Biocatalysis in biphasic systems based on ionic liquids

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Abstract

Biocatalytic processes find increasing applications in industry due to the high efficiency of biotransformations, and renewable nature of the enzymes and substrates. Enzymatic biocatalysis is an attractive alternative to conventional chemical catalysis, with corresponding advances now allowing a wide range of biotransformations. However, to make the enzymatic process sustainable, it is essential to separate the product and reuse the biocatalyst while maintaining its efficiency. Liquid-liquid biphasic systems appear as alternative approaches to integrate the enzymatic reaction, product recovery and reuse of the biocatalyst and phase constituents. Moreover, to expand the range of biocatalytic reactions, ionic liquids and deep eutectic solvents have emerged as alternative reaction media due to their unique properties, in particular their ability to maintain enzymes active and stable.

In this chapter, we outline novel opportunities for using enzymes in biphasic systems based on ionic liquids and deep eutectic solvents and discuss the key points and scientific developments expanding the application of biocatalysis.

Key Words:

Biphasic systems; Biocatalysis; Enzymes; Integrated process; Ionic liquids, Deep Eutectic solvents.

1. Introduction

The demand by industry to meet the directives of the green chemistry agenda is promoting intense activity in the field of biocatalysis [1–5]. Biocatalysis allow high selectivity, biodegradability, and mild reaction conditions. For this reason, a number of biocatalytic applications and remarkable developments has been observed in several industrial sectors, from chemical and pharmaceutical industries to food products [6–14]. In parallel, enzymology has become one of the most effective and important areas focused on the research and development sectors of biocatalysis. However, the high structural complexity is the main disadvantage associated with enzymes at industrial levels [15]. The creation of highly active, robust and stable biocatalysts, while avoiding biocatalyst deactivation are the primary difficulties faced in industrial settings. Modern biotechnology, using protein engineering, and directed evolution are creating a revolution, resulting in significant advances in enzymes used in the aforementioned industries [6].

Enzymes have been recognized as highly effective catalysts since they are proven to be very useful in preventing various nasty chemicals forming in conventional processes [4]. Thus, enzymes are also fully compatible with modern standards for safety, health, and the environment, being considered green catalysts [8]. Biotechnological advances enabled the enzymes to be "made to measure" with novel activity and adaptations to different process conditions, allowing for a wide range of uses.

In this chapter, we present an overview of biphasic systems (BS) as alternative platforms for biocatalysis. Moreover, this chapter intends to highlight the multidisciplinary approach required for the success of this subject, bringing together the scientific and industrial communities to accomplish its application at industrial level.

2. Biocatalysis in biphasic systems

Over the past five decades, enzymes as biocatalysts have become one of the pillars of biotechnology with numerous applications due to their mild reaction conditions, high selectivity and biodegradability [6,16]. Even though they have excellent catalytic properties, enzymes have some characteristics that are not suitable for such applications, insofar as: they are unstable and high cost [17]. Although procedures based on the use of enzymes are replacing conventional chemical methods, the commercialization of some enzymes for industrial applications is still at a low pace due to their high costs, low efficiency and operational catalytic and storage problems [18]. Therefore, rigorous efforts have been made by the scientific community to improve enzymes and to replace unfavorable reactions with green and clean technologies, increasing their performance while developing a more sustainable industrial process [19].

The use of BS constituted by two immiscible solvents, such as water and a water-immiscible organic solvent, offers several advantages in biocatalysis. Their main use has been the separation of the enzyme from the products of the reactions, but it may also be the separation of the enzyme from the reactants if these are deleterious towards the enzyme. Usually, the organic phase is used to recover the product formed, while the aqueous phase contains the enzyme [20,21], as illustrated in Fig. 1.

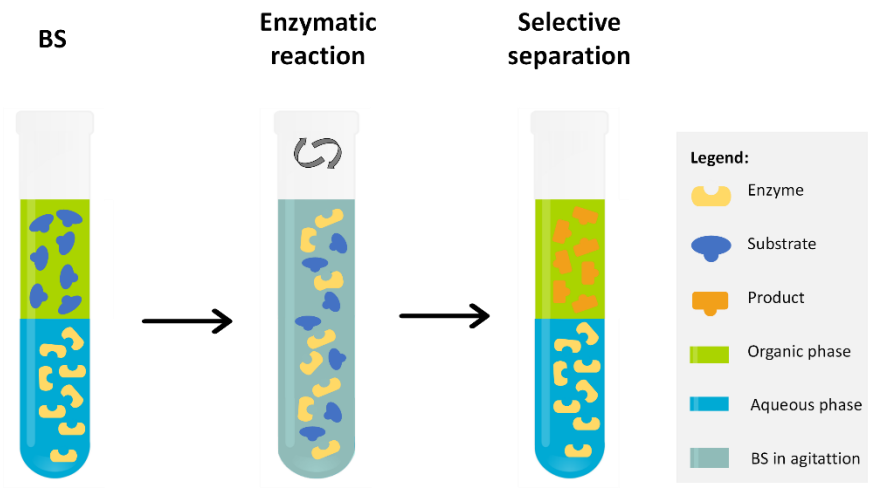

Figure 1. Schematic representation of a biocatalytic reaction in a heterogeneous medium using BS.

Due to the peculiar characteristics of BS, they are an attractive alternative offering several advantages, such as high yields, high selectivity and simultaneous possibility of purification. In fact, the application of BS as a medium for biocatalysis allows for the integration of the enzymatic reaction, separation/purification of products and the reuse of the enzyme, while keeping the technological simplicity and a low cost [22]. The organic solvents commonly used are ethyl acetate and hexane, mainly due to their immiscibility in aqueous media [23]. Table 1 summarized examples of the use of BS as media for biocatalysis.

Table 1. Examples of biocatalytic reactions in BS.

Enzyme and source	Substrate	IL BS	Application/ Product	Ref
Laccase from *Polyporus versicolor*	17β-estradiol	Phosphate buffer	Oxidation of steroids	[24]
Laccase from *Trametes pubescens*	17β-estradiol	Ethyl acetate and acetate buffer	Oxidation of steroids	[25]
Porcine pancreas lipase type II	Ibuprofen and sorbitol	Hexane and water	Synthesis of bioactive derivatives of ibuprofen	[26]
Peroxidase from *Bjerkandera adusta*	Anthracene	Silicone oil and water	Degradation of anthracene	[27]
Laccase from *T. versicolor*	Anthracene	Silicone oil and water	Degradation of anthracene	[28]
Lipase B from *Candida antarctica*	2-phenethyl acetate	Dioxane and water	Transformation of 2-phenethyl acetate to 2-phenylethanol and acetate	[29]
Lipase B from *C. antarctica*	1-phenethyl acetate	Organic solvents (acetone, acetonitrile, (1,4)-dioxane and tetrahydrofuran) and water	Transformation of rac-1-phenylethyl acetate to (R)-1-phenylethanol and (S)-1-phenylethyl	[30]

Lugaro *et al*. [24] were one of the first authors to use this approach. They showed that laccase from *Polyporus versicolor* catalyzed the 17β-estradiol oxidation in a heterogeneous medium using a BS composed of water and ethyl acetate (EtOAc). The steroidal substrates and metabolites were solubilized in the phase composed of EtOAc while the enzyme remained in the aqueous phase. A similar work was developed by Nicotra *et al.* [25], where the 17β-estradiol was oxidized into two dimers (1c and 1d) by laccase from *Trametes pubenscens*, also using a BS composed of EtOAc. The authors characterized the chemical structure of the products obtained by RP-TLC, NMR and RP-HPLC analysis, showing that the structures of the Lugaro's reaction products were poorly characterized and ambiguously determined [25].

Ibuprofen is a poorly water-soluble drug. To improve its bioavailability, a modification in the chemical structure of ibuprofen was proposed by Zappaterra *et al.* [26]. In their work, porcine pancreatic lipase B was used for the direct esterification of ibuprofen and sorbitol to produce the ibuprofen sorbitol ester, a water-soluble drug. The enzymatic reaction occurred in a hexane-water BS, where the organic solvent acted as a solubilizer for ibuprofen an the aqueous phase acted as the solvent for sorbitol [26]. The impact of agitation speed on ibuprofen esterification with sorbitol was investigated at various agitation speeds (from 100 to 600 rpm). The results demonstrated that 400 rpm was the best speed since it lead to the highest conversion yield value obtained of 60% [26].

Also within the BS perspective is the two-phase partitioning bioreactor (TPPB). This approach to biphasic biocatalysis consists of applying the two-phase system in a bioreactor, comprising an organic solvent that allows high concentrations of the target water-immiscible substrates to be dissolved and the aqueous phase containing the biocatalyst. Here the substrate migrates from the organic to the aqueous phase at low concentrations, where the enzymatic reaction takes place, as illustrated in Fig. 2 [28,31]. This approach is to be preferred whenever high concentrations of substrate can affect the performance or stability of the enzyme.

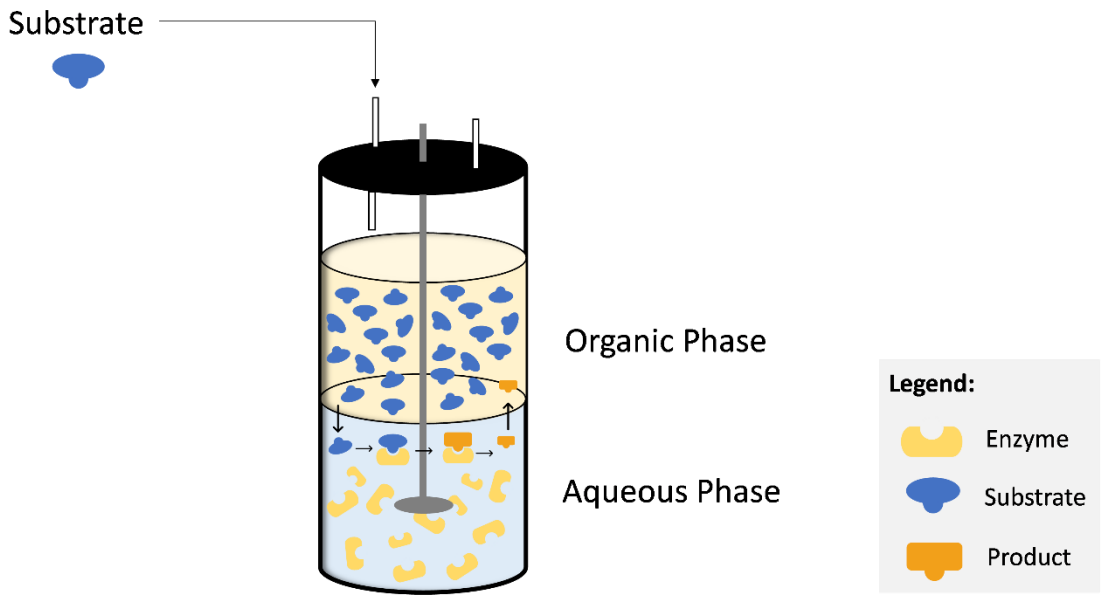

Figure 2. General scheme for two-phase partitioning bioreactors (TPPB) concept.

Several works using the TPPB have been reported, such as the enzymatic degradation of anthracene, a polycyclic aromatic hydrocarbon (PAH) [27]. Anthracene was chosen as the poorly soluble substrate and its degradation was carried out by the enzyme versatile peroxidase (VP) from *Bjerkandera adusta* [27]. The effects of solvent (silicone oil) viscosity, agitation rate, and surfactants (Tween 80 and Triton X-100) were evaluated on the removal of anthracene, as well as the mass transfer rate. It was found that in the presence of Triton X-100, the mass transfer coefficients increased from 0.37 to 0.52 min^{-1} when the agitation rate was increased from 200 to 250 rpm. This means that the addition of the surfactant improved the mass transfer of the substrate from the organic to the aqueous phase. At the optimized conditions (silicone oil viscosity of 50 cSt, 250 rpm with the addition of Triton X-100) it was obtained an anthracene oxidation of 88% [27].

Another work evaluated the degradation of anthracene by laccase from *Trametes versicolor* in a TPPB [28]. The organic phase comprised silicone oil and surfactant (Triton X-100) saturated with anthracene. The combined configuration of the TPPB operating with silicone oil as an immiscible solvent allowed to achieve a conversion rate of 16

μmol/L.h of anthracene. Moreover, the results revealed that Triton X-100 benefited the laccase stability [28]. In addition, the reuse of silicone oil to dissolve more anthracene was evaluated in three consecutive cycles of reaction, and a percentage of anthracene removal of 97% was attained. It was also proved that the distribution of anthracene between organic and aqueous phases was facilitated by adding Triton X-100, since it improved the mass transfer of anthracene from organic to the aqueous phase [28].

In addition to BS as a medium for heterogeneous biocatalysis, Broering *et al.* [29] developed organic–aqueous tunable solvent (OATS) systems. In this approach, the hydrophobic substrate is converted by the enzyme in a single liquid phase (homogeneous biocatalysis). Then, CO_2 is added to induce the BS formation while allowing the selective separation of the product from the enzyme. The hydrophobic product preferentially migrates to the organic phase, while the hydrophilic enzyme partitions to the aqueous rich phase, allowing an easy recovery of the product and the biocatalyst reuse [29], as sketched in Fig. 3. The main advantage of this methodology is to solve some issues related to the heterogeneous biocatalysis approach (catalytic reaction in a two-phase system), such as lower reaction rates due to interphase mass-transfer limitations [29], and a slight loss in enzymatic activity due to the continuous stirring to increase the interfacial contact area between the two phases [29]. Thus, OATS combine the high reaction rates with a simple method for biocatalyst reuse and product separation [29]. However, for improved performance, some requirements must be designed and optimized, namely the use of an OATS mixture providing an acceptable enzyme reactivity in the CO_2-pressurized separation process, in addition to the enzyme partition to the aqueous phase, while reaction products are retained in the organic phase [29]. As proof of concept, a BS constituted by water and dioxane with responsive behavior through the pressurization with CO_2 was developed. This system was evaluated using the enzyme lipase B from

Candida antarctica and 2-phenethyl acetate as substrate, allowing 80% chiral product recovery in the organic phase while displaying less than 10% of apparent biocatalyst activity loss after recycling six times [29].

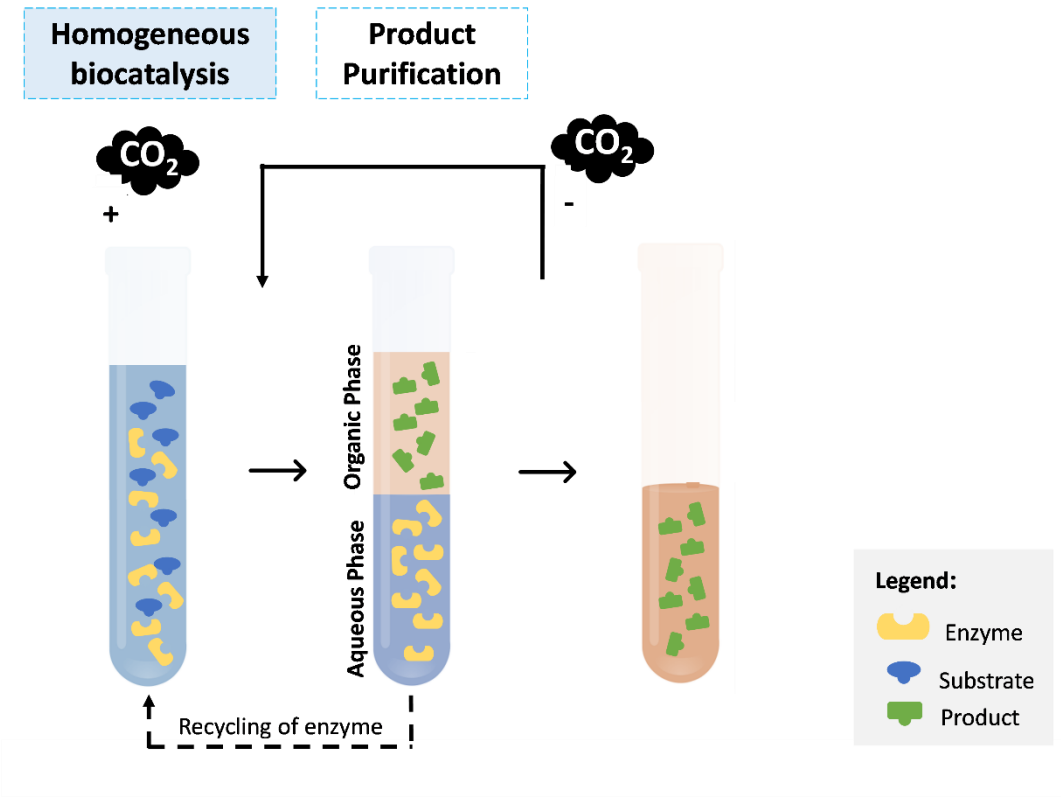

Figure 3. A proposed organic–aqueous tunable solvent (OATS) process for biocatalyst reuse. Adapted from Broering *et al.* [29].

In another work using OATS, the transformation of rac-1-phenylethyl acetate to (R)-1-phenylethanol and (S)-1-phenylethyl was performed using lipase [30]. Different solvents, namely acetone, acetonitrile, (1,4)-dioxane and tetrahydrofuran were studied, from which the OATS based on (1,4)-dioxane was considered to be the most suitable to promote the enzymatic reaction due to its high saturation concentration (17.7 ± 0.8 mM) and increase in the enzymatic reaction rate (0.014 ± 0.001 s^{-1}) of 1-phenylethyl acetate [30].

In summary, BS proved to increase the solubility of hydrophobic substrates and, therefore, promote mass transfer, enhancing the overall substrate conversion. The biocatalysis of hydrophobic substrates can be favored by the presence of a hydrophilic phase, where enzymes are preferentially solubilized, while the hydrophobic phase enhances the substrate solubility. However, it is important to take into account that depending on the organic solvent, high volatility and toxicity may be present, characteristics that are not desired in the development of a sustainable process, without mentioning that they can affect the performance of biocatalysts, and as demonstrated by several authors [24,32,33].

2.1. Ionic liquids as alternative solvents in biphasic systems

Depending on the type of the biocatalyst, its stability may be limited; therefore, the use of alternative solvents such as ionic liquids (ILs) may improve their performance when properly designed [34–46]. ILs are ionic compounds that belong to the molten salts group, present a melting temperature below 100°C by general definition. ILs are typically composed of a large and unsymmetrical cation and an organic or inorganic anion [47]. The low melting temperatures of ILs result from the weak intermolecular interactions derived from the large size ions and their charge distribution, and lack of an ordered crystalline structure [47]. The ionic nature of ILs is responsible for some of their unique properties, such as their negligible vapor pressure under atmospheric conditions, low flammability, high thermal and chemical stabilities, large liquid temperature range, high ionic conductivity and excellent microwave-absorbing ability. Among the large range of ILs that can be synthesized, the most studied are nitrogen-based and phosphonium-based [48], with some examples of their cation chemical structures given in Fig. 4. The cation can be of a different nature and additionally designed by changing the size of the alkyl

side chains and the addition of functional groups [49]. Furthermore, the anion can have a different chemical nature, such as halogens, sulphates, cyano-based, fluorinated, *etc*. (Fig. 4).

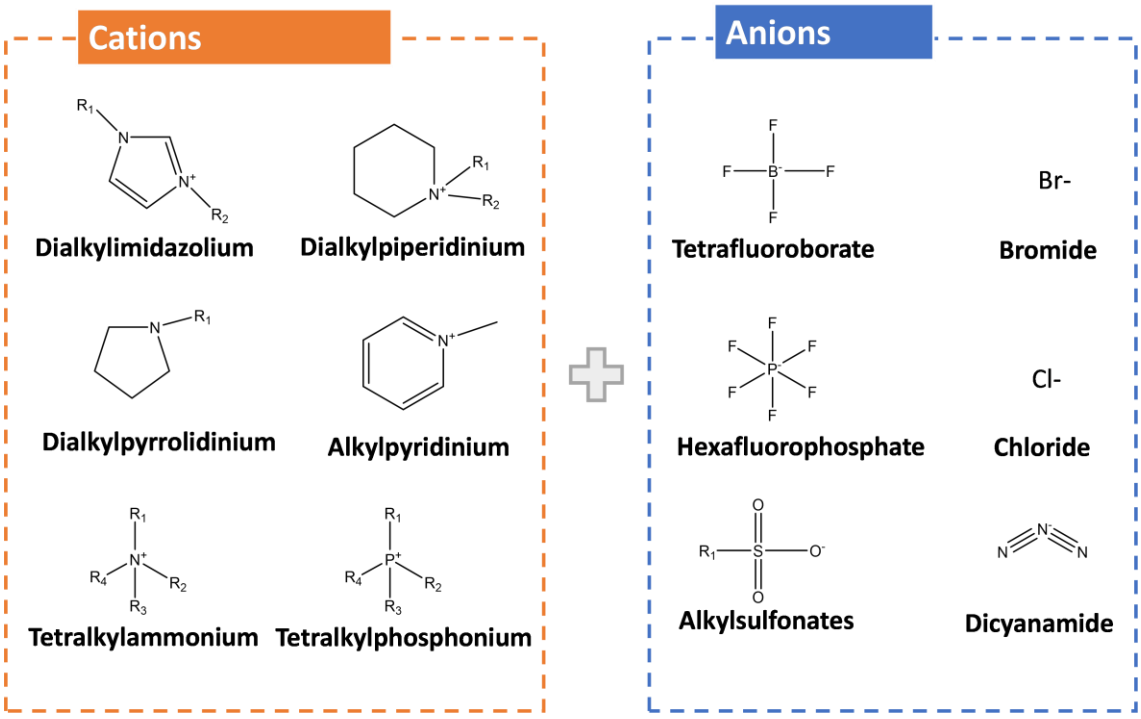

Figure 4. Chemical structures of common IL cations and anions usually used in biocatalysis.

Based on the vast number of cation/anion combinations, it is possible to tune their physicochemical properties aiming at designing a specific IL for a target application, and so, ILs are commonly described as "designer solvents". This feature overcomes the limited selectivity of common volatile organic solvents, allowing the design of more effective solvents for extraction purposes and more efficient separation platforms. Based on these excellent chemical properties, ILs are well-known reaction media. In addition, when properly designed, ILs are a friendly alternative (green solvents) due to their combination of non-volatility and non-flammability [50], eliminating solvent losses to the atmosphere, and consequently decreasing both the environmental footprint and the

cost of the process [50]. However, the negligible vapor pressure is not enough to assure that these compounds are in fact "green", although losses to the atmosphere are completely prevented when compared to traditional volatile organic solvents. Properties such as toxicity, cytotoxicity and biodegradability must also be accessed. For instance, even the most hydrophobic ILs have a non-negligible miscibility with water, which can result in the contamination of aqueous streams [51]. In recent years, several studies were conducted to evaluate the toxicity and biodegradability of ILs [52–58], either by the combination of different anions and cations or by changing the alkyl side chain length and number of alkyl groups at the cation ring. These studies showed that the ILs toxicity is primordially determined by the cation nature and increases with the increase of the length of the alkyl side chain (increase in hydrophobicity) [57,58]. Although ILs cannot enter into the environment by evaporation, they can enter into the biosphere by water streams. Therefore, the synthesis of "greener" ILs and studies on their applications are nowadays one of the major topics of research within the ILs community [59]. Starting materials must be non-toxic and ideally should be renewable. Low-cost synthetic routes and easy preparation should also be filled. In this direction, some novel ILs have been reported, such as those composed of cholinium- [60,61] and glycine-betaine-based cations [62,63], combined, for example, with anions derived from amino acids and carboxylic acids [60,61] (**Fig. 5**).

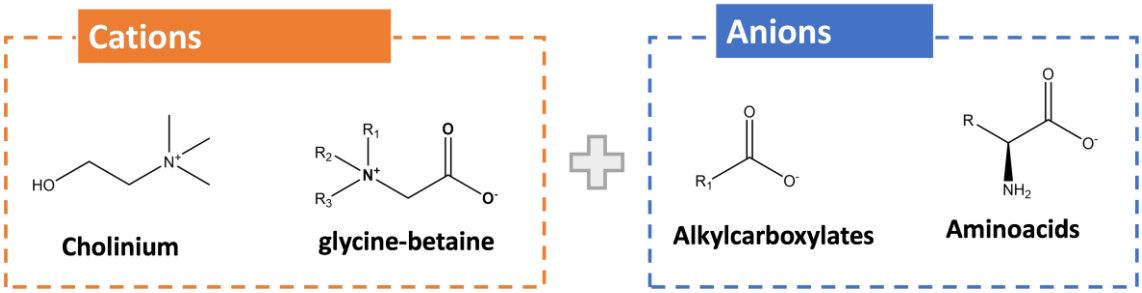

Figure 5. Chemical structures of "greener" IL cations and anions.

The use of ILs in biocatalysis bring many advantages. Compared to some organic solvents, when suitably designed, ILs do not inactive enzymes [64] and no changes occur in the enzyme structure [65]. The stability and improved activity of enzymes in ILs lead a large number of works, as detailed in recent review articles [34–36,39–46]. These works point out the importance of ILs in biocatalytic reactions for enzyme-related applications. Depending on the IL chosen, the bioprocess can be improved due to high conversion rates and high selectivity [66]. For example, some enzymes in ILs composed of $[BF_4]^-$, $[PF_6]^-$, and $[NTf_2]^-$ anions retain activity, while in ILs constituted by Cl^-, NO_3^-, $[CF_3SO_3]^-$, $[CF_3CO_2]^-$ or $[CH_3CO_2]^-$ anions lead to decreases in the enzyme activity [64]. More recently, Bisht *et al.* [38] demonstrated the use of "greener" ILs, namely cholinium-based ILs, as potential media for enzymes, since these ILs lead to a remarkable enhanced activity and improved stability of enzymes. Besides, ILs can also alter the polarity of the media, which can improve substrates solubility or alter substrate specificity, leading to faster enzymatic reactions, and reduce by-products formation by suppressing side reactions [64,67]. ILs can also enhance enantioselective of enzymatic reactions [64]. The works found in the literature studying enzymatic catalysis in BS composed of ILs and salts/buffers are summarized in Table 2.

Table 2. Examples of ionic liquid biphasic systems (IL BS) in biocatalysis.

Enzyme and source	Substrate	IL BS	Application/ Product	Ref
E. coli whole-cell nitrilase	o-chloromandelonitrile (CMN)	[BMIM][PF_6] and Phosphate buffer	Hydrolysis of CMN to racemic o-chloromandelic acid	[68]
Saccharomyces cerevisiae whole-cell Pyruvate decarboxylase	Benzaldehyde and glucose	[BMIM][PF_6] and fermented broth	Synthesis of (R)-phenylacetylcarbinol	[69]
Recombinant β-glucuronidase from *Aspergillus oryzae*	Glycyrrhizin	[BMIM][PF_6] and acetate buffer	Synthesis of β-D-mono-glucuronide-glycyrrhizin and glycyrrhetinic acid	[70]
Epoxide hydrolases from mung beans	Styrene oxide	[BMIM][PF_6] and phosphate buffer	Biosynthesis of chiral vicinal diols	[71,72]
E. coli whole-cell P450 monooxygenase	1, 4-fluorothioanisole 3, ethyl phenyl sulfide 5, methyl p-tolyl sulfide 7, and 4-methoxythioanisole 9	[$P_{6,6,6,14}$][NTf_2] and potassium phosphate buffer	Asymmetric sulfoxidations of substrates	[73]
E. coli whole cells co-expressing yeast reductase and glucose dehydrogenase from *Bacillus subtilis*	3-chloro-1-phenyl-1-propanone	[BMIM][NTf_2] and Tris-HCl buffer	Biosynthesis of (S)-CPPO	[74]

In the study reported by Zou *et al.* [68], a novel integrated *in situ* enzymatic process using *E. coli* whole-cell nitrilase-catalyzed asymmetric hydrolysis of nitriles was developed by the introduction of a biocompatible ionic liquid biphasic system (IL BS), composed of 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) and potassium phosphate buffer (10% (v/v)). This work applied a BS with ILs to enhance the nitrilase-catalyzed asymmetric hydrolysis of o-chloromandelonitrile (CMN) into racemic

o-chloromandelic acid (R-CMA). A water immiscible IL was applied to form a biphasic reaction system. The effects of substrate concentration on the asymmetric hydrolysis of CMN to R-CMA in aqueous and [BMIM][PF_6]-buffer BS were investigated. The results proved that the BS supported higher substrate concentration than the single aqueous phase. Besides, the hydrolysis reaction in [BMIM][PF_6]-buffer BS led to higher enzyme activity. Finally, it was shown that the *E. coli* whole-cell nitrilase retained 85% of activity after 7 cycles reaction [68].

The synthesis of the pharmacological precursor (R)-phenylacetylcarbinol ((R)-PAC) was carried out in the BS constituted by the IL [BMIM][PF_6] and the fermentation broth, using *Saccharomyces cerevisiae* whole-cell as the biocatalyst for the *in situ* reaction with pyruvate decarboxylase [69]. The *S. cerevisiae* cells present toxicity to the substrate benzaldehyde; thus, the BS was used to maintain the substrate in the hydrophobic IL phase, while the reaction occurs in the fermentation broth phase. When comparing biphasic biotransformation with the traditional monophasic system, the yield and productivity of (R)-PAC increased 1.5 times, whilst the benzyl alcohol (by-product) was reduced [69].

Recombinant β-glucuronidase from *Aspergillus oryzae*, immobilized on zinc-based nanoparticles, was used to catalyze the biotransformation of glycyrrhizin into β-D-mono-glucuronide-glycyrrhizin followed by its transformation into glycyrrhetinic acid [70]. The enzyme activity was independently evaluated in buffer and IL media, and the best activity was achieved in 20% (v/v) of [BMIM][PF_6]. Then, the reaction was studied in a BS composed of [BMIM][PF_6] and acetate buffer, where β-glucuronidase presented high stability and improved performance than in pure IL [70]. The BS also presents the advantage to allow the final product purification since glycyrrhizin and β-D-mono-glucuronide-glycyrrhizin were soluble in the buffer-rich phase, while glycyrrhetinic acid

migrated to the IL-rich phase [70]. In this work, the recovery of IL was studied, and 76% of IL was recovered and reused after eight repeated batches without affecting the enzymatic reaction [70].

Cross-Linked Enzyme Aggregates (CLEAs) was used to hydrolysis styrene oxide by epoxide hydrolases in an IL BS [71,72]. The enzymatic reaction was evaluated in different ILs families, such as the imidazolium-, pyrrolidinium-, piperidinium-, phosphonium- and cholinium-based ones. Among them, both works [71,72] verified that [BMIM][PF_6] was the IL presenting the best biocompatibility with the enzyme, and was thus selected to prepare a BS with phosphate buffer [71,72]. The enzymatic reaction presented a yield of 49% and an enantiomer selectivity of 96-97% [71,72]. Moreover, Yu *et al.* [71] went one step further, performing a control with an n-hexane BS for comparison, observing that substrate concentration in the IL BS was up to 4.0 times higher than that in the n-hexane-based one. Finally, the enzyme reuse was evaluated during 5 cycles of reaction, showing that the IL BS maintained 70% of the initial activity, while the n-hexane BS only maintained 31% of the initial enzyme activity [71].

As an example of a bio-oxidation, Gao *et al.* [73] demonstrated an aqueous-IL BS to increase the enantioselectivity and productivity of asymmetric sulfoxidations catalyzed by sulfide monooxygenases P450 (1, 3, 5, 7 and 9). *Escherichia coli* (P450pyrI83H-GDH) co-expresses monooxygenase and glucose dehydrogenase, and was designed for asymmetric sulfoxidations of thioanisole 1, 4-fluorothioanisole 3, ethyl phenyl sulfide 5, and methyl p-tolylsulfide-methoxythioanisole 9, respectively. Because the substrates and their products are hazardous to cells and inhibit sulfoxidation, a biphasic potassium phosphate-IL buffer system was designed to address these issues. The inhibition of reactions, as well as the toxicity of substrates and products to cells, were investigated, and all of them were avoided by using the potassium phosphate buffer-[$P_{6,6,6,14}$][NTf_2] BS.

This represents a biphasic reaction system with excellent biocompatibility with cells and high solubility with substrates and products [73]. The sulfuroxidations of 1, 3, 5, 7, and 9 used *E. coli* cells in potassium phosphate-[$P_{6,6,6,14}$][NTf_2] buffer at a 3:1 ratio (v/v). In such a BS, > 99% of substrates and 35% to 60% of products remained in IL, significantly decreasing substrate toxicity and inhibiting product toxicity. [$P_{6,6,6,14}$][NTf_2] demonstrated good biocompatibility for *E. coli* cell growth and the capacity to shield the cells from much of the substrate toxicity, making it an appropriate co-solvent in the KP buffer BS. In summary, the BS with IL provides an effective reaction platform for bio-oxidation, while improving cell and enzyme compatibility [73].

(S)-3-Chloro-1-phenyl-1-propanol ((S)-CPPO) is a chiral building block for the synthesis of pharmaceutical drugs. There are enantioselective enzymes capable of transform 3-chloro-1-phenyl-1-propanone (3-CPP) in (S)-CPPO; however, this substrate presents low solubility in aqueous media [74]. Choi *et al.* [74] proposed the use of an IL BS to overcome this limitation. *E. coli* whole cells co-expressing the enzyme methylglyoxal reductase (YOL151W) and glucose dehydrogenase from *Bacillus subtilis* were used as biocatalysts to perform the transformation of 3-CPP in (S)-CPPO using glucose as a cofactor. After a selection from a variety of ILs, [BMIM][NTf_2]-Tris-HCl buffer BS was chosen for the reaction using Tween 40 as an additive. In the proposed BS, 3-CPP was solubilized in the [BMIM][NTf_2] phase, while glucose was dissolved in the buffer phase, and the biocatalyst located on the interface, where the reaction occurred. After 8h of reaction, 100 mM (S)-CPPO was synthesized with an enantiomeric excess of >99% [74].

2.2. Deep eutectic solvents as alternatives solvents in biphasic systems

Deep eutectic solvents (DES) are an emerging type of alternative solvents that resemble ILs, preferabaly biomass-derived, that are formed by eutectic combinations of a hydrogen-bond acceptor (HBA) (typically a quaternary ammonium halide salt) with a hydrogen-bond donor (HBD), such as saccharides, alcohols or carboxylic acids [75]. The formation of a DES is characterized by their melting point, which present reduced melting temperatures than both forming compounds, which may be liquid at room temperature [75,76]. If properly designed, DES present improved solvent ability and peculiar physicochemical properties, which are additionally adjusted by shifting its composition [77]. The fact that biodegradable and inexpensive compounds can be used to form DES and their peculiar physical proprieties have triggered their research in the area of biocatalysis, with an increasing number of applications being reported [76,78–81]. DES have been evaluated as alternative solvents or co-solvents in biocatalysis. DES properties can influence the biocatalyst structure and activity and the biocatalytic process, such as their viscosity and water content [78,81,82]. When well designed, DES can improve enzyme stability and activity, enhancing reaction efficiency [82]. More information can be found in the works from Xu *et al.* [82], Tan and Dou [81] and Pätzold *et al.* [78]. Xu *et al.* [82] identified the downstream separation of the target compound from DES as one of the challenges for this solvent to be applied. Here, BS enter as a promising option to include DES as a solvent in biocatalysis reactions and as an integrated step to separate the target compounds. So far, all examples found in the literature explored lipase-catalyzed reactions in DES, which are summarized in Table 3.

Table 3. Examples of deep eutectic solvents biphasic systems (DES BS) in biocatalysis.

Enzyme	Substrate	ABS composition	Application/ Product	Ref
Penicillin acylase G from *Escherichia coli*	7-ADCA and PGME	PEG and ammonium sulfate or dextran	Synthesis of cephalexin	[92]
Penicillin acylase G from *E. coli*	7-ADCA and PGME	Copolymers: P_{ADBA} and P_{MDB}	Synthesis of cephalexin	[93]
Penicillin acylase G from *E. coli*	7-ADCA and PGME	PEG and phosphate buffer	Synthesis of cephalexin	[94]
Urease *Canavalia ensiformis*	Urea	PEG and dextran	Production of $(NH_4)_2CO_3$	[95]
Urease from *C. ensiformis*	Urea	PEG and dextran	Study of cell mineralization $CaCO_3$	[96]

Lipase B has been used to synthesized biodiesel [83,84]. However, the typical enzymatic reaction has some limitations, such as mass transfer and accumulation of the by-product glycerol. The use of BS to overcome these problems has been explored as recently reported by Merza *et al.* [83]. In this work, biodiesel was produced by lipase B from *Candida antarctica* using waste oils as substrate [83]. The reaction occurred in DES composed of cholinium chloride and glycerol ([Ch]Cl:Gly), followed by the introduction of n-hexane to extract the fatty acids methyl esters produced. The content of water was also studied, with the results showing that [Ch]Cl:Gly (1:2) with 3 wt% water allowed to achieve the high yield of 44%. When comparing the use of the DES [Ch]Cl:Gly with the typical IL [BMIM][PF_6], a 63% higher yield was observed and a 71% higher yield with the addition of 3 wt% water [83]. The enzyme reuse was analyzed in 5 consecutive cycles, *i.e.*, the reuse of the DES system was improved by removing the by-product glycerol and using 1-butanol as a washing solvent [83].

In the same line, Zhang *et al.* [84] produced biodiesel using Novozym 435 (immobilized lipase B from *C. antarctica)* and yellow horn seed oil as substrate in DES media with microwave irradiation method. The authors evaluated 11 DES as alternative solvents in the reaction, and compared the results with t-butanol and the [BMIM][BF_4] IL, being the best conversion yield of 75% achieved in ChCl:Gly (1:2). BS was formed with DES, where the substrate acts as the upper phase; after the reaction, the upper phase was the biodiesel produced and the bottom phase was the DES phase, with the enzyme partitioning between the two phases [84]. Under the optimum conditions (8% of *Novozym 435*, methanol/oil ratio 6:1, microwave power 400 W, 50 ℃, and reaction time 120 min), 95% conversion yield was achieved [84]. Furthermore, the recovered enzyme was used for four successive reaction cycles with a small enzyme activity loss (10% decline of conversion efficiency was observed after two times recycling). However, the conversion yield still kept 80% above and did not dramatically decrease until the 5^{th} recycling [84]. The decrease of the catalytic activity of the enzyme after 4 runs might be due to the structure destruction of the enzyme caused by methanol and long-time stirring [84].

Syldatk and co-workers [85] studied a reaction system based on a DES consisting of [Ch]Cl and several sugars to perform the enzymatic synthesis of glycolipids [85]. The DES-based solvent system acts simultaneously as substrate and solvent and is also suitable for the enzymatic synthesis of novel tailor-made glycolipids [85]. All DES were prepared by mixing equimolar ratios of [Ch]Cl with one of the sugar components. The mixtures were constantly stirred and heated at 100°C until a colorless liquid appeared. The reactions studied were performed by adding lipase Novozyme 435 and fatty acid to the corresponding DES [85]. BS was formed after the reaction by adding ethyl acetate for glycolipid extraction, while the enzyme remains in the DES phase. With this reaction system, it was possible to form a DES-based on [Ch]Cl and levoglucosan and to

successfully use it with lipase for an enzymatic synthesis reaction [85]. This finding should help to create a novel class of glycolipids with more beneficial characteristics [85]. It is also interesting to note that the used lipase was not specific for one glycolipid product. Besides sugar-mono-laurates, many polyacrylate sugars were formed during synthesis [85].

3. Biocatalysis in aqueous biphasic systems

Albertsson introduced, in the mid-1950s, aqueous biphasic systems (ABS) as a separation technique [86], being a particular type of BS. ABS are an efficient and clean approach for biocatalysis since both phases are mostly composed of water, and thus may be a biocompatible medium for biologically active molecules, like enzymes [50]. ABS are formed when two water-soluble components, (*e.g.* polymer–polymer, polymer–salt or salt–salt combinations) are mixed in water above given concentrations, leading to the formation of a biphasic aqueous system [50]. In general, the compounds will compete for water molecules with the formation of hydration complexes, with the salting-out effect leading to the formation of ABS.

For the design of an effective ABS in biocatalytic reactions, their phase diagrams and respective tie-lines are required. This information is crucial to define ABS mixture compositions able to form two-phase systems and know the coexisting phases compositions. All ABS have a singular phase diagram under a group of conditions, like temperature, pressure and pH. Fig. 6 depicts an example of a triangular and an orthogonal representation of the phase diagram for an ABS composed of a polymer, a salt, and water, and the respective binodal curve (points TCB, Fig. 6) [50]. The binodal curve (points TCB in Fig. 6) represents the separation between the miscible and immiscible regions, *i.e.*, above the binodal curve is located the biphasic or heterogeneous region (the mixture

suffers phase separation and forms new two coexisting phases), while below it is the monophasic or homogeneous region [87]. The larger the biphasic region, the higher the ability of the phase-forming components to form BS. Three mixture compositions at the biphasic region are also identified as X, Y and Z in Fig. 6. These mixtures are located along the same tie-line (TL), meaning that all these mixtures present the same top ($T_{Polymer}$, T_{Salt}) and bottom phase compositions ($B_{Polymer}$, B_{Salt}). The determination of the TLs is usually carried out by a gravimetric approach as proposed by Merchuk *et al*. [88], which has been described in detail in several articles [89,90]. The tie-line length (TLL) is a numerical indicator of the composition difference between the two phases and it is often used to correlate with the trends observed in solutes partitioning between the phases. The critical point of the ternary system is Point C, where the two binodal nodes meet, *i.e.*, the compositions of the two coexisting phases become equal and the BS ceases to exist.

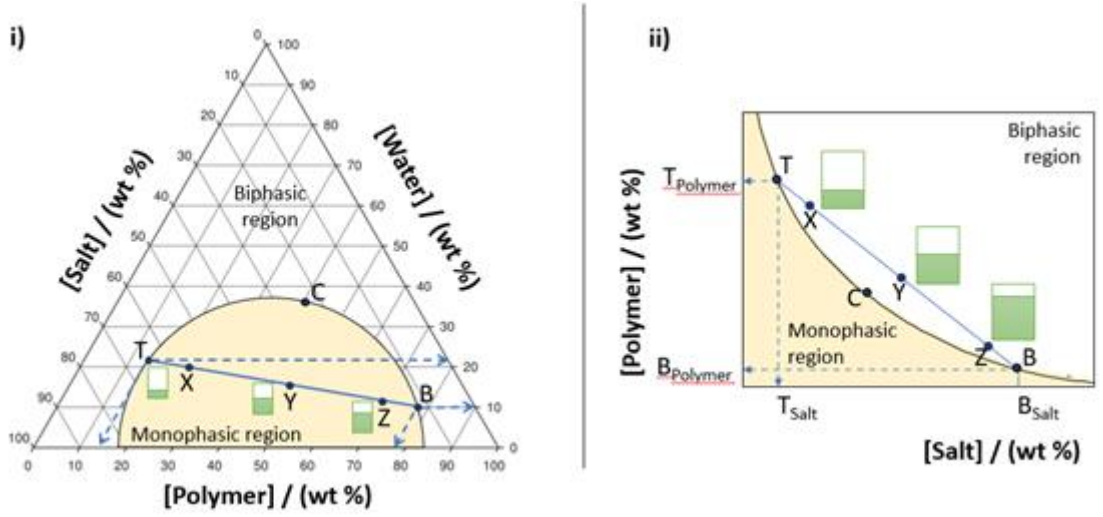

Figure 6. Schematic representation of an ABS phase diagram in an (i) triangular and (ii) orthogonal representation. TCB - Binodal curve, C - Critical point, TB - Tie-line, T - composition of the top phase, B - composition of the bottom phase, and X, Y and Z – initial mixture compositions of biphasic mixtures.

The factors that influence the formation of two-phases in ABS and have an impact on the bioreactions are molecular weight and concentration of polymer, hydrophobicity, pH and temperature [50,91]. If properly designed, the simplicity of ABS, water-rich environment, improved biocompatibility and low cost of phase-forming compounds make them appropriate for biocatalysis. Comparing to classical BS, the aqueous media present in both phases is adequate for aqueous soluble biocatalyst, substrates and products. In addition, ABS can combine production, extraction and recovery techniques into a single step when the biocatalyst is retained in one phase and the reaction product in the opposite phase. Recent developments are found in the literature regarding the use of ABS for biocatalysis, as summarized in Table 4.

Table 4. Examples of aqueous biphasic systems (ABS) in biocatalysis.

Enzyme	**Substrate**	**ABS composition**	**Application/ Product**	**Ref**
Penicillin acylase G from *Escherichia coli*	7-ADCA and PGME	PEG and ammonium sulfate or dextran	Synthesis of cephalexin	[92]
Penicillin acylase G from *E. coli*	7-ADCA and PGME	Copolymers: P_{ADBA} and P_{MDB}	Synthesis of cephalexin	[93]
Penicillin acylase G from *E. coli*	7-ADCA and PGME	PEG and phosphate buffer	Synthesis of cephalexin	[94]
Urease *Canavalia ensiformis*	Urea	PEG and dextran	Production of $(NH_4)_2CO_3$	[95]
Urease from *C. ensiformis*	Urea	PEG and dextran	Study of cell mineralization $CaCO_3$	[96]

The work of Guisan *et al*. [92] studied the reaction between 7-amino-deacethoxy-cephalosporanic acid (7-ADCA) or phenyl glycine methyl ester (PGME) with penicillin acylase G (PGA) from *Escherichia coli* in an ABS composed of polyethylene glycol (PEG) (600-20,000) and ammonium sulfate or dextran to produce cephalexin, a soluble compound. Different compositions of PEG (top-rich phase) and ammonium sulfate or dextran (bottom-rich phase) were evaluated. It was found that the highest partition coefficient (K = 23.0) for cephalexin occurred in the ABS constituted by PEG 600/ammonium sulfate (3 M), at pH 6.5, which allowed a synthesis yield of 90% of cephalexin. Using the same enzyme (PGA) and substrates (7-ADCA and PGME) Li *et al*. [93] evaluated the ABS composed of two copolymers (P_{ADBA} and P_{MDB}) in the production of cephalexin. First, the authors verified that the partition coefficient of cephalexin in this ABS was 2.57. Then, the synthesis of cephalexin in the ABS achieved a yield of 98.2%, significantly higher than the enzymatic reaction in phosphate buffer media (73.6% yield). Finally, the recyclability of phase components (copolymers) was successfully achieved (P_{ADBA} recovery = 97.4% and P_{MDB} recovery = 97.2%) by changing the pH of the system [93], showing the use of ABS in biocatalysis to be a promising approach to be applied in the biochemical industry.

Besides all the advantages that ABS offers for biocatalysis, its potential can be improved if the enzymatic reaction occurs in a continuous mode. For this, a microfluidic device can be used [94,95]. In this technology, a parallel individual laminar flow containing phase constituents, enzyme and substrate are introduced in a double Y-branched microfluidic device to continuously promote the enzyme reaction and product separation (**Fig. 7**). The reaction product generated is diffused into the preferential phase, while the biocatalyst is retained in another phase. Then, the phases are separated at the end of the microfluidic device [94,95].

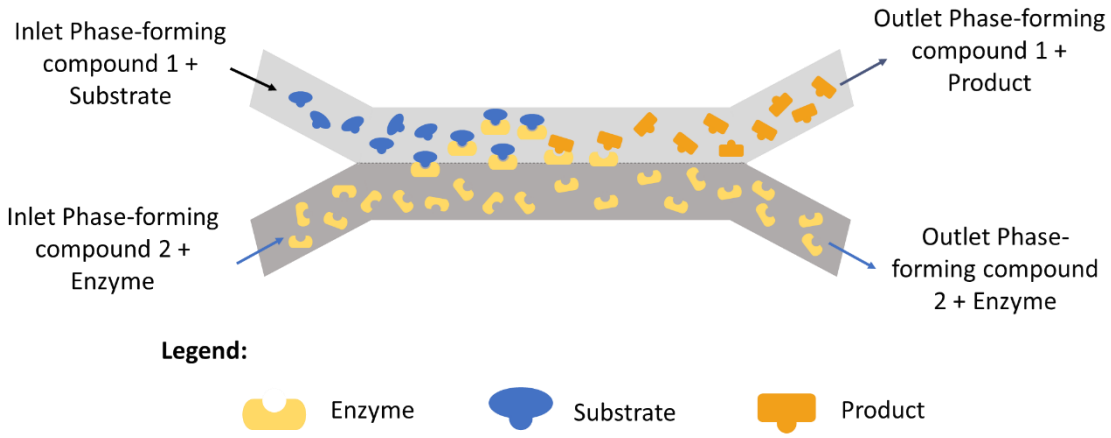

Figure 7. General scheme of an ABS microfluidic device for enzymatic reaction and product separation.

Using microfluidics, a continuous-flow process based on a properly designed ABS (constituted by PEG 4000 and phosphate buffer) was evaluated in the production of cephalexin [94]. In this study, the device offered the advantage of recycling the reaction phase containing the enzyme since the PGA preferentially migrated to the salt-rich phase, while cephalexin migrated to the PEG-rich phase. On the other hand, continuous dialysis of the recycled phase was required to avoid enzyme precipitation. At the optimized conditions, the cephalexin concentration at the outlet was initially 40 mM and then decreased to 35 mM after 300 min. With phase and enzyme recycling, the microfluidic device operated for 5 h in continuous mode. Comparing to the previous work of Guisan *et al*. [92], where the same reaction and conditions were performed in a classical ABS, the main advantage of using a microfluidic device was to combine the cephalexin production and separation in a single step and continuous mode, with enzyme recycling [94]. Another example of biocatalysis in microfluidic is the production of ammonium carbonate by the conversion of urea with urease from *Canavalia ensiformis* [95]. Compared with the conventional ABS, a reaction rate 500 times higher was obtained in

the microfluidic system under agitation. Moreover, the conversion rate of the substrate to the product was improved with the recirculation of the enzyme, obtaining an increase of about four times in four cycles [95].

ABS are also being used in cellular biology studies due to their capability to simulate organelles in liquid environments within less complex systems by forming artificial microcompartments for biomolecules and bioprocesses [97]. This approach permits understanding natural bioprocesses in cells and their interactions with the environment. To understand the environment interactions and phase compartmentalization on enzymatic mineralization on cells, Cacace and Keating [96] studied the enzymatic hydrolysis of urea to produce calcium carbonate ($CaCO_3$). The reaction was catalyzed by urease from *Canavalia ensiformis* in an ABS composed of PEG 8 000 and dextran 10 000. Urease has preference for the dextran-rich phase ($K = 0.12$), where the enzymatic reaction takes place and $CaCO_3$ precipitates, despite Ca^{2+} concentrations being the same in both phases. In this work, the volume of the dextran-rich phase was reduced while maintaining the same ABS volume, which improves local reaction rates (47% increase in $CaCO_3$ precipitated) [96].

3.1. Biocatalysis in ionic-liquid-based aqueous biphasic systems

Rogers *et al.* [49] first reported the formation of ABS constituted by inorganic salts and ILs. Since then, IL-based ABS have been used as novel alternatives for diverse applications and have been the focus of a significant amount of research, as critically reviewed by Freire *et al.* [50]. Among these applications, biocatalysis has been highlighted in the last years. While water is the traditional solvent for biocatalysis, some enzymes have been shown to be more active in hydrated ILs [37,67,98]. Thus, IL-based ABS have been proposed as viable alternatives for biocatalysis (as summarized in Table

5) with a set of other important advantages such as: lower viscosities than polymer phases, quick phase separation, and high and tailored extraction efficiency. When ILs are used in ABS, the polarity of both phases of the ABS may be adjusted, the separation process can be improved, and the biomolecule partition can be regulated [50,99,100].

Table 5. Examples of IL-based ABS in biocatalysis.

Enzyme and source	Substrate	IL-based ABS	Application/ Product	Ref
Lipase B from *C. antarctica*	1-phenylethyl acetate	[BMIM][BF_4] and NaPi Buffer	Hydrolysis of 1-phenylethyl acetate	[101]
Laccase from *T. versicolor*	Dye	PPG 400 and [Ch][DHC]	Degradation of dye	[61]
Laccase from *T. versicolor*	Rutin	PEG 600 and [Ch][DHph]	Synthesis of oligorutin	[60]
Laccase from *T. versicolor*	ABTS	Ammonium-based ZIs and PEG	Oxidation of ABTS	[87]

Meyer *et al.* [101] proposed a thermomorphic ABS composed of ILs and salt, forming a homogeneous media for the biocatalysis reaction, followed by an increase of temperature to occur phase separation and thus separate products and the enzyme. After testing several cholinium-, imidazolium- and pyridinium-based ILs, an ABS constituted by [BMIM][BF_4] and sodium phosphate (NaPi) buffer has selected to perform an enzymatic reaction with Lipase B from *C. antarctica* [101]. The hydrolysis of 1-phenylethyl acetate was performed with 49.9% conversion and 99.9% enantiomeric excess. The enzyme was reused for 6 consecutive reaction cycles with minimal activity losses [101]. However, it should be remarked that this IL is not the best option in the formation of the ABS since tetrafluoroborate-based ILs are not water-stable compounds, *i.e.*, they can hydrolyze in contact with water [102].

Trying to overcome some of the toxicity and biodegradability concerns associated to imidazolium-based ILs, ABS formed by biocompatible phase-forming components, *e.g.* constituted by cholinium-based ILs and polymers, have been developed by Ferreira *et al.* [61]. The authors studied the use of IL-based ABS for the decolorization of dyes. The enzymatic degradation occurred in the BS using laccase as the biocatalyst. Among the phase constituents evaluated, PEG 400, cholinium dihydrogen phosphate ([Ch][DHP]) and cholinium dihydrogen citrate ([Ch][DHC]) were selected since the biocatalyst presented improved performance when compared to polypropylene glycol (PPG 400) and cholinium acetate ([Ch][Acet]). The partition of the dye and the enzyme was evaluated, and it was found that the enzyme and the dye/products partitioned to opposite phases. After optimizing the best ABS conditions and composition for Remazol Brilliant Blue R (RBBR) decolorization, the system composed of PPG 400 and [Ch][DHC] was selected to evaluate the recovering and reuse of the IL-rich phase containing the biocatalyst, as summarized in Fig. 8. The phase containing the biocatalyst was able to perform six consecutive cycles of dye decolorization with a decolorization of RBBR (>96%).

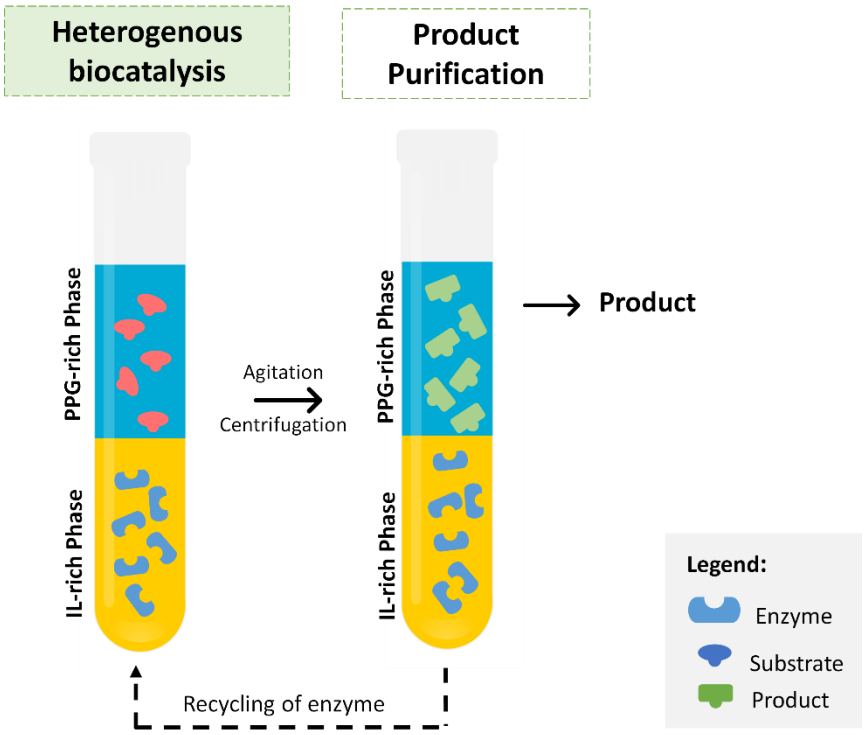

Figure 8. Integrated process using IL-based ABS for dye decolorization and enzyme reuse. Adapted from Ferreira *et al.* [61].

In the same line, Muñiz-Mouro *et al.* [60] showed the viability of cholinium-based ABS as an integrated platform for laccase-catalyzed processes. Although the laccase-catalyzed polymerization of rutin is a promising strategy, since it allows the control of the polymeric structure by the nature of the reaction medium, the biocatalyst currently represents one of the highest costs of the process. PEG-IL-based ABS have been investigated with the aim of combining the biocompatibility and low toxicity of PEGs, with increased efficiency and selectivity of the separation step associated with IL-based ABS, while decreasing the viscosity of the media when compared to polymer-based ABS [60]. Notably, using PEG 600 and [Ch][DHP] as ABS constituents, it was successfully achieved an adequate separation of oligorutin for the PEG-rich phase (60% of extraction efficiency of oligorutin ($EE_{\text{oligorutin}}$)) and of the enzyme to the IL-rich phase. The synthesis

of oligorutin by laccase was performed in a biphasic medium that allowed simultaneously to carry out the reaction, the product recovery, and the enzyme reuse without any further external stimuli [60]. The synthesized oligorutin migrated to the PEG-rich top phase (67% of $EE_{\text{oligorutin}}$), which was then recovered by separating the two phases after the first cycle of reaction. The IL-rich phase containing laccase (95% of EE_{laccase}) was recovered and reused up to three times in a new biocatalytic reaction cycle.

The use of ILs as alternative solvents and constituents of ABS is a promising topic under development. Therefore, there is still much space to research and to carry out the development of such a friendly process, for both the environment and enzymes. Other alternative solvents based on ions, namely zwitterions (ZIs), have also been considered as replacements for classical organic solvents and ILs. ZIs are compounds where a cation is covalently bonded to an anion, preventing the occurrence of ion exchanges between the coexisting phases in an ABS [103]. The authors shown that ABS can be formed by ZIs and polymers, and that these systems display a thermoreversible behavior [87]. These ABS allow the extraction and separation of the enzymatic reaction products for the polymer-rich phase, while the enzyme migrated to the zwitterion-rich phase (Fig. 9) [87]. The biocatalytic reaction occurred in a homogeneous media, with subsequent formation of the BS induced by small changes in temperature, which led to the complete separation of the enzyme from the products in a single-step. Ferreira *et al*. [87] also demonstrated that these ABS allow the reuse of the phase containing laccase at least 5 times without decreasing the catalytic activity.

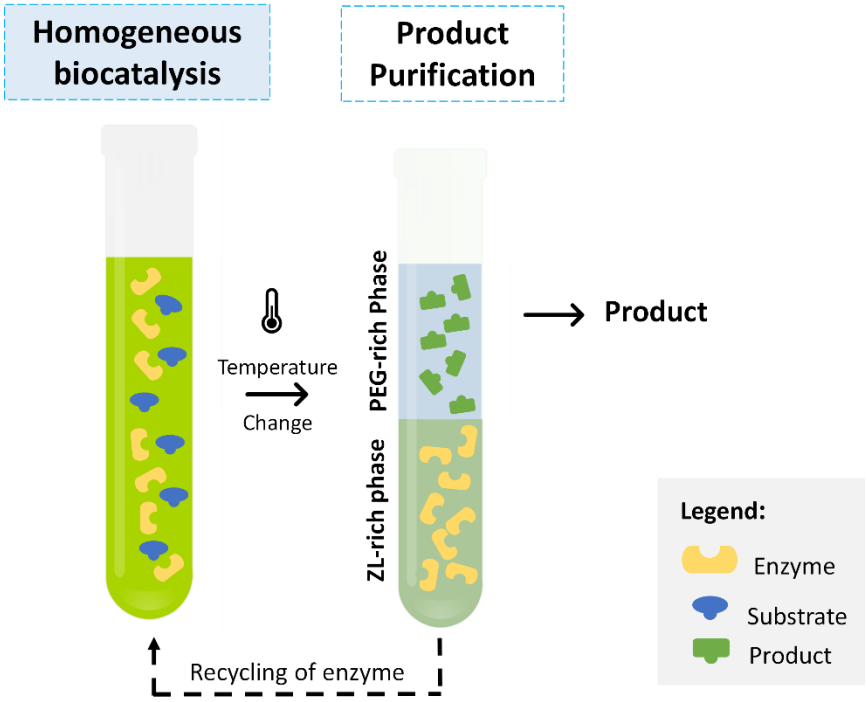

Figure 9. Flowchart of the integrated reaction-separation process developed, including the enzyme and ZI-rich phase recyclability. Adapted from Ferreira *et al.* [87].

4. Concluding Remarks

Based on the available literature biphasic systems can be a sustainable platform for biocatalytic reactions; they allow an easy recovery and reuse of the biocatalyst while ensuring the reaction products separation. When properly designed, biphasic systems can be biocompatible, emerging as novel alternatives in the field of biocatalysis under "greener" conditions. Suitable ILs and DES have emerged as excellent solvents for enzymatic reactions due to their tunable ability, especially for insoluble or partially soluble substrates in water and other common organic solvents. Moreover, the combination of ILs or DES with enzymes in biphasic systems provides excellent reaction yields, allowing integrated processed to be developed, efficiently separating the products and recycling the phase containing the biocatalyst. More important, some works proved

that enzymes can be easily recovered from the biphasic system and reused without significantly losing activity. In summary, the published studies show that biphasic systems are promising platforms as simultaneous reaction media and separation processes for several processes. However, their use and application are still limited to the laboratory scale. Thus, scale-up studies should be carried out to evaluate the scalability of the process to be implemented at an industrial scale. Moreover, since ILs and DES can be tailored to fit the requirements of other biocatalytic processes, we expect that BS based on ILs or DES may gain recognition, attract further interest, and play a significant role in developing enzymatic sustainable bioprocesses.

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