Aqueous two-phase systems: An attractive technology for downstream processing of biomolecules

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Today, industry demands fast and economic downsteam processes for the partitioning and purification of biomolecules as well as those processes that give high yield and high purity of the product. Therefore, in light of the above demands, aqueous two-phase systems are an ideal technology where clarification, concentration, and partial purification can be integrated in one step. Moreover, this method can be made highly selective and can be easily scaled up, thus allowing wider biotechnological applications.

Aqueous two-phase system: A general account

Downstream processing is an integral part of any product development, and the final cost of the product depends largely on the cost incurred during extraction and purification techniques. The conventional techniques used for product recovery, for example precipitation and column chromatography, are not only expensive but also result in lower yields. Furthermore, since solid–liquid separation by centrifugation or filtration results in some technical difficulties, for example filter fouling and viscous slurries[1], there is an ongoing need for new, fast, cost-effective, ecofriendly simple separation techniques. Thus, for separation of biomolecules, aqueous two-phase systems (ATPS) offer an attractive alternative that meets the above-mentioned requirements as well as the criteria for industrially compatible procedures. Hence, it is increasingly gaining importance in biotechnological industries[2]. The advantage of using this technique is that it substantially reduces the number of initial downstream steps and clarification, concentration, and partial purification can be integrated in one unit. Furthermore, scale-up processes based on aqueous two phase systems are simple, and a continuous steady state is possible.

Aqueous two-phase system was developed in Sweden during mid-1950s for the separation of macromolecules, and cells and organelles[3]. These systems were initially applied to the separation of plant organelles and viruses. Since then, attention has been directed towards widening its application scenario. During the last two decades, lot of work has been done to develop feasible separation processes using aqueous two-phase systems for various biological materials, and proteins and recombinant proteins[4].

An aqueous two-phase system is an aqueous, liquid–liquid, biphasic system which is obtained either by mixture of aqueous solution of two polymers, or a polymer and a salt. Generally, the former is comprised of PEG and polymers like dextran[5,6], starch[7], polyvinylalcohol[8], etc. In contrast, the latter is composed of PEG and phosphate or sulphate salts. This polymer–salt system results in higher selectivity in protein partitioning, leading to an enriched product with high yields in the first extraction step.

Since these phase components are inert towards biological materials, these can be employed for partitioning of biomolecules, and cell organelles and whole cells as well. The basis of partitioning depends upon surface properties of the particles and molecules, which include size, charge, and hydrophobicity. Moreover, the most characteristic feature of the two-phase system is that the water content in it is as high as 85–99%, which when complemented with suitable buffers and salts results in providing a suitable milieu for biological materials, as well as in an easy scale-up possibilities[9]. In addition, the low surface tension between the two phases results in partitioning of proteins possible without any loss in their activity. The content of polyols present in most aqueous phase media helps to stabilize the enzymes by reducing the water content[10]. Also, the small droplets, which are generated in such a phase system give short distances and large surface areas, facilitating mass transfer[11]. The necessary separation of the two immiscible liquid phases, which is relatively slow under unit gravity, can be enhanced by centrifugation[12]. Therefore, the mechanical separation step can be replaced by an extraction process which is thermodynamically controlled and enables

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the separation of cells and cell debris from soluble proteins by partitioning into opposite phases under suitable conditions[13].

Partitioning of the two phases is a complex phenomenon, taking into account the interaction between the partitioned substance and the component of each phase. A number of different chemical and physical interactions are involved, for example hydrogen bond, charge interaction, van der Waals' forces, hydrophobic interaction and steric effects[11]. Moreover the distribution of molecules between the two phases depends upon the molecular weight and chemical properties of the polymers and the partitioned molecules[14] of both the phases.

The distribution of molecules between the two phases is characterized by the partition coefficient, K_{part}, defined as the ratio of the concentrate in the top (C_{top}) and bottom (C_{bottom}) phase, respectively.

$$K_{part} = C_{top}/C_{bottom}. \quad (1)$$

The ability of a certain substance to partition in such a phase system may be described as the sum of different factors[15] which can be expressed as:

$$\ln K_{part} = \ln K_{el} + \ln K_{hydrophobic} + \ln K_{hydrophilic} + \ln K_{conformation} + \ln K \text{ of other factors}, \quad (2)$$

where K_{el}, $K_{hydrophobic}$, $K_{hydrophilic}$ and $K_{conformation}$ denote partition factors due to electrical, hydrophobic, hydrophilic and conformation effects respectively. Thus, different factors of the system can be manipulated in order to achieve the desired effect[16].

Aqueous two phase systems meet all the characteristics of an ideal extraction technology, specially for proteins, since it is less time consuming and has the potential to give high yield and high purity, involving low investment, less energy, and lower labour costs. However, it is not being commercially exploited, since most of the phase-recycling processes have not been defined. To adopt this technique for large-scale processing therefore involves developing recycling operations to make it economically feasible[5]. This would also save cost of effluent treatment and diminish environmental load[8]. However, for high-priced pharmaceuticals, produced by recombinant technology, the cost of phase-forming chemicals is tolerable in view of their high yield, volume reduction and enrichment obtained by the extraction step.

Thus, in light of the above, only those aqueous two phase systems are desirable where back extraction of the product has been achieved to facilitate recycling of the phase components. Guan *et al.*[17] have developed a back extraction system using 20% (w/w) PEG-phosphate ester and 10% (w/w) potassium phosphate system, at pH 6.0, which gives 76% yield of recombinant interferon $\propto$ 1 from *Escherichia coli*. The purification of a mixture of two lipases produced by *Bacillus stearothermophilus* SB-1 with complete recycling of both the phase components has also been accomplished in our laboratory[18].

Aqueous two-phase systems offer an attractive technology for large-scale protein purification as well, but more efficient phases for effective and economical downstream processing still need to be developed. Generally, PEG/dextran systems are employed which are expensive due to the high cost of dextran. Crude, unfractionated dextran has therefore been used as a cost-effective substitute for dextran in enzyme extraction[6] and in bioconversions[2]. However, a major drawback in the industrial application of dextran is its high molecular weight and high viscosity. Therefore, PEG/salt systems have been preferred for large-scale enzyme extraction. Though this system is inexpensive, its application is limited due to presence of high salt concentrations which may denature the purified enzyme[5]. This therefore led to trying out inexpensive substitutes of dextran, like derivatives of starch[2], cellulose, polyvinyl alcohol[8], hydroxypropyl starch (HPS)[7] and ethyl hydroxy ethyl cellulose (EHEC). These are not only inexpensive but can be used at lower concentrations as well. Thus, the major thrust in improving aqueous two-phase systems for large downstream processing has been towards developing newer polymers which are inexpensive and can be used in low concentrations.

Novel phases

Aqueous two-phase systems can become an even more effective partitioning and purification technique with the introduction of novel phases, for example microemulsion phases are attractive protein-extracting media[20]. The partitioning can be modulated over 3–4 orders of magnitude through controlling pH, ionic strength and surfactant concentration[21]. Recent trends in magnetically enhanced phase separation have also been applied to aqueous two-phase systems by adding ferrofluid or iron oxide particles to the phases[22]. It was observed that magnetic additives completely distributed in dextran-rich phases. In addition, the dextran phase could be kept stationary in a column system, while the PEG phase could be easily piped through the column. A semi-continuous three-step separation of enzymes, e.g. lactate dehydrogenase and β-galactosidase, has been developed using a magnetic field where separation was achieved in less than an hour[23].

Another strategy used was to enhance selective partitioning by the formation of agarose beads in aqueous two-phase systems[24]. The top phase is formed by a copolymer of PEG and PPG (Synperonic F-68, ICI Petrochemicals and Plastics Division) and the lower phase consists of hydroxyethylated agarose, gelling at low temperatures. Agarose is melted at an elevated temperature followed by decrease in temperature, and subse-

quently the agarose is mixed with synperonic under constant stirring. After a short time, the temperature is rapidly lowered to 20°C to make the final agarose gel. Gel beads with diameters ranging from 20 to 200 µm can be obtained by controlling the stirrer speed. Agarose beads, based on aqueous two-phase systems, have suitable interfacial tension for the production of beads with a size relevant for use both in chromatography, and for the entrapment of cells. Furthermore, this is not restricted to agarose alone but can be employed in the production of beads made of other gel-forming polymers like alginate and carragenan.

Spray columns of novel phases forming components of PEG/dextran derivatives are another attractive device due to their simplicity and ease of construction and operation. Joshi *et al.*[25] studied spray columns in which the polymer phase was dispersed into fine droplets by passing through a nozzle at the lower end of a tube filled with another phase.

Some other new types of aqueous two phase systems are being developed in which a thermoseparating polymer serves as a phase-forming component. Binary aqueous solutions of a thermoseparating polymer split into two equilibrium phases above a critical temperature, referred to as the cloud point[26] (CPT). The most common class of water-soluble-thermoseparating polymers are the random copolymers of ethylene oxide (EO) and propylene oxide (PO), henceforth, collectively called EOPO polymers. These two polymer solutions demix into two macroscopic phases when heated above a critical temperature. While one of the phases is enriched in the polymer, the other phase gets depleted. One of the main advantages of using these polymers in aqueous two-phase system is the possibility that after a separation step wherein the target biomolecules have been partitioned to the EOPO-rich phase, the system is heated above the CPT which enables both polymer recycling and its removal from the target production solution without involving any costly separation methods.

Cosolutes, such as salts or surfactants, are often added to enhance the partitioning of biomolecules in aqueous two-phase extraction[26]. The aim is to take advantage of their stronger partitioning into one of the phases, and their ability to exhibit specific interactions such as electrostatic interactions and hydrophobic attractive forces, with the target biomolecule. Another approach that is being used to concentrate enzymes is the use of extreme volume ratios of phases. The conditions are standardized in order to preferentially partition the desired enzyme in the smaller phase[27].

Applications

Apart from the large-scale purification of extracellular proteins, the aqueous two phase systems can be applied to the following as well: (i) separation of membrane proteins, for example cholesterol oxidase and bacteriorhodopsin[28]; (ii) for structural analysis of the biological membranes such as thylakoid membranes[29]; (iii) for the concentration and purification of viruses[30]; and (iv) for bioremediation[31]. It can also be used for retroviral vectors purification as an apt substitute for microfilteration, ultrafiltration and chromatography protocols[32].

Besides partitioning and purification, two-phase systems have also been used for extractive bioconversions. The biocatalysts (enzymes or microorganisms) are partitioned to one of the phases and the product is extracted from the reaction compartment, and thus product inhibition can be avoided. This process has been used to enhance the production of lactic acid by *Lactobacillus* sp. by reducing the end-product inhibition[33]. It has also been used in small-scale conversion of cellulose[6] and starch[19] to glucose, as well as for butanol, acetic acid and butyric acid formation[34] by *Clostridium acetobutylicum*. Tjerneld *et al.*[6] have carried out semicontinuous hydrolysis of substrates in aqueous two-phase systems based on crude dextran and PEG over a period of more than 450 h. Using this system, the enzyme could be recycled.

Thus, aqueous two-phase systems offer an effective extraction process for biomolecules. It is characterized by short process times, high yield, and high productivity. It has the option for continuous and automated operation. It is an economical technology with low investment energy and labour cost and has great potential for modification. Further studies are required to understand the mechanism involved in partitioning of biomolecules.

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Received 20 March 1999; revised accepted 14 May 1999