Novel Bioengineering Strategies for the Development of Processes for the Recovery of Proteins

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

Original scientific paper
Received: June 16, 2003
Accepted: February 1, 2004

The increasing demand for new pharmaceutical products has encouraged manufacturers to seek new bioengineering strategies for the development of competitive biotechnological processes for the recovery and purification of proteins. The new trend in the development of recovery systems, exploits novel approaches of bioprocess integration and intensification. In this paper, some of the achievements of the practical implementation of such bioengineering strategies for the development of biotechnological processes using defined experimental vehicles, are addressed. An arbitrary selection of bioprocesses using aqueous two-phase systems (ATPS) and expanded bed adsorption (EBA) to implement these novel approaches is reported with the aim of establishing the benefits of such strategies. The successful cases of intracellular protein recovery from baker's yeast, the development of a highly simplified process to c-phycocyanin recovery from *Spirulina maxima* and the radical approach for recovering inclusion body proteins are discussed. It is proposed that the trend of the practical application of the novel bioengineering strategies for the recovery of protein products will address the current process requirements. Such trend will give impetus to the development of bioseparation systems, and will draw attention from industries needing to develop new, and improve existing, commercial bioprocesses.

Keywords:
Bioengineering strategies, protein recovery, process integration, bioprocess intensification

Introduction

The increasing need to rapidly and economically bring new biopharmaceutical products to market using scalable and efficient technologies, has encouraged manufacturers to seek new strategies for the development of competitive biotechnological processes for the recovery and purification of proteins. It is clear that the commercial success of new biotechnological processes strongly depend on the adequate definition of the primary recovery an purification steps. Furthermore, novel approaches that reduce the necessary time of process scale up and transfer technology in pharmaceutical industry will facilitate the generic industrial implementation of new biotechnological processes. In this context, the new trend in the bioengineering strategies for the development of recovery systems, exploits novel approaches of bioprocess integration and intensification.[1–4] Currently, bioprocess integration has received attention from the practical and commercial point of view. Bioprocess integration, wherein two unit operations are combined into one in order to achieve specific goals not effectively met by discrete processes, offers considerable potential benefits for the recovery of proteins.[1,2] In the same line of research, bioprocess intensification involves the development of recovery processes (avoiding excessive number of unit operations) oriented to increase the flow of the required biological suspensions to obtain commercial products.[2–4]

From the technologies available for the recovery of proteins, aqueous two-phase systems (ATPS) and expanded bed adsorption (EBA) offer process characteristics to be considered as interesting candidate for the implementation of process integration and bioprocess intensification strategies.[1–4] It is evident that the relationship between integration and intensification of bioprocesses results in the use of both strategies simultaneously as the predominant trend. However, there is currently little evidence of successfully implementation of these bioengineering strategies for the development of biotechnological processes for the recovery of proteins.[5]

The current paper focuses on the achievements obtained from the practical implementation of the integration and intensification bioengineering strat-

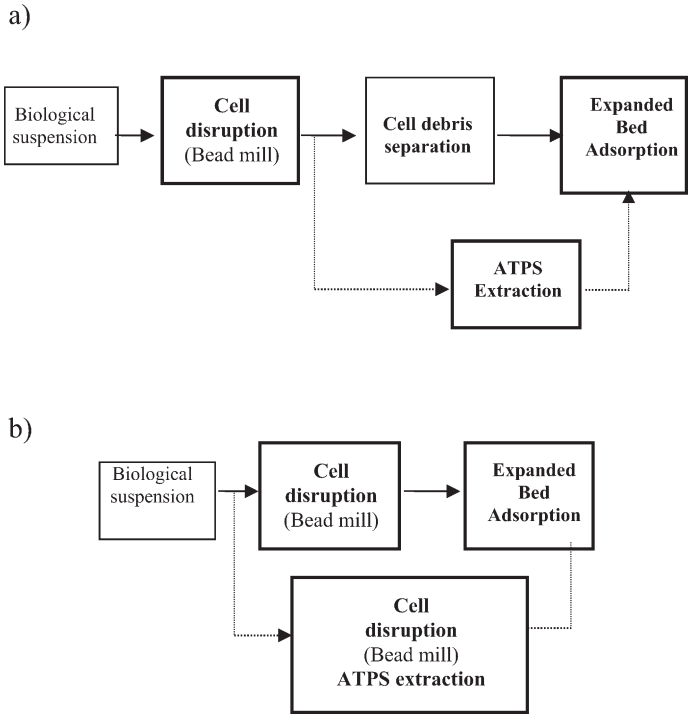
*Address for correspondence: Marco Rito-Palomares, Departamento de Tecnología de Alimentos, Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM), Ave. Eugenio Garza Sada 2501-Sur, Monterrey, NL 64849, México.
Tel: 00 52 81-8358-2000 (4821); Fax: 00 52 81-8328-4322;
Email: mrito@itesm.mx

egies for the development of biotechnological processes using defined experimental systems. An arbitrary selection of bioprocesses using ATPS and EBA to implement these novel approaches is reported to establish their benefits. The experimental vehicles used involve (i) the recovery of intracellular proteins from baker's yeast, (ii) the development of a highly simplified process to c-phycocyanin recovery from *Spirulina maxima*, and (iii) a radical approach for recovering inclusion body proteins. Furthermore, the potential benefits of the biotechnological processes developed for the simplified scale-up and commercialization are discussed.

Process integration strategies for the recovery of intracellular proteins

Processes for the recovery of intracellular proteins generally involve the release of the product by mechanical or chemical disruption, followed by removal of cell fragments and some contaminants by cross-flow membrane filtration or high speed centrifugation. However, at large scale the achievement of quantitative elimination of cell fragments with filtration and/or centrifugation may be difficult. In addition, negative impact on the recovery process and stability of the target product may be caused by the complex nature of the products and contaminants present inside de cell. The use of ATPS extraction and EBA can alleviate such difficulties by following a conventional route for the recovery of intracellular proteins exploiting these techniques (see Figure 1a). Furthermore, process integration of cell disruption and primary recovery unit operations may enhance both the yield and quality of intracellular protein products.[1,2]

Direct product capture has been achieved by integrating cell disruption with fluidised bed adsorption[1–2] for the recovery of intracellular glyceraldehydes 3-phosphate dehydrogenase (G3PDH) and other proteins from bakers' yeast. However, the use of high concentration of biomass from the cell disruption device (> 20 % wet w/v) strongly increased the expansion of the bed for a given flow rate evidenced process difficulties. The use of ATPS represents an attractive alternative to achieve process integration for the recovery of products in three major areas; (i) extractive bioconversion,[6] (ii) extractive fermentation[7] and (iii) integration of cell disruption and primary purification step. This latest novel alternative, involves the cell disruption in ATPS to achieve the goals of process integration for the recovery of intracellular proteins (see Figure 1b). In this context, the generic process applicability of such strategy has been reported before,[2] using the recovery of G3PDH from baker's yeast as a representative model system to compare the performance of conventional discrete processes and a fully integrated operation. The scheme of process integration proposed in this latest study proved that simultaneous disruption and ATPS extraction produced a process for the primary recovery of intracellular proteins from yeast. In particular, operating conditions were reported that facilitate the recovery of the intracellular enzyme directly and rapidly from disrupted yeast in a single operation. It is clear that process economics benefits are associated with the reduction of unit operations (see systems I and II in Table 1). Although, further studies to address the use of ATPS for process integration are essential, the potential of the integration of cell disruption with ATPS for the direct recovery of specific intracellular protein targets has been demonstrated.



F i g . 1 – *Recovery of intracellular proteins using a conventional route (a) and process integration strategy (b). The (a) diagram represents the conventional route in which the removal of cell debris is needed prior to the use of expanded bed adsorption or the product release and primary ATPS recovery in a discrete units operations. The (b) diagram represents the direct integration of cell disruption and expanded bed adsorption or the simultaneous cell disruption and ATPS recovery in one single unit operation.*

Bioprocess intensification: a novel process for the recovery of high-value products

Currently, manufacturers are seeking competitive advantages through bioprocess intensification to develop scalable and efficient biotechnological

Table 1 – *Comparison of the number of unit operations involved in a conventional and integrated processes for the recovery of biological products*

System	Process goal	Number of unit operations			Reference
		Conventional route	Integrated process with EBA	Integrated process with ATPS extraction	
I	Recovery of intracellular protein from yeast	3	2	1	[1, 2]
II	Recovery of G3PDH from yeast	3	2	1	[1, 2]
III	Recovery of c-phycocyanyn from *Spirulina maxima*	9	–	4	[8, 9]
IV	Processing of inclusion body proteins	8–10	3	3	[3, 4]

The number of unit operations involve primary recovery and purification steps. Reports exploiting the use of expanded bed adsorption (EBA) for the recovery of c-phycocyanin from *Spirulina maxima* are not known to the author. In the integrated processes the use of aqueous two-phase system (ATPS) extraction substitute the use of EBA.

processes to bring rapidly and economically new pharmaceutical products to market. Such situation offers an attractive alternative to exploit certain well know fermentation processes to generate high-value products of considerable economical interest. In this context, the production of c-phycocyanin (a blue-colored protein) by *Spirulina maxima* represents a very interesting case because both the industrial and commercial value of this product are considerable. The commercial value of food grade c-phycocyanin (purity of 0.7, defined as the relation of 620 nm to 280 nm absorbance) is around \$0.13 USD mg^{-1}, whilst that of reactive grade c-phycocyanin (purity of 3.9) varies from \$1 to 5 USD mg^{-1}. In contrast, the commercial value of analytical grade c-phycocyanin (purity greater than 4.0) can be as high as \$15 USD mg^{-1}.[8]

The recovery of c-phycocyanin from *Spirulina maxima* has been attempted previously.[8] However, the resulting protocols have not been enable to reach the maximum purity of the product (greater than 4.0) and have been characterized by an excessive number of unit operations (i.e. ten unit operations, see Figure 2a). Consequently, affecting product yield. Furthermore, the scale up of these procedures raises complications associated to the stage

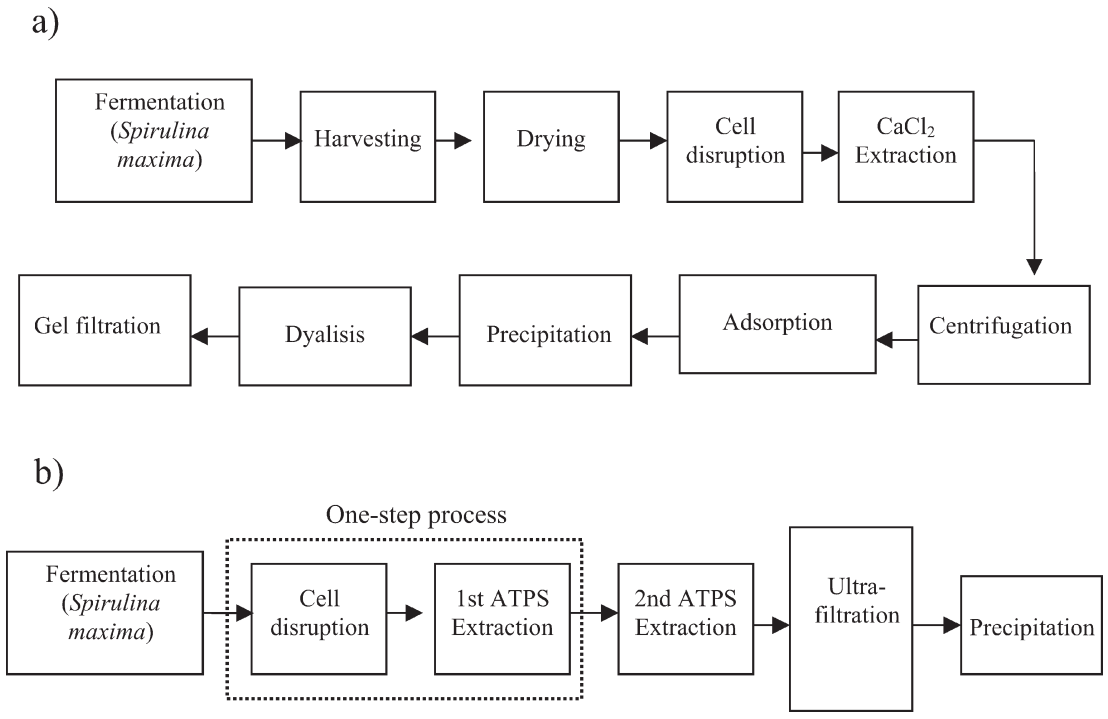


Fig. 2 – *Direct comparison of the existing protocol for c-phycocyanin recovery produced by Spirulina maxima (a) with the new proposed process (b). In the new proposed process, the product release and primary ATPS extraction can be achieved in situ in the mechanical cell disruption device (bead mill).*

used, for example the use of hand-milling and the use of chromatography. To overcome some of these disadvantages attributed to established c-phycocyanin purification protocols, the use of aqueous two-phase systems (ATPS) has been suggested as an attractive alternative for the recovery of c-phycocyanin produced by *Spirulina maxima*. The use of ATPS for the recovery of protein products from fermentation broth has been addressed before.[7]

Recently, a report on the purification of c-phycocyanin from *Spirulina maxima* cultures using ATPS, was published.[9] This report presented the successful development of a greatly simplified process for the purification of c-phycocyanin (see Figure 2b). The use of process intensification approach involving two-stage ATPS, ultrafiltration, precipitation and process integration strategy resulted in the development of the bioprocess to obtain highly purified c-phycocyanin (greater than 4.0) in only four unit operations. Specifically, the new process integrated cell disruption and the primary recovery with ATPS in one single unit operation (Cisneros M and Rito-Palomares M, 2003. *Journal of Microbiology and Biotechnology, Submitted*) 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 (see system III in Table 1). Although, additional successful cases of the use of ATPS for process integration are needed to raise the attention towards this bioengineering strategy, the way in which the developed process greatly simplifies the recovery and purification of the protein product is evident (see Figure 2). The reduced process steps together with the nature of the unit operations of the resulting prototype biotechnological process together with the necessary control, and monitoring aspects of the process will necessarily facilitate its rapid scale up and commercialization.

New strategies to develop processes for recovering inclusion bodies proteins

Typical process route for recovering inclusion body proteins involves release and collection of inclusion bodies by mechanical cell disruption (i.e. high-pressure homogenisation) and by centrifugation, respectively. Inclusion bodies are then solubilized in a strong denaturant prior to refolding through removal of denaturant. The entire process is complicated by the need for multiple cell disruption passes to reduce cell debris size[10] and by the need to wash this debris from the inclusion bodies by repeated centrifugation,[11] possibly with the use of detergents and other chemical agents (see Figure 3a).

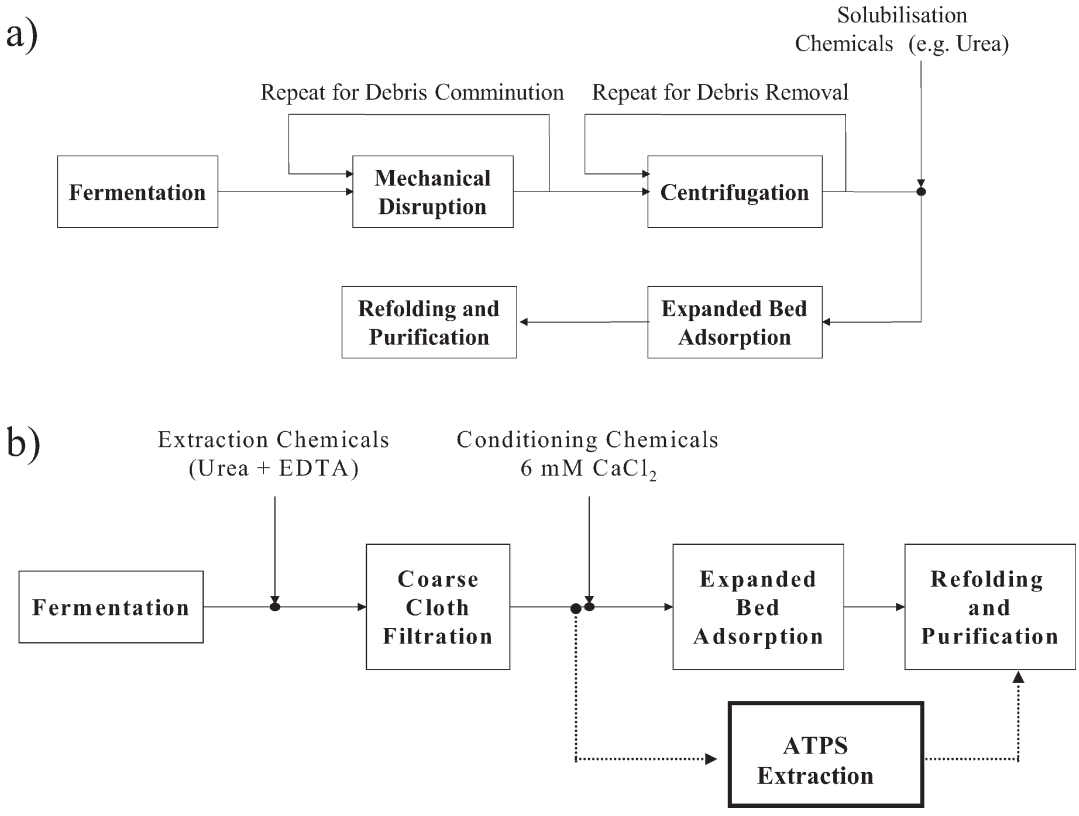


Fig. 3 – *Production of denatured protein competent for refolding, exploiting the traditional process route (a) and the potential new process involving novel strategies (b)*

The complexity of separating solids of similar particle diameter together with the multi-step nature of this conventional method, can result in low process yield and high process cost. Furthermore, the use of large amount of stainless steel tanks is also viewed as a significant process disadvantage. Consequently, despite the fact that the formation of inclusion bodies is usually associated with the high expression yield and with protection of the protein from *in vivo* proteolysis, inclusion bodies are perceived as an undesirable outcome of expression.

In attempts to overcome some of the disadvantages attributed to established methods for processing inclusion bodies, different approaches have been proposed.[12–14] However, these strategies have been disadvantaged by problems associated to efficiency at large scale and complications of subsequent processing.[3] A different bioengineering strategy involving the *in situ* dissolution of periplasmic inclusion bodies with subsequent recovery of the soluble protein by ATPS, has been reported.[15,16] Furthermore, this strategy has been extended to achieve *in situ* dissolution of cytoplasmic inclusion bodies using chaotrope (urea) and EDTA.[17–19] This new bioengineering strategy that exploit the use of a chemical extraction method overcome many of the limitations associated with conventional inclusion bodies processing methods. In addition, it has been reported that the extraction of inclusion bodies with this mechanical method was equivalent to that from mechanical disruption.[18] Furthermore, potential coupling of chemical extraction with expanded bed adsorption and aqueous two-phase extraction (as primary capture methods; see Figure 3b), has been demonstrated for the recovery of the major capsid protein (L1) of human papillomavirus (HPV) type 16, expressed as inclusion body in *E. Coli*.[3,4]

A direct comparison of the new proposed strategy with the existing process involving repeated homogenisation and centrifugation (see Figure 3 and system IV in Table 1), highlights the superiority of the current approach. This novel process greatly simplifies the traditional way in which proteins expressed as inclusion bodies can be recovered, with significant scope for generic commercial application. It is clear that, for certain products, this bioengineering strategy opens the way to further bioprocess intensification. Particularly, for new interesting proteins whose potential production using conventional processes is not economically feasible.

It is anticipated that the use of conventional strategies for the development of biotechnological processes for the recovery of interesting recombinant proteins, specifically those that are currently being considered as potential vaccine candidates (e.g. structural proteins, virus-like particles), will result in multi-unit operations processes. Such processes will be characterized by the use of expensive chromatography steps that will eventually raise concerns regarding the economic feasibility of the processes at commercial scale. It is clear that the opportunities offer by the novel bioengineering strategies outlined in this paper will simplify the resulting prototype processes for the recovery of new emerging protein products. It is expected that, the new biotechnological processes produced using the proposed novel strategies of integration and intensification will involve the use of a reduced number of unit operations, which will provide benefits concerning the investment and operation cost.

Conclusion

The above novel bioengineering strategies outline the possibilities for biotechnological process development to better service the scale up and commercial requirement established by the manufacturers to bring to market new biopharmaceutical protein products. However, it is not immediately clear how fast such possibilities might be achieved. It is apparent that little attention has been paid in the development of efficient and scalable bioseparations processes for protein recovery. There is little evidence that traditional bioengineering approaches for protein recovery are achieving optimal results for the new range of biopharmaceutical products. It is expected that the trend in the development of recovery systems will involve the use of the novel strategies of bioprocess integration and intensification for the purification of new high-value products or the optimisation of conventional biotechnological processes. It is anticipated that the generic implementation of such strategies to bioseparation developments will identify process constraints and failures that will need to be addressed. However, it is clear that the potential opportunities offer by the novel bioengineering strategies, outlined in this paper, will draw attention from industries for commercial applications.

ACKNOWLEDGEMENTS

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

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