Chapter 15

Green Precipitation with Polysaccharide as a Tool for Enzyme Recovery

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INTRODUCTION

The two major types of macromolecules in biological systems are proteins and polysaccharides, which are responsible for cell structure, energy storage/production or enzymatic reactions. Moreover, these two macromolecules exert essential structural functions in the food systems, acting as stabilizers for emulsions and foams, and being the most important gelling and thickening agents (Tolstoguzov, 1991). However, such structural functions are hugely affected by the interactions between these important macromolecules and other components in food systems. The interactions between protein-polysaccharide was initially described by Bungenberg de Jong and co-workers in 1949, where they studied the complex formed by gelatine-acacia gum coacervation and published through the years several updates on this complex, drawing attention to protein/polysaccharide complexes and coacervates and the possibility of their application to foods (Khamanga, 2017). It was only after 1981 that studies were published describing the complex formation between proteins and polysaccharides, as well as, their interactions. Larionova, et al. in

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1981 described the features of the formation of soluble polyelectrolytes complexes between carboxymethyl ethers of cellulose and dextran with basic proteins.

Until the year 2000 more than 40 scientific studies were published describing the interaction between different proteins and different polysaccharides, showing the increasing interest in water-soluble polymeric complexes. After this era, the scientific community-maintained an interest in the interaction between these macromolecules, but the field of study was reoriented towards the development of technologies to be applied in the food industry. One of the most cited studies reported by Ye in 2008 described the importance of the application of proteins and polysaccharide in the food industry and the key role that these complexes have in the structure and stability of processed food. In addition, the authors analyzed the development of novel food processes and products by giving a brief overview of recent research work on the complexation between milk proteins and polysaccharides and the application of the complexes in the dairy industry. On the other hand, Le Bourvellec and Renard in 2012 went one step further and studied the interactions between polyphenols and both proteins and polysaccharides, by giving a review of the methods used to study the interactions, as well as, the extension of each kind of interactions.

In recent years, the primary interest has been focused on the downstream process through the application of polysaccharides for complex formation and their application for protein isolation and purification. Thus, studies in scaling up have grown significantly by the demand for large amounts of enzymes to be used in biotechnological processes.

The enzyme industry has been growing in the past few years because of the increasing interests in the application of most environmentally friendly techniques. Enzymes are suitable for applying in several industries, food, pharmaceuticals, animal feed, and others with less impact on the market. In enzyme production, the scaling process represents the major problem since the extraction of enzymes comes from a large amount of biomass from the fermentation of homogenates and microbial suspension. On the other hand, other classes of enzymes can be extracted from natural sources, such as fruits and vegetables, since these sources naturally produce enzymes as natural secondary metabolites. Bioseparation steps for the recovery of the final product can account for up to 70% of overall production costs. Thus, many of the traditional processes are no longer applied to such industries, since they present several negative aspects, such as high costs, negative environmental impact, the presence of hard chemicals and a short life in the final products.

The recent trend of replacing linear economy by circular economy has opened the opportunity to explore the valuable compounds present in industrial waste. An important source of such waste is the agro-food industry that produces and processes tons of food daily, generating a high percentage of waste rich in bioactive compounds. By transforming this waste into a by-product prone to be valued new economic income can be generated, for example, functional ingredients can be obtained for various applications.

For establishing competitive biotechnological processes for enzyme extraction and purification, several research groups have devoted scientific research on the improvement of more “natural” techniques, such as bioseparation based on

polysaccharides and natural polyelectrolytes, using their capacity to produce non-soluble complexes with different protein groups, the enzymes being the most important. Nowadays, biotechnology has been improving the application of bioseparation in scaling up for application in large amounts for industrial application.

This chapter reviews the enzyme's isolation and purification through precipitation using polysaccharides and natural polyelectrolytes for production of non-soluble complexes. Additionally, future trends in this field and the applications of such techniques to industries for bioseparation of industrial enzymes will also be covered.

POLYSACCHARIDES

Polysaccharides are carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages, which by hydrolysis release the constituent monosaccharides, such as glucose. Since polysaccharides are one of the significant macromolecules, they are present in all structures having different functions in nature. Furthermore, in addition to its natural functions and nutritional properties, polysaccharides have different physicochemical properties that make them very interesting from a technological standpoint.

Many polysaccharides are used to store energy in organisms, typically fold together and can contain many monosaccharides in a dense area. Moreover, as the side chains of the monosaccharides establish as many hydrogen bonds as possible between themselves, water cannot intrude the molecules, making them hydrophobic. They not only store the energy but allow for a change in the concentration gradient, which can influence the holding capacity of nutrients and water. All polysaccharide preparations are polydisperse, containing molecules with different degrees of polymerization, hence they can be organized by structure, by shape (linear or branched), by monomeric units (homoglycans, di-heteroglycans, tri-heteroglycans, tetra-heteroglycans, and penta-heteroglycans) and by charge (neutral, anionic and cationic). The charge has a significant effect on the ionic polysaccharides since these macromolecules adopt a fully extended shape (due to Coulombic repulsion) and thus impart high viscosity to their solutions. The viscosity and other properties of such molecules have a great interest for further applications in different areas.

Anionic polysaccharides are polymers of sugar acid (e.g., alginate); sugar units biosynthesized with anionic substituents such as sulfate groups (e.g., carra-geenan); or carboxyl groups that are substituted through a chemical reaction (e.g., carboxymethylcellulose). The presence of these anionic groups increases the polarity and water solubility, though the intrinsic charge weakens intermolecular associations between polymer chains due to repulsion (Nieto, 2016).

Alginates are linear, unbranched polymers, containing β-(1-4)-linked L-guluronic acid (G) units, and are therefore highly anionic and very polar polymers. These polymers are present in the cell wall of brown algae as calcium, magnesium and sodium salts of alginic acid.

Carrageenan is a collective term for sulfated polysaccharides extracted from certain species of red seaweed of the family, Rhodophyceae. Major commercial sources are *Eucheuma spinosum, Eucheuma cottonii, Gigartina* spp., *Chondrus*

crispus, and *Hypnea* spp. In terms of grades, four commercial types of carrageenan extracts are available: kappa I, kappa II, iota and lambda types. Therefore, different seaweeds produce different carrageenans fractions, with one type being more predominant in any species. Carrageenans are made up of alternating galactopyranosyl dimer units linked by alternating β-(1-4) and α-(1-3) glycosidic bonds. The sugar units are sulfated either at C2, C3 or C6 of the galactose or C2 of the anhydrogalactose unit.

Carboxymethylcellulose or CMC is cellulose ether produced by reacting the alkali cellulose with sodium monochloroacetate, under controlled conditions. There are many degrees of substitution producing different properties of CMC. Hence, many grades of CMC are in the market to perform different functions. The structure of CMC involves carboxymethyl substitution of the native cellulose polymer at the C2, C3, or C6 positions of anhydroglucose units.

These described polysaccharides are very interesting for the industry given the different applications for which they have been studied. Likewise, due to the greater understanding in this field, other anionic polymers can be considered for technological applications. Until now some polysaccharides such as xanthan gum and pectin have been reported. Xanthan gum is a polysaccharide produced industrially by a microbial fermentation of bacterium *Xanthomonas campestris* involving a carbohydrate substrate and other growth-supporting nutrients. The molecular structure consists of a linear glucose chain linked by β-(1-4) glycosidic bonds like cellulose and possesses a trisaccharide side chain attached through O3 of alternate glucose units in the main chain (Fan, 2007).

Pectin is a soluble heterogeneous polysaccharide containing linear chains (Sonia et al., 2014). It is mainly produced commercially from citrus peels and apple pomace. It has been agreed that pectin is not a regular chain but has a backbone made up of α(1-4)-linked D-galacturonic acid residues, and a more significant proportion of these galacturonic acid residues is methyl esterified naturally (Pérez et al., 2014).

On the other hand, there are only a few examples of cationic polysaccharides, such as chitosan, where the amino group confers a positive charge when the polysaccharide is dissolved in an acidic solution. Several other grades of powders such as low methoxy, amidated pectin and cationically modified guar that contain $-NH_2$ groups are also available in the market (Nieto, 2016).

Chitosan is commercially produced by the deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs, shrimps, oysters and other mollusk shells) and cell walls of fungi. Nearly all chitin and chitosan produced commercially are chemically extracted from the sources mentioned above; chitin can also be produced from shell waste fermentation with microorganisms or with the aid of enzymes. Chitosan is poly-β-(1-4)-2-amino-deoxy-D-glucopyranose.

PROTEINS

Proteins are complex macromolecules that makeup almost 70% by weight of living cells and play roles in their fundamental structure and function. A large number of

proteins are known (they have been isolated, purified and studied), of which the majority varies between 5000 to several million Daltons in weight, and are present in most processed and natural foods.

Proteins have an extraordinary diversity of functions and can be arbitrarily classified into two main categories: "structural proteins" and "proteins with biological activity". Structural proteins are present in all tissues of animals and some vegetables, internal organs, cell membranes and intracellular fluids. Its function depends mainly on its fibrous structure. Among them, can be mentioned collagen, elastin, keratin, etc. Proteins with biological activity have an active role in all biological processes, with enzymes being their most notable exponents.

Among proteins, enzymes are presented as the most interesting from the industrial standpoint, interest that increases year by year. Enzymes act as catalysts for the chemical reactions in biological systems. These offer much more competitive processes compared to chemical catalysts. Several enzyme-based processes have been developed to produce different valuable products since biocatalysis was first introduced nearly a century ago (Bruggink et al., 1998). Consequently, the use of enzymes has expanded to the manufacture of pharmaceutical intermediates and fine chemicals (Griengel et al., 2000; Hills, 2003).

The rise in the prices of traditional commodities such as oil has led industries to look for alternative sources of raw materials, such as biomass. Besides, an increase in social awareness concerning environmental problems has led to public pressure on green technologies, actively demanding the replacement of chemical processes by cleaner, safer and more ecological catalytic processes (Benkovic et al., 2003). In this sense, enzymatic biocatalysis has rapidly been replaced by traditional chemical processes in many areas, and it is expected that this substitution may be accelerated through the development of new technologies in enzymatic engineering (Choi et al., 2015).

The application of enzymes to produce various types of chemical and biological substances have become a proven technology in the chemical, pharmaceutical, food, cosmetic, textile and paper industries. The chemical and pharmaceutical industries found that biocatalysis is faster, less expensive and a safer process, with a reduction in the number of reaction steps and the amount of produced waste (Huisman et al., 2013; Tomsho et al., 2011). In the food industry, biocatalysis has been used to produce raw materials and final products for a long time (Fernandes, 2010). However, most uses of biocatalysis have focused on hydrolytic reactions for scaling, solubility improvement and clarification. With the increasing demand for nutritional aspects, a great deal of attention has been paid to the functionality of foods beyond the primary function of nutrient supply. A recent trend in the food industry is to develop functional foods such as probiotics, antioxidants, low-calorie sweeteners and rare sugars (Nagaoka et al. 2008; Coscueta et al., 2016; Hajfathalian et al., 2017; Anal, 2017). Likewise, recently the cosmetic industry has faced a challenge due to the growing demands of consumers of natural and ecological cosmetics (Ansorge-Schumacher et al., 2013).

Consequently, the cosmetic industry promotes basic research and ecological processes using enzymes to develop more effective cosmetic products. In the textile industry, before the conversion into cloth and yarns, cotton undergoes

several processes, including refining, bleaching, dyeing and polishing (Queiroga et al., 2007). These processes consume large amounts of energy, water and resources, discharging vast amounts of waste. For the development of cleaner processes, the use of enzymes is proliferating. In the pulp and paper industries, different enzymes are used to improve the quality of the pulp by eliminating pitch in wood, lignin and hemicellulose, which are typical impurities (Maijala et al., 2008). Also, the chemical process of pulp manufacture requires the addition of a large number of alkaline chemicals and chlorine (Fu et al., 2005), which has been replaced with enzymatic processes, avoiding the generation of elemental chlorine and significantly reducing the amount of waste that causes ozone depletion and acidification, as well as high energy consumption.

The growing demand for enzymes has led to the emergence of an industry around these macromolecules. Enzymes are not only obtained from natural sources but also are designed and produced in small biological factories. Enzyme engineering was due to the discovery of a genetic code, which opened the possibility of obtaining proteins on a large scale from various organisms in which they do not occur naturally. The proteins obtained in this way are known as recombinant proteins. These can be produced by microorganisms such as bacteria, fungi, viruses and yeasts, as well as in cultivable cell lines of insects, plants and mammals (Janasson et al., 2002; Palomares et al., 2004), protein expression systems "without cells" and animals and transgenic plants (Farrokhi et al., 2009). Overexpression of recombinant proteins offers the advantage of producing large amounts of proteins of interest with characteristics such as natural protein and with relative ease and quickly.

Enzymes are water-soluble molecules that are naturally mixed with many other compounds of a different nature, along with many other similar proteins (some of them with undesired catalytic activity). The catalytic activity of contaminating enzymes may decrease the regio or enantioselectivity as well as the specificity of the desired "biocatalyst". Thus, to be used, the enzymes of interest must be separated from their natural matrix. The separation strategies used for this purpose can involve long and tedious processes, or include a single chromatographic step (Barbosa et al., 2015). However, even in the best of cases, this can have a negative economic impact on the final cost of the biocatalyst. Even in the overexpression of recombinant proteins, despite the aforementioned advantages, the same complications of downstream processing occur. That is why in recent decades much work has been done with a focus on the search for simpler and more effective alternative purification operations depending on the final objective of the enzyme application (Campos et al., 2019; Barbosa et al., 2011; Rocha et al., 2012; Woitovich et al., 2016).

COMPLEX FORMATION BETWEEN POLYSACCHARIDES AND PROTEINS

The complex formation between polysaccharides and proteins is a phenomenon that allows reaching the energy equilibrium, leading to a decrease in the total electrostatic free energy of the mixture (Tolstoguzov, 1991). The electrostatic

interactions that occur between macromolecules with net opposite charges lead to the formation of complexes, which aggregate up to reach sizes and superficial properties that lead to phase separation (Schmitt et al., 2011).

Interactions between proteins and polysaccharides may result in three different thermodynamic consequences: co-solubility, incompatibility or complexing. Generally, the decrease in free energy results from a positive enthalpic contribution occurring from the electrostatic interactions between the biopolymers, and the negative entropic contribution arises from the release of water molecules due to the compaction of biopolymers. Therefore, the complex formation is driven mainly by electrostatic interactions and short-range interactions such as hydrophobic, van der Waals forces or hydrogen bonds might be secondarily involved in the formation of the protein-polysaccharide complexes (Schmitt et al., 2009). Therefore polyelectrolytes interact strongly with proteins due to the substantial electrical charge. Initially, the studies mainly looked at the interactions with synthetic polyelectrolytes, but later natural polyelectrolytes (polysaccharides) were incorporated into these studies, increasing the possibilities of processing and the interest in the formation of complexes between natural polysaccharides and proteins.

The attraction between the natural polysaccharides and proteins can be affected by physicochemical parameters, such as pH value, ionic strength, protein/polysaccharide ratio, polysaccharide linear charge density, protein surface charge density, and stiffness of the polysaccharide chain (Schmitt et al., 2011; Schmitt et al., 2009; Ru et al., 2012). The interaction between protein/polysaccharide occurs when the pH > p*I* (p*I* – isoelectric point) and initiates at the first critical pH (pH_c). Usually, the complex formation occurs within a pH range, therefore, in the second critical pH ($pH_{\varphi 1}$) there is an abrupt increase in turbidity showing the continued aggregation of soluble complexes into insoluble protein/polysaccharide complexes due to charge neutralization (Cooper et al., 2005). A complex formed by the electric charges can transfer between the soluble and non-soluble state by the "simple" shift of pH, as shown in Fig. 15.1. With an acid-base titration curve, it is possible to see the turbidity formation and identify the critical pH of the complex formation. Another way to study these interactions is through dynamic light scattering, Campos et al., in 2016 studied the pH shift that occurs in complex systems between bromelain (*Ananas comosus*, pineapple enzyme) and carrageenan (a natural anionic polysaccharide), and described the hydrodynamic radius (Hd), as well as, the intensity percentage of the complexes formed. A Hd maximum and intensity maximum was achieved at pH between 4.6 and 4.8, which was indicative of complex formation; this range of pH was identified as the first critical pH (pH_c). Two other critical pH values were identified. The second critical point was at pH $\leq$ 3.75 ($pH_{\varphi 1}$) when the association between the macromolecules started. The third critical point pH $\geq$ 6.7 ($pH_{\varphi 2}$) was the point where insoluble complexes dissociate into soluble complexes or interaction between protein molecules and polysaccharides chains do not occur. Between these two critical pH points the range of non-soluble complex formation (Campos et al. in 2016). The same behavior was reported by Duran et al., in 2018 who studied the interaction between carrageenan and quinoa proteins (*Chenopodium quinoa* Willd). They reported that the main protein of the quinoa seed isolate was soluble at alkaline pH due to its negative

net charge and had stability over a wide range of pH. In addition, they reported a precipitation process by secondary aggregation. This complexation occurs through hydrogen bonds and hydrophobic interactions, but also through covalent disulfide bridges that are known to be formed by quinoa proteins.

Several studies also showed the evaluation of enzymes complexed with polysaccharides by fluorescence spectroscopy, thus analyzing the effect of complexation on the structure of the enzyme. These studies should be added to this kind of experimental research, to better understand the implication of the folding of the enzymes on their activities. Campos et al.,[38] evaluated the complex between carrageenan and bromelain by fluorescence spectroscopy and the results showed that carrageenan did not negatively affect the microenvironment of tryptophan residues present in bromelain since the fluorescence spectrum did not change when compared with control bromelain(Campos et al., 2016; 2019).

In summary, and considering the specific case of enzymes, it can be said that the enzyme-polysaccharide complex formation induces a more ordered structure, so the thermodynamic stability of the complex is higher than that of the polysaccharide or enzyme alone. As a result, an elevation of the protein melting temperature on complexation with polysaccharide is observed, it is influenced by the hydrophobicity and electric charge density of polysaccharide and protein. As polysaccharides are excluded from the protein domain, increasing the hydration of the macromolecule, leads to thermal stability being observed. Moreover, this stabilization preserves the enzymatic activity through time especially when the enzyme is stored in solution for long periods.

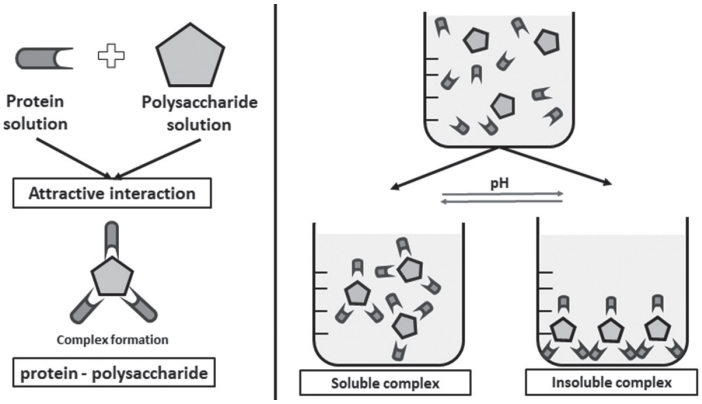

Figure 15.1 Diagram of protein-polysaccharide complex formation, transfer between soluble complex to insoluble complex (*Adapted from* Campos, et al.[38]).

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APPLICATION OF BIOSEPARATION IN THE ISOLATION OF ENZYMES

A process of enzyme bioseparation refers to the recovery, isolation and purification of these proteins from a complex matrix, from animal, vegetable or microbial sources.

In the last years, the bioseparation problem has become relevant due to the growing need to have large quantities of enzymes in different production processes, since it has been described as a viable alternative for replacing the traditional process in the industrial environment. The purification process of enzymes always involves four principal steps: i) removal of cell and cell fragments from the environment in which they are found (clarification); ii) concentration or purification of low resolution (primary recovery); iii) high-resolution purification (allows the separation of the interesting molecule from others with similar features); iv) packaging of the final product.

An ideal bioseparation process should be mainly simple, fast and of low cost since it will represent about 70% of the total product cost (Liam and Mota, 2003). Moreover, it should combine the high extraction yield and selectivity, but also, must ensure reproducibility at the industrial scale up. Besides the selectivity in bioseparation would depend mainly on the final application of the product. This is what will determine the purity, the required concentrations and even the permitted levels for different types of impurities. In this sense, for example, the enzymes to be applied in the medical and pharmaceutical areas present more restrictive directives, so the selectivity in the process must be more significant.

Enzyme bioseparation can be divided into two main groups: i) high yield and high purity grade, such as affinity chromatography; or ii) high yield and low purity grade, such as precipitation, aqueous two-phase systems, ultrafiltration, among others. The latter is the easiest to scale up with a lower cost of application, and mainly refers to precipitation, because of the natural ingredients applied, as polysaccharides, and the simple equipment required for the process. Another aspect to consider in bioseparation processes, mainly on a macro scale, is the ecological one. When handling a large amount of biomass, it is necessary to apply methods that are not aggressive to the environment, that is, they generate the least possible amount of waste material, which is, also biodegradable or non-toxic. In ideal cases, these methods must be able to produce by-products instead of waste, i.e., material that can be valorized when implemented in other processes or as a product in itself.

AFFINITY PRECIPITATION—A tool for green downstream

Green chemistry is the key to sustainable development. Green technologies are emerging as the best alternative due to their reduction in energy consumption, allowing the use of alternative resources and guaranteeing high quality and safe products. A process can be classified as a green process if integrated technologies allow the reduction of negative impacts on the environment, by reduction of water and energy requirements, as well as, reduction of processing time throughout the productions steps, which includes the downstream stage. As an example, natural enzyme isolation can be very difficult because usually it is present in a very low concentration and has to be separated from a complex biomass, requiring several stages in a process for enzyme separation and isolation, and is one of the crucial steps of precipitation. It is important to highlight that precipitation is a part of green chemistry, that is, using natural compounds as precipitants in sustainable conditions, we can refer to this methodology as “green precipitation”.

In recent years, the search for solutions for the scaling of protein isolation and purification methods for biotechnological processes has increased significantly as a result of the growing demand for enzymes. In traditional methods for enzyme separation in large volumes, approximately 90% use a high percentage of inorganic salts or organic solvents, with ammonium sulfate being the most common precipitant. This salt is used in the range of 40–70% of the saturation level to separate 50% of the total enzyme content. The problem of the application of this salt is that it has a high cost and a high negative impact in the process, as well as on the environment.

Affinity precipitation was first described in 1970 by Mosbach et al. in 2003. Their work was based on the observation of an insoluble complex formed by lactate dehydrogenase (LDH) with a polyelectrolyte, which was added in such a way as to precipitate, this being a direct consequence of the affinity interaction between a multivalent enzyme and a bifunctional ligand.

The precipitation with natural polyelectrolytes (green precipitation), considering the desirable conditions for the enzyme bioseparation, is a method that has been favorably positioned in recent years. It allows the recovery of a macromolecule of a complex mixture using certain natural polymeric ligands capable of interacting specifically with the target molecule and reversibly precipitate with certain stimuli. This bifunctionality of the ligand is the basis of the purification strategy (Hilbrig and Freitag, 2003), since in a first step, the ligand in its soluble form interactions with the protein of interest, producing the formation of the complex and therefore becomes insoluble and separates by decantation. Then, by means of an appropriate stimulus (change of some environmental conditions, i.e., pH, temperature, ionic strength and the presence of certain co-solutes) the dissociation of the complex is induced, allowing the precipitation of the free ligand (for recycling) and the recovery of the protein in soluble form.

Green precipitation presents several advantages such as easy scaling, a possibility of continuous processing, simplicity in the necessary equipment and low necessary concentrations of ligand (polysaccharide). On the other hand, its main disadvantage is the low sensitivity in some instances, since co-precipitation can occur with other molecules, which can be considered as impurities, which require coupling with other methodologies to obtain a purer product. In addition, green precipitation can be combined with other novel and green methods such as aqueous two-phase systems, taking advantage of the combined properties of both methodologies, resulting in an increase in selectivity and also allowing, in the same step, to extract and separate more than one compound of interest (Teotia and Gupta, 2004; Leong et al., 2017; Rocha et al., 2016).

Conclusion

During the past few years the production and commercialization of industrial enzymes have been growing, due to the enormous progress in the upstream process. The projections show continuous growth in the enzyme production, more prominently in those of natural origin. This is mainly related to the development of new strategies to overcome the problems related to the downstream operations of the production process (extraction, separation and purification). The recent

innovation in bioseparation, more precisely in green precipitation, has been driven both by the imperative need to transform food industrial waste into by-products and in turn add value, as well as by the need to reduce operational costs by increasing the general yield of the processes. Thus, it is necessary to implement these new integrated techniques to further optimize yields. Although, a greater interdisciplinary contribution is still needed to reach an efficient scale up that allows transferring these technologies in an adequate way to the industry. The recovery of high-value natural ingredients, mainly enzymes, from by-products through a successful application of green precipitation operations will result in a better and more efficient economy. This application will lead to savings that will benefit manufacturers and consumers, and also the environment. In a perfect system, where everything is framed under sustainable practices bioprocessing should set an example to other industrial sectors.

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