric point, which therefore makes this process highly pH dependent and electrostatic in nature (Lovrien, Goldensoph, Anderson, & Odegaard, 1987). In this regard, $(\text{NH}_4)_2\text{SO}_4$ saturation is of critical importance and plays a major

role in TPP, as it is responsible for protein–protein interaction and precipitation. This is possibly partly related to the nature of the protein itself, as an inverse relationship has been reported between the molecular weight of a protein and the amount of $(\text{NH}_4)_2\text{SO}_4$ required to precipitate the protein (Pike & Dennison, 1989). In this earlier study, the authors used a set of standard proteins with known physicochemical properties to monitor their behavioural changes under various conditions of TPP. Thus, $(\text{NH}_4)_2\text{SO}_4$ must be optimized to obtain the maximum recovery or yield of the desired macromolecules.

Generally, one starts with a minimum salt concentration of 20% (w/v) to optimize the partitioning parameters of the TPP process (Dennison & Lovrien, 1997). As the concentration of the salt 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 the other protein surfaces (Choonia & Lele, 2013; Dennison, 2011; Gagaoua et al., 2014; Wati et al., 2009). However, with an excessive increase in salt concentration, the degree of purification factor and activity recovery of enzymes and proteases might be decreased significantly (Duman & Kaya, 2013; Gagaoua et al., 2014; Gagaoua, Hoggas, & Hafid, 2015). For example, the $(\text{NH}_4)_2\text{SO}_4$ saturation (40%–60%) resulted in the 16.2% yield of nattokinase from *Bacillus natto* NRRL-3666 with a 1.46-fold purification. A further increase in salt saturation (>80%) reduced the yield (0.9%) and purity (0.23-fold) (Garg & Thorat, 2014).

6.2 IMPACT OF *t*-Butanol (CRUDE EXTRACT TO *t*-BuOH RATIO)

As presented earlier, the peculiarity of TPP compared to the conventional ‘salting out’ is the use of *tert*-butanol. *t*-Butanol is the only organic solvent for partitioning in TPP that will be discussed in this chapter, since most of the reports published on TPP have used this solvent because it gives the best results (Chew et al., 2018; Dennison, Moolman, Pillay, & Meinesz, 2000; Gagaoua et al., 2014, 2017; Gagaoua & Hafid, 2016; Gagaoua, Hoggas, & Hafid, 2015). Also, it has been shown since the earlier studies that large quantities of enzymes sustained their activities in *t*-butanol/water mixtures (Tan & Lovrien, 1972), a peculiarity that is confirmed by many studies (Chew et al., 2018; Gagaoua & Hafid, 2016; Nadar et al., 2017).

t-Butanol or *t*-BuOH is a C4 nonionic kosmotrope that is very soluble and miscible in water, but after the addition of enough salt, like $(\text{NH}_4)_2\text{SO}_4$, becomes hydrated and acts as a differentiating solvent. Owing to its larger molecular size and branched structure, *t*-BuOH does not easily permeate inside the folded macromolecules and hence does not cause denaturation. This was supported by X-ray diffraction studies with proteinase K that has shown no presence of *t*-BuOH inside the protein (Singh et al., 2001). At temperatures of 20–30°C, *t*-BuOH imparts significant kosmotropic and crowding effects and enhances TPP (Dennison, 2011). *t*-BuOH is normally completely miscible with water and is much less flammable than hexane, methanol or ethanol, which are used in conventional extraction (Harde & Singhal, 2012).

Also, these organic solvents displayed higher deactivation and lower interfacial precipitation via hydrophobic interactions with proteins (Dennison & Lovrien, 1997). The optimum ratio of *t*-BuOH in the TPP process presumably arises as a result of two factors. If the amount of *t*-BuOH is lower, it does not adequately synergize with $(\text{NH}_4)_2\text{SO}_4$ (Gagaoua & Hafid, 2016). If it is higher, it is likely to cause protein denaturation (Dennison, 2011; Dennison et al., 2000; Mondal et al., 2006). It is noteworthy that increased amounts of *t*-BuOH do not increase the yield of the target macromolecule during the TPP process, because, as the amount of the cosolvent increases, the viscosity of the solution also increases. This increase in viscosity might lead to a decrease in molecular mobility, hence, lowering the interactions of the molecules. For these reasons, the appropriate ratio of *t*-BuOH to crude slurry should be chosen at a constant concentration of salt to set up an optimal TPP separation process. The above clearly showed why *t*-BuOH is considered to be the best organic solvent for TPP as it can easily bind to the macromolecules, hence, increasing their buoyancy and helping efficient separation.

Overall, sulphate ion and *t*-BuOH are known to be excellent protein structure markers or kosmotropes (Dennison, 2011; Dennison & Lovrien, 1997). Kosmotropes, inversely to chaotropic agents, cause water molecules to favourably interact, which also stabilizes intramolecular interactions in the macromolecules, especially proteins (Moelbert, Normand, & De Los Rios, 2004).

6.3 IMPACT OF pH

As mentioned, the pH of the medium is another important parameter affecting the TPP system, as it causes ionization of acidic or basic amino acids present in proteins and influences other aspects of macromolecules (Alvarez-Guerra & Irabien, 2014a; Dennison, 2011; Gagaoua & Hafid, 2016). It was reported that TPP systems usually sharply change around the isoelectric point (*pI*) of macromolecules, mainly with proteins, and the efficiency of the system depends on this parameter notably in the presence of an electrolyte like $(\text{NH}_4)_2\text{SO}_4$. It was postulated that the distribution and partitioning of macromolecules in TPP systems change with pH due to electrostatic interactions between phases and charged macromolecules.

In TPP, and in the case of protein recovery, if the system pH is above the isoelectric point (*pI*) of the protein, hydrophilic amino acid residues are charged negatively and the protein will acquire net negative charge moving it to the bottom of the aqueous phase. Under these conditions, proteins remain in the lower aqueous phase (Fig. 4). However, if the system pH is below the *pI* of the target protein, there is maximum precipitation or accumulation of protein in the interfacial precipitate phase (Gagaoua et al., 2014; Gagaoua & Hafid, 2016; Gagaoua, Hoggas, & Hafid, 2015). This may be due to the development of a positive charge on the surface of the protein at a low pH that facilitates binding of $(\text{NH}_4)_2\text{SO}_4$ to the cationic sites, thus greatly enhancing the effect of electrostatic screening and minimizing the electrostatic repulsion between like-charged protein molecules or protein-stabilized emulsion drops. It has been reviewed that changes in pHs facilitate the change in net charge of target molecules and influence partitioning of proteins

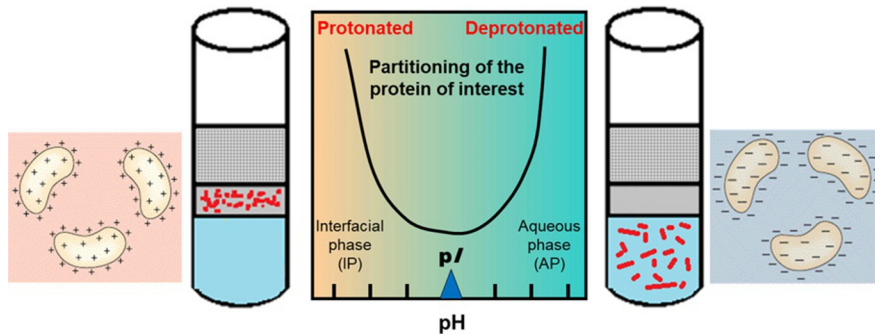


FIG. 4

Basis of macromolecules (protein) partitioning under Three Phase Partitioning system. When the pH of the system is much lower than the pI of the protein, there is protein target accumulation in the interfacial phase (*left*). When the pH of the system is much higher than the pI of the protein, there is protein target accumulation in the aqueous phase (*right*).

(Dennison, 2011). This basis of macromolecule (protein) partitioning under the TPP system is depicted in Fig. 4. Accordingly, it was reported that the solubility of proteins was maximized when TPP was carried out at the pI of the protein (Pike & Dennison, 1989). Hence, in light of the earlier reasons, it is important to investigate cautiously the effects of pH on the TPP process and to define the optimal pH conditions for the partitioning that would enhance both the purification fold and the recovery of the target macromolecules, namely proteins and enzymes.

6.4 IMPACT OF TEMPERATURE

Temperature is another important physical parameter that does not have a great effect on the amount of the recovered macromolecules, but did impact the configuration and overall stability of enzymes and proteases (Chew et al., 2018; Dennison & Lovrien, 1997; Gagaoua & Hafid, 2016). It is hypothesized that the highest levels of the partitioning process operates at low temperatures, a condition which is considered favourable as the heat generated from the solvent or salt precipitation can be dissipated quickly, thus, ensuring minimal protein denaturation (Chew et al., 2018; Gagaoua et al., 2016; Yan et al., 2017).

According to Nadar et al. (2017), at lower temperature (4°C) the recovery yield or activity may be similar to that obtained at room temperature which is postulated to be due to the low movement of particle solvent (Gagaoua et al., 2016; Gagaoua, Hafid, et al., 2015). Other studies showed that a temperature range of 20–40°C may be optimal; however, this is difficult to confirm in light of the complexity of the process parameters involved (Garg & Thorat, 2014; Sharma & Gupta, 2001a). Indeed, at very high temperatures, and apart from the denaturation of proteins, the volatility of *t*-BuOH leads to its low availability during separation, resulting in a reduced synergistic effect with $(\text{NH}_4)_2\text{SO}_4$, hence, producing a lower yield.

For these reasons, the temperature effect at different intervals must be optimized after having validated the parameters described earlier. For example, if we refer to our recent work on cucumisin (Gagaoua et al., 2017), the effect of temperature on the partitioning behaviour increased with an increase in the temperature from 5 to 20°C. The maximum recovery and purification fold of 155%–157% and 4.78–4.82 were found from 10 to 20°C, respectively. The probable reason behind this may be, as already explained, at 10–20°C temperature *t*-BuOH imparts significant kosmotropic and crowding effects and enhances the partitioning process. The decrease in purification factor and activity at temperatures above 25°C was recognized to be due to thermal deactivation of the macromolecule. Thus, it seems that the TPP system would be very adaptable to separate macromolecules from extremophiles, such as molecules of microorganisms from hot sources (Ait Kaki El-Hadef El-Okki, Gagaoua, Bourekoua, et al., 2017; Loureiro, Braia, Romanini, & Tubio, 2017; Omidinia, 2012).

7 VARIANTS OF TPP

Studies on the usage of TPP for the extraction and purification of macromolecules, especially proteins and enzymes, either as a one-step purification methodology or combined with other techniques have been successfully implemented during the last 2 decades (Alvarez-Guerra & Irabien, 2014a; Chew et al., 2018; Gagaoua & Hafid, 2016; Mondal et al., 2006; Nadar et al., 2017; Yan et al., 2017). Furthermore, a two-step TPP was also used for better separation of the phases and to enhance the overall fold purity of recovered macromolecules (Jain et al., 2004; Raghava et al., 2008; Rajeeva & Lele, 2011; Sadaf et al., 2018; Saxena et al., 2007). Most of these protocols showed that TPP extraction is less expensive than chromatographic methods and the number of unit operations involved in the chromatographic protocols was reduced.

To the knowledge of the author, to date, six variants of the TPP system have been reported, which are used for the recovery of different macromolecules from various sources including microorganisms from atypical environments. These variants include macroaffinity ligand-facilitated TPP (MLF-TPP) (Mondal, Sharma, & Gupta, 2003; Mondal, Sharma, Lata, & Gupta, 2003; Sharma & Gupta, 2002a; Sharma et al., 2003a), enzyme-assisted TPP (EA-TPP) (Gaur et al., 2007; Nadar et al., 2018; Varakumar et al., 2017), ultrasound-assisted TPP (UA-TPP) (Avhad et al., 2014; Chougale et al., 2014; Pakhale & Bhagwat, 2016), microwave-assisted TPP (MA-TPP) (Kulkarni & Rathod, 2015; Saifuddin & Raziah, 2008), metal-ion assisted TPP (MIA-TPP) (Gupta, Jain, & Roy, 2002; Roy, Mondal, & Gupta, 2014; Singh & Singh, 2003) and ionic liquid-based TPP (IL-TPP) (Alvarez-Guerra & Irabien, 2014a, 2015; Alvarez-Guerra, Irabien, Ventura, & Coutinho, 2014). These TPP variants were exploited as process intensification tools to improve the extraction yield and purity of different macromolecules as can be seen in Table 1 and Fig. 5. In the following, they are briefly described by giving examples of their uses and the main principles behind each of them.

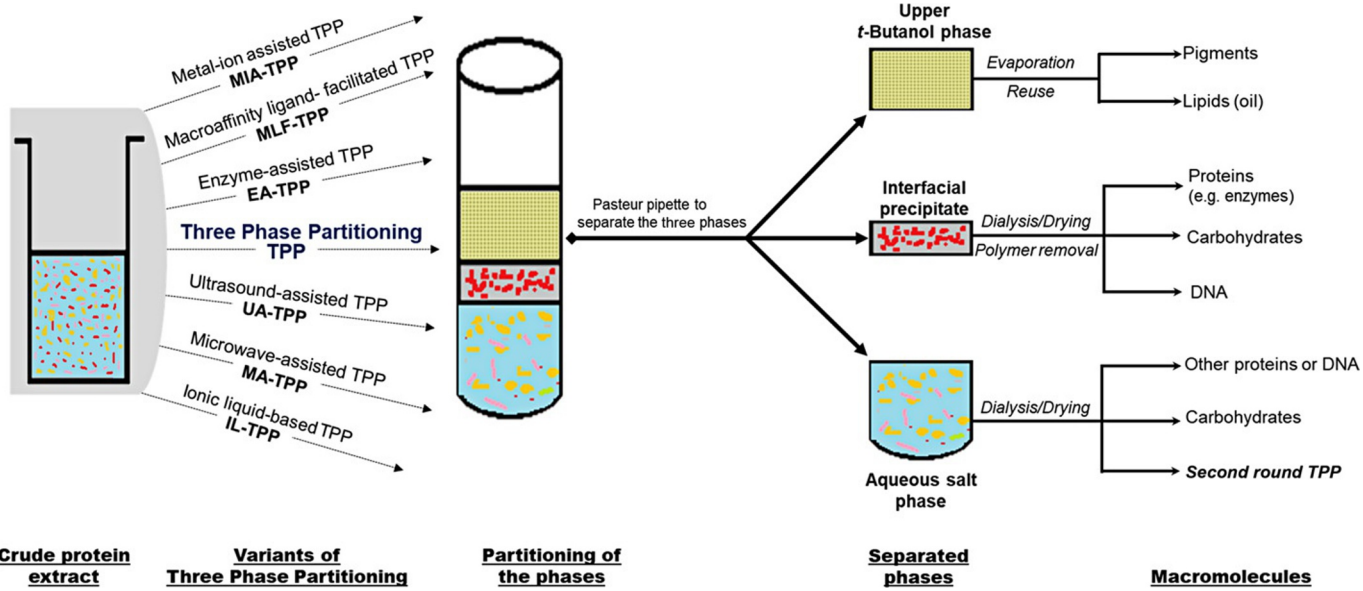


FIG. 5

Overall schematic representation of the different variants of Three Phase Partitioning system applied for the separation and purification of biomolecules.

7.1 MACROAFFINITY LIGAND-FACILITATED TPP (MLF-TPP)

In macro (affinity ligand)-facilitated TPP (MLF-TPP) (Sharma & Gupta, 2002a), the polymer–protein complex is precipitated in a similar way as in TPP (Fig. 5). According to Roy et al. (2014), the complex precipitated as an interfacial layer between upper organic solvent phase and lower aqueous phase, that can be separated, and the purified macromolecule can then be dissociated and recovered from the precipitate. MLF-TPP, as a technique closely related to affinity precipitation, was developed as a general technique of protein separation, which combines the advantages of affinity recovery with those of TPP resulting in a more selective separation of the target protein from crude slurry. It consists of two main steps: (i) the binding of the protein with a suitable smart polymer (Mondal & Gupta, 2006; Roy & Gupta, 2003) and (ii) precipitating the polymer–protein complex as an interfacial layer when subjected to a mixture of $(\text{NH}_4)_2\text{SO}_4$ and *t*-BuOH (Mondal, Sharma, Lata, & Gupta, 2003). The macroligand can bind selectively to the target macromolecule, which allows obtaining the affinity complex as an interfacial precipitate (Roy et al., 2014; Yan et al., 2017). The mixture can be separated into two sections: the *t*-BuOH layer and the remaining dissolved precipitate in the aqueous and salty phase. The smart polymers that are used for MLF-TPP were described as reversible soluble–insoluble polymers that precipitate upon the application of an appropriate stimulus, which may for example involve a change in pH or temperature or the addition of a chemical polymer species (Roy et al., 2014; Roy & Gupta, 2003).

The types of macroaffinity ligands that are used were categorized as naturally occurring polymers such as alginate and its ester constitutes, chitosan and *k*-carrageenan; and synthetic polymers like eudragit S-100, L-100, methylmethacrylates, etc. (for review and detailed protocol of MLF-TPP, see Roy et al., 2014). MLF-TPP showed numerous advantages, such as high selectivity, low cost, ease in scaling up in addition to the elimination of preclarification of crude extracts (Chew et al., 2018; Gautam, Mukherjee, Roy, & Gupta, 2012). MLF-TPP has successfully purified proteins with conformations closer to native structures compared to the simple TPP, and to date it has been implemented to recover numerous enzymes from several microorganisms, some of which are from atypical environments (Gautam et al., 2012; Mondal, Sharma, & Gupta, 2003; Mondal, Sharma, Lata, & Gupta, 2003; Sharma & Gupta, 2002a; Sharma et al., 2003a; Sharma, Roy, & Gupta, 2004). As summarized in Table 1, it has been used to purify several enzymes such as glucoamylase, pullulanase, α -amylase, pectinase, cellulase and xylanase.

7.2 ENZYME-ASSISTED TPP (EA-TPP)

The use of enzymes in food, pharmaceutical and other industries has long been recognized for its effectiveness, both technologically and economically. A comprehensive review (Marathe, Jadhav, Bankar, & Singhal, 2017) has recently detailed the peculiarities and advantages of enzyme-assisted extraction of bioactives which aims to achieve high product yields, reduce by-product formation and avoid severe

operational conditions. To date, EA-TPP was developed for the separation of various biomolecules such as polyphenols, oils, polysaccharides, flavours and colourants (Fig. 5 and Table 1) (for review, see Marathe et al., 2017; Nadar et al., 2018). EA-TPP was extensively used for the recovery of molecules from different plant sources, however, to the knowledge of the author; this promising technique has not yet been applied to macromolecules from microbial sources.

Enzyme-assisted extraction of biomolecules seems to be a potential alternative to conventional solvent extraction methods and is gaining more attention because it is an efficient, sustainable and eco-friendly extraction technology (Nadar et al., 2018; Puri, Sharma, & Barrow, 2012). EA-TPP has so far been tested for extraction of edible oils from *Mangifera indica* with the help of Protizyme™ (Gaur et al., 2007), and the authors obtained a yield of 98% compared to TPP alone (82%) using the same conditions (Sharma, Khare, & Gupta, 2002). Similarly, Harde and Singhal (2012) also used EA-TPP for the extraction of forskolin (diterpene) from *Coleus forskohlii* roots. The extraction yield was found to be increased from 30.83% by TPP to 83.85% by EA-TPP. This technique was further used to recover oils from *Jatropha curcas* L. seed kernels (Shah, Sharma, & Gupta, 2004) and oleoresin from ginger (*Zingiber officinale*) rhizomes (Varakumar et al., 2017). EA-TPP can therefore be a promising approach for the extraction of biofuels, oils, biosurfactants and other value-added products from extremophiles including *Archaea* (Barnard, Casanueva, Tuffin, & Cowan, 2010; Cameotra & Makkar, 1998; Coker, 2016; Derguine-Mecheri, Kebbouche-Gana, Khemili-Talbi, & Djenane, 2018; Kebbouche-Gana et al., 2009; Khemili-Talbi, Kebbouche-Gana, Akmuoussi-Toumi, Angar, & Gana, 2015; Siegert, Sitte, Galushko, & Krüger, 2014; Wentzel, Lewin, Cervantes, Valla, & Kotlar, 2013). For example, TPP was used for the recovery of surfactin (a bacterial cyclic lipopeptide recognized as one of the most effective biosurfactants) from fermentation broths by a hybrid TPP and membrane filtration process (Chen et al., 2008). Moreover, microalgae are also gaining attention due to their ability to produce high amounts of oil or proteins (Halim, Gladman, Danquah, & Webley, 2011), for which EA-TPP was also efficiently applied (Li et al., 2013; Reddy & Majumder, 2014; Waghmare, Salve, LeBlanc, & Arya, 2016).

For instance, it seems that the high cost of the enzyme is the main limiting factor for the use of EA-TPP in large scale. However, the increasing environmental concerns together with the application of TPP and other downstream techniques for the purification of enzymes of interest are likely to make EA-TPP a viable tool in the future for the recovery of the above-described molecules from microorganisms of extreme and atypical environments.

7.3 ULTRASOUND-ASSISTED TPP (UA-TPP)

Ultrasound irradiation has been used in numerous separation protocols to enhance mass transfer during extraction, fermentation, biocatalysis, adsorption, APTS and very recently TPP (Avhad et al., 2014; Dahmoune et al., 2015; Gharat & Rathod, 2013; Guo, Han, Zhang, Wang, & Zhou, 2012; Sharma & Gupta, 2004;

Tavanandi, Mittal, Chandrasekhar, & Raghavarao, 2018). The application of ultrasonic assisted extraction in processing technology is recognized to be of great interest for enhancing extraction of components from different material sources (Vilkhu, Mawson, Simons, & Bates, 2008), including extremophile enzymes (De Castro et al., 2008; de Pascale et al., 2008). In the case of combining ultrasonication with the TPP process, UA-TPP is relatively novel and less explored but seems to be a viable tool as TPP is dependent on the mass transfer phenomenon (Fig. 5 and Table 1). According to Avhad et al. (2014), ultrasonic wave induces formation of gas-filled bubbles and collapse of these bubbles imparts vicious shockwave and mechanical shear to surrounding environments.

The positive effect of UA-TPP compared to conventional TPP is promising as it brings many advantages by reducing the process operating time, increasing the recovery and purification fold. UA-TPP was applied for the extraction of different molecules (Table 1) such as oil from almond, apricot and rice bran (Sharma & Gupta, 2004), oil from *J. curcas* L. seed kernels (Shah et al., 2004), astaxanthin from *Paracoccus* sp. (Chougale et al., 2014), polysaccharides from *Corbicula fluminea* (Yan, Wang, Qiu, Wang, & Ma, 2018) and oleanolic acid and ursolic acid from *Ocimum sanctum* (Vetal et al., 2014). It was further used for the extraction and purification of enzymes such as polyphenol oxidase from waste *Solanum tuberosum* (Niphadkar & Rathod, 2015), Serratiopeptidase from *Serratia marcescens* (Pakhale & Bhagwat, 2016) and a fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672 crude broth (Avhad et al., 2014). In this later study, the authors reported an efficient selective partitioning of the fibrinolytic enzyme in the bottom phase leaving contaminant proteins in the upper and intermediate phases. The authors reported that at optimum conditions, i.e., 80% $(\text{NH}_4)_2\text{SO}_4$ saturation, pH 9.0, broth to *t*-BuOH ratio 0.5 (v/v), TPP achieved a 7.98-fold purification with 45% recovery of fibrinolytic enzyme after 1 h of conventional stirring. However, EA-TPP showed an increase in fold purity of fibrinolytic enzyme from 7.98 to 16.15 at optimized conditions of irradiation with 25 kHz frequency, 40% duty cycle, 150 W rated power during 5 min. Thus, EA-TPP not only decreases the time of operation of TPP but also is able to double the purity.

7.4 MICROWAVE-ASSISTED TPP (MA-TPP)

Microwave-assisted extraction is a technique combining microwave energy and traditional solvent extraction (Dahmoune, Nayak, Moussi, Remini, & Madani, 2015), to heat polar solvents in contact with solid samples and to partition compounds of interest between the sample and the solvent, reducing both extraction time and solvent consumption (Kapoor, Butler, Pandhal, & Vaidyanathan, 2018). Thus, the extraction occurs as a result of changes in the cell structure caused by electromagnetic waves leading to the production of high extraction rates and better results with lower costs. Microwave-assisted extraction offers the possibility for extraction of biochemical components including lipids, pigments, carbohydrates,

vitamins and proteins (Chikhouné et al., 2017; Kapoore et al., 2018; Phongthai, Lim, & Rawdkuen, 2016). Recently, microwave-assisted extraction combined with TPP (MA-TPP) has been used for laccase purification from *Trametes hirsuta* (Patil & Yadav, 2018). The authors reported that there is a significant lowering of processing time (7 min) compared to conventional TPP (60 min). As summarized in Table 1, MA-TPP has been occasionally applied for the effective extraction of lipase from *Candida rugosa* (Saifuddin & Raziah, 2008) and mangiferin from the leaves of *M. indica* (Kulkarni & Rathod, 2015). The study on lipase by Saifuddin and Raziah (2008), using MA-TPP, demonstrated a remarkable increase in the initial reaction rates of transesterification by further 1.6 times, hence giving a combined increase in activity of about 5.4 times. These studies highlighted that the application of microwave irradiation can compete favourably with the conventional TPP techniques, and might be easily adapted for the extraction/purification of bioactives from microorganisms of atypical environments.

7.5 METAL-ION ASSISTED TPP (MIA-TPP)

Immobilized metal affinity chromatography (IMAC), known also as metal affinity-based separation, is the most widely used method to purify proteins according to their affinity to specific metal ions (Gaberc-Porekar & Menart, 2001; Gupta & Roy, 2002). This technique exploits the affinity of (i) proteins containing natural surface-exposed histidine, cysteine and tryptophan residues and (ii) recombinant proteins with engineered histidine tags or histidine clusters, at pH values around neutral to form complexes with chelated metal ions like Zn^{2+} , Cu^{2+} , Ni^{2+} , Cd^{2+} , Hg^{2+} , Co^{2+} and Fe^{2+} (Sarawathy & Ramalingam, 2011). The proteins can then be eluted by reducing the pH, increasing the mobile phase ionic strength or adding ethylenediamine tetraacetic acid (EDTA) to the mobile phase. It is believed that this technique is more suitable for the purification of recombinant proteins as poly-histidine fusions and for membrane proteins and protein aggregates where detergents or high-ionic strength buffers are required (Falcicchio, Levisson, Kengen, Koutsopoulos, & van der Oost, 2014; Karakus, Uslu, Yazici, & Salih, 2016).

Based on these characteristics, TPP has been proposed to be interfaced with a metal affinity-based step to selectively recover proteins with surface histidine residues present at the appropriate distance (Gupta & Roy, 2002; Roy & Gupta, 2002). Among the proteins which have been investigated for MIA-TPP, we found Soybean trypsin inhibitor that contains surface histidine residues (Galaev, Kumar, Agarwal, Gupta, & Mattiasson, 1997; Gupta, Jain, et al., 2002). Using conventional TPP, Soybean trypsin inhibitor was recovered in the interfacial precipitate (using 50% $(NH_4)_2SO_4$ saturation) giving a purification fold of 4.0 (Roy & Gupta, 2002). However, carrying out TPP with 30% $(NH_4)_2SO_4$ saturation left 91% Soybean trypsin inhibitor activity in the aqueous phase. Subjecting the dialyzed aqueous phase of this TPP to a second cycle in the presence of suitable concentrations of Zn^{2+} or Cu^{2+} ions instead of $(NH_4)_2SO_4$, gave considerably enhanced purification

yield recovery. An activity recovery of 66% was obtained with 0.18 mM Zn^{-2} and the protein was purified 12-fold, and with 0.20 mM Cu^{-2} , a 73% activity recovery with 13-fold purification was obtained. MIA-TPP was later used to purify turnip peroxidase in the presence of Cu^{2+} with excellent results like those described in the earlier example (Singh & Singh, 2003).

These studies showed the potential of MIA-TPP to overcome the drawbacks of the chromatographical affinity techniques with great potential for industrial purification of enzymes in large quantities. Therefore, since IMAC is already used for (hyper)thermophilic enzymes, the MIA-TPP technique would be suggested to extract/purify macromolecules from microorganisms that grow in atypical environments that produce proteins with surface-exposed histidine, cysteine and tryptophan residues (Falicchio et al., 2014). Indeed, several quantities of His-tagged (hyper)thermophilic proteins were already purified by the IMAC approach. The technique was mainly applied to purify (hyper)thermozymes belonging to a number of enzymatic classes and subclasses, such as hydrolases (van Lieshout et al., 2012; van Lieshout, Faijes, Nieto, van der Oost, & Planas, 2004), kinases (Verhees et al., 2002), isomerases (Akerboom et al., 2003), carboxylesterases (Levisson, van der Oost, & Kengen, 2009), aldolases (Wolterink-van Loo et al., 2007), dehydrogenases (van der Oost et al., 2001), catalase–peroxidases (Kengen, Bikker, Hagen, de Vos, & van der Oost, 2001) and oxidases (Kengen, van der Oost, & de Vos, 2003).

7.6 IONIC LIQUID-BASED TPP (IL-TPP)

IL-TPP is a novel technique for macromolecule recovery that combines characteristics of IL-ATPS and TPP (for review and detailed protocol, see Alvarez-Guerra & Irabien, 2014a). According to the studies by Alvarez-Guerra's research group, IL-TPP uses the same ionic liquid/salt systems of IL-ATPS; and it promotes the partitioning of the target protein in the liquid–liquid interface in the same way as TPP. Therefore, the performance of IL-TPP is very similar to TPP, but the *t*-BuOH is replaced by the ionic liquid and other salts can be considered besides $(\text{NH}_4)_2\text{SO}_4$. IL-TPP can overcome the main drawbacks of both IL-ATPS and TPP techniques. The accumulation of the protein of interest at the interface makes its recovery easier, and the process is simplified because the back extraction step is not required, so the main disadvantage related to the use of IL-ATPS is avoided (Alvarez-Guerra & Irabien, 2014a). This technique was extensively described in recent years for the concentration and recovery of different protein molecules, but focused on the study of lactoferrin and bovine serum albumin (Alvarez-Guerra & Irabien, 2014b, 2015; Alvarez-Guerra, Ventura, Coutinho, & Irabien, 2014). It has been reported that the recovery of ionic liquid in IL-TPP can be further enhanced by concentrating the salt-rich phase by means of vacuum evaporation (Alvarez-Guerra, Irabien, et al., 2014). The vacuum evaporation can be performed at room temperature and this avoids the degradation of the ionic liquid and protein in the salt-rich phase. On the other hand and as described in Section 1 for ATPS,

IL-ATPS is 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 (Freire et al., 2012; Yan et al., 2017). Further details concerning IL-TPP as an emerging alternative tool for the recovery and purification of macromolecules from extremophiles can be found in the extensive review by Alvarez-Guerra and Irbien (2014a).

8 APPLICATIONS OF TPP AND ITS POTENTIAL USES TO TARGET MACROMOLECULES FROM MICROBIAL OF ATYPICAL ENVIRONMENTS

As stated earlier, TPP is a simple and fast purification technique, found to be very effective for the recovery of a wide range of macromolecule solutions including DNA, and expressed proteins. Even though TPP was first developed as an upstream technique, it has been used for both upstream and downstream macromolecule purification processes and is also commonly used as a one-step, a two-step or a combined purification method with other tools as previously detailed in Section 7 (Chew et al., 2018; Dennison, 2011; Gagaoua & Hafid, 2016; Mondal et al., 2006; Yan et al., 2017). Originally, TPP was used to purify numerous enzymes (Table 1) and its efficiency as an emerging technique for enzyme purification is evident from the reported increase in the total activity of enzymes in several studies (Boucherba et al., 2017; Chew et al., 2018; Dennison, 2011; Gagaoua et al., 2014; Gagaoua et al., 2016; Gagaoua et al., 2017; Gagaoua & Hafid, 2016; Gagaoua, Hafid, et al., 2015; Gagaoua, Hoggas, & Hafid, 2015). In all cases, TPP allowed high recovery and purity levels of the purified proteins and enzymes (Table 1).

Table 1 lists some examples of TPP and its variants that have been used for the extraction of a myriad of macromolecules from different sources including microorganisms of atypical environments. For example, TPP was successfully and widely used to purify enzymes from fermentation broth as well as from natural sources such as α -galactosidase from *Aspergillus oryzae* (Dhananjay & Mulimani, 2008, 2009), inulinase from *Aspergillus niger* (Kumar, Premkumar, et al., 2011) and invertase from *Saccharomyces cerevisiae* (Akardere et al., 2010). Laccase, an enzyme used by food industries for baking, fruit juice and wine processing, was also recovered from different microbes such as submerged cultures of *Ganoderma* sp. WR-1 (Rajeeva & Lele, 2011), *T. hirsuta* (Patil & Yadav, 2018), *Pleurotus ostreatus* (Kumar et al., 2011; Kumar, Sathyaselvabala, Premkumar, Vidya Devi, & Sivanesan, 2012) and *Coriolopsis trogii* (Liu et al., 2015). Other enzymes were also reported such as β -galactosidase from *Lactobacillus acidophilus* (Choonia & Lele, 2013), lipase from *Chromobacterium viscosum* and *Thermomyces lanuginosa* (Kumar et al., 2015; Roy & Gupta, 2005), nattokinase from *B. natto* NRRL-3666 (Garg & Thorat, 2014), superoxide dismutase from *Kluyveromyces*

marxianus (Simental-Martinez et al., 2014), serratiopeptidase from *S. marcescens* (Pakhale & Bhagwat, 2016), α -amylase from *Bacillus amyloliquefaciens*, *A. oryzae* and *Rhizopus oryzae* FSIS4 (Ait Kaki El-Hadef El-Okki, Gagaoua, Bourekoua, et al., 2017; Lovrien et al., 1987; Mondal, Sharma, Lata, & Gupta, 2003), naringinase from *Aspergillus brasiliensis* (Shanmugaprakash et al., 2015), pullulanase from *Bacillus acidopullulyticus* (Mondal, Sharma, & Gupta, 2003) and many others.

Gu et al. showed also the effectiveness of TPP for the extraction of α -galactosidase from *A. niger* by comparing it with ATPS (Gu et al., 2012). The system comprised of PEG/phosphate allowed a high purification factor of up to 3.25-fold with 84.41% activity recovery. The TPP system based on *t*-BuOH and $(\text{NH}_4)_2\text{SO}_4$ exhibited even better purification fold (6.27) with 97.21% activity yield recovery (Gu et al., 2012). As introduced earlier, ATPS which is considered an efficient and economical strategy has already been applied as an aqueous system for the purification of enzymes from extremophiles such as the hyperthermophilic archaeal amylolytic enzyme (MJA1, a thermostable α -amylase) from the hyperthermophile *Methanococcus jannaschii* (Li & Peeples, 2004).

Furthermore, we have recently applied TPP for the recovery of an extracellular thermostable xylanase from the *Bacillus oceanisediminis* strain SJ3 newly recovered from Algerian soil (Boucherba et al., 2017). The results indicated that the xylanase of *B. oceanisediminis* was recovered in the interfacial precipitate phase of TPP system using the optimum parameters of 50% $(\text{NH}_4)_2\text{SO}_4$ saturation with 1.0:1.5 ratio of crude extract:*t*-BuOH at pH of 8.0. The xylanase was recovered with 3.48 purification fold and 107% activity recovery. From further extremophiles, a strain of *Trichoderma citrinoviride* AUKAR04 identified based on the morphological and 5.8S ribosomal RNA sequencing, was found to produce a cocktail of enzymes such as xylanase, carboxymethyl cellulase (CMCase) and β -1,3-glucanase (Periyasamy et al., 2017). These enzymes were partially purified by TPP, which recovered the maximum activities of xylanase (99.8%) with 5.7-fold, CMCase (96.5%) with 5.5-fold and β -1,3-glucanase (98.4%) with 5.6-fold purification. The authors indicated the potential application on bioconversion of lignocellulosic biomass into fermentable sugars by the enzyme cocktail produced by TPP from *T. citrinoviride* AUKAR04 for sustainable production of bioethanol (Periyasamy et al., 2017). From a newly fungal *R. oryzae* FSIS4 strain isolated from the wheat seed cultivated in an arid area from Algerian Sahara, the largest hot desert and the third largest desert in the world (Ait Kaki El-Hadef El-Okki et al., 2017) we have recently recovered using TPP a new thermostable α -amylase (Ait Kaki El-Hadef El-Okki, Gagaoua, Bourekoua, et al., 2017). A purification fold of 14.94 and a recovery yield of 168.8% were obtained. The study indicated clearly that the recovered α -amylase is a potential candidate for future applications in the bread-making industry and in other food biotechnology applications due to its interesting physicochemical characteristics. Similarly, but using MLF-TPP, Mondal et al. purified three α -amylases from wheat germ (55-fold), *B. amyloliquefaciens* (5.5-fold) and porcine pancreas (10-fold) with recovery yields of 77%, 74% and 92%, respectively (Mondal, Sharma, Lata, & Gupta, 2003).

On another hand, the TPP system was used to purify numerous proteases. Proteases, which hold the first place in the world market of enzymes, play an important role in biotechnology. In many sectors and namely food industries, proteases are frequently used in their production steps (Kasana, Salwan, & Yadav, 2011). The bulk of these enzymes comes from microbial sources, and extracellular proteases are of commercial value with multiple applications in various industrial sectors (Gupta, Beg, Khan, & Chauhan, 2002; Gupta, Beg, & Lorenz, 2002). Accordingly, extremophilic microorganisms such as thermophiles are an example of important sources of biodiversity and thermostable proteases, as well as other enzymes of great value (Haki & Rakshit, 2003; Saggi & Mishra, 2017). Among the proteases that were purified by the TPP system, we found the psychrothermotolerant alkaline protease isolated from *Bacillus pumilus* MP27 from Southern ocean water samples (Baweja et al., 2017; Baweja, Tiwari, Singh, Nain, & Shukla, 2016). This protease was partially purified by single-step TPP with purity fold of 16.65 (Baweja et al., 2017). The enzyme was found to be highly stable within a range of temperature and pH, maintaining 52.25% of its activity at 50°C and 92% at pH 12. The protease with a molecular weight of ~53kDa exhibited an exceptional activity along with tested detergents, showing 98% stability with SDS (10mg/mL) and ~99% stability with Tide detergent (7mg/mL). As the enzymes from cold-adapted organisms are generally labile under ambient conditions, their purification was efficiently and rapidly done using a gentle separation technique by applying the aqueous separation techniques described earlier (Asenjo & Andrews, 2011; Yan et al., 2017).

TPP also provides a reliable and an efficient alternative for the separation of various carbohydrate polymers with a simultaneous useful modification in their characteristics, extraction of oils, pigments and small organic molecules. This has been extensively discussed earlier for the different TPP variants including the proposals to be used for molecules from microorganisms of atypical environments. Indeed, a newly halophilic *Bacillus subtilis* subsp. *spizizenii* isolated from the Sambhar Salt Lake, Rajasthan, has been very recently used to extract by the means of TPP a bioactive constituent responsible for antibacterial and antifungal activities against numerous test microorganisms (Yadav et al., 2017). The recovered bioactive constituent (not yet identified and described as a compound with molecular ion of m/z 301.14) showed activities against *Proteus vulgaris*, *B. subtilis*, *A. niger*, *Rhizopus oligosporus* and *Penicillium chrysogenum* (Yadav et al., 2017). The authors reported that the compound has very high antimicrobial activity of 35 mm zone of inhibition against *B. subtilis*, exhibiting 1.75 times more antibacterial activity.

Following the same efficiency, TPP has been very recently employed to extract and separate an antioxidative polysaccharide–protein complex (PSP) from *C. fluminea* (Wang, Qiu, Wang, Ma, & Yan, 2017). The authors working under the following optimal TPP conditions: 20% (w/v) ammonium sulphate concentration, 1.5:1.0 (v/v) *t*-butanol to crude extract ratio, 30min and 35°C obtained a high extraction yield (9.0%) compared to conventional enzyme extraction (7.6%). The authors reported that the antioxidant activities of PSP were more evident than those of enzyme–PSP *in vitro*.

Finally, TPP has been successfully applied as a rapid purification method to extract DNA from pork meat (Janosi & Szamos, 2001), meat matrices containing soybean flour (Ujhelyi, Gelencser, & Szamos, 2011), wheat (Szamos, Aubrecht, & Gelencser, 1998) and *Escherichia coli* (Shabani, 2009).

And last but not least, TPP was applied in further applications related to the construction of biosensors (Adanyi, Szamos, Szabo, & Varadi, 1999), development a putrescine biosensor based on putrescine oxidase from *Micrococcus rubens* (Boka, Adanyi, Szamos, Virag, & Kiss, 2012) and for recombinant green fluorescent protein (GFPuv) extraction from *E. coli* (Jain et al., 2004; Vessoni Penna, Ishii, Cholewa, & de Souza, 2004). In other studies, the TPP system was efficiently adapted as a tuning permeabilization of microbial cells (Raghava & Gupta, 2009). In the work of these authors, it has been shown that a preincubation step in which cells were exposed to *t*-BuOH was found to tune the selectivity of permeabilized cells of *Thermus thermophilus*, *S. cerevisiae* and *E. coli*.

CONCLUSIONS

In this chapter, I showed that alternative bioseparation technologies, known as aqueous recovery systems, are making inroads as emerging tools for the separation of different macromolecules from a myriad of biological sources including microorganisms from atypical environments. With the discovery of extremophilic microorganisms which are able to grow in a broad variety of conditions, and more particularly the last 3 decades have seen the isolation of many novel hyperthermophilic, halotolerant, alkaliphiles, acidophiles, psychrophiles and oligotrophs species (Dopson, Ni, & Sleutels, 2016; Ravot, Masson, & Lefèvre, 2006; Shrestha et al., 2018), microbiologists quickly understood the strong potential of fast and versatile aqueous systems for prepurification, extraction or purification of their biomolecules. To date, TPP techniques are not extensively applied to separate molecules of interest from extremophiles, probably due to the lack of knowledge of the TPP by microbiologists that were the first to identify the new microorganisms. Consequently, the development of reliable, simple and fast purification techniques is highly welcomed both at the research and at industrial levels. From the above-described peculiarities of TPP, I recommend this emerging separation technique as an early-stage exploratory research tool for the screening of macromolecules (e.g. enzymes) and bioactives from extremophiles and other microbial sources. Moreover, to fulfil the demand of a growing market for industrial enzymes or other bioactives, TPP and its variants can be easily used as innovative downstream methods for purification of catalytically active enzymes, especially thermozyms. TPP is sufficiently suitable for the recovery of proteins at high levels of purity and stability to accommodate the purification of biomolecules from extremophiles that possess extraordinary properties that function under severe conditions. Thus, the development of rapid and straightforward techniques such as TPP for the extraction of these macromolecules will certainly be beneficial in terms of reducing operating cost and simplifying the techniques required to obtain the targeted molecules.

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