Yau *et al. Bioresour. Bioprocess.* (2015) 2:49
DOI 10.1186/s40643-015-0078-0

Bioresources and Bioprocessing
a SpringerOpen Journal

REVIEW

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Current applications of different type of aqueous two-phase systems

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Abstract

In recent year, aqueous two-phase system (ATPS) has become a proven tool used in separation and purification technology. The application of ATPSs in clarification, partitioning and partial purification of biomolecules and bioproducts had showed the rapid development. This method is able to give high recovery yield and high purity in a single step. The ATPS shows characteristics of high selectivity and is easily to scale up. Therefore, ATPS offers an attractive alternative that meets the requirements of the high demand in industry processes and it is also beneficial in terms of economic and environmental protection. In the past, a lot of works and researches have been done in order to develop feasible separation processes using different types of ATPSs and their applications in numerous product separations. This paper aims to review on the recent literature works in the development of different type of ATPSs and their applications in novel separations and purifications of biomaterials.

Keywords: Aqueous two-phase system, Bioproducts recovery, Partitioning, Purification, Recovery yield

Background

Aqueous two-phase system (ATPS) is a liquid–liquid fractionation method. The method is based on incompatibility of two aqueous solutions such as a polymer/salt system [e.g. polyethylene glycol (PEG) and potassium phosphate], a polymer/polymer system (e.g. PEG/dextran) (Show et al. 2013; Rosa et al. 2010), an ionic liquid (IL) and a salt system, and a low molecular weight alcohol and a salt system (Albertsson 1987; Ruiz–Ruiz et al. 2012). Furthermore, micellar and reverse micelle ATPS can also be formed with ionic and/or non-ionic surfactants solution (Ruiz–Ruiz et al. 2012). ATPS can be modified with affinity ligands, which is able to increase the recovery yields and purification folds significantly (Ruiz–Ruiz et al. 2012).

An ATPS, which can provide the non-toxic and low operational cost isolation process besides fast and large-scale purification, has become an ideal technology for separation process (Tang et al. 2014). The ATPSs can be used as a one-step process that allows the removal of contaminant components and at the same time, separating out the desired target products. The separation is based on the selective partitioning of the target products between the two phases (Rosa et al. 2010). Basically, the separation is dependent on the parameters which are related to the system properties. The ATPSs have been successfully applied in the downstream recovering of biopharmaceutical products by manipulating the complexity of the physical and chemical interactions involved in the partitioning process (Rosa et al. 2010).

The design of experiment is a very useful method in finding the optimal purification conditions. This method also can be used to analyse the influence of different parameters involved in the purification process and to evaluate the interactions between the parameters (Rosa et al. 2010). Furthermore, an advanced screening technique that allows a fast evaluation of parameters dependencies and robustness can be employed to establish the optimal conditions to recover the target molecules effectively (Rosa et al. 2010). Benavides, Rito-Palomares (2008) and Rito-Palomares (2004) have suggested a useful strategy for the development of aqueous two-phase extraction (ATPE). Generally, this strategy is divided into

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four main stages. The first stage is initial physicochemical characterisation of the feedstock, followed by second and third stages where the type of ATPS and system parameters are selected, respectively. The final stage is to evaluate the effect of process parameters on the product recovery and purity (Rosa et al. 2010). The applications of ATPS containing different phase combinations have been reported by Chen et al. (Chen et al. 2014), including the separation and purification of various biological products (Chen et al. 1999), metal ions (Rogers and Bauer 1996), antibodies (Azevedo et al. 2009), antibiotics (Jiang et al. 2009), small organic species (Willauer et al. 2002), nano- and micro-solid particulates (Helfrich et al. 2005) and also as green media (Chen et al. 2005). Different type of APTSs that have been applied into novel partitioning and recovery of various products in different fields will be further reviewed and discussed in this paper.

Applications of ATPSs

Application of polymer/polymer ATPS

A new method of in situ product recovery called extractive bioconversion has been introduced. This technique combines the product formation and separation into single-step process and concurrently, removes the products from the biocatalysts (Zijlstra et al. 1998; Ng et al. 2013). These biocatalysts can be reused for the product recovery from the phase separation of ATPS (Zijlstra et al. 1998; Ng et al. 2013). By incorporating ATPS into extractive bioconversion, the yield of recovered product in enzymatic synthesis can be improved. The product degradation and inhibition which normally happen during the conventional product recovery can be eliminated by rapid removal of the product using extractive bioconversion in ATPS (Ng et al. 2013; Daugulis 1988; Freeman et al. 1993). This method has been demonstrated by Ng et al. (2013) in investigating the synthesis and recovery of cyclodextrins (CDs) from *Bacillus cereus* cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) (Lin YK et al. 2015). An extractive bioconversion in ATPS consisted of 7.7 % (w/w) PEG 20,000 and 10.3 % (w/w) dextran T500 with volume ratio (VR) of 4.0 were selected as the optimum recovery system of CDs. The enzymatic conversion of starch is occured in dextran-rich bottom phase of system. The CDs produced from the systhesis were transferred to the PEG-rich top phase. Higher partition coefficient of CDs was achieved by removing the top phase product and adding starch every 8 h to repeat the CDs systhesis process. The experiments showed an average total CDs concentration of 13.7 mg/mL (composed of 4.77 mg/mL of α-CD, 5.02 mg/mL of β-CD and 3.91 mg/mL of γ-CD) was effectively recovered in the top phase (Ng et al. 2013).

In year 2014, a very impressive new finding in an attempt to remove the metallic single-walled carbon nanotubes (SWCNTs) (Tang et al. 2014) from semiconducting SWCNTs by using a polymer/polymer ATPS has been reported Tang et al. (2014). The rolled up graphene sheets of a nanotube structure is strongly affected by its electrical properties in electronic applications. The electrical conductivity of SWCNTs can be metallic or semiconducting. To separate the metallic SWCNTs from semiconducting SWCNTs, the PEG is dissolved in *N*-methylpyrrolidone (NMP) while dextran is dissolved in water with 1 % cetyltrimethylammonium bromide (CTAB). NMP and cationic surfactant CTAB are added to the system to disperse SWCNTs, meanwhile dextran will wrap around a SWCNT. CTAB is an amine-bearing surfactant. The selective adsorption of amine onto semiconducting SWCNTs is applied based on the affinity of CTAB on semiconducting SWCNTs and reverse micellar effect. The study reveals that dextran-rich bottom phase contains more semiconducting species while metallic SWCNT is more abundant in the PEG-rich top phase. The finding was proven by infrared spectroscopy analysis where CTAB only present in the dextran-rich bottom phase and metallic-SWNCT can be recovered from dextran-rich bottom phase.

Application of polymer/salt ATPS

The improvement of upstream technologies in manufacturing of biopharmaceutical products has raised the demand and interest to develop a cost-effective and efficient downstream separation technology. Large-scale ATPE is becoming an essential tool for purification of biopharmaceutical products such as interleukin, human growth hormone (hGH), insulin-like growth factor and monoclonal antibodies (Rosa et al. 2010).

The polymer/salt ATPS have been used to purify the interleukin-18 binding protein (IL-18BP) from serum-free Chinese hamster ovary (CHO) cells supernatant. IL-18BP is an important regulator of immune system. For instance, the experiment conducted by Kornmann, Baer (2008) shows that the PEG/sulphate ATPS is an ideal system where the IL-18BP was successfully partitioned to the polymer-rich phase at 2 mL scale in the first cycle. The experiment continued with 10 mL scale at the second cycle to find the optimum pH and concentrations. These were important to obtain the highest recovery and purity of IL-18BP from serum-free CHO cells supernatant (Rosa et al. 2010). The results showed that the best performing ATPS was composed of 11.25 % PEG 10,000 (w/w)/11.25 % Na_2SO_4 (w/w) at pH 5. The IL-18BP was recovered up to 98 % in the PEG-rich phase with a final purity of 92 % (Rosa et al. 2010). At the same time,

Kornmann, Baer (2008) also assessed the purification of IL-18BP directly from crude unclarified harvest. The process performance was found to be insignificantly influenced by the presence of cells. The optimised ATPS had been scaled up to 100-fold and showed no significant difference in process performance. The final purity of 86 % of IL-18BP had recovered (Kornmann and Baer 2008).

An ATPS which consisted of PEG 4600/ammonium sulphate has been developed to recover and purify the recombinant growth hormone antagonists (GHA) from *Escherichia coli* cells homogenate (Rosa et al. 2010). GHA is an antagonist for hGH. Initially, the extraction was performed at 30 g-scale with 8 % (w/w) PEG 4600 and 10 % (w/w) ammonium sulphate. Then, the extraction was followed by adding 40 % (w/w) of PEG solution to bottom phase to extract the remaining GHA. The results showed that 89 % of GHA in the PEG-rich phase was recovered. By applying Alfa Laval LAPX 202 continuous disc stack centrifuge, the process performance was able to scale up to about 1000-folds. All the GHA has been recovered in PEG-rich phase after two stages of extraction process in this modified centrifuge which function as a purifier for continuous processing (Rosa et al. 2010).

In order to supply a safe and biologically active form of therapeutic protein, a selective removal of process-related impurities are required. The development of the cost-effective and non-chromatographic separation technique such as ATPS has been seen as a potential alternative (Rosa et al. 2010; Azevedo et al. 2009; Asenjo and Andrews 2011; Bhambure et al. 2013). A novel aqueous two-phase-assisted platform for the efficient removal of process related to impurities associated with *E. coli*, namely host cell proteins (HCP) and nucleic acids (DNA) have been conducted by Bhambure et al. (2013). The purified granulocyte colony stimulating factor (GCSF) has chosen as model biotherapeutic protein product in this study. The study observed that HCP which was produced by *E. coli* DH5 alpha null cells are preferentially partitioned (Lan et al. 2013) in the salt-rich bottom phase in 8 % PEG 6000– 2 % sodium sulphate (system I) and 10 % PEG 6000–11 % potassium phosphate (system II). The same study also observed that nucleic acids (DNA) are preferentially partitioned to the salt-rich bottom phase too (Bhambure et al. 2013; Duarte et al. 2007; Luechau et al. 2009a; 2009b). Meanwhile, the purified GCSF protein was found to partition in the PEG-rich top phase. Product recovery for system I was 25.1 $\pm$ 2.3 %, whereas and for system II was 30.6 $\pm$ 0.75 %. The results from analysis showed that 99.5 % of product has been recovered with the concentrations of HCP and DNA that less than 100 ppm and 10 mg/mL were obtained, respectively (Bhambure et al. 2013).

A study of the recovery and partial purification of fibrinolytic activities from oven-dried and freeze-dried basidiocarps of *Auricularia polytricha* has been conducted by Mohamed Ali et al. (2014). Fibrinolytic activity is defined as the ability of proteolytic enzymes such as nattokinase, streptokinase or urokinase to dissolve the fibrin in blood clots. These proteolytic enzymes are often used as pharmaceuticals to treat cardiovascular disease and stroke. Both fibrin plate assay and Folin-spectrophotometric method were used to assess the effect of processing methods on fibrinolytic activity. The fibrinolytic activities of freeze-dried *A. polytricha* basidiocarps' extract exhibited a higher fibrinolytic activity of 3.88 U/mL compared to the oven-dried one (2.19 U/mL). The PEG 8000 and phosphate-based ATPS was employed to further concentrate and recover fibrinolytic crude enzyme. All the system parameters including the molecular mass and concentration of PEG, concentration of salt and crude and pH was evaluated in order to maximise the recovery of the fibrinolytic activity of *A. polytricha*. With a PEG 8000 20.0 % (w/w):phosphate 11.6 % (w/w) VR of 1.5, at pH of 7.0 together with 25 % (w/w) of crude extract from freeze-dried *A. polytricha*, sporocarps have shown a purification fold of 7.01-fold. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) has been conducted to characterise the fibrinolytic enzymes. In fibrin plate assay, the crude and concentrated fibrinolytic enzyme showed positive result for fibrinolytic activities. The enzyme has a molecular weight of 66 kDa based on SDS-PAGE result (Mohamed Ali et al. 2014).

The use of poly (ethyleneglycol)/poly (acrylate)/salt system has been successfully explored in the purification of protein and plasmid DNA. This application has become the most recent advance and alternative method in isolation of small DNA fragments that generated from polymerase chain reaction (PCR) (Matos et al. 2014). The main problem of PCR is to obtain the high purity of PCR product as contaminants such as enzymes or larger DNA molecules will be present in final product. The use of commercial kits in purification of PCR products are often result in low recovery which will cause the problem on subsequent steps in molecular biology work (Chen and Thomas 1980; Bellot et al. 2011). The research showed that ATPS method can be selected to isolate the DNA molecules larger than 5000–7000 bp. The PCR products showed the strong partition at between the phases in a two-step extraction process. The DNA is partitioned in PEG-rich phase and the impurities will be partitioned to poly (acrylate)-rich phase at primary extraction. The PEG-rich phase from primary extraction will be used to form the PEG/salt two-phase system in secondary (back) extraction. Finally, the purified target DNA will

be strongly partitioned to salt-rich phase and the phase is almost free polymer. The system proven the fast and effective tool in purified the small DNA molecules that less than 4000 bp and able to obtain the high yield (Matos et al. 2014).

Application of thermo-separating polymer ATPS

The application of thermo-separating polymers in forming ATPS has deeply been discussed (Ng et al. 2012). Thermo-separating copolymers were firstly discovered in early 1990s which consisted of ethylene oxide (EO) and propylene oxide (PO) that are able to form ATPS with dextran (Berggren et al. 1995; Johansson et al. 1999). By heating up an aqueous solution above the cloud point (lower critical solution temperature, LCST), the linear and non-ionic EOPO random copolymers will be thermo-separated into two phases from aqueous solution (Ariff et al. 2011; Walter 1986; Jönsson and Johansson 2003; Johansson et al. 1997; Pereira et al. 2003). In this system, the desired target protein is separated to the EOPO-rich top phase at the primary ATPS. Once the temperature increases above the LCST, the EOPO-rich top phase will be removed from the primary system to form the secondary ATPS (Show et al. 2012). A target protein will be partitioned to the top phase, leading the formation of concentrated EO/PO solution at the bottom phase after the secondary ATPS is formed (Show et al. 2011). Since the polymers and salt component can be recycled and reused in subsequent or new primary ATPS extraction (Johansson et al. 1999; Jönsson and Johansson 2003; Johansson et al. 1997), the cost of purification process can be significantly reduced, and at the same time, the environmental pollution can be minimised by reducing the use of harmful organic solvent and salts (Chen et al. 2014). A typical example of the application of thermo-separating polymer ATPS is the use of an EOPO/salts ATPS to extract lysozyme from hen egg white (Dembczynski et al. 2010) and to recover CGTase of *B. cereus* (Ng et al. 2012).

Show et al. (2011) developed an aqueous two-phase flotation (ATPF) (Lee et al. 2015) that formed by EOPO copolymer and ammonium sulphate to recover the *Burkholderia cenocepacia* strains ST8 lipase directly from fermentation broth (Show et al. 2012). In this study, ATPF combines solvent sublation and ATPE system (Dembczynski et al. 2010; Pei et al. 2009), which is based on the interaction process between the hydrophilic group (hydroxyl or glucosan) in surface-active compounds and the hydrophobic group (phenyl or alkyl) in water that are adsorbed to the bubbles surfaces of an ascending nitrogen gas stream. The nitrogen gas bubbles will then dissolve in the top polymer layer of the solution. The average separation efficiency is 76 % and purification fold is 13 % higher under the optimal conditions. The study also indicated that 75 % of EOPO polymers had been recovered (Show et al. 2011). By using this ATPF, *B. cepacia* lipase was satisfactorily purified in single downstream processing step. This technique also obtained a high concentration coefficient, easy separation and operation, low usage of organic solvent, high separation efficiency and low impact to environment (Show et al. 2011).

The thermo-separating polymer ATPS study was extended to investigate the feasibility of recovering lipase of *B. cenocepacia* strain ST8 and recycling the phase components in ATPS. Lipase (Tan et al. 2015) was successfully recovered from the polymer-rich top phase in the system of EOPO 3900 with 48.5 % (w/w) tie-line length, VR of 2.3 and 20 % (w/w) of crude load at pH 7 in primary ATPS (Show et al. 2012). In secondary system, the concentrated solution of EOPO copolymer was recovered in the bottom phase, while lipase was collected at the top phase. The aqueous salt solution from the primary system will then be mixed with the concentrated polymer solution from the secondary system to form a new ATPS system. The result showed an average purification factor of 14 with the yield as high as 99 % (Show et al. 2012).

In another work, EOPO and phosphate is used to develop a new ATPS to separate and recover the ciprofloxacin (CIP), a type of fluoroquinolone antibiotic. The extraction efficiency of CIP is 97.7 % by using 3.0 mL of 80 % (w/w) EOPO and 7.0 mL of 55 % (w/w) K_2HPO_4 at pH 11 (Chen et al. 2014). Meanwhile, an CIP extraction efficiency of 85.6 % is achieved in the second extraction. The phase-forming components are able to recycle and reuse for not more than two times (Chen et al. 2014). This new method is successful in determining the CIP in real samples analysis of milk, egg and shrimp with 6.8 mg/g as limit of detection (LOD) in HPLC–UV detection system (Chen et al. 2014).

An ATPS with the combination of EO50PO50 containing 50 % (w/w) EO and 50 % (w/w) PO, and K_2HPO_4 was used to extract lysozyme from hen egg white. The experiment was performed over four successive extractions in two stages by using the recovery and recycling of phase components. The primary two-phase system of 40 % EO50PO50 and 10 % K_2HPO_4 (w/w) was formed at the first stage. The lysozyme partitioned to the top copolymer-rich phase and the contaminant proteins still remained in the bottom phosphate-rich phase at this stage (Dembczynski et al. 2010). The secondary two-phase system was composed of a concentrated copolymer solution and a lysozyme solution after induced with temperature at second stage. The specific activity of

lysozyme obtained from these four successive extractions was within the range of 38,438–42,907 U/mg of protein (Dembczynski et al. 2010).

Application of alcohol/salt ATPS

The alcohol/salt ATPS is formed by mixing two immiscible phases which are composed of alcohol and salt solutions. By removing the alcohol using evaporation, the target protein can be easily extracted and recovered (Guan et al. 1996; Tianwei et al. 2002). The alcohol/salt ATPS has less toxicity impact to the environment and is cheaper (Zhi and Deng 2006) compared to the conventional ATPSs. The alcohol/salt ATPS have been used to purify the intracellular human recombinant interferon-α2b (IFN-α2b) from *E. coli*. IFN-α2b purification by nine biphasic systems with combination of alcohol-based top phase (ethanol, 1-propanol and 2-propanol) and salt-based bottom phase (ammonium sulphate, dipotassium hydrogen phosphate and monosodium citrate) were studied by Lin et al. (2013). The optimum conditions for the purification of IFN-α2b was achieved in ATPS composed of 18 % (w/w) of 2-propanol and 22 % (w/w) of ammonium sulphate in 1.0 % (w/w) of sodium chloride. The purification factor of IFN-α2b obtained is 16.2 with the yield of 74.6 % (Lin et al. 2013).

An alcohol/salt-based ATPS was also applied to purify serine proteases from mango (Mangifera Indica Cv. Chokanan) peel by Amid et al. (2012). In this experiment, different types and concentrations of alcohol (e.g. 1-propanol, 2-propanol, and ethanol) and different types of salt (e.g. sodium citrate, potassium phosphate, and ammonium sulphate) were used to investigate their effects on the purification and selective separation of serine protease at different pH and different concentration of NaCl. The serine protease purification achieved a partition coefficient of 64.5 and a selectivity of 343.2 by employing an ATPS of 16 % (w/w) of 2-propanol, 19 % (w/w) of potassium phosphate and 5 % (w/v) of NaCl at pH 7.5. The study also reported that 96.7 % of serine protease can be recovered with a purification factor of 11.6 (Amid et al. 2012).

An innovative method named as microwave-assisted aqueous two-phase extraction (MAATPE) has been formed by integrated between ATPE that had a high electric constant and demixing effect with high efficiency of microwave-assisted extraction (MAE). This is a first novel MAATPE technique developed by Zhang et al. (2015) which has been applied in extraction of alkaloids from *Sophora flavescens* Ait. The ATPS which consists of ethanol/ammonia sulphate was selected as it showed the low viscosity, easy demixing, solvent recycling, environmental-friendly process compared to other phase-forming components and surfactants which are mostly high viscosity and will caused the difficulty to form transparent solutions in the preparation of ATPS. The response surface methodology is used to optimise the experimental conditions. The final optimised conditions were determined at solvent-to-material ratio 60:1, temperature 90 ℃, extraction time 5 min and microwave power 780 W for the ATPS composed of 28 % (w/w) of ethanol and 18 % (w/w) of $(NH_4)_2SO_4$. The MAATP showed the less impurities with a better yield of 63.78 ± 0.45 mg/g and a higher recovery (92.09 ± 0.14 %) are obtained in the extraction and purification of alkaloids. At the same time, the MAATPE proven can improve the demixing and mass transfer from solid–liquid extraction to liquid–liquid extraction with the interaction of microwave and ATPS (Zhang et al. 2015).

Application of aqueous two-phase affinity partitioning (ATPAP)

Aqueous two-phase affinity partitioning (ATPAP) is formed when at least one phase forms component of the system is chemically modified by attaching an affinity ligand which is specific to the target molecule (Ruiz–Ruiz et al. 2012). In this system, a selective interaction occurs between the specific ligands in one of the polymeric phases of system and the partition of the desired protein such as cellular fragment or biomolecule towards the modified polymer-rich phase can be achieved (Cordes et al. 1987). Alternative, by adding the free ligands to a conventional ATPS, a partition shift of the desired molecule also can be induced without the chemical modification of the phase-forming component in ATPAP (Azevedo et al. 2009). In 1975, the selective partition of S-23 myeloma proteins in a modified dextran–polyethylene oxide (PEO) of ATPAP system has been investigated by Flanagan and Barondes (1975). Four additional parameters in ATPAP, which must be considered to optimise ATPAP partitioning (Ruiz–Ruiz et al. 2012), have been identified including: 1) pH, 2) ionic strength in polymer/polymer or polymer/salt systems, 3) nature and concentration of affinity ligand and 4) concentration of modified polymers.

Rosa et al. (2007) have conducted a study on the partitioning of human immunoglobulin (IgG) in a polymer/polymer and polymer/salt ATPS with the use of functionalised PEGs. The work aims to compare the partitioning of human IgG in polymer/polymer and polymer/salt system using several different ligands, and to investigate the respective selectivity of these ligands to the human IgG. The hydrophobic, electrostatic and biospecific effects through interaction of ligands with human IgG are the basis factors to be considered when selecting the suitable ligands. The different chain lengths of PEGs can be functionalised with either acidic or basic end groups

(Rosa et al. 2007). Initially, the selectivity studies were conducted with pure IgG using the non-functionalised systems. The results showed that the target protein is partitioned to bottom phase. Afterwards, the systems containing pure proteins, an artificial mixture of proteins and a CHO cells supernatant were used to perform the selectivity tests with the most suitable ligands. The results showed that PEG/phosphate system was not desirable for the affinity partitioning of IgG. The diglutaric acid functionalised PEGs (PEG–COOH) were chosen in this study and it exhibited great affinity to IgG where all IgG were completely recovered in the top phase by employing 20 % (w/w) of PEG 150–COOH and 40 % (w/w) PEG 3350–COOH in PEG/dextran system. The result showed that with the application of artificial mixture of proteins such as human serum albumin (Chow et al. 2013) and myoglobin, PEG 3350–COOH did not exhibit affinity to IgG. However, IgG was able to recover with a yield of 91 % in PEG 150–COOH system. The best recovery yield of 93 %, a selectivity of 11 and a purification factor of 1.9 of the IgG from CHO cells supernatant were achieved in a PEG/dextran system when PEG 150–COOH was used. Furthermore, a 60-fold increase in selectivity was obtained when this functionalised PEG was added to the ATPS compared to the systems without functionalised PEG. In other work, triazine-based IgG affinity ligands coupled to the PEGs with carboxylic acid end groups were chosen because the ligands are expected to mimic the effect of the Cl^- ion and are able to selectively extract IgG to the top phase (Rosa et al. 2007). The modified triazines show affinity towards IgG which observed by Teng et al. (1999) have promoted several of PEGs functionalised with triazine ligands (Rosa et al. 2007).

An attempt to optimise the affinity partitioning conditions of papain in ATPS was carried out by Ling et al. (2010). Papain (EC3.4.22.2) is a cysteine from latex Carica papaya and it is widely used as industrial enzyme (Ling et al. 2010; He et al. 1082). In this system, reactive red 120 was added as free affinity dye ligand. Five parameters including pH, concentrations of ammonium sulphate, sodium chloride, dye and PEG, which affect the papain partitioning in ATPS, were tested by using response surface methodology. The results revealed that the optimum condition of the system was determined as PEG of 0.102 g/mL, sodium chloride of 25 mg/mL, ammonium sulphate of 0.17 g/mL, reactive red 120 of 4.5 μg/mL at pH 8.0. A ratio of specific activity of papain partitioning between top and bottom phase, KU, value is 1.92 with a recovery yield of 50.6 %.

Application of ionic liquid-based ATPS

Ionic liquids are potential green solvents to replace the existing volatile organic solvents (VOCs). Typically, ILs and ionic molten salts (melting point below 100 ℃) are carbon compounds containing one nitrogen atom which have positive charge (imidazolium or pyridinium) by which the organic cation is counter-balanced by an organic or inorganic anion (He et al. 1082). Imidazolium- and pyridinium-based ILs were reported to have highest UV–Vis absorption wavelengths at 210 and 264 nm, respectively, and it is an useful information in determining the concentration of ILs (He et al. 1082). ILs exhibit very low vapour pressure, excellent solvation quality, variable viscosity range and thermal stabilities (Anderson et al. 2002). Up to now, ILs have been widely used in the applications of synthesis (Fukushima et al. 2003), catalysis (Welton 1999), chromatographic separation (Ding et al. 2004; Vaher and Koel 2005), extraction processes (Carda-Broch et al. 2003; Liu et al. 2003), and mass spectrometry analysis (Mank et al. 2004) in chemistry, biotechnology and environmental engineering field. Recently, a new IL/salts ATPS system was developed to recycle, metathesis and determine the distribution ratios of short-chain alcohols (He et al. 1082).

The study done by He et al. (1082) was aimed to develop an environmentally friendly extraction method based on IL/salt system as a new pretreatment procedure to couple with a reversed-phase high-performance liquid chromatography (RP-HPLC) for simultaneous concentration and analysis of testosterone (T) and epitestosterone (ET) in human urine (Antov et al. 2006). A hydrophilic IL, 1-butyl-3-methylimidazolium chloride [(C4mim)Cl], has been selected as the phase-forming IL to study the phase characteristics of ATPS such as phase diagrams and effect of salts (He et al. 1082). By using 3 mL of urine, the experiment began with a single hydrolysis, deproteinisation and extraction step. Finally, the experiment was continued by direct injection of the IL-rich upper phase into HPLC system for further analysis. The method has been successfully analyse the T and ET in human urine with a detection limit of 1 ng/mL and linear range of 10–500 ng/mL for both compounds by optimising the extraction conditions (type and amount of salts, concentration of analytes, and temperature). Extraction efficiencies for both analytes were determined as 80–90 % in the integrated extraction at optimal conditions within 67–600 ng/mL of steroid concentration and 10–50 ℃ of temperature (He et al. 1082).

Another application of IL-based ATPS is to remove the textile dyes in wastewater. The dyes are highly stable and resistant to biodegradation because of the complex aromatic structures and synthetic origin of dyes (Kiran et al. 2006; Aksu and Tezer 2005). The release of large amount of dyes through aqueous effluents can cause environmental and economic problems. In this context, environmental regulations have becoming stricter in the concern of

discharging the dyes from aqueous effluents (Robinson et al. 2001; Hessel et al. 2007). The work done by Ferreira et al. (2014) has proposed a novel approach to remove dyes from aqueous discharges by using IL-based ATPS. The partition coefficients and extraction efficiencies of a set of textile dyes (chloranilic acid, Indigo Blue and Sudan III) have been carried out by using ATPS composed of hydrophilic ILs (phosphonium- and imidazolium-based) and an inorganic (aluminium sulphate) or organic salt (potassium citrate). An evaluation of the IL structure, the nature of the salt and the pH of the aqueous medium has been assessed from the data of experiments. A selection of IL and salt that leads to the complete extraction of the three dyes to the IL-rich phase in a single-step procedure has been evaluated from the data analysis. The recovery of dyes will allow the reuse of IL-rich phase. All the partition coefficients and/or extraction efficiencies values reported in this research suggested that the same IL-rich phase can be used up to 800 times without reaching saturation. The saturation limit of the IL-rich phase with Sudan III is 2.12 g/kg IL at 25 ℃ (Ferreira et al. 2014).

Besides the application of thermo-separating polymer ATPS, the IL-based ATPS has been successfully tested in purification of lipase in the latest development. The study is done by Souza et al. (2015) to develop the ATPS that formed with cholinium-based ionic liquid– ILs (or salts) and tetrahydrofuran (THF) to purify the lipase from *Bacillus* sp. ITP-001. The cholinium-based salts that consist of cholinium chloride, cholinium bitartrate and cholinium dihydrogencitrate together with THF were used to form the ATPS. The lipase from *Bacillus* sp. ITP-001 is produced by submerged fermentation. The dialysed solution containing the lipase was then used to prepare the ATPS after the pre-purification steps. The optimum conditions for this purification were determined to be 40 % (w/w) THF and 30 % (w/w) cholinium bitartrate at 25 ℃. The result showed a lipase yield of 90.0 ± 0.7 % with a purification factor of 130.1 ± 11.7-fold (Souza et al. 2015).

Application of aqueous surfactant two-phase, ASTP

An new aqueous surfactant two-phase (ASTP) is formed with the solution to divide into two immiscible aqueous phases when cationic and anionic surfactants are mixed at a concentration higher than critical micelle concentration (CMC) and at certain molar ratio of cationic and anionic surfactant compositions (Weschayanwiwat et al. 2008). This novel ASTP extraction system has becoming popular since it also presents a phase separation that mimics to the conventional non-ionic surfactants. ASTP can be formed at low surfactant concentrations and compositions (Weschayanwiwat et al. 2008). The molar ratio of surfactants play an important role in ASTP system (Yin et al. 2002). The aggregates in the phases can exist in different forms including spherical micelles, rodlike micelles or vesicles by simply using different surfactant concentrations and compositions (Weschayanwiwat et al. 2008).

In conventional ASTP extraction, when non-ionic surfactant is heated above the cloud point temperature, the dehydration of detergent will cause phase separation to occur. The detergent-based systems or cloud point extraction (CPE) systems have been introduced and used in conventional ASTP extraction process (Selber et al. 2004). One is the surfactant-rich and the other is the surfactant-dilute phase. In the small volume of surfactant-rich phase, the organic contaminants will partition into the surfactant-rich phase, then aggregate and concentrate. At the same time, a small amount of organic contaminants known as remediated water will remain in the surfactant-dilute phase (Weschayanwiwat et al. 2008). By using a vacuum stripper, the surfactant-rich phase can be recycled and reused if the organic contaminants have high enough volatility (Weschayanwiwat et al. 2008). This kind of ATPS system is mostly used to separate the hydrophobic and amphiphilic molecules based on the solubilisation and partitioning of membrane bound substances. Hinze and Pramauro (1993), Sanchez-Ferrer et al. (1994) have listed all the membrane proteins that have been solubilised from cells including plants and neural cells, archaebacteria, eubacteria and yeasts (Selber et al. 2004). The surfactants mixtures always exhibit superior properties compared to the single surfactants (Kang et al. 2001) and the effectiveness of extraction (Khan and Marques 1999) is also improved which is vital for many types of technical applications (Weschayanwiwat et al. 2008).

ASTP system, which consists of a cationic surfactant [e.g. dodecyltrimethylammonium bromide (DTAB)] and an anionic surfactant [e.g. alkyldiphenyloxide disulphonate (DPDS)] has been used to extract benzene from wastewater. The experiment showed that phase separation occured when the DTAB:DPDS molar ratio range from 1.6:1 to 2.4:1 was used. The lowest CMC value with greatest synergism which mean that the highest negative values of the micellar interaction parameter and the highest extraction efficiency were showed with 2:1 molar ratio of DTAB:DPDS at zero charge of the surfactant

aggregates. Compared to ASTP extraction using non-ionic surfactants, the partition ratio of benzene is 48 and 72 % of the benzene is extracted into the surfactant-rich phase at a total surfactant concentration of 50 mM phase solution in a single-stage extraction (Weschayanwiwat et al. 2008).

Another example of ASTP system is demonstrated by Selber et al. (Selber et al. 2004) where the target protein consisted of fungal cellulose endoglucanase I (EGI) and amphiphilic protein hydrophobin I (HFBI) from *Trichoderma reesei* was chosen. A part of cellulose-binding domain at EGI was replaced by HFBI and this genetically modified engineering protein is named as EGIcore-HFBI. This fusion protein is expressed homologously under the command of the *cbh1* promoter. The objective of this work was to examine the technical feasibility of purification of this engineered protein in large scale by using detergent-based ATPS. A non-ionic polyoxyethylene Agrimul NRE 1205 was selected as phase-forming polymer. Agrimul BRE 1205, which is derived from plant oil rich in C12 lipids, has a large extent of C12 chains with small components of C14, C16 and C18 chains. The cloud point at 2 % of Agrimul NRE 1205 in water is at 22 ℃ (Selber et al. 2004). A direct scale up was completed in downstream process from 10 mL to 1200 L. The cultivation was scaled up from 7 L to 1500 L.

Application of ATPS with the combination of carbohydrates

A technique to use mono- and disaccharides to prepare novel ATPS has been developed recently. One of these studies was carried out by de Brito Cardoso et al. (2013) where acetonitrile and carbohydrates are used to prepare ATPS. Acetonitrile is a colourless aprotic solvent and it forms homogeneous mixture when it is mixed with water. The acetonitrile molecules do not strongly interact between each others. However, they are likely to form hydrogen bond networks with water molecules (Takamuku et al. 1998). The carbohydrates molecules [general formula of $(CH_2O)x$] are non-charged. They are biodegradable, non-toxic and renewable feedstock and show an inherent salting-out character (de Brito et al. 2013). The acetonitrile/carbohydrate system has been applied to partition the common antioxidant, vanillin. The ternary phase diagrams were examined at 298 K. The effect of the carbohydrate structure on the liquid–liquid or phase separation ability was evaluated. Numerous of carbohydrates were investigated, including monosaccharides (glucose, mannose, galactose, xylose, arabinose, and fructose), disaccharides (sucrose and mannose) and also commercial food-grade of sucrose, fructose and glucose. However, the main weakness of using food-grade sugar is the tendency of phase separation in reducing the two-phase region which is due to the impurities effect. The vanillin extracted is migrated towards the acetonitrile-rich phase with partition coefficients higher than 3.0, and vanillin has been recovered up to 91 % in a single-step ATPS. Carbohydrates are proven to be a potential substitute of inorganic salts and polymers in the formation of new ATPS. For instance, Wang et al. (2008) have used sugars with the combination of acetonitrile in the formation of ATPS. Also, Wu et al. (2008) and Freire et al. (2011) have applied the use of sugars in combination with the ILs.

An ATPS study on the affinity separation in partitioning *Cellulomonas fimi* β-mannanase (EC 3.2.1.78) containing a mannan-binding module has been investigated by Antov et al. (2006) using polysaccharides from galactomannan and hydroxypropyl starch. Affinity partitioning of β-mannanase was achieved because of the biospecificity of the mannan-binding module towards the top phase which contained galactomannan. A pH of 8 was used in the experiment in order to inhibit enzyme degradation at the phase containing galactomannan. By lowering the pH to 6 (optimal pH) of β-mannanase, the top phase polymer was removed from degradation by the enzyme. Utilisation of alternative polysaccharide polymers in forming ATPS can improve the partitioning of biological compounds with special interest in plant cell wall polysaccharide degrading enzymes that contain a carbohydrate-binding module (CBM). The functions of CBM are to bind an enzyme to its target substrate and to enhance its action (Tunnicliffe et al. 2005). The study has introduced a new method in affinity partitioning of enzymes that consists of CBMs based on the utilisation of the natural affinity in one of the phase-forming components at the polymer two-phase systems (Antov et al. 2006).

Application of triblock copolymers ATPS

The triblock copolymer ATPS has been discovered based on a hydrophobic complexant for metal separation and purification (Rodrigues et al. 2008). Basically, the triblock copolymers are built by two EO blocks and one PO block, and they have a general structure of (EO)x(PO)y(EO)x. When the copolymers are dissolved in an aqueous solution at critical temperatures and concentrations, a micelle is formed where the core is dominated by hydrophobic units [poly (propylene oxide), PO] surrounded

by a crown of hydrophilic units (ethylene oxide, EO) (de Lemos et al. 2013). These cores are able to dissolve the water-insoluble complexing agent and hydrophobic metal complex. An ATPS formed by a triblock copolymer of PEO–PPO–PEO [poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide)], L64, with an average molar mass of 2900 g/mol and 40 % EO, which corresponds to the composition (EO)13(PO)30(EO)13, and an electrolyte ($MgSO_4$, Li_2SO_4 or $Na_2C_4H_4O_6$) in water at 298 K were used to investigate the separation of copper and zinc by de Lemos et al. (2013). The 1-(2-pyridylazo)-2-naphthol (PAN) acted as an extracting agent in this separation. The factors that affect the metal separation including the amount of extractor added of the ATPS electrolyte, the pH and the composition of the system. The optimum separation ($S_{Cu'Zn} = 204$) of Cu and Zn was obtained using the L64 + $Na_2C_4H_4O_6$ ATPS at pH 3.0 with a tie-line length of 41.28 % (m/m) and a PAN/metal molar ratio of 3.0. The copper stripping percentage achieved a maximum value (% S = 83.3 %) by using 0.076 mol/kg of H_2SO_4 in this ATPS at thermodynamic condition (de Lemos et al. 2013).

ATPSs composed of macromolecule and electrolyte were used to investigate the separation of metallic ions. These works are mainly based on the PEO (2000 g/mol) and inorganic anions (CO_3^{2-}, SO_4^{2-}, OH^- or PO_4^{3-}) (Rogers and Bauer 1996; Rogers et al. 1993). Most of the ions are separated into the salt-enriched phase (Silva et al. 2006), except pertechnetate anion (TcO_{4-}) and the pentacyanonitrosylferrate anion $[Fe(CN)_5(NO)]^{2-}$ or nitroprusside $[Fe(CN)_5(NO)]^{2-}$ (da Silva et al. 2008). The pertechnetate anion is partitioned into the polymer-rich phase. This is due to the reason of hydrophobic nature and small value of estimated hydration Gibbs free energy ($\Delta hyd G$) in pertechnetate anion (Rogers and Zhang 1996). On the other hand, the $[Fe(CN)_5(NO)]^{2-}$ anion is separated and entered to polymer-rich phase due to strong interaction between the anion and the EO segments of the PEO (Silva et al. 2006). Triblock copolymers-based ATPS has been applied in the separation of the hydrophilic and hydrophobic biomolecules (Bolognese et al. 2005; J-h et al. 2007). The behaviour (Chow et al. 2015) of copolymer solution is highly depending on the EO/PO ratio and the molar mass of the macromolecules. Critical micellization temperature (CMT) and the critical micellization concentration (CMC) are the temperature and concentration where the copolymer molecules began to self-associate, respectively (da Silva et al. 2008). The ATPS composed of potassium phosphate, macromolecules—poly (ethylene oxide) (1500 g/mol) and triblock copolymers, PEO–PPO–PEO were used to study the separating behaviour of $[Fe(CN)_5(NO)]^{2-}$ and $[Fe(CN)_6]^{3-}$ anions by da Silva et al. (da Silva et al. 2008). Two copolymers with different hydrophobicity consist of L35 (50 % EO and 1900 g/mol) and F68 (80 % EO and 8400 g/mol) were used. The parameters of the temperature, tie-line length and phases hydrophobicity of the partition coefficient for the $[Fe(CN)_5(NO)]^{2-}$ and $[Fe(CN)6]^{3-}$ anions in each ATPS were examined. The result found that the partition coefficient of anions follows the order of L35< F68< PEO relatively. The decrease in partition coefficient of both anions when the temperature was increased indicated that an exothermic process occurred in the separating process. The movement of anions to the top phase is an enthalpic process which is proved by the thermodynamic parameters obtained from Van't Hoff equation and calorimetric measurements (da Silva et al. 2008).

Summary of the application of ATPSs

All types of ATPS that have been reviewed in this paper have been summarised in Table 1 for comparison of recovery yield, purification factor (PF), purity, fold and selectivity or separation efficiency.

Conclusions

ATPS has been reported as an attactive alternative or platform in downstream processing that can be adopted by industries due to its high capacity and easily scalability. However, the limited knowledge in the partitioning mechanism and poor understanding of the experimental design, installation and operation of ATPSs are the factors that still hindered its application. More efforts are needed to increase the popularity of the application of ATPSs in the downstream purification and recovery processes in order to replace the conventional techniques for economical and environmental sustainability.

In future, the ATPS is expected to rapidly evolve experimentally and theoretically to become a powerful technology in separation science with many useful applications in the industries. The ATPSs will be modified and improved to overcome the disadvantages and challenges facing in this technique. More novel types of ATPS will be discovered and integrated with other

Table 1 Summary of the types and comparison results of ATPS from literature reviews

No.	Type of ATPS	Recovery yield	PF	Purity	Fold	Selectivity/ separation efficiency	Reference
1.	PEG 20,000/dextran T500	87–95 %					(Ng et al. 2013)
2.	PEG 10,000/Na_2SO_4	98 %	2.3	2 %	100		(Kornmann and Baer 2008)
3.	PEG 4600/ammonium sulphate	89 %			1000		(Hayenga and Valex 2002)
4.	PEG 6000/sodium sulphate and potassium phosphate (dibasic)	>99.5 %					(Bhambure et al. 2013)
5.	PEG 8000/phosphate				7.01		(Mohamed Ali et al. 2014)
6.	Poly (ethyleneglycol)/poly (acrylate)/salt	>95 %					(Matos et al. 2014)
7.	EOPO copolymer/ammonium sulphate	99 %			13 %	76 %	(Show et al. 2011)
8.	EOPO copolymer/potassium phosphate	99 %	14				(Show et al. 2012)
9.	EOPO coplymer/phosphate	82–97 %				97.7 %	(Chen et al. 2014)
10.	EO50PO50/K_2HPO_4	71 %	3.3 ± 0.36				(Dembczynski et al. 2010)
11.	Alcohol/salt	74.6 %	16.2				(Lin et al. 2013)
12.	Alcohol/salt	96.7 %	11.6			343.2	(Amid et al. 2012)
13.	MAATPE (ethanol/ammonia sulphate + microwave-assisted extraction, MAE)	92.09 ± 0.14 %					(Zhang et al. 2015)
14.	PEG 150-COOH/dextran	93 %	1.9		60	11	(Rosa et al. 2007)
15.	PEG/ammonium sulphate, reactive red 120 as free ligand	50.6 %				80–90 %	(Ling et al. 2010)
16.	$[C_4mim]Cl$(IL)/salt					80–90 %	(He et al. 1082)
17.	$[C_2mim][CF_3SO_3]$; $[C_4mim][CF_3SO_3]$; $[C_4mim][Tos]$; $[C_4mim][N(CN)_2]$; $[P_{4444}]Br$; $[P_{4441}][CH_3SO_4]$; $[Pi_{(444)1}][Tos]$; and $[P_{4444}]Cl$ (ILs)/ inorganic salt $Al_2(SO_4)_3$ and organic salt $K_3C_6H5O_7.H_2O$					98 %	(Ferreira et al. 2014)
18.	Cholinium-based ionic liquid—ILs (or salts) and tetrahydrofuran	90.0 ± 0.7 %			130.1 ± 11.7		(Souza et al. 2015)
19.	Cationic surfactant (DTAB) and an anionic surfactant (DPDS)					72 %	(Weschayanwiwat et al. 2008)
20.	Sucrose, D−(+)−maltose, D−(+)−glucose, D−(+)−mannose, D−(+)−galactose, D−(+)−xylose, L−(+)−arabinose, and D−(−)−fructose/acetonitrile	91 %					(de Brito et al. 2013)
21.	Galactomannan and hydroxypropyl starch, reppal PES 200/PEG 40,000	83 %	3				(Antov et al. 2006)

technologies as a promising alternative which can be used as commercial applications in recovering the high value products.

Authors' contributions

YKY carried out the aqueous two-phase system studies, participated in the sequence alignment and drafted the manuscript. CWO carried out the bioseparation studies. EPN participated in the sequence alignment. JCJ and TCL participated in the design of the study and performed the statistical analysis. PLS conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Acknowledgements

The author wishes to thank Leong Yoong Kit from Department of Chemical and Environmental Engineering, Faculty of Engineering, University of Nottingham Malaysia Campus for editing the English of the manuscript. This study was supported by Ministry of Science, Technology and Innovation, Malaysia (02-02-12-SF0256), Fundamental Research Grant Scheme (FRGS/1/2015/SG05/UNIM/03/1)and Prototype Development Research Grant Scheme (PRGS/2/2015/SG05/UNIM/03/1).

Competing interests

The authors declare that they have no competing interests.

Received: 15 October 2015 Accepted: 13 December 2015
Published online: 28 December 2015

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