BIOSEPARATIONS IN AQUEOUS TWO-PHASE SYSTEMS

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Bioseparations conducted in aqueous two-phase systems offer a great number of advantages over the conventional separation techniques. Among them the most relevant are rapid mass transfer due to low interfacial tension, rapid and selective separation, easiness of operation mode, reliability in scale-up, biocompatibility and environment-friendly features, and possibility of process integration when applied in biomolecule production. Upon overcoming the major problem - mostly empirical establishment of operating conditions, bioseparations in aqueous two-phase systems will become a necessary step in both existing and newly developed bioprocesses for the primary recovery of products.

KEYWORDS: Bioseparations; aqueous two-phase system; partition; extraction, downstream processing

INTRODUCTION

During the last several decades, a rapid development of different separation techniques in biochemistry and related fields has occurred, having the great impact on the advances made in biotechnology and product recovery. It is a well known fact that a greater part, some estimation says 60-90% (1), of the cost of biotechnology process is expended on downstream processing. That is why is not surprising that a major step in designing any successful bioprocess is the proper development of an efficient method for the isolation and purification of the target product. Such downstream processing must ensure in the first step that the product is separated from of other compounds. Moreover, for the products like proteins, enzymes, nucleic acids, antibiotics, antigens, etc. biological activity must be preserved up to the last purification step by the selection of a separation technique that is specific and compatible to the product.

The conventional downstream processing techniques currently being used have several disadvantages - low selectivity and degree of resolution, difficulties in scale-up, high
energy consumption, especially for viscous slurries, and possible denaturation of biomolecules by extraction utilising organic solvents. In addition, organic solvents are generally expensive and pose potential environmental hazard. Bioseparation operations have long been dominated by chromatographic techniques, despite limitations of high cost, batch operations, low throughput and complex scale-up (2).

Among the various alternatives, separation in aqueous two-phase system (ATPS) seems to be one of the most attractive, giving the solution to the most of problems listed above. According to the literature (3), bioseparation in ATPS belongs to bulk separations, marked by phase changes, and these techniques are roughly classified by their industrial maturity and resolution potential as suitable for selected industrial separations at the present.

Aqueous two-phase system provides a technically simple, easily scalable, energy efficient and mild separation technique for recovery of biomolecules (4). The mild environment of ATPS and low interfacial tension combined with the ease of scale-up and continuous operation makes it well suited for the large-scale separation and purification of biological macromolecules. When applied in biotechnological processes they allow process integration, as simultaneous separation and concentration of the target product are achieved (5). That is why bioseparations in aqueous two-phase systems are recognized as potential and powerful primary separation and purification step in the overall product recovery train.

FORMATION OF AQUEOUS TWO-PHASE SYSTEMS

An aqueous two-phase system is formed by mutual incompatibility of two-polymers or a polymer and a salt in aqueous solutions (4). Spontaneous separation of the phases occurs beyond a critical concentration of these components, resulting in two phases, enriched with respect to one of the components. The composition of an aqueous two-phase system is often illustrated by a phase diagram, a great number of them being determined and presented in the literature (4,6). The composition of an ATPS depends on the type of phase system constituents and their concentration, polymer molecular weight, pH, temperature and dissolved salts.

High molecular weight dextran and polyethylene glycol are the two polymers that have been widely used so far because of their well-known physical and chemical characteristics. Dextran is non-toxic and has a stabilising effect on biomolecules and microbial cells, so they can be grown and kept viable for a longer time, resulting in a higher product yield. Low-molecular-weight polyethylene glycols (PEGs) have been found to be inhibitory to cell growth (7), possibly due to their interactions with the cell walls of microorganisms.

A more economical alternative to the PEG/dextran system is PEG/salt ATPS and they have been extensively used for the protein purification from the crude feedstocks (8). The system has marked hydrophobic difference between the phases and, although it provides large partition selectivity, a high concentration of salts may be toxic to the microbial cells (9). Nevertheless, a few cultivations have been carried out in the PEG/salt systems with success, where the inhibitory effect of the salt has been found to be minimal (7,10,11).

Application of most widely used polymer/polymer system, PEG/dextran, is expensive at a larger scale. This problem may be overcome by using crude dextran (12,13) or
cheaper non-toxic polymers (14) instead of fractionated dextran. Another approach with the aim of decreasing the cost of polymer/polymer systems is to recycle polyethylene glycol which can be accomplished by its back-extraction by salt in two-phase system integration (15,16).

New developments in composition of ATPS include phase forming components that are easier to process than classical PEG/dextran or PEG/salt systems. Phase comprising thermoseparating polymers (17) and pH-sensitive polymers (18) offer the advantage of a simple back-extraction of the target species from the polymer-rich phase after the initial extraction by either raising the temperature or changing the pH to precipitate out the polymer.

**FACTORS AFFECTING PARTITIONING**

The basis of separation in ATPS is the selective distribution of substances between the two phases. This distribution is governed by a number of so-called environmental factors, relating to the properties of the phase system, then by the properties of the substance, and, finally, by the interaction between them, thus making the prediction of partitioning a difficult task.

The environmental factors include the influence of phase forming polymer type and its concentration (13,19), molecular weight of polymers (13,29), the tie-line length and phase volume ratio (21,22) and salt type and its concentration (23-25) and pH (20,22,24).

The properties of target substance that influence the partitioning include its molecular weight, net charge, hydrophobicity and other surface properties. Generally, small molecules are more evenly distributed between the phases; the partitioning of macromolecules is extremely variable, whereas that of particles is usually directed to one phase or to the interface (4). Since the influences of variables such as hydrophobicity and net charge of substance is mutually dependent their final effect is difficult to differentiate (26). However, some experiments with genetically engineered proteins clearly made the difference in their partition behaviour due to the differences in net charge (27) and hydrophobicity (28).

The multiplicity of factors contributing to partitioning makes the system very powerful, in contrast to the other established separation techniques like centrifugation, electrophoresis and so on, allowing the fractionation of molecular or particulate species differing very slightly from each other. Thus, the partition can be made more selective by manipulating the system properties to make a particular kind of interaction predominant. Moreover, some proteins show the so-called optimal behaviour, resulting in high yield and purification, if the partition is occurring in phase diagram regions in both polymer/salt (29) and polymer/polymer systems (22), where the differences between composition of phases are lower i.e. near the plait point.

**AQUEOUS TWO-PHASE SYSTEM AS A TOOL FOR BIOSEPARATION**

Both polymer/polymer and polymer/salt ATPS have advantages over conventional water-organic solvent systems for bioseparations. Since the bulk of both phases consists of water, aqueous two-phase systems form a gentle environment for biomaterials. The interfacial tension is extremely low (30), 0.0001 - 0.1 mN m⁻¹, compared with interfacial tension
between water and an organic solvent, which is usually in the range of 5 - 50 mN m⁻¹. This allows creation of high interfacial contact area of the dispersed phases and thus an efficient mass transfer. Furthermore, the polymers are known to have a stabilising influence on the particle structure and the biological activities (4). That are the reason for the aqueous two-phase systems to be recognised as biocompatible an environment-friendly.

**Analytical application of ATPS**

The sensitivity of partitioning in ATPS due to different surface properties and conformation of both soluble and particulate material has made this technique a very useful analytical tool for a variety of applications in analytical procedures. A particular advantage of the partition technique, besides the sensitivity and speed, is that it can allow the analysis of macromolecular and cell structures and that can be accomplished without sophisticated equipment.

The most powerful application of ATPS, very important for researchers in biochemistry, microbiology and biomedicine, has been its ability to fractionate cells into subpopulations, to probe their charge and hydrophobic surface properties, and to trace cell surface alteration occurring as a result of both normal and abnormal in vivo processes such as differentiation, maturation and aging, and also due to in vitro treatments (31,32).

Partitioning in ATPS under varying conditions of pH and salt has been useful for the estimation of surface charge and isoelectric point of proteins (33). Moreover, aqueous two-phase systems partitioning promises to be a convenient tool to study protein surfaces and detecting the changes in it as a result of mutations of amino acid residues (34).

Partition in aqueous two-phase systems have found its application as analytical procedure in the case of rapid quantification of antigen in the so-called Partition affinity ligand assay (PALA) (35), and also for sample preparation prior to the analytical procedure (36).

**ATPS for product recovery in biotechnology**

It is well known that cultivation and bioconversion processes are often subject to product inhibition and to a need of biocatalyst reutilization. Besides, in any cultivation process the target biomolecule has to be recovered from a mixture of a large number of interfering substances. Extractive cultivations and bioconversions that are conducted in ATPSs, as processes which integrate production and in situ product recovery, are the methods of choice to overcome the low product yield and potential toxicity for cells and denaturations of enzymes when using organic solvents in the extractions.

Cultivation system based on ATPS offer the possibility of replacement of the more difficult mechanical separation of cells from product by an extraction process that allows partition into opposite phases. These extractive cultivations have been applied for the production of extracellular enzymes (23,37,38), even more an enhanced enzyme production (23,39) and changed fungal morphology (38) were observed in aqueous two-phase systems in comparison to homogeneous cultivations.

The potential of aqueous two-phase systems for conducting the processes of extractive cultivation for obtaining the bioproducts such as antibiotics (9), toxin (40), aroma compounds (41), organic molecules of low molecular weight (42,44) and hydrogen (45) has also been demonstrated. In addition, there are reports on cultivations of both animal
(46, 47) and plant cells (48) for production of valuable metabolites in aqueous two-phase system.

Most biotechnological products, especially from submerged cultivations, are obtained in very dilute solutions, so the first desirable step in their recovery should be concentration. A two-phase system is able to carry out not only such concentration by extraction of most of the target substance to the phase with smaller volume compared to the original solution (16) but also its concomitant purification since the impurities may be concentrated to a lesser extent.

Extraction in ATPS offers in many cases better alternative to existing technology in the early processing stages of large-scale isolation of proteins from crude homogenisate (8). Moreover, if properly optimised, it also provides integration of cell debris removal, concentration and partial purification, thus reducing the number of downstream processing steps and improving the overall yield and economical feasibility of biotechnological process, which is especially important in the production of high valuable recombinant proteins (7, 49).

ATPS has proven to be a convenient medium for conducting extractive bioconversions, in which integration of bioconversion and product removal is achieved with the possibility of repeated use of the biocatalyst. In this way, the improvement of the productivity of biotechnological processes may be accomplished for products of hydrolysis of starch (50), cellulose (51) and pectin (52), as well as for bioconversion of pharmacologically active compounds (53).

Low productivity of biotechnological processes can be overcome in ATPS if it comes as a result of product inhibition (42), then if the product is unstable in the prolonged contact with biocatalyst (53) or if the product is very toxic (54), by their immediate partition into opposite phases.

**Improvement of the selectivity of extraction**

It is of great interest to increase the selectivity of product partitioning in order to make extraction predictable, as well as to achieve sufficient purification. Introducing an affinity partitioning, a separation process involving a biospecific affinity ligand, offers the advantage of improved selectivity besides all advantages of ATPS itself, listed above. Such affinity ligands are able to bind specifically and reversibly with the target protein in one of the phases of the system (55).

A relatively recent approach that has been applied for enhancement of the selectivity of extraction is genetic modification of the target protein with a fusion peptide or protein to target its partitioning into desired phase of a predetermined two-phase system (34). In the same context, new advances in two-phase systems showed that partition yield can be improved by fusing target proteins with a hydrophobic tag (56).

**Application of ATPS to process integration**

It is very important in biotechnology to achieve process integration combining the steps of production and recovery of the bioproduct in order to facilitate the development of scalable and efficient bioprocess. Furthermore, such integrated bioprocess needs to address the need to rapidly yield products in a state suitable for the validation, polishing, formulation and delivery operations. In this context, the application of ATPS, allowing in
product recovery and biocatalyst retention, to process integration represents an attractive alternative for the recovery of products in three major areas of research - extractive bioconversion, extractive cultivation and integration of cell disruption and primary purification step of intracellular products. The use of process intensification approach involving ATPS and process integration strategy results in a reduced number of unit operation and improvement of the overall volumetric productivity, and concomitant improvement in the effectiveness of the bioprocess.

CONCLUSION

Aqueous two-phase systems offer simple and powerful media for conducting bioseparations from the point of view of separation efficiency, easiness of scale-up and biocompatibility. Nevertheless, to achieve their wider use, it is necessary to overcome the poor understanding of the molecular mechanisms governing the behaviour of substances in ATPS and mostly empirical establishment of operating conditions. Consequently, bioseparation in aqueous two-phase systems will become a necessary step in both existing and newly developed bioprocesses for the primary recovery of products.

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Многобројне су предности извођења биосепарационих процеса у воденим двофазним системима у односу на конвенционалне сепарационе технике. Међу најважније свакако спадају ефикасан пренос масе омогућен ниским међуповршинским напоном, брза и селективна сепарација, једноставност у извођењу, поузданост приликом преношења параметара процеса у веће размере, биокомпатибилност и нешкодљивост по животну средину, као и могућност интергисања процеса у производњи комерцијално важних биомолекула.

Превазилажењем главног недостатка - углавном емпиријског одређивања оперативних услова, несумњиво је да ће биосепарације у воденим двофазним системима наметнути као незаобилазан корак у примарном издвајању производа биотехнолошких процеса, како постојећих, тако и будућих.