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Recovery of Biological Products in Aqueous Two Phase Systems

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***Abstract*—Recovering of biological products, such as enzymes, proteins, nucleic acids, amino acids and microorganisms, from the contaminants requires many steps, each removal of the contaminants, isolating of target product and purification of product closer to the demand. For the success of the large scale production, there is a need for improving process economic, efficient and delicate downstream technique enough to preserve the properties of bio-products. Aqueous two phase systems (ATPSs) have overcome these demands and emerged as a powerful method for the downstream processing of bio-products. This paper is intended to encourage a starting idea for beginners in using ATPSs technique with biological products. Factors contributing to the partition behavior of biomolecules between the two phases including polymer molecular weight and concentration, biomolecule size, surface charge, pH and temperature are presented.**

***Index Terms*— Aqueous two phase systems (ATPSs), Biomolecule, Partitioning.**

I. Introduction

Downstream processing of biological products from fermentation broth is an important step of production. It is considered to be the most expensive part of process production [1]. The conventional techniques such as chromatography, electrophoresis and precipitation have been widely employed. However, these methods are considerable cost, providing low yields and not suitable for large scale production. Recently, one of the most economical downstream processing for biomolecules recovery is an aqueous two phase systems (ATPSs).

ATPSs have been an attractive technique for recovery of biological materials over other methods since it constitutes gentle environmental condition containing high water content in both liquid phases up to 70-90% [2]. The interfacial tension between the two phases is low [3], resulting in high mass transfer. Many polymers used in the system have stabilizing effects on the biological activity and structure of proteins and enzymes [2]. Thus, the denaturizing of labile biomolecules possibly decreases. This technique is also straightforward and requires relatively simple equipments which are easy to operate [4]. Moreover, the conditions for separation on a large scale do not considerably change from small scale, thus easy in scale-up and reliable.

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With regard to these advantages, the two phase systems have been applied in several fields such as recovery of biopharmaceuticals, environmental remediation, proteins purification and extractive bioconversion. However, a major drawback of ATPSs is the lack of knowledge on the mechanism involved in the partitioning process and poor understanding of the technique [5]. Then it is difficult to predict phase equilibrium and product partitioning. The cost of phase forming polymer is also high [6] and the wastewater streams generated from polymer-salt system have high concentrations of phosphate or ammonium ions.

The present article is intended to encourage a starting idea for beginners in using ATPSs technique. This was done to provide a better focus on mechanism of biomolecules partitioning between the phases, the type of phase composition, factors affecting biomolecule partitioning behavior, application and its future perspectives.

II. Mechanism Of Partitioning

The ATPSs can be formed by combining aqueous solutions of two incompatible polymers or from a mixing solution of polymer and salt above critical concentration. Two liquid layers are obtained at equilibrium. The first polymer predominates in one phase and the second polymer or salt predominates in the other phase. Generally, the biomolecules are more evenly distributed between the phases. The distribution is concerned by many parameters relating to the phase system, physico-chemical properties of biomolecule and theirs interaction. After adding solution to the system, mixing and phases settle, the partitioning of target product should be one side whereas the undesirable particles such as cells, cell debris, other proteins and contaminants distribute to the opposite phase. A general characteristic of phase forming and molecules partitioning between the phases is shown in Fig.1.

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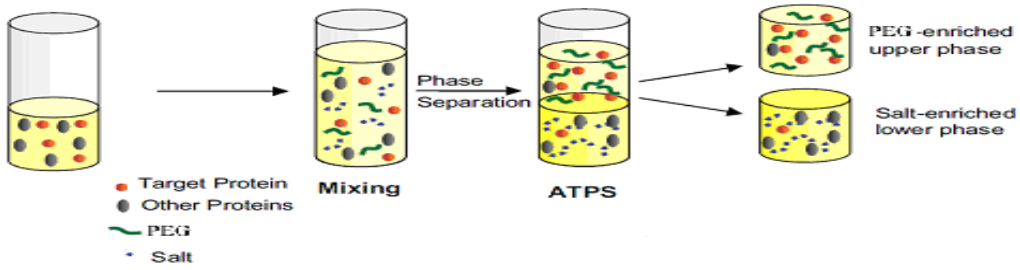

Fig. 1. The schematic distribution of molecules in aqueous two phase systems (adapted from [7])

The mechanisms that cause the uneven distribution of biomolecules are largely unknown. The partitioning behaviour of the molecule between the two phases is uncertain phenomena due to the involvement of many factors in the interactions between the biomolecules and phase-forming components such as hydrogen bond, charge interaction, van der Waals' force, hydrophobic interaction and steric effect [8, 9]. The net effect of these interactions is likely to be different in the two phases and therefore the biomolecules will partition into one phase where the energy is more favorable. The relationship between the partition coefficient (K) and ΔE at equilibrium can be expressed as:

$$K = \exp\left(\frac{\Delta E}{kT}\right) \tag{1}$$

where ΔE (J) is the energy needed to move biomolecules from one side to the other, k is the Boltzman constant and T (K) is the absolute temperature. ΔE depends on the size of the partitioned molecule.

The partition coefficient, K is also defined as the ratio of biomolecules concentration in the top phase (C_T) to in the bottom phase (C_B) (as shown in eq. (2)). If the coefficient is higher than 1, the molecules prefer the top phase and if lower than 1 is in the bottom phase.

$$K = \frac{C_T}{C_B} \tag{2}$$

The partition coefficient has also been found empirically to depend on several factors, which act roughly independent. Therefore, the overall partition coefficient may be written as:

$$\ln K = \ln K_0 + \ln K_{el} + \ln K_{hfob} + \ln K_{biosp} + \ln K_{size} + \ln K_{conf} \tag{3}$$

where el, hfob, biosp, size and conf stand for electrochemical, hydrophobic, biospecific, size, and conformational contributions to the partitions coefficient respectively and $\ln K_0$ includes other factors [10].

In addition to partition coefficient, other parameters (eq. (4)-(7)) are evaluated in order to understand the distribution behavior of target molecules between the phases. Phase volume ratio (R_V) is defined as the ratio of volume in the top phase (V_T) to that in the bottom phase (V_B).

$$R_V = \frac{V_T}{V_B} \tag{4}$$

Yield recovery from top phase (R_T) and from bottom phase (R_B) are calculated in order to evaluate the purification ratio (PR) from aqueous two phase system, according to following equations:

$$R_T = \frac{100}{1 + \frac{1}{R_V K}} \tag{5}$$

$$R_B = \frac{100}{1 + R_V K} \tag{6}$$

$$PR = \frac{\text{Recovery of target protein}}{\text{Recovery of total protein}} \tag{7}$$

III. Type Of Phase Composition

Liquid phase system can be obtained when one or more polymers are dissolved in water in the presence or absence of low molecular weight solutes. Different types of phase composition have been classified in two main groups. First group contains two different polymers while the other contains only one polymer and one salt in an aqueous solution.

The phase system based on two polymers occurs from the unfavorable energy of interaction. It appears when segments of one polymer contact segments of the other polymer or the polymer segments bond strongly to each other. This kind of phase separation is known as complex coacervation. The favorable interactions are due either to electrostatic interactions between positive charges on one polymer and negative charges on the other, or to strong hydrogen bonding between segment pairs. The examples of polymer used in preparation ATPSs are polyethylene glycol (PEG), dextran, polypropylene glycol, polyvinylpyrrolidone, and hydroxypropyldextran. Generally, PEG and dextran are preferably employed in ATPSs preparation among polymers because they perform desirable physical properties with non toxicity [11] and dextran has a stabilizing effect on microbial cells [12]. Zijlstra et al. [13] designed the system containing PEG, dextran and culture medium to support the long-term growth of animal cells. The hybridoma cells were successfully cultured and partitioned to the PEG phase. Johansson and Reczey [14] used PEG and dextran system to concentrate and purify β-glucosidase from *Aspergillus niger*. They found too high concentrations of both polymers forced all soluble proteins into the bottom phase (dextran-rich phase) and provided low solubility in concentrated PEG solution. It was concentrated up to 700 times and purified 2-3 times.

Although PEG and dextran system is a commonly use, the high cost of fractionated dextran is a limitation for its application in large scale process. Antov [15] cultivated *Polyporus* squamosus in ATPSs which composed of PEG and crude dextran in order to produce endo- and exo-pectinase. The products were enriched in the top phase with

partition coefficient about 2.45. Although crude dextran provided similar properties with dextran and less expensive, crude dextran was difficult to handle and removed from the system due to its high molecular weight fractions and high viscosity.

Other alternative inexpensive substitutes of dextran such as derivatives of starch, methylcellulose, cellulose, ethyl hydroxyl ethyl cellulose, agarose, guar gum and polyvinyl alcohol can be used with lower concentration as well. For example, Almeida et al. [16] achieved in purification of cutinase from ATPSs composed of PEG and a crude hydroxypropyl starch with presence of sodium chloride or sodium sulphate in the system at pH 4.0. Oliveira et al. [17] studied the partition behaviour of trypsin in PEG and cashew-nut tree gum. The recovery of maximum trypsin was obtained in the cashew-nut tree gum phase with the system consisted of PEG 8000 at pH 7.0 and 1.0 M NaCl. da Silva and Meirelles [18] used maltodextrin, which was a low-cost starch derivative, to replace dextran in partitioning behavior of bovine serum albumin, α-lactoalbumin and β-lactoglobulin with polypropylene glycol (PPG). Most proteins partitioned preferentially to the PPG phase.

The other phase system based on only one polymer in the presence of low molecular weight solutes (salt) is usually preferred for large scale operation since salt is much cheaper than dextran and the phases have a lower viscosity, then it is easier to handle and a shorter time for phase partitioning is required [4]. Many types of salt, such as potassium dihydrogen phosphate, potassium chloride, sodium dihydrogen phosphate, sodium carbonate, sodium citrate, magnesium sulphate and ammonium sulphate, can be used in forming ATPSs with polymers, especially with PEG [19]. The most common polymer-salt used is PEG and phosphate salt (generally sodium or potassium phosphate) system due to low cost, widely employ in the past and current application, and suitable range of system pH from 6-9 under which the system is stable [5]. However, selecting salt also depends on its effect with interesting product because different salts affect the water structure and hydrophobic interactions differently [20]. Although the application of polymer-salt system is inexpensive and provides large partition selectivity [12], the system is limited in the presence of high salt concentrations which probably causes protein denature and inhibits cell growth. Furthermore, the recycling of salt from ATPSs is poorly investigated. Then, it is difficult to dispose in large amount without environmental problem.

IV. Factors Affecting Biomolecule Partitioning Behavior

The partitioning of biomolecule in ATPSs depends on many variables such as type and concentration of polymers, the surface properties of particles and temperature. The role of these factors on partitioning behavior is discussed in the following sections:

A. Polymer molecular weight

The molecular weight of phase polymers influences biomolecule partitioning both by altering the phase diagram and by changing the number of polymer-biomolecule interactions. In general, increasing the molecular weight of the phase polymers resulting in the distribution of biomolecule towards more strongly into the other phase as the repulsive interactions between the polymer and biomolecule become stronger. When the same molecules are added into phase system with different molecular weight of polymer, theirs partition coefficient decrease as molecular weight increase. The reason of this phenomenon is that an increase in molecular weight of polymer results in an increase in the chain length of the polymer and the exclusion effect, which lead to the reduction in the free volume. Thus, polymer acquire a more compact conformation with intramolecular hydrophobic bonds and hindered the partition of biomolecule into the top phase [21]. Madhusudhan et al. [22] studied the extraction of alcohol dehydrogenase (ADH) from yeast in PEG-salt system. The free volume in the PEG phase significantly reduced with an increase in PEG molecular weight from 600 to 20,000 resulted to the ADH selectively partition to the bottom phase because of the volume exclusion effect. A similar observation was found in partitioning of α-galactosidase from *Aspergillus oryzae* [1]. However, at very low molecular weight of polymer is also unsuitable to use in phase forming because the exclusion effect decreases and as a result the polymer can induce all the particles including undesired molecules to the polymer phase [21]. Therefore, the selection of the proper molecular weight of polymer is the key point in this technique. Furthermore, the differential partitioning of biomolecule may influence by the interfacial tension between the phases [23, 24]. Decreasing the molecular weight of phase forming polymers also decreases interfacial tension [25].

B. Polymer concentration

In general, an increase in polymer concentrations relates to high density, refractive index and viscosity of the phase. Thus high concentration of polymer provides large difference in properties between the phases. In case of polymer-salt system, lower concentration of salt is required for ATPSs preparation when using the higher the concentration of polymer. The role of molecular weight also concerns with concentration used in phase forming. The higher the molecular weight of the polymer, the lower the concentration required for phase separation. The viscosity of the phase is affected by the molecular weight of polymer. Since the viscosity of a polymer solution mainly depends on the concentration. High viscosity might impact further process. The viscosity of one phase might be decreased by employing a higher molecular weight of the polymer. The interfacial tension between the two phases of polymers system is very small in comparison to the interfacial tension between an aqueous phase and an organic solvent phase. The interfacial tension is dependent on the polymer and salt composition. When the polymer concentration is increased, the composition of the phase system is removed from the critical point and the interfacial tension is increased [2]. As a result, the biomolecules will favour more to the top or bottom phase. For example, Babu et al. [26] found that increasing the concentration of PEG 1500 from 12 to 18% (w/w) resulted in increase partitioning of polyphenol oxidase to the bottom phase. The ionic composition strongly affects the behaviour

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of phase system containing polyelectrolyte whereas in the phase diagram of non-ionic polymer-polymer system is little. In general, polymer concentration is required for phase separation to form when the salt concentration is increased [2].

C. Biomolecule surface properties

The surface properties of biomolecule, such as surface net charge, molecular weight, shape, surface hydrophobic and the existence of specific binding site, affect the partitioning [17]. The actual surface of biomolecule, which contacts the surrounding solution, may be quite different from the overall material properties. For example, the surface of globular protein is made of different types of amino acid which generally contains both polar and non-polar in nature. The polar and non-polar groups incorporated in the side chain of the amino acids cause a different hydrophobicity and hydrophilicity. The partitioning is related to the surface properties of the material. The proteins are charged or can be charged modification of overall molecule at different pH values [9]. When the solution pH changes from acidic value to basic value, the protein becomes less positively or more negatively charged [21]. In general, for PEG-salt system, negatively charged protein should prefer the PEG rich phase while the positively charged protein distributes in salt phase [27, 28]. Shang et al.[29] studied the partitioning behavior of four amino acids with different side chains, (cysteine, phenylalanine, methionine, and lysine) in PEG-phosphate system. Lysine exists as a cation in the pH range 6.5-8.0. Other amino acids are anions at the same condition. In the two phase systems, the distribution ratios of lysine were the lowest among the four amino acids. That means the electrostatic interaction between lysine cation and salt anion is the biggest. Thus, lysine preferred to be in the bottom phase. Amino acids with negative charges preferred to be in the top phase. That was probably due to the repulsion caused by the salt anions.

At the isoelectric point of the protein, the sum of all the charges on the protein is zero. All other pH values the protein has a net charge. Thus, partitioning of a protein in a two-phase system frequently depends on the net biomolecule charge, which is a function of the solution. The pH changes may also induce conformational changes in the structure of the protein, causing also a change in protein partitioning behavior. This behavior can be explained on the basis of Albertsson's equation. The partition coefficient of a charged biomolecule is influenced by electrostatic and non-electrostatic (van der Waals) molecular interactions as follow:

$$\ln K_p = \ln K_p^0 + \frac{F\Delta\psi Z_P}{RT} \quad (8)$$

where K_p and K^0_p are the partition coefficient at given pH and the isoelectric point (pI) and $\Delta\psi$ is the difference of interfacial potential between the top and bottom phases (ψ_{top}-ψ_{bottom}) which influences the partitioning behavior of target biomolecule. The Z_P, F, R and T represent the net protein charge, Faraday constant, universal gas constant and absolute temperature, respectively.

For example, when the pH increases above the pI of β-galactosidase in range 4-5 [30], their charge became negative and strongly interacted with PEG-rich phase. The partitioning behaviour is similar to α-galactosidase from Aspergillus oryzae [1]. Gautam and Simon [31] found that the β -glucosidase surface became positively charged when the pH of the system was increased from 6.0 to 8.0 ($pI_{glucosidase}$=8.7). Thus, the β–glucosidase likely distributed in the salt phase. However, at very extreme pH, the protein might be denatured. A denatured protein has a significantly greater surface area than the native protein, and the exposed surface is much more hydrophobic, causing also different partitioning [10].

Different molecular weights of biomolecules also have an effect on partitioning. It found that biomolecule with higher molecular weights are more influenced by changes in the molecular weight of polymers than those with small molecular weights.

D. Temperature

The effect of temperature is very different for each phase system relying on the type of polymer used. For example at high temperature, it is easily to form two phase with small concentration of PEG or salt whereas in case of PEG and dextran system, two phases will easily form at lower temperature [2]. An increase in temperature results in increased differences in the phase composition. It enhances the concentration of PEG and salt in the top and bottom phase respectively. Consequently, the number of water molecules available for solute salvation in the bottom phase decrease due to an increase in salt concentration. This also reduces the solubility of biomolecules in the phase. The partition coefficient of the biomolecules probably influences by this variation in the phase compositions. Naganagouda and Mulimani [1] indicated that the partition coefficient of α-galactosidase in PEG-salt system increased with temperature from 25 to 55 ^{0}C.

Furthermore, increasing temperature can destroy the bonds of biomolecule. As these bonds are weakened and broken, the biomolecule becomes more flexible structure. Water in two phase systems can interact and form new hydrogen bonds with the functional group of the biomolecules. The presence of water further weakens nearby hydrogen bonds by causing an increase in the effective dielectric constant near them. As the structure is broken, hydrophobic groups are exposed to the solution. As a consequent, losses in solubility of molecule are observed.

V. Application Of Aqueous Two Phase Systems

ATPSs can be considered as an integrated technique where extraction, concentration and primary purification are in a single unit operation. The application of two-phase systems has been focused on the recovery of biomaterials from fermentation broths and biological extracts. Normally, the biological products are present in the broth at low levels or in dilute form and have to be concentrated, isolated and purified from other constituents of broth. The two phase systems can complete these requirements in such a way that most of the desired biomaterials are removed to a phase with a small volume compared to the original. The interfering substances

and contaminants, such as cells, cell debris, RNA, carbohydrate and lipid, should partition into the other phase based on surface properties of the particles and molecules including size, shape, surface net charge, hydrophobicity and the existence of specific binding sites [8]. A simultaneous primary purification may also be achieved.

In recent years, the use of ATPS processes for protein extraction and primary purification, in particular enzyme purification has considerably increased (Table 1). The ATPSs are not only restricted to enzymes purification from microbial cells but also enzymes from more complex raw materials like animal tissues and plant cells. Furthermore, ATPSs have been applied for separation of membrane bound cholesterol oxidase from *Nocardia rhodochrous* [32] which normally are rather difficult and time-consuming to purify, the purification of plasmid DNA from *Escherichia coli* cell lysate [33] and the extraction of small molecular weight compounds such as

amino acids [29]. The ATPSs are also an option for the downstream processing of therapeutic proteins for example monoclonal antibodies, growth factors and hormones [34]. Another interesting examples of this technique to non-protein product include extraction of metal ions from aqueous solution [35, 36], removal of food coloring dyes from textile plant waste [37], removal of chromium (III) [38], extraction and purification of betalains (pigment) [39] and recovery small organic molecules [40].

TABLE 1. AQUEOUS TWO PHASE SYSTEMS OF ENZYMES

Enzyme	Production source	ATPS	Recovery yield (%)	Purification factor	Reference
α-galactosidase	*Aspergillus oryzae*	PEG-phosphate	87.7	3.60	[1]
β-glucosidase	*Aspergillus niger*	PEG-dextran	85-95	3.30	[14]
	Trichoderma reesei	PEG-phosphate	>92	-	[31]
Xylose reductase	*Candida mogii*	PEG-phosphate	103.5	1.89	[41]
Bromelain	Pineapple	PEO-PPO	79.5	1.25	[42]
		PEG/phosphate	90	2.70	[26]
Lysozyme	Chicken egg white	PEG-sulfate	70	7.60	[43]
Protease	*Bacillus subtilis* TISTR25	PEG-phosphate	96.3	6.10	[44]
Laccase	*Agaricus bisporus*	PEG-phosphate	95	2.48	[45]
Lipase	*Aspergillus terreus*	PEG-phosphate	100	12	[46]
Phenylalanine dehydrogenase	Recombinant *Bacillus badius*	PEG-$(NH_4)_2SO_4$ -NaCl	95.85	474.3	[47]
Xylanase	*Bacillus pumilus*	PEG-phosphate	98	33	[48]

ATPS processes are employed in integration of the upstream operation of fermentation and downstream recovery processes. It is called as extractive bioconversion. The concept of extractive bioconversion in ATPSs is immediately to transfer the products from theirs bioconversion when they are formed [6]. The biocatalyst employed for conversion presents in one phase and the products are either distributed among the two phase or they remove preferably to the biocatalyst free phase. Thus, removal of product without losing biocatalyst can possibly be completed. ATPSs have shown potential for improving yield and productivity of bioconversion. Since toxicity and product from bioconversion are instantly separated resulting in an increase in the productivity and decreasing the degree of product inhibition [5]. For example, the fermentative production of 6-pentyl-a-pyrone (6PP), which is aroma compounds produced by *Trichoderma harzianum*, caused inhibition of the microbial growth as a result the production of 6PP was limited. The attempt on minimization of this effect during fermentation in ATPSs was success [49]. Li et al [50] investigated the extractive bioconversion of starch using ATPS. The use of ATPS gave a higher maltose yield from 19.0 to 15.5 mg ml^{-1} compared to the control. Higher hydrolysis rates were found in the two-phase systems where

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glucose produced from starch hydrolysis was removed into the top phase with a simultaneous reducing the degree of glucose inhibition. More examples of extractive bioconversion in two-phase systems are presented in Table 2.

TABLE 2. EXAMPLES OF EXTRACTIVE BIOCONVERSION IN ATPSS

Bioconversion product	Reference
Cultivation of *Polyporus squamosus* in PEG and crude dextran for production of pectinase	[15]
Hydrolysis of starch using amylase in thermoseparating polymer-based aqueous two-phase systems	[50]
Bioconversion of cellulose by *Trichoderma viride* in dextran and polyethylene glycol (PEG) system	[51]
Synthesis of cephalexin by using penicillin G acylase in PEG-magnesium sulfate system	[52]
Bioconversion of penicillin G to 6-aminopenicillanic acid (6-APA) in ATPS consisting of PEG and potassium phosphate solution	[53]
Production of xylanase by the thermophilic fungus *Paecilomyces thermophila* J18 in solid-state fermentation using ATPS	[54]
Production of pectinases by *Polyporus squamosus* in aqueous two-phase system (PEG-crude dextran)	[55]
The use of ATPS for simultaneous biosynthesis and purification of two extracellular *Bacillus* hydrolases	[56]

The extractive bioconversion in ATPSs has also overcome the degradation of product such as the production of antibiotic decrease after closing maximum bioconversion due to the prevalence of product degradation over synthesis [57]. Furthermore, the subsequent downstream processing steps are probably eliminated in number as it is an integration of product removal with that of bioconversion.

VI. Conclusions And Future Perspectives

It can be concluded that the aqueous two phase systems have been successfully applied in the upstream and downstream processing of several biological materials. The partitioning of biomolecules in the system is dependent on the properties of the protein as well as on the two aqueous phases.

The system composed of polymer-salt has been widely used due to several advantages, including: higher selectivity, lower cost, and lower viscosity in biomolecule partitioning in comparison with polymer-polymer systems. In the polymer–salt systems also have a wide application and the range of system pH (from 6 to 9) under which the two phase systems are stable.

However, the high consumption of phase-forming components and their impact on wastewater treatment is a relevant issue for the application of these systems on a large scale. Although PEG is biodegradable and non-toxic, the polymer costs need to be evaluated the process economics. Moreover, salt disposal (e.g. phosphate, ammonium) can be problematic due to enhancing eutrophication phenomenon in water. This limitation may be conducted by recycling the

polymers and salts or choosing other salts used in the process such as sodium citrate which is biodegradable and non-toxic salt. The further study for solving these problems should be considered.

The application of ATPSs for large scale manufacturing has not been fully encouraged since the limitations of ATPS arise from poor understanding of theoretical mechanism on phase equilibrium and protein partitioning, the cost of phase forming polymers and the isolation of biomolecule from the phase-forming compounds. The developments in the field of ATPS can be achieved by combining some downstream processing techniques such as ion-exchange chromatography, gel filtration, precipitation and ultrafiltration for further product purification.

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