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BIOMOLECULES EXTRACTED BY ATPS: PRACTICAL EXAMPLES

BIOMOLÉCULAS EXTRAÍDAS POR SBAS: EJEMPLOS PRÁCTICOS

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Abstract

The actual biotechnology industry demands fast and economic upstream and downstream processes to purify biomolecules. In this context, different purification techniques, that offer both high recovery and purity to the final product, have been assayed by different research groups. Liquid-liquid extraction with aqueous two-phase systems is one of the most studied methodologies for bio-separation. This technique presents several advantages such as mild conditions of working, cost-effectiveness, short-time consumption and high recovery percentage of the final product. With the aim to present a comparison of liquid-liquid extractions with other techniques, several aqueous two-phase extraction processes of biomolecules are presented in this review. We presented the advantages and disadvantages of them as of the compared systems. In general, the highest final product purities are achieved when different methodologies are combine, being the chromatographic ones the most applied in the last stages for the high purification factor obtained after them. Alternative methodologies, such as aqueous two-phase systems (ATPS), *i.e.*, PEG/salts or ionic liquids; aqueous two-phase micellar systems, using solvents and surfactants; and extractive fermentation with ATPS, are relevant for both cost-effectiveness and time-saving of the purification process.

Keywords: *liquid-liquid extraction, downstream processing, biomolecules, polymers, surfactant.*

Resumen

La industria biotecnológica actual exige procesos rápidos y económicos para la producción y la purificación de biomoléculas. En este contexto, diferentes técnicas separativas que ofrezcan un alto rendimiento y una alta pureza del producto final han sido evaluadas por diferentes grupos de investigación. La extracción líquido-líquido con sistemas bifásicos acuosos (SBAs) es una de las metodologías más estudiadas para bio-separación. Esta técnica presenta varias ventajas, tales como condiciones suaves de trabajo, alta relación costo-beneficio, cortos tiempos de consumo y alto porcentaje de recuperación del producto final. Con el objetivo de comparar extracciones líquido-líquido con otras técnicas de separación, diferentes procesos de extracción de biomoleculas serán presentados en este trabajo de revisión. Adicionalmente, se presentaran las ventajas y desventajas de cada uno de ellos. En general, los más altos grados de pureza del producto final fueron obtenidos al combinar diferentes metodologías, siendo las cromatográficas las más aplicados en las últimas etapas debido a los elevados factores de purificación obtenidos después de ellas. Metodologías alternativas como por ejemplo, SBAs basados en mezclas de PEG/sal o liquídos iónicos/sal, sistemas micelares de dos fases acuosas (SMDFAs) formados por solventes o surfactantes; fermentación extractiva utilizando SBA, también resultan ser relevantes debido a sus bajos costos y cortos tiempo en sus procesos de purificación.

Palabras clave: extracción líquido-líquido, procesos de purificación, biomoléculas, polímeros, surfactante.

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1 Introduction

The wide variety and combinations of production processes employed to purify biomolecules from different biological sources are known as bioseparation or downstream processing (Shukla *et al*., 2007). As it comprises a key point for the commercial development of biotechnological products (Kelley 2009), bioseparation protocols should be designed in order to reach the best cost/benefit ratio. A conventional downstream process includes an initial clarification step (removal of cells and enrichment of the target molecule), an intermediary purification (first capture of target molecule) and a polishing (final product with high degree of purity) (Shukla *et al*., 2007). Each step may even comprise several unit operations depending on the biological source complexity and/or the final purity degree required. Unfortunately, the use of a multi-step procedure generally leads to low recovery yields, thus impacting in the final product costs (Low *et al*., 2007; Kurasawa *et al*., 2013). For large-scale purification, the drawbacks of employing several purification steps is even more significant due to the large space and buffer volumes required for downstream operations (Aldington & Bonnerjea 2007). As a result, alternative methodologies that allow several purification steps to be integrated, offering simple procedures, are being evaluated (Low *et al*., 2007). Important candidates include precipitation, aqueous two-phase systems (ATPS) and expanded bed chromatography. These techniques present the advantage of integrating clarification and intermediary purification, avoiding the use of a multi-step protocol (Lan *et al*., 2011; Rosa *et al*., 2013). On the other hand, liquid-liquid extraction and expanded bed adsorption can even integrate upstream and downstream processes. These last methodologies are currently known as extractive bioconversion.

In this review, we discuss and compare different downstream processing methodologies based mainly on liquid-liquid extraction. Additionally, we offer our point of view on which one of them is the most suitable. The text is structured with a briefly explanation of polymeric, micellar, reverse micelle and ionic liquid-based aqueous two phase system, followed by the comparison of ATPS biomolecules purification examples with other techniques. It should be noticed that most of the examples cited in this paper are based on previous works developed by our research group.

2 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a purification technique, which consists of the transfer of certain components from one phase to another when immiscible or partly soluble liquid phases are brought into contact with each other (Mazzola *et al*., 2008). The conventional process of LLE uses water-organic solvent two-phase systems. However, it is known that there are some limitations to use of organic solvents particularly in large-scale applications, due to solvents toxicity, their cost, their impact on the environment, and due to the fact that organic solvents may lead to irreversible product degradation (Dermiki *et al*., 2009). In this respect, the liquid-liquid extraction by aqueous two-phase systems (ATPS) has been proved to be a valuable technique for separating and purifying biomolecules, organelles, membranes, as well as whole cells, from complex media. In comparison to other separation techniques, aqueous two-phase extraction offers many advantages, such as a short processing time, low energy consumption, environmentally friendly and it is relatively ease to scale-up (Liu *et al*., 2011). Furthermore, the high water concentration (between 80-90%) in such systems favors the stability of biologically active molecules during the separation process, in comparison to the two-phase systems with organic solvents (Hatti-Kaul 2001). On the other hand, extractive bioconversion using aqueous two-phase systems (ATPS) seems to be a very attractive method for the integration of fermentation and downstream processing of extracellular proteins (Pandey & Banik 2011). Several ATPS formats have been studied since the first report announced by Beijerinck in 1896, which showed that the combination of gelatin, agar and water in certain proportions forms two immiscible aqueous rich phases (Beijerick 1896). Nonetheless, it was only in 1958 that Albertsson (1958) reported the ability of ATPS formed by polyethylene glycol (PEG) and dextran to separate biomolecules from aqueous media. Among the ATPS studied, we can mention: polymeric (ATPS-P), micellar (ATPS-M), reverse micellar (ATPS-RM) and ionic liquid-based (ATPS-IL). The next section will summarize each ATPS technique.

2.1 Liquid-liquid extraction in polymeric aqueous two-phase systems

Polymeric aqueous two-phase system (ATPS-P) is a liquid-liquid extraction technique formed by polymers and salt solutions, or polymer/polymer solutions mixtures. On these systems two water-soluble solutes separate into two immiscible aqueous-rich phases based on polymer-polymer, polymer-salt or salt-salt solute combinations (Ventura *et al*., 2013). This system has the advantage to be formed simply by the mixture of the proper components (polymer, salt). Although the price of the polymers, can be high when compared with salts cost, some cheap alternatives polymer are being studied, such as tree gum (Benavides & Rito-Palomares 2008), xanthan (Chethana *et al*., 2006) and starch derivatives (Lin *et al*., 2003). Another alternative is the recycling of the polymer (Pereira *et al*., 2012). These systems are usually used in batch mode, and would greatly improve process overall costs if used in continuous mode. A good review about the constrains of this approach can be find elsewhere (Espitia-Saloma *et al*., 2013), as a detailed example (Rosa *et al*., 2013).

2.2 Liquid-liquid extraction in micellar aqueous two-phase systems

Surfactants solution can form micellar aqueous two-phase systems (ATPS-M). Surfactants are amphiphilic molecules composed of a hydrophilic or polar moiety, known as head, and a hydrophobic or nonpolar moiety, known as tail. The surfactant head can be charged (anionic or cationic), dipolar (zwitterionic), or non-charged (nonionic) (Rangel-Yagui *et al*., 2005). Surfactants can form micelles solutions and these systems are based on the property that some micelle solutions present to phase separate into a micelle-rich and a micelle-poor phase, under certain conditions such as temperature, pH and ionic strength (Liu *et al*., 1996). Hence they offer hydrophobic and hydrophilic environments at the same time, allowing selectivity on the partitioning of molecules and contaminants according to their hydrophobicity (Nikas *et al*., 1992; Rangel-Yagui *et al*., 2003; Andrade *et al*., 2011). Further information about the basis of this technique can be found in the literature (Mazzola *et al*., 2008).

2.3 Liquid-liquid extraction in reverse aqueous two-phase micellar systems

As the aqueous two-phase micellar systems, the reverse systems (ATPS-RM) are surfactants-based. However, reverse micelles form spontaneously reversible spherical aggregates of amphiphilic molecules in non-polar liquids (Hasmann *et al*., 2007). Nanometer-sized water pools are formed by a monolayer of surfactants molecules entrapping water (Li & Cao 2014). In this way, reverse micelles are formed in organic solutions and depending on the water content of the system, may have a small or large water core for hosting molecules. These aggregates can be used to solubilize different molecules according to their hydrophobicity, size and charge (Storm *et al*., 2014). Additional information about the technique can be found in the literature (Mazzola *et al*., 2008).

2.4 Liquid-liquid extraction in ionic liquid-based aqueous two-phase systems

Ionic liquids, designed also as green solvents, comprise a group of novel molecules which are widely studied due to their characteristics such as negligible volatility, large liquid range, high thermal capacity, chemical stability and strong solvent property (Pramanik *et al*., 2011). Ionic liquids have been already applied in biocatalysis, electrochemistry, and bio-separations (Yu *et al*., 2011). In recent years ionic liquids have been investigated as novel aqueous two-phase systems (ATPS-IL) (Li *et al*., 2012b). The ionic liquids are typically comprise inorganic or organic cations and organic anions, usually liquid at room temperature, low vapor pressure and present a great structural variety. Due to ionic liquids characteristics, the systems composed of them do not suffer from high viscosity and from formation of opaque aqueous solutions or are limited to a narrow range of polarities (Ventura *et al*., 2013). Such extraction system has been successfully used to extract proteins, enzymes, antibiotics, spices, and drug molecules (Tan *et al*., 2012).

3 Biomolecules extracted by ATPS

Different biomolecules and cellular components have been purified by ATPS. In this section it will be discussed and compared ATPS purification methods applied to extracted or purified biomolecules produced by biological systems. The section is divided, for organization purpose, in classes of biomolecules: enzymes, antibiotics, colorants, pDNA and LPS. It is worth noticed that LPS was included as extracted biomolecule, but it was studied as a contaminant removed from other biomolecules.

3.1 Enzymes

Several hydrolytic and non-hydrolytic enzymes have been successfully purified. In this sub-section we will discuss some practical examples of enzymes purification applying ATPS and to compare them with other techniques.

3.1.1 Bromelain

Bromelain is a mixture of proteolytic enzymes present in all tissues of the pineapple (Ananas comosus Merr.), and it is known for its clinical therapeutic applications, food processing, and as a dietary supplement (Novaes *et al*., 2013). Rabelo *et al*., (2004) evaluated bromelain purification with ATPS-P formed by poly(ethylene oxide) (PEO)-poly(propylene oxide) (PPO)-poly(ethylene oxide) (PEO) block copolymers. The best result showed 79.5% enzyme activity recovery, a purification factor of 1.25 in the top phase and 1.4 partition coefficient.

Hebbar *et al*. (2008) used reverse micellar system composed of cationic surfactant n-hexadecyltrimethyl-ammonium (CTAB) to recovery bromelain. The extraction was performed at different pHs, the results were as follow: pH 9.0 - activity recovery of 132%, purification factor of 1.7-fold; pH 8.0 - activity recovery of 106%, purification factor of 5.2-fold and 45% forward extraction efficiency; pH 10.0 - activity recovery of 81% and purification factor of 1.2-fold.

Novaes *et al*. (2013) extracted bromelain of pineapple waste employing ATPS-P formed by PEG and poly(acrylic acid) (PAA). In this work, bromelain partitioned preferentially to the top/PEG-rich phase and, in the best condition [8% of PEG and PAA, PEG 2,000 g/mol, PAA 15,000 g/mol, and 6% of Na_2SO_4 at 30ºC] achieved a yield of 335.27% with a purification factor of 25.78.

An alternative to reach higher results is combined techniques. In this sense, Coelho *et al*., (2013)

purified bromelain through an unconventional ATPS-P which integrates fractional precipitation by ammonium sulfate. With this new technique, the authors obtained a purification factor of 11.80 with a 66.38% activity yield, in a system composed by PEG concentration of 10.86% and 36.21% ammonium sulfate saturation. ATPS formed by PEG/phosphate buffer was evaluated by Babu *et al*., (2008) and a bromelain activity recovery of 228% and a purification factor of 4.0 was achieved.

Bromelain was also extracted by precipitation techniques. Soares *et al*., (2012) studied bromelain purification through ethanol precipitation method. Employing two-step precipitation, 30-70% (v/v) ethanol, it was possible to purify bromelain with a purification factor of 2.28 with a 99.2% recovery yield. Devakate *et al*., (2009) studied the purification applying ammonium sulfate precipitation and ion-exchange chromatography. The group obtained the best precipitation results using 40-70% of ammonium sulfate, achieving 2.97 purification factor and recovering 69.7% of the enzyme from crude extract. Cation-exchange chromatography presented better results than precipitation with a purification factor of 10 and 84.5% recovery.

Amid *et al*. (2011) expressed bromelain in the BL21-AI *Escherichia coli* strain. The recombinant bromelain was then purified in a single step using immobilized metal affinity chromatography (IMAC), specifically a nickel-NTA column. This single step purification method increased bromelain preparation purity by about 41-fold. Table 1 summarizes the results obtained for bromelain as shown in this section.

3.1.2 Collagenase

Collagenase is an enzyme traditionally produced by microorganisms in the extracellular matrix. In this way, it takes one step to separate the target biomolecule from the cells and other step to purify it from other contaminants presented in the fermented broth. A variety of methods and strategy were applied to collagenase purification.

Collagenase from *Penicillium aurantiogriseum* was purified by Rosso *et al*. (2012) using ATPS-P formed by PEG/phosphate, which obtained a 376.8% yield in the top phase with a 14.7 purification factor. Wu *et al*. (2010) employed the strategy of three-step procedure (ammonium sulfate precipitation, and two gel filtrations) and achieved a 31.53-fold purity with a 7% yield from crude extract. Liu *et al*. (2010), using a combination of ammonium sulfate precipitation, ion-exchange chromatography and gel filtration, obtained a lower result of purification factor (20.4) but a higher yield (25.2%), when compared with the values obtained by Wu *et al*. (2010). On the other hand, higher values to both parameters, yield (67.21%) and purification factor (30.34), were obtained by Jain and Jain (2010), whom used only ammonium sulfate precipitation as a purification method. Baehaki *et al*. (2012), combined ammonium sulfate precipitation and ion-exchange chromatograpy to improve the collagenase purification, but lower results were achieved, purification factor of 26.3, with a 2.6% yield from the crude extract. Moreover, liquid-liquid extraction with use of polymer (ATPS) achieved interesting results, since it is a simple and easy method to work with and presented the best results as the results obtained by Rosso *et al*. (2012). Table 2 summarizes the results obtained for collagenase as shown in this section.

3.1.3 Lysozyme

Lysozyme (muramidase, EC 3.2.1.17) is widely distributed in animal tissues and secretions. Apart from that, lysozyme exists abundantly in hen egg white constituting 3.5% of the total egg white proteins. Due to its antimicrobial activity this enzyme is utilized as a food preservative, and some pharmaceutical applications (Dembczynski *et al*., 2012). Lysozyme is another example of enzyme that is widely employed to ATPS extraction assays. Lu *et al*. (2013) assessed lysozyme purification from hen egg using PEG 4,000/potassium citrate aqueous two-phase system (ATPS), observing an increase in lysozyme recovery with higher salts concentration.

Table 1. Bromelain extraction/purification results from different techniques

Technique	Yield (%)	Purification factor	Partition coefficient	Reference
ATPS-polymeric	79.50	1.25	1.40	Rabelo *et al*. (2004)
ATPS-reverse micellar (pH 9.0)	132.00	1.70	-	Hebbar *et al*. (2008)
ATPS-reverse micellar (pH 8.0)	106.00	5.20	-	Hebbar *et al*. (2008)
ATPS-reverse micellar (pH 10.0)	81.00	1.20	-	Hebbar *et al*. (2008)
ATPS-polymeric	335.27	25.78	-	Novaes *et al*. (2013)
Unconventional ATPS-polymeric	66.38	11.80	-	Coelho *et al*. (2013)
ATPS-polymeric	228.00	4.00	-	Babu *et al*. (2008)
Ethanol precipitation	99.20	2.28	-	Soares *et al*. (2012)
Ammonium sulfate precipitation	69.70	2.97	-	Devakate *et al*. (2009)
Ion-exchange chromatography	84.50	10.00	-	Devakate *et al*. (2009)
IMAC	-	41.00	-	Amid *et al*. (2011)

ATPS: aqueous two-phase systems; IMAC: immobilized metal affinity chromatography.

Table 2. Collagenase extraction/purification results from different techniques.

Technique	Yield (%)	Purification factor	Reference
ATPS-polymeric	376.80	14.70	Rosso *et al*. (2012)
Ammonium sulfate precipitation and two gel filtrations	7.00	31.53	Wu *et al*. (2010)
Ammonium sulfate precipitation, ion-exchange Chromatography and gel filtration	25.20	20.40	Liu *et al*. (2010)
Ammonium sulfate precipitation	67.21	30.34	Jain and Jain (2010)
Ammonium sulfate precipitation and ion-exchange chromatograpy	2.60	26.30	Baehaki *et al*. (2012)

ATPS: aqueous two-phase systems.

Table 3. Lysozyme extraction/purification results from different techniques.

Technique	Yield (%)	Purification factor	Reference
ATPS-polymeric	103.0	21.11	Lu and co-workers (2013)
ATPS-polymeric	57.5	-	Dembczynski *et al*. (2012)
ATPS-polymeric	100.0	-	Johansson *et al*. (2008)
Affinity precipitation	80.0	28.00	Shen and Cao (2007)
Membrane-affinity chromatography	79.0	-	Chiang *et al*. (1993)
Dye ligand-immobilized composite membrane	82.0	25.40	Yilmaz *et al*. (2005)

ATPS: aqueous two-phase systems.

Furthermore, the purification factor and specific activity were increased to higher PEG 4,000 concentrations, achieving 21.1 and 103%. Dembczynski *et al*., (2012) studied an optimization method to identify the system composition for the most efficient separation conditions to lyzozyme from hen egg white in the aqueous two-phase system $EO_{50}PO_{50}$/potassium phosphate. The influence of phosphate, copolymers of ethylene oxide and propylene oxide ($EO_{50}PO_{50}$), NaCl concentration and pH on the partition coefficient and extraction yield of lysozyme were evaluated. The best results in lyzozyme partitions (45) and yield (57.5%) were found with $EO_{50}PO_{50}$, potassium phosphate and NaCl concentrations of 17.40, 22.67% and 0.85 M, respectively and for pH 9.0. Nevertheless, the most important factor analyzed was the NaCl concentration influencing the most on lysozyme partition and yield. Another polymeric system composed of PEG2,000/NaPA8,000 containing 6% Na_2SO_4, partitioned lysozyme to the PEG-rich top-phase (K equals 6.5), with a yield next to 100% (Johansson *et al*., 2008).

Another technique used for lysozyme purification was affinity precipitation. Affinity precipitation technique has tremendous potential and is a powerful technique for protein purification (Kumar *et al*., 2008). One of the main advantages of affinity precipitation is its easy scalability (Roy *et al*., 2005), and that it may provide a pure protein in a single step (Gawande & Kamat 1999). It is a cost-effective process, does not need special equipment and the consumption of polymer is minimal, as most of it can be recovered (Gawande and Kamat, 1999). Shen and Cao (2007) synthesized by radical polymerization a thermo-sensitive N-alkyl substituted polyacrylamide polymer which was applied to affinity precipitation of lysozyme from egg white, wich purification factor of 28 and yield of 80%.

Other interesting techniques about lysozyme purification were also studied by other authors. Chiang *et al*. (1993) studied the isolation and purification of lysozyme from hen egg white using two-step procedure. The egg white was processed by sequential dilution and diafiltration using a UF membrane (molecular weight cut-off 300 kDa). The membrane process increased the specific activity of lysozyme 6-fold, and recovered 96% of lysozyme activity. The permeate from diafiltration was further purified by affinity chromatography using chitin as adsorbent, which yielded a product of specific activity of 70,400 U/mg protein, and the overall lysozyme recovery was 79%. This process used phosphate to help separation between lysozyme and other proteins, however the existence of phosphate in the residual egg white would limit its further uses. Nevertheless, the method provides a potential alternative in lysozyme separation.

Yılmaz *et al*., (2005) investigated separation and purification of lysozyme from aqueous solution and egg white in poly(hydroxyethylmethacrylate)/chitosan (pHEMA-chitosan) composite membrane prepared by UV-initiated photopolymerisation. A dye ligand (i.e. Reactive Green 19) was immobilized onto the membrane. The lysozyme adsorption capacity of the RG 19 immobilized membrane was 60.8 mg/mL. The adsorption capacity of the plain composite membrane was 7.2 mg/mL. The lysozyme was purified 25.4-fold in a single step with a recovery 82%. Finally, the authors concluded that repeated separation/desorption processes showed that the dye ligand-immobilized composite membrane gave efficient separation

of lysozyme from egg white in aqueous solution, and it could be used in large-scale applications. Table 3 summarizes the results obtained for lysozyme as shown in this section.

3.1.4 Xylanolytic complex

Xylanolytic complex are a discrete, multifuncional and multienzyme complex found in some xylan-degrating microorganisms. Rodrigues *et al*., (1999) described the transfer of an extracellular xylanase from *Penicillium janthinellum* by using reversed micellar two phase system of the anionic surfactant sodium bis(2ethylhexyl) sulfosuccinate (AOT) and the following factors influence: pH, temperature, surfactant concentration and buffer concentration. The highest enzyme recovery was around 10%, suggesting that for xylanase extraction by reversed micelles other type of surfactant, perhaps cationic, could be more appropriate (Rodrigues *et al*., 1999). Therefore, xylanase recovery by reversed micelles using cationic surfactant N-benzyl-N-dodecyl-N-bis(2-hydroxyethhyl)ammonium chloride (BDBAC) was evaluated under different experimental conditions and was obtained a 27% recovery (Rodrigues *et al*., 1999). The partition behavior of *P. janthinellum* xylanase has also been studied in aqueous polymeric two-phase systems.

Xylitol dehydrogenase was extracted from crude extracts of Candida guilliermondii in BDBAC-reversed micelles in isooctane, by a two-step procedure, with recovery yield around 121% and 2.3 purification factor (Cortez *et al*., 2004). Another approach using a cationic surfactant evaluated the effectiveness of liquid-liquid extraction by CTAB reversed micelles in purifying xylitol dehydrogenase and xylose reductase in two different phases of the micellar system, with recovery yields around 100% for both enzymes, and 1.8 and 5.6 purification factor, respectively (Cortez *et al*., 2006).

Panagiotou *et al*., (2002), extracted xylitol dehydrogenase from *Fusarium oxysporum* with 1.6% yields and a purity increase of 15-fold employing multi-step strategies which included salt precipitation and successive chromatography steps (Panagiotou *et al*., 2002).

Xylanolitic complex was also purified with other techniques as in the work of Cortez *et al*., (1998) which carried out precipitation assays of xylanase and total protein by using sodium sulfate. In the precipitations performed with sodium sulfate, the maximal xylanase recovery (71.8%) was attained at 25% concentration. At higher salt concentrations, the enzyme dissolved once more in the supernatant and the recovery yield decreased. The total protein precipitation curve showed a different behavior, in comparison to the xylanase. The highest recovery level (68%) was observed at 40% salt concentration.

Cortez and Pessoa (1999) carried out fractionated ethanol precipitations to separate β-xylosidase from the total proteins present in the fermented medium (xylanolitic complex). Results showed adequate selective separation of the xylanolitic enzymes. Some differences in solubility between total xylanase and β-xylosidase were observed. The highest β-xylosidase recovery yield and the highest total xylanase yield were achieved with ethanol concentrations of 60 and 80%, respectively. At 60% ethanol concentration, a slight decrease in the xylanase solubility was observed, and at 80% ethanol concentration, almost 100% of the total xylanase was precipitated. At pH 4.6 and 7.0, about 85% of the total xylanase was recovered, whereas at pH 5.9 and 6.3, about 95% of the total xylanase was recovered. The results obtained in this study (74% of β-xylosidase and 80% of total xylanase recovery) revealed that ethanol fractional precipitation is a proper technique for purify enzymes produced by *Penicillium janthinellum* from sugar cane bagasse. This technique does not affect the kinetic characteristic of the enzyme and provides a solution with low ionic strength, which is desirable for further purification steps.

Another method applied to xylanase is affinity precipitation. It can be selectively precipitated with a methyl methacrylate polymer like Eudragit S-100, which becomes insoluble below pH 5.0, its solubility change is reversible and can be used as an affinity ligand for number of enzymes (Gawande & Kamat 1999; Roy *et al*., 2005). Roy *et al*., (2005) had a 95% recovery of xylanase activity with 42-fold purification.

The selective precipitation of xylanase from an aqueous phase containing mixtures of xylanase and cellulase was studied by Shin *et al*., (2004) using an ionic surfactant as precipitating ligand and a polar organic solvent as recovery solvent. From the four ionic surfactants tested, only sodium di-(2-ethylhexyl) sulfosuccinate (AOT) showed a complete removal of xylanase at pH 4.5. The recovery of xylanase from the xylanase-AOT complex was a strong function of the type and the volume of the polar solvent and of the concentration of sodium acetate buffer in the final aqueous solution used to solubilize the recovered xylanase. With ethanol as a recovery solvent, a recovery of about 80% was obtained. The cellulase activity in the recovered xylanase was below the detection limit. These authors demonstrated that an ionic surfactant can recover enzymes from aqueous solutions without loss in their activity.

Gupta *et al*., (1994) studied xylanase from *Thricoderma. viride* and purified 4.2 fold by precipitation with a commercially available enteric polymer Eudragit S-100. Electrophoretic analysis also indicated removal of contaminant proteins. The enzyme could be recovered in more than 89% yield and the binding of the enzyme to the polymer was predominantly by electrostatic interaction.

For this group of enzymes the selective precipitation shown greater results and if precipitation agents can be recover at the end it could be an interesting technique to be applied in large-scale purification. Nevertheless, in the selective precipitation studies presented, it was used commercially available enzymes for the assays. ATPS system are known for its capacity to handle complex media, even in the presence of cells, as in extractive fermentations

(Pandey & Banik 2011). Table 4 summarizes the results obtained for xylanolytic complex as shown in this section.

3.1.5 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme, belonging to the important class of $NADP^+$-dependent dehydrogenases, which catalyzes the first step of the pentose phosphate pathway, i.e. the oxidation of glucose-6-phosphate (G6P) to gluconolactone 6-phosphate, using $NADP^+$ as hydrogen acceptor (Puchkaev *et al*., 2003). A system composed of PEG/phosphate, was able to recover 97.7% of G6PD from *Saccharomyces cerevisiae* cell homogenate, increasing purity by 2.28-fold (Ribeiro *et al*., 2007). Rangel-Yagui *et al*., (2003) demonstrated that the use of a two-phase aqueous mixed micellar system composed of the nonionic surfactant *n*-decyl tetra(ethylene oxide) ($C_{10}E_4$) and the cationic surfactant CnTAB (alkyltrimethylammonium bromide, n = 8, 10, or 12) can improve significantly the partitioning behavior of the net-negatively charged enzyme G6PD on the one obtained in the two phase aqueous $C_{10}E_4$ micellar system. Overall, the two-phase aqueous mixed ($C_{10}E_4$/C_{10}TAB) micellar system yielded the highest G6PD partition coefficient (7.7), with a 71% yield in the top phase, providing the optimal balance between the denaturing effect and the electrostatic attractions for the three cationic surfactants examined.

G6PD industrial purification has been carried out through multiple-step processes based on chromatography technology. This technique provided recovery 98% yield and a purification factor of 103 in expanded bed ion-exchange chromatography (Chang *et al*., 1995). Gómes-Manzo *et al*., (2013) produced a his-tagged human G6PD in *E. coli* and the purification was through in a single chromatographic step by immobilized metal affinity (Nickel Sepharose) with a 58% yield and specific activity of 224 IU/mg.

Tejedor *et al*., (1986) studied the precipitation profiles of G6PD, phosphofructokinase (PFK) and 6-phosphogluconate dehydrogenase (6PGD) in different PEG concentration and pHs. Precipitation generally occurred between narrow limits of polyethylene glycol. Particular conditions (%PEG and pH) for the selective enzyme enrichment have been determined. For G6PD, all specific activities in precipitated obtained at pH 5.0 are higher than in the original hemolysate (10.7×10^{-2} U/mg). In contrast, precipitates obtained at pH 6.0 or 7.0 have lower specific activities than the original hemolysate. This means that, at pH 6.0 and 7.0, the supernatants became enriched in G6PD. The highest G6PD enrichment (5-fold) in pellet corresponds to that of 90% yield (6% PEG and pH 5.0). Under these conditions the enzyme could also be separated from 6PGD and, therefore, this precipitate would be the most appropriate for a preliminary isolation of G6PD. Since such a precipitate also contains 80% of original PFK, a previous PFK precipitation would be necessary at 6% PEG pH 6.0, after which G6PD would be precipitated (6% PEG, pH 5) from the supernatant. According to these authors the enrichment degree of each enzyme by using the above mentioned conditions is adequate for an initial step of purification. Nevertheless, a more selective isolation of each enzyme can be expected through the manipulation of precipitation curves horizontally along the PEG axis by varying solution conditions.

As shown above, both liquid-liquid purification and precipitation may play an important role in G6PD purification, but it would be in the first step in the purification process to aid chromatography steps. Both techniques presented a high extraction yield and were able to partially purified G6PD. Table 5 summarizes the results obtained for glucose-6-phosphate dehydrogenase as shown in this section.

3.1.6 Glucose oxidase

Glucose Oxidase (GOX) is a flavoprotein which catalysis the oxidation of glucose to gluconic acid and hydrogen peroxide, using molecular oxygen as the electron acceptor (Chen *et al*., 2013). GOX by CTAB reversed micelles from raw and centrifuged cell homogenates of Aspergillus niger enriched with commercial enzyme was studied by Ferreira *et al*., (2005). The recovery yield obtained from centrifuged homogenate (92.7%) was compared with the one obtained from raw homogenate (94.3%), thus demonstrating that cell debris removal is not necessary to obtain satisfactory extraction performances (Ferreira *et al*., 2005).

Table 4. Xylanolytic complex extraction/purification results from different techniques.

Biomolecule	Technique	Yield (%)	Purification factor	Reference
Xylanase	ATPS-reverse micellar	10.0	-	Rodrigues *et al*. (1999)
Xylanase	ATPS-reverse micellar	27.0	-	Rodrigues *et al*. (1999)
Xylitol dehydrogenase	ATPS-reverse micellar	121.0	2.3	Cortez *et al*. 2004
Xylitol dehydrogenase	Salt precipitation and successive chromatography steps	1.6	15.0	Panagiotou *et al*. (2002)
Xylanase	Sodium sulfate precipitation	71.8	-	Cortez *et al*. (1998)
β-xylosidase	Ethanol precipitation	74.0	-	Cortez and Pessoa (1999)
Xylanase	Affinity precipitation	80.0	-	Shin *et al*. (2004)
Xylanase	Polymer precipitation	89.0	4.2	Gupta *et al*. (1994)

ATPS: aqueous two-phase systems.

Table 5: Glucose-6-phosphate dehydrogenase extraction/purification results from different techniques.

Technique	Yield (%)	Purification factor	Reference
ATPS-polymeric	97.7	2.28	Ribeiro *et al*. (2007)
ATPS-micellar	71.0	-	Rangel-Yagui *et al*. (2003)
Expanded bed ion-exchange chromatography	98.0	103	Chang *et al*. (1995)
IMAC	58.0	-	Gómes-Manzo *et al*. (2013)

ATPS: aqueous two-phase systems; IMAC: immobilized metal affinity chromatography.

Table 6: Glucose oxidase extraction/purification results from different techniques.

Technique	Yield (%)	Purification factor	Reference
ATPS-reverse micelle precipitations, ultrafiltration, anion-exchange and size-exclusion chromatographies	94.3	-	Ferreira *et al*. (2005)
Anion-exchange and gel filtration chromatographies	10.3	8.6	Simpson *et al*. (2007)
	9.0	89.3	Kelley and Reddy (1986)

ATPS: aqueous two-phase systems.

Simpson *et al*. (2007) purified glucose oxidase from *Penicillium sp*. using many steps (precipitations, ultrafiltration, anion-exchange and size-exclusion chromatographics) with 10.3% yields and purification of 8.6-fold.

Kelley and Reddy (1986) studied the glucose oxidase purification from *Phanerochaete chrysosporium* combininng anion-exchange and molecular size chromatographies. The authors achieved purification of about 90-fold. In the DEAE-Sephadex step, about 60% of the total glucose oxidase activity present in crude cell extracts was recovered in the 0.25 M NaCl eluate, giving about a 5-fold increase in specific activity. The next Sephacryl S-300 step produced 38-fold enrichment in specific activity and 41% recovery of total activity. The elution profile from the DEAE-Sepharose column showed that a single protein peak had all the glucose oxidase activity, with 89.3-fold enrichment in specific activity and enzyme recovery of 9%.

Since reversed micelles extraction can be conducted with homogenate, it may be applied as a clarification step. The results presented may indicate that combining techniques as reversed micelles extraction and a chromatographic step may possess the capacity to do purification of GOX. Table 6 summarizes the results obtained for glucose oxidase as shown in this section.

3.1.7 Inulinase

In one of the first investigations, the enzyme inulinase was extracted from *Kluyveromyces marxianus* into a reversed micelle phase of the cationic surfactant BDBAC in isooctane/hexanol with an 87% recovery yield of and a 2.8 purification factor (Pessoa Jr & Vitolo 1998).

In regard to inulinase purification, different methods of reaching this goal can be found in the Literature, such as expanded bed (Pessoa Jr & Vitolo, 1998; Kalil, Maugeri-Filho & Rodrigues, 2005), liquid-liquid extraction by reversed micelles (Pessoa Jr & Vitolo, 1998), cation-exchange chromatography (Kalil *et al*., 2010), two-step approach based on precipitation with ethanol followed by ultrafiltration (Golunski *et al*., 2011) and use of four steps: ultrafiltration, DEAE Sepharose fast flow anion-exchange column chromatography, gel filtration and filtration (Li *et al*., 2012). A comparison between the results obtained in all cited methods showed that liquid-liquid extraction promoted the worst results (20.4% enzyme activity recovery). All the other methods promoted enzyme activity recovery between 67.5 and 93%, and the ion-exchange expanded bed chromatography achieved the highest purification factor, of 10.4.

Using the anion-exchange expanded bed chromatography technique with diethylaminoethanol (DEAE), inulinase was recovered directly from the cultivated medium of *Candida kefyr*. The highest enrichment factor obtained was 4.3, concentration factor of 2.8 and 93.1% enzyme recovery. The selectivity and specificity of the chromatographic technique were higher than those of the other techniques tested for this enzyme (Pessoa Jr & Vitolo 1998).

Kalil *et al*., (2005) also purified inulinase from *Kluyveromyces marxianus* using cation-exchange expanded-bed chromatography; the overall yield of inulinase activity was 78%, with a 10.3 purification factor. The same group, in 2010, used cation-exchange chromatography to purify inulinase produced by *K. marxianus*, and obtained results lower than in 2005: 67.5% recovery with a 6.6-fold purification factor (Kalil *et al*., 2010). Golunski and coworkers (2011) using a two-step approach based on ethanol precipitation followed by ultrafiltration obtained a 5.5-fold purification of the crude extract from *K. marxianus*,

with 86.1% yield. Table 7 summarizes the results obtained for Inulinase as shown in this section.

3.2 Antimicrobials

3.2.1 Clavulanic acid

Clavulanic acid (CA) is a beta-lactamic molecule that presents weak antibiotic activity, but is a potent beta-lactamases inhibitor (Haga *et al*., 2013). The first attempt on CA extraction employing aqueous two-phase systems was performed by Videira and Aires-Barros (1994). The authors studied the aqueous two-phase system composed of PEG and potassium phosphate. The results revealed that this molecule showed high affinity with the PEG-rich phase with partition coefficients ranging from 1.5 to 114 and high recoveries (75%). By increasing the tie-line length and pH, it raised the potassium clavulanate partition coefficient in all conditions tested and 99% of the clavulanate was recovered in the PEG phase. Silva *et al*., (2009) evaluated a similar system to that used for Videira and Aires-Barros (1994), to purify CA from fermented broth of *Streptomyces clavuligerus*, attaining a 100% yield and a 1.5-fold purification factor. Later on, the same authors, Silva *et al*., (2012), studied the strategy of combined methods for the purification of CA from fermented broth of amino acids: first, ATPS composed of PEG/potassium phosphate followed by an anion-exchange chromatography. As a result, a concentration factor of 2 and 100% of purification in relation to the amino acids lysine, proline, histidine and tyrosine was achieved. Viana-Marques *et al*., (2011) studied the extractive fermentation of CA by *S. clavuligerus* using aqueous two-phase systems composed of PEG and phosphate salts. In that work, the CA extraction in stirred flasks was evaluated by a 2^{4-1} fractional factorial design followed by a 2^2 central composite design. The variables investigated were PEG molar mass, concentrations of PEG and phosphate salts and agitation intensity. The results revealed that agitation intensity and PEG molar mass were the most significant variables to the process. So, the authors optimized the process and the best results were showed in terms of partition coefficient (K = 8.2), CA yield in the PEG-rich phase (η = 93%) and productivity (P = 5.3 mg/L.h). Furthermore, studies in the optimized condition to scale-up the process, e.g. bench-scale fermenter, were carried out and revealed better results than the ones obtained in stirred flask. As an alternative to the aqueous two-phase systems based on polymers/salts, a novel inexpensive and stable ATPS composed of PEG and sodium polyacrylate (NaPA) supplemented with NaCl and Na_2SO_4 was investigated by Pereira *et al*., (2012) to extract CA from fermented broth. The authors evaluated the influence of PEG-molecular size and polymer concentrations on commercial CA partitioning at 25 °C. The data showed that commercial CA was preferentially partitioned for the PEG-rich phase with a partition coefficient varying from 1 to 12 depending on the system composition. The partition to the PEG phase was increased in the systems with high polymer concentrations. Moreover, the salt Na_2SO_4 caused higher CA preference for the PEG-phase than NaCl. The systems with a 10% of PEG 4,000, 20% of NaPA 8,000 and 6% of Na_2SO_4 composition were selected as the optimal ones in terms of recovery of CA from fermented broth of *S. clavuligerus*. The partition coefficient (9.15) were competitive with commercial extraction methods of CA (11.91).

However, liquid-liquid extraction by micellar systems did not promote similar results to the ATPS ones. Santos *et al*. (2011) employed mixed micellar systems composed of Triton X-114 and AOT, achieving partition coefficient 1.48 and 86.3% recovery in the micelle-rich phase. Andrade *et al*. (2011) employed the surfactants $C_{10}E_4$ and DDAO, with a 52% recovery and removal of 70% of the contaminant proteins. Further investigation was led by Haga *et al*. (2013), who used $C_{10}E_4$ with the addition of CTAB or AOT surfactants. In these systems, a decrease in the partition coefficient value was achieved: 0.87, for the mixed micellar systems, 1.44 was obtained with CTAB/$C_{10}E_4$, while 0.78 was observed with AOT/$C_{10}E_4$.

3.2.2 Tetracicline

Another antibiotic molecule, tetracycline, was extracted from the fermented broth of *S. aureofaciens* by Pereira *et al*. (2013). The authors compared the conventional ATPS PEG/Na_2SO_4 and [Ch]Cl/K_3PO_4 with ATPS composed of PEG and cholinium-based salts, e.g. liquid ionic, which are very recent systems (Freire *et al*., 2012).

Table 7: Inulinase extraction/purification parameters from different techniques.

Technique	Yield (%)	Purification factor	Reference
ATPS-micellar	90.0	3.0	Pessoa Jr and Vitolo (1998)
Anion-exchange expanded bed chromatography	93.1	05.8	Pessoa Jr and Vitolo (1998)
Cation-exchange expanded-bed chromatography	74.0	10.4	Kalil *et al*. (2005)
Cation-exchange expanded-bed chromatography	67.5	06.6	Kalil *et al*. (2010)
Ethanol precipitation and ultrafiltration	86.1	05.5	Golunski *et al*. (2011)

ATPS: aqueous two-phase systems.

Table 8: Antimicrobials extraction/purification results from different techniques.

Biomolecule	Technique	Yield (%)	Purification factor	Reference
Clavulanic acid	ATPS-polymeric	75.0	-	Videira *et al*. (1994)
Clavulanic acid	ATPS-polymeric and anion-exchange chromatography	100.0	1.5	Silva *et al*. (2009)
Clavulanic acid	ATPS-polymeric (extractive fermentation)	93.0	-	Viana-Marques *et al*., (2011)
Clavulanic acid	ATPS-polymeric	55.0	-	Pereira *et al*. (2012)
Clavulanic acid	ATPS-micellar	86.3	-	Santos *et al*., (2011)
Clavulanic acid	ATPS-micellar	52.0	-	Andrade *et al*. (2011)
Clavulanic acid	ATPS-micellar	21.5	-	Haga *et al*. (2013)
Tetracycline	ATPS-ionic liquid-based	higher than 80.0	-	Pereira *et al*. (2013)
Nisin	ATPS-micellar	-	5.6	Jozala *et al*. (2008)

ATPS: aqueous two-phase systems.

Both systems were efficient to extract tetracycline from the fermented broth. However, the latter promoted extraction efficiencies higher than 80%. The authors have observed that not only the nature of liquid ionic but also the pH of the medium had a significant influence on the partition of tetracycline, and the phase that the target biomolecule was recovered relied on the cholinium-based salt used. According to the authors, these systems are applicable to extract tetracycline from complex medium and can be envisaged as valuable platforms to be applied at industrial level by pharmaceutical companies.

3.2.3 Nisin

Nisin is an extracellular antimicrobial peptide that belongs to the class of bacteriocins and was discovered in 1928. It is produced by *Lactococcus lactis*, and it is used as a food preservative. Nisin use is approved by the Food and Drug Administration (FDA) and generally recognized as safe (GRAS), meeting the requirements of safe food with fewer chemical additives (Jozala *et al*., 2012).

The partitioning behavior of nisin in ATPS-M was also investigated, and the results showed an effective separation of the biomolecule from other compounds present in the fermentation broth. The separation was attained using an ATPS-M formed of Triton X-114, and the separation method was capable of extracting nisin into the micelle-rich phase, while removing the majority of the impurities to the micelle-poor phase, obtaining a partition coefficient close to 2 (Jozala *et al*., 2008).

Jozala *et al*., (2012) studied the addition of salts in Triton X-114 ATPS-M to investigate nisin partitioning behavior, and the influence of electrolytes. In the presence of only buffer, partition coefficient values were around 3, nevertheless in the presence of Mg_2SO_4 and $(NH_4)_2SO_4$, nisin showed partition coefficient of 5.6 and 5.4, respectively. These results demonstrated that, aqueous two-phase system can be optimized, even with a simple addition of electrolytes. Table 8 summarizes the results obtained for antimicrobials as shown in this section.

3.3 Colorants

Ventura *et al*. (2013) evaluated the recovery of red natural colorants from the fermented broth of *Penicillium purpurogenum* employing aqueous two-phase systems based on ionic liquid, specifically imidazolium and quaternary ammonium using a potassium citrate buffer, as the salt component. In order to achieve optimum conditions, the authors used different pH, chemical structure and concentrations of liquid ionics and salt. The systems [N2,2,2,2]Br-based ATPS promoted a higher capacity of isolating the colorants from the proteins. Moreover, it was observed that the red colorants partitioning is favored using short tie-lines and higher pH. The authors achieved 24.4 partition coefficients and 96.6% yield.

Another natural dye carmine partitioning was studied by Mageste *et al*. (2009) employing aqueous two-phase systems prepared by mixing aqueous solutions of polymer (poly(ethylene oxide) - PEO) or copolymer (L35) with aqueous salt solutions (Na_2SO_4 and Li_2SO_4). The experiments were carried out as a function of polymer molar mass, pH, hydrophobicity, system tie-line length and nature of the electrolyte. The authors observed that the partition of carmine dye was dependent not only on the electrolyte nature but also on the pH of the system; and it was achieved a partition coefficient of 300 in the best conditions, which is significantly high, demonstrating the potential of this technique to recover carmine dyes. The results of partition with the salt Li_2SO_4 was better than the ones achieved with Na_2SO_4. Carmine molecules were concentrated in the polymer-rich phase. The authors used statistical tools to optimize the process; the variables analyzed were concentration of $Li_2SO_4$11 and PEO 1500 and pH under the response partition coefficient of carmine. The maximum partition coefficient of carmine was obtained with PEO and sulfate concentrations equal to 28.16% and 11.57%, respectively, and the parameter that had the highest influence on the carmine partition to the top phase was the PEO concentration.

3.4 pDNA

Plamidial DNA (pDNA) as well as RNA recovery was also studied by using ATPS. Johansson *et al*., (2012) demonstrated a ATPS-polymeric that yield in the final salt phases is 60-70%. pDNA presented some attraction with polyacrylic polymer at pH 5.0-6.0, and a repulsive interaction above pH 6.0, probably due the formation greater charges both molecules.

Rahimpour *et al*., (2006) presented two different aqueous two-phase systems to be optimal for plasmid yield recovery (100% pDNA and 32% RNA) and one for RNA depletion (78% pDNA and 23% RNA), both using PEG 400/citrate. The best phase yield differences between pDNA and RNA were of 83%, achieved by Wiendahl *et al*., (2012), using PEG/PO4 system and the genetic algorithms. However, a better separation of pDNA and RNA (83%) was achieved by Wiendahl *et al*., (2012) using PEG/PO_4 system and genetic algorithms.

Ebrahimpour *et al*., (2010), used a chromatographic process of stable expanded beds which enables plasmid DNA isoforms such as supercoiled (sc) or open circular (oc) to be recovered directly from lysate with cell debris and key impurities such as chromosomal DNA, RNA, proteins and endotoxins, without the need for prior removal of suspended solids. He achieved 73.1% sc and 15.4% oc recoveries. The growth to full potential of applications for expanded bed technology in bioproduct recovery may be considered to be currently limited by the availability of suitable adsorbents and columns in terms of efficiency and cost (Oelmeier *et al*., 2011).

In contrast to labor and time-consuming chromatographic techniques, particulate adsorbents are a very promising alternative for rapid isolation of plasmid DNA from bacteria (Paril *et al*., 2009). To date, cationic micro and nanoparticle-based adsorbents with high binding capacity towards plasmid DNA have been developed (Chiang *et al*., 2005; Paril *et al*., 2009). However, such cationic adsorbents are intrinsically low-selective and readily adsorb other forms of nucleic acids along with plasmid DNA. Shakhmaeva *et al*., (2011), proposed an effective method of purifying supercoiled plasmid DNA from contaminating nucleic acids using water suspension of nanosized, negatively charged multi-layered CNTs (carbon nanotubes). The method is suitable for fine purification of supercoiled plasmid DNA preparations after chromatographic or particle-based isolation.

Recent developments in the field of pDNA vaccine research include an improved method for pDNA purification with a pre-column removal of impurities by selective metal cation-induced precipitation (Ongkudon & Danquah 2011). It has been found that addition of $CaCl_2$ into the bacterial cell lysate can be used to selectively precipitate RNA from plasmid DNA but, at a reasonably high concentration, thus making it economically unattractive. Furthermore, an extra purification stage, such as chromatographic removal, needs to be integrated to remove a small proportion of non-precipitated RNA (Eon-Duval, Gumbs & Ellett 2003). Endotoxins also show better interaction with free metal ions than pDNA, suggesting that there is a huge potential for selective removal of endotoxins using metal ions to be integrated into a commercial pDNA production line (Ongkudon & Danquah 2011). One of the major concerns on cation-induced pDNA purification is the binding of cations on pDNA molecules, which could affect its yield as well as its biological functionality. A study, however, has found that treatment with EDTA could potentially selectively resolubilise pDNA back into its native form, hence leading to high yield and enhancing further downstream processes (Tan *et al*., 2007).

3.5 LPS

Bacterial endotoxins are lipopolysaccharides (LPS) derived from the outer membranes of gram-negative bacteria. The highly pyrogenic nature of bacterial endotoxins made the terms of pyrogen, endotoxin, and LPS synonymous. Endotoxin is known to cause reactions in animals with symptoms of high fever, vasodilation, diarrhea, and in extreme cases, fatal shock (Erridge *et al*., 2002; Ogikubo *et al*., 2004).

There is a possibility to remove endotoxins present in protein solutions using ATPS-M (Aida & Pabst 1990; Magalhães *et al*., 2007). Above critical micellar concentration of surfactants (CMC), endotoxins can be accommodated in the micellar structure by non-polar interactions of alkyl chains of lipid A and the surfactant tail groups, and are consequently separated from the water phase (micelle-poor phase). By performing Triton X-114 phase separation, endotoxin levels in recombinant proteins derived from *E. coli* have been reduced by as much as 98% of the original amount in a single step (Lopes *et al*., 2011).

The study led by Ongkudon and Danquah (2011) concluded that selective endotoxin removal can conveniently be carried out at a pH condition similar to that of alkaline-lysed cell lysate and at a low $ZnSO_4$ concentration. It was also reported that this method provided ease of subsequent plasmid DNA purification. Due to protein-endotoxin interactions, endotoxin removal from protein solutions requires techniques that result in strong interactions with endotoxin, such as affinity chromatography, which has proven to be one of the most effective methods (Anspach 2001; Wei *et al*., 2007; Sakata *et al*., 2011; Li *et al*., 2011); however, this method is not highly reproducible and may be followed by a significant loss of the product being purified. In addition, the adsorption capacity of adsorbents is generally low (Lee *et al*., 2003).

In addition, previous studies regarding large-scale purification have shown that Triton X-114 phase separation can also be applied for removal of higher concentrations of LPS from large-scale protein purification (Aida & Pabst 1990; Cotten *et al*., 1994; Adam *et al*., 1995; Liu *et al*., 1997; Reichelt *et al*., 2006; Rozkov *et al*., 2008; Jensen

et al., 2008). However, the low concentration obtained and the thermal sensitivity of target proteins brings difficulties to the industrial purification of biomolecules, which usually involves high cost techniques. Lopes *et al*. (2011) employed ATPS-M to remove LPS from preparations, which contained recombinant protein of pharmaceutical interest (GFPuv). The mass balance results were of approximately 100% and recovery of the target protein in the dilute phase ended in approximately 100%. In condition with 4.0% (w/w) TX-114/buffer at 60.0°C, the partition coefficient for pure GFPuv (K_{GFPuv} = 13.85) was lower than for cell homogenate (K_{GFPuv} = 15.00). A purification factor (FP) of 10 fold of the target biomolecule was obtained. The removal of LPS in a single purification step to safe levels is extremely difficult, due to the complex chemical composition of these molecules.

Mercaldi *et al*., (2008) successfully employed ATPS-M containing Triton X-114 for LPS removal and hydrophobic proteins. Results showed a reduction of more than 90% of LPS, with final LPS concentration of 44.00 EU/mL in the product. Cheng *et al*., (2008) showed that removing LPS by adding 1% Triton X-114 to the SP-Sepharose column washing buffer could reduce the final LPS level of purified hG31P protein (human G31P) from 13,500 EU/mg to 54.00 EU/mg. Rozkov *et al*., (2008) also reported the use of Triton X-114 to remove LPS from preparations containing plasmids expressed in cell cultures; these authors achieved 95% of LPS removal with two cycles and 99% with three extraction cycles. According to Schädlich *et al*., (2009), LPS concentration per mg/L could be reduced by additional 99% to 15.00 EU (and 8.00 EU after two extraction cycles). In addition, 86% of L1N10 protein (vaccine against HPV infection) was recovered after one extraction using TX-114 and 83% of the protein in the second one. In order to reduce LPS molecules to a tolerable limit, the ATPS-M should be integrated, for instance, by using an affinity chromatography process. This process could ensure high-resolution removal of reminisce.

Lopes *et al*. (2012) applied IMAC with Ca^{+2}-IDA to remove most of the lipopolysaccharides (LPS) contaminants (higher than 90%) from the end product (rGFP), with a substantial advantage in time, effort, and production costs. The highest adsorption capacity obtained was 2,677,061 EU/mL. Factors such as pH and ionic strength were essential to reach an effective LPS removal for contaminant levels; this technique is recommended for the removal of contaminating LPS present in different steps of a purification process at concentrations between values lower than 100 EU/mL and 100,000 EU/mL.

On the other hand, several purification methods have been developed to remove LPS, such as ultrafiltration (Yamamoto & Kim 1996; Jang *et al*., 2009), anion-exchange chromatography (Chen *et al*., 2009), cation-exchange chromatography (Kunioka & Choi 1995; Morimoto *et al*., 1995), affinity resins (Lowe *et al*., 2011), histidine (Matsumae *et al*., 1990), two phase micellar extraction (Liu *et al*., 1996; Nikas *et al*., 1992), and Polymyxin B (Karplus*et al*., 1987; Petsch & Anspach 2000). Ion-exchange chromatography uses the ability of charge titration to separate LPS from the protein of interest. Histidine and other affinity resins can also nonspecifically bind the LPS. Ultrafiltration does not scale well, due to relatively low flow rates, and often leads to large product loss. Polymyxin B is also not suitable for products destined for intravenous use, because the antibiotic is physiologically active in humans (Damais *et al*., 1987). Every protein presents different problems during purification and endotoxin removal, but Lowe *et al*., (2012) developed a general method that worked well in endotoxin reduction for several His-tagged proteins, even using the C41DE3 *E. coli* strain, which has an unusually thick cell wall and LPS layer (Chen *et al*., 2009). The authors found that pretreatment with deoxycholic acid and Triton-114, prior to loading onto the first affinity column, greatly reduce the endotoxin load of the protein preparation. Additionally, using a similar wash while the protein is bound to an immobilized metal affinity chromatography (IMAC) column eliminates most of the endotoxin, and levels can be reduced to below the limit of detection on an anion-exchange chromatography (AXC) column.

A more recent method is the use of magnetic particles to extract different biomolecules, with the potential to remove along-side LPS. This is a simple methodology that involves the addition of magnetic microspheres into complex biological feedstock in order to purify the target molecule (Safarik & Safarikova 2004). Moreover, the addition of specific ligands on the microsphere surfaces can largely improve the separation selectivity (Peter*et al*., 2009). Indeed, several magnetic microspheres are commercially available to capture tagged proteins (Malpiedi *et al*., 2013). For example, Gray *et al*., (2010) used MagneHis^{TM} (Promega) Protein Purification System, from Promega, to purify His-tagged proteins directly from *E. coli* lysates. This methodology demonstrated high operational productivity, even for unclarified feedstock, achieving similar values with clarified medium (Lan *et al*., 2011).

Table 9: LPS removal results from different techniques.

Technique	Product purified	LPS removal (%)	References
ATPS-M/Triton X-114 single step	GFP	98	Lopes *et al*. (2011)
Chromatography with Triton X 114 in buffer	hG31P protein	99.6	Cheng *et al*. (2008)
ATPS-M/Triton X-114 - 2 steps	pDNA	95	Rozkov *et al*. (2008)
IMAC	GFP	90	Lopes *et al*. (2012)

ATPS: aqueous two-phase systems; IMAC: immobilized ion affinity chromatography.

Even though there is an extended use of magnetic separation at micro and preparative scale procedures, mainly as a consequence of its simplicity and the elimination of clarification steps, most of magnetic separation still has not been industrialized because presents limited capacity and more of them cannot work continuously (Lindner *et al*., 2010). Table 9 summarizes the results obtained for LPS removal as shown in this section.

Conclusion

The potential of purification processes has been studied during a long time and each one has its value. The mechanism of partitioning is complex and relates on knowledge of some physicochemical characteristics of both the purification system and the product of interest. In this review, the importance of some standard, simple and robust techniques of separation for recovery of biological products was highlighted. As it could be noticed, for the majority of biomolecules the separation purity increases when two, three or more combined purification techniques are used. Chromatographic methods still remain the most efficient, however, with different support types. New extraction methods, as aqueous two-phase systems of PEG-based salts and cholinium, *i.e*., liquid ionics; micellar aqueous two-phase systems using solvents and surfactants; extractive fermentation with ATPS are relevant for both their cost-effectiveness and time-saving of the purification process reducing the number of steps. In this case, the cost-benefit aspects are very important and should be taken into account in the biomolecules purification process. These extraction methods have more economic advantages, since the components used for the process are relatively low and quite available when compared to chromatographic methods, as polymers, salts, nonionic surfactants, among others constituents.

Among these advantages, ATPS can be employed for partitioning of whole cells and a different biomolecules types, such as: enzymes, antimicrobials, colorants, virus, nucleic acids, recombinant proteins, DNA plasmids, antibodies, antigens, growth factors, hormones, lipopolysaccharides and others. With simple operation, non-denaturing (70-80% w/w of water content), rapid mass transfer and selective separation aqueous two-phase system (ATPS) is an attractive technique to be used as the initial separation step of biomolecules, for it has presented high recovery yields (around 70-200%, in the case of enzyme activity recover) and even adequate purification factors (nearly 1.5 to 5.0), which could be improved with subsequent high-resolution steps. In addition, ATPS has a low intrinsically associated running cost, seeing that scale-up processing is readily obtainable from lab-scale experiments, beyond, can be an ecofriendly simple separation techniques that allows recycling all the components and so diminish environmental load.

Although, some biological products extracted through scale-up process using ATPS were not successfully achieved. Failures were observed in polymer/polymer ATPS with high viscosity as well as slow segregation, low especially polyethyleneglycol (PEG)/salt system causing corrosion of equipment and precipitation of target product.

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Abbreviations

BM, bone marrow; CD, cluster of differentiation; CFC, colony forming cell; EDTA, Ethylenediaminetetraacetic acid; EPO, erythropoietin; FBS, fetal bovine serum; Flt 3, flt3 ligand; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HSC, hematopoietic stem cell; IMDM, Iscove's modified Dulbecco's medium; IL, interleukin; MNC, mononuclear cells; PBS, phosphate buffered saline; RB, roller bottle; RT, room temperature; SCF, stem cell factor; UCB, umbilical cord blood.

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