Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 807 (2004) 3–11
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Practical application of aqueous two-phase partition to process development for the recovery of biological products

Marco Rito-Palomares*

Departamento de Tecnología de Alimentos, Centro de Biotecnología, Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM), Ave. Eugenio Garza Sada 2501-Sur, Monterrey, NL 64849, Mexico

Available online 10 February 2004

Abstract

The practical application of aqueous two-phase systems (ATPS) to process development has been exploited for several years for the recovery of biological products. Unfortunately, this has not resulted in an extensive presence of the technique in commercial processes. Some of the main identified reasons for such situation involve the full understanding of the mechanism governing phase formation and the behaviour of solute partitioning in ATPS processes, the cost of phase forming polymers and the necessary extended time to understand the technique for process development. 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 increasing use for the recovery of high-value products and the recent development of alternative low cost ATPS, are discussed. It is proposed that the potential trend in the application of ATPS processes for the recovery of biological products will involve the recovery of high-value bio-particulate products with medical applications. This proposed trend in the application of ATPS will address the urgent need to rapidly and economically bring new biopharmaceutical products to market using scaleable and efficient bioprocess technology.

Keywords: Reviews; Aqueous two-phase systems; Partitioning; Process integration; Proteins

Contents

1. Introduction

Aqueous two-phase systems (ATPS) have been extensively exploited to process different biological sources for the recovery of biological products. It has been established that ATPS form when combinations of hydrophilic solutes (polymers or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations. In general, the research in ATPS can be divided into two major areas. The elucidation of the mechanistic molecular understanding of solute partitioning in ATPS is the main focus of

* Tel.: +52-81-8328-4132; fax: +52-81-8328-4136.
E-mail address: mrito@itesm.mx (M. Rito-Palomares).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.01.008

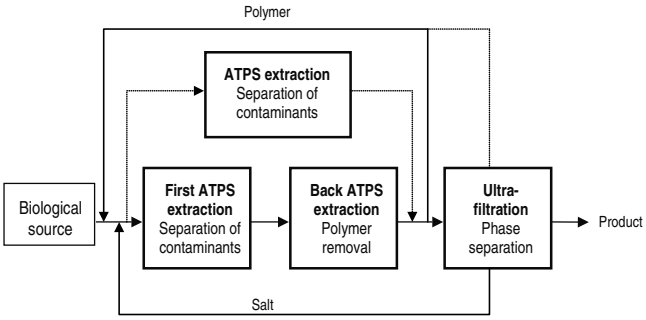

Fig. 1. Simplified representation of one- and two-stage ATPS process with ultra-filtration. Chemical forming phases are mixed with the biological suspension to form the first two-phase system. The bottom phase containing the major contaminants is discarded and the top phase further processed by ultra-filtration. 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 ultra-filtration unit. The phosphate rich permeate generated can be concentrated and recycled.

one of the major areas of research, whilst the other is concerned with the practical implementation of the technique to process development. The area that addresses the molecular understanding of partitioning, generally involve the use of model systems (characterised with the purified target product in an aqueous environment) to establish the rules that can predict the behaviour of solutes in ATPS. Although, advances have been achieved with respect to solute partition in ATPS, additional knowledge is needed to fully understand the phenomena. On the other hand, it has been proved that the practical application of ATPS for the recovery of biological products from different sources, generates robust, easy to scale and bio-compatible extraction processes. Such processes produce fractions from a variety of biological suspensions in a state suited for further purification and extraction of selected products (e.g. target enzymes).

ATPS process development for the recovery of biological products most simply involve the design of extraction stages. A one-stage ATPS process (Fig. 1) is characterised by an extraction stage that 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 target product. The potential commercial value of the product is easily compromised by the relative high concentration of polymer in the top phase. Therefore, further processing of the top phase by, for example ultra-filtration is required to remove the polymer from this stage. 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. 1). In the second extraction stage (back extraction), the product of interest is partitioned to a bottom salt-rich phase which enables re-use of the polymer-rich top phase. Further processing of the bottom phase by ultra-filtration yields a product concentrate.

The practical application of aqueous two-phase partition to process development has been exploited for the recovery of biological products for more than 30 years. Unfortunately, this has not resulted in an extensive use of the technique in commercial processes (but see [1]). Such situation 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, the factors that disadvantage the technique for commercial adoption, need to be addressed.

This paper focuses on presenting a practical overview to highlight the importance of ATPS processes for the recovery of biological products. A critical analysis of selected ATPS processes reported during previous years is summarised 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 alternative ATPS developed, are presented. General rules defining a practical strategy for the development of extraction ATPS processes are given in this review. Furthermore, the additional ATPS applications and the expected potential trend of the practical application of ATPS for the recovery of biological products are discussed.

2. ATPS processes for the recovery of proteins

A critical analysis of reports [2–15] dealing with the use of ATPS in bioproducts recovery exhibited that the application of ATPS for the recovery of proteins has resulted in processes designed as primary purification operations. Such processes have been characterised by single or multi-staged operations. From the total product recovery and economic view point, it is clear that the definition of one-stage ATPS primary recovery processes are preferred. Furthermore, the majority of multi-staged operations processes developed have exploited two-stage systems. In this context, three types of ATPS (i.e. polymer–salt, polymer–polymer and others) have been traditionally used (see the selection of the processes described in Table 1). In the polymer–salt systems, polyethylen-glycol (PEG)–phosphate ATPS are commonly used, 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 are stable. In these extraction systems, the product of interest is concentrated in a phase that contains predominantly water and increased concentration of one of the phase forming components, which in the majority of the cases is PEG. As a representative example of the successful development of an extraction process that exploit ATPS is the processing of an *Aspergillus niger* culture filtrate for the recovery of the extra-cellular enzyme β-glucosidase that resulted in a top phase with the protein concentrated up to 700 times [6]. The total product recovery for β-glucosidase was in the range of 85–95% with a concentration factor of 60–720 times. An additional case of

Table 1
General characteristics of selected ATPS processes to protein recovery

Type of ATPS	Biological origin	Target product	Extraction steps	Product recovery (%)	Reference
Polymer–polymer					
PEG–dextran	*Aspergillus niger*	β-Glucosidase	1	95	[6]
PEG–starch	Wheat	α-Amylase	1	75	[10]
PEG–HPS	*Sccharomyces cerevisiae*	Alcohol dehydrogenase	1	77–100	[14]
(EO–PO)–Reppal	Recombinant *E. coli*	Apolipo-protein	1	85–90	[9]
Polymer–salt					
PEG–phosphate	Bovine blood	BSA	2	85	[13]
	Brewers' yeast	Piruvate kinase	2	75	[12]
	Cheese whey	α-Lactoalbumin	2	65	[11]
	Aspergillus awamori	Glucoamylase	2	96	[8]
	Bovine brain	Prion proteins	2	N.r.	[15]
	Serum free	IgG	2	100	[3]
	Spirulina maxima	c-Phycocyanin	2	87	[54]
	Bakers' yeast	G3PDH	1	73	[44]
	E. coli	L1	1	65	[16]
	Bacillus pumilus	Alkaline xylanase	1	98	[4]
	Transgenic milk	Human al-antitrypsin	1	91	[5]
PEG–citrate	*E. coli*	Penicillin acylase	1	92	[7]
	Commercial source	Porcine insulin	1	N.r.	[2]
PEG–sulphate	Trangenic milk	Human al-antitrypsin	1	91	[5]

HPS, hydroxylpropyl starch; EO, ethylene oxide; PO, propylene oxide; N.r., not reported.

the application of ATPS is the recovery of the recombinant apolipoprotein A1 expressed in *Escherichia coli* [9]. To process the filtrate from *E. coli* fermentation, thermoseparating polymers (ethylene oxide (EO) and propylene oxide (PO)) and starch were used. Apolipoprotein A1 was partitioned to the top EO–PO rich phase and the contaminant 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. Recently, the non-optimised recovery of viral coat L1 protein (e.g. 65%) produced by recombinant *E. coli* was reported using an one-stage ATPS process [16]. These reports and others [2–15] clearly demonstrated the benefits of ATPS processes at bench scale for the recovery of products. Furthermore, the type of proteins 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, piruvate kinase, porcine insuline, apolipoprotein; Table 1), which demonstrates the flexibility and potential generic application of this technique at bench scale. In this context, one of the major advantages of ATPS is their suitability to process suspensions with high concentration of biomass (up to 50% (wet w/v)) without compromising capacity or resolution. Furthermore, the complex nature of the biological sources (biological suspensions, fermentation broths, commercial sources, etc.) processed exploiting ATPS demonstrated the robustness and generic application of the technique.

Clearly, the success of ATPS in the efficient generation of bench-scale prototype processes with potential commercial application has been proved by the existence of numerous reports dealing with the recovery of a large number of biological products. However, a very well known characteristic of this novel technique is the lack of large-scale ATPS as a part of downstream commercial processes. This may be attributed to the fact that the knowledge of the mechanism of solute partitioning in ATPS is limited. Consequently, industries exhibit a reluctance to embrace this technique as a part of their own processes. As a result reports dealing with the commercial adoption of ATPS are not common. However, it is well known the classical report proving the success of the application of ATPS at large-scale (10,000 l fermentation) for the recovery of periplasmic IGF-I [1]. Such success, resulted in one of the few industrial cases known, in which the commercial adoption of ATPS for the recovery of a biological product has been achieved.

The disadvantages of batch operations (and the complications associated to the implementation of ATPS processes in a continuous mode of operation) and the lack of equipment needed for some processes, may also explain the absence of ATPS at commercial scale. Furthermore, an important limitation of this technique, that enhance the existing reluctance to adopt it for commercial purposes, is the absence of "commercial kits" (as in the case of conventional technologies), that facilitate the evaluation of ATPS processes at bench-scale. Consequently, 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. Additionally, the relative high need for chemicals to form working ATPS has saddled this technique with an unfavourable economic image [12,13]. This is relevant since the implementation of ATPS processes not only depends on technical potential and feasibility, but also depends strongly on process economics.

3. Practical strategies to ATPS process development for the recovery of biological products

Practical strategies to design ATPS extraction processes are needed to overcome the poor understanding of the molecular mechanism governing the behaviour of solute in ATPS. A practical approach favours the predictive design of extraction stages using this technique. Traditionally in ATPS process development, for each extraction process, operating conditions need to be empirically established. The application of practical strategies minimises the necessary time for the design of ATPS extraction stages. As initial step, general process conditions can be selected base upon experience in the partitioning of solute in ATPS. 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 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 this review, as a relevant contribution to the field, practical strategies (as applied to techniques of precipitation and ion exchange) involving a basic knowledge of the technique, are presented (see Fig. 2) to facilitate the development of extraction ATPS processes. It is anticipated that the proposed practical approach to process development will give basic rules for the initial establishment of preliminary ATPS process conditions. The general selection of the type of ATPS considers one of the different systems: (i) polymer–polymer, (ii) polymer–salt, and (iii) other ATPS. Selection of polymer–polymer (e.g. polyethylen-glycol–dextran) systems are defined by process economic considerations. In this case, the high cost of one of the polymers (i.e. dextran) conditioned the application of these types of systems to process in which the cost of the product of interest is considerable and compensate that from the chemical forming phase. The use of others type of ATPS is discussed in this review. Due to the low cost of the chemicals forming phases, the initial selection of a polymer–salt ATPS has been preferred. Consequently, it is suggested as a first step of the practical strategies to process development. PEG–phosphate ATPS represents the type of systems most widely exploited in the area. Such situation is associated to the knowledge accumulate with these systems and to their stability on the basis of phase formation [13,17]. Low molecular weight of PEG (e.g. 1000 or 1450 Da) is initially preferred to concentrate the majority of cells, debris and 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 [18] or it can be obtained from previous reports ([19] for example). 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.

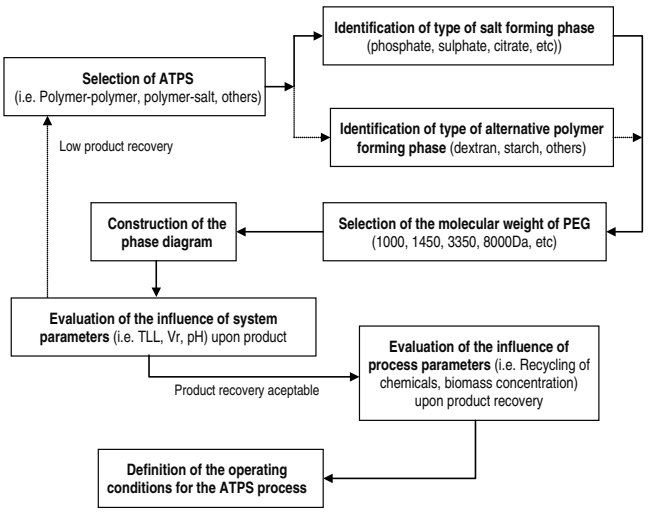

Fig. 2. Practical strategies to ATPS process development for the recovery of biological products. Strategies for the use of ATPS for the development of extraction processes presented here required knowledge of the basic concepts of the technique.

3.1. Influence of system parameters upon the product partition behaviour in ATPS

To evaluate the influence of system parameters upon partition behaviour, it is suggested to examine the behaviour of product recovery from the top phase when TLL is increased and Vr equals to 1 and pH equals to neutrality (or to the value that gives more stability to the target product) are kept constant. 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, both 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, the impact of changing system pH upon product recovery is evaluated. In this latest case, TLL and Vr should be kept constant at the selected values. Here again, the system pH that results in an increase in product recovery must be considered. The general practical strategies outline here has been exploited for the development of recent ATPS processes (as examples see references [7,11,16,20]). It is important to consider that, 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 needs to be examined. In contrast, once the conditions for an acceptable product recovery are obtained, it can be anticipated that a prototype ATPS process is preliminary defined. To further characterise the established ATPS process, the influence of process parameters (e.g. recycling of chemical forming

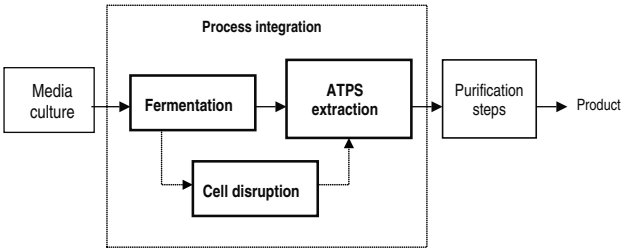

Fig. 3. Simplified representation of process integration of ATPS and fermentation for intra- and extra-cellular products. The flow diagram represents the extractive fermentation ATPS process in which, the production and the recovery of the target product can be integrated in one single unit operation. Alternative to it is represented in the integrated process of cell disruption and ATPS for the recovery of intracellular products.

phases, biomass concentration, stability of the phase formation, etc.) upon process performance needs to be addressed.

3.2. Influence of process parameters upon the product partition behaviour in ATPS

The specific characteristic of each extraction process strongly define the selection of the process parameters to be evaluated. Phase formation in polymer–polymer ATPS, has been attributed to the hydrated surfaces of each species, which are sufficiently incompatible to generate phase separation [21,22]. In contrast, phase separation in polymer–salt systems was initially associated with differing interactions with the ether dipoles of the polymer chain [23]. However, such explanation has not been widely adopted. Thus, the descriptive mechanisms in systems composed of polymer and salt remain unclear. In an attempt to generate practical knowledge regarding polymer–salt phase separation, interesting work of phase separation in these ATPS using model experimental systems, characterised by the sole presence of the target product, has been performed (for details see previous reviews [24,25]). The phenomena of polymer–salt phase separation in ATPS loaded with biological suspensions, has been addressed from a practical view point in recent publications [26–28]. These reports concluded that, certain process parameters such as the type of biological suspension, the definition of the continuous phase in the ATPS [26,27] and the geometry of the equipment influenced the rate of phase separation [28]. Processing of complex biological suspension demands the evaluation of the influence of such factors or parameters on the ATPS process performance to further facilitate the predictive design of extraction systems [29]. A relevant aspect of the recovery of recombinant proteins that exploit the use of urea to process the product, is the evaluation of the influence of such denaturant on the ATPS. In this latest respect, Ramsch et al. [30] have critically addressed the behaviour of the modified ATPS that involved the presence of up to 30% (w/w) concentration of urea in the systems. 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 waste water treatment may be reduced by recycling the phase components [12,18]. In this context, the author has demonstrated the feasibility of phase recycling for different ATPS processes [12,13]. It is evident that the development of robust ATPS extraction stages with potential implementation at commercial scale will require the evaluation of the effect of process parameters upon the performance of the designed ATPS processes.

4. Alternative ATPS for the development of processes to protein recovery

Alternative ATPS have been recently developed to improve some of the advantages of ATPS. A selection of some of the new types of ATPS reported and their applications is illustrated in Table 2. In an attempt to reduce the cost of certain polymers such as dextran, the use of crude starch modified as a substitute for the bottom phase of a defined ATPS has been suggested [10,14,20]. However, poor purification of the target product has been the result in some cases of the use of these modified systems [20]. In contrast, ATPS that exploited the use of a copolymer of ethylene oxide and propylene oxide (ucon) proved to be efficient for the development of recovery process [24,31,32]. Furthermore, the use of ucon favours the potential recycling of the chemicals forming phases by altering the process temperature. In the search for alternative low cost chemical forming phases for

Table 2
Selection of alternative ATPS exploited for the recovery of proteins

Type of ATPS	Practical application	Reference
PEG (or other polymer) and an alternative compound		
PEG–cashew nut tree gum	Characterisation of the system using purified BSA	[33]
PEG–dye ligand and dextran	Recovery of IgG and hybridoma in an extractive fermentation system	[35]
PEG–HPS	Processing of recombinant *E. coli* homogenate for the recovery of cutinase	[20]
PEG (or dextran)–IAA–Cu(II)	Recovery of membrane (cytochrome b03, ubiquinol oxidase) proteins from *E. coli*	[34]
Alternative ATPS		
Poly-VI/VCL-modified starch	Processing of wheat meal for the recovery of α-amylase	[10]
EOPO and Reppal	Recovery of recombinant apolipoprotein A1 from *E. coli*	[9]
Benzoyl dextran–ucon	Purification of 3-phosphate glycerate kinase from Bakers' yeast	[24,31,32]

HPS, hydroxylpropyl starch; VI/VCL, vinylcaprolactam; PEI, polyethylenimine; EOPO, ethylene oxide propylene oxide; IAA, iminoadiacetic acid.

ATPS, the use of novel or exotic compounds such as cashew nut tree gum has been reported [33]. The great disadvantage of this latest type of ATPS is associated with the need of a formal and complete characterisation of the ATPS (i.e. construction of the phase diagram, partition behaviour of commercial proteins in the systems, etc.). In addition, low process efficiency is a common characteristic of these ATPS as compared with that from commercial or traditional ATPS [33]. 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 behaviour.

Modification of the PEG rich phase to enhance selectivity of the ATPS towards the product of interest, has been made (see Table 2). These modifications include, among others, the use of metal ions (such as Cu(II); [10,34]) and the use of dye ligands [35]. In particular, the use of Cu(II) resulted in an alternative ATPS for the development of a process in which the protein (cytochrome b03) of interest was affinity partitioned into the modified PEG-rich phase. In the case of a PEG–dye ligand system, it was successfully exploited for the extractive fermentation for the recovery of IgG [35]. 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 selective partitioned to the modified PEG rich phase, the problem of efficiently separation of the polymer from the product still remained. Consequently, the complexity associated in the preparation of the modified systems, the cost of such process and the effect on the overall process recovery are relevant factors to be considered for the generic application of these alternative ATPS.

5. Application of ATPS to process integration

There is considerable interest by manufacturers of bioproducts to achieve process integration of the upstream operations of fermentation and downstream recovery processes to facilitate the development of scaleable and efficient bioprocesses. Such bioprocesses need to address the urgent need to rapidly yield products in a state suitable for the validation, polishing, formulation and delivery operations. The approach of process integration that attempts to achieve specific objectives not efficiently met by discrete processes by combining two operations into one is considered as one of the process strategies with potential benefits for the recovery of biological products. In this context, the application of ATPS to process integration represents an attractive alternative 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 (see Fig. 3). The application of ATPS for extractive bioconversion exploited for more than a decade has been discussed in a recent review [36]. This report concluded that although the extensive application of ATPS in this particular area, 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 behaviour. However, it also implied that the extended application of extractive bioconversion to high-value protein products, together with the development of low-cost alternative ATPS will give a new impetus to this technology in the near future.

In the context of process integration, extractive fermentation exploiting the use of ATPS represents an attractive technology to remove the product of interest from the fermentation broth as it is formed or produced (see Fig. 3). Application of ATPS to extractive fermentation 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 feasible to obtain the product in a cell-free stream. Recently, extractive fermentation using ATPS have been developed for the recovery of different protein products [37–41] that resulted in an increase in the productivity. Furthermore, the use of ATPS in extractive fermentation has been exploited to address process disadvantages of conventional process (characterised by discrete operation of fermentation and primary product recovery) such as product inhibition [17] and product hydrolysis [42]. In the first case, the successful removal of aroma compounds (i.e. 6-pentyl alpha pyrone) from the media culture of *Trichoderma harzianum* is reported whilst in the case of product degradation, water soluble antibiotics were efficiently extracted 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, it is clear that extractive fermentation processes are limited for the recovery of extra-cellular products. Therefore, the recovery of intracellular products, in which cell disruption is mandatory, needs a different strategy.

Process integration of cell disruption and primary recovery could enhance product yield and quality. Product sequestration at cell disruption could be achieved using fluidised bed adsorption (FBA). In this context, it has been reported the successful integration of cell disruption and FBA for the recovery of intracellular proteins from yeast [43]. In this line of research the application of ATPS to process integration for the recovery of intracellular proteins, represents an attractive alternative (see Fig. 3). A recent report [44] dealing with the integration of cell disruption and ATPS proposed an integration scheme for the recovery of the intracellular protein glyceraldehydes-3-phosphate-dehydrogenase (G3PDH) from yeast, that clearly proved the potential development of a prototype process, in which simultaneous disruption and aqueous two-phase extraction is achieved. Although, further studies to address the potential application of ATPS to process integration as a primary step for the recovery of intracellular proteins are essential, it is clear that process economics benefits are associated to the reduction of unit operations by the proposed approach of process integration.

Table 3
Current alternative applications of ATPS

ATPS	New application	Reference
PEG–dextran	Separation of substances from cells that inhibits the polymerase chain reaction (PCR)	[51]
	Extractive bioconversion	[36]
EOPO–dextran	Extractive fermentation for the recovery of lactic acid from *Lactococcus lactis*	[49,50]
PEG–phosphate	Recovery of viral coat proteins from recombinant *E. coli*	[16]
	Preparation of highly purified fractions of small inclusion bodies	[53]
	Recovery of aroma compounds under product inhibition conditions	[17]
PEG–sulphate	Drowning-out crystallisation of sodium sulphate	[45]
	Recovery of metal ions from aqueous solutions	[47]
	Recovery of food coloring dyes from textile plant wastes	[46]
	Partition of small organic molecules	[48]

EOPO, ethylene oxide propylene oxide.

6. Potential trend in the application of ATPS processes

Traditionally, the practical application of ATPS processes for the recovery of biological products has been focussed mainly on the primary purification of proteins. It is anticipated that a new potential trend of this technique is emerging and defined by the recent re-direction of the type of target products that can be recovered by the application of ATPS. Recently, the use of ATPS processes has been extended to non-protein products (see Table 3). In the context of large-scale application of ATPS, a novel procedure of drowning-out crystallisation of sodium sulphate using ATPS is reported to obtain crystal of pure anhydrous salt [45]. In this case, the phases are recycled allowing the design of a continuous process. Furthermore, the early success using simplified systems obtained in the recovery of metal ion, colouring dyes and small organic molecules using ATPS processes [46–48], represents an interesting case to address some of the environmental concern of industries.

The potential trend in the application of ATPS processes for the recovery of biological products will extend to the food and cosmetic industry. The application of ATPS in these industries will emphasis the development of protocols to recover compounds of commercial significance. In this context, the in situ recovery of aroma compounds with ATPS has been addressed [17]. 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 involve the use of biotechnological technologies. The problem of product inhibition exhibited in the production of certain aroma compounds (such as 6-penthyl alpha pyrone produced by *T. harzianum* [17]) can be addressed by the continuous removal of the product of interest from the fermentation broth using and extractive phase with ATPS. The bio-compatibility of ATPS facilitate the integration with fermentation step to address a problem not specific to the production of aroma compounds (see lactic acid production in references [49,50]).

A practical novel application of ATPS has also been the use of the technique to improve certain analytical techniques (e.g. polymerase chain reaction (PCR); [51]). In this case, the removal of inhibitory substance from cells was possible using a PEG–dextran system. It is evident that, the increase in the number of such type of applications, 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 the commercial interest in the technique. The potential use of ATPS for medical applications [16,52,53] that is currently developing as a new novel application and the emerging trend of combine application of ATPS with conventional and non-conventional techniques [54,55] will definitively draw attention from bioprocess industries. The practical application of ATPS processes for the recovery of high-value products will also give new impetus to the technology. In this context, the particular case of the recovery and purification of a protein product of commercial value of US$ 15 mg^{-1} (c-phycocyanin produced by *Spirulina maxima*) using ATPS reported by the author [56], represent an important example of this type of application. This report presented the successful development of a greatly simplified process for the purification of c-phycocyanin. The use of process intensification approach involving two-stage ATPS, ultra-filtration, precipitation and process integration strategy resulted in the development of the bioprocess to obtain highly purified c-phycocyanin in only four unit operations. Specifically, the new process integrated cell disruption and the primary recovery with ATPS in one single unit operation and eliminates the need for chromatography steps. Herein again, it is clear that process economics benefits are associated with the significant reduction of unit operations, that will necessarily facilitate the rapid scale up and commercialisation of the prototype process.

The successful recovery of small inclusion bodies and viral coat protein from complex homogenates highlights a generic role that ATPS techniques will play in the recovery and purification of new bio-particulate products (viral and plasmid gene therapy vector, particulate protein vaccines, etc.; [16,52,53]). Furthermore, it is anticipated that the development of biotechnological processes exploiting ATPS using a process integration approach to facilitate the interaction between fermentation and bioseparation steps [57] will

be a dominant trend in the field. It is clear that, the proposed trend in the application of ATPS will address the urgent need to rapidly and economically bring new biopharmaceutical products to market using scaleable and efficient bioprocess technology.

7. Conclusion

It is evident that the extensive laboratory application of ATPS processes for the recovery of protein products has been characterised by the lack of commercial processes exploiting the technique. The existence reluctance from industries to exploit ATPS is justified by the necessary time involved in the learning process of the technique, the poor understanding of the mechanism governing partition of solutes in the systems and the cost of phase forming polymers. The development of alternative ATPS to improve the selectivity of the processes has not resulted in a wide adoption of such systems. The reasons of the reduced application of these systems include the complexity of the construction of the new forming phase polymers and the low process recovery reported. However, it is anticipated that, the use of low-cost ATPS, the considerable potential to achieve process integration and the increasing application of ATPS for the recovery of high-value products is opening new areas of opportunity. It is clear that the urgent need from manufactures to bring to market new high-value bio-particulate products with medical applications will draw attention for commercial applications. Consequently, ATPS processes as a necessary step for the primary recovery of biological products will be an important option for the new bioprocesses of the pharmaceutical industries.

Acknowledgements

The author wishes to acknowledge the financial support from the ITESM research chair (CAT 005).

References

[1] R.A. Hart, P.M. Lester, D.H. Reifsnyder, J.R. Ogez, S.E. Builder, Biotechnology 12 (1994) 1113.
[2] J.G.L.F. Alves, L.D.A. Chumpitaz, L.H.M. da Silva, T.T. Franco, J. Chromatogr. B 743 (2000) 235.
[3] B.A. Andrews, S. Nielsen, J.A. Asenjo, Bioseparation 6 (1996) 303.
[4] M.A. Bim, T.T. Franco, J. Chromatogr. B 743 (2000) 349.
[5] D.P. Harris, A.T. Andrews, G. Wright, D.L. Pyle, J.A. Asenjo, Bioseparation 7 (1997) 31.
[6] G. Johansson, K. Reczey, J. Chromatogr. B 711 (1998) 161.
[7] J.C. Marcos, L.P. Fonseca, M.T. Ramalho, J.M.S. Cabral, J. Chromatogr. B 711 (1998) 295.
[8] N.M. Minami, B.V. Kilikian, J. Chromatogr. B 711 (1998) 309.
[9] J. Persson, L. Nystrom, H. Ageland, F. Tjerneld, J. Chromatogr. B 711 (1998) 97.
[10] N. Pietruszka, I.Y. Galaev, A. Kumar, Z.K. Brzozowski, B. Mattiasson, Biotechnol. Prog. 16 (2000) 408.
[11] M. Rito-Palomares, M. Hernandez, J. Chromatogr. B 711 (1998) 81.
[12] M. Rito-Palomares, A. Lyddiatt, J. Chem. Technol. Biotechnol. 75 (2000) 632.
[13] M. Rito-Palomares, C. Dale, A. Lyddiatt, Process Biochem. 35 (2000) 665.
[14] A. Venancio, C. Almeida, J.A. Teixeira, J. Chromatogr. B 680 (1996) 131.
[15] S.G. Walker, C.J. Dale, A. Lyddiatt, J. Chromatogr. B 680 (1996) 91.
[16] M. Rito-Palomares, A. Middelberg, J. Chem. Technol. Biotechnol. 77 (2002) 1025.
[17] M. Rito-Palomares, A. Negrete, E. Galindo, L. Serrano-Carreon, J. Chromatogr. B 743 (2000) 403.
[18] H. Hustedt, K.-H. Kroner, M.-R. Kula, Applications of phase partition in biotechnology, in: H. Walter, D.E. Brooks, D. Fisher (Eds.), Partitioning in Aqueous Two-Phase Systems; Theory, Methods, Uses and Application in Biotechnology, Academic Press, Orlando, FL, USA, 1985, p. 529.
[19] M. Rito-Palomares, A. Lyddiatt, J. Chromatogr. B 680 (1996) 81.
[20] M.C. Almeida, A. Venancio, J.A. Teixeira, M.R. Aires-Barros, J. Chromatogr. B 711 (1998) 151.
[21] N.L. Abbott, D. Blankschtein, T.A. Hatton, Bioseparation 1 (1990) 191.
[22] P.-A. Albertsson, Partition of Cell Particles and Macromolecules, first ed., Wiley, New York, USA, 1986.
[23] J.G. Huddleston, A. Veide, K. Kohler, J. Flanagan, S.-O. Enfors, A. Lyddiatt, Tibtech 9 (1991) 381.
[24] H. Cabezas, J. Chromatogr. B 680 (1996) 3.
[25] H.-O. Johansson, G. Karlstrom, F. Tjerneld, C.A. Haynes, J. Chromatogr. B 711 (1998) 3.
[26] J.C. Merchuk, B.A. Andrews, J.A. Asenjo, J. Chromatogr. B 711 (1998) 285.
[27] M.H. Salamanca, J.C. Merchuk, B.A. Andrews, J.A. Asenjo, J. Chromatogr. B 711 (1998) 319.
[28] C. Solano-Castillo, M. Rito-Palomares, J. Chromatogr. B 743 (2000) 195.
[29] M. Rito-Palomares, L. Cueto, J. Chromatogr. B 743 (2000) 5.
[30] C. Ramsch, L.B. Kleinelanghorst, E.A. Knieps, J. Thommes, M.-R. Kula, Biotechnol. Prog. 15 (1999) 493.
[31] M. Lu, P.-A. Albertsson, G. Johansson, F. Tjerneld, J. Chromatogr. B 680 (1996) 65.
[32] J. Persson, L. Nystrom, H. Ageland, F. Tjerneld, J. Chem. Technol. Biotechnol. 74 (1999) 238.
[33] L.A. Sarubbo, L.A. Oliveira, A.L.F. Porto, H.S. Duarte, A.M.A. Carneiro-Leao, J.L. Lima-Filho, G.M. Campos-Takaki, E.B. Tambougi, J. Chromatogr. B 743 (2000) 79.
[34] U. Sivars, J. Abramson, S. Iwata, F. Tjerneld, J. Chromatogr. B 743 (2000) 307.
[35] G.M. Zijlstra, M.J.F. Michielsen, C.D. de Gooijer, L.A. van der Pol, J. Tramper, Bioseparation 7 (1998) 117.
[36] G.M. Zijlstra, C.D. de Gooijer, J. Tramper, Curr. Opin. Biotechnol. 9 (1998) 171.
[37] Y.X. Guan, Z.Q. Zhu, L.H. Mei, Separ. Sci. Technol. 31 (1996) 2589.
[38] N. Kulkarni, A. Vaidya, M. Rao, Biochem. Biophys. Res. Commun. 255 (1999) 274.
[39] C. Li, O.Y. Fan, J.H. Bai, Biotechnol. Lett. 22 (2000) 843.
[40] J. Sinha, P.K. Dey, T. Panda, Biochem. Eng. J. 6 (2000) 163.
[41] J. Sinha, K.P. Dey, T. Panda, Appl. Microbiol. Biotechnol. 54 (2000) 476.
[42] O. Hernandez-Justiz, R. Fernandez-Lafuente, M. Terreni, J.M. Guisan, Biotechnol. Bioeng. 59 (1998) 73.
[43] H. Bierau, Z. Zhang, A. Lyddiatt, J. Chem. Technol. Biotechnol. 74 (1999) 208.
[44] M. Rito-Palomares, A. Lyddiatt, Chem. Eng. J. 87 (2002) 313.

[45] M.E. Taboada, T.A. Graber, J.A. Asenjo, B.A. Andrews, J. Chromatogr. B 743 (2000) 101.
[46] J.G. Huddleston, H.D. Willauer, K.R. Boaz, R.D. Rogers, J. Chromatogr. B 711 (1998) 237.
[47] R.D. Rogers, A.H. Bond, C.B. Bauer, J. Zhang, S.T. Griffin, J. Chromatogr. B 680 (1996) 221.
[48] R.D. Rogers, H.D. Willauer, S.T. Griffin, J.G. Huddleston, J. Chromatogr. B 711 (1998) 255.
[49] J. Planas, A. Kozlowski, J.M. Harris, F. Tjerneld, B. Hahn-Hagerdal, Biotechnol. Bioeng. 66 (1999) 211.
[50] J. Planas, V. Varelas, F. Tjerneld, B. Hahn-Hagerdal, J. Chromatogr. B 711 (1998) 265.
[51] P.-G. Lantz, F. Tjerneld, B. Harn-Hagerdal, P. Radstrom, J. Chromatogr. B 680 (1996) 165.
[52] G.M.F. Braas, S.G. Walker, A. Lyddiatt, J. Chromatogr. B 743 (2000) 409.
[53] S.G. Walker, A. Lyddiatt, J. Chromatogr. B 711 (1998) 185.
[54] Y. Li, R. Beitle, Biotechnol. Prog. 18 (2002) 1054.
[55] C. Rangel-Yagui, H. Lam, D.T. Kamei, D.I.C. Wang, A. Pessoa, D. Blankschtein, Biotechnol. Bioeng. 84 (2003) 445.
[56] M. Rito-Palomares, L. Nuñez, D. Amador, J. Chem. Technol. Biotechnol. 76 (2001) 1273.
[57] J. Thommes, M. Halfar, H. Gieren, S. Curvers, R. Takors, R. Brunschier, M.-R. Kula, Biotechnol. Prog. 17 (2001) 503.