Chapter 3

Lipase Production and Purification from Fermentation Broth Using Ionic Liquids

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3.1 INTRODUCTION

The downstream processing of biotechnological products consists of different unit operations, which depend on the product characteristics, the required level of purification, and whether the product is intracellular (formed inside the cell) or extracellular (secreted into the surrounding medium). If biological cells are not involved in the production stage, the reaction medium can be directly concentrated and the target product purified; otherwise, a first step of cellular lysis may be required to release the target product, followed by a cell, or cell debris, separation.

The first step thus involves the separation of cells from the fermentation broth, which is normally carried by common techniques such as centrifugation and filtration (Krieger et al., 1999). The various stages of the downstream processing include the concentration, extraction, purification, and polishing of the compound of interest. The main difference between the product concentration and purification is that the latter involves the separation of unwanted components, which closely resemble the product in its physical and chemical form. Because the number of techniques required for the complete purification is often high and complex, this step is the most expensive in a (bio)product production. The final task is the (bio)product polishing, that is, the preparation of the purified product in a high purity level or in other words, the complete isolation of the target product from the main components/contaminants present, in a stable form, portable and convenient to use. A scheme of a bioprocess is summarized in Fig. 3.1.

In 2014, nearly 6500 enzymes were known, of which more than 200 were commercialized. The outstanding increase in the enzymes sale, allied to the improved understanding of their production biochemistry, fermentation process design, and recovery technologies, has promoted an increase in the number of
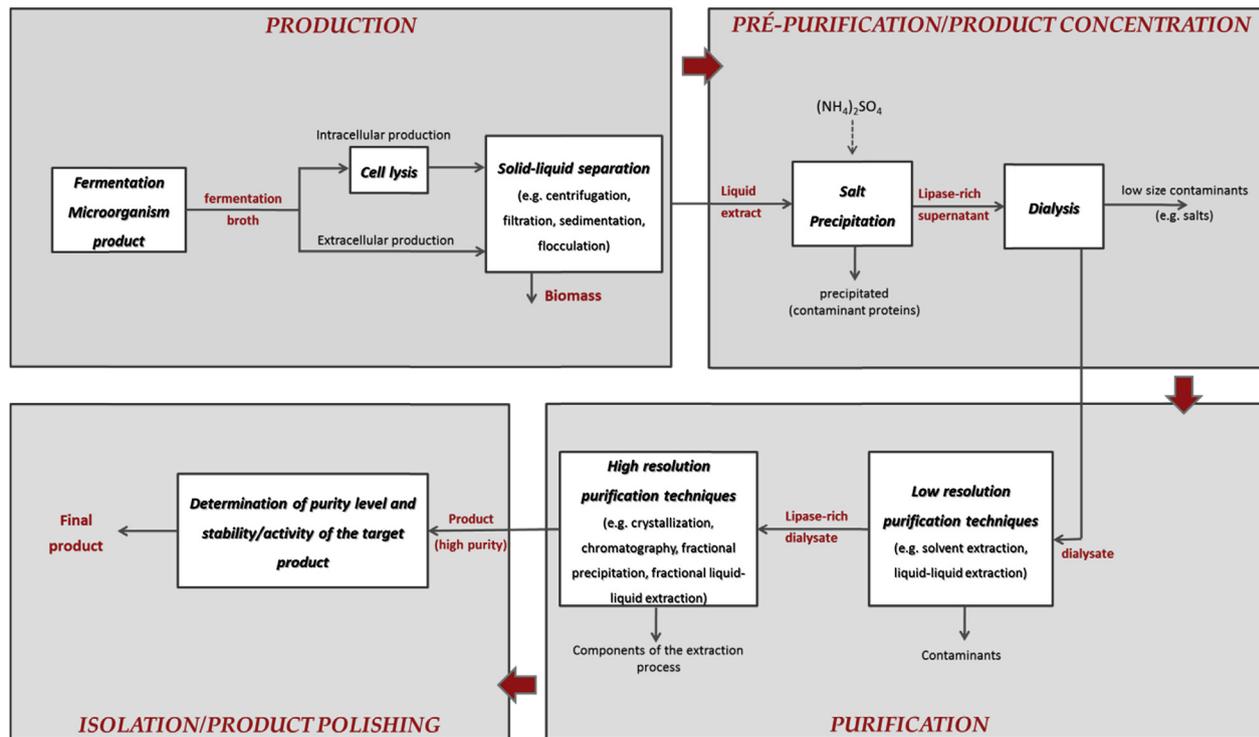
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FIGURE 3.1 Scheme representing the production and purification steps of a general target microbial product.

enzymes that can be affordably produced. Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3), which hydrolyze ester linkages of glycerides at the water−oil interface, are one of the most important and well-known classes of enzymes already in literature (data collected from the *ISI Web of Knowledge* indicates that more than 122,000 articles were published with *lipase* as one of the topics, and from this set 54 are reviews focused in the *lipase + production + purification* (Isi Web)). Considering the wide number of lipase applications (Sharma et al., 2001), namely in leather and cosmetics processing, food, animal feed, pulp and paper processing, textile industry, biotechnological and chemical fields, and their ability to catalyze a plethora of reactions in aqueous and nonaqueous media (esterification, alcoholysis, and acidolysis (Sharma et al., 2001; Otero et al., 2005; Byun et al., 2007)), the focus on these compounds has been increasing in the last years, enabling them to emerge as a key class of enzymes. This accrued interest is also related with their chemo-, regio- and enantio-specific behavior. Meanwhile, the downstream processing of lipases is equally difficult and expensive, since this step is crucial to obtain the enzymes with a high purity level while maintaining their enzymatic activity and stability behavior. The most conventional extraction technologies are still being extensively used for protein purification; however, these methodologies are characterized by a significant number of drawbacks, namely difficulties in scale-up, high production costs, and lack of suitable biocompatible solvents. These issues raise concerns not only from scientists working in the field, but also from the industries responsible for the lipase production and the companies that actually use (or could use) lipases in their processes, due to the consequent cost of this raw material. Therefore, cost-effective methods that can continuously separate, concentrate, and purify proteins are of great commercial interest. Because lipases are of microbial origin (Thakur, 2012), their production is done by means of microbial fermentation processes (Saxena et al., 2003). Most commercial applications do not require homogeneous lipase preparations, however a certain degree of purity can enable their efficient and successful usage. Despite the versatility of the lipase production and purification conditions, these steps were not studied and optimized until recently.

The traditional methods to purify macromolecules involve, as mentioned above, several stages, normally following the ammonium sulfate precipitation, dialysis, ionic and affinity chromatography and/or electrophoresis. The search for inexpensive production systems capable of producing large quantities of lipases has resulted in the development of new technology platforms using different steps of purification, based on diverse extraction methodologies. The main developments were carried taking into account some heuristic rules that will dictate the success of the extraction/purification technology applied, namely:

1. The mass transfer and equilibrium should be fast and reached by relatively low energy inputs.
2. The extraction process should be quick and highly selective.

3. The separation process should be preferentially carried out at room temperature to minimize the energy costs of the purification step.
4. Novel extraction processes should be more economical than those currently applied.
5. The purification processes should not affect the chemical structure, activity, and stability of lipases.
6. The scale-up process should be possible and predictable from the lab to the pilot and industrial scales.
7. The continuous mode should be possible to apply, aiming at promoting the processing of higher amounts of the fermentation broth and the continuous purification, causing a consequent decrease in the overall costs associated.
8. The extraction processes should be designed as much as possible to be implemented and (easily) incorporated along with the production step, aiming not only at the continuous purification but also simultaneously promoting the continuous production and purification tasks (extractive fermentation).

To better understand the idea behind this work, it is necessary to briefly distinguish the different techniques already applied in the extraction/purification of microbial lipases, mainly considering their division in technologies of low and high resolution. In this context, a brief description and analysis of the principal techniques (chromatographic processes) will be presented with the objective of highlighting the importance of seeking new alternative purification platforms for the extraction/purification of lipases.

The most common purification processes used at the time of this writing are the chromatographic and liquid−liquid extraction techniques, commonly represented by polymeric or alcoholic aqueous biphasic systems (ABS), aqueous micellar two-phase systems (AMTPS), or aqueous two-phase flotation (ATPF) systems. Some reviews (Saxena et al., 2003; Antonian, 1988; Aires-Barros et al., 1994; Palekar et al., 2000) have already described the results achieved with these various technologies and it is not our intention to extensively discuss these processes here. This work will focus on the use of ABS based in ionic liquids (ILs) on lipase purification presenting the use of IL-based ABS, their advantages, disadvantages, and the main results reported in literature.

Most lipases produced by microorganisms are extracellular, implying that, independently of the microorganism used, the step of cellular lysis is avoided and only a first step of cell separation is required, which is included in the pretreatment/prepurification of the fermentation broth (Fig. 3.1; Cramer and Holstein, 2011). This step is normally carried out by low resolution processes, such as a centrifugation or filtration, to eliminate the biomass. Following the removal of the cells, a precipitation is applied in the liquid extract, as described in Fig. 3.1, by saturation with salts (commonly ammonium sulfate) or some organic solvents (normally ethanol or acetone), aiming at the

elimination of a significant fraction of contaminants, in particular proteins (Cramer and Holstein, 2011). Some authors propose the use of ultrafiltration to promote the concentration of the fermentation broth, followed by the precipitation (Palekar et al., 2000). The lipase-rich supernatant obtained is then introduced in a dialysis system to eliminate small contaminants, namely cell debris or salts used in the fermentation process and/or in the precipitation of proteins. As final output of the pretreatment task, a dialysate is obtained for posterior use in the purification step, richer in lipase, and free of the most common and highly concentrated contaminants, which will then be used in the purification process. The extent of the separation/purification processes varies with the order and resolution of each purification step. The most common techniques used in the purification are the chromatographic processes, membranes and immunopurification, and the focus of this work, ABS, AMTPS, and ATPF. Various works (including reviews (Saxena et al., 2003; Palekar et al., 2000; Gupta et al., 2004)) have already demonstrated the capabilities of those different purification processes, although the number of works dealing with the use of membranes and techniques of immunopurification (Saxena et al., 2003) is very limited.

Summing up, this chapter focuses on the description and critical analysis of the most recommended techniques already employed in the lipase production and mainly in its purification. Associated or not with the utilization of the vulgar technologies, the common liquid—liquid extraction techniques normally applied in lipase purification and their suitability are assessed and properly discussed. This chapter then discusses improvements in the performance of the liquid—liquid technologies by including ILs as separation and extraction agents. Finally, a critical analysis based on the main conclusions will be presented and future perspectives and challenges will be addressed.

3.2 COMMON METHODS OF LIPASE EXTRACTION

3.2.1 Common Separation Techniques

In most lipase purification plants, chromatography is used to achieve the level of purity required, which is entirely dependent on the final application. Hence, the use and combination of different chromatographic processes may be considered (Krieger et al., 1999). Ion exchange chromatography, gel filtration, and affinity chromatography are three of the best known and most applied chromatographic techniques (Table 3.1). The ion exchange practice (Krieger et al., 1999; Veeraragavan et al., 1990) is extensively used, due to its high applicability.

Other strong ion exchanger groups and Q-Sepharose (Palekar et al., 2000) were also reported. The ion exchange is most commonly used due to its high capacity for loaded protein (Pimentel et al., 1994). This technique is normally

TABLE 3.1 Chromatography Techniques Commonly Applied in the Protein Purification According to Their Main Properties

Properties	Technique
Size	Gel filtration (size exclusion) chromatography
Charge	Ion exchange chromatography
Hydrophobicity	Hydrophobic interaction chromatography
	Reversed phase chromatography
Ligand specifity	Affinity chromatography

characterized by the great degree of lipase purification, due to the establishment of strong electrostatic interactions between the enzyme and the gel (Krieger et al., 1999). In the work reported by Krieger et al. (1999), the lipase was recovered in the form of a high molecular aggregate after gel filtration chromatography, which is the second chromatographic technique most investigated in terms of purification, sometimes being used more than once per process. Schmidt-Dannert et al. (1994, 1996) started with the analysis of a lipase from the thermophilic *Bacillus thermocatenulatus* (DMS 730) by the application of some chromatographic techniques, namely the ion exchange chromatography on Q-sepharose. The authors concluded that by applying one step of hexane extraction, methanol precipitation, and ion exchange chromatography, the lipase was 67-fold more concentrated (Schmidt-Dannert et al., 1994). Some other works (Veeraragavan et al., 1990; Schmidt-Dannert et al., 1996; Borkar et al., 2009; Kumar et al., 2012; Ghanem et al., 2000; Imamura and Kitaura, 2000; Litthauer et al., 2002; Snellman et al., 2002; Abdou, 2003; Taipa et al., 1992) appeared with similar approaches using chromatographic techniques; the results were considered by the authors a success (purification factors using the ammonium sulfate precipitation and different chromatography schemes between 10- and 3028-fold). Gel filtration and affinity chromatography are also important processes used in more than 60% and 27% of the purification apparatus, respectively (Saxena et al., 2003; Palekar et al., 2000; Gupta et al., 2004). The number of works dealing with these techniques is significant, with the chromatographic techniques being used as purification platforms, principally for biopharmaceuticals (some of them also enzymes) where the level of purity required is normally extremely high and the techniques are well described, and for which the knowledge generated allows the manipulation of the process even at large scales.

However, for the lipase, a different scenario is found; the usual procedures are of deficient performance, mainly because they promote a decrease in the lipase activity (Gupta et al., 2004), they are difficult to manipulate, principally

due to the high number of chromatographic units connected in the same process, which results in time-consuming processes with low final yields. The affinity chromatography can be applied in an early stage of the purification process, however the materials are expensive and thus, gel filtration or ion exchange chromatography are preferred due to the lower costs associated with their consumables. Moreover, although gel filtration represents lower purification capacities for loaded proteins, it can be applied in both cases, as an initial step of concentration or in the final contribution for the product polishing.

3.2.2 Liquid—Liquid Extraction

The liquid—liquid extraction technologies described here are represented by three particular types of ABS, particularly those based in alcohols, polymers (systems normally called by ABS), copolymers (systems normally referred to as aqueous two-phase flotation or ATPF), or surfactants (used in the study of aqueous micellar two-phase systems—AMTPS). ABS are normally described as systems formed as the result of the incompatibility between aqueous solutions of two polymers, or a polymer and a salt of high ionic strength. The works on the subject suggest that when the two polymers (most often one of them being polyethylene glycol (PEG) (Saxena et al., 2003; Molino et al., 2013; Mazzola et al., 2008; Hamel and Hunter, 1990; Raja et al., 2011)) are mixed, large aggregates are formed, their affinity for the water molecules is changed, and thus, the polymers tend to separate into two different aqueous phases due to steric exclusion. The same exclusion behavior can be achieved when one polymer is mixed with a high concentration of salt since the salt will interact with the water molecules present, promoting in the same way the phase separation (Diamond and Hsu, 1992). With promising results found for these techniques when applied in the extraction/purification of proteins, these systems were widely investigated and explored, considering not only the system components, but also the main conditions applied in the purification processes, namely temperature, pH, and concentration of the main components used in the preparation of the extraction system (Zaslavsky et al., 1986). The ABS are thermodynamically described by the phase diagrams, presenting the binodal curves and the respective tie-lines (Mazzola et al., 2008).

The binodal curve (red line in Fig. 3.2) represents the borderline between the monophasic and biphasic regions, which means that from these data it is possible to determine the entire region with extraction potential (biphasic). The tie-lines (black lines in Fig. 3.2) allow the description of the compositions of the phases (top and bottom) in equilibrium. The phase diagrams can be used for two different purposes/interpretations. The thermodynamic approach is the first, and the second, no less important, is the interpretation from the point of view of the partition/purification of molecules. The first idea that should be
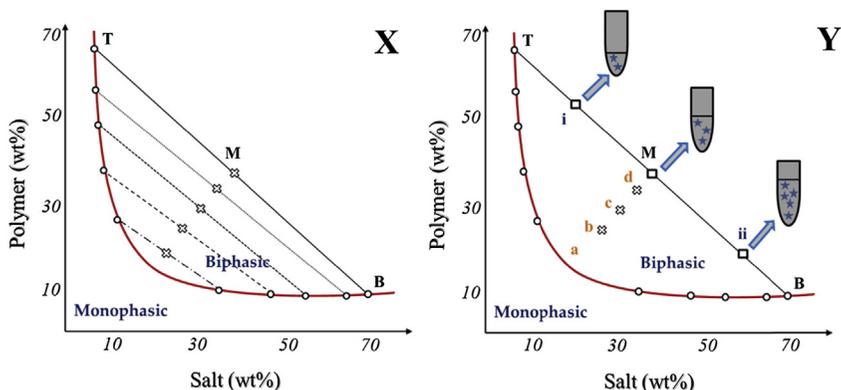
FIGURE 3.2 Illustration of a general phase diagram in mass fraction percentage (wt%) composed of a generic polymer and a salt. In the system, the binodal curve (*red line* (dark gray in print versions)) and different tie-lines (*black lines*) are described for a fixed temperature. (X) In the phase diagram *T* represents the top phase composition, *B* the bottom phase composition, and *M* the overall system composition. (Y) The small letters *i* and *ii* represent extraction points (biphasic point) in the same tie-line of *M* mixture point, *a*, *b*, *c*, and *d* also represent extraction points, but now corresponding to different tie-lines.

clarified is that each point in the biphasic region is different and is part of an extensive number of potential points of extraction with different properties and characteristics, which leads to a large range of conditions to be applied in the extraction of a target molecule. Specifically, two distinct scenarios can occur when discussing a phase diagram. First of all, if different mixture points are considered randomly in the biphasic region (points *a*, *b*, *c*, *d*, *M*), different extraction systems will be formed, as well as different tie-lines, which means that distinct ABS are formed with distinct phase compositions. From the point of view of the extraction process, different extractions are promoted, with different partition and purification phenomena of the solute occurring, since the interactions in both phases and between all system components are different. On the other hand (second scenario), if the same tie-line is considered (Fig. 3.2Y) and different points are chosen (points *M*, *i*, and *ii*), distinct extraction points are obviously formed and characterized by the same top and bottom phases in terms of compositions. In this second scenario, the extraction of one solute is also promoted, but the interaction between all components and the solute will be the same.

In both scenarios some consideration should be taken into account: first, these rules are applied if the same solute is being considered or if the same conditions of temperature and pH are promoted; otherwise, different interactions and (entropic or hindrance) effects can occur (Willauer et al., 2002). Second, the same components can be used, meaning the same pairs of polymer/polymer, polymer/salt, alcohol/salt, IL/salt, or IL/polymer; if not,

different phase diagrams will be investigated and consequently, a different response will be achieved in terms of partition/purification process. Third, it is mandatory to take into account the complexity of the sample feed where the solute of interest is present (in particular when the extraction or purification represents the capture of the solute from the fermentation broth), since the balance of interactions between the target molecule to be purified, the main contaminants, and each one of the components of the extraction system may be different, consequently imposing new rules in the choice of the ABS type and the extraction point in the biphasic region. Summing up, different conditions, components, and solutes may give a distinct response in terms of partition, concentration, and purification, which is normally pointed out as one of the most important reasons for the use of ABS as part of downstream processing or as downstream processes (Molino et al., 2013). Moreover, ABS are generally pointed out as processes capable of providing a mild environment (meaning higher amounts of water), they are nonvolatile, and they require the use of (sometimes) cheap components, allowing an easy scalability. However, they continue to be underestimated as relevant separation techniques by the academic and industrial communities, mainly due to the system complexity (at least three components to form the system), and poor understanding of the partition mechanisms (several interactions controlling the partition phenomenon, few techniques providing a deep and fundamental analysis of those interactions, allowing a complete and controlled manipulation of this extraction platform), leading to a labor-intensive, time-consuming, and high-cost process development (Rosa et al., 2010).

The discrepancies between opinions of different authors concerning the use and applicability of ABS are apparent. Notwithstanding, as shown in Fig. 3.3, the number of studies using these systems, and thus their potential applications, is increasing, which means that the confidence of the scientific community in their potential and applicability keeps growing, and this explains why the number of relevant publications keeps increasing each year. Fig. 3.3 gives evidences that the number of articles dealing with ABS in different fields is significant (1338 reported in the *ISI Web of Knowledge* by July, 2014). These works report different analysis, from the fundamental (based principally in the intermolecular interactions) point of view, passing through the thermodynamic analysis, and culminating in the study and design of distinct applications. The domain of application is one of the key fields, because the importance of the fundamental point of view and the thermodynamic design appears to understand how to manipulate only the systems having as background the final application. The idea of describing a new methodology is of crucial importance, the capacity of the user to properly control the technology is of extreme importance but only taking into consideration the success of the application. In the field of ABS the set of applications is summarized in three principal domains: as product concentration, extraction, and purification methodologies (Molino et al., 2013; Freire et al., 2012; Goja et al., 2013).
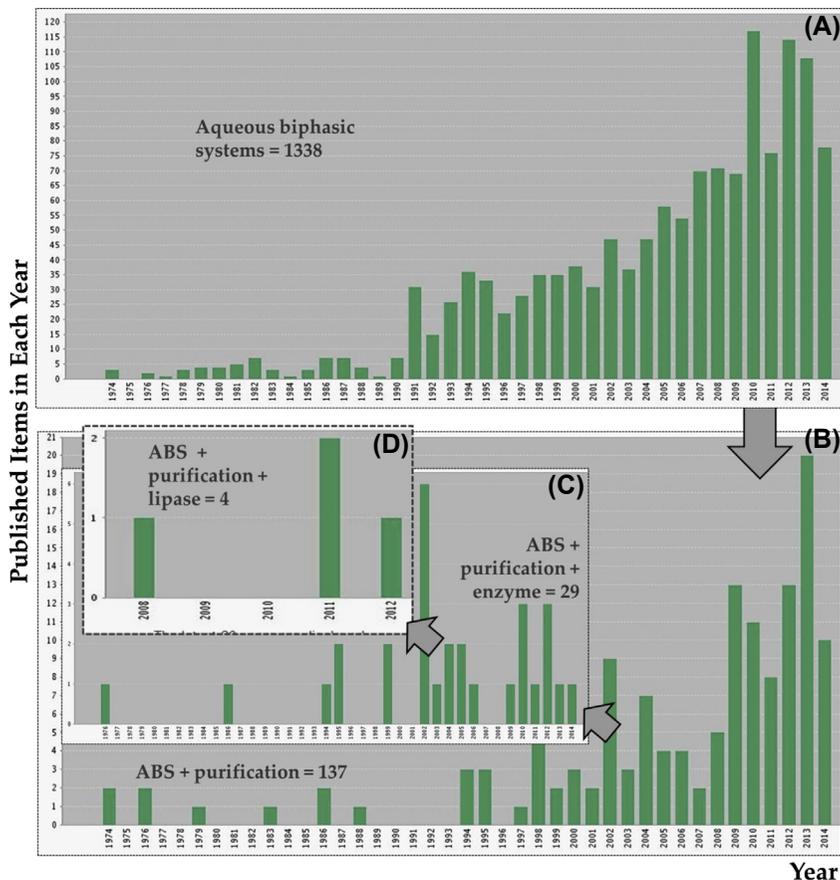
FIGURE 3.3   Brief search in the *ISI Web of Knowledge* on the domains: (A) Aqueous biphasic systems (1338 works); (B) Aqueous biphasic systems + purification (137 works); (C) Aqueous biphasic systems + purification + enzyme (29 works); (D) Aqueous biphasic systems + purification + lipase (4 works). The graphics exposed show the evolution of the ABS field since 1974 and demonstrate the development considering the technique and its application in different fields (1338 works), including the extraction of enzymes (29 works) and, in particular, lipases (4 works).

Considering the purification, a vast range of products have been studied, namely virus, biopharmaceuticals, genetic material (eg, nucleic acids), pollutants (eg, endocrine disruptors), added-value compounds from terrestrial and marine raw materials (eg, fucoxanthin and R-phycoerythrin from brown and red macroalgae), therapeutic proteins, and finally, microbial enzymes (Molino et al., 2013; Freire et al., 2012; Goja et al., 2013). Currently those are the main focus on the use of ABS basically because they are describing compounds whose purification is still very complicated and expensive, imposing the demand for other technologies with high efficiency and capable of maintaining

the activity and chemical structure of the products purified. Most reports in the field describe the use of polymeric systems, where the impact of distinct polymers, their molecular weights, polymer + polymer and polymer + salt combinations, temperatures, and pH values were evaluated (Aires-Barros et al., 1994; Gupta et al., 2004; Molino et al., 2013; Raja et al., 2011; Rosa et al., 2010, 2011).

The partitioning of enzymes in ABS depends on (1) (intrinsic conditions) the physicochemical properties of the solute (eg, protein hydrophobicity/hydrophilicity, charge, and size), and (2) (extrinsic conditions) the ABS type, components, and concentrations of the components applied in the extraction. A plethora of ABS will be shown and discussed in this work. Their selection was based on systems already applied in terms of lipase purification (Zhang and Liu, 2010; Ooi et al., 2009a; Bassani et al., 2010; Zhou et al., 2013; Khayati and Alizadeh, 2013; Li et al., 2010; Ooi et al., 2011a). Thus, ABS with alcohols (Table 3.2), polymers (Table 3.3), surfactants (Table 3.4), and copolymers (Table 3.5) will be reviewed here.

3.2.2.1 Alcohol/Salt-Based ABS

Ooi et al. (2009b) have applied for the first time ABS based in alcohols and salt systems to recover a lipase derived from *Burkholderia pseudomallei*. In this work, nine ABS comprised of ethanol, 2-propanol and 1-propanol; and ammonium sulfate, potassium phosphate, and sodium citrate, were evaluated for their effectiveness/success in recovering the lipase. The lipase from *B. pseudomallei* was successfully purified using a 2-propanol/potassium phosphate ABS in a single-step procedure. A purification factor of 13.5 and a yield of 99% were achieved, without compromising the enzyme activity.

TABLE 3.2 Extraction and Purification of Lipase Using Alcohol-Based ABS

Lipase derived from *Burkholderia pseudomallei* (Ooi et al., 2009b)	
Phase former agents	1-propanol, 2-propanol, ethanol + potassium phosphate, ammonium sulfate, sodium sulfate, NaCl (additive)
Conditions studied	Concentration of the phase former agents Addition of various concentrations of NaCl (additive)
Advantages	Easy recovery of the alcohol, lower costs, enzyme activity is maintained
Disadvantages	Lower purification factors
Maximum extraction and purification performance	PF = 13.5, yield = 99%

TABLE 3.3 Extraction and Purification of Lipase Using Polymer-Based ABS

Intracellular lipase from *Trichosporon laibacchii* (Zhang and Liu, 2010)

Phase former agents	PEG, K_2HPO_4, NaCl (additive)
Conditions studied	PEG molecular weight, different PEG 4000/K_2HPO_4 combinations, pH
Advantages	Combination of extraction and enzyme immobilization approaches Ease of scale-up Possible recycling of the phase components
Disadvantages	20% of enzyme activity loss
Maximum extraction and purification performance	$K = 7.61$; PF = 5.84

Lipase from *B. pseudomallei* (Ooi et al., 2009a)

Phase former agents	PEG, K_2HPO_4, NaCl (additive)
Conditions studied	PEG molecular weight, phase composition, tie-line length, volumetric ratio, feed of crude fermentation broth, pH
Advantages	Single-step approach
Disadvantages	
Maximum extraction and purification performance	PF = 12.42; yield = 93%

Lipase from *B. pseudomallei* (Ooi et al., 2011a)

Phase former agents	PEG, dextran T500, ammonium sulfate, sodium phosphate, magnesium sulfate, potassium phosphate, sodium citrate
Conditions studied	PEG molecular weight, PEG concentration
Advantages	Coupling the upstream and the downstream by applying ABS; extractive fermentation approach was capable of maintaining the viability of the cells in 7 cycles of the repetitive ATPS fermentation Effective approach in terms of process costs and processing time
Disadvantages	Only dextran T500 is suitable to perform the extraction
Maximum extraction and purification performance	Yield = 92.1% (single step)

***C. rugosa* (Lip1) (Bassani et al., 2010)**

Phase former agents	PEG, potassium phosphate salt, NaCl (additive)
Conditions studied	PEG molecular weight, PEG concentration, temperature, NaCl concentration, volumetric ratio, feed of crude fermentation broth

TABLE 3.3 Extraction and Purification of Lipase Using Polymer-Based ABS—cont'd

Advantages	The polymer is not promoting any changes in the secondary and tertiary structures or in the enzyme activity
Disadvantages	Lower purification factors
Maximum extraction and purification performance	Enzyme recovery = 78.3%, PF = 2.3
Porcine pancreatic lipase (PPL) (Zhou et al., 2013)	
Phase former agents	PEG, potassium phosphate dibasic, potassium phosphate buffer
Conditions studied	PEG molecular weight, PEG and salt concentration, temperature
Advantages	
Disadvantages	
Maximum extraction and purification performance	$K = 12.7$, extraction efficiency = 94.7%, PF = 4
Lipase from *R. aglutinis* (Khayati and Alizadeh, 2013)	
Phase former agents	PEG, potassium oxalate, potassium sodium tartrate
Conditions studied	PEG molecular weight, salt, feed of crude fermentation broth
Advantages	
Disadvantages	
Maximum extraction and purification performance	PF = 13.9, yield = 71.2%
Porcine pancreatic lipase (Li et al., 2010)	
Phase former agents	P_{NNC}, P_{ADB}
Conditions studied	P_{NNC} concentration, pH, neutral salts (additives) and their concentrations, pure and crude lipase
Advantages	The recycling of the phase former agents is performed by pH adjustments
Disadvantages	
Maximum extraction and purification performance	PF = 18, recovery of lipase = 83.7%

TABLE 3.4 Extraction and Purification of Lipase Using AMTPS

Lipases A and B from *Chromobacterium viscosum* (Vicente et al., 1990)

Phase former agents	AOT-based reverse micelles, benzene as the organic phase
Conditions studied	Single step extraction of lipase A, back extraction of lipase B, number of steps
Details of the process	Two lipases were removed
Maximum extraction and purification performance	Lipase A: PF = 4.3 and enzyme recovery = 91% Lipase B: PF = 3.7 and enzyme recovery = 76%

Yeast-lipase (Yu et al., 2003)

Phase former agents	AOT-based reverse micelles, isooctane as the organic phase
Conditions studied	pH, AOT concentration, salt concentration (ionic strength), phase—volume ratio, stirring time
Details of the process	In this work the back extraction of the lipase was studied keeping into consideration the following conditions: Ethanol (cosolvent) concentration, pH, stirring time, and pH
Maximum extraction and purification performance	Enzyme extraction = 100% Back-extraction: Yield = 68%

Lipase from *Aspergillus niger* (Nandini and Rastogi, 2010)

Phase former agents	CTAB, isooctane, potassium phosphate buffer
Conditions studied	Forward and backward extraction were studied, taking into account the salt and surfactant concentrations and salt concentration and pH, respectively
Details of the process	Both forward and backward extractions were studied and optimized
Maximum extraction and purification performance	Lipase activity recovery $\geq$ 78%, PF $\geq$ 4.0

Lipase from the *Burkholderia* sp. ST8 strain (Ooi et al., 2011b)

Phase former agents	Triton X-114, Pluronic L31, Pluronic L61, Pluronic L81, Pluronic L121
Conditions studied	Pluronic L81 concentration, addition of additives (salts), back extraction of lipase
Details of the process	The cloud-point temperatures were determined for the surfactant and copolymers
Maximum extraction and purification performance	PF = 7.2 single step of purification, polishing step yield = 89%, K between 0.34 and 4.5

TABLE 3.5 Extraction and Purification of Lipase Using ATPF Systems	
Lipase from *Burkholderia cepacia* strain ST8 (Show et al., 2011, 2012, 2013)	
Phase former agents	EOPO copolymer
Conditions studied	Polymer molar mass, ammonium sulfate concentration, pH, the amount of the loaded crude feedstock, initial volume of EOPO phase situated in the top of the aqueous solution, concentration of EOPO, initial volume of aqueous phase, nitrogen flow rate, flotation time
Details of the process	The recycling of the components was also studied
Maximum extraction and purification performance	Extraction efficiency = 99%

The authors believe that the use of alcohol/salt-based ABS proved to be effective for the purification of solvent-tolerant lipase, not only because of the significant purification parameters obtained without compromising the enzyme activity and structure, but also because the recovery of the alcohol is easy, potentially decreasing the costs associated with the entire process of production and purification. Unfortunately, no more studies were developed and of course, the effect of other process conditions remain unknown, namely, pH values and temperatures of the extraction system, the isolation of the lipase, and the scale-up of the process, making possible the study and implementation of the commercial recovery process.

3.2.2.2 Polymer/Salt-Based ABS

Zhang and Liu (2010) reported the partial purification of an intracellular lipase from *Trichosporon laibachii* by applying different polymeric-based ABS. In this work, the impact of different conditions, in particular the PEG molecular weight, the system's phase compositions, and the pH were evaluated on the lipase partition. The best results obtained in this work reported a partition coefficient of 7.61, with an activity recovery of 80.4%, and a purification factor of 5.84 (PEG 4000 (12 wt%) + K_2HPO_4 (13 wt%), at pH 7 and with 2.0 wt% of NaCl). In this work, a new approach was reported consisting of the combination of ABS with the enzyme immobilization. The main results suggest that this new integrated methodology can be an advantageous and useful technique for the purification of lipases (the in situ immobilization of the lipase in the PEG phase resulted in a highest immobilized lipase activity of 1114.6 U/g). The authors (Zhang and Liu, 2010) claim that this process can be considered cost-effective as well as time-saving, because it is fast and simple, involving a small number of steps (the immobilization step is directly performed in the top PEG-rich phase to achieve an in situ immobilization).

Zhang and Liu (2010) ended their analysis calling the attention to the relative easiness of the scale-up process and possible recycling of the phase components, evidencing that the whole process can be designed to be cost-effective.

In the same line of work, Ooi et al. (2009a) have determined the effect of different ABS based in distinct PEG polymers in terms of their purification capacity. The main focus of this work was the direct purification of a lipase produced by *B. pseudomallei*. The migration behavior of the lipase was observed taking into consideration several parameters such as the phase composition, the tie-line length, the volumetric ratio (V_r), the feed of crude fermentation broth, the system pH and, finally, the addition of neutral salts. The optimum conditions for the direct purification of lipase from the fermentation broth were optimized as follows: ABS based in PEG 6000 + potassium phosphate system at pH 7; a TLL of 42.2 wt%, allowing the fixation of the compositions of the phases in terms of all components and, consequently, of the interactions; a V_r of 2.70, where the same components' compositions in both top and bottom phases and the reconcentration of the lipase in the polymeric phase are achieved; 1 wt% of NaCl used as additive (acts in the polarity of the phases, allowing the differential migration of the lipase); for 20 wt% of crude load. Based on this system, the maximum lipase purification factor achieved was 12.42-fold, with a yield of 93%. The same group developed (Ooi et al., 2011a) an extractive fermentation process for the same lipase, using polymeric-based ABS. This process consisted in the simultaneous development of the cell cultivation and the downstream processing of the extracellular lipase derived from *B. pseudomallei*, in two distinct partitioning systems. The best results found for this integrated process were achieved for the ABS composed of 9.6 wt% of PEG 8000 and 1.0 wt% of dextran T500. The process allowed the biomass accumulation in the bottom phase whereas the lipase was preferentially concentrated into the top phase. A yield of 92.1% was achieved in a single step. The potential of coupling the upstream fermentation with the downstream processing by applying ABS is very attractive, since the purification of the lipase in repeated cycles of extractive fermentation can be achieved, which is ideally effective in terms of process costs (recycling of the cells/biomass for subsequent fermentations) and processing time (the simultaneous purification during batch fermentation was promoted).

Bassani et al. (2010) have determined the interaction between a lipase from *Candida rugosa* (Lip1) and PEG polymers of different molecular weights, through the utilization of fluorescence and circular dichroism. These studies were then applied to the analysis of the enzyme partition mechanism by applying distinct ABS based in PEG (2000, 4000, 8000, and 10,000) and the potassium phosphate salt (at pH 7). The main results presented in this work (Bassani et al., 2010) describe a decrease of the partition coefficients with the PEG molecular weight showing that the enzyme migration is driven by the excluded volume effect and not by the unfavorable Lip1−PEG interactions

(notice the preferential migration of the lipase for the PEG-rich phase). The presence of the polymer was not responsible for any significant changes in the secondary and tertiary lipase chemical structures or in its biological activity. Lip1 was preferentially concentrated in the PEG-rich layer being the PEG 2000, the system in which the best enzyme recovery (78.3%) and the highest purification factor (2.3) were achieved (Bassani et al., 2010).

Zhou et al. (2013) have also studied the use of polymeric-based ABS for the purification of porcine pancreatic lipase (PPL) from crude PPL using PEG and the inorganic salt potassium phosphate dibasic or potassium phosphate buffer (pH 7). Behind the phase diagrams study at room temperature, the authors have evaluated the preferential partition of PPL for the PEG-rich phase, and have concluded that the preference of the lipase for the more hydrophobic phase was independent of the molecular weight of the PEG, when PEG 1000 and PEG 1500 were applied. However, for systems based in PEG 4000, the lipase was more concentrated in the phosphate-rich phase, indicating that important interactions between the lipase and the salt and/or water were created. The authors (Zhou et al., 2013) concluded that at low PEG molecular weight, the PEG-lipase interactions controlled the partition phenomenon, but at high PEG molecular weights, the exclusion phenomenon promoted by the polymer is more important than the polymer−lipase interaction, since the lipase was excluded from the polymer-rich phase. The authors mentioned that the enzyme was efficiently purified in PEG 1500/potassium phosphate (17/13 wt%) systems at pH 7 and 4°C, with an enzyme partition coefficient of 12.7, an extraction efficiency of 94.7%, and a purification factor around 4.

In the same year Khayati and Alizadeh (2013) studied the application of ABS, again based in PEG and various salts (potassium oxalate and potassium sodium tartrate), at pH 6.6 and 24 °C, and directly applied them to the cell-free fermentation broth containing the lipase of interest produced by *Rhodotorul aglutinis*. The most appropriate system to perform the purification of the lipase from the fermentation broth (12.5%, v/w) was composed by PEG 4000 (17.5 wt%) + potassium oxalate (12.5 wt%), being the enzyme partitioned for the PEG (top)-rich phase with a maximum purification factor of 13.9 and lipase yield of 71.2%. The authors concluded that the partition coefficient of the lipase (K_e) decreased with the PEG molecular weight increase. Having these results in mind, it is possible to conclude that probably the fermentation contaminants are somehow forcing the lipase salting-out from the phase where the main contaminant proteins are presented, which means the increase of the purification factors, when these are compared with the purification factors obtained in previous works.

One key problem of the ABS is that the phase-forming polymers could not be efficiently recycled (Li et al., 2010). The lack of efficient recycling strategies imposes high costs to the process and increased environmental pollution. In this context, Li et al. (2010) studied the use of ABS composed by the pH-sensitive copolymer, the P_{ADB}, and the light-sensitive copolymer, P_{NNC}. The top phase is

rich in P_{NNC} and the bottom phase is enriched in P_{ADB}. In this work, the impact of various process parameters, such as the concentration of the phase-forming copolymers, the system pH, and the different types and concentrations of salts on the migration profile of the lipase were evaluated. Considering the main results of the direct lipase purification from the crude material, it was found that the lipase was purified from crude material with 83.7% of recovery and a purification factor of approximately 18-fold (Li et al., 2010). The authors concluded that the lipase partition coefficient could be effectively controlled by using some inorganic salts at certain concentrations. Because those systems were designed by using a light-sensitive copolymer (P_{NNC}) and a pH-sensitive copolymer (P_{ADB}), the recycle of P_{ADB} was carried out by adjusting the pH of the system to its isoelectric point ($pI = 4.1$). On the other hand, the light-sensitive P_{NNC} was eliminated by laser irradiation at 488 nm (Li et al., 2010). In conclusion the authors proposed novel ABS with potential application in the biotechnological industry in particular, as a downstream technology, with reduced costs and limiting any environmental impact.

3.2.2.3 Surfactant-Based ABS: Aqueous Micellar Two-Phase Systems

The liquid−liquid extraction process by applying reverse micelles consists of two fundamental steps: (1) a forward extraction in which the lipase is transferred from an aqueous solution into a reverse micellar organic phase and (2) a back extraction process, where the protein is released from the reverse micelles and transferred into an aqueous phase to be recovered (Taipa et al., 1992; Lee and Chong, 2011). The general process is described by the entrapment of the lipase units in the water pools inside the micelle structure, thus avoiding the direct contact of the macromolecule with the organic solvent, and avoiding one of the most important sources of nonfavorable interactions with the enzyme responsible for its denaturation. In this context, some authors truly believe that this technology can be extensively applied as a downstream process (Molino et al., 2013; Basheer and Thenmozhi, 2010). In 1990, Vicente et al. studied the selective separation and purification of a lipolytic preparation from *Chromobacterium viscosum* by applying AOT-based reverse micelles with benzene as the organic phase. During the production via fermentation, two lipases were produced; lipase A was purified from the original crude enzyme preparation by 4.3-fold with a recovery of 91% and lipase B by 3.7-fold with a recovery of 76%. Later, Yu et al. (2003) used sodium bis(2-ethylhexyl) sulfosuccinate (AOT) reverse micelles in isooctane for the separation of yeast-lipase by a two-step procedure. Different conditions were used in this study, namely the pH and ionic strength of the aqueous phase, the surfactant concentration, the phase volume ratio, the temperature, the stirring time, and finally the cosolvent concentration used in the back-extraction of the lipase. The complete extraction (100%) of the lipase was achieved in the forward extraction using 250 mmol/

L of AOT (surfactant), 0.05 mol/L of KCl in the initial aqueous phase, a stirring time of 10 min, and room temperature. The back extraction was also optimized, resulting in 68% of back extraction yield and 45% of activity recovery yield for the lipase, obtained for 0.5 mol/L of KCl in the second aqueous phase, 3% (v/v) of ethanol, a stirring time ranging from 15 to 30 min at room temperature, and pH 8.0 (Yu et al., 2003).

Later in 2010, Nandini and Rastogi investigated AMTPS based in the cationic surfactant cetyltrimethylammonium bromide (CTAB). They studied the effect of various process parameters on both forward (salt and surfactant concentrations) and backward (salt concentration and pH) extraction processes of a lipase from its crude extract. Both the yield and purification parameters were optimized using a response surface methodology. The results showed that the optimum processing conditions were salt concentration of 0.16 M, surfactant concentration of 0.20 M, and pH 9 for forward extraction, and salt concentration of 0.80 M and pH of 7.23 for the backward extraction, leading to a value of lipase activity recovery ($\geq$78%) and a purification factor $\geq$ 4.0 (Nandini and Rastogi, 2010).

Ooi et al. (2011b) have studied the efficiency of a temperature-induced AMTPS composed of a single nonionic surfactant (Triton X-114) and Pluronics (triblock copolymers) on the partitioning and recovery of a lipase from the *Burkholderia* sp. ST8 strain. In this work, the clouding phenomenon (cloud-point temperature) and the performance of the lipase partitioning in these AMTPS were the principal parameters investigated. Pluronic L81 showed the best results in terms of the lipase partition efficiency to the micelle-rich phase. Based on AMTPS composed of 24 wt% of Pluronic L81 and 0.5 wt% of potassium chloride (KCl), a purification factor of 7.2 was achieved. Meanwhile, the lipase was consecutively extracted from the micelle-rich phase to a new aqueous solution aiming to eliminate the presence of the surfactant from the lipase bulk, as part of the polishing approach, by applying a potassium thiocyanate (KSCN) solution. The yield of the polishing step obtained was around 89% and the partition coefficients of 0.34 and 4.50 were achieved for lipase and surfactant, respectively. The separation of the surfactant from the lipase was achieved ($K_{\text{surfactant}} = 4.50$) and its back extraction was accomplished in 80% (Ooi et al., 2011b).

3.2.2.4 Aqueous Two-Phase Flotation Systems

ATPF is a purification methodology, which combines the use of ABS and solvent sublation (Bi et al., 2010a,b). This technique is based on the use of surface-active compounds with a hydrophilic group (hydroxyl or glucosan) and a hydrophobic group (phenyl or alkyl) in water, which are adsorbed to the surface of nitrogen bubbles of an ascending gaseous stream. The bubbles are then dissolved in the polymer placed on the top of the aqueous solution as depicted in Fig. 3.4 (scheme adapted from (Bi et al., 2010a; Show et al., 2011)). The main advantages of the technique are related with the high
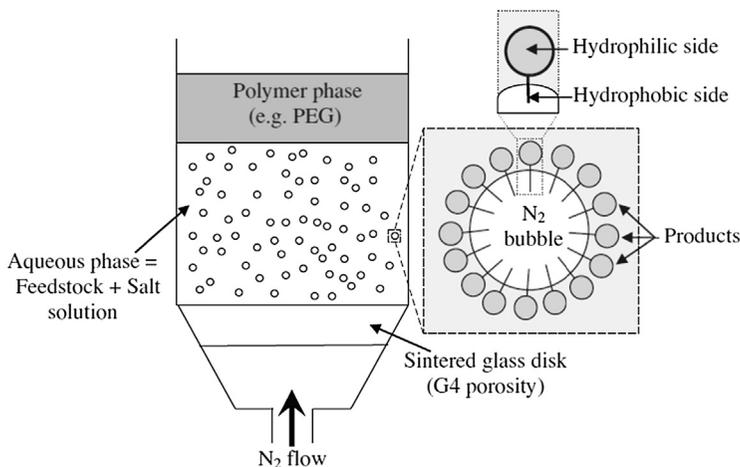
FIGURE 3.4 Illustrative scheme of the ATPF technique representing the use of a nonrecyclable polymer and a nonspecific salt solution. *Adaptation from Show, P.L., Tan, C.P., Anuar, M.S., Ariff, A., Yusof, Y.A., Chen, S.K., Ling, T.C., 2011. Direct recovery of lipase derived from* Burkholderia cepacia *in recycling aqueous two-phase flotation. Sep. Purif. Technol. 80, 577−584.*

concentration coefficient, soft separation, the low dosage of organic solvents required, high separation efficiency, simple operation, and, according to some experts, the low environmental impact (Bi et al., 2009).

Show et al. (2011, 2012, 2013) used this technique having as the main purpose the recovery and purification of lipases. In the first approach, the authors have used ATPF systems composed of a thermo-sensitive ethylene oxide-propylene oxide (EOPO) copolymer and one ammonium sulfate salt for the recovery of lipase from *Burkholderia cepacia* strain ST8 directly from the fermentation broth. Different strategies were considered in terms of the conditions studied, namely the variation of the polymer molar mass, the concentration of ammonium sulfate, the pH of the system, the amount of the loaded crude feedstock, the initial volume of EOPO phase situated in the top of the aqueous solution, the concentration of EOPO, the initial volume of aqueous phase, the nitrogen flow rate, and finally, the flotation time. According to the authors, the lipase was successfully purified from the fermentation broth and then easily separated by using the EOPO copolymer in the design of the ATPF systems. Considering the optimal ATPF' conditions, the authors were capable of efficiently separating and purifying the lipase between 13% and 99%, respectively (Show et al., 2011, 2012, 2013).

These results showed that there was no relevant variation of the lipase specific activities between the products recovered by the application of ATPF systems with fresh and recycled chemicals. Another important approach developed in this work was the recycling of the ATPF phase-forming components, polymer and salt, considered effective in terms of costs associated,

processing time (reduction of the operation time), and environmental impact (the phase components are introduced again in the formation of new ATPF systems) (Show et al., 2011, 2012, 2013).

3.3 LIPASE EXTRACTION BY IL-BASED ABS

In the mid-2010s, novel ABS based in ILs were proposed by Rogers and coworkers (Gutowski et al., 2003). Commonly described as room temperature liquid salts with uncommon properties (Earle et al., 2006; Esperança et al., 2010; Seddon, 2003), ILs have attracted attention from both academia and industry in downstream processes and analytical techniques. Their crescent interest relies on their unique properties, such as the negligible vapor pressure and lack of flammability, as well as their high chemical and thermal stabilities, low melting points, and a large liquidus temperature range (Freire et al., 2012; Brennecke and Maginn, 2001; Aparicio et al., 2010). However their major advantage is a unique capability to solvate a huge variety of solutes, of a wide range of polarities, and to have a significant impact upon their solubility in water. ILs are thus capable of solubilizing several classes of compounds, from polar to nonpolar, organic to inorganic, biocompounds to metals, which means that they can cover the whole hydrophilicity−hydrophobicity (polarity) range. By fine-tuning their characteristics and properties it is possible to develop novel and more effective ABS for a given separation, which is a crucial issue for their use as part of the downstream processing of biocompounds.

It has been shown that the application of ILs as phase forming agents in ABS boosts the extractive performance and the selectivity parameters of a wide range of compounds (Freire et al., 2012). Since the first report on the use of ILs to form ABS by Rogers and coworkers (Gutowski et al., 2003), a large number of works have been dealing with the study of IL-based ABS and prompted the publication of a major critical review (Freire et al., 2012) of the field. This review addresses three major points concerning the IL-based ABS: (1) the interpretation of the main interactions contributing to the formation of ABS; (2) the study of a large plethora of ABS with different components, considering in particular the effect of the IL structure and various types of *salting-out* agents on the phase equilibria of ABS; the most important (3) applications of IL-based ABS, in which the extraction of biomolecules and other added-value compounds are included. Despite the number of articles published on the subject, the authors suggest that there is still room for improvement that should be accomplished regarding not only the study of new IL-ABS, but also their potential applications, principally in terms of exploring and understanding their extractive potential. While most studies are focused on the variation of the structural features of the IL and the type of salt employed (Freire et al., 2012), these could focus on cheaper and more environmentally friendly compounds using either quaternary ammonium and cholinium-based ILs (e Silva et al., 2014; Pereira et al., 2013a; Shahriari et al., 2013) and

citrate-based or other organic salts of a renewable nature (e Silva et al., 2014; Passos et al., 2012). It should be emphasized, however, that the IL-based ABS known in 2015 are well beyond the IL-salt combinations, already being reported the use of amino acids (Domínguez-Pérez et al., 2010; Zhang et al., 2007), carbohydrates (Freire et al., 2011; Wu et al., 2008a,b,c; Chen et al., 2010), polymers (Pereira et al., 2013b,c; Tomé et al., 2014), and even surfactants (Bhatt et al., 2013; Gong and Zhu, 2014; Vicente et al., 2014).

Despite the number of publications on IL-based ABS, the works regarding the use of IL-based ABS in the extraction and purification of lipases are few. Deive et al. (2011) proposed the use of IL-based ABS to extract the *Thermomyces lanuginosus* lipase (*Tl*L). The enzyme activity was monitored for the systems studied, based in imidazolium ILs with distinct alkyl chain lengths and combined with the chloride, alkylsulfate, alkylsulfonate, and acetate anions. Several operating conditions influencing the lipase activity and the ABS partition were studied, namely the temperature, the pH, the deactivation kinetics, and the water content. The kinetic of the *Tl*L deactivation was investigated and ATR-FTIR studies were carried, aiming at the identification of the *Tl*L structure when exposed to the selected ILs. The main results reported allowed the identification of the optimal conditions, which were identified as the use of ABS based in the 1-ethyl-3-methylimidazolium ethylsulfate ($[C_2MIM][EtSO_4]$) combined with the potassium carbonate (K_2CO_3)—Fig. 3.5—due to the capacity to maintain the enzyme native
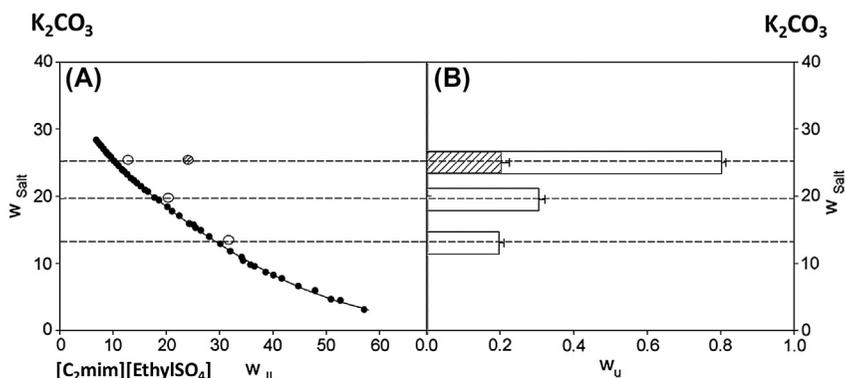

FIGURE 3.5   Summary of the results by Deive et al. (2011), taking into account the high enzyme activity achieved. (A) Phase diagram of the ABS composed of $[C_2mim][EthylSO_4]$ + K_2CO_3 + water. The *black dots* are the experimental data and the *solid line* the fitting of the binodal curve. The *white and striped dots* represent the compositions of the biphasic mixtures used in the extractions. (B) In the extraction experiments, the *white bars* represent the enzyme recovered in the IL-rich phase and the *hatched bar* represents the enzyme recovered at 25% salt content and 27% of IL content. *Adapted from Deive, F.J., Rodriguez, A., Pereiro, A.B., Araujo, J.M.M., Longo, M.A., Coelho, M.A.Z., Lopes, J.N.C., Esperanca, J.M.S.S., Rebelo, L.P.N., Marrucho, I.M., 2011. Ionic liquid-based aqueous biphasic system for lipase extraction. Green Chem. 13, 390−396.*

structure (the same structure found for the enzyme in water), which was confirmed by the ATR-FTIR data and the deactivation kinetics analysis. The authors concluded from the lipase extraction efficiencies found (99% of activity) that the use of IL-based ABS constitutes a powerful and promising separation alternative to conventional methods (Deive et al., 2011).

In the same year, Ventura et al. (2011, 2012a) developed an integrated work of optimization (Ventura et al., 2011) and application to the extraction and purification of a lipase from the fermentation broth (Ventura et al., 2012a). In a first step, the authors studied the *Candida antarctica* lipase B (CaLB) partitioning in several ABS composed of distinct IL chemical structures and three phosphate inorganic salts, aiming at the identification of the best IL for the enzyme purification (Ventura et al., 2011). For that purpose, different families of ILs were tested, namely pyridinium, piperidinium, pyrrolidinium, and imidazolium. Included in the imidazolium structures, different anions, namely triflate [CF_3SO_3], dicyanamide [$N(CN)_2$], methanesulfonate [CH_3SO_3], and chloride (Cl), and several alkyl chain lengths, from C_2 to C_8, were investigated. For each system studied, the enzyme partitioning between the two aqueous phases was measured and the purification factor and the enzyme recovery parameters were determined. The authors identified that the maximum lipase purification and recovery were obtained for an octyl side chain associated to the imidazolium cation, the [$N(CN)_2$] anion and ILs belonging to the aromatic pyridinium family. However, the additive characteristics were not observed for the extraction parameter, since the tailored [C_8pyr][$N(CN)_2$] was tested, and the extraction results were not efficient (purification factor $= 0.998 \pm 0.002$). In this context, the best results were achieved with [C_8mim]Cl with a purification factor of 2.6 ± 0.1 and an enzyme recovery of $(95.9 \pm 0.2)\%$ at the salt-rich phase.

Following this optimization study and considering the ABS with the best results in terms of purification factor and extraction efficiency (Fig. 3.5), a new study was conducted, aiming at the production and purification of an extracellular lipolytic enzyme produced by *Bacillus* sp. ITP-001 (Ventura et al., 2012a). The direct contact of the bacteria with the ILs was avoided and the separation and purification steps were performed using the fermentation broth after the end of the production stage. This work was the first report of a comprehensive study where the integration of IL-based ABS in the process of production and purification of a lipase was shown. The first step on the prepurification stage was a salt precipitation with ammonium sulfate ($(NH_4)_2SO_4$), followed by dialysis. During the salt precipitation using ($(NH_4)_2SO_4$), a significant amount of contaminant proteins was removed and the lipase was concentrated in the supernatant. Following the salt precipitation, the supernatant was passed through a dialysis system aimed at removing the low molecular weight compounds, including the inorganic salts used in the salt precipitation and fermentation processes. After this step, the results showed a small decrease in the enzymatic activity, justified by losses of enzyme during the dialysis process. The purification step was then

performed by applying four distinct ABS based in four hydrophilic ILs, namely 1-butyl-3-methylimidazolium chloride [C$_4$mim]Cl, 1-butyl-3-methylimidazolium chloride [C$_4$mim]Cl, 1-butyl-3-methylimidazolium dicyanamide [C$_4$mim] [N(CN)$_2$], 1-methyl-3-octylimidazolium chloride [C$_8$mim]Cl, and 1-butyl-3-methylpyridinium chloride [C$_4$mpyr]Cl, conjugated with the phosphate buffer solution composed of K$_2$HPO$_4$ and KH$_2$PO$_4$ at pH 7. In this work, a buffer was used to keep the pH during the entire process of purification, since the pH can have a big influence in both the enzyme activity and its partition (Ventura et al., 2011; Barbosa et al., 2011; Perumalsamy et al., 2007).

Finally, the ability of IL-based ABS in the purification of the lipase produced by submerged fermentation was evaluated and compared against some conventional PEG-based ABS. The results suggest that the enzyme purification was mainly controlled by the alkyl chain length, followed by the cation core and the anion moiety. Both high purification factors and enzyme recovery efficiencies at the salt-rich phase were obtained for all systems (90.6 $\pm$ 0.1 $< R_B^E <$ 96.14 $\pm$ 0.08)%, in accordance with the previous optimization report (Ventura et al., 2011). The maximum purification and recovery parameters were obtained for the [C$_8$mim]Cl-based ABS (Fig. 3.6). The purification of the *Bacillus* sp. ITP-001 lipase was also investigated, using ABS based in polyethylene glycol (PEG 8000) and potassium phosphate (Barbosa et al., 2011). Despite some differences in the operation conditions of the two systems (distinct inorganic salts used, different temperature and pH), the results obtained when IL-based ABS are investigated are in general superior, with purification factors ranging from 37 to 51, against purification factors below 30 for the PEG-based ABS.

As highlighted in these works (Ventura et al., 2012a; Barbosa et al., 2011), another advantage of the IL-based ABS when compared with PEG-based systems is the low viscosity of the aqueous phases on these systems. The dynamic viscosity of the top and bottom phases was measured at room temperature for several IL-based and polymer-based ABS. The results suggest that the viscosity of the salt-rich (bottom) phase for IL-ABS (6.11−19.70 mPa s) is low and similar to the polymer-based ABS (6.96−8.27 mPa s). Meanwhile, the viscosity of the IL-rich phases (4.96−8.91 mPa s) is comparable with those of the salt-rich phases (6.11−19.70 mPa s), while the viscosity of the PEG-rich phase is normally higher, and in this particular case is larger by an order of magnitude (26.67−134.57 mPa s) (Ventura et al., 2012a). This is a relevant advantage, since the lower viscosities make the fluid transport and the mass transfer between both phases easier, helping in the solute partition. These results, associated with the performance of the IL-based ABS, appear as excellent advantages of this purification technology.

One of the disadvantages often cited about the use of IL-based ABS is the high cost of some ILs. To overcome this problem we are currently studying novel approaches using ABS based in ILs, in which lower amounts of ILs are
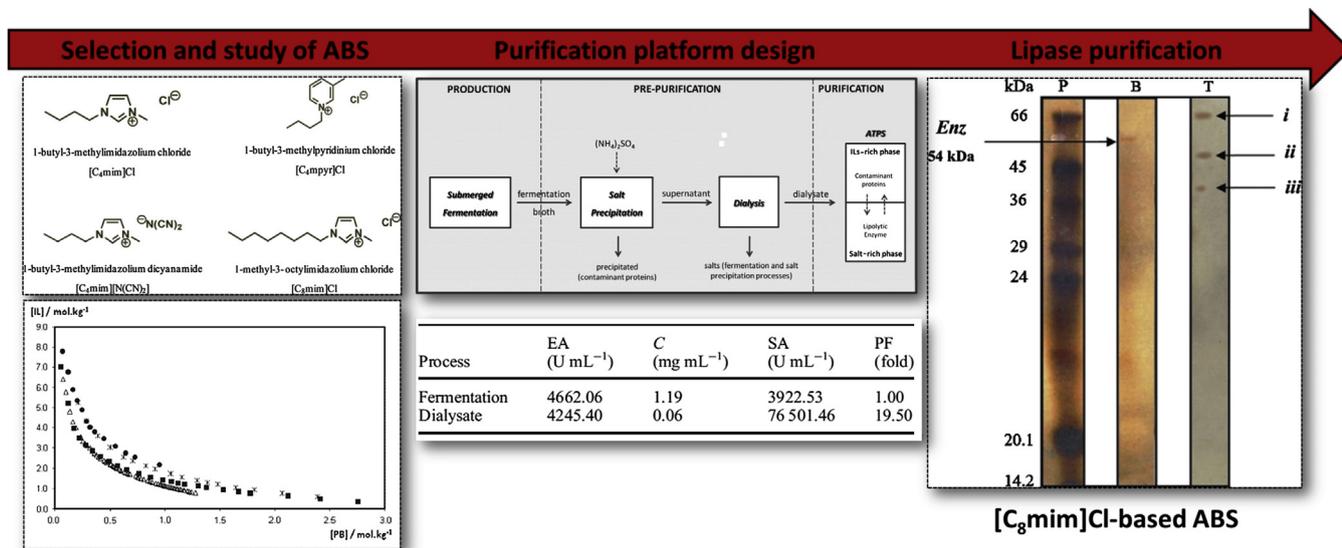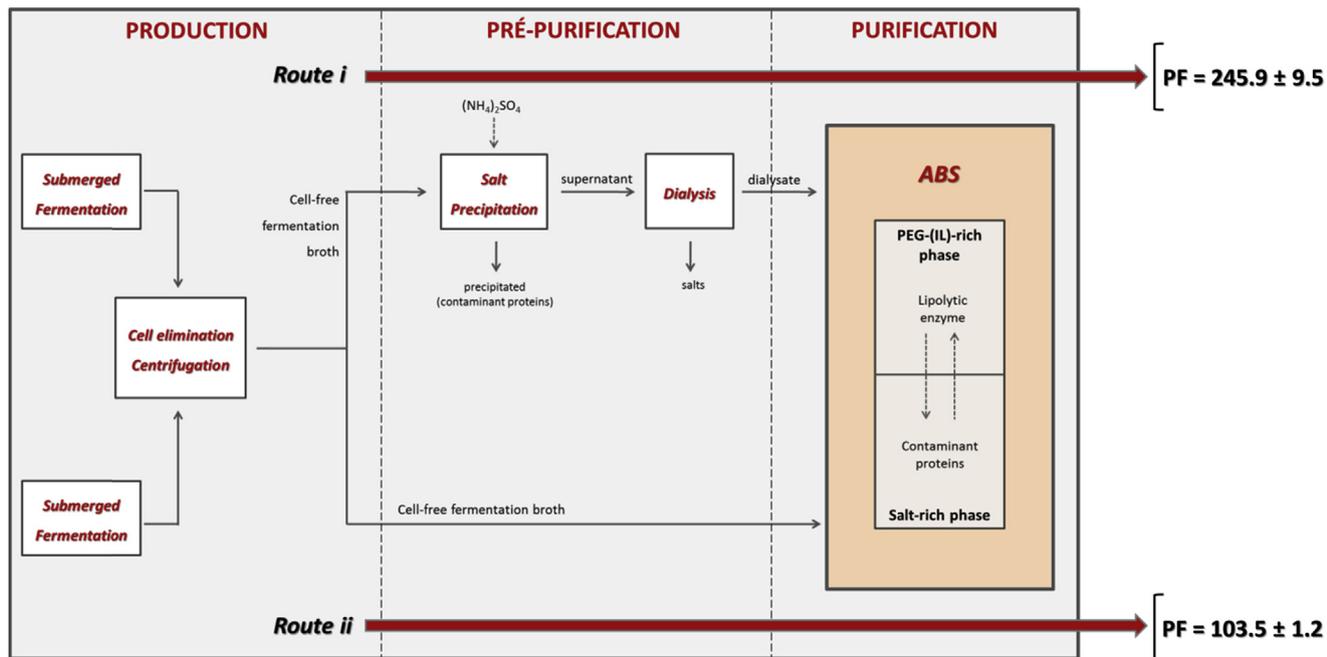
FIGURE 3.6 Main route selected for the study of the production and purification of a lipolytic enzyme produced by *Bacillus* sp. ITP-001 via submerged fermentation applying IL-based ABS (Ventura et al., 2012).

required. In this context, ILs are applied as additives or adjuvants in the formation of ABS (Pereira et al., 2010; de Souza et al., 2014a). In a recent work (de Souza et al., 2014b) imidazolium-based ILs as adjuvants (at a concentration of 5 wt%) in ABS composed of PEG polymers (1500, 4000, 6000, and 8000 g/mol) and potassium phosphate buffer (pH 7) were applied in the purification of the *Bacillus* sp. ITP-001 lipase. After a preliminary optimization carried out with commercial CaLB (de Souza et al., 2014b), the best results were transposed to the purification of the lipolytic enzyme from *Bacillus* sp. ITP-001. The results indicate that it was possible to purify the commercial lipase, despite its high purity, 5.2 times and the produced lipase 254 times, using [C_6mim]Cl as adjuvant. Besides the modification proposed by this work (de Souza et al., 2014b) concerning the amount of IL used in the preparation of the extractive system, another change was possible. This new study considered the analysis of the importance of the prepurification route of salt precipitation and dialysis. The ABS with [C_6mim]Cl as adjuvant (system with best purification capacity) was thus tested in terms of extraction efficiency and purification factor for both routes (de Souza et al., 2014b). ***Route ii*** is described by the direct use of the fermentation broth in the preparation of the ABS, as depicted in Fig. 3.7. This figure shows the two approaches investigated. The numerical results reported in Fig. 3.7 indicate that in both cases a significant purification of the lipase is achieved, since the purification factors are very high. When the two routes are compared, it is possible to evaluate the effect of the prepurification step, since the purification factors can be significantly improved when the salt precipitation and the dialysis are excluded from the purification process (de Souza et al., 2014b) ($PF_{Route\ i} = 103.5 \pm 1.2$-fold and $PF_{Route\ ii} = 245.9 \pm 9.5$-fold). Moreover, a comparison between the various works investigating the purification of this lipolytic enzyme from *Bacillus* sp. ITP-001 (Ventura et al., 2011; de Souza et al., 2014b) shows that the highest purification factors were achieved by the application of ABS using ILs as adjuvants ($PF_{Route\ i} = 245.9 \pm 9.5$-fold and $PF_{Route\ ii} = 103.5 \pm 1.2$-fold) (de Souza et al., 2014b) when compared with the IL-ABS ($PF = 51 \pm 2$-fold) (Ventura et al., 2012a) and polymer-based ABS ($PF = 30$-fold).

3.4 MAIN CONCLUSIONS

In this work, some of the recent developments in downstream processing with an emphasis on the purification of lipases are addressed. Bacterial lipases are mostly extracellular bioproducts and their production by fermentation is a strategic factor for their future commercialization on a large scale. However, their production is affected by various conditions, namely nutritional and physicochemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation, and dissolved oxygen concentration (Aires-Barros et al., 1994). Lipases are important for many

FIGURE 3.7 Schematic representation of the two routes considered in the most recent works for the production and purification of the lipolytic enzyme produced by *Bacillus* sp. ITP-001 via submerged fermentation: *Route i* represents the classic approach, characterized by a prepurification step, including precipitation with $(NH_4)_2 SO_4$ followed by dialysis; *Route ii* represents the novel approach without prepurification.

applications and their purification is, consequently, a field of utmost importance. Despite the fact that some of the commercial applications do not require highly pure homogeneous lipase preparations, an adequate degree of purity is essential for their industrial application, because it enables their most efficient and successful use in industries such as fine chemicals, pharmaceuticals, and cosmetics. The industrial strategies for downstream processing, in particular for the purification scheme employed, should be inexpensive or low cost, fast, with high yields easy scale-up. Until 2015, the principal technologies considered are the chromatographic techniques associated with the use of salt precipitations. However, liquid–liquid extraction, in particular the use of aqueous biphasic systems, aqueous two-micellar systems, and aqueous two-phase flotation systems, may lead to improved downstream processes. The results of the purifications achieved when those techniques are considered can be very diverse, as shown in Table 3.6. The main results discussed here also suggest that the chromatographic techniques, depending on their position on the process and the complexity of the fermentation broth, can be advantageous, in particular when these techniques are used in the final steps of purification (product polishing). The results reported in Table 3.6 suggest that the best results regarding the purification of lipases were obtained by using

TABLE 3.6 Purification Factors Obtained by the Various Works Reviewed, Taking into Account the Distinct Liquid–Liquid Purification Techniques Employed

Purification Technique	Purification Factor Range	References
Chromatography	10–3028	Saxena et al. (2003), Palekar et al. (2000), Gupta et al. (2004), Veeraragavan et al. (1990), Schmidt-Dannert et al. (1996), Schmidt-Dannert et al. (1994), Borkar et al. (2009), Kumar et al. (2012), Ghanem et al. (2000), Imamura and Kitaura (2000), Litthauer et al. (2002), Snellman et al. (2002), Abdou (2003), and Taipa et al. (1992)
Common ABS		
Polymer-ABS	4–30	Zhang and Liu (2010), Ooi et al. (2009a), Bassani et al. (2010), Zhou et al. (2013), Khayati and Alizadeh (2013), and Li et al. (2010)
Alcohol-ABS	13.5	Ooi et al. (2009b)
Surfactant-ABS	3.7–7.2	Vicente et al. (1990), Yu et al. (2003), Nandini and Rastogi (2010), and Ooi et al. (2011b)
IL-ABS	37–246	Deive et al. (2011), Ventura et al. (2012a), and Ventura et al. (2011)

chromatographic techniques. However, it should be noticed that these values were the result of a complex scheme of purification obtained by the combination of several steps of purification based on salt precipitation and various chromatographic techniques.

Concerning the polymeric-ABS, the main conclusions are that the partitioning phenomenon is normally influenced by the type and molecular weight of the polymers, the pH, the addition of salts, or by temperature modifications, among others (Molino et al., 2013). The advantages of ABS are the reduction on the volume treated, their high extraction capacity, the low time required to promote the partition of the solute, and also the fact that these technologies are considered relatively straightforward to scale up. Meanwhile, from all technologies employed, the use of IL-based ABS are one of the most promising approaches shown, which is justified by the high purification factors achieved, though these should be taken with care given the limited number of studies and systems investigated.

Different approaches were proposed regarding the use of IL-based ABS, namely the simple ABS composed of ILs + salt + water, or ABS using ILs as additives composed of polymer + salt + water + IL. The results analyzed suggest that the most promising techniques are based on the use of ABS where the ILs are applied as adjuvants (higher purification factors). However, the number of works contemplating the application of IL-based ABS are still limited and more studies are required to create a more complete picture, to allow the development of a deeper understanding on the mechanisms of interaction between the main components of the ternary/quaternary systems and the partition of the solvents, and the optimization of the best IL systems to be applied. For example, the study of more hydrophobic ILs as adjuvants should be carried by applying non-aromatic acyclic families such as quaternary ammonium, phosphonium, and cholinium. Moreover, the operational conditions should be investigated, namely the pH, temperature, or even other polymeric-ABS. Besides these studies, the design of new schemes (conjugation of various purification techniques defined for a specific lipase with a specific application) should be addressed.

The purification schemes should have potential for being applied in a continuous process, with extraction capacity and high selectivity for the desired product. As aforementioned, several purification approaches were already proposed in literature (Saxena et al., 2003; Antonian, 1988; Aires-Barros et al., 1994; Gupta et al., 2004). From those, the number of works investigating the use of conventional chromatographic techniques and common aqueous biphasic systems, based principally in polymers and simple salts, is significant, despite the lower purification factors achieved. This means that these works need to be planned taking into consideration the process design, because it is notorious that the use of isolated techniques was not very successful until now. A deeper understanding is required for the success of the lipase purification schemes or for the success of purification processes in

general, which we believe must be based in an appropriate case-by-case design and development of the purification platform. Most works analyzed here provide clear evidence that the extent of purification varies with the number and the order of the purification steps, and also that these aspects have been evaluated through different purification protocols pursued by several researchers. The main constraints about the purification schemes being proposed is that they often rely on purification strategies with low yields, long time periods, and low selectivity.

3.5 CRITICAL ANALYSIS AND FUTURE CHALLENGES

The main objective of this work was the description and analysis of novel methodologies based on ABS used to purify lipases after their production by microorganisms. Despite the significant efforts made by various researchers, the number of works reported is limited but the results are promising. In this sense, the selection or design of the best purification scheme for a specific lipase is yet extremely difficult. As a result, more studies are required and should be performed to fulfill the experimental gaps and the lack of knowledge. As discussed, the chromatographic techniques can be advantageous not only due to its most popular characteristics, namely the high yields of extraction and the reduced time spent in the extraction operations, but also because of the possibility to conjugate several techniques performing different schemes of purification, allowing the optimization of the purification capacities (Krieger et al., 1999). However, the best results are not satisfactory and, in this context, new alternatives of purification or even new schemes are demanded, principally to replace those with negative effects on the lipase activity and stability. Besides the decrease in the enzyme performance, some of these technologies are not easy to manipulate, due to the high number of purification units connected in the same purification process, which results in the development of more time-consuming processes with low final yields. Alternative and more amenable techniques are being developed, mainly considering the application of liquid−liquid extraction technologies.

The use of ABS (a widely used technology) was validated and widely applied in the extraction, concentration, and purification of many solutes, including lipases. Associated with these technologies, several properties are often highlighted as advantages of ABS, namely the low interfacial tension, lower process time and energy, and the higher amounts of water present (diminishing the of enzyme inactivation) and their capacity to manipulate various solutes (depending of the main interactions acting in the partition phenomenon). Moreover, ABS are advantageous downstream processes because they are appropriate for a continuous operation regime, they have scale-up potential, it is easy to incorporate them in general purification platforms, and finally, the phase forming components are nontoxic and highly biodegradable. However, the selectivity, one of the most important problems

associated with the purification strategies, remained a major challenge. In this sense, IL-based ABS with a great variety of different cations, anions, and alkyl chains to be conjugated, and different salts, carbohydrates, and amino acids, to be used as salting-out species, appeared and have been investigated (Freire et al., 2012). One of the major advantages of IL-based ABS is the large range of polarities achieved when different anion/cation/alkyl chain combinations are conjugated (Freire et al., 2012). Playing with the polarity of both aqueous phases, the principal interactions can be manipulated and, consequently, the selectivity enhanced. Other aspects were also improved by the combination of ILs and ABS, namely the lower viscosities of the phases (Freire et al., 2012). However, for certain cases, the lipase activity and stability can be negatively affected due to the presence of some particular ILs and also, when the main contaminants present in the fermentation broth are very similar in terms of chemical structures, the extraction/purification selectivity remains an issue.

An advantageous procedure, in our opinion, is the development and use of new ABS based in ILs with reduced capacity to interact with proteins and lipases, and ILs with surfactant nature, capable of auto-aggregating in micellar systems. The main idea behind these systems is the same described for regular AMTPS, the formation of micellar systems able to separate into two aqueous phases, promoting the migration of the lipase between the two phases, depending on the main interactions in control, but this time, by adding ILs with long alkyl chains (with tensioactive nature). The first efforts in this direction were recently carried where different long alkyl chain-based ILs were applied as cosurfactants in the formation of AMTPS based in the nonionic surfactant Triton X-114 and the McIlvaine buffer (Vicente et al., 2014). This work looks at ILs as a new class of tunable cosurfactants, three distinct families being studied here, namely imidazolium, phosphonium, and quaternary ammonium. These families were conjugated with different lengths of alkyl chain and various anions aimed at studying the binodal curves of the novel IL-AMTPS. In this work, the impact of the IL absence/presence, the concentration and the structural features of distinct ILs on the binodal curves construction, was studied. The main results obtained regarding the binodal curves studied provide evidence that the presence of ILs has an important effect on the T_{cloud}, since the binodal curves position seems to be highly dependent on the ILs hydrophobic/hydrophilic character. Aiming at evaluating their applicability as extraction systems, studies considering the partition of two model (bio)molecules, namely the protein Cytochrome c and the dye Rhodamine 6G, were performed. The results reported in this work put in evidence of the potential that small amounts of IL can have in the selective purification of different molecules, which is in our opinion opening new doors in the proper development of successful downstream technologies.

Besides the usual conditions that can be changed in all classes of ABS described in this work, namely temperature, pH, concentration of phase former components, type of polymer, salt, IL, and different cation/anion/alkyl chain

combinations, some important issues still need to be addressed regarding the final objective of the downstream processing, the industrial application. The use of ILs needs to be improved, since some of the most common ILs used to prepare ABS can deactivate lipases (Ventura et al., 2012b; de los Rios et al., 2007; Kaar et al., 2003; Klähn et al., 2011). Another question normally raised by industry is the high price of some ILs, which means that at lab scale it is possible to apply the ILs as solvents, but when the scale is increased, the costs associated may not be compatible with industrial use, even if the target product is an added-value compound. The scale-up is one big issue and four major questions need to be answered before the purification process design: (1) how much lipase is required and what is the demand in terms of purity level; (2) what is the source of the lipase; (3) what is the scheme optimized at lab scale; and (4) what equipment is available at the industrial facilities. The design is clearly very important, and we believe that the concept of extractive fermentation may be advantageous regarding the purification of lipases.

As well-known, the fermentation processes are constantly hampered by a variety of problems, side reactions, production of intermediates or contaminants, originated from the accumulation of products in the bioreactor. Thus, the integration of fermentation and a primary product separation step will have a positive impact on the productivity/yield of the fermentation processes (Ooi et al., 2011a), and in the overall costs, since the product recovery costs and effluent treatment costs will be reduced, as a result of the use of a more concentrated feedstock. Meanwhile, several conditions need optimization, such as the selection of a suitable solvent, taking into account the biocompatibility between solvents and microorganisms; the development of appropriate models to the mass transfer; the heat transfer; the partitioning of different solutes and contaminants; the best conditions of temperature, agitation, and pH; and also the incorporation of models and parameters into a process simulator aiming at the optimization of the whole process. With all these requirements satisfied, it will be possible in the future to design more efficient integrated platforms of production and purification of lipases (capable to guarantee their required level of purify). The process modeling may have a crucial role in the purification of lipases, not only in the implementation of extractive fermentation processes, but also in the study and implementation of a continuous purification regime. Since ABS and AMTPS are liquid−liquid separation processes, they are excellent candidates for microfluidic separation techniques, a field that is in quick expansion since 2000.

Miniaturization, as a process intensification methodology (which means increased mass and heat transfer), allows for the binding of the high surface-to-volume ratio, aiming at developing efforts to achieve higher yields over shorter periods of time, higher product purity, and better process control along with the reduction of cost and equipment associated with downstream processing (Woodcock et al., 2008; Keoschkerjan et al., 2004) and the possibility for parallel operations (Berg et al., 2010; Jahnisch et al., 2004;

Teh et al., 2008). Other economic benefits, improvement of intrinsic safety, and reduction of the environmental impact can be achieved, as well as the benefits of moving from batch to continuous mode. These microfluidic devices have important advantages such as savings in time, space, materials, costs, and a higher control of the operator over the purification system. Due to their flexibility as manufacturing processes, they can be potentially designed for the required chemistry in contrast to the conventional technologies (Dietrich, 2009). Despite the reduced number of studies using this technology, the attempts reported in literature were a huge success in terms of selectivity and yield (Meagher et al., 2008; Pohar et al., 2012). Recently, microfluidic separation devices and ABS based in ILs were already tested and applied in purification schemes (Novak et al., 2012). This microscale equipment seems also to be a promising device for parallel processing (SooHoo and Walker, 2009; Znidarsic-Plazl and Plazl, 2007), which could be applied with success in the large-scale purification of lipases from the fermentation broth.

Studies contemplating the recycling of the phase components (polymers, ILs, and salts) are being developed (Zhang and Liu, 2010; Freire et al., 2012). In the particular case of ILs, three main approaches are being considered, namely their recycling, regeneration, or reuse (Freire et al., 2012; Claudio et al., 2013, 2014; Alvarez-Guerra et al., 2014). The results suggest that it is possible to successfully recycle and reuse various ILs applied in the extraction of several biomolecules, including proteins and even when these ILs are applied as solvents in solid−liquid extraction technologies (Claudio et al., 2013). Meanwhile, more studies are required, contemplating the reuse, recycling, and regeneration of these solvents and other phase separation agents (polymers, salts and surfactants), mainly considering the particular case of processes used to purify lipases. In this context, other studies are mandatory in our opinion, namely the investigation on the recovery of the lipase from the presence of the ABS and AMTPS separation agents, which will be an important task to be developed regarding the lipase polishing. The treatment of effluents originated from the lipase polishing should also be considered for future studies, because this is not only important in terms of technological scheme but also regarding the total costs of these purification processes. Indeed, not only the technological background is important but also the economic point of view; engineers and researchers should take into account the necessity to properly develop the economic analysis in parallel with the technological description. In this sense, the number of sequential operations necessary to achieve the desired purity of a lipase should be taken into account, since this is one of the main conditions with relevant contribution to the overall cost of the downstream scheme. Furthermore, the capital investment and the amount of consumables needed for each step as well as the individual time required for each operation and the amount and expertise of the human resources are key factors promoting the economic profile of the downstream processes.

Summing up, it will be possible in the future to project the production and purification of specific lipases for a certain application with total control of the technological, economic, and quality conditions required for the success of each industrial process, under the concept of Quality by Design, defined as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management," adapted from (Rathore and Winkle, 2009). The main aim will be to design a quality product and establish a robust manufacturing process that can consistently deliver the intended performance of the product.

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