I
Methodology

Asymmetric Organic Synthesis with Enzymes. Edited by Vicente Gotor, Ignacio Alfonso, and Eduardo García-Urdiales
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Medium Engineering

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1.1
Introduction

'Efficiency' and 'selectivity' are the two keywords that better outline the outstanding performances of enzymes. However, in some cases unsatisfactory stereoselectivity of enzymes can be found and, in these cases, the enantiomeric excesses of products are too low for synthetic purposes. In order to overcome this limitation, a number of techniques have been proposed to enhance the selectivity of a given biocatalyst. The net effect pursued by all these protocols is the increase of the difference in activation energy ($\Delta\Delta G^{\neq}$) of the two competing diastereomeric enzyme–substrate transition state complexes (Figure 1.1).

The enantioselectivity of biocatalytic reactions is normally expressed as the enantiomeric ratio or the *E* value [1a], a biochemical constant intrinsic to each enzyme that, contrary to enantiomeric excess, is independent of the extent of conversion. In an enzymatic resolution of a racemic substrate, the *E* value can be considered equal to the ratio of the rates of reaction for the two enantiomers, when the conversion is close to zero. More precisely, the *E* value is defined as the ratio between the specificity constants (k_{cat}/K_M) for the two enantiomers and can be obtained by determination of the k_{cat} and K_M of a given enzyme for the two individual enantiomers.

However, considering practical limitations, that is, the availability of optically pure enantiomers, *E* values are more commonly determined on racemates by evaluating the enantiomeric excess values as a function of the extent of conversion in batch reactions. For irreversible reactions, the *E* value can be calculated from Equation 1 (when the enantiomeric excess of the product is known) or from Equation 2 (when the enantiomeric excess of the substrate is known) [1a]. For reversible reactions, which may be the case in enzymatic resolution carried out in organic solvents (especially at extents of conversion higher than 40%), Equations 3 or 4, in which the reaction equilibrium constant has been introduced, should be used [1b].

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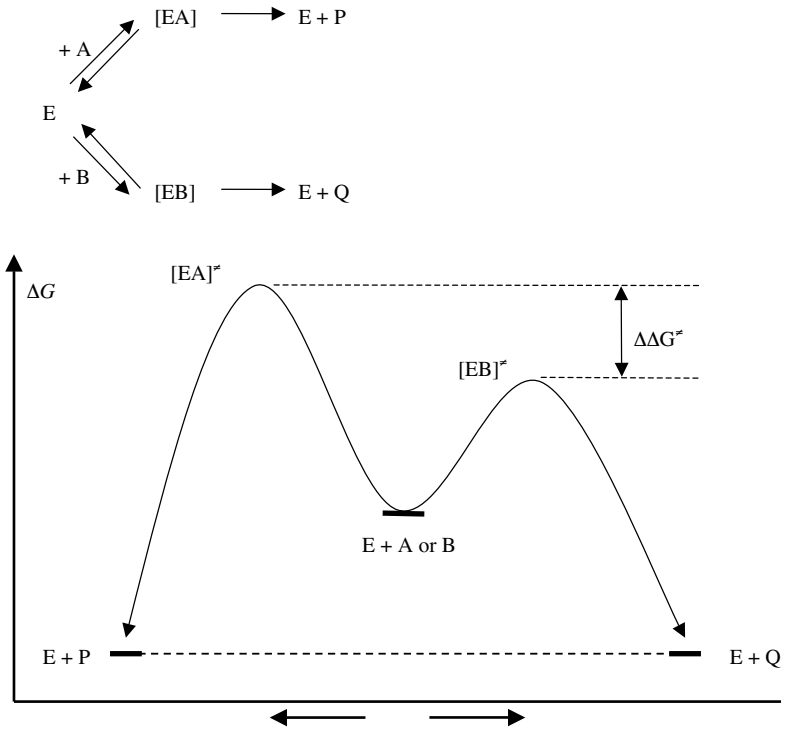


Figure 1.1 Energy diagram for an enzyme-catalyzed enantioselective reaction. E = enzyme; A and B = enantiomeric substrates; P and Q = enantiomeric products; [EA] and [EB] = enzyme–substrate complexes; $\Delta\Delta G^{\neq}$ = difference in free energy; $^{\neq}$ denotes a transition state.

For obtaining both the product and the remaining substrate in high enantiomeric excess in one reaction step, the *E*-value needs to be high, usually around or more than 100.

$$E = \frac{\ln\,[1-c(1+ee_p)]}{\ln\,[1-c(1-ee_p)]} \tag{1}$$

$$E = \frac{\ln\,[(1-c)(1-ee_s)]}{\ln\,[(1-c)(1+ee_s)]} \tag{2}$$

$$E = \frac{\ln\,[1-(1+K)c(1+ee_p)]}{\ln\,[1-(1+K)c(1-ee_p)]} \tag{3}$$

$$E = \frac{\ln\,\{1-(1+K)[c+ee_s(1-c)]\}}{\ln\,\{1-(1+K)[c-ee_s(1-c)]\}} \tag{4}$$

Where c is conversion of substrate, ee_p and ee_s are enantiomeric excesses of product (P) and remaining substrate (S), $K = (1 - c_{eq})/c_{eq}$, and c_{eq} = conversion at equilibrium.

Owing to the logarithmic relationship between E and $\Delta\Delta G^{\neq}$, a small increase in $\Delta\Delta G^{\neq}$ produces a dramatic change in E. For instance, when $\Delta\Delta G^{\neq}$ is increased by only 1 kcal mol^{-1} approximately, the enantiomeric excess of the product is enhanced from ~80 to 95% [2].

$$\Delta\Delta G^{\neq} = -RT \cdot \ln E \tag{5}$$

The stereochemical outcome of a biocatalyzed reaction is predetermined by the enzyme, the reactant substrates, and also by the reaction conditions (temperature, pH, solvent, additives, etc.). Accordingly, each of them offers a possibility to modulate the enzymatic performances. In this chapter we are going to focus on the latter. With this regard, significant literature examples have shown how a careful tuning of the chemical structure of the substrates as well as of the reaction conditions (temperature and pH) might help to reach the synthetic goals in terms of the enantiomeric excesses of the products (see, for instance, Ref. [3,4]). However, the parameter that has been mostly investigated is the chemical composition of the reaction medium, to the point that the so-called 'medium engineering' has emerged in the last years as an important area of research and biotechnological development for industrial applications [for previous reviews see 5]. In this chapter, experimental evidences of the influence of this parameter on enzymatic enantioselectivity are discussed. As will be shown in the following text, most of the literature data are related to the exploitation of hydrolases (lipases, esterases, proteases); however, some significant examples related to other classes of enzymes have also been reported.

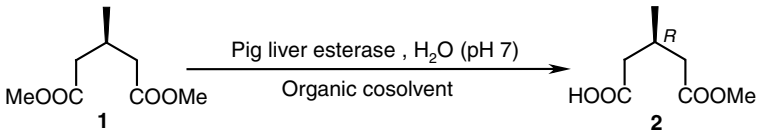
1.2 Modulation of Enzyme Enantioselectivity by Medium Engineering

The term 'medium engineering', that is the possibility to affect enzyme selectivity simply by changing the solvent in which the reaction is carried out, was coined by Klibanov, who indicated it as an alternative or an integration to protein engineering [5a]. Indeed, several authors have confirmed that the enantio-, prochiral-, and even regioselectivity of enzymes can be influenced, sometimes very remarkably, by the nature of the organic solvent used.

In the following text, examples of solvent effects on enzyme selectivity, referred either to systems based (i) on water-miscible organic cosolvents added to aqueous buffers or (ii) on organic media with low water activity, are discussed.

1.2.1 Selectivity Enhancement by Addition of Water-Miscible Organic Cosolvents

Initially, biocatalysis was being conducted in neat aqueous solutions because of the general notion that this environment is optimal for maintaining the enzyme conformation most suitable for binding and catalysis. However, because of the limited water-solubility of many organic substrates, it has been suggested to add varying proportions of water-miscible organic cosolvents to achieve an enhanced concentration



Scheme 1.1

Table 1.1 Influence of cosolvents on the asymmetric hydrolysis of the prochiral diester (**1**) catalyzed by pig liver esterase.

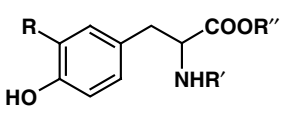
Organic cosolvent, v/v	Temperature (°C)	Enantiomeric excesses of (**2**)
None	20	79
Acetonitrile, 5%	20	70
Acetone, 5%	20	72
Dimethylsulfoxide, 5%	20	81
Methanol, 5%	20	88
Methanol, 20%	−10	97

of the substrate *in the reaction medium* [6]. Quite soon, however, it became clear that these cosolvents can also influence the stereoselectivity of the biocatalysts.

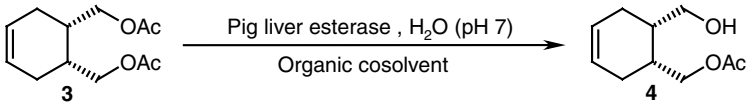
This area of research was pioneered by Bryan Jones and coworkers in the late 1970s and 1980s [7]. For instance, in the 1980s they showed that the pig liver esterase (PLE)-catalyzed hydrolysis of the model prochiral compound 3-methyl-glutarate dimethyl ester (**1**) to give the (*R*)-monoester (**2**) was significantly effected by the added cosolvent (Scheme 1.1 and Table 1.1)[7b], the higher enantiomeric excess value of (**2**) being obtained in the presence of 20% v/v MeOH and at low temperature.

In another significant example published in the same period, Guanti and coworkers described the desymmetrization of the meso cyclohexene diester (**3**) to give the hemiester (**4**), whose enantiomeric excess increased from 55 to 96% when moving from plain buffer to the same buffer containing 10% v/v *t*-BuOH (Scheme 1.2 and Table 1.2 [8]).

Finally, as an 'old' example of kinetic resolution of racemic mixtures, mention must be made on the report of Kise and Tomiuchi on the significant effect of acetonitrile on the enantioselectivity of different proteases toward the kinetic resolution of aromatic amino acid ethyl esters (**5**–**8**). For instance, (L)-DOPA (**8**) was obtained with 99% ee in the presence of 90% v/v acetonitrile [9].



5 R = R′ = H ; R″ = Et
6 R = H ; R′ = Ac ; R″ = Et
7 R = OH ; R′ = H ; R″ = Et
8 R = OH ; R′ = H ; R″ = H



Scheme 1.2 .

Table 1.2 Influence of cosolvents on the asymmetric hydrolysis of the *meso*-diester (**3**) catalyzed by pig liver esterase.

Organic cosolvent, v/v	Relative rate of hydrolysis	Enantiomeric excesses of (**4**)
None	100	55
Dimethylsulfoxide, 20%	70	59
Dimethylsulfoxide, 40%	28	72
Dimethylformamide, 20%	35	84
tert-Butanol, 5%	70	94
tert-Butanol, 10%	44	96

In a very recent report related to a different group of enzymes, it has been shown that cosolvents, especially methanol, can improve to a great extent the enantioselectivity of three Baeyer–Villiger monooxygenases (phenylacetone monooxygenase, 4-hydroxyacetophenone monooxygenase, and ethionamide monooxygenase) when using organic sulfides as the substrates; depending on the enzyme and on the nature of the solvent and the substrate used, reversal of enantiopreference was also observed [10].

Several other examples have been reported in the literature and most of them have been already reviewed (see, for instance, [11]). However, it must be mentioned that, of course, the selectivity enhancement via addition of water-miscible organic cosolvents may not be taken for granted, as sometimes this approach may be unsuccessful [11a].

The discussion on possible rationales of this phenomenon has been reported at the end of this chapter.

1.2.2 Selectivity Enhancement in Organic Media with Low Water Activity

1.2.2.1 Organic Solvent Systems

In the third example of the previous paragraph [9], the reaction conditions described are similar to those used for the enzymatic transformations in 'bulk' organic media, an area that was pioneered by Klibanov and coworkers in the 1980s [12] and later investigated and synthetically exploited worldwide [13].

In these systems, solid enzyme preparations (e.g. lyophilized or immobilized on a support) are suspended in an organic solvent in the presence of enough aqueous buffers to ensure catalytic activity. Although the amount of water added to the solvent (as a rule of thumb $<5\%$ v/v) may exceed its solubility in that solvent, a visible discrete aqueous phase is not apparent because part of it is adsorbed by the enzyme. Therefore, the two phases involved in an organic solvent system are a liquid (bulk organic solvent and reagents dissolved in it) and a solid (hydrated enzyme particles).

The interest and success of the enzyme-catalyzed reactions in this kind of media is due to several advantages such as (i) solubilization of hydrophobic substrates; (ii) ease of recovery of some products; (iii) catalysis of reactions that are unfavorable in water (e.g. reversal of hydrolysis reactions in favor of synthesis); (iv) ease of recovery of insoluble biocatalysts; (v) increased biocatalyst thermostability; (vi) suppression of water-induced side reactions. Furthermore, as already said, enzyme selectivity can be markedly influenced, and even reversed, by the solvent.

However, in most cases enzymes show lower activity in organic media than in water. This behavior has been ascribed to different causes such as diffusional limitations, high saturating substrate concentrations, restricted protein flexibility, low stabilization of the enzyme–substrate intermediate, partial enzyme denaturation by lyophilization that becomes irreversible in anhydrous organic media, and, last but not least, nonoptimal hydration of the biocatalyst [12d]. Numerous methods have been developed to activate enzymes for optimal use in organic media [13].

Before discussing the 'medium engineering' phenomenon and its synthetic relevance in details, it is useful to offer a brief overview of the 'fundamentals' of biocatalysis in organic media.

1.2.2.2 Enzyme Properties in Organic Solvents

Enzyme activity in organic solvents depends on parameters such as water activity, pH control, substrate–product solvation, enzyme form, and nature of the solvent.

Water Activity It became apparent quite soon that enzyme activity was higher in hydrophobic solvents than in hydrophilic ones and, indeed, Laane *et al.* [14] found that there was a direct correlation between activity and solvent hydrophobicity, expressed as log *P* (where *P* is the partition coefficient of a given solvent between *n*-octanol and water). Soon after, Zaks and Klibanov [12c] demonstrated, with three unrelated oxidoreductases, that enzyme activity was more or less coincident in the various solvents, provided that the amount of water bound to the protein was the same. The maximal enzymatic activity for the three examined enzymes was attained at about 1000 molecules of water/enzyme molecule, that is, roughly a monolayer of water on the surface. A more rigorous way to correlate enzyme activity with the water present in the reaction medium was proposed by Halling [15] and Goderis [16], who expressed the water in the medium no longer in terms of content but in terms of thermodynamic water activity (a_w). Water activity is correlated to mole fraction of water (χ_w) and water activity coefficient (γ_w) by the equation $a_w = \chi_w \cdot \gamma_w$. Since, in first approximation, the water activity coefficient (γ_w) increases as a function of solvent hydrophobicity, it follows that a given value of a_w will be obtained at a lower water concentration in a hydrophobic than in a hydrophilic medium. Therefore, to attain the same level of enzyme hydration, or of a_w, in the enzyme, less water is necessary in hydrophobic than in hydrophilic solvents.

Because of the fundamental role played by water, it is clear that the control of a_w in the enzymatic reactions carried out in organic solvents is of paramount importance and, as a consequence, different methods have been developed to this end, all of them based on the knowledge that some salts have a fixed hydration state, which gives a well-defined a_w [17].

pH and pH Control It is well known that the protonation state of the various groups of an enzyme is important for enzyme activity. However, while in water protonation is simply controlled by pH adjustments, this is not the case in organic solvents, where even the pH concept itself is challenged. A simple way to control the initial state of enzyme protonation was developed by Zaks and Klibanov [12a] who showed that enzyme activity in organic solvents was markedly dependent on the pH of the solution from which the enzyme was recovered (by lyophilization or precipitation).

This methodology, the so-called 'pH adjustment', is now widely applied and is perfectly suitable when there is no alteration of acid–base concentration in the course of the reaction. If this is not the case, as, for instance, in the synthesis, hydrolysis, or aminolysis of esters, enzyme protonation and activity might be deeply influenced. To overcome this drawback, the use of buffering systems involving highly hydrophobic acids and their sodium salts, and highly hydrophobic bases and their hydrochlorides, has been proposed [18]. The main disadvantage of these buffer systems is that they are appreciably soluble only in solvents with a relatively high polarity.

Enzyme Form Proteins are practically insoluble in most organic solvents; therefore, in the absence of any special treatment, they are usually present as a solid suspension. This simplifies catalyst–product separation and enzyme reutilization.

The simplest way to prepare a biocatalyst for use in organic solvents and, at the same time, to adjust key parameters, such as pH, is its lyophilization or precipitation from aqueous solutions. These preparations, however, can undergo substrate diffusion limitations or prevent enzyme–substrate interaction because of protein–protein stacking. Enzyme lyophilization in the presence of lyoprotectants (polyethylene glycol, various sugars), ligands, and salts have often yielded preparations that are markedly more active than those obtained in the absence of additives [19]. Besides that, the addition of these ligands can also affect enzyme selectivity as follows.

Adsorption on solid matrices, which improves (at optimal protein/support ratios) enzyme dispersion, reduces diffusion limitations and favors substrate access to individual enzyme molecules. Immobilized lipases with excellent activity and stability were obtained by entrapping the enzymes in hydrophobic sol-gel materials [20]. Finally, in order to minimize substrate diffusion limitations and maximize enzyme dispersion, various approaches have been attempted to solubilize the biocatalysts in organic solvents. The most widespread method is the one based on the covalent linking of the amphiphilic polymer polyethylene glycol (PEG) to enzyme molecules [21].

Enzyme Kinetics and Stability Kinetic studies, carried out mostly with hydrolases, have shown that enzymes in organic solvents follow conventional models [12a, 22].

Several reports have indicated that enzymes are more thermostable in organic solvents than in water. The high thermal stability of enzymes in organic solvents, especially in hydrophobic ones and at low water content, was attributed to increased conformational rigidity and to the absence of nearly all the covalent reactions causing irreversible thermoinactivation in water [23].

1.2.2.3 Medium Engineering

In the following text, a few examples of solvent effects on enzyme selectivity are discussed, starting from the 'classical' works published by Klibanov's group.

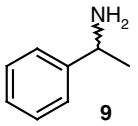
In a first report [24], the enantioselectivities of various proteases were evaluated by comparing the biocatalyzed hydrolysis of 2-chloroethyl esters of *N*-acetyl-L- and D-amino acids in water and their transesterification with *n*-propanol in butyl ether. By comparing the ratio of the k_{cat}/K_M values for the L- and D-enantiomers in the two reactions, a remarkable relation of the proteases enantioselectivity was observed: apparently, in this case, the organic solvents 'destroyed' the selectivity of the tested enzymes. This finding

Table 1.3 Influence of the organic solvent on the enantioselectivity of the protease subtilisin in the kinetic resolution of the racemic amine (**9**) (expressed as the ratio of the initial rate of acylation of the pure enatiomers, v_S/v_R).

Organic solvent	Selectivity (v_S/v_R)
Toluene	0.95
Octane	1.3
Acetonitrile	1.4
Carbon tetrachloride	1.5
Ethyl acetate	1.6
Butyl ether	1.8
Pyridine	2.5
Dimethylformamide	2.9
Tetrahydrofuran	3.5
tert-Amyl alcohol	4.1
3-Methyl-3-pentanol	7.7

was synthetically exploited for the preparation of peptides containing D-amino acids, compounds that, in water, could not be substrates for these enzymes [25].

Later on the crucial role played by the solvent was enlightened in the protease-catalyzed resolution of racemic amines [26]. As shown in Table 1.3, the ratio of the initial rates of acylation of the (*S*)- and the (*R*)-enantiomers or racemic α-methyl-benzylamine (**9**) varied from nearly 1 in toluene to 7.7 in 3-methyl-3-pentanol. Similarly, the same authors found a significant solvent effect for the subtilisin-catalyzed transesterification of racemic 1-phenylethanol (**10**) using vinyl butyrate as acyl donor (Table 1.4 [27]).



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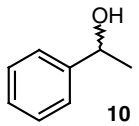

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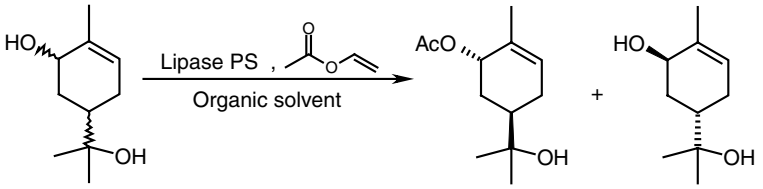
Table 1.4 Influence of the organic solvent on the enantioselectivity of the protease subtilisin in the kinetic resolution of the racemic alcohol (**10**) (expressed as the enatiomeric ratio *E*, that is the ratio of the specificity constants of the two enatiomers, $(K_{cat}/K_M)_S/(K_{cat}/K_M)_R$).

Organic solvent	$(K_{cat}/K_M)_S$	$(K_{cat}/K_M)_R$	*E*
Dioxane	170	2.8	61
Benzene	13	0.24	54
Triethylamine	330	6.9	48
Tetrahydrofuran	230	5.8	40
Pyridine	43	1.4	31
Dimethylformamide	1.4	0.16	9
Nitromethane	16	3.3	5
Acetonitrile	48	16	3

Of course, the influence of organic solvents on enzyme enantioselectivity is not limited to proteases but it is a general phenomenon. Quite soon, different research groups described the results obtained with lipases [28]. For instance, the resolution of the mucolytic drug (±)–*trans*-sobrerol (**11**) was achieved by transesterification with vinyl acetate catalyzed by the lipase from *Pseudomonas cepacia* adsorbed on celite in various solvents. As depicted in Scheme 1.3 and Table 1.5, it was found that *t*-amyl alcohol was the solvent of choice: in this medium, the selectivity was so high ($E > 500$) that the reaction stopped spontaneously at 50% conversion giving both (+)-*trans*-sobrerol and (−)-*trans*-sobrerol monoacetate in 100% optical purity [29].



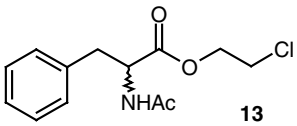
(±)-*trans*-**11** (1*S*, 5*R*)-**12** (1*R*, 5*S*)-**11**

Scheme 1.3 .

Table 1.5 Influence of the organic solvent on the enantioselectivity of the lipase PS (from *Pseudomonas* species) in the kinetic resolution of racemic *trans*-sobrerol (**10**).

Organic solvent	***E***
Tetrahydrofuran	69
Acetone	142
Dioxane	178
3-Pentanone	212
tert-Amyl alcohol	518

In all the reported examples, the enzyme selectivity was affected by the solvent used, but the stereochemical preference remained the same. However, in some specific cases it was found that it was also possible to invert the hydrolases enantioselectivity. The first report was again from Klibanov's group, which described the transesterification of the model compound (**13**) with *n*-propanol. As shown in Table 1.6, the enantiopreference of an *Aspergillus oryzae* protease shifted from the (L)- to the (D)-enantiomer by moving from acetonitrile to CCl_4 [30]. Similar observations on the inversion of enantioselectivity by switching from one solvent to another were later reported by other authors [31].



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When discussing the role of reaction medium on enzyme enantioselectivity, the potential effects of (i) water activity [5b,13f,32], (ii) enzyme form, and (iii) pH, should

Table 1.6 Influence of the organic solvent on the enantioselectivity of the protease from *A. oryzae* subtilisin in the kinetic resolution of the racemic amino acid (**12**) (expressed as the ratio of the initial rate of acylation of the pure enatiomers, v_S/v_R).

Organic solvent	Selectivity (v_S/v_R)
Acetonitrile	7.1
Dimethylformamide	5.7
Pyridine	4.3
Tert-Butanol	1.7
Acetone	1.3
Tetrahydrofuran	1.3
Cycloexanone	1.1
Methylene chloride	0.88
3-Octanone	0.73
Nitrobenzene	0.60
tert-Butyl acetate	0.44
Methyl *tert*-butyl ether	0.34
Cyclohexane	0.27
Toluene	0.26
Octane	0.24
Carbon tetrachloride	0.19

not be neglected. As for the first point, the best way to avoid water effects is to carry out experiments at controlled water activity, which indeed has been done in numerous cases [for a review see [5b,13a]]. When the effects of water activity (or water content) have been specifically investigated, the results were rather contradictory since increases, decreases, or, most often, no variation in the enantioselectivity as a function of the water present in the reaction medium were observed [5b,13e,33,34]. Contradictory results were also obtained for enzyme form, since no influence [35] or influence [36] on enzyme enantioselectivity was reported. In the latter case, the large differences in stereoselectivity as a function of *Candida antarctica* lipase B form were ascribed to mass transport limitations, with lyophilized preparations more prone to this problem than immobilized preparations [36]. Considering pH, it has been demonstrated that alcohol dehydrogenase enantioselectivity is affected by pH variations in aqueous buffers [37]. However, a study carried out in organic solvents showed that the hydrolytic enzyme cutinase was not influenced by pH changes [34].

1.2.3 Rationales

The experimental evidences that 'medium engineering' might represent an efficient method to modify or improve enzyme selectivity (alternative to protein engineering and to the time-consuming search for new catalysts) were immediately matched by the search for a sound rationale of this phenomenon. The different hypotheses formulated to try to rationalize the effects of the solvent on enzymatic enantioselectivity can be grouped into three different classes. The first hypothesis suggests that

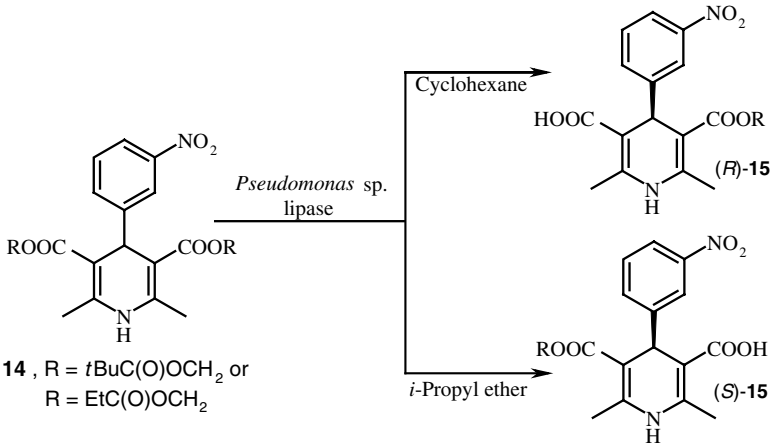
the solvent, depending on its polarity, could modify the biocatalyst conformation and, thus, influence the selectivity by altering the molecular recognition process between substrate and enzyme [27].

The second group of studies tries to explain the solvent effects on enantioselectivity by means of the contribution of substrate solvation to the energetics of the reaction [38]. For instance, a theoretical model based on the thermodynamics of substrate solvation was developed [39]. However, this model, based on the determination of the desolvated portion of the substrate transition state by molecular modeling and on the calculation of the activity coefficient by UNIFAC, gave contradictory results. In fact, it was successful in predicting solvent effects on the enantio- and prochiral selectivity of γ-chymotrypsin with racemic 3-hydroxy-2-phenylpropionate and 2-substituted 1,3-propanediols [39], whereas it failed in the case of subtilisin and racemic *sec*-phenetyl alcohol and *trans*-sobrerol [40]. That substrate solvation by the solvent can contribute to enzyme enantioselectivity was also claimed in the case of subtilisin-catalyzed resolution of secondary alcohols [41].

In a third model it has been proposed that solvent molecules could bind within the active site and, depending on their structure, interfere with the association or transformation of one enantiomer more than the other one [28a,42,43]. For instance, a correlation between the enantiomeric ratio *E* and the van der Waals volume of the solvent molecules was observed in the resolution of 3-methyl-2-butanol catalyzed by *C. antarctica* lipase B; the van der Waals volume was suggested as one of the parameters that govern solvents effects on enzyme selectivity [44]. Additionally, significant evidence at a molecular level came from a series of crystallographic data reported by Klibanov [45]. In these works, whose main goal was to demonstrate that the tertiary structure of enzymes was the same once the proteins had been dissolved in water or suspended in organic solvents, it was shown that molecules of the organic solvents were able to penetrate into the active site of the protease subtilisin, displacing some of the water molecules present. Quite significantly, molecules of different solvents were found to be located in different regions of the active site and, additionally, this happened not only in pure organic solvents but also in mixtures of organic solvents and water (i.e. dioxane–water 40 : 60 v/v) [45b].

On the basis of these results, it is not surprising that the search for an exhaustive rationale for the 'medium engineering' effect is still the object of scientific debate. Furthermore, it should be pointed out that despite the aforementioned experimental results supporting the second and third type of hypotheses, both of them lack fully reliable predictive value and are not sufficient by themselves to explain every case. In fact, the second hypothesis appears to be valid only for the specific enzyme and substrate investigated each time, or when the formation of solvent–enzyme complexes is proposed (third hypothesis), no generalization is possible because of the large number of possible solvent–enzyme complexes and because each complex might behave differently depending on the nature of the substrate.

However, whatever the mechanism of action is, the effect of solvents on enzyme selectivity is sometimes really dramatic. For example, Hirose *et al.* [42] reported that in the *Pseudomonas* species lipase-catalyzed desymmetrization of prochiral



Scheme 1.4 Asymmetric hydrolysis of dihydropyridine diesters: influence of solvent on lipase stereochemical preference.

dihydropyridine dicarboxylates (i.e. **14**), the (*S*)-monoesters as high as 99% ee were obtained in water-saturated di-isopropyl ether, whereas the (*R*)-isomers were formed preferentially (88–91% ee) in water-saturated cyclohexane (Scheme 1.4).

1.2.4
Modulation of Enzyme Selectivity: New Trends of Research

The aspects of medium engineering summarized so far were a hot topic in biocatalysis research during the 1980s and 1990s [5]. Nowadays, all of them constitute a well-established methodology that is successfully employed by chemists in synthetic applications, both in academia and industry. In turn, the main research interests of medium engineering have moved toward the use of ionic liquids as reaction media and the employment of additives.

1.2.4.1 Ionic Liquids

Ionic liquids, which can be defined as salts that do not crystallize at room temperature [46], have been intensively investigated as environmentally friendly solvents because they have no vapor pressure and, in principle, can be reused more efficiently than conventional solvents. Ionic liquids have found wide application in organometallic catalysis as they facilitate the separation between the charged catalysts and the products.

In recent years ionic liquids have also been employed as media for reactions catalyzed both by isolated enzymes and by whole cells, and excellent reviews on this topic are already available [47]. Biocatalysis has been mainly conducted in those room-temperature ionic liquids that are composed of a 1,3-dialkylimidazolium or *N*-alkylpyridinium cation and a noncoordinating anion [47a].

The polarity of common ionic liquids is in the range of the lower alcohols or formamide, and their miscibility with water varies widely and unpredictably and is

not strictly correlated with their polarity [47a]. Even though the miscibility of ionic liquids and organic solvents is not yet well documented, generally they mix with lower alcohols and ketones, dichloromethane, and THF, whereas they do not mix with alkanes and ethers [48].

The possibility of using solvents with high polarity, such as that of many ionic liquids, increases the solubility of polar substrates and extends the range of applications of biocatalysis. Hydrolases such as lipases (from *C. antarctica* and from *P. cepacia*), proteases (thermolysin, α-chymotrypsin), esterases, and glycosidases have been used in plain ionic liquids, whereas oxidoreductases such as formate dehydrogenase, peroxidases, laccases, and Baker yeast have been employed in water-ionic liquid mixtures or in water-ionic liquid biphasic systems [47]. Both increased enzyme stability and activity have been reported as compared to conventional organic solvents media. For example, α-chymotrypsin, lipase from *C. antarctica* and esterase from *Bacillus stearothermophilus* were found to be 17, 3, and 30 times more stable in ionic liquids than in organic solvents [47b].

Several reports deal also with the effects of ionic liquids on enzyme enantioselectivity, which is the subject of this chapter. Although in several cases there was no change or even a decrease in enantioselectivity compared to organic solvents [47], in other cases improved enantioselectivity was observed [47,49–56]. In the following text, the latter cases will be examined in some detail.

Lipases from *C. antarctica* and *P. cepacia* showed higher enantioselectivity in the two ionic liquids 1-ethyl-3-methylimidazolium tetrafluoroborate and 1-butyl-3-methylimidazolium hexafluoroborate than in THF and toluene, in the kinetic resolution of several secondary alcohols [49]. Similarly, with lipases from *Pseudomonas* species and *Alcaligenes* species, increased enantioselectivity was observed in the resolution of 1-phenylethanol in several ionic liquids as compared to methyl *tert*-butyl ether [50]. Another study has demonstrated that lipase from *Candida rugosa* is at least 100% more selective in 1-butyl-3-methylimidazolium hexafluoroborate and 1-octyl-3-nonylimidazolium hexafluorophosphate than in *n*-hexane, in the resolution of racemic 2-chloro-propanoic acid [51].

The examples described so far refer to experiments carried out in plain ionic liquids. However, it has been proved that ionic liquids are also capable of positively influencing enzyme enantioselectivity when employed in mixtures with aqueous buffers or conventional organic solvents. For example, *N*-ethyl-pyridinium trifluoroacetate improved the selectivity of subtilisin Carlsberg in the resolution of several amino acid esters in comparison with acetonitrile; both the ionic liquid and acetonitrile were present in 15% concentration in an aqueous buffer [52]. A subsequent study demonstrated that subtilisin enantioselectivity was related to the kosmotropicity of individual cations and anions of ionic liquids and that it was higher at higher values of the overall ionic liquid kosmotropicity [53]. Ionic liquids–*tert*-butanol cosolvent systems markedly boosted the activity, stability, and enantioselectivity of *C. antarctica* lipase B in the resolution of racemic *p*-hydroxy-phenylglycine methyl ester [54] and, similarly, cosolvent systems consisting of ionic liquids and chloroform or *tert*-butanol increased the selectivity of the same enzyme in the resolution of racemic methyl mandelate [55]. Finally, the three-component system

made of ionic liquid–isopropanol–water gave the best results in the enantioselective hydrolysis of prochiral diester malonates [56].

1.2.4.2 **Additives**

Basically, there are three ways to tune enzyme enantioselectivity by means of 'additives': (i) the additives are placed in the reaction medium together with the organic solvent, the enzyme, and the reagents; (ii) the additives are co-lyophilized with the biocatalyst before use in the organic solvent; (iii) the additives are complexed with the substrates before their transformation in the organic medium.

One example that refers to the first case is the resolution of racemic 2-phenyl-4-benzyloxazol-5(4*H*)-one (**12**) by alcoholysis with *n*-butanol catalyzed by *C. antarctica* lipase B or by *Mucor miehei* lipase [57]. The enantioselectivity of both enzymes was improved by addition to the organic medium of an organic base such as triethylamine or of a solid-state buffer such as 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid and its sodium salt. The positive effect of the additives on the enantioselectivity (and activity) of the two enzymes was attributed to the control of the protonation state of the biocatalysts [54]. Similarly, the addition of a small amount of an aqueous solution containing metal ions (LiCl or $MgCl_2$) to an organic solvent medium, remarkably enhanced (up to 200 times) the enantioselectivity of *C. rugosa* lipase in the resolution of racemic 2-(4-substituted phenoxy) propionic acids [58]. EPR experiments suggested that the increase in enzyme selectivity brought about by the metal ions was due to an enhancement of the reaction rate for the *R*-enantiomer combined with a decrease of the rate for the *S*-enantiomer [58].

In the second way by which additives can influence enzyme enantioselectivity, it has been shown that including excess salt (e.g. KCl) during lyophilization can enhance not only the activity of subtilisin Carlsberg by more than 20 000 fold, but also its enantioselectivity toward *N*-acetyl-phenylalanine methyl ester [59]. The changes in selectivity reflected the activity for the (L)-enantiomer, whereas the activity for the (D)-enantiomer was mostly unaffected, which suggests that the favored reaction is more sensitive to the structural integrity of the salt-treated enzyme [59]. Enhanced enantioselectivity (and activity) in organic solvents was also observed for lipase from *P. cepacia* co-lyophilized with crown ethers and cyclodextrins [60]. Co-lyophilization with cyclodextrins improved the enantioselectivity of subtilisin Carlsberg and *C. rugosa* lipase as well [61]. The cyclodextrin effect was ascribed to the preservation of the enzyme active site and not to the formation of additive–substrate or –product complexes [61]. Finally, co-lyophilizing horseradish peroxidase with numerous amino acids influenced enzyme's subsequent stereoselectivity in the sulfoxidation of methyl phenyl sulfide in 2-propanol. The greatest effect was observed with D-proline, which increased enzyme selectivity from a level that was synthetically meaningless to a useful one [62].

An example that refers to the third method additives can be employed is described below. Markedly enhanced enantioselectivity was reported for *P. cepacia* lipase and subtilisin Carlsberg with chiral substrates converted to salts by treatment with numerous Bronsted–Lowry acids or bases [63]. This effect was observed in various organic solvents but not in water, where the salts apparently dissociate to regenerate

the free substrates. It should be emphasized that in some instances the enzymes exhibited virtually no enantiopreference toward a free substrate but a profound one toward its salt [63].

1.3 Conclusions and Outlooks

Nowadays biocatalysis is a well-assessed methodology that has moved from the original status of academic curiosity to become a widely exploited technique for preparative-scale reactions, up to the point that the so-called 'industrial biotechnology' (to which biocatalysis contributes to the most extent) is one of the three pillars of the modern sustainable chemistry.

As shown in this chapter, by focusing on the modulation of enzyme selectivity by medium engineering, quite simple modifications of the solvent composition can really have significant effects on the performances of the biocatalysts. The main drawback remains the lack of reliable predictive models. Despite the significant research efforts (particularly in the last decade), it is likely that a reasonable foresight of the enantioselective outcome of an enzymatic transformation will continue to be based solely on a careful analysis of the increasingly numerous literature reports.

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