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REVIEW

Recovery of biotechnological products using aqueous two phase systems

Win Nee Phong,[1] Pau Loke Show,[2] Yin Hui Chow,[3] and Tau Chuan Ling[1,*]

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia,[1] Bioseparation Research Group, Department of Chemical and Environmental Engineering, Faculty of Engineering, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia,[2] and Faculty of Built Environment, Engineering, Technology and Design, Taylor's University, No. 1 Jalan Taylor's, 47500 Subang Jaya, Selangor, Malaysia[3]

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Aqueous two-phase system (ATPS) has been suggested as a promising separation tool in the biotechnological industry. This liquid-liquid extraction technique represents an interesting advance in downstream processing due to several advantages such as simplicity, rapid separation, efficiency, economy, flexibility and biocompatibility. Up to date, a range of biotechnological products have been successfully recovered from different sources with high yield using ATPS-based strategy. In view of the important potential contribution of the ATPS in downstream processing, this review article aims to provide latest information about the application of ATPS in the recovery of various biotechnological products in the past 7 years (2010–2017). Apart from that, the challenges as well as the possible future work and outlook of the ATPS-based recovery method have also been presented in this review article.

[**Key words:** Aqueous two-phase system; Biotechnological products; Downstream processing; Recovery; Liquid-liquid extraction technique]

Aqueous two-phase system (ATPS) separation technique was first developed by a Swedish biochemist, P. A. Albertsson in 1986 (1). Since then, this liquid-liquid extraction technique has become a powerful bioseparation tool (1,2) and has been extensively exploited to process a range of biotechnological materials such as proteins, enzymes, phytochemicals, nucleic acids, and pigments (3,4). In ATPS, two immiscible phases can be formed by mixing two water miscible solutes beyond critical concentrations (5). Typical ATPS mixture consists of two different polymers [ethylene oxide propylene oxide co-polymer (EOPO), polyethylene glycol (PEG), polyacrylates, dextran] or a combination of polymer/a low molecular weight alcohol and kosmotropic salt (phosphate, citrate, and sulfate).

ATPS is renowned as an efficient, economical and versatile emerging technique for the bioprocessing of biotechnological products. Over the last two decades, ATPS has gained increasing interest due to its potential in circumventing some of the technical limitations exist in the downstream processing. This system demonstrates a good ability in eliminating most of the contaminants in the early steps of recovery process. It has been reported extensively that intracellular compounds can be separated from cell debris (5) or from the mixture of other soluble components (6,7) effectively using ATPS. Another author also agreed that ATPS has more versatility over the conventional solvent extraction methods in the downstream processing of biomolecules (3). For instance, this well-established method is not only able to extract target compound by partitioning them into one of the phases, but also capable of concentrating the target compound by partitioning them into the smaller volume of the extraction phase (8). A schematic illustration of product recovery using ATPS is presented in Fig. 1.

Overall, ATPS represents an interesting advance in bioseparation process and has been proposed as a promising alternative technique to recover diverse biomolecules from various sources. In this review article, we first describe the latest information about the recovery of various types of biotechnological products based on ATPS strategies in the past 7 years (2010–2017), then we study the challenges and future perspective of the ATPS-based recovery method.

ADVANTAGES OF ATPS

The use of conventional extraction method particularly chromatography-based method involves complex scale-up, batch operation, low capability in process integration, laborious processing cycles, require high energy input and high cost (9). On the other hand, the removal of cell debris by filtration or centrifugation may be challenging when processing high biomass load due to heterogeneous distribution of particle size and high viscosity (10). On top of that, a higher number of operational steps would incur higher overall cost owing to the loss of some amount of target compound in each processing stage.

In response to the increasing market demand of biotechnological products, a more versatile and economical bioseparation process which can offer higher process throughput, shorter processing time and scalability is necessary to cope with the current rate-limiting downstream processes (11). However, it should be noted that the selection of an appropriate separation method is crucial in the downstream processing. Otherwise, inappropriate extraction technique could negatively affect the biofunctionality of target

* Corresponding author. Tel.: +60 3 79674104; fax: +60 3 79674178.
E-mail addresses: tauchuan.ling@gmail.com, tcling@um.edu.my (T.C. Ling).

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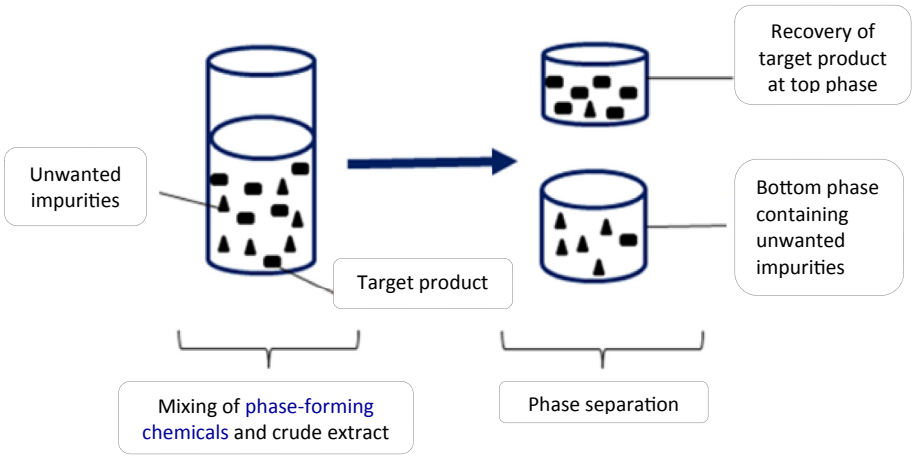

FIG. 1. Schematic view of recovery of target product based on ATPS concept.

molecules and would therefore limit their application to a great extent (12).

Alternatively, ATPS exhibits application potential with great technical and economic advantages in downstream processing. Several discrete stages which involve separation, concentration into smaller volume and purification can be reduced and substituted by ATPS, allowing the overall recovery process become more energy-efficient and cost-effective (3). Compared to the conventional method, the use of ATPS for bioseparation offers many advantages (2). These include simple process operation, rapid separation, high selectivity, low energy consumption and low cost (3,8,13–16). ATPS has also been able to offer a biocompatible environment for the isolation of biotechnological products due to the presence of high water content in both phases (1,17). The extremely low interfacial tension of this system (between 0.0001 and 0.1 dyne/cm) creates high interfacial contact area of the dispersed phases, which in turn, enhances the efficiency of the mass transfer (1).

Chemical cost is considered one of the dominant cost factors for large-scale bioseparation process. The use of inexpensive phase components in ATPS would make the whole downstream processing more economical. Problem of downstream pollution may also be avoided by recycling the ATPS phase components (2). Extraction using ATPS is relatively rapid and the processing capacity of this technological simple technique is quite high. Due to the simplicity and reliability of scaling-up approach, the extent of ATPS extraction to industrial scale application is feasible and practical (8,11,13).

Influence of parameters on the partitioning behavior of ATPS The selective distribution of the compounds to be separated between the two phases serves as the basis of partitioning in ATPS. In ATPS, the partition profile of the solutes depends on different physicochemical interactions between the biomaterial and the phase forming chemicals (18). Interactions such as van der Waals' forces, hydrogen bond, electrostatic interactions, steric effects, hydrophobicity, biospecific affinity interactions as well as conformational effects between the phase components and the substances contribute to the partitioning of the particular substance (1).

To achieve an effective bioseparation process, the partitioning behaviour of biomolecule in ATPS can be influenced by changing some of the dominating factors such as molecular weights and size of polymers, type and composition of phase component, type of ions in the system, the addition of neutral salts like NaCl, tie line length (TLL), system temperature and pH (3,5,13).

ATPF integration process Downstream processing represents the key economic constraint to the large production of biotechnological products at lower cost (19). As part of the downstream processing, isolation and extraction continue to be a significant challenge towards the commercial production of biotechnological products. When developing an economically practical recovery process, aspects such as recovery, operational cost, throughput, biocompatibility, recyclability, upscaling need to be considered to favour the economic feasibility of the recovery process (20,21). For environmental benefits and long-term sustainability, all the processing stages should be easily adopted and simplified without the involvement of extensive energy input.

The concept of implementing process integration into the downstream processing of biotechnological products at commercial level offers considerable potential benefits and is of great economic and practical interest (22–24). Through process integration, several downstream processes such as separation, concentration and extraction can be integrated into one single step and this could reduce waste and processing time, lessen energy consumption and consequently cut the overall production cost (6–8,11,13).

Owing to its flexibility and biocompatibility, ATPS can be combined with other separation methods or processes to overcome commonly observed issues in biotechnological processes like low productivity, expensive operation and long processing time. Application of ATPS in extractive bioconversion, extractive fermentation or extractive disruption is one of the elegant emerging examples of process integration.

Incorporating ATPS into extractive bioconversion or extraction fermentation could improve the efficiency of biotechnological processes. In general, both strategies involve the continuous removal of bioproduct from its site of production via bioconversion or fermentation to the opposite phase simultaneously during processing (10,25).

On the other hand, the direct incorporation of cell disruption treatment with recovery process can be achieved in the ATPS extractive disruption. The flow diagrams of conventional discrete process and integrated process (extractive disruption) are illustrated in Fig. 2.

TYPES OF BIOPRODUCTS

Protein There is an increasing request for a large number of new proteins due to their versatile applicability in various sectors like food, medical, pharmaceutical and chemical industries.

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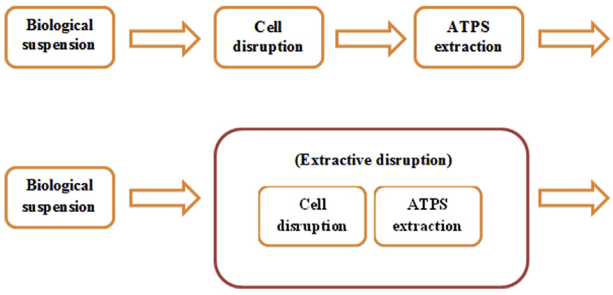

FIG. 2. Simplified representation of two flow diagrams in downstream processing for comparison. The upper flow diagram represents the conventional discrete process in which the cell disruption process is followed by an ATPS-based extraction process. On the other hand, the integrated process (extractive disruption) wherein cell disruption and ATPS were integrated into one step is presented in the lower diagram.

Technological advancement in upstream processing has led to the production of proteins of interest from various sources in higher yield. However, the use of chromatography for protein separation requires high purification cost at large scale, thus this has become a bottleneck in the biotechnology industry (26,27).

The potential application of ATPS as a primary recovery step of recombinant proteins from transgenic plants has been studied. A research group proposed a novel simplified ATPS-based method to recover a type of recombinant human protein expressed in alfalfa. Granulocyte-colony stimulating factor (rhG-CSF) was used as a model protein in this study. It was reported that at pH 7.0, ATPS comprising of 16.1% PEG 8000 and 10.0% potassium phosphate was the best extraction condition. About 88% of rhG-CSF can be recovered in the top phase by the system. On the contrary, 93% of alfalfa contaminant proteins was found to be accumulated not only in the bottom phase but also at the interface (28).

Recently, a new type of eco-friendly solvent, known as deep eutectic solvent (DES) has attracted a great deal of attention as promising green media in the extraction and separation of target compounds from various sources. DESs are good substitutes of ionic liquids (ILs). Apart from possessing similar properties with ILs such as excellent electrolyte, stability, negligible volatility at room temperature and high solubilization ability; DESs are generally inexpensive and can be synthesized easily than ILs (29). A series of recent experiments demonstrated that DES-based ATPS could replace IL-based ATPS to offer new possibilities in the separation of proteins (30,31). For instance, ATPS formed by betaine-based deep eutectic solvent and phosphate solution were successfully applied in the extraction of protein. In this work, the extraction efficiency of up to 99.82% can be achieved after optimization whilst the back extraction efficiency could reach 32.66%. FT-IR spectra was used to examine the functionality of protein and the results showed that the conformation of protein can still be well-retained after the extraction process (31).

The exploitation of microalgae for protein source is promising, but the downstream processing of microalgae is rather time-consuming and challenging. This is because the thick cell walls of microalgae need to be treated to facilitate the release of intracellular compounds before the subsequent extraction process. To simplify the overall downstream processing, the concept of ATPS extractive disruption was adopted in a recent research. The recovery of 84.23% total protein from microalgae using process integration in which cell disruption and ATPS were integrated into one step was reported (11). This approach would allow efficient downstream processing at shorter time, thus reducing the chance of undesirable target modification or degradation. As a result, both the yield and molecular quality of bioproducts can be improved (32,33).

Enzyme The development of an efficient recovery technique without negatively affecting the native structure and biofunctionality of enzyme is crucial for the economic viability of its biotechnological application (34). Up to date, enzymes like protease, Rubisco, β-mannanase, lipase from various sources have been successfully recovered by ATPS with higher purity and higher yield compared to the traditional recovery techniques which are laborious, costly and difficult to scale up.

The use of ATPS in the lipase recovery process is gaining attention in the recent decades. ATPS could potentially be industrialized as a feasible recovery process for lipase derived from microbes. An extractive fermentation based on ATPS strategy was developed to simultaneously cultivate and extract lipase produced by *Burkholderia pseudomallei* in a single step operation. Based on the results, ATPS formed by 9.6% PEG 8000 and 1.0% Dextran T500 was proposed to be the best condition for the extractive lipase production. It was observed that the lipase predominately partitioned to the top phase while the biomass mainly gathered in the bottom phase. Finally, highly active lipase was obtained with a total yield of 92% (35).

Nonetheless, one of the limitations of using PEG/dextran or polymer/salt as the phase-forming components in the extractive fermentation is that most of them cannot be recycled effectively. The poor recyclability of the phase-forming components would lead to environmental pollution and expensive operation, thereby restricting the application of ATPS in the downstream processes. To improve the recyclability of the ATPS-based extractive fermentation process, a novel method using a thermo-responsive smart polymer called ethylene oxide-propylene oxide (EOPO) was developed. EOPO is a type of themosperating polymer and it will form two clear layers when it reaches above critical solution temperature. With this special property, it can be recovered and recycled easily (Fig. 3) (36). Adopting the similar approach (35), the extracellular lipase was successfully produced by *Burkholderia cepacia* and recovered from the fermentation mixture using EOPO in a single step. The optimal condition for lipase production was achieved when the extractive fermentation in ATPS composed of 10% ethylene oxide-propylene oxide (EOPO) was optimally controlled at pH 8.5, 200 rpm speed and 30 °C. A high lipase yield of 99% was obtained from the optimized system. Attributed to its simple, rapid and recyclable characteristics, it can thus be concluded that the process integration of the lipase production and purification process is a promising and attractive approach (37).

Besides the microorganism of *Burkholderia* species, the extraction of extracellular lipase released by other species of microorganisms has also been reported. It was reported that 71.2% of lipase produced by *Rhodotorula glutinis* was obtained in the top phase of PEG4000/oxalate potassium ATPS (38). Souza et al. studied the extraction of lipase using ATPSs composed of different phase-forming components in 2014 (39) and 2015 (40). About 90% of lipase can be purified from *Bacillus* by tetrahydrofuran (THF)/cholinium bitartrate ATPS (39). On the other hand, a lipase yield of 96.4% produced by *Bacillus* was successfully recovered using ATPS composed of THF/potassium phosphate (40). It was found that ATPS formed by PEG 1500 and potassium phosphate was superior to traditional methods (ultrafiltration and precipitation with acetone and kaolin) in purifying crude lipase extract produced by *Yarrowia lipolytica* IMUFRJ 50682. The purification fold of lipase was greater than 40 when the ATPS was optimized under the condition of pH 6 and 4 °C. The purified lipase extract showed good thermostability up to 50 °C and good stability at a wide pH range after the extraction process (41). Another recent study also aimed at improving the sustainability of the recovery process of lipase produced by *B. cepacia* ST8 via ATPS. In this work, a high recovery yield of 94.0 % with a purification factor of 22.4 was successfully obtained by ATPS composed of [N4,4,4,4][BES] + $(NH4)_2SO_4$ (42).

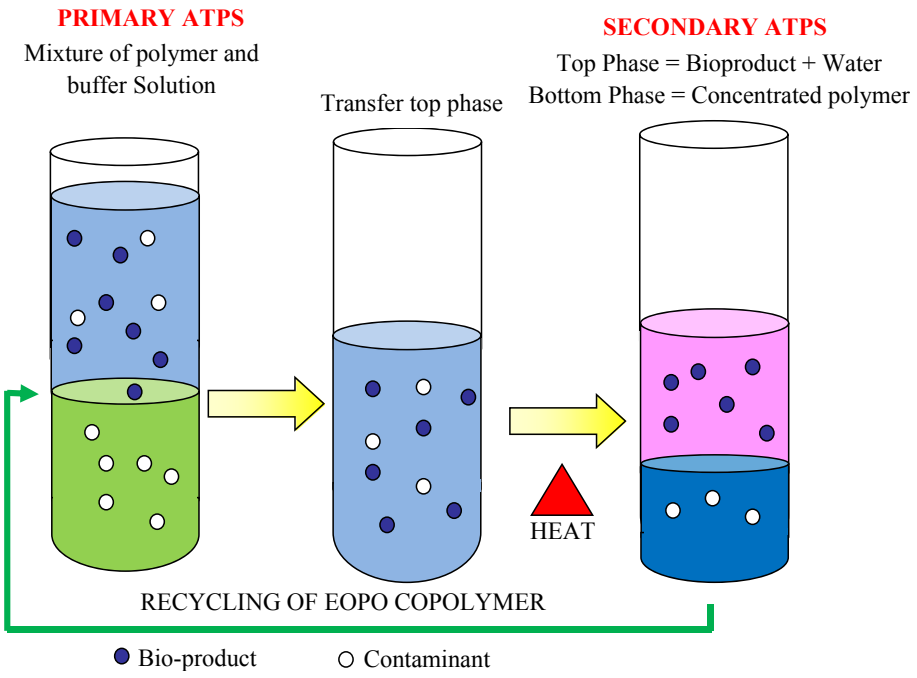

FIG. 3. This is a schematic diagram of the recycling ATPS using thermos-separating polymer. In the primary ATPS, the top phase contains desired bio-products will be separated to another test tube. Then it will be heated above critical solution temperature and form two phases. This will be called secondary ATPS. The top phase consists only water and pure bio-products while the bottom phase consists of concentrated polymer which can be re-used for a new ATPS. Modified from Show et al. (36).

Apart from lipase, numerous studies have also been conducted to investigate the recovery of various enzymes using ATPS-based extraction strategy. In a recent research, PEG 8000/citrate ATPS was proposed as a promising alternative to chromatographic techniques as a primary purification step to recover extracellular protease from solid state fermented of *Aspergillus tamarii* URM4634 for industrial applications, with activity yield and purification factor achieved 55.8% and 3.95, respectively (43).

β-Mannanase derived from *Bacillus subtilis* ATCC 11774 was successfully isolated by PEG 6000/potassium citrate ATPS. The addition of 3% of [Bmim]BF_4 as an adjuvant greatly improved the recovery yield of β-mannanase, showing about 26% higher in the recovery yield (89.65%) compared to ATPS without IL (44).

Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) which can be found in photosynthetic plants, appears to be a new protein ingredient for food market. In order to develop the best Rubisco extraction system, the performances of three different ATPSs namely PEG 400/Potassium citrate, PEG 400/Cholinium dihydrogen phosphate and Iolilyte 221PG/potassium citrate ATPSs were investigated. Processing parameters like type of phase-forming components, pH and tie-line length (TLL) were optimized and resulted in about 80−100% of partition efficiencies. Overall, the experiment showed that Iolilyte 221PG/citrate ATPS was the most efficient in separating Rubisco but stability studies revealed that PEG-based ATPSs were better in maintaining the functional properties of Rubisco (45).

Monoclonal antibodies Due to the inherent high specificity, therapeutic monoclonal antibodies (mAbs) like IgG are widely applied to treat various diseases and disorders such as cancer, transplant rejection, immune and inflammatory disorders (46). Nonetheless, the current downstream processing of IgG faces extreme challenges arise from the progressive development in the upstream processes. With the continuous increase in IgG titers, the rate-limiting chromatography-based downstream processing of IgG has been considered as the bottleneck in producing commercially viable therapeutic products. Hence, the incorporation of different types of ATPS to the typical upstream and downstream processes used by the antibody manufacturers has been attempted extensively in the recent years to improve the process throughput, scalability, processing time and cost.

In 2014, a research team simultaneously clarified and captured the anti-CD34 mAb from hybridoma cell cultures using ATPS formed by 7% PEG 6000 and 5% dextran at pH 3, and with the addition of 150 mM NaCl (47). Using this system, 84% of IgG was recovered in the top phase while the soluble protein and hybridoma cells were separated to the bottom phase and interface, respectively. Also, the use of low acidic system pH not only enables effective viral inactivation but also the transferability of this strategy for the recovery of other mAbs as it can lower the tendency of IgG precipitation (47).

Phase-forming component can be coupled with ligand which has an affinity for specific mAb to enhance the differences in polarity and selectivity between the two-aqueous phases. An unique hybrid extraction process which combines the ATPS and the magnetic fishing technique to purify antibodies from the cell culture fluids was proposed. Magnetic particles (MPs) coated with gum Arabic coupled with the affinity ligand, aminophenyl boronic acid (GA-APBA-MP), were added to the PEG/dextran ATPS. The protein purity and recovery yield were 98% and 92%, respectively. This excellent combination reduces the duration of the phase separation process and the consumption of MPs considerably when compared to the ATPS for the former and the magnetic fishing technique for the latter (48).

Recently, an affinity dual ligand which is based on a choline binding polypeptide tag (C-LytA) fused to the synthetic antibody binding Z domain (LYTAG-Z) was used to integrate the clarification of cell and the primary recovery of IgG into a single step. The efficiency of the LYTAG-Z in improving the separation of IgG from various types of crude feedstock to the ligand-rich phase was evaluated in four kinds of ATPS [i.e., PEG/dextran, PEG/phosphate, EOPO/polyacrylic acid sodium salt (NaPAA) and EOPO/dextran].

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Results showed that the LYTAG-Z worked more effectively in which the bottom phase of the ATPS was composed of dextran. Using this approach, an IgG purity of 42% and 89% of IgG was successfully recovered in the PEG-rich top phase of 7% PEG 3350/6% dextran ATPS while separating the impurities and cells to the opposite phase. The ligand can be recovered for further usage by decreasing the system pH to pH values less than 3 during the subsequent back-extraction step to break the binding between the IgG and ligand. Although such affinity partitioning approach imposed an added burden to the downstream processes as it requires an additional dissociation step to eliminate the ligand-bearing polymer from the product, the expense of these functionalized phase-forming components can be compensated by the cost of the final IgG product (49).

Capsaicin Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a very powerful alkaloid that has been widely used in medicine. The ingestion of capsaicin has shown a positive effect on the human health due to its unique biological and pharmacological activities. Extraction of capsaicin using microwave-assisted extraction, ultrasound-assisted extraction, organic solvent extraction, supercritical fluid extraction, acid and alkali extraction, and enzymatic extraction present some technical limitations and are thus not suitable for large scale application.

Alternatively, ATPS appears to be a feasible method for capsaicin extraction at commercial level. For this reason, recent studies have been focusing on the use of ATPS for capsaicin extraction. Various studies showed that the capsaicin yields obtained from *Capsicum* are relatively high using ATPS composed of cholinium chloride/acetonitrile (90.57%) (50), ethylene oxide-propylene oxide (EOPO)/dipotassium phosphate (95.5%) (51) and ethanol/sodium phosphate (85.6%) (52).

Cyclodextrins The extraction of a novel carbohydrate, cyclodextrins (CDs) has been attempted using ATPS extractive bioconversion technique. Extractive bioconversion in ATPS is an efficient in situ product recovery technique, which integrates bioconversion and extraction into one step by instantly isolating bioproducts from biocatalysts once they are formed (53,54).

Simultaneous synthesis and recovery of CDs using extractive bioconversion with cyclodextrin glycosyltransferase (CGTase) enzyme derived from *Bacillus cereus* in PEG/dextran ATPS was examined in 2013. In this study, repetitive batch of CDs synthesis was performed by replenishing top phase components and adding starch every 8 h. Enzymatic bioconversion of starch occurred mainly in dextran-rich bottom phase, whilst the bioproduct, CDs accumulated in the top phase. At the end of the experiment, CDs with a total yield of 13.7 mg/mL was obtained under the optimum condition of 7.7% PEG 20,000 and 10.3% dextran T500, 20% CGTase and 6% sago starch. Overall, this study demonstrated the feasibility of employing ATPS in the extractive bioconversion of cyclodextrins for the prolonged production of CGTase without requiring complicated set-up (55).

The recovery of CDs using ATPS extractive bioconversion has gained increasing interest. Following this, PEG/salt ATPS was employed in the extractive bioconversion of gamma-cyclodextrin (γ-CD) from soluble starch with *B. cereus* CGTase. It was observed that the γ-CD predominantly migrated to the top phase, whilst CGTase gathered in the salt-rich bottom phase. Under the optimum condition of ATPS containing 30.0% PEG 3000 and 7.0% potassium phosphate, 81.88% of γ-CD was recovered in the top phase after 60 min of bioconversion process. The possibility of recycling the CGTase enzyme was also investigated in this study. Experiment showed that CGTase was successfully recycled for three times in the repetitive batch processes. This clearly indicated that this method is cost-efficient and environmentally friendly for the production and purification of γ-CD (54).

Pigments The feasibility and efficiency of ATPS as a preliminary purification of natural anthocyanin pigments were proven experimentally. Different types of ATPSs such as ethanol/ammonium sulfate ATPS (56), sodium dihydrogen phosphate/ethanol ATPS (57), PEG 4000/magnesium sulfate ATPS (58) have been tested with different plant sources like mulberry (56), grape juice (57) and red cabbage (58), respectively.

Majority of the free sugars were successfully removed during the extraction process. More importantly, anthocyanins extract obtained by ATPS showed relatively high stability (57), better colour quality (58) and high antioxidant ability compared to that by the conventional extraction (80% ethanol aqueous solution containing 0.01% HCl) (56). All the experimental results indicated that the purification of anthocyanin pigments using ATPS-based technique is feasible and attractive.

Chlorogenic acid Chlorogenic acid holds promise in many aspects of health due to its pharmacology properties. The ATPS extraction has also been applied to the recovery of chlorogenic acid. Blueberry leaves could be the new potential resource for chlorogenic acid extracts due to the abundance of this bioactive compound in the leaves. A DES-based ATPS was developed to recover chlorogenic acid from blueberry leaves. This study demonstrated that DES-based ATPS has a great potential to extract chlorogenic acid at pilot scale. In this work, ATPS formed by 26.72% ChCl-1,3-Butanediol and 35.11% K_2HPO_4 at pH 3 and temperature 35 °C was optimal to the recovery process. Using the developed method, it was observed that the recovery of chlorogenic acid by DES-ATPS achieved 96.18% with a purity of 74.5% (29).

Ginsenosides Ginsenosides are the important pharmacological constituent of *Panax ginseng* C. A. Mey. A new extraction method based on IL-ATPS to recover ginsenosides from *P. ginseng* C. A. Mey was developed. It was reported that 35% [C4Tr]Br, 20% NaH_2PO_4, 3% ginseng extracts, 42% water was the optimal ATPS composition for the recovery of ginsenosides. Under the optimum condition, the extraction efficiency of ginsenosides in the IL-rich phase reached 99.5% in one step. Antioxidant activities of ginsenosides before and after the ATPS extraction process have also been investigated in this study. Overall, the results clearly indicated that the developed method is conducive for the bioactivity improvement of ginsenosides and exhibits potential application in the pharmacology industry (12).

Nucleic acids ATPS-based extraction strategy has also been successfully employed in the separation of nucleic acids. The feasibility of scaling up the ATPS for plasmid DNA (pDNA) purification has been investigated. A recovery of 97.4% of pDNA was successfully obtained in a tenfold scale up. This clearly indicated that the ATPS-based recovery process holds a great promise in the large-scale recovery of pDNA (59).

A two-step extraction based on ATPS was used to isolate amplified DNA fragments generated during in vitro DNA polymerase chain reactions (PCR) in 2014. The PCR products partitioned very strongly between the phases during the extraction process. In the first step, the DNA migrated to the PEG-phase. Back-extraction was performed in the second step, where the DNA strongly partitioned to a salt-rich phase. On the other hand, it is interesting to note that larger DNA molecules were precipitated in the interphase of ATPS. As a result, small DNA fragments of less than 4000 bp was successfully purified from the PCR mixtures. This study demonstrated that the developed method is a rapid and cost-effective purification technique for DNA products (60).

Extraction of pDNA from an alkaline bacterial cell lysate has been investigated using ATPS composed of 12% polyethylene glycol and 12% sodium sulfate. To improve the purification and recovery yields of pDNA, a modified oligonucleotide was added to the system as an affinity ligand. It was observed that the addition of affinity

ligand had a significant effect on the partitioning behaviour of pDNA, but the partitioning of other intracellular compounds like RNA and protein content were not affected. Under optimum condition, about 67% of pDNA was reported to be recovered in the top phase (61).

Phenolic compounds Natural phenolic compounds from plants constitute a major class of plant secondary metabolites with a large structural diversity. These include phenolic acids, tannins, flavonoids, curcuminoids, coumarins, quinones and lignans. Due to their antioxidative, antimutagenic, anticarcinogenic and anti-inflammatory properties, the role of phenolic compounds in the human health and food industry has become increasingly important (62). Separation and determination of the individual and total content of phenolic compounds have thus been widely studied.

Several recent studies showed that ATPS has been successfully employed for the recovery of phenolic compounds. The extraction of phenolic compounds from *Eucalyptus globulus* wood veneers to be used as natural antioxidants was studied using ATPS composed of PEG2000 and ammonium sulfate. The influence of several parameters on the extraction efficiency was studied. It was found that the yield of the total phenolic compounds increased with the extraction time, temperature as well as the amount of solvent used. On the contrary, ATPS composition did not give any significant impact on the total phenol yield. Phenolic compounds accumulated in the PEG-rich top phase and the presence of phenolic compounds with potential antioxidant activity was detected by RP-HPLC-ESI-TOF (63).

Ultrasound can be incorporated with ATPS to enhance the extraction process. Ultrasound-assisted ATPS extraction method has been successfully used to extract polyphenolic compounds from *Aronia melanocarpa* pomace. In this study, the influence of processing parameters such as ammonium sulfate concentration, ethanol-water ratio, ultrasonic time and power on the extraction yields were investigated. Then, the process was further optimized by Box-Benhnken design with response surface methodology (RSM). Under optimal extraction condition, the yields of total phenolics and total flavonoids were 68.15 ± 1.04 and 11.67 ± 0.63 mg/g, respectively (64).

Following the same approach, an ultrasound-assisted ethanol/ammonium sulfate ATPS was attempted in the extraction of bioactive components from wheat chaff. In ATPS consisted of 23.8% ammonium sulfate, 24.3% ethanol and 1.2% wheat chaff with the aid of ultrasound waves (500 W, 30 Hz, 10 min), extraction yields of phenols and sugars reached 2.67 mg/g and 16 mg/g, respectively. During the extraction process, it was observed that the phenol compounds and xylooligosaccharides predominantly partitioned toward the ethanol-rich top phase and salt-rich bottom phase, respectively. Results revealed that about 96% extracted sugars accumulated in the bottom phase, whilst the top phase recovered approximately 64% phenols (65).

A recent study showed that the solvent used in ATPS plays a role in maintaining the functionality of the bioactive compounds. A novel ultrasound-assisted ionic liquid-based extraction technique to recover polyphenols from Chinese purple yam was developed. The developed method was then compared with the regular ethanol-based ultrasound-assisted extraction. It was found that the use of ionic liquid in polyphenols was superior to that of the extract from the former in terms of the free radical scavenging activity and total antioxidant activity. The results concluded that IL-UAE could potentially be a green and rapid technique for efficient extraction of polyphenols with low solvent consumption (66).

Stevioside There is a growing demand for natural sweeteners as a replacer to artificial sweeteners in the food and beverage products. The applicability of ATPS in the extraction of stevioside has also been investigated in 2017. In this study, choline chloride was combined with potassium phosphate to establish an ATPS for the extraction of stevioside. The effects of pH, composition of the phase forming components and temperature on the partitioning of stevioside were investigated. Based on the experiment, 24% [Ch]Cl and 30% K_3PO_4 at pH 13.7 and 298 K was found to be the optimal ATPS composition. In general, hydrophilic properties of molecules and phases are the main determinants in the separation of target molecules in ATPS. As a hydrophilic glycoside, stevioside prefers to stay in the less hydrophobic phase, namely the salt-rich phase. This explained the tendency of stevioside moving toward the bottom salt-rich phase as observed in this study. Overall, the results demonstrated that the developed method can be quite successful for the extraction of stevioside (67).

The recovery of different types of biological products by ATPS as discussed above is summarized in Table 1.

CHALLENGES AND FUTURE PERSPECTIVE

ATPS provide simple and powerful media for bioseparation of biotechnological products. Despite the definite advantages of ATPS, the wide commercial adoption of ATPS as a part of downstream processes at pilot scale is still not extensive. The reluctance to embrace ATPS by industries is mainly due to the complexity in understanding the interrelated interactions between ATPS compounds as well as difficulty in predicting the mechanism governing the phase formation and partition of biological materials, making the method development wholly empirical (16,68). Understanding forces governing partitioning of solutes in ATPS would allow a better process predictability and optimization. Extensive research with the aid of modern analytical techniques, statistical tool or reliable mathematical modelling is thus required (10). Recently, a semi-empirical model based on continuum electrostatics was applied to predict the protein partitioning characteristics in different polymer/polymer systems. Besides showing a good predicting ability on proteins partitioning, this model could significantly decrease the number of experiments needed for optimization of protein partitioning in ATPS (26).

Many different types of ATPS have been developed in order to improve some advantages of the existing ATPS (16). For example, PEG rich phase are modified to improve selectivity of the system towards target biomolecule through the use of metal ions or dye ligands (16). Another author also agreed that the alteration of ATPS with affinity ligands have resulted in substantial increases in the recovery yield and purity of biological products. However, strategies for biological or chemical displacement of the target molecule from the affinity ligand have been poorly addressed and this may represent an exciting new area to explore (69). Considerable attention to this area would definitely result in the emerging of more versatile and economical phase systems with advance applications.

One of the major trends is that there is gaining interest in using new raw materials with benign, eco-friendly, low cost and recyclable characteristics to form ATPS. Salts like phosphates and sulfates would pose waste water treatment problems if the salts are not subjected to recycling. Hence, efforts have been putting into the replacement of these salts by biodegradable salt like citrate (43). The use of alternative recyclable phase formers in ATPS may also help to reduce the cost of operation in downstream processing. This has driven the use of 'smart' polymers such as thermoresponsive polymer that allows easy recycling as the phase forming chemicals in ATPS (37). Apart from that, more studies are also focusing on the utilization of non-conventional solvents like deep eutectic solvents (DESs) and ionic liquids (ILs) in ATPS-based extraction. However, experiments on their extraction efficiency to a wide range of

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TABLE 1. Summary of different types of target compounds recovered by ATPS.

Target compound	Source	Phase component	Recovery yield (%)	Purification factor	Reference
Granulocyte-colony stimulating factor (rhG-CSF)	Alfalfa	PEG 8000/potassium phosphate	88	–	28
Bovine serum albumin (BSA)	Model protein	Betaine-based deep eutectic solvent/phosphate	99.82	–	31
Total proteins	Microalgae	Methanol/potassium phosphate	84.23	–	11
Lipase	*Burkholderia pseudomallei*	PEG 8000/Dextran T500	92	–	35
Lipase	*Burkholderia cepacia*	Ethylene oxide-propylene oxide (EOPO)	99	–	36
Lipase	*Rhodotorula glutinis*	PEG 4000/oxalate potassium	71.2	13.9	37
Lipase	*Bacillus*	Tetrahydrofuran (THF)/cholinium bitartrate	90	130.1	38
Lipase	*Yarrowia lipolytica* IMUFRJ 50682	PEG 1500/potassium phosphate	–	>40	40
Lipase	*Burkholderia cepacia* ST8	[N4,4,4,4][BES]/$(NH4)_2SO_4$	94.0	22.4	41
Protease	*Aspergillus tamarii* URM4634	PEG 8000/citrate	55.8	3.95	42
β-Mannanase	*Bacillus subtilis* ATCC 11774	PEG 6000/potassium citrate, with the addition of 3% of [Bmim]BF_4	89.65	3.01	43
Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco)	Spinach	PEG 400/Potassium citrate, PEG 400/Cholinium dihydrogen phosphate and Iolilyte 221PG/potassium	72.1–98.8	–	44
IgG	Hybridoma cell	PEG 6000/dextran	84	–	46
Antibodies	Chinese Hamster Ovary (CHO) cell culture fluids	PEG/dextran	92	–	47
IgG	Commercially available cell culture fluids	PEG 3350/dextran	89	–	48
Capsaicin	*Capsicum oleoresin*	Ethylene oxide-propylene oxide (EOPO)/dipotassium phosphate/ethanol	95.5	–	50
Capsaicin	*Capsicum frutescens*	Cholinium chloride/acetonitrile	90.57	3.26	49
Capsaicin	*Capsicum chinense* var.cumari-do-Pará	Ethanol/sodium phosphate	85.6	5.2	51
Cyclodextrins (CDs)	Enzymatic conversion of starch by *Bacillus cereus* cyclodextrin glycosyltransferase	PEG 20000/dextran T500	–	–	54
gamma-Cyclodextrin (γ-CD)	Enzymatic conversion of starch by *Bacillus cereus* cyclodextrin glycosyltransferase	PEG 3000/potassium phosphate	81.88	–	53
Anthocyanin	Mulberry	Ethanol/ammonium sulfate	85.8	–	55
Anthocyanin	Grape juice	Sodium dihydrogen phosphate/ethanol	99	–	56
Anthocyanin	Red cabbage	PEG4000/magnesium sulfate, followed by forward osmosis	–	–	57
Chlorogenic acid	Blueberry leaves	ChCl-1,3-butanediol/K_2HPO_4	96.18	–	29
Ginsenosides	*Panax Ginseng* C. A. Mey	[C4Tr]Br/NaH_2PO_4	99.5	–	12
Plasmid DNA (pDNA)	Alkaline bacterial cell lysate	Polyethylene glycol/sodium sulfate	67	–	60
Phenolic compounds	*Eucalyptus globulus* wood veneers	PEG2000/ammonium sulfate	–	–	62
Phenolic compounds	*Aronia melanocarpa* pomace	Ultrasound-assisted ammonium sulfate/ethanol	–	–	63
Phenols compounds	Wheat chaff	Ultrasound assisted ethanol/ammonium sulfate	64	–	64
Stevioside	Stevia	Choline chloride/thetripotassium phosphate	95.02	–	66

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biotechnological products are not extensively reported. It is believed that extensive research on these areas would lead to a revolution in separation technology.

New possibilities of ATPS are being explored to extend its generic application and to maximize its application with a wider adoption. Through integration of ATPS with other promising tools, it is anticipated that the use of bioprocessing design which involves lesser unit operations would make ATPS an important option in the processing of biotechnological products (16).

Last but not least, the current trend of bioprocessing is also going towards biorefinery concept that allows the simultaneous separation of two compounds in a single step of extraction process. This could possibly trigger the development of a new ATPS-based strategy with more versatile application and thus open door for a wide range of biomolecules recovery at commercial level.

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