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The Practical Application of Aqueous Two-Phase Processes for the Recovery of Biological Products

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Abstract Although the generic implementation of aqueous two-phase systems (ATPS) processes for the recovery of biological products has been exploited for several years, this has not resulted in a wide adoption of the technique. The main reasons involve the poor understanding of the mechanism governing phase formation and the behavior of solute partitioning in ATPS processes, the cost of phase forming polymers, and the necessary extended time to optimize the technique. In this review paper, some of the practical disadvantages attributed to ATPS are addressed. The practical approach exploited to design ATPS processes, the application to achieve process integration, the extended use for the recovery of high-value products, and the recent development of new low-cost ATPS, are discussed. It is proposed that the trend of the practical application of ATPS processes for the recovery of biological products will involve the purification of new high-value bioparticulate products with medical applications. Such a trend will give new impetus to the technique, and will draw attention from industries needing to develop new, and improve existing, commercial processes.

Key words: ATPS processes, recovery of biological products, proteins, process integration

Aqueous two-phase systems (ATPS) form when combinations of hydrophilic solutes (polymers or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations. Current research in ATPS can be divided into two major areas. One is strongly concerned with a mechanistic molecular understanding of solute partitioning in ATPS, whilst the other focuses upon practical implementation of the technique for process development. The practical application of ATPS for the recovery of biological products from different sources, generates robust, easy to scale up, and biocompatible extraction processes. Such processes produce fractions from a variety of biological suspensions in a state suited for further

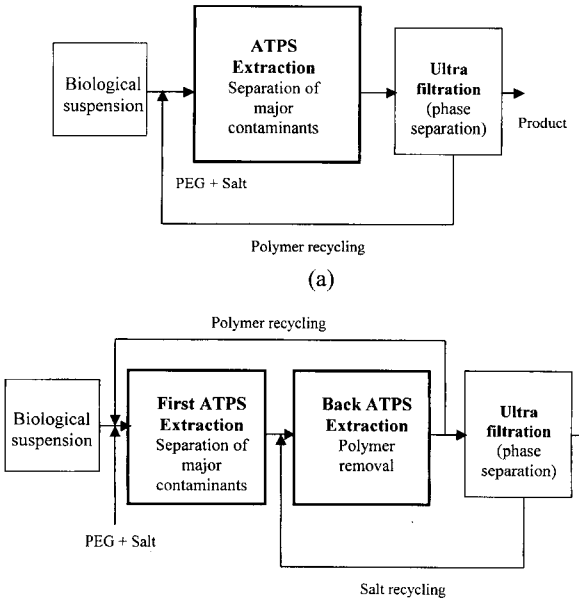

Fig. 1. Simplified representation of one-stage (a) and two-stage (b) ATPS process with ultrafiltration.
Chemical forming phases are mixed with the biological suspension to form the first two-phase system. The bottom phase containing the major contaminants is discarded and the top phase further processed by ultrafiltration. The polymer-rich permeate can be recycled. In the two-stage ATPS process, the top phase from the first extraction is mixed with fresh salt to form the second two-phase system. The second PEG-rich phase is recycled, whilst the bottom phase containing the product of interest is passed through an ultrafiltration unit. The phosphate-rich permeate generated can be concentrated and recycled.

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purification and extraction of selected products (e.g. target enzymes).

The development of ATPS processes will most simply involve the design of ATPS extraction stages. In a typical one-stage ATPS process (Fig. 1a), the extraction stage yields a bottom phase containing particles (cells or cell debris) and contaminants (e.g. RNA, carbohydrates, lipids) and a top polymer-rich phase containing the product of interest. The high concentration of polymer in the top phase compromises the value of the product in that state. Consequently, the polymer from this stage must be removed by further processing the top phase by, for example, ultrafiltration. In the case of a two-stage ATPS process, the first extraction eliminates the bottom phase particles and contaminants from the feedstock and generates a top phase enriched in the target soluble product (Fig. 1b). In the second extraction stage (back extraction), the product of interest is partitioned to a bottom salt-rich phase which enables reuse of the polymer-rich top phase. Further processing of the bottom phase by ultrafiltration yields a product concentrate.

The generic implementation of ATPS for the development of extraction processes for the recovery of biological products has been exploited for more than 30 years. However, this has not resulted in widespread commercial application of the technique. The absence of ATPS in industry may be attributed to several factors including the cost of phase-forming polymers, a lack of knowledge of the technique, and poor understanding of the mechanism governing phase formation and solute partition. Although successful exploitation of ATPS for the recovery of desired products have been proved, reports of commercial adoption are not common [12].

The current review focuses on generating a practical understanding of ATPS processes for the recovery of biological products. An arbitrary selection of the various ATPS processes reported during the last five years is summarized here to establish the benefits of the technique at bench scale. The attractive role of ATPS in process integration, and a general examination of the recent new ATPS developed, are presented. General rules defining a practical approach for the development of extraction ATPS processes are given in this review. Furthermore, the new ATPS applications and the expected potential trend of the practical application of ATPS for the recovery of biological products are discussed.

The Practical Application of ATPS Processes for the Recovery of Proteins

Recently, several ATPS processes have been designed as primary purification operations, which have been characterized by single stage or multi-staged operations [4, 5, 7, 11, 17, 24, 26, 27, 29, 34–36, 47, 48]. For economic reasons, the majority of the latest type of processes development have exploited two-stage systems. From the arbitrary selection of the processes described in Table 1, it is clear that the three types of ATPS (i.e. polymer-salt, polymer-polymer, and others) have been used. Within the polymer-salt systems, PEG-phosphate ATPS are preferred due to several process advantages, including low cost, wide past and current application, and the range of system pH (from 6 to 9) under which the ATPS is stable. In all cases, the resulting phase with the product of interest contain predominantly water and increased concentration of one of the phase-forming components. As an example, the extracellular enzyme β-glucosidase produced by *Aspergillus niger* and present in a

Table 1. A simplified selection of the recent ATPS processes developed for the recovery of protein.

ATPS used	Biological source	Product of interest	Number of ATPS steps	Product recovery (%)	Reference
PEG-phosphate	Serum-free	IgG	2	100	[5]
	Aspergillus awamori	Glucoamylase	2	96	[26]
	Bovine blood	BSA	2	85	[36]
	Brewers' yeast	Pyruvate kinase	2	75	[35]
	Cheese whey	α-Lactoalbumin	2	65	[34]
	Bovine brain	Prion proteins	2	N. r.	[48]
	Bacillus pumilus	Alkaline xylanase	1	98	[7]
	Transgenic milk	Human al-antitrypsin	1	91.0	[11]
PEG-citrate	*E. coli*	Penicillin acylase	1	92	[24]
	Commercial source	Porcine insulin	1	N. r.	[4]
PEG-sulfate	Trangenic milk	Human al-antitrypsin	1	91	[11]
PEG-dextran	*Aspergillus niger*	β-Glucosidase	1	95	[17]
PEG-starch	Wheat	α-Amylase	1	75	[29]
PEG-HPS	*Saccharomyces cerevisiae*	Alcohol dehydrogenase	1	77–100	[47]
(EO-PO)-Reppal	Recombinant *E. coli*	Apolipoprotein	1	85–90	[27]

HPS=Hydroxylpropyl starch, EO=Ethylene oxide, PO=Propylene oxide.
N. r.=not reported.

culture filtrate was concentrated up to 700 times by two-phase partitioning [17]. The recoveries of β-glucosidase were in the range of 85–95% with a concentration factor of 60–720 times. A further example of the application of ATPS is the recovery of the recombinant apolipoprotein A1 expressed in *E. coli* [27]. In this case, the filtrate from *E. coli* fermentation was added to thermoseparating polymers (ethylene oxide-EO and propylene oxide-PO) and starch. Apolipoprotein A1 was partitioned to the top EO-PO rich phase and the contaminating proteins to the bottom starch phase. The recoveries of the recombinant apolipoprotein were in the range of 85–90% with a purification factor of 2.5–2.7. The protein products that have been recovered with acceptable process yield (i.e. 65–100%; see Table 1) using ATPS varies from low to high value products (e.g. BSA, pyruvate kinase, porcine insulin, apolipoprotein; Table 1), which demonstrates the flexibility of this technique. In addition, the different nature of the biological sources (biological suspensions, fermentation broths, commercial sources, etc.) that have been processed by exploiting ATPS proves the robustness and generic application of the technique. In this context, one of the major advantages of ATPS is their suitability for processing of suspensions of up to 50% (wet w/v) without compromising capacity or resolution.

The recovery of a large number of biological products using ATPS has proved the success of this technique in the efficient generation of bench-scale prototype process with potential commercial application. However, the lack of large-scale ATPS as a part of downstream processes is a very well known characteristic of this novel technique. This may be attributed to a reluctance to embrace this technique by industries due to the fact that the knowledge of the mechanism of solute partitioning in ATPS is limited. As a result, the reports dealing with the commercial adoption of ATPS are not common. However, there is a very interesting report in which a commercial adoption of ATPS for the recovery of a biological product has been shown [12]. Such report proved the success of the application of ATPS at large-scale (10,000 l fermentation) for the recovery of periplasmic IGF-I.

Alternative explanations for the absence of ATPS at commercial scale may involve the disadvantages of batch operation (and the complications associated with the implementation of ATPS processes in a continuous mode of operation) and the lack of equipment needed for some processes. Furthermore, the absence for this technique of "commercial kits" (as in the case of conventional technologies), that facilitate the evaluation of ATPS processes at bench-scale, enhance the existing reluctance to adopt this technique for commercial purposes. The lack of commercial kits implies that the process developments mostly rely on "in house" designs, which may raise issues of process reproducibility and robustness. This is an important aspect of the technique that needs to be addressed. Furthermore, the implementation of ATPS processes not only depends on technical potential and feasibility, but also depends strongly on process economics. The relative high need for chemicals to form working ATPS has saddled this technique with an unfavorable economic image [35, 36].

Examination of the New ATPS for the Recovery of Proteins

In order to improve some of the advantages of ATPS, new types of ATPS have recently been developed. Table 2 illustrates a selection of some of the new types of ATPS reported and their applications. The use of crude starch modified as a substitute for the bottom phase of a defined ATPS [3, 29, 47] attempted to reduce the cost of certain polymers such as dextran. However, in some cases, the use of these modified systems resulted in a poor purification of the target product [3]. In contrast, ATPS that exploited the use of Ucon [19, 23, 28] proved to be efficient for the development of recovery process. In addition, these new types of systems present potential for the recycling of the chemicals-forming phases by altering the process temperature. In the search for new low cost chemical forming phases for ATPS, the use of novel or exotic compounds such as cashew nut tree gum has been suggested [40]. The great disadvantage of this latest type of ATPS is associated with the need for a formal and

Table 2. Selection of the new type of ATPS exploited for the recovery of biological products.

New type of ATPS	Application	Reference
Benzoyl dextran-Ucon	Purification of 3-phosphate glycerate kinase from Bakers' yeast	[19, 23, 28]
PEG-HPS	Purification of cutinase from recombinant *E. coli*	[3]
PEG-cashew nut tree gum	Study of the behavior of commercial BSA	[40]
PEG (or dextran)-IAA-Cu(II)	Recovery of membrane (cytochrome b03, ubiquinol oxidase) proteins from *E. coli*	[44]
Poly-VI/VCL - modified starch	Purification of α-amylase from wheat meal	[29]
PEG-dye ligand and dextran	Extractive fermentation for the recovery of IgG and hybridoma	[51]
PEG (or EO-PO)-(PEI)	Recovery of lactic acid	[30, 31]
EO-PO and Reppal	Recovery of recombinant apolipoprotein A1 from *E. coli*	[27]

HPS=Hydroxylpropyl starch, VI/VCL=vinylcaprolactam, PEI=polyethylenimine.
EO-PO=Ethylene oxide propylene oxide, IAA=iminoadiacetic acid.

complete characterization of the ATPS (i.e. construction of the phase diagram, partition behavior of commercial proteins in the systems, etc.). In addition, these ATPS are characterized by low process efficiency as compares with that from commercial or traditional ATPS [40]. Such a situation can be explained by the low purification of the compounds used to form the phases and the resulting effect on the product partition behavior.

To enhance selectivity of the ATPS toward the product of interest, modification of the PEG-rich phase has been made (Table 2). These modification include, among others, the use of metal ions (such as Cu(II); [29, 44]) and the use of dye ligand [51]. The use of Cu(II) resulted in new ATPS for the development of a process in which the protein (cytochrome b03) of interest was affinity partitioned in the ATPS. In the case of a PEG-dye ligand system, it was successfully exploited for the extractive fermentation for the recovery of IgG [51]. However, it is clear that the potential application of such highly modified ATPS focuses to the recovery of high-value products, in which the cost of the final product can compensate that of the chemical forming phases. Furthermore, once the product of interest has been selectively partitioned to the modified PEG-rich phase, the problem of efficiently separating the polymer from the product still remained. Consequently, the generic application of these new ATPS is limited by the complexity associated in the preparation of the modified systems, the cost of such process, and the effect on the overall process recovery.

Application of ATPS to Process Integration

Currently, the achievement of process integration of the upstream operations of fermentation and the downstream recovery processes, associated with the manufacture of bioproducts, is raising interest. The approach of process integration that attempts to combine two operations into one, in order to achieve specific objectives not efficiently met by discrete processes, is considered as one with potential benefits for the recovery of biological products. In this context, the use of ATPS represents an attractive alternative to process integration for the recovery of products in three major areas of research: (i) extractive bioconversion, (ii) extractive fermentation, and (iii) integration of cell disruption and primary purification step (Fig. 2). The application of ATPS for extractive bioconversion has been exploited for more than a decade. However, this has not yet resulted in a wide commercial application. Some of the reasons involve the cost of the phase-forming polymer and the complexity of ATPS behavior. A recent review in this particular area [50] implied that the extended application of extractive bioconversion to high-value protein products, together with the development of low-cost ATPS, will give a new impetus to this technology.

Recovery of products exploiting the use of ATPS in extractive fermentation represents an attractive technology

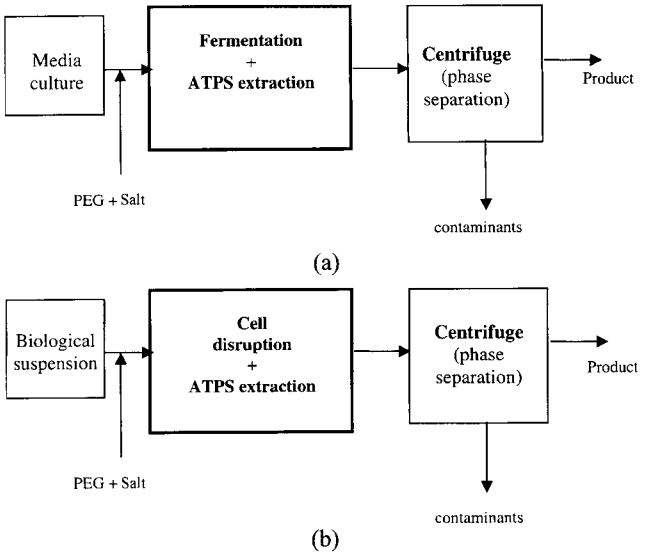

Fig. 2. Simplified representation of process integration of ATPS and fermentation (a) and ATPS and cell disruption (b).
The upper flow diagram represents the extractive fermentation ATPS process, in which the production and the recovery of the target product can be integrated in one single unit operation. The lower one represents the integrated process of cell disruption and ATPS for the recovery of intracellular products.

to remove the product from the fermentation broth as it is formed (Fig. 2a). Extractive fermentation in ATPS is a meaningful approach to overcome low product yield in a conventional fermentation process, and by proper design of the two-phase systems, it is possible to obtain the product in a cell-free stream. Recently, extractive fermentation using ATPS have been developed for the recovery of different protein products [10, 20, 22, 42, 43] that resulted in an increase in the productivity of the processes. Furthermore, the use of ATPS in extractive fermentation has been exploited to address the process disadvantages of conventional processes (characterized by discrete operation of fermentation and product recovery) such as product inhibition [37] and product hydrolysis [13]. In the first case, the successful removal of aroma compounds from the media culture is reported, whilst in the case of product degradation, the contaminants were efficiently removed from the fermentation broth. It seems that the practical application of ATPS for extractive fermentation represents a very interesting alternative to overcome existing problems. However, extractive fermentation processes are limited for the recovery of extracellular products. Therefore, the recovery of products, in which cell disruption is mandatory, needs a different approach.

Direct product sequestration at cell disruption could enhance product yield and quality. Such product capture could be achieved using fluidized bed adsorption (FBA). In this context, the successful integration of cell disruption and FBA for the recovery of intracellular proteins from

yeast has been reported [6]. In this line of research, the use of ATPS represents an attractive alternative to process integration for the recovery of intracellular proteins (Fig. 2b). The reports dealing with the integration of cell disruption and ATPS are not common (M. Rito-Palomares and A. Lyddiatt, 2002. *Chem. Eng. J. Accepted*). The scheme of process integration proposed in this latest study for the recovery of intracellular proteins clearly proved that simultaneous disruption and aqueous two-phase extraction produced a process for the primary recovery of intracellular proteins from yeast. It is clear that process economics benefits are associated with the reduction of unit operation by the approach of process integration. However, this report also concluded that further studies, to address the potential of ATPS for process integration as a primary step for the recovery of intracellular proteins, are essential.

New Applications of ATPS Processes

For the last decade, the practical application of ATPS processes for the recovery of biological products has been focussed on the primary purification of proteins. A recent redirection of the type of target products that can be produced by the application of ATPS may define a new trend of this technique. Recently, the use of ATPS processes has been extended to nonprotein products (Table 3). In this context, the potential application of ATPS processes for the recovery of metal ion, coloring dyes, and small organic molecules [14, 38, 39], although the successful results were obtained using simplified systems, represents an interesting case to address some of the environmental concern of industries. In the context of large-scale application of ATPS, a novel procedure of drowning-out crystallization of sodium sulfate using ATPS was reported to obtain crystal of pure anhydrous salt [46]. In this case the phases are recycled, allowing the design of a continuous process.

The application of ATPS has also been extended to the food and cosmetic industries, in which the recovery of compounds of commercial significance (e.g. aroma compounds) has been addressed [37]. The low cost of the ATPS represents an attractive alternative to the conventional route that exists for the production of these products. Furthermore, it confers the denomination of natural products, since their production involves the use of biotechnological technologies. The production of aroma compounds such as 6 penthyl-alpha pyrone produced by *Trichoderma harzianum* [37] often exhibits the problem of product inhibition. Such problem can be alleviated by the continuous removal of the product of interest from the fermentation broth. This problem, that it is not specific to the production of aroma compounds (see lactic acid production in references 30, 31), may be addressed by exploiting the biocompatibility of ATPS in an integrated process with fermentation.

ATPS has also been exploited for the improvement of certain analytical techniques (e.g. polymerase chain reaction; [21]). In this case, the removal of inhibitory substance from cells was possible using a PEG-dextran system. The increase in the number of this type of application, the necessary development of commercial kit for the generic implementation of ATPS, together with a practical approach for the predictive ATPS process design, are essential to attract commercial interest in the technique. The potential use of ATPS for medical applications [8, 49], which is currently developing as a new novel application of ATPS processes, will definitively draw attention from industries. In this context, the successful recovery of small inclusion bodies from complex homogenates highlights a generic role that ATPS techniques might play in the recovery and purification of new bioparticulate products (viral and plasmid gene therapy vectors, particulate protein vaccines, etc. [8, 49]).

Practical Rules on the Development of ATPS Processes for the Recovery of Biological Products

In the context of process design, the poor understanding of the molecular mechanism governing the behavior of solute in ATPS severely limits the predictive design of extraction stages using this technique. Consequently, for each extraction process, operating conditions need to be empirically established. In order to minimize the necessary time for the design of ATPS extraction stages, a practical

Table 3. Selection of the new current applications of ATPS.

ATPS	New application	Reference
PEG-phosphate	Recovery of aroma compounds	[37]
	Generation of highly purified preparations of small inclusion bodies	[49]
PEG-sulfate	Drowning-out crystallization of sodium sulfate	[46]
	Recovery of metal ions from aqueous solutions	[38]
	Recovery of food coloring dyes from textile plant wastes	[14]
	Partition of small organic molecules	[39]
PEG-dextran	Separation of polymerase chain reaction (PCR)-inhibitory substances from cells	[21]
	Extractive bioconversion	[50]
EOPO-dextran	Extractive fermentation for the recovery of lactic acid from *Lactococcus lactis*	[30, 31]

EOPO=Ethylene oxide propylene oxide.

approach is needed. On the basis of experience in the partitioning of solute in ATPS, general process conditions can be selected. However, the accumulation of certain experience in ATPS represents a major disadvantage for the generic and wide application of this technique. It is clear that the researchers interested in the use of ATPS need to become experts in the area before starting the design of defined experiments. Such situation is explained due to the lack of reports detailing the necessary steps for the application of ATPS.

In order to facilitate the development of extraction ATPS processes, a practical approach (as applied to techniques of precipitation and ion exchange), involving a basic knowledge of the technique, is presented here as one of the most important outcomes derived from the present review (Fig. 3). For the general selection of the type of ATPS to be used, three different systems can be considered: (i) polymer-polymer, (ii) polymer-salt, and (iii) other ATPS. The application of polymer-polymer (e.g. polyethylene-glycol (PEG)-dextran) systems are in some cases limited by process economic considerations associated with the high cost of one of the polymers (i.e. dextran). The use of others type of ATPS have been discussed before in this review. Due to the low cost of the chemicals-forming phases, the initial selection of a polymer-salt ATPS is suggested. PEG-phosphate ATPS represents the type of systems most widely exploited in the area. Such situation may be associated with the knowledge accumulated with these systems and to their stability on the basis of phase formation [36, 37]. The selection of a low molecular weight of PEG (e.g. 1,000 or 1,450 Da) is preferred to concentrate the majority of contaminants in the lower phase. Once the general selection of the ATPS has been achieved, a phase diagram is needed. This can be constructed using the cloud point method [16] or it can be obtained from previous reports [2]. The phase diagram is essential for the evaluation of the influence of the system parameters (i.e. tie line length (TLL), phase volume ratio (Vr), and pH) upon the recovery of the target product, to define the operating conditions of the ATPS process.

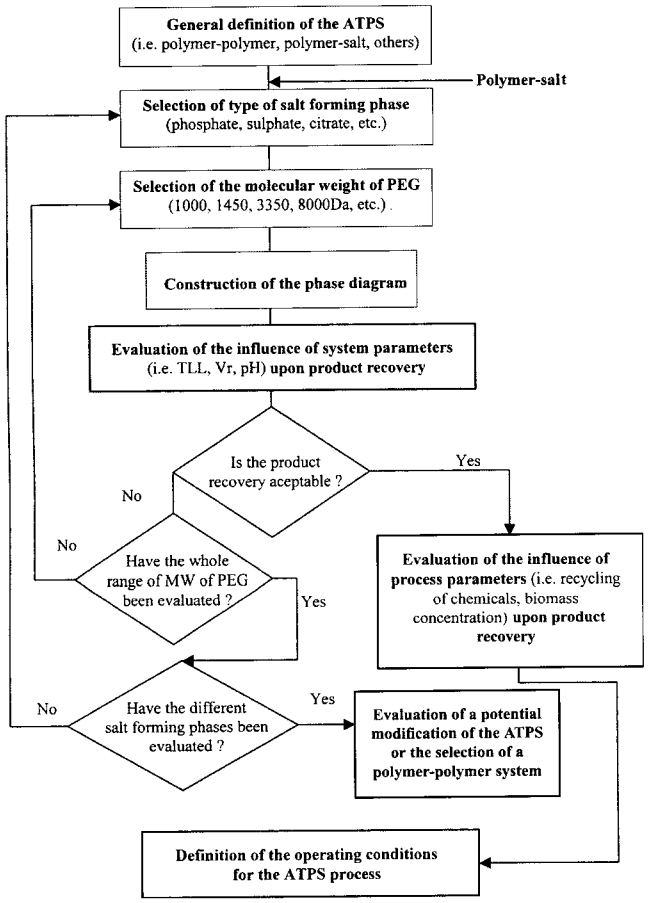

Fig. 3. General sequential rules suggested for the development of ATPS processes for the recovery of biological products.
General rules and strategies for the use of ATPS for the development of extraction processes presented here require knowledge of the basic concepts of the technique.

Influence of System Parameters Upon the Product Partition Behavior in ATPS

The behavior of product recovery from the top phase, when TLL is increased and Vr equal to one and pH equal to neutrality (or to the value that gives more stability to the target product) are kept constant, needs to be examined. The TLL that results in the highest product recovery must be selected. Once the system TLL has been selected, the effect of changing Vr (the use of Vr values less and greater than one are suggested) upon product recovery should be evaluated. In this case, TLL (at the selected value) and pH are kept constant. If improvement in product recovery is obtained, then the new Vr value should be adopted. Finally, by keeping constant TLL and Vr at the selected values, the impact of changing system pH upon product recovery is evaluated. Here again, the system pH that results in an increase in product recovery must be considered. The suggested approach has been exploited for the development of recent ATPS processes (as examples, see references 3, 24, 34). If after the manipulation of the different system parameters the product recovery achieved is not acceptable, then a change of the conditions of the selected ATPS (e.g. selection of polymer-polymer system or a modified ATPS) as it is illustrated in Fig. 3 is suggested. In contrast, once the conditions for an acceptable product recovery are obtained, it can be assumed that a prototype ATPS process is preliminary-defined. To further characterize the established ATPS process, the influence of process parameters (e.g. recycling of chemical forming phases, biomass concentration, stability of the phase formation, etc.) upon process performance is needed.

Influence of Process Parameters Upon the Product Partition Behavior in ATPS

The selection of the process parameters to be evaluated depends on the specific characteristic of each extraction process. The phenomenon of phase formation in polymer-polymer ATPS has been attributed to the hydrated surfaces of each species, which are sufficiently incompatible to generate phase separation [1, 2]. In contrast, the descriptive mechanisms in systems composed of polymer and salt remain unclear. However, it has been suggested that phase separation is associated with differing interactions with the ether dipoles of the polymer chain [15]. For further discussion of phase separation in polymer-salt systems in model system (characterized by the sole presence of the target product), see previous reviews [9, 18]. In the case of complex systems (e.g. biological suspensions), recent publications [25, 41, 45] addressed such phenomena. These reports concluded that the type of biological suspension, the definition of the continuous phase in the ATPS [25, 41], and the geometry of the equipment influenced the rate of phase separation [45]. The processing of complex biological suspension requires the evaluation of the influence of such suspensions on the ATPS process performance [33]. For example, in the case of the recovery of recombinant proteins, the use of urea needs to be considered. In this latest respect, Ramsch *et al.* [32] have developed a modified ATPS that involves the presence of urea in the systems up to 30% w/w concentration of urea. The potential of such systems for the recovery of recombinant proteins is evident. In the context of the consumption of chemicals, the corresponding problems of costs and wastewater treatment may be reduced by recycling the phase components [16, 35]. In this context, the author has demonstrated the feasibility of phase recycling for different ATPS processes [35, 36]. It can be anticipated that the evaluation of the effect of process parameters upon the performance of the designed ATPS process will result in the development of robust ATPS extraction stages with potential implementation at commercial-scale.

CONCLUSION

The widespread laboratory application of ATPS processes for the recovery of protein products has not resulted in a commercial adoption of the technique. The reason for such reluctance from industries to exploit ATPS include the poor understanding of the mechanism governing partition of solutes in the systems, the necessary time involved in the learning process of the technique, and the cost of phase-forming polymers. Although new ATPS to improve the selectivity of the processes have been developed, the complexity of the construction of the new forming phase polymers and the low process recovery have disadvantaged such systems. The development of new low-cost ATPS, the practical application to achieve process integration, and the extended use for the recovery of high-value products will give new impetus to the technique. It is expected that the trend of the practical application of ATPS processes for the recovery of biological products will involve the purification of new high-value bioparticulate products with medical applications, which will draw attention from industries for commercial applications.

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