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RECENT DEVELOPMENTS IN AQUEOUS TWO-PHASE EXTRACTION IN BIOPROCESSING

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1. Introduction

Many fermentation processes do not see the light of day due to the high cost of downstream processing methods for the recovery of many biomolecules, which are of importance in industrial and academic research. Especially with the recent developments opening up new avenues toward the production of many such biomolecules, an increased interest has arisen in the development of efficient downstream processing methods for their separation, concentration and purification from fermentation and cell culture media. In conventional methods like centrifugation and even modern methods like electrophoresis and column chromatography, scale-up problems are enormous, making them uneconomical or prohibitively expensive unless the product is of high value. There are many methods for separating proteins, carbohydrates, and lipids, while there are fewer methods to handle complexes of proteins, membranes and cell organelles, and there is a paucity of methods for separating intact viable cells. Therefore there has been a need for alternative approaches to bioseparation problems.

Aqueous Two-Phase Extraction (ATPE) is one such method. Even though this technique has been known for quite some time, it has gained importance for industrial exploitation only recently. Unlike conventional liquid-liquid extraction (which is widely used in the chemical industry) involving organic and aqueous phases or pairs of organic phases, ATPE employs two aqueous phases. ATPE has been successful to a large extent in overcoming the limitations of conventional extraction such as poor solubility of proteins in organic solvents and the tendency of organic solvents to denature proteins/enzymes. ATPE has been recognized as a versatile technique for downstream processing of biomolecules such as proteins,

enzymes, viruses, cells, cell organelles, and other biological materials.[1,2,3,4] ATPE offers many advantages such as a biocompatible environment, low interfacial tension, low energy, easy scale-up, and continuous operation. More importantly, partitioning depends on differences in surface properties and does not depend on size, shape or density of the separand except under specific conditions.[5,6,7] Further, the equipment and methods of conventional organic aqueous phase extraction used in the chemical industry can be easily adapted to ATPE. However, ATPE is not selective enough to provide the extreme purity usually desired. Another main reason that ATPE has not reached industry is perhaps the slow demixing rate of the phases. Thus, ATPE has been recognized as a potential primary purification step in the overall protein/enzyme recovery train[8,9] in which the final purification is achieved by methods such as chromatography or crystalization. ATPE is also effective and efficient for the removal of contaminating materials and undesirable byproducts such as nucleic acids and polysaccharides.[5]

In this review we emphasize a few recent developments but do not attempt a comprehensive review of ATPE as such. Among recent developments, affinity partitioning is one of the most promising areas for the application of ATPE for the purification of biomolecules by means of attaching dye ligands[6] or metal affinity ligands [10] to the phase forming polymers. Extractive bioconversion using aqueous two-phase systems (ATPSs) is another recent development that can improve a number of existing processes to make them economically viable. This technique provides a novel means for the simultaneous production and purification of a bioproduct obtained through the use of enzymes or microorganisms. Recognizing the potential of ATPE, efforts are now in progress to develop or discover more efficient and economical ATPSs for large-scale purifications. The main deterrent to the widespread industrial application of use of ATPE is perhaps the slow demixing rates of the well mixed individual aqueous phases during/after the extraction. Another interesting development is the application of electric fields for increasing the phase demixing rate. and for selective extraction.[11] ATPE is gradually finding applications

also in biorelated areas such as food technology (e.g.: fat separation from cheese whey) and for purification of plant based bioproducts.

In the past three decades a wealth of information has been reported in the literature concerning various aspects of ATPE but the engineering aspects have received scant attention when compared to the scope of physicochemical studies and biological applications of ATPE. In the present review the emphasis is on recent developments in the engineering area of ATPE following a brief review introducing ATPSs. Further, ATPE can be employed not only for the separation of soluble materials (proteins/enzymes) but also for particulate matter such as cells, organelles and membranes.[1,3,4] However, the present article emphasises ATPE pertaining to proteins/enzymes.

2. Aqueous Two-Phase Systems

a. Formation of Aqueous Two-Phase Systems

ATPSs are of two types, polymer/polymer type and polymer/salt type. Some of the commonly used phase systems are listed in Tables I and II. Both components of these systems are separately miscible in water in all proportions and also with each other at low concentrations. As the concentration increases above a certain critical value phase separation occurs. The curve formed by joining these critical concentrations is called the binodal or phase diagram. Each ATPS is characterized by an exclusive phase diagram that indicates the equilibrium composition for that particular system and constitutes the most fundamental data for any type of extraction. A typical phase diagram is shown in Figure-1. Bamberger *et al.*[12] discussed in detail the methods for the construction of these phase diagrams. Albertsson[1], Diamond Hsu [6], and Zaslavasky [4] have compiled phase diagrams for a number of systems. The published phase diagrams indicate plait points for each ATPS. The formation of two phases typically requires total system concentrations of Polymer-1 in the range 5-12 % (wt/wt), and Polymer-2 concentration in the range 8-20 % (wt/wt), depending strongly on the molecular weights of these polymers. Similarly, for polymer-salt

TABLE I : POLYMER/POLYMER TWO-PHASE SYSTEMS

Polymer 1	Polymer 2
Polyethylene glycol	Dextran, Ficoll, Pullulan, Polyvinyl alcohol
Polypropylene glycol	Dextran, Hydroxypropyl dextran
	Polyvinyl pyrrolidone, Polyvinyl alcohol
	Methoxypolyethylene glycol
Polyvinyl alcohol	Dextran, Hydroxypropyl dextran
	Methyl cellulose
Polyvinylpyrrolidone	Dextran, Hydroxypropyl dextran
	Methyl cellulose
Methyl cellulose	Dextran, Hydroxypropyl dextran
Ethylhydroxyethyl cellulose	Dextran
Hydroxypropyl dextran	Dextran
Ficoll	Dextran
Ficoll	Agarose

TABLE II: POLYMER/SALT TWO-PHASE SYSTEMS

Polymer	Salt
Polyethylene glycol	Potassium phosphate, Sodium sulphate Sodium formate, Sodium potassium tortrate, Magnesium sulphate, Ammonium sulphate
Polypropylene glycol	Potassium phosphate
Methoxypolyethylene glycol	Potassium phosphate
Polyvinylpyrrolidone	Potassium phosphate

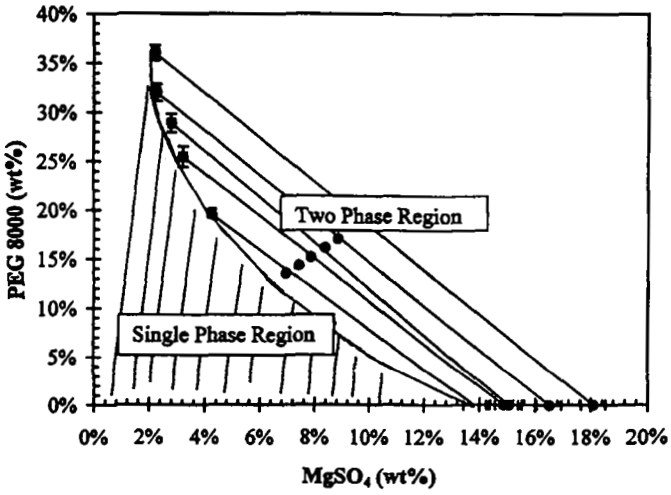

Figure 1.
Phase diagram of the PEG 8000-magnesium sulfate two-phase system at 4 °C. The error bars shown are based on triplicate analysis.[10]

systems, two phases are formed when the polymer concentration is in the range 8-16 % (wt/wt) and salt concentration must be as high as 10 %(wt/wt), which typically exceeds 2M.

b. Factors Affecting Aqueous Two-Phase Systems

The hydrophobicity, molecular weight and concentration of the phase forming polymers in the case of polymer/polymer type systems and, the type of salt and its concentration in the case of polymer/salt type systems, influence the formation of ATPS. The lower the molecular weight, the higher is the concentration of the polymer required for phase formation and vice-versa. Temperature has a considerable influence on the phase diagram. Detailed analysis of these aspects is available in the literature.[1,2,3,4,6]

c. Physico-Chemical Properties

The physical properties of ATPS such as density, viscosity and interfacial tension determine the phase separation rate and also contribute to the biomolecule partition behavior. Methods of measuring some of these properties have been given by Albertsson,[1] Walter *et al.*,[2] and Zaslavsky. [4] Researchers have measured these properties for the systems they use but most of them have not reported them. There is a need for the systematic measurement and reporting of the physical properties of these aqueous two-phase systems. Such an attempt was made by Snyder *et al.*[13] regarding polymer/salt type two-phase systems. However, in the case of polymer/polymer type ATPSs such compilations of data still appear to be unavailable.

3. Partitioning in Aqueous Two-Phase Systems

a. Factors Affecting Partitioning

The basis for separation by two-phase systems is the selective distribution of biomolecules between the phases which is quantified by the partition coefficient. This coefficient depends on many parameters such as biomolecule size, choice of polymers (or salt) and their molecular weight, concentration of polymers (or salt) in the phases, biomaterial surface properties, pH, biomolecule concentration (at high concentrations) and system temperature. Detailed analysis of these aspects is available in the review articles available in literature.[5,6,7]

Although a considerable amount of research has gone into studying partitioning in ATPSs, many of the physical phenomena concerning these complex systems are not yet completely understood. For instance, the significant effect of simple organic salts on the partition coefficient of the separand was unexplained until Hartounian *et al.*[14] developed recently a thermodynamic model combining the UNIQUAC and Debye-Huckel equations.

b. Models for Partitioning

Mathematical models are of immense use in the prediction of the partitioning behavior of a given biomolecule without the measurement of an inordinately large

number of parameters. Mathematical models associated with ATPSs are broadly categorized into three types: 1. Lattice models, which are based on a lattice representation of the polymer solutions within each of the coexisting phases; 2. Virial models, which use a virial type expansion in the concentration of the components of the system to describe thermodynamic properties; and 3. A scaling thermodynamic approach which utilizes recently-developed polymer-physics concepts aimed at describing polymer solution behavior.[8] The quantitative modeling of biomolecule partitioning in ATPS is an extremely complex problem. Excellent reviews are available by Baskir *et al.*,[15] and Diamond and Hsu,[16] who analyzed the existing models while comparing them with their own models. A deeper thermodynamic basis of such predictive models has been offered by Kabiri and Cabezas[17] and King *et al.* [18]

Despite the availability of many models in the literature, there is a need for more research in this area. Models which examine the influence of protein surface properties, such as surface charge and hydrophobicity, are needed. Furthermore, investigation of whether the interaction of water with phase polymers, buffering salts, and proteins plays a key role in protein partitioning[15] must be performed. Water is not an inert solvent and is highly structured in the liquid state and able to engage in multiple noncovalent interactions (eg. hydrogen bonding with a single segement of the hydrophilic polymers). Hence thhe Flory-Huggins interaction coefficients must be determined by enthalpic contributions from the chain segments.[19]

4. Recent Developments

Recent research on ATPSs is focussed on the two main issues associated with their ultimate application to industrial processes. These issues are specificity and scale-up. The following sections briefly summarize selected research efforts toward these goals.

a. Affinity Partitioning

Affinity partitioning (AP) is based on the preferential/biospecific interaction between the molecule and affinity polymer derivative. The interaction results in a

biomolecule-polymer derivative complex which selectively partitions to one of the phases leaving the contaminating substances or proteins in the other phase. Most of the reported investigations regarding affinity partitioning pertain to polymer/polymer type ATPSs.[6] Very few reports are available on polymer/salt type ATPSs[20] mainly due to the interference of high salt concentrations with the biospecific interactions.

Affinity partitioning is influenced by many factors such as the ligand concentration and its binding characteristics[21], concentration and molecular weight of polymers, pH, temperature, salt type and concentration, number of ligands per molecule, etc.[22] Although a comprehensive theory capable of predicting the effects of all these parameters is yet to be developed, a few theories are available that account for the effects of ligand concentration and the binding and number of ligands per molecule.[23,24,25] The application of Affinity Partitioning to the purification of biomolecules requires the synthesis of a specific polymer-ligand. The proteins that bind the polymer-ligand can be selectively separated from a heterogeneous mixture by means of counter-current distribution employing affinity partitioning. Numerous examples are reported in the literature.[6]

Affinity partitioning is also performed at large scale. Cordes and Kula[26] purified formate dehydrogenase (FDH) using Affinity Partitioning directly from cell homogenates of *Candida biodinii* using procion red HE3B as PEG-ligand. To separate FDH from PEG-ligand, potassium phosphate was added to this top phase resulting in PEG/phosphate system. The FDH partitioned to the lower salt rich phase, while the PEG-ligand remained in the top phase, which was separated and recycled. To remove the trace amounts of the ligand complex from the salt phase containing the product, fresh PEG was added to form again a PEG/salt system. Upon separation of the phases, the top phase was recycled and the bottom phase containing FDH was subjected to ultrafiltration (UF) and lyophylization to provide the final product. Tjerneld *et al.*,[27] also performed a large-scale affinity partitioning for the purification of lactate dehydrogenase (LDH) from pig muscle using a PEG/hydroxypropyl starch system with procion yellow HE-3G as ligand. Economic analysis indicated that the cost of purification was only about 10-20 % of the cost of LDH.

Despite the differences in the type of ligand (dye or hydrophobic and other ionic groups), certain features of AP are universal and independent of the type of ligand. These are understood better by the thermodynamic models of Flanagan and Barondes,[23] Cordes *et al.*,[24] Brooks *et al.*,[28] and Baskir *et al.*,[15]. Kopperschlager and Birkenmeier[29] discussed the theoretical considerations of affinity partitioning and the nature of affinity ligands and their mode of coupling. They suggested affinity partitioning of proteins as a tool for studying protein-ligand interaction.

Affinity Partitioning highlights the primary objective of maximizing the selectivity and yield of ATPE. However, a need still exists for a complete understanding of affinity partitioning accounting for the unequal binding strength of the ligands in each of the phases, multiple and unequal binding of ligands to the biomolecules of interest, and the influence of the non-ligand carrying polymer on the binding of the ligands to the proteins/enzymes.[8]

b. Metal Affinity Partitioning

Metal affinity partitioning exploits the affinity of transition metal ions for electron-rich amino acid residues, such as histidine and cysteine, that are accessible on the surfaces of proteins. When the metal ion is partially chelated and coupled to a linear polymer, such as PEG, the resulting polymer-bound metal chelate can be used to enhance the partitioning of metal binding proteins into the polymer-rich phase of a PEG-salt or PEG-dextran aqueous two-phase system. Since most proteins favor the salt-rich heavy phase of an aqueous two-phase system, metal affinity partitioning can be a very efficient and selective means of isolating and purifying a metal-binding protein from a crude mixture.[30] However, in practice they are yet to be used to isolate proteins from crude mixtures.

On the other hand, a variety of recombinant proteins have been isolated from *E. coli* using immobilized metal affinity chromatography (IMAC). In most cases, the desired protein had been isolated in a single chromatographic step from clarified cell lysate without any pretreatment. The one-step isolation of *D*-xylose isomerase (XI) from *Actinoplanes missouriensis* on a copper-chelated Sepharose Fast Flow gel is a

good example.[31] In some cases, a significant amount of host protein was observed to bind. Haymore *et al.*,[32] observed the binding of high-affinity *E. coli* proteins on a copper-chelated immino diacetate (IDA)-Trisacryl gel during the isolation of recombinant bovine somatotropin variants engineered with high-affinity histidine-containing binding sites. Binding of host proteins also occurred on Ni(II)-IDA during for the isolation of human HIV-1 reverse transcriptase expressed in *E. coli*.[33] Binding of *E.coli* proteins is reported to be less severe for Zn(II) and Ni(II) and require less stringent elution conditions[10]. From each of these cases, it is possible to identify a useful heuristic for a selection of the best metal for use in an affinity chromatography. Weakly binding proteins are easily separated from more tightly binding *E. coli* proteins by use of a copper chelating gel, since higher affinity binding favors retention of the contaminating proteins. On the other hand, fusion proteins engineered with histidine affinity binding sites have a higher association constant than host proteins and are easily isolated on Zn(II) and Ni (II) chelating resins which only weakly bind *E. coli* proteins. One may conclude that recombinant proteins with affinities that are similar to those of host proteins will prove difficult to isolate by IMAC. Immobilized metal ion affinity partitioning enabling excellent extraction of proteins in PEG/Salt and PEG/DX systems has been reported.[34,35]

Suh metal affinity partitioning model

Suh and Arnold[25] extended the general affinity partitioning model of Cordes, *et al.*,[24] to the modeling of metal affinity partitioning by including the inhibition of ligand binding due to protonation of the histidine binding site to form a non-coordinating protonated imidazole. The protein is assumed to have n equivalent surface histidine binding sites characterized by a metal ligand association constant, K_L, and a hydrogen ion association constant K_H (which comes from the pK_a of the histidine residue). By summing over all possible combinations of bound and unbound protein they obtained the following expression for the total protein concentration in the presence of metal ligand, $[P_{tot}]$,

$$[P_{tot}] = (1 + K_H[H] + K_L[L])^n [P] \quad \text{(4b-1)}$$

where $[L]$, $[H]$, and $[P]$ are the unbound ligand, hydrogen ion, and unbound protein concentrations, respectively. In the absence of ligand the total protein concentration, $[P_o]$, becomes

$$[P_o] = (1 + K_H[H])^n [P] \quad \text{(4b-2)}$$

The partition coefficients K and K_o are, by definition,

$$K = \frac{[P_{tot}]'}{[P_{tot}]''} \quad \text{and} \quad K_o = \frac{[P_o]'}{[P_o]''} \quad \text{(4b-3)}$$

where the single and double primes denote the top and bottom phases, respectively. The subscript *'o'* refers to properties of the system in the absence of ligand.
Combining eqs.(4b-1), (4b-2), and (4b-3) yields

$$\frac{K}{K_o} = \left\{\frac{[P_{tot}]'}{[P_{tot}]''}\right\}\left\{\frac{[P_o]''}{[P_o]'}\right\} = \left[\frac{1 + K_L'[L]' + K_H'[H]'}{1 + K_L''[L]'' + K_H''[H]''}\right]^n \left[\frac{1 + K_H''[H]''}{1 + K_H'[H]'}\right]^n \quad . \quad \text{(4b-4)}$$

If one assumes that hydrogen ion distributes evenly between the two phases and that K_H is the same in the two phases, then eq. (4b-4) becomes

$$\frac{K}{K_o} = \left[\frac{1 + K_L'[L]' + K_H[H]}{1 + K_L''\dfrac{[L]'}{K_P} + K_H[H]}\right]^n \quad , \quad \text{(4b-5)}$$

where $K_P = [L]'/[L]''$ is the ligand partition coefficient in the two-phase system. Eq. (4b-5) is the Suh metal affinity partitioning model.

Application of the Suh model requires an expression for the free ligand concentration. Since the ligand is either bound to the protein or free in solution, a

material balance on the total metal ligand concentration $[L_{tot}]$ can be written:

$$[L_{tot}] = \left\{[L]' + [L^*]'\right\}\left(\frac{R}{R+1}\right) + \left\{[L]'' + [L^*]''\right\}\left(\frac{1}{R+1}\right) \quad , \qquad (4b\text{-}6)$$

where $[L^*]$ is the bound ligand concentration in the two-phase system and R is the phase volume ratio defined as the top phase liquid volume divided by the bottom phase liquid volume. Suh assumed that it is reasonable to ignore the concentration of bound ligand and simplified eq.(4b-6) to

$$[L_{tot}] = [L]'\left(\frac{1}{R+1}\right)\left(R + \frac{1}{K_P}\right) \quad . \qquad (4b\text{-}7)$$

Combining eq. (4b-5) and eq. (4b-7) yields

$$\frac{K}{K_o} = \left[\frac{1 + K_L' \dfrac{(R+1)}{\left(R + \dfrac{1}{K_P}\right)}[L_{tot}] + K_H[H]}{1 + K_L'' \dfrac{(R+1)}{(K_P R + 1)}[L_{tot}] + K_H[H]}\right]^n \quad . \qquad (4b\text{-}8)$$

Eq. (4b-8) is the expression used by Suh to model metal affinity partitioning of cytochrome *c* and myoglobin in PEG/dextran aqueous two-phase systems.

Extended metal affinity model

The assumption of negligible ligand binding is valid only at low pH, where few sites are capable of binding, and at low total ligand concentration. For conditions in which the fraction of bound metal ligand is significant, eq. 4b-6 can be written as

$$[L_{tot}] = \left\{[L]' + n\theta_L'[P_{tot}]'\right\}\left(\frac{R}{R+1}\right) + \left\{[L]'' + n\theta_L''[P_{tot}]''\right\}\left(\frac{1}{R+1}\right) \quad , \qquad (4b\text{-}9)$$

where the fraction of sites bound by metal ligand, θ_L, is given by

$$\theta_L = \frac{K_L[L]}{1 + K_H[H] + K_L[L]} \quad . \tag{4b-10}$$

In terms of the total protein concentration in the system, eq.(4b-9) can be rewritten as

$$[L_{tot}] = [L]'\left(\frac{RK_P + 1}{K_P(R+1)}\right) + [P_{tot}]\left[\left(\frac{KR}{RK+1}\right)n\theta'_L + \left(\frac{1}{RK+1}\right)n\theta''_L\right] \tag{4b-11}$$

Eq. (4b-11) simplifies to eq. (4b-8) when the fractional site saturation is very small, i.e. as $\theta_L \rightarrow 0$. When the ligand partitions to the PEG-rich phase, which is the usual case, $K_P >> 1$ and $\theta''_L << 1$. Eq. (4b-11) simplifies to

$$[L_{tot}] = [L]'\left(\frac{R}{R+1}\right) + [P_{tot}]\left(\frac{KR}{RK+1}\right)n\theta'_L \quad . \tag{4b-12}$$

Combining eq. (4b-12) with the metal affinity model, eq. (4b-5) gives

$$\frac{K}{K_o} = \left[\frac{1 + K_L\left(\frac{R+1}{R}\right)\left([L_{tot}] - [P_{tot}]n\theta'_L\left(\frac{KR}{KR+1}\right)\right) + K_H[H]}{1 + K_H[H]}\right]^n . \tag{4b-13}$$

Eq. (4b-13) along with the fractional site saturation equation (eq 4b-10) comprise an extended form of the Suh model applicable for metal affinity protein partitioning in PEG/salt aqueous two-phase systems. This model is useful for predicting metal affinity protein partitioning in which the target protein is the only significant partitioning protein in the system and there are no additional sources of metal binding inhibition.

Guinn [10] successfully achieved the partitioning of recombinant Hb from crude cell lysate using Cu (II) IDA-PEG in a two-phase system of PEG and magnesium sulfate. A three-step synthetic scheme for the preparation of Cu(II)IDA-PEG from methoxy-PEG (MPEG) is shown in Figure-2. To our knowledge, this is the first recorded attempt to successfully apply metal affinity partitioning techniques to the isolation of a recombinant protein from crude cell lysate. Successful demonstration

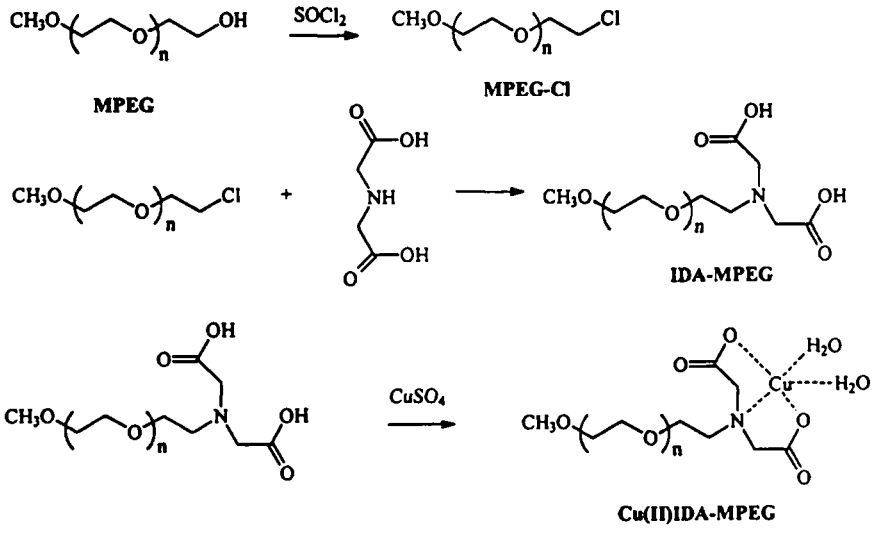

Figure 2.
Three-step synthetic scheme for the preparation of Cu(II)IDA-PEG from methoxy-PEG (MPEG).[10]

of this technology sets the stage for its potential commercial use in the isolation of native and non-native metal-binding proteins. The purity of the extracted PEG-phase as measured by SDS-PAGE, is remarkably high, suggesting that the metal chelate Cu (II) IDA-PEG is able to selectively recognize rHb through multiple site binding. After the extraction, dissociation of the rHb-Cu (II) IDA-PEG complex could be accomplished by lowering the pH, the addition of a competing electron-donor such as imidazole or NH_4Cl, or by the addition of a strong chelator such as EDTA. The last approach is preferable since complete chelation of copper is desirable to prevent oxidation of the protein. Following dissociation, the PEG phase containing rHb can be applied directly to a chromatographic polishing step or, first, extracted back into a salt-rich phase prior to chromatography, depending on the separation mechanism (ion exchange, size exclusion, etc.). Using either approach, it may be possible to recover and reuse PEG and metal chelate.

c. Metal Affinity Solubilization

Proteins have been isolated by precipitation for well over one hundred years. The technique has found wide applicability because it is simple to apply, easy to scale, and economical.[36] Typically, the goal is bulk removal of protein contaminants from a crude mixture. Fractional precipitation schemes have been devised as a means of selectively precipitating proteins.[37] The precipitated protein can be either an undesirable contaminating protein or the target protein of interest. The selectivity of fractional precipitation schemes can be enhanced by the use of affinity precipitants that function by binding selectively to the desired protein. Precipitation generally occurs as a result of extensive interprotein crosslinking. [38]

Guinn [10] has described a unique mechanism for selectively enhancing the solubility of a target protein under conditions leading to the quantitative precipitation of proteins. The method, which is called metal *affinity solubilization*, exploits the reversible binding of a metal-chelating polymer ligand for acidic amino acid residues on the protein surface. When the protein is bound by a suitable number of polymer ligands, the solubility of the protein in high concentrations of the same polymer can be greatly enhanced. Protein precipitation in the presence of poly(ethylene glycol) is through the mechanism of volume exclusion, which involves non-specific interactions between the polymer and protein. A novel protein isolation approach is presented by Guinn [10] in which Cu(II)IDA-PEG is reversibly bound to the protein to sterically prevent precipitation and selectively solubilize the protein in the presence of PEG as precipitant. The following thermodynamic model is also presented which accurately relates the protein-polymer interaction coefficient with the decrease in excluded volume due to bound polymer.

Protein solubility decreases logarithmically with salt or polymer concentration in a consistent and predictable manner, according to the Cohn equation,

$$\log S = K - \beta C \qquad \text{(4c-1)}$$

where S is the protein solubility (g/L) and C is the precipitant concentration (% w/v).[37,39] By equating the chemical potential of the precipitated protein with that of the same protein in solution at equilibrium the protein solubility can be written,

$$\ln S = \ln S' + dS' - aC \tag{4c-2}$$

where the concentrations are expressed in moles/liter.[40, 41] The constants d and a are the protein-protein and protein-PEG interaction coefficients, respectively, while S' is the intrinsic protein solubility in the absence of PEG. The interaction coefficient a and second virial coefficient d can be related to the volumes mutually excluded by neighboring molecules through the Flory[42] dilute solution theory. Edmond and Ogston[41] considered the simplified view that the solutes could be treated as hard spheres with co-volumes given by

$$U_{pol-pol} = 10^3 d = \frac{4\pi N}{3}\left(2r_{pol}\right)^3 \tag{4c-3}$$

$$U_{pol-pro} = 10^3 a = \frac{4\pi N}{3}\left(r_{pol} + r_{pro}\right)^3 \tag{4c-4}$$

where U represents the molar excluded co-volumes for pairs of spherical molecules, r_{pol} and r_{pro} are the equivalent radii (cm) for the polymer and protein molecules, respectively, and N is Avagadro's number. Eq. (4c-4) suggests that a, and therefore the slope of the solubility curve, depends only on the relative sizes (molecular weights) of the polymer and protein. So with these ideas in mind, the binding of a polymer to the surface of a protein, as in metal-affinity extraction, has the effect of increasing the likelihood of overlap in the molecular domains of the protein and polymer since the interaction between the bound polymer and solvent is energetically favorable. When the polymer-bound protein contains pegylated regions in which interaction with the polymer in solution is favorable, steric exclusion is less effective.

The greater the protein surface coverage with polymer the less effected the protein is by PEG concentration. Since the polymer-bound protein occupies a region of space in which it was formally excluded, the co-volumes mutually excluded can be written,

$$U_{pol-pro} = 10^3 a = \frac{4\pi N}{3}\left(r_{pol} + r_{pro}\right)^3 - \frac{4\pi N}{3} b\left(r_{pol}\right)^3 \qquad \text{(4c-5)}$$

where b is the number of bound polymer ligands. The interaction coefficient a in the presence of metal chelating polymer can be determined using eq. (4c-5) for appropriate hard sphere radii. Atha and Ingham[40] approximated these radii from diffusivity data for various proteins. It is possible to estimate the hard sphere radii from the slope and intercept of the solubulity curve in the absence of metal chelate by application of eq. (4c-3) and eq. (4c-4). Recasting eq. (4c-2) in the form of the Cohn equation we get,

$$\log S = \log S'_{app} - \beta C \qquad \text{(4c-6)}$$

where the intercept is given by,

$$\log S'_{app} = \log S' + d\left(\frac{S'}{2.3 M_{pro}}\right) \qquad \text{(4c-7)}$$

and the slope is given by,

$$\beta = \frac{a}{0.23 \tilde{M}_{pol}} \qquad \text{(4c-8)}$$

Thus metal-affinity solubilization is consistent with polymer solution theory and can be expected to obey a modified version of the Cohn equation.

d. Microgravity Extraction Research

Over the past several years, there has been considerable interest in the use of the microgravity environment of earth orbit as a laboratory for understanding the role of surface forces in liquid-liquid phase separation. The importance of surface wetting forces on the phase separation of aqueous two-phase systems in microgravity

was observed during Space Shuttle flight STS-26 in October 1988. Separation experiments were performed in a Plexiglas hand-held phase partition experiment (PPE) module consisting of 18 chambers filled with PEG and dextran solutions in plastic extraction cavities.[43,44,45]

It is possible that surface forces have only a minor influence on phase demixing. Phase demixing is driven by the thermodynamic gain in free energy associated with the reduction in total surface area of the dispersed phase. Thus, phase demixing will occur spontaneously unless the emulsion is in some way stabilized by the presence of surfactants. Sedimentation due to an imposed body force serves only to increase the frequency and effectiveness of drop-drop and drop-interface collisions and coalescence that lead to a reduction in total surface area. Surface wetting forces are short-range forces, extending over a few molecular diameters and would not be expected to play an important role in increasing coalescence rates.

In microgravity, the coalescence of dispersed phase droplets results in a decrease in dispersed phase volume. Since the continuous phase is incompressible, the decrease in droplet volume induces a flow of the continuous phase toward the droplet. In the absence of buoyancy forces, assuming the dispersed phase droplets are uniformly distributed, there would be an increase in the collision efficiency of droplets due to the effect of local flow. Thus in microgravity, as in unit gravity, coalescence and phase separation is spontaneous and enhanced by the local flow induced by droplet coalescence. This scenario would help to explain why phase separation was possible for some of the systems in STS-26 using the PPE module but not for others and why low viscosity and surface tension had such a large positive effect on demixing rates.[46] Due to the combined effects of Brownian motion and the local flow induced by droplet coalescence there is a greater probability that PEG-rich phase droplets (typically the dispersed phase in dextran/PEG systems) would impact the container walls and remain there. If the continuous phase is dextran it would prevent the dispersed phase from contacting the container walls. The sole effect of surface wetting forces in microgravity would seem to be to give phase separation a directionality.

Evidence of surface tension-driven phase separation in microgravity and its potential processing applications led to the development of a reusable platform, the ORSEP, for conducting multi-stage extraction using aqueous two-phase systems for both terrestrial and space-based processing applications. A schematic representation of the ORSEP is shown in Figure-3. The extractor consists of two disk-shaped plates into which 24 cylindrical cavities have been machined along two concentric circles of the plate faces. When the plates are joined and securely bolted together to form a leak-tight seal, the cavities can be aligned to form an extraction chamber. Access to the chambers is through a small, stoppered port above each cavity of the top plate.

In order to exploit surface tension driven phase separation, the top cover plate is fabricated from a hydrophobic plastic (e.g. Plexiglas, Lexan, etc.) while the bottom plate is machined from stainless steel. The entire unit is mounted to a base consisting of 24 miniature stirbar drivers, which are intended to provide adequate mixing by means of a stirbar installed in each chamber.

Multi-stage extraction using the ORSEP is intended to mimic the procedure known as "Craig extraction" or counter-current distribution (CCD). As illustrated in Figure-4 this technique provides a means of isolating and purifying a mixture of solutes by sequentially contacting the top phase of each chamber with the bottom phase from the adjacent chamber, in a manner that mimics counter-current extraction.

The fraction of original solute, added in the first stage, in chamber r after n total transfers is,

$$f(r,n) = \frac{n!}{r!(n-r)!} p^r (1-p)^{n-r} \qquad \text{(4d-1)}$$

where p=E/(E+1) and $E = KR$. The phase volume ratio, R, is defined as the ratio of the top phase volume to the bottom phase volume, and the solute partition coefficient, K, is defined as the ratio of the concentration of solute in the top phase to that in the bottom phase. Multi-stage partitioning of a solute results in a solute concentration profile that closely approaches a Gaussian distribution. Suitable mixing design was provided in a second-generation extractor, ADSEP (Advanced

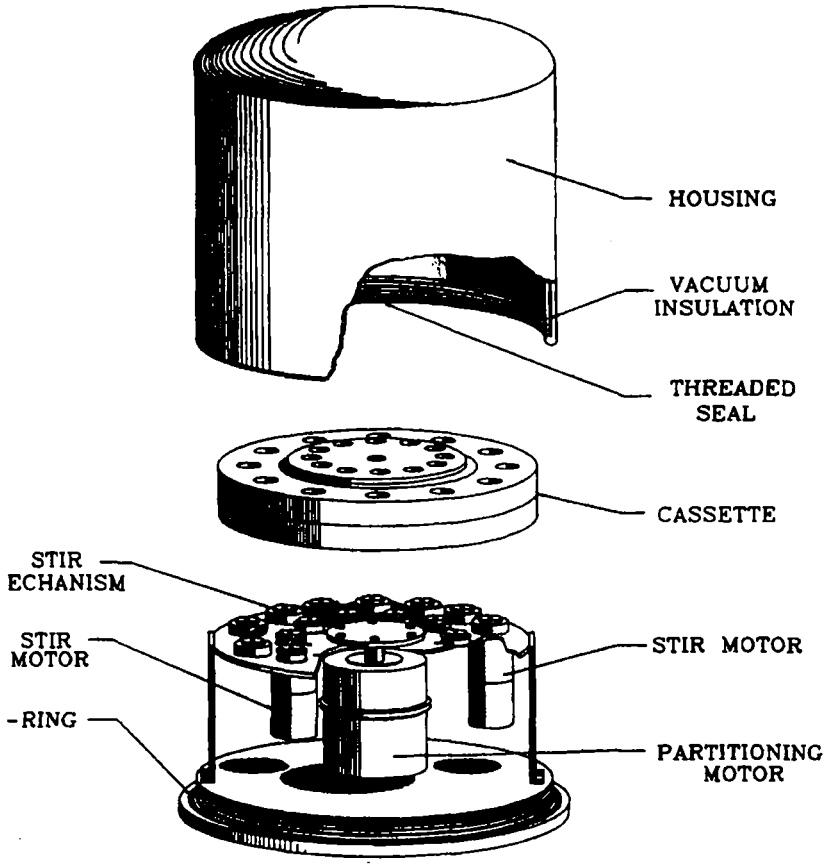

Figure 3.
Schematic representation of the ORSEP multi-stage countercurrent distribution apparatus.

Separator), for the necessary vertical bulk motion required to acieve adequate momentum transfer between the two fluid phases [10].

e. Counter-Current Distribution

Counter-current distribution (CCD) has gradually evolved into a preferred multi-stage contacting method for aqueous two-phase partitioning of biological

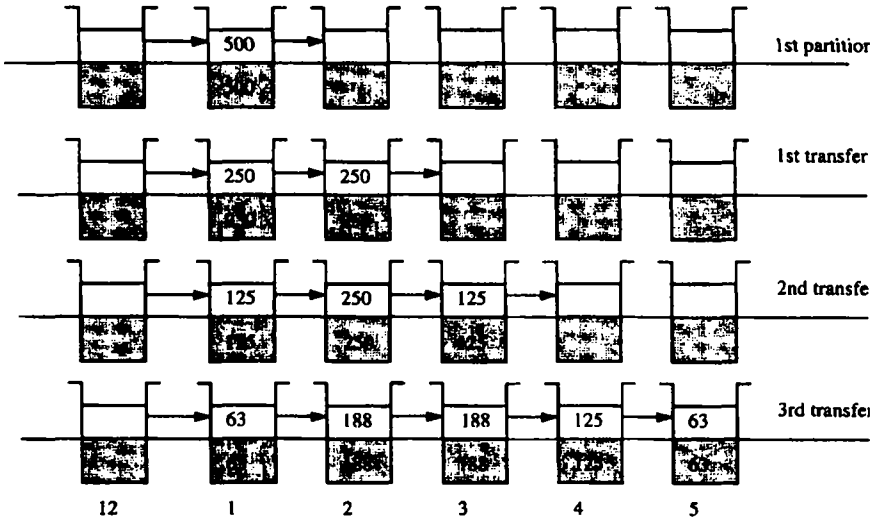

Figure 4.

A schematic representation of Craig extraction.[10]

materials.[47] However, scale-up of this technique beyond the laboratory has been hampered by the lack of a comprehensive model that accounts for the influence of non-idealities in the partitioning process. Counter-current distribution of a mixture of solutes has been modeled as an equilibrium staged process which generates a solute concentration profile that can be represented by a Gaussian error distribution. However, aqueous two-phase CCD is an inherently non-equilibrium process and can result in non-ideal behavior due to such factors as (1) non-alignment of the phase interface and phase cut location, (2) incomplete phase demixing, (3) incomplete solute mass transfer, and (4) perturbation of the phase system composition as a result of solute loading and system dilution. In general, a perturbation of the phase composition in the feed stage due to these and other factors will result in a stage-to-stage variation in the solute partition coefficient (K), the phase volume ratio (R), and the total system volume (V) which can translate into a significant deviation from ideal CCD performance. Guinn[10] developed a comprehensive material balance

model for non-ideal CCD and tested it using the ORSEP multi-staged extractor described earlier.

In stage-independent CCD operation K, R, and V do not vary from stage to stage. However, stage-independent CCD operation is approached only for those limited cases for which solute loading has a negligible impact on phase system composition, total system volume, and phase volume ratio and for which mass transfer and phase demixing have reached equilibrium. CCD has been modeled by Guinn [10] as an equilibrium staged process that generates a solute concentration profile that can be described by a Poisson distribution. A solute material balance model was developed as a tool to evaluate CCD extractor performance which extends the extends the existing model to account for the non-ideal behavior. Begining with the assumption of stage-independent partitioning, he developed the general equations which describe solute partitioning in CCD but take into account the effects of liquid-interface and phase cut nonalignment on CCD performance. The model can then be systematically generalized for non-ideal operation by applying an empirical demixing model to account for non-equilibrium phase demixing and a model to account for the effect of solute partitioning and feed stage dilution on the perturbations of the phase volume ratio. Perturbation of the feed stage due to any of these non-equilibrium events will be conveyed as a deviation from ideal CCD operation to each successive stage.

It was shown [10] that CCD performance depends on alignment of the interface and phase cut, extent of demixing, and feed composition, and the modified model is useful in predicting multi-stage solute partitioning by countercurrent distribution under these non-ideal conditions using single-stage partitioning data. Application of the model can assist in improving the outcome of a CCD separation experiment.

Mathematical Model

In a CCD apparatus, phase transfer is accomplished by slicing the two-phase system along the plane joining the upper and lower chamber halves. In the unlikely

event that the phase interface is aligned with this phase cut plane, Eq. 4d-1 can be applied to predict the solute composition in each phase. A more general treatment takes into account the non-alignment of the liquid interface and phase cut.

Three distinct volume regions are defined as shown in Figure-5 for two adjacent extraction stages of a typical CCD apparatus containing a total of n extraction stages. Light phase is transferred from the left adjacent cavity (n-1) to the right cavity (n) by slicing the phase system at the junction of the two extractor rings (along the heavy horizontal line in the figure). In general, the liquid-liquid interface may be above or below the phase system cut as indicated by the volume V^*. The total liquid volume is V and the volume of the lower cavity half, V_b, is fixed by the actual geometry of the extractor.

If x and y are the mass concentrations of the solute in the light and heavy phases respectively and L and H are the volumes of the light and heavy phases then the solute balance over stage n following transfer r is,

$$\left[\left(V - V_b - V^*\right)x + V^* y\right]_{n-1,r-1} - \left[\left((V - V_b - V^*\right)x + V^* y\right]_{n,r-1} = \left[Lx + Hy\right]_{n,r} - \left[Lx + Hy\right]_{n,r-1} \quad \text{(4e-1)}$$

Rearranging, an equation for x in stage n after the r transfer is obtained:

$$x_{n,r} = \frac{\left\{\left[V - V_B - V^*\left(\frac{K-1}{K}\right)\right]x\right\}_{n-1,r-1} - \left\{\left[V - V_B - V^*\left(\frac{K-1}{K}\right) - \frac{(RK+1)V}{K(R+1)}\right]x\right\}_{n,r-1}}{\left\{\frac{(RK+1)V}{K(R+1)}\right\}_{n,r}} \quad \text{(4e-2)}$$

where the partition coefficient K=x/y and the phase volume ratio R=L/H. The mis-alignment volume $V^* = H - V_B$ is zero by definition when the liquid interface is at or below the plane of the phase cut. For stage-independent operation, K, V, and R are constant and Eq. (4e-2) can be rewritten,

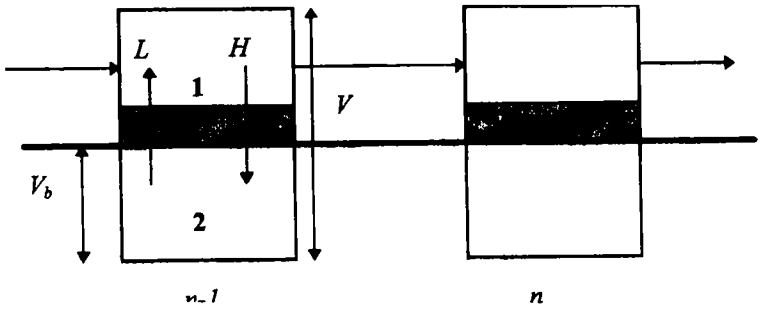

Figure 5.

Definition of terms for the CCD extractor model. The lower cavity half below the thick horizontal line are stationary while the upper halves move from left to right. The volume of the lower cavity half, V_b, is fixed by the geometry of the cavity. V represents the total fluid volume and V^* is the volume of lower phase that would be carried over if the interface is above the phase cut plane. L and H show the direction of light and heavy phase movement during demixing.

$$x_{n,r} = \frac{\left[V - V_B - V^*\left(\frac{K-1}{K}\right)\right]x_{n-1,r-1} - \left[V - V_B - V^*\left(\frac{K-1}{K}\right) - \frac{(RK+1)V}{K(R+1)}\right]x_{n,r-1}}{\left[\frac{(RK+1)V}{K(R+1)}\right]} \quad \text{(4e-3)}$$

f. Extractive Bioconversion

Extractive bioconversion employing aqueous two-phase systems can improve certain existing bio-processes to make them economically viable.[48] This technique provides an interesting means for the simultaneous production and purification of a bioproduct obtained through the use of enzymes or microorganisms. Similar to Affinity partitioning, which attempts to isolate the target protein and contaminating material in opposite phases, the objective of extractive bioconversion is to exploit an ATPS in which the substrate or the biocatalyst (enzyme or microorganism) partitions to one phase and the product (e.g., protein or steroid) partitions to the opposite phase. The purpose of separating the product from the substrate is not only to purify but also to increase the rate of reaction/conversion if the biocatalytic reaction is inhibited by

the product.[1] Similarly, if the biocatalyst is inhibited by the substrate, the system conditions can be manipulated in such a way that the substrate and the biocatalyst partition to opposite phases.[49] Integration of bioconversion and downstream processing steps not only increases the productivity of the bioprocesses but also provides the possibility of running the bioconversion in a continuous mode.[50,51] A generic conceptual diagram of an aqueous two-phase extractive bioconversion process is shown in Figure-6.

By proper selection of parameters the cells or enzymes can be restricted to only one of the phases in an ATPS. This provides a method for immobilizing the biocatalyst in a rather simpler manner without the use of any harmful chemical treatment, keeping it in solution at all times. The partition behavior of enzymes, substrate and product could be utilized to create a temporary immobilized system, providing an efficient extraction of the product from the site of its production . At the same time, diffusional resistances, which could be critical in immobilized enzyme systems, are minimized by the absence of a solid phase for immobilization. The easily obtained small droplets or large interfacial area (due to the low phase interfacial tensions) facilitate mass transfer even for macromolecules, and the high content of polymers in the medium helps to stabilize the enzymes. Mattiasson[52] demonstrated the feasibility of carrying out enzymatic conversions in ATPS by performing the process in one of the phases and continuously removing the product to the other phase, thus providing a convenient system which economically uses the soluble enzymes for the degradation of macromolecular substrates. In this context Wennestern *et al.*[53] studied the conversion of starch to glucose, using the substrate starch as the affinity ligand to keep the enzymes in the bottom phase, in a system composed of PEG/DX. At the beginning of the run the starch concentration was 14-17% and, of this available starch 90% conversion could achieved. The hydrolysis of cellulose is a long-standing problem of efficient biomass conversion.

A number of extractive bioconversions relating to the conversion of complex compounds such as cellulose to simpler compounds such as alcohol, using ATPS have been reported.[54,55,56,57,58,59] In these studies mixer-settler type contactors were

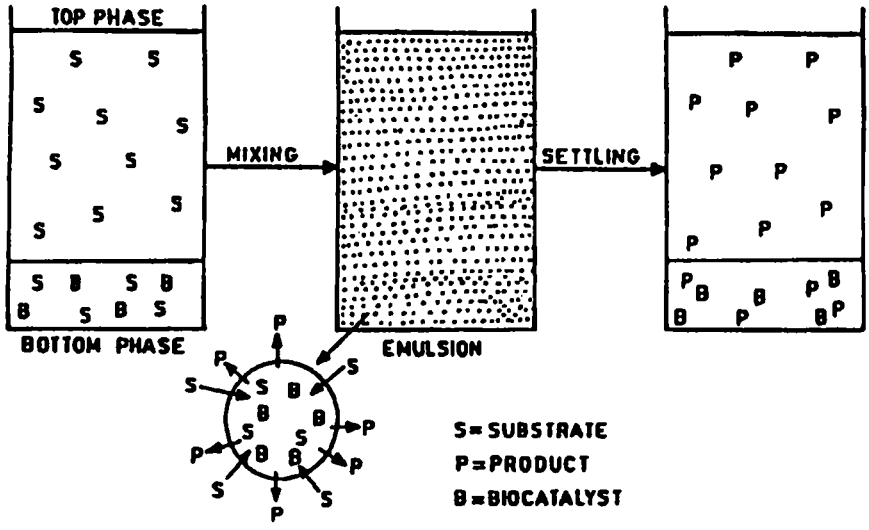

Figure 6.
A generic conceptual diagram of an aqueous two-phase extractive bioconversion process. Reprinted from 'Enz.Microb.Technol., 12, E.Andersson, A.C.Johansson and B.Hahn-Hangerdal, Alfa-amylase production in aqueous two-phase systems with *Bacillus subtilis*, 333-338, 1985,[64] with permission from Elsevier Science.

employed. Tjerneld *et al.*,[60,61] investigated the semicontinuous hydrolysis of cellulose in the PEG/crude Dextran system using endo-β-gluconase and β-gluconase from *Tricoderma reesii.* In their experimental design, the top and bottom phases were contacted in a mixer, and solid cellulose was added intermittently. The mixture was pumped to a settling unit where the phases were separated and recycled. The top phase could also be removed for subsequent purification of glucose. This process was run in semibatch mode for more than 450 hrs, with a starting cellulose concentration of 75 g per liter, and about 50 g per liter of glucose could be produced at dilution rates in the range of 0.006 – 0.12 per hr.

Since extractive bioconversion involves the integration of bioconversion with one or more downstream processing step(s), such as extraction using ATPS, UF etc.,

an opportunity is provided to explore new types of industrially relevant bioreactor designs.[50, 62] When both of the steps are used in series, the UF membrane is protected from contact with particulate matter in the medium, contributing to an improved membrane life. Kuhn[63] produced ethanol (with about 90 % theoretical yield over 10 cycles) in a PEG/DX system with baker's yeast, which was added to a mixture of glucose and malt. Andersson *et al.*,[64] performed repeated batch processes In PEG/DX systems using *Bacillus subtilis* for the production of α-amylase which partitioned to the top phase (80%) while the bacterial cells partitioned to the bottom phase. Most of the bioconversions have been performed using polymer/polymer type ATPSs. Lee and Chang[49] were among the first to successfully employ the PEG/Potassium phosphate system for the production of acrylamide from acrylonitrile using *Brevibacterium*. The selective partitioning of the product into the top phase was found to reduce the inhibition of the active bacterial enzyme by both the substrate and the product. Hayashida *et al.*,[65] reported the hydrolysis of soluble starch by glucoamylase and β-amylase in the PEG/DX system, showing that the enzymatic reactions were not influenced by the presence of polymers. Mukataka *et al.*,[66] reported extractive bioconversions using the PEG/DX system for enzymatic hydrolysis of casein protein. Rates of casein photolysis by α-chymotrypsin and by trypsin are reported in single phase and ATPSs.

g. New Phase Systems

Though PEG/DX is the most popular system for ATPE research, the prohibitive cost of purified DX severely limits the use of this system on an industrial scale. Many cheaper polymers were explored and found suitable for the purification of proteins and enzymes.[21] Thus, polymer-salt type ATPSs, with faster phase demixing rates and lower cost, have gained popularity in the recent years.

In general, lowering the temperature facilitates the phase separation due to reduced solubility of the phase forming components. A contrary observation, not readily predicted, was made by Stewart and Todd[67] who observed that saturated solutions of NaCl at elevated temperatures (about 60 °C) form ATPSs with PEG. This

system is suitable for the separation of flavorings, such as amino acids, dipeptides, and nucleotides, from acid hydrolysates used in the food industry; however, this system cannot be used for most protein separations due to high temperatures required to form the ATPS and due to the denaturing properties of high NaCl concentrations. The PEG/NaCl two-phase extraction provides a product lower in salt than that obtained by directly spray drying neutralized acid hydrolysates.

Two-phase partitioning is evolving as a promising technique for the isolation of DNA. In isolating nucleic acids from biological sources, the action of released nucleases and other enzymes used to be inhibited. Chaotropic agents, such as guanidine isothiocyanate, and detergents, such as sodium dodecyl sulfate disrupt the protein structure and inhibit enzyme activity. By using an ATPS of PEG/Salt- type containing chaotropic agents and detergents, Cole[68] showed that nucleic acids partition to the salt phase in high amounts, while protein and cellular constituents concentrate in the PEG phase or precipitate at the interface. This technique is quickly evolving into only a few steps; hence, it is amenable to automation. The polymer/salt system [68] was superior to polymer/polymer type systems [1, 2] for the partitioning of nucleic acids because the former is a less viscous system in which phase separation is more rapid.

Glotova *et al.* [69] reported a new phase system of skim milk proteins/sodium salt of carboxymethyl cellulose (CMC)/water. The authors studied the concentration of skim milk proteins in this ATPS and the effects of low degrees of polymerization (200 and 500) and substitution (0.5 and 0.8) of CMC. The maximum protein concentration in the protein phase was 15 %, which represented 85 % of the maximum possible milk protein yield.

Terstappen *et al.* [70] explored the use of detergent based ATPSs for the isolation and purification of lipase from *Pseudomonas cepacia.* They investigated the partitioning of prokaryotic and eukaryotic extracellular lipases in detergent-based ATPSs . They observed that prokaryotic lipase preferred the detergent-rich coacervate phase, while eukaryotic enzymes were largely excluded from this phase, possibly due to their glycosylation. The utility of this extraction technique was tested using culture broth from which about 76% of extracellular lipase could be extracted into the

coacervate phase in a single step, leading to a 4-fold concentration of lipase and a purification factor of about 24.

In order to improve the economics of ATPE alternate phase systems are being explored for different applications. The choice of the polymers is influenced to a large extent by regulatory requirements in addition to the technical aspects of the phase system. If this were not the case, any hydrophilic polymer could be exploited to form ATPS for the purification of biomolecules. PEG, DX and MDX are non-toxic substances and have been accepted for food and pharmaceutical applications.[71]

Preliminary studies on the immiscibility of various gelling polymers were carried out in the context of long-term preservation of separated phases (Table-3). Significant combinations in which the top phase gelled included mixtures of Ficoll and low-melting agarose. In an extension of the 19th-century observations of Beijerinck[72,73] that agar and gelatin form immiscible solutions, mixtures of agarose and gelatin were also found to form two phases, both of which form gels. Both phases can be liquified at temperatures consistent with bioproduct activity, including cell viability. Certain molecular weight maltodextrins[74] form gels after separation from polyethyleneglycol.[75]

h. Electrokinetic Demixing

Pairs of aqueous phases involved in ATPE are characterized by high viscosities, low interfacial tensions and similar densities.[1, 2] The resulting slow demixing of these phases has been counteracted by centrifugation, column contacting or electrokinetic demixing - each having its own drawbacks.[9] Raghavarao *et al.* [76, 77] observed that electrokinetic demixing increases demixing rates of ATPSs (up to 100 ml) more than five fold in a manner that depends on field strength, field polarity, concentration of partitioning anion and phase composition. Electrokinetic demixing is also potentially useful in situations such as low gravity where an additional force has to be introduced for the demixing of the equilibrated phases.[78] Operation of electrokinetic demixing at commercial scale requires further understanding of the fundamentals involved in the process.

TABLE III: AQUEOUS TWO-PHASE SYSTEMS THAT GEL ON PHASE SEPARATION

Polymer 1	Polymer 2
LE* Agarose, 2 %	Dextran (70 K), 10 %
LE Agarose, 2 %	PEG (8 K), 20 %
SeaPlaque™, 2 %	Dextran (70 K), 10 %
SeaPlaque™, 2 %	Ficoll (400 K), 10 %
Sea Prep™, 2 %	Ficoll(400 K), 10 %
HGT*Agarose, 2 %	Ficoll(400 K), 10 %
LE Agarose, 2 %	Ficoll(400 K), 10 %
SeaPlaque™, 0.5 %	Ficoll(400 K), 5 %
SeaPlaque™, 0.75 %	Ficoll(400 K), 5 %
SeaPlaque™, 1.0 %	Ficoll(400 K), 5 %
Sea Plaque™, 1.0 %	Ficoll(400 K), 7.5 %
Agar, 1.0 %	Gelatin, 10.0 %
Sea Plaque™, 1.0 %	Gelatin, 10.0 %
PEG (8 K), 4.2 %	Maltodextrin (3.6 K), 18.0 %
PEG (8 K), 4.4 %	Maltodextrin (3.6 K), 26.5 %

* LE = low electroosmotic mobility
* HGT = high gelling temperature

When two polymers are dissolved in aqueous solution at concentrations that cause phase separation, certain dissolved ions such as phosphate are unequally partitioned between the phases[79] leading to an electrical potential across the interface[12] and an apparent electrokinetic potential at the surface of the dispersed phase droplets.[80,81] As a consequence of the latter, droplets of dispersed phase move in the continuous phase in the presence of an externally applied electrical field.[11,45,77] It is therefore possible to control demixing rates by application of an electric field to ATPS emulsions of appropriate ionic composition, and Brooks and Bamberger[78] initially demonstrated enhanced emulsion clearing at the 1-ml scale. They demonstrated qualitatively, by monitoring the system turbidity in a 1-ml

chamber, that electrophoretic mobility of the phase droplets enhances the phase demixing.

The effects of the electric field strength, phosphate ion concentration, temperature, field polarity and phase composition on the demixing rate of PEG/DX and PEG/MDX systems were evaluated quantitatively. It was found that an optimum field strength of around 25 V/cm exists at which the demixing is most rapid and, in an optimized system, causes a 2-fold decrease in demixing time relative to that at zero field using normal polarity (anode at the top of the column) and a 6-fold decrease in demixing time relative to that at zero field at 25 $\pm$ 2° C in the case of reverse polarity (anode at the bottom, electric field opposing gravitational settling).[76,77]

Brooks and coworkers[78,80] measured drop electrophoretic mobilities in ATPSs. They were surprised to discover that the sign of the droplet mobilities was opposite to that predicted from the phosphate partitioning and the Donnan potential. They also found mobility to be directly proportional to drop radius - a contradiction of standard colloid electrokinetic theory.[82] Levine[80] and Brooks *et al.*,[81] proposed a hypothesis that a dipole potential at the phase boundary oriented in a way that reverses the potential gradient locally is responsible for the idiosyncrasy of the electrokinetic mobilities of ATPS droplets. For a general situation concerning either a solute/solid sphere/droplet the velocity due to all driving forces namely, diffusion, sedimentation and electrophoresis during electrophoresis is given by [83]

$$-v = \left(\frac{k_B T}{3\pi\eta_C d}\right)\frac{1}{C}\frac{dC}{dz} + \frac{d^2(\rho_S - \rho_C)g}{18\eta_C} + \left(\frac{k_B ZF}{3\pi\eta_C dR}\right)\frac{d\phi}{dz} \qquad \text{(4h-1)}$$

where Z is surface net charge of the solute sphere or phase droplet; R is the universal gas constant; ρ_S and ρ_C are densities of solute sphere or dispersed phase droplet and of solvent or the continuous phase, respectively; n_C is viscosity of the continuous phase, F is Faraday's constant and ϕ is the applied electric potential.

In the specific case of liquid droplets (like the individual phase droplets of ATPSs) the above equation has been modified by Raghavarao et. al., [84] considering the droplet internal circulation as per Hadamard and Rybczynski's equation[85] and the internal diffuse double layer as indicated by Frumkin[86] and Levich.[86] On incorporation of these modifications, the equation will take the following form:

$$-v = \left(\frac{k_B T}{3\pi\eta_C d}\right)\frac{1}{C}\frac{dC}{dz} + \left(\frac{d^2(\rho_D - \rho_C)g}{18\eta_C}\right)\left[\frac{3\eta_D + 3\eta_C}{3\eta_D + 2\eta_C}\right] + \left(\frac{d\sigma_E E}{2(3\eta_D + 2\eta_C + \sigma_E{}^2/\lambda)}\right) \quad \text{(4h-2)}$$

where ρ_D is the dispersed or droplet phase density, E is the electric field strength (V/cm), σ_E is the surface charge density (C/cm^2) and λ is a function of K_D and K_C, the conductivities of the dispersed or droplet phase and continuous phase, respectively.

In the case of ATPSs the size of the droplet is much larger than typical colloid particles (up to at least two orders of magnitude), and the drops grow in size due to coalescence during electrokinetic demixing. Hence the contribution from diffusion can be ignored, and the equation further reduces to

$$-v = \left(\frac{d^2(\rho_D - \rho_C)g}{18\eta_C}\right)\left[\frac{3\eta_D + 3\eta_C}{3\eta_D + 2\eta_C}\right] + \left(\frac{d\sigma_E E}{2(3\eta_D + 2\eta_C + \sigma_E{}^2/\lambda)}\right) \quad \text{(4h-3)}$$

The buoyant drop migration velocity (v_G), in the absence of an electric field, due to gravity is given by the first term in the above equation while the second term is zero. In horizantal analytical microelectrophoresis, electrophoretic velocity (v_E) is given by the second term while the first term is zero, since v_E and v_G are orthogonal. The contribution from the buoyant or sedimentation velocity [first term in equation (4h-3)] comes into effect in the case of phase demixing in a contacting column even in the presence of an electric field. Thus a critical drop diameter (d_C), above which buoyant motion dominates over electrophoretic motion can be obtained by equating the two terms in equation (4h-3) giving

$$d_C = \left(\frac{3\sigma_E E \eta_C (3\eta_D + 2\eta_C)}{g(\rho_D - \rho_C)(\eta_D + \eta_C)(3\eta_D + 2\eta_C + \sigma_E^2/\lambda)} \right) \tag{4h-4}$$

and electrophoretic mobility (μ_E) can be obtained from the elctrophoretic velocity (second term in equation 4h-3) as

$$\mu_e = -\frac{v_E}{E} = \left(\frac{d\sigma_E}{2(3\eta_D + \eta_C + \sigma_E^2/\lambda)} \right) \tag{4h-5}$$

Raghavarao *et al.*,[84] have measured the electrophoretic mobilities of individual phase droplets suspended in their opposite phase for a PEG/DX system using a micro-electrophoresis unit. These values compared well with the predicted mobilities obtained from electrokinetic theory. Effective electrophoretic mobilities estimated from electrokinetic demixing data in a column compared well with predicted and experimentally measured values of electrophoretic mobility. It was confirmed that the droplet electrophoretic mobility increased with increasing drop diameter, and the effective electrophoretic mobility (obtained from demixing data) also increased with an increase in pH and partitioning anion (phosphate) concentration. Further, the dependence of demixing rate on pH and partitioning anion (phosphate) concentration was characterized. Increasing the concentration of phosphate ion charges by increasing pH increases the demixing rate of the PEG/DX system at a given applied DC electric field. This observation and others were used to draw inferences about the electric double layer and the transport of drops during demixing. Analysis of these data[84] was found useful in gaining insight into two paradoxes of electrokinetic demixing of aqueous two-phase systems: (1) The direction of migration of drops is the opposite of that predicted by colloid electrokinetics and (2) The phase demixing rate increased irrespective of the sign of the applied electric field.

The paradox concerning the direction of mobility in relation with the phospate ion partitioning and resulting Donnan potential, noticed by Brooks and

coworkers[78,80] in microelectrophoresis could be explained based on the electroosmotic flow generated due to the diffuse double layer at the droplet interface. Electroosmotic flow inside a drop is schematically shown in Figure-7. Similarly the observed increase[78,80] in mobility with an increase in droplet diameter, pH and phosphate concentration also could be explained by the electroosmotic flow and the model equations given above.

Further, the observed increase in phase demixing rates in a column[76,77] also could be explained by this electroosmotic flow model. In the case of normal polarity the electrophoretic motion supplements the buoyant rise or fall velocities of the phase drops, thereby enhancing the phase demixing rate. Interestingly, in reverse polarity, though the field pulls the phase drops against buoyant motion, it assists their growth in the dispersion zone until d_C (eq 4h-4) is exceeded and buoyancy takes over causing faster phase demixing. As the buoyant velocity of a drop is proportional to the square of its diameter while the electrophoretic velocity is proportional to its diameter, the demixing rate is actually faster in reverse polarity than in normal polarity.

However, there is a need for a systematic furhter theoretical study to identify and quantify the forces, including the force associated with the electroosmotic flow inside the droplet, in order to establish the force balance for a droplet under electro kinetic motion.

i. Electro-extraction

As both the phases of ATPSs are electrically conductive, application of electric fields in these systems gives rise to electrokinetic mass transfer of charged species. Thus ATPS was shown to function as a medium for electrophoretic separation with the two-aqueous-phase interface providing stability against convection and facilitating product recovery.[87] Proteins have been directed into either the top or the bottom phase of PEG/DX system employing 20-50 V/cm electric fields perpendicular to the phase interfaces. Binary protein mixtures were separated in both

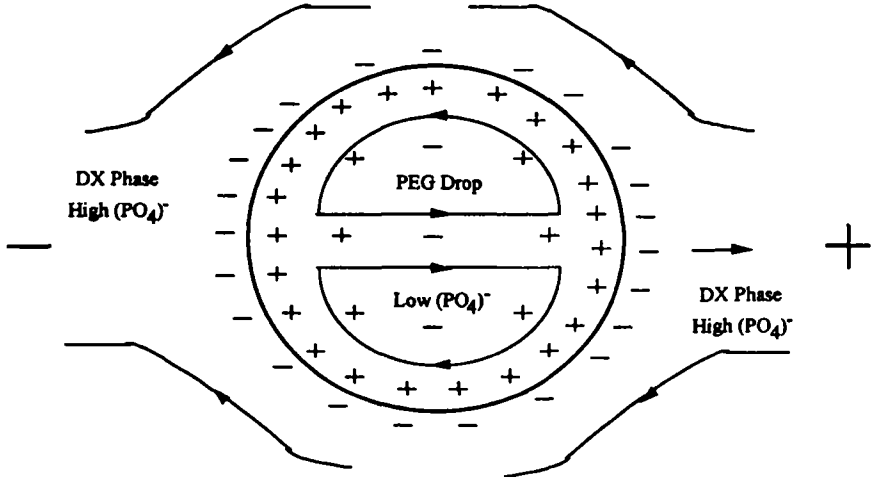

Figure 7.

Schematic representation of electroosmotic flow inside a droplet. (Arrows indicate the predicted direction of electrokinetically induced fluid flow in the drop and in the continuous phase).

batch and continuous modes by operating between isoelectric points and directing oppositely charged proteins into opposite phases.

Several studies have reported improved extractive separation by the application of electric fields to both traditional organic solvent extraction systems and aqueous two-phase systems.[87,88,89,90,91] Scott and Wham[88] applied an electric field to create emulsion with high interfacial area for efficient contacting with the continuous phase and also to induce coalescence in a novel counter-current extractor. Increased mass transfer was achieved due to the altered convection currents within and around the oscillating aqueous drops dispersed in a continuous, nonconducting, organic phase under the influence of a pulsating electric field.[90] Electroextraction with an applied field of 250 V/cm was successfully employed to recover citric acid from water using n-butanol (saturated with water) as solvent.[92]

Levine and coworkers[93,94] reported that electrophoretic transport of proteins across the interface of ATPSs is greatly impeded in one direction. They indicated that the electrophoretic transfer of proteins is readily acheived if the protein is migrating from its less-preferred phase to its more-preferred phase and that proteins do not migrate in the opposite direction, that is, from the more-preferred to the less-preferred phase under similar conditions. However, Theos and Clark[87] reported that protein can be made to transfer electrophoretically across the aqueous two-phase boundary in both the directions.

Electroextraction appears to be a promising technique for commercial scale electrophoretic separations due to the several advantages it offers over free solution methods, such as controlling starting composition, limiting convective mixing, and facilitating product isolation.[87]

5. Wider Application of Aqueous Two-Phase Systems

ATPE is also applicable in the area of food technology. Chen[95] utilized ATPS as a means of clarifying cheddar cheese whey. Fat in cheese whey could partition exclusively into the bottom phase of a PEG/KH2PO4 system resulting in a clear top phase containing whey proteins. This method should remove approximately 98% of whey proteins. Ogawa *et al.*,[96] carried out the isolation of high phytin containing particles from rice bran using a combination of differential centrifugation and ATPE using a PEG/DX system. The isolated particles consisted mainly of phytic acid, potassium and magnesium, as determined from the chemical composition and electron microscopic observations. Other potential food applications of ATPE include enriching soybean and corn endosperm proteins.[97]

The partitioning behavior of six Calvin-cycle enzymes from a spinach (*Spinecea oleracea*) chloroplast (multienzyme complex) extract was studied using a PEG/DX system by Persson and Johansson.[98] These weak complexes may dissociate during conventional extraction techniques, and ATPE is useful for detection and isolation of such complexes. Isolation of plant enzymes is frequently hampered by

the presence of phenolic compounds, pigments, and mucilages. Vilter[99] exploited ATPE, which has great advantage with respect to yield, product purity and processing time to overcome these problems. ATPE may open new avenues in plant research, especially for obtaining enzymes from plant materials. Thus enzymes can be produced from sources that have been avoided until now due to the difficulties encountered in processing.

Chen[100] studied the influence of several system parameters on partitioning of bovine α-lactalbumin and β-lactoglobulin in the PEG/K_2PO_4 system. These proteins from cheddar cheese whey could be separated by differential partitioning, and essentially pure β-lactoglobulin and 84 % pure α-lactalbumin m, n, & k could be obtained simultaneously from cheddar cheese whey in a single liquid-liquid extraction step. Soluble potato cresolase monophenol monooxygenase was partially purified using Triton X-114 in two-phase partitioning.[101] Potato was homogenized in 200 mM EDTA and 6 % (w/v) Triton X-114. The enzyme was purified fivefold, with a yield of 18, and 97 % of the phenols removed from the enzyme extract by this method, avoiding post-purification browning of the enzyme. ATPSs were also investigated to overcome the problem of product inhibition in acetone-butanol-ethanol fermenta-tion.[102] An industrial grade DX and hydroxypropyl starch were tested as copolymers with PEG to form two-phase fermentation broth and the performance was compared with a single-phase conventional fermentation through a series of batch runs. The yield was observed to increase by 24 %.

Thus ATPE is finding more and more applications in many areas of pharmaceutical, food and other related areas as well as biotechnology.

6. Scale-up Aspects

It has been demonstrated that scale-up is easier in the case of ATPE than in other processes for the large scale purification of proteins.[5,27,103,104,105,106] Technical feasibility has reported up to the 100,000 liter scale.[107] The equilibrium between the phases is known to be diffusion controlled, and thus in turn dependent on viscosities of the individual phases of the ATPSs.

However, the low interfacial tensions of these systems facilitate achieving equilibrium. It was shown that scale-up by a factor of 25,000 could be accomplished with ease if proper conditions were provided for adequate mass transfer. It is important to note that the partition coefficient for most enzymes is independent of their concentration in the individual phases over a fairly wide concentration range and scale of operation. This result is crucial for the purpose of scale up. For the commercial use of ATPE, experiments can be conducted in small-scale equipment, and the results obtained at this level can be directly applied to large-scale equipment. Further, the scale up is facilitated by the availability of equipment and machinery used for extraction technology in the chemical industry e.g., mixer-settlers, column contactors and continuous counter current apparatus, which can be easily adapted for ATPE.

Two processes have been published in detail for the large-scale isolation and purification of FDH from yeast and LDH from bacteria.[26,106] During scale-up all concentrations were increased linearly according to the amount of cell homogenate (up to 200 kg) involved in the extraction systems. Variations in the quality of the starting materials, such as a difference of about 50 % in FDH activity in the yeast cells, could be accommodated with out any changes in process parameters.

Since the enzyme or any other biologically active material stays in solution at all times, ATPE lends itself to a continuous operation which allows a decrease in processing time and improves the productivity considerably as pointed out by Dunhill and Lilly.[108] However, the biggest difficulty encountered in developing continuous processing was high capacity of the method which requires large amounts of cells and other materials. Veide *et al.*,[109] used a single-step ATPE (employing a PEG/K_2PO_4 system) process to achieve 75% yield in a large scale (375 liter) isolation of β-galactosidase from *E.coli*. Another *large-scale* extraction (24 kg of cells) reported is that of D-LDH from *Lactobaccilus confusus*, using two-stage ATPE followed by UF and chromatography to achieve a 3-fold enrichment.

Strandberg *et al.*,[110] reported a pilot scale production process for a recombinant protein, consisting of fermentation, cell harvest, disintegration,

extraction, diafiltration, and freeze drying. The purification scheme, including extraction in a PEG/phosphate system, yielded an overall recovery of 37%. Pilot scale trials were reported for the extraction and purification of enzymes from animal tissue by Boland et al. [111] while discussing the general applicability of ATPE, its economics, and its potential industrial applications.

A two-stage ATPE was reported for extraction of Fumarase from baker's yeast. They indicated a recycling strategy which resulted in higher specific activity of the enzyme while lowering the PEG requirements by 50 % and phosphate by 12%.[112] Joshi *et al.*,[113] have suggested a procedure for the design and scale-up of column contactors. The hydrodynamic and mass transfer characteristics are found to be independent of column diameter (> 50 mm) and column height (> 300 mm). Further, it was observed that the values of k_la vary linearly with the fractional holdup. These results were utilized in their stepwise scale-up procedure.

7. Economics

One of the critical factors in the industrial purification of enzymes and proteins using ATPE is the selection of appropriate systems. Most of the large-scale purifications reported in the literature use either PEG/DX or PEG/Salt systems. These systems have a number of desirable characteristics, such as suitable physical properties, non-toxicity, and biodegradability and the approval of regulatory authorities.

However, the high cost of fractionated DX (approximately $ 500 /kg) and the high salt concentrations (of the PEG/Salt systems) have necessitated a search for suitable alternatives. Kroner *et al.*[21] showed the technical feasibility as well as the cost effectiveness of the application of the PEG/Crude DX system for large-scale enzyme purification (crude DX costs $15 /kg). However, the high viscosity and polydispersity of the crude DX phase even after the partial hydrolysis of crude DX, remains a serious drawback of this system. Tjerneld *et al.*,[114] developed a PEG/Hydroxypropyl starch (HPS) system (HPS costs $ 20 /kg) which had characteristics similar to that of PEG/DX systems and was successfully applied to

large-scale purification of pig muscle protein.[27] Ethylhydroxyethyl cellulose is another inexpensive phase-forming polymer that forms ATPS with HPS or DX at a very low total polymer concentration.[27] Due to the high viscosity and the resulting long time required for individual phase separation, this system could not be explored on a large scale.[6]

At this juncture, the PEG/MDX system[74,115] appears to be the most cost-effective ATPS (MDX costs only $1 /kg) although large-scale operations have yet to be carried out using this system. The productivity and economics of a purification process improves considerably, especially for intracellular enzymes, when ATPE is employed in place of conventional methods, as was indicated by Kroner *et al.*.[21]

The cost of waste treatment is another important economic factor.[116] Phase-forming polymers like PEG, DX and MDX are biodegradable and non-toxic; however, salt (sulfates and phosphates) disposal could be problematic. PEG recycling can be easily integrated into a process and, depending on the tie line length, up to 90-95% of the polymer has been recovered and recycled.[117] If required an intermittent cleaning step can be included. Along with the PEG phase of the secondary system, a large proportion (about 25% of the salt added) of salt is also recycled. Another 25% of the salt can be separated from the product stream (the salt rich phase) of the secondary system by UF[118] during final product purification. Salt separation from the primary bottom phase containing cell debris, soluble and insoluble enzyme/protein etc. is difficult to accomplish by mechanical separation techniques such as centrifugation. However, salt could be extracted by forming another ATPS in mixtures of aliphatic alcohols, salt and water.[116] Using 3 or 4 stages in a counter-current extraction 95% of salt can be removed from the polymers and cell debris. After separation, alcohol and salt can be recycled to the process[119] thus improving the economics.

8. Conclusions

Downstream processing in many bioprocesses accounts for a large share of the total cost. ATPE appears to be a promising technique in the train of downstream

processing steps. It is tolerant of particulate matter and can reduce to a large extent the volume of the process stream, while simultaneously concentrating and purifying the desired product/biomolecule. This process stream of reduced volume can be subjected to more expensive and selective purification steps such as chromatography for the desired final purification. Thus ATPE could be an attractive complementary step to more selective downstream processing steps. Some successful applications of ATPE on large/industrial scale have been demonstrated.

Electrokinetic demixing and Electroextraction appear to overcome the main drawbacks of ATPE, namely slow rate of phase demixing, low selectivity and inadequate control over the partitioning behavior of the desired biomolecule. The goal of developing effective downstream processing methods employing ATPSs is an interdisciplinary effort involving a combination of microbiologists, biochemists and biochemical engineers. In principle ATPE offers the advantage of easy adaptation of the extraction equipment already used in the chemical and pharmaceutical industries to achieve efficient extraction; however the drop dynamics, phase demixing rates and mass transfer aspects of traditional contacting equipment still needs to be studied in more detail employing various ATPSs and real systems involving actual fermentation broths and raw mixtures.

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