7 Biocatalytic Reactions in Ionic Liquids

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ABSTRACT

Today biocatalytic reactions are widely used in industrial processes. The spectrum of applications has grown since the discovery that enzymes are active even in organic solvents. The main advantages of organic solvents are increased solubility of hydrophobic substrates, easy product purification, possible enzyme stabilising effects, and suppression of side reactions such as hydrolysis. But biocatalysts show a lack of activity and stability in protic and polar solvents, which is a common problem using enzymes. The hope of scientists for ionic liquids is to close this gap. Many studies have been carried out since early reports published in 2000. Many applications show good activities, yields, and/or selectivities for single and multiphase systems. In addition to that, many researchers deal with the question about influence of ionic liquids and impurities such as water on the biocatalytic reactions. Another field of interest is the use of whole-cell systems, as they allow the use of a greater variety of enzymes because of their integrated cofactor regeneration. In order to use a whole-cell system, several important points have to be taken into consideration, such as finding an appropriate solvent, catalyst, and downstream process. In addition to these points, the toxicity of ionic liquids against cells is also very important, and several reports give an overview of which ionic liquids are biocompatible.

7.1 INTRODUCTION

Biocatalysts, such as enzymes and whole-cell systems, have been used for a long time in human history. In ancient Mesopotamia, China, and Japan,

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biocatalysts were used to refine food or to produce alcoholic drinks. Researchers in the nineteenth century investigated enzymes and carried out the first syntheses, Emil Fischer developed the lock-and-key principle, and Croft Hill synthesised isomaltose with the aid of an enzyme [1, 2]. In the twentieth century, enzymes were mostly used and investigated in aqueous solution, but the first studies about enzymes in organic solvents were published in the late 1980s [3–5]. The main advantages of organic solvents are increased solubility of hydrophobic substrates, easy product purification, possible enzyme-stabilising effects, and suppression of side reactions such as hydrolysis. Nowadays biocatalysts are widely used in varying industrial processes. β-Tyrosinase is applied as biocatalyst in the production of L-dopa, and dextrine is synthesised by an amylase [6, 7]. Biocatalysts work excellently in non-polar and aqueous solvents but show a lack of activity and stability in protic and polar solvents, which is a common problem using enzymes. The hope of scientists is for ionic liquids to close this gap. The first promising studies showed comparable activity and stability with conventional media [8, 9]. To the present day, many studies and reviews are dealing with biocatalytic synthesis in ionic liquids, although there is still a need to clarify the relationships between structure, stability, and activity.

7.2 ENZYMES IN IONIC LIQUIDS

In 2000, Erbeldinger et al. reported the synthesis of aspartame from carboxybenzoxy-L-aspartate and L-phenylalanine methyl ester chloride. The special feature of this reaction was the unusual choice of solvent using a thermolysin-catalysed reaction in $[C_2mim][PF_6]$. The initial enzyme activity and yield were of the same order of magnitude as in conventional solvents. Surprisingly, the stability of the enzyme was much higher than in the commonly used ethyl ethanoate [8]. The study was ground-breaking for research in the field of biocatalysis in ionic liquids and led to a rapid increase of publications.

The first enzymes that were applied in research were lipases. This Enzyme Class (EC 3.1.1.3) already showed good activity and stability in organic solvents and established new ways of synthesis, for instance, esterification of fatty acids with alcohols. A fundamental problem of commonly used organic solvents is insufficient solubility of the substrate. Polar substances dissolve better in polar than in non-polar media. In contrast, enzymes show only small or no activity in polar solvents because the enzyme competes for the needed water in order to sustain its conformation [10]. This effect depends on the nature of both the enzyme and the solvent. High substrate solubility for polar substances was achieved by using ionic liquids as solvent, which additionally were believed to offer good enzyme activity. Park and Kazlauskas discovered a possibility to improve and control selectivity [11]. They solvated glucose in the ionic liquid $[C_2mim][BF_4]$, and acetylated it with the aid of *Candida*

antarctica Lipase B (CaLB) and vinyl acetate. The problem with using common organic solvents is the higher solubility of the product than of the precursor, resulting in a further acetylation and a decreasing yield. With the use of 1-(2-methoxyethyl)-3-methyl-3*H*-imidazolium chloride ($[C_1OC_2mim][BF_4]$), a mono-acylated product was isolated with a yield of 99% and a selectivity of 93%. The main advantage, in contrast to propanone as solvent, is the 100 times higher solubility of glucose. In general, lipases showed the highest tolerance to ionic liquids among all researched enzymes. Ionic liquids with the general structure $[C_n mim]Y$ and $[C_n py]Y$ ($n = 2$–10, $Y^- = [BF_4]^-$ or $[PF_6]^-$) were similarly efficient as the organic solvents methyl *tert*-butyl ether, toluene, and dioxane. For these investigations, lipases CaLB and PCL (*Pseudomonas cepacia* lipase) were used [12–14]. Lipases are able to catalyse a broad range of common and uncommon types of reactions in ionic liquids. Esterification, trans-esterification, acylation of amines, and the aminolysis have been reported [13, 15–19]. Some of these reactions are characterised by good enantioselectivities and yields.

Besides lipases, other enzyme classes have also been investigated. Among others, glycosidases, esterases, phosphatases, laccases, glucooxidases, and dehydrogenases have been utilised as biocatalysts in ionic liquids [18, 20–24]. The very first described biocatalysis in an ionic liquid was carried out with the protease thermolysin, as already mentioned before. Studies with α-chymotrypsin showed a very good conversion for a trans-esterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol in different ionic liquids. The enzyme activity in the ionic liquid $[C_2mim][N(SO_{-2}CF_3)_2]$ had a value of $13.6\,U\,mg^{-1}$ and hence was in the same range as found in the organic solvent 1-propanol ($23.2\,U\,mg^{-1}$). Half-life and conversion increased for all ionic liquids used, as opposed to a system containing 1-propanol as solvent in which denaturation took place within 30 minutes after addition of enzyme. Moreover, the amount of product formed was only 25% [25]. Table 7.1 shows a small selection of important biocatalytic syntheses in ionic liquids out of a large number of published papers.

New interesting possibilities open up with two-phase systems for biocatalysis in ionic liquids, since dehydrogenases, for example, are dependent on cofactors and show no activity in pure ionic liquid systems. By using a biphasic system, it is possible to overcome this restriction. While enzyme and cofactors remain in the aqueous phase, product and substrate are present in the ionic liquid phase, a good distribution coefficient provided [26, 27]. Further advantages are simplified catalyst separation, recycling of enzyme solutions, and improved conversions. In two-phase systems composed of $H_2O/[C_4mim][PF_6]$, thioanisol was oxidised by peroxidase and glucose oxidase. The stereoselectivity was comparable with purely aqueous systems, accompanied by a largely improved solubility of the substrates. The isolation of products and substrate from the ionic liquid phase is essential, and a principal problem. The often proposed distillation works only for volatile compounds. Otherwise, remaining involatile compounds limit the recyclability of the ionic liquids.

TABLE 7.1 A Small Selection of Important Biocatalytic Syntheses Reported in Ionic Liquids

Enzyme Class	Enzyme	Ionic Liquid[a]	Comment	Reference
Oxidoreductase				
1.1.1.1	Alcohol dehydrogenase	1	Biphasic, improved yield	[24]
1.10.3.2	Laccase	4-9	Good activity and stability	[88, 89]
1.11.1.7	Horseradish peroxidase	3,6	Used in sol–gel and w/IL emulsion	[90, 91]
Hydrolase				
3.1.1.3	*C. antarctica* Lipase B	1,2,6,10,11	Good stability, activity, and yield	[13, 16, 46]
3.1.3.1	Alkaline phosphatase	12,salts	Influence of salts on activity	[21, 92]
3.2.1.21	Cellulase	13,14	Used on crop samples	[85, 93]
3.2.1.23	β-Galactosidase	1,2,6,15,16	Whole-cell-system good possibility	[20, 52]
3.4.21.1	α-Chymotrypsin	2,6,10,11	Substrate and alkyl-length influence	[25]
3.4.21.62	Subtilisin	17	Increased yield and ee%	[94]
3.4.24.27	Thermolysin	2	First published synthesis in ionic liquid	[8]
3.5.1.4	Peptid amidase	15,18	Amidation of H-Ala-Phe-OH	[95]
Lyase				
4.1.2.37	Hydroxynitril lyase	6,10,19	High yield with high ee%	[96, 97]
Isomerase				
5.1.2.2	Mandelate racemase	15,16,20	Influence of a_w on synthesis	[98]

[a] Ionic liquids are designated: (1) $[C_4mim][N(SO_2CF_3)_2]$, (2) $[C_4mim][PF_6]$, (3) $[C_8mim][N(SO_2CF_3)_2]$, (4) $[C_4mim]Br$, (5) $[C_4mim]Br$, (6) $[C_4mim][BF_4]$, (7) $[C_2mim][C_2H_5SO_4]$, (8) $[C_2mim][CH_3SO_4]$, (9) $[C_2mim][C_1OC_2OC_2OSO_3]$, (10) $[C_2mim][BF_4]$, (11) $[C_2mim][N(SO_2CF_3)_2]$, (12) $[C_2H_5NH_3][NO_3]$, (13) $[C_2mim][CH_3COO]$, (14) $[C_2mim][(C_2H_5)_2PO_4]$, (15) $[C_1mim][CH_3SO_4]$, (16) $[C_4mim][C_8H_{17}SO_4]$, (17) $[C_2py][CF_3COO]$, (18) $[C_4mim][CH_3SO_4]$, (19) $[C_5mim][BF_4]$, and (20) $[C_8mim][PF_6]$.

Additional purification steps may overcompensate the positive effects, leading to higher costs at the end [23].

Many enzymes tolerate ionic liquids in their reactions only to a certain amount. Beyond this, most of them will become deactivated or denaturated. It has to be tested, for the given combination of enzyme and ionic liquid, whether or not this is a reversible or irreversible process. But even when denaturation may occur, a better process performance may be achieved, for example, higher yields by suppression of side reactions. For a final evaluation, a careful investigation of thermodynamic and kinetic properties of the reaction system is mandatory.

7.3 SINGLE-PHASE AND MULTIPHASE SYSTEMS FOR BIOCATALYSIS IN IONIC LIQUIDS

There are many options using an ionic liquid in a biocatalytic system. First of all, they can be employed as a single-phase system by adding pure ionic liquid. Then, it is possible to create a biphasic system by combining a water-immiscible ionic liquid, such as $[C_4mim][PF_6]$, with water or buffer. Ionic liquids can also be used as additives and solubilising agents, and as water-in-ionic liquid (w/IL) micro-emulsions.

Not many enzymes tolerate high concentrations of ionic liquids if they are utilised in a single-phase system. Mainly lipases are suitable for such reactions, as already discussed earlier. In addition, enzymes are scarcely dissolved in ionic liquids and form suspensions. But advantages of a single-phase system include the good solubility of polar substances and the suppression of side reactions. Disadvantages include the problems of product isolation and recycling of ionic liquids. However, products with a low boiling point can be evaporated from the reaction mixture and product isolation can be eased, implicating another two advantages: the reusability of the reaction mixture is provided for, and removal of the reaction products leads to a shift of the chemical equilibrium towards the products [28, 29].

By using a two-phase system, it is possible to simplify downstream processes to a further extent. A biphasic system is generally assembled from an aqueous phase and an ionic liquid phase containing a water-immiscible ionic liquid such as $[C_4mim][PF_6]$ or $[C_4mim][N(SO_2CF_3)_2]$. Organic substrates and products should dissolve better in ionic liquids, while the enzymes remain in the aqueous buffer solution. By this means, high product yields and good enzyme activities are achieved and inhibition by-products or substrates are avoided. The application to different classes of enzymes besides hydrolases and lipases is an additional advantage, since cofactor regeneration is easier and the partial inactivating effect of the ionic liquids can be reduced severely in a two-phase systems. The mass transfer limitation can be considered a negative aspect in biphasic systems. Due to the high viscosity of ionic liquids, insufficient mixing can lead to a limitation of mass transfer, inducing decreased enzyme activity

[9]. But in an experiment with a well-mixed system, the ionic liquid [C_4mim][PF_6], having a high viscosity of 397 mPa s, showed higher initial reaction rates than [C_4mim][$N(SO_2CF_3)_2$], which has a viscosity of only 27 mPa s [30].

A promising new possibility of increasing the solubility of enzymes in ionic liquids is w/IL. The enzyme is stabilised in this system, containing buffer and water-immiscible ionic liquid, with the aid of surfactants. Nano- or micro-sized water domains are formed [31]. This layer of water protects the enzymes from the damaging impact of solvent while maintaining good stability and activity. Currently, a major problem is the low solubility of surfactants in ionic liquids. This limitation can be overcome by producing new surfactants with better solubility properties or by adding an organic solvent as solubiliser. Moniruzzaman et al. reported the first example for an enzymatic reaction in a microemulsion of [C_8mim][$N(SO_2CF_3)_2$] with the help of sodium bis(2-ethylhexyl) sulfosuccinate (AOT). The hydrolysis of 4-nitrophenyl butanoate by the lipase *Burkholderia cepacia* was investigated, and a higher activity than in a microemulsion of isooctane (water-in-oil [w/o] emulsion) was achieved [32]. In a more recent study, ternary mixtures of [C_4mim][PF_6]/H_2O/Tween20 were the centre of research, and their stability and activity were determined. A conversion of nearly 100% was obtained in a mixture of 80% IL, 15% Tween20, and 5% water. Furthermore, different compositions of the ternary system were compared with each other and with a w/o emulsion consisting of AOT and hexane. All tested w/IL emulsions provided higher yields and mostly better activities while showing vastly improved stabilities compared with a purely aqueous system [33]. Still, the use of surfactants could lead to additional efforts necessary for downstream processing.

Hussain et al. demonstrated another application for ionic liquids in a biocatalytic system. To gain higher substrate solubility, the work group added 20% of water-miscible or water-immiscible ionic liquids to a fermentation of 6-bromo-β-tetralone to (S)-6-bromo-β-tetralol. Especially interesting was the fact that water soluble [C_2mim][4-$CH_3C_6H_4SO_3$] allows, besides a high yield, a good initial activity and also an excellent substrate solubility. The prevented aggregation of yeast cells and thus better suspension is the proposed reason for obtaining improved results (compared with a 10% ethanol solution) [34].

7.4 INFLUENCE OF IONIC LIQUIDS ON ENZYME AND SUBSTRATE

Ionic liquids have the extraordinary ability to solubilise natural materials such as cellulose, carbohydrates, and amino acids [8, 35–37]. In particular, the dissolution of cellulose and other biopolymers makes it possible to find new or enhanced ways of utilisation and production of basic chemicals from nature.

Enzymes do not often dissolve in pure ionic liquids, but form a suspension in the case of a well-mixed system. Although enzyme solvation in hydrophilic ionic liquids such as [C_4mim][NO_3], [C_4mim][$MeSO_4$], or [C_4mim][lactate] can

be achieved, no observable catalytic activity with lipases has been found. These ionic liquids break up protein–protein bonds, resulting in a denaturation of the enzymes. So far the understanding is that the deactivation is mainly due to the anion. A few publications have dealt with the potential of ionic liquids for renaturation of proteins. The reversible denaturation of lysozyme in $[EtNH_3]$ $[NO_3]$, for example, has been investigated by fluorescence spectroscopy [38].

In general, a solvent should interact as little as possible with substrates, products, and the biocatalyst itself. But ionic liquids cannot be considered completely inert. A weak acidity at the H2-proton within the ring was discovered in 1-alkyl-3-methylimidazolium ionic liquids [39]. Most of the commonly used anions showed neutral or weak basic characteristics. These have the ability to interact with acidic or basic elements of reaction mixtures and can affect parameters such as activity and stability. To explain and understand the interaction between protein and ionic liquid, many researchers refer to the Hofmeister series, since ionic liquids dissociate in sufficient amounts of water into cations and anions. For 40 years, it has been known that enzyme activity follows the series developed by Hofmeister. Kosmotropic anions and chaotropic cations have been discovered as protein stabilising agents (see Figure 7.1). Kosmotropic anions compete for water that is associated with the enzyme. Thus, the surface area of the enzyme decreases and unfolding of the enzyme structure is inhibited. Chaotropic anions, on the other hand, bind to the protein–water interface due to their high polarisability, and consequentially destabilise the enzyme [40, 41].

The situation becomes more complex when ionic liquids are used as pure solvent with a small amount of water. Chaotropic anions such as $[BF_4]^-$ or $[PF_6]^-$, in contrast to kosmotropic anions, compete only weakly with the water at the protein–water interface [40, 42, 43]. In this case, the enzyme needs the water to maintain its conformation, meaning that kosmotropic anions are destabilising. Generally, the Hofmeister series shows only poor correlation when using a pure ionic liquid: for example, the chaotropic anion $[NO_3]^-$ has a negative impact on lipase activity. Obviously, the hydrophobicity is much

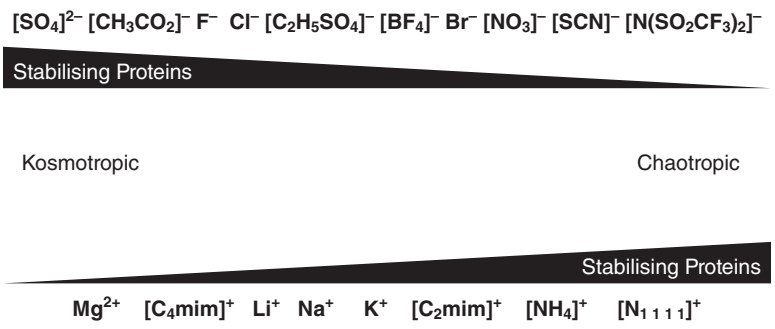

Figure 7.1 Hofmeister series to describe the influence of ionic liquids on proteins [40, 42, 43].

more dominant, resulting in retained activity of enzyme suspensions in ionic liquids. Nevertheless, there is a publication reporting the impact of protein denaturants and stabilisers on water structure, claiming that the Hofmeister effects are more or less a coincidence [44]. However, it can be concluded that the Hofmeister series is much more suitable for understanding the influence of ionic liquids on enzyme activity than the log *P* value. The often used polarity scales for ionic liquids using Reichhardt's dye or other tools seem not to be useful to describe ionic liquid–protein interactions.

7.5 WATER CONTENT AND WATER ACTIVITY

Water content plays a major role in enzyme-catalysed reactions in organic media. Every enzyme needs a minimum of water to maintain its conformation and unfold their ternary structures. A sphere of protecting water molecules is discussed in different publications. Of importance, a complete removal of water from the organic solvent induces a severely reduced enzyme activity [10, 17, 45]. For a long time, no relation between water content in different organic solvents and enzyme activity could be detected, until Peter Halling and his colleagues described a correlation with water activity. It was found that an enzyme showed the same activity when, besides the temperature, the same water activity was used in different organic solvents instead of the water content [10]. The thermodynamic water activity a_w is the free amount of water in a substance, which is in direct contact with the surrounding atmosphere. This can be described and measured by the quotient of water vapour pressure of the substance and of pure water:

$$a_w = \frac{p_w}{p_{ws}},$$

where a_w is the water activity, p_w is the water vapour pressure of the substance (in pascals), and p_{ws} is the saturated vapour pressure of the pure water (in pascals).

In a similar way, the water content of ionic liquids should be also controlled carefully, when used as reaction media for biocatalysts. However, in order to compare ionic liquids with each other and with organic media, researchers should work with water activity instead of water content. This will lead to more consistent data and reliable parameters for enzyme activity, stability, and conversion [46]. Garcia et al. published a comparative study about the influence of non-conventional media on biocatalysis and used defined water activities for comparing the results (see Figure 7.2) [47]. In addition, it has to be considered that ionic liquids contain some residual water, even after drying, due to their relative hydrophobicities. Furthermore, many ionic liquids are hygroscopic and attract water as long as they are not kept under water-free conditions [48, 49].

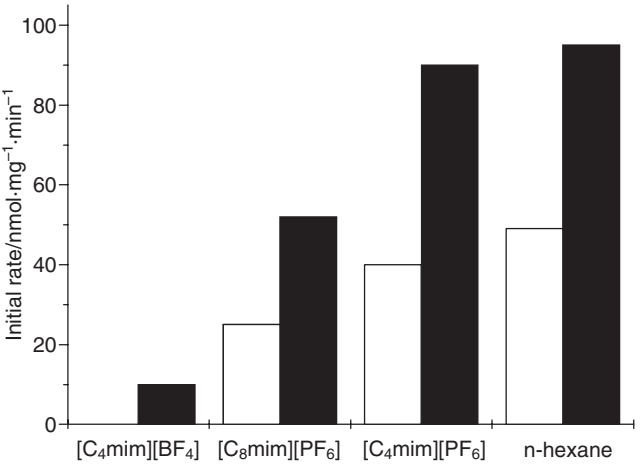

Figure 7.2 Initial rates of trans-esterification for immobilised cutinase in different solvents, $a_w = 0.2$ (white), $a_w = 0.7$ (black) [47].

While Karl Fischer titration is an easy and fast opportunity to receive information on the current water content in a liquid system, there is no adequate method for the determination of water activity. Devices for the measurement of water activity often need large sample volumes and also a considerable amount of time, since the ionic liquid must equilibrate with its vapour phase above. It is possible to avoid this problem by plotting the measured water activity versus water content for a solvent at a given temperature. By means of these data, it is possible to determine the water activity in this solvent by measuring only its water content. It has to be kept in mind that all compounds, not only the solvent, in a mixture contribute to the water content. Another problem, which has yet to be solved, is adjusting a defined water activity in a system. Equilibration with a saturated salt solution is a commonly used technique (see Figure 7.3). A vessel with the ionic liquid or organic solvent is placed in another larger sealed vessel, containing an oversaturated salt solution. The vapour phase over the saturated salt solution equilibrates with the vapour phase above the solvent. The equilibrating vapour phase is in contact with the solvent and is also subject to equilibration, resulting in a solvent with a specific water activity, which is dependent on the different salts used in the saturated salt solution. Still, this method needs a great amount of time for equilibration.

7.6 IMPURITIES

Especially at the beginning of research into the area of ionic liquids, many results were difficult to reproduce. A possible reason was, and still is today,

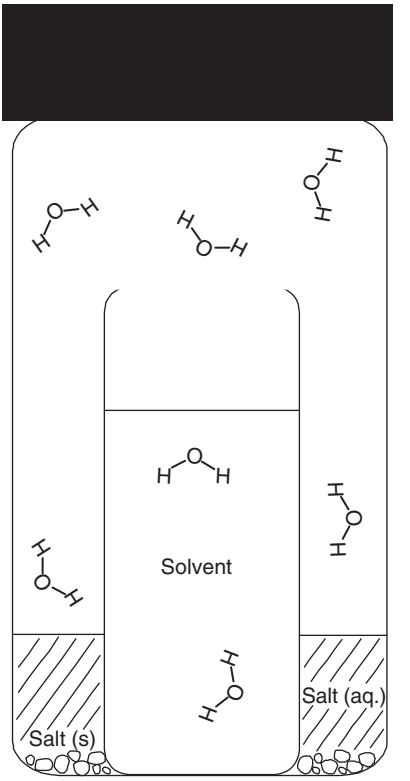

Figure 7.3 Method for adjusting water activity in a solvent system.

impurities in ionic liquids. An excellent example is the varying of melting temperature from 5.8 to 15 °C of the ionic liquid $[C_4mim][BF_4]$ [50]. Widegren et al. also report that viscosities vary around 30% at different temperatures [51]. These impurities can consist of solvents and reagents used in the synthesis of ionic liquids, remaining salts, as well as water. They decrease the viscosity and influence the melting point. Ionic liquids, polluted with starting substances, can also have a significant impact on the biocatalytic reaction since they could affect both the activity and the stability of the enzyme. Consequently, every synthesis should be followed by a purification step. Evaporation in vacuum or extraction may be a sufficient method to remove impurities. Another possibility lies in a change of the synthetic procedure. Conversion in metathesis reactions, for example, is strongly dependent on the salt used for precipitation [50]. As long as all groups work with the same high quality of ionic liquids, reproducibility and comparability of results can be achieved.

7.7 BIOCATALYSIS IN WHOLE-CELL SYSTEMS

While lipases and hydrolases easily catalyse reactions in pure ionic liquids, enzymes such as dehydrogenases can only be active in two-phase systems or in one-phase systems with water-miscible ionic liquids. These enzymes require cofactors for their regeneration. But cofactors are expensive and should not inhibit the reaction in any way. This problem can be avoided by using whole cells as biocatalysts since they have integrated cofactor regeneration. Whole cells are used in many examples to produce fine chemicals, often with high regio- and enantioselectivities. Besides, wild-type organism recombinant cells with specific properties are used, which could be resistant to solvents or able to achieve improved yields and activities. Despite the many advantages, there are still problems to be solved in whole-cell biocatalysis. In general, similar rules should be applied for whole-cell systems and isolated enzymes. However, organisms have additional possibilities for interaction with the surrounding environment.

To set up a successful whole-cell biotransformation, the following points have to be addressed:

(i) For the desired reaction, a suitable whole-cell biocatalyst has to be found or designed with the help of genetic engineering.
(ii) Other enzymes present in the cell may cause undesirable side reactions.
(iii) Downstream processing of the reaction is complex and depends on whether the product is sequestered inside the cells or excreted from the cells into the solvent. Moreover, a contamination with cell residue, biosurfactans, polynucleotides, and polysaccharides is possible.
(iv) Accumulation of substrates and products in the cells leads to inhibition of the reaction. Overall cell catalytic activity declines subsequently.
(v) As long as product concentration is low, either due to limited solubility or due to low productivity, large volumes of aqueous solution have to be processed with all associated negative aspects, such as waste and energy consumption.

To overcome these problems, a biphasic system can be applied. Products and precursors will be extracted into the organic phase, while fermentation takes place in the aqueous phase. *In situ* extraction is strongly dependent on the solvent; it should provide a high log *P* value, dissolve products and precursors better than the buffer phase, and should not be cytotoxic or harmful to humans and the environment. Few solvents come into consideration because of this specification. Long-chained alkyl alcohols, aldehydes, and ketones are possible media for fermentation. By making the correct choice, substrate and products remain in the organic layer, while the biocatalyst is located in the

aqueous phase. A limitation by catalyst inhibition is avoided, and additional side reactions such as hydrolysis in esterifications are suppressed.

The potential of ionic liquids for use with whole cells has also been tested quite early. However, a prediction as to which ionic liquid is more suitable for a catalytic system is difficult since results in several studies are contradictory. Ionic liquids should fulfil the same requirements as organic solvents. First, high distribution coefficients for substrates and products are desired to overcome inhibition. With a better distribution into the ionic liquid phase, better yields and selectivity can be expected. As opposed to organic solvents such as toluene aggregation into the interphase of a two-phase system was not observed. This characteristic is beneficial for downstream processing. In this context, well-mixed phases are necessary to eliminate mass transfer limitations due to the high viscosity of ionic liquids. Otherwise, poorer conversions and slower initial reaction rates than in organic systems will be observed [9]. If the ionic liquid is dispersed in the system as fine spheres, the mass transfer will not be limited. Another possibility to decrease this limitation is the use of more hydrophilic substrates resulting in a higher concentration of substrate in the aqueous phase [30].

Experiences with whole-cell systems in ionic liquids are mostly based on empirical data. Scientists report biocatalysis with a good conversion but a poor enantiomeric excess, or the other way around. But there are also counter-examples. In a system containing buffer phase, $[C_4mim][N(SO_2CF_3)_2]$, and cells of *Lactobacillus kefir*, twice the yield with constant high enantiomeric excess was obtained. This system was scaled up to a 200-cm^3 system, with comparable results [28, 52].

Lou et al. used a whole-cell system from *Saccharomyces cerevisiae* for the reduction of acetyltrimethylsilane, $CH_3C(O)SiMe_3$, in order to produce enantiopure (S)-1-trimethylsilylethanol, $CH_3CH(OH)SiMe_3$ (see Figure 7.4). The hydrophobic $[C_4mim][PF_6]$ and the hydrophilic $[C_4mim][BF_4]$ were used. In contrast to a hexane/buffer system and pure aqueous buffer system, better initial activities, yields, and enantiomeric excess were achieved (see Table 7.2). Both ionic liquid systems could be recycled at least six times without losing noteworthy activity [53].

Alongside extractive fermentation, extraction is also a possible downstream process. Important for an effective extraction is the distribution coefficient of product in the ionic liquid, K_d:

$$K_d = \frac{m_a([A_i]-[A_f])}{m_s[A_f]},$$

where m_a is the mass of the aqueous phase (in grams), m_s is the mass of the solvent phase, $[A_i]$ is the initial concentration of solute in aqueous phase (in $mol \cdot l^{-1}$), and $[A_f]$ is the final concentration of solute in the aqueous phase (in $mol \cdot l^{-1}$).

Ionic liquids have also been tested for selective extraction of natural compounds among others [54]. The ionic liquid $[P_{6\,6\,6\,14}][N(SO_2CF_3)_2]$ has relatively

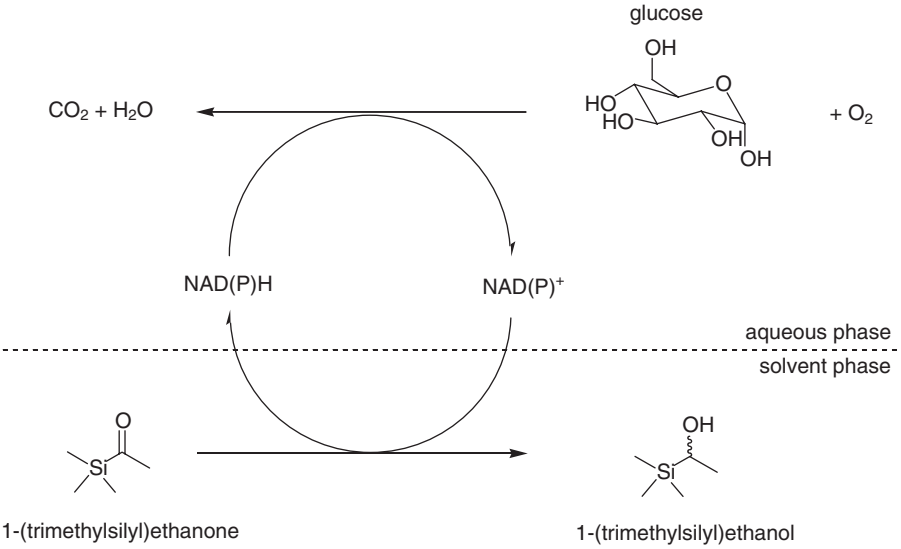

Figure 7.4 Conversion of prochiral acetyltrimethylsilane to (S)-1-trimethylsilylethanol with *Saccharomyces cerevisiae* [53].

TABLE 7.2 Reduction in Acetyltrimethylsilane (ATMS) to (S)-1-Trimethylsilylethanol with *Saccharomyces cerevisiae*, Buffer: Tris-HCl [53]

Solvent	pH	*c*(ATMS)/ mM	Initial rate/ $mM\,h^{-1}$	Yield/%	ee/%
Buffer (aq.)	7.3	14	4.88	84.1	82.7
Hexane:buffer (2:1)[a]	8.0	42	1.45	97.4	95.4
$[C_4mim][PF_6]$:buffer (1:6)[a]	7.3	84	63.4	99.9	99.9
$[C_4mim][BF_4]$:buffer (10%)	7.3	77	74.5	99.2	99.9

[a] Formation of a biphasic system.

high distribution coefficients at pH 11 for ethanoic, lactic, and succinic acid, with $K_d = 6, 14$, and 23, respectively [55]. Amino acids were also extracted well from different ionic liquids, adding dicyclohexano-18-crown-6 to the ionic liquid phase formed by $[C_4mim][PF_6]$. The distribution coefficient for tryptophan was 76: 96% of amino acid was recovered. Similar results were obtained with a model solution for a fermentation broth [56].

The extraction of penicillin G from an aqueous two-phase system containing ionic liquid, water, and $Na[H_2PO_4]$ showed an extraction yield of 93% with $[C_4mim]Cl$, and 93.7% with $[C_4mim][BF_4]$. The yield from fermentation at pH

5–6 was 91.5% [57]. Furthermore, aniline, benzoic acid, 4-toluic acid, and erythromycin were extracted with the help of [C_4mim][PF_6] [9, 58]. However, some of the examples also suffer from the problem of isolation of the product from the ionic liquid phase, and for its recycling.

7.8 ENVIRONMENTAL IMPACT OF IONIC LIQUIDS

If ionic liquids are to be used in biocatalytic synthesis, information about their toxicity must be gathered. Toxicity data are necessary to evaluate survivability of the microorganisms in the fermentation broth, but additionally to allow a risk assessment for the environment. Objects of investigation are, besides the intended or unintended contamination of the environment, the behaviour and deposition of ionic liquids within the environmental cycle of matter. Synthesis of ionic liquids is the first problematic step in evaluation of toxicity. Basically, the very low vapour pressure of ionic liquids has the benefit of producing less air pollution than any organic solvent. Many different solvents are used during the synthesis and recovery of ionic liquids, resulting in atmospheric loading with pollutants and reducing the advantage of low vapour pressure [59]. Investigations concerning toxicity and biodegradability discovered a massive threat for aquatic organism from several ionic liquids [60–62]. The toxicity of imidazolium- and pyridinium-based ionic liquids was found to be dependent on the length of the alkyl chain. In conclusion, the longer the side chain, the higher is the toxicity of an ionic liquid (see Table 7.3). 1-Octyl-3-methylimidazolium bromide, for example, shows toxicity towards *Vibrio fischerii* exceeding the EC_{50} value of toluene and benzene, whereas 1-butyl-3-methylimidazolium shows almost no toxicity at all [63].

It should be kept in mind that, similar to the work with enzymes, impurities may be responsible for the toxicity effect. This might be especially important when reading early studies because producers of ionic liquids had to learn how

TABLE 7.3 Toxicity of ionic liquids with a culture from *V. fischerii* [63]

Ionic Liquid	log *P*	EC_{50}/ppm
1-Octyl-3-methylimidazolium bromide	0.80	1.77
1-Hexyl-3-methylimidazolium bromide	0.15	6.44
Toluene	2.73	31.7
1-Butyl-3-methylpyridinium dicyanamide	–2.40	98.0
Benzene	2.13	108
1-Butylpyridinium chloride	–	440
1-Butyl-3-methylimidazolium chloride	–2.40	897
Trichloromethane	1.97	1,199
1-Methylimidazole	–	1,218
1-Butyl-3-methylimidazolium bromide	–2.48	2,248
Methanol	–0.74	101,068

to improve purity and had to install appropriate tools for quality control. Ionic liquids show a particular behaviour that influences their environmental fate, as well as the analysis of ionic liquids. Depending on the concentration and nature of solvent, ionic liquids form aggregates or cluster. Of course, the type of cation and anion influences this as well. The property could be verified by mass spectrometry, conductometry, and simulation [64–66]. These clusters might be able to explain the solubility effect of hydrophilic compounds when present in aqueous phase, especially since addition of salt normally leads to decreasing solubility (salting out).

These disadvantages led to an ecological revision of the handling of ionic liquids, with the result that more and more scientists try to focus their attention on exploring environmental friendly ionic liquids. Successes were achieved in ecological balance by changing to a microwave-based solvent-free reaction control for the synthesis of 1-alkyl-3-methyl imidazolium salts [67]. Regarding the regeneration of ionic liquids from reaction systems, Keskin et al. described methods and results for the extraction of ionic liquids with supercritical CO_2 [68]. In order to reduce the toxicity of ionic liquids themselves, the application of ions found in nature or gained from natural substances is beneficial. Several publications report the successful synthesis of ionic liquids in which either cations or anions are represented by amino acids [69, 70]. Additionally there are attempts to increase the biodegradability of ionic liquids [71]. In the area of environmental sustainability, more research has yet to be done, especially with respect to the unknown toxicity of numerous compounds. The Centre for Environmental Research and Technology of the University of Bremen (UFT) created a comprehensive database that provides information, among other things, about the hazardous behaviour of various ionic liquids [72].

Speaking of environmental aspects, it is also essential to turn attention towards the biocatalytic reactions in ionic liquids. Many studies just cover the influence on the environment, but not the fermentation of ionic liquids. Biocompatibility of solvents in a two-phase system is an important factor. It is important to differentiate between molecular toxicity and phase toxicity. If a solvent has a direct impact on the cell or the enzyme, it is defined as molecular toxicity. The solvent can modify cell walls, inhibit enzymes, denaturise proteins, or expand membranes by inclusions. Phase toxicity occurs when fundamental nutrients or substrates, necessary for the function of an organism, are extracted into the solvent phase, leading to an inhibition of cell growth. By forming a solvent layer around the biocatalyst, mass transfer can be reduced. Another possibility of phase toxicity is extraction of essential cell components from the cell, leading to cell death [73, 74].

Prediction of cell toxicity in ionic liquids is rather difficult as well. Some organisms within a species tolerate ionic liquids better than others. *Lactobacillus delbruekii* showed, in the presence of $[C_6mim][PF_6]$, a relatively high activity of 94% (in relation to the aqueous system), while other *Lactobacillus* species indeed showed activity, but only in range of 4–80% [75]. An interesting concept in whether an organism tolerates a solvent is the critical log *P* value

(log P_{crit}). This parameter has to be determined for individual organisms. A log P value higher than the log P_{crit} of the cell indicates a biocompatible solvent for this organism. For instance, a cell with a log P_{crit} of 2 needs a more hydrophilic medium than a cell with a log P_{crit} of 3. This concept could be useful in finding evidence for compatible ionic liquids [76].

But aside from all behaviour of cells or ionic liquids, processes are also responsible for toxicity. Ionic liquids such as [C_2mim][BF_4] and [C_4mim][PF_6] show a different behaviour depending on process conditions. If a solid agar plate is used, [C_4mim][PF_6] will be less toxic than [C_2mim][BF_4], whereas, in a suspended medium, [C_4mim][PF_6] is more toxic. An explanation for this phenomenon is based on the diffusion of ionic liquid to the cell cultures. Diffusion of hydrophobic [C_4mim][PF_6] to the solid agar plate is hindered, but in a well-mixed suspension a large contact area can be established between organism and ionic liquid so that the toxic effect of [C_4mim][PF_6] becomes more important [77].

As a general rule, the toxicity of ionic liquids in fermentations has a similar effect to that in the environmentally relevant toxicity. Long alkyl chains are more toxic than short chains [78]. Cornmell et al. wanted to know where exactly ionic liquids affect the cell, and used Fourier transform infrared spectroscopy (FT-IR). Cells were exposed to different ionic liquids, lysed, and afterwards separated and analysed. It was experimentally verified that toxic ionic liquids accumulate in the lipid membrane of the cell and cause membrane disruption. The process seems to proceed slower with biocompatible ionic liquids [79].

Closely related to the evaluation of toxicity and environmental behaviour is the abiotic and biotic degradation of ionic liquids. There has been very little work done so far, and published data indicate that, depending on their nature, ionic liquids might not be easily degradable [62, 71]. Studies are again hampered by aggregate formation and the difficulty for exact analysis. For widening industrial applications, these data must be generated by the producers of ionic liquids under the regulations of REACH (**R**egistration, **E**valuation, **A**uthorisation and restriction of **CH**emical substances) [80]. For a final judgment of the beneficial application for ionic liquids in biotechnology, a complete life cycle analysis or eco-efficiency analysis has to be done. BASF has published this analysis for their BASIL process [81]. So far, only limited progress has been made for the use of ionic liquids in biotechnology. This is due to the fact that there are insufficient data available on the early development step in the laboratory. Nevertheless, first attempts have been published [82].

7.9 CONCLUDING REMARKS AND FUTURE ASPECTS

In the past 10 years, a large number of ionic liquids and biocatalysts have been tested for applicability in reactions. The partially inconsistent results give an ample scope for future research in that field. Understanding the interaction of

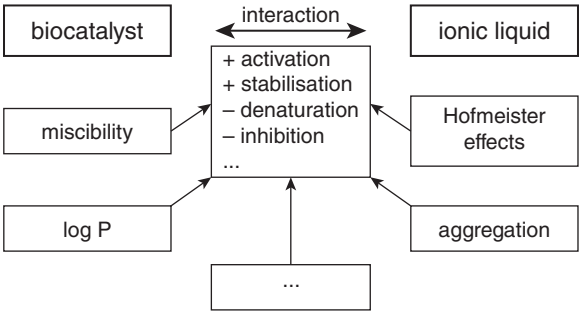

Figure 7.5 Interaction between ionic liquids and biocatalyst and possible influence.

ionic liquids with biocatalysts will be the preferred focus in the future (see Figure 7.5). Nevertheless, some advantages of ionic liquids compared with organic solvents can be crystallised from all of these studies:

(i) Ionic liquids are able to dissolve polar and hydrophilic substrates, which are not soluble in organic liquids, such as amino acids and carbohydrates.
(ii) High thermal stability (up to 400 °C) allows higher temperatures for syntheses.
(iii) Improved stability for many enzymes.
(iv) Separation of substrate and products by extraction with organic solvents enables a recycling of ionic liquids that still contains the catalyst.

New approaches for reducing limitations, such as activity loss in ionic liquids, include the immobilisation of enzymes in micro-emulsions in w/IL. Another possibility is preparation of functionalised ionic liquids that are designed to the requirements of the biocatalytic system. An alternative path is screening for new enzyme types and the subsequent change in protein structure in order to find ideal enzymes for a specific ionic liquid. Ilmberger et al. cloned a cellulose, which already showed activity in the wild type, multiple times, and increased enzyme activity [83]. Furthermore, ionic liquids are used to improve existing syntheses selectively, since their positive properties (such as substrate solubility) are very useful. The enhancement of a synthesis for enantiopure alcohols from different racemic phenyl ethanols, for instance, is described in the literature [84]. Ionic liquids could play a central role if their strength is utilised. Datta et al.'s work shows a hyperthermophilic cellulase tolerating up to 15% of $[C_2mim][O_2CCH_3]$ and still retaining high enzymatic activity in Avicel and a natural matrix. This cellulase could now be used for the hydrolysis of cellulose which was extracted with the help of ionic liquids. This way, expensive purification steps in the downstream processing of ionic liquid-dissolved

cellulose could be eliminated [85]. A successful evaluation of the impact of the environmental behaviour of ionic liquids will be crucial. But a strong exchange with other disciplines might also lead to a better understanding of their implications for biotechnology. Examples are simple models being able to predict the properties of ionic liquids, such as melting point or distribution behaviour [86, 87].

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