

# Strategies for Stabilization of Enzymes in Organic Solvents

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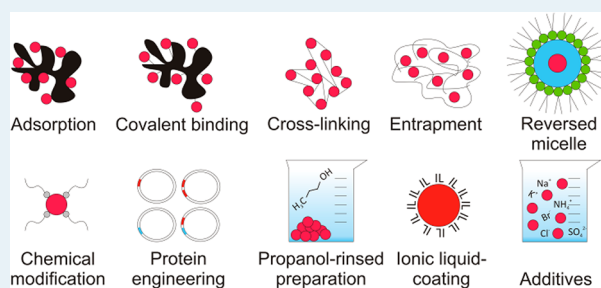
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## Supporting Information

**ABSTRACT:** One of the major barriers to the use of enzymes in industrial biotechnology is their insufficient stability under processing conditions. The use of organic solvent systems instead of aqueous media for enzymatic reactions offers numerous advantages, such as increased solubility of hydrophobic substrates or suppression of water-dependent side reactions. For example, reverse hydrolysis reactions that form esters from acids and alcohols become thermodynamically favorable. However, organic solvents often inactivate enzymes. Industry and academia have devoted considerable effort into developing effective strategies to enhance the lifetime of enzymes in the presence of organic solvents. The strategies can be grouped into three main categories: (i) isolation of novel enzymes functioning under extreme conditions, (ii) modification of enzyme structures to increase their resistance toward nonconventional media, and (iii) modification of the solvent environment to decrease its denaturing effect on enzymes. Here we discuss successful examples representing each of these categories and summarize their advantages and disadvantages. Finally, we highlight some potential future research directions in the field, such as investigation of novel nanomaterials for immobilization, wider application of computational tools for semirational prediction of stabilizing mutations, knowledge-driven modification of key structural elements learned from successfully engineered proteins, and replacement of volatile organic solvents by ionic liquids and deep eutectic solvents.

**KEYWORDS:** biocatalysis, enzyme stability, organic solvents, chemical modifications, enzyme immobilization, protein engineering



## INTRODUCTION

Industrial biocatalysis is recognized as one of the key drivers of the chemical industry.<sup>1</sup> Enzymes possess several desirable qualities for a wide range of applications, ranging from synthesis of pharmaceutical intermediates to large-scale production of biofuels from renewable sources.<sup>2</sup> Hydrolases, particularly lipases, represent the most important industrial biocatalysts. This is primarily due to their ability to convert a wide spectrum of substrates; their high stability at extreme temperatures; their high chemo-, regio-, and enantioselectivity; and no requirements for cofactors.<sup>3</sup> Several companies use lipase-catalyzed reactions to produce useful intermediates.<sup>4–7</sup> However, the advantages of biocatalysts, such as their biodegradability, high specificity, and activity under mild conditions, reflect the fact that enzymes have evolved to work in cellular environments and are therefore usually intolerant of harsh industrial process conditions.<sup>4,8</sup> Although water is considered as the solvent of life, it is a rather poor solvent for most synthetic reactions.<sup>9</sup> Organic solvents are usually required to increase the solubility of hydrophobic substrates, shift the thermodynamic equilibrium to favor synthesis over hydrolysis, and suppress water-dependent side reactions.<sup>10</sup> However, the majority of natural

enzymes display lower catalytic efficiency in organic solvents compared with native aqueous solutions.

There are two possible solutions to cope with the insufficient tolerance of enzymes toward organic solvents: (i) optimizing the process conditions to the available biocatalyst and (ii) preparing biocatalysts that can function under ideal process conditions.<sup>11</sup> While in the past, process conditions used to be designed around the limitations of the enzyme, today enzymes are usually engineered to suit the harsh process specifications.<sup>10</sup> During the past three decades, numerous studies have shed light on the effects of organic solvents on enzyme structure and function. Molecular dynamics simulations have complemented experimental data and provided insights into the deleterious effects of organic solvents, such as rigidification of enzyme conformations, loss of crucial water from the protein structure, solvent penetration into the active site, and damage to the protein structure.<sup>9,12</sup> Increased understanding of molecular changes in enzyme structure and catalytic mechanism in

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Table 1. Classification of Enzyme Stabilities

type of stability	techniques	parameter <sup>a</sup>	definition
thermodynamic (difference between the free energies of the folded and unfolded conformations of a protein)	circular dichroism spectroscopy, intrinsic fluorescence spectroscopy, near-ultraviolet spectroscopy of aromatic residues, NMR spectroscopy, Fourier transform infrared spectroscopy, small-angle X-ray scattering, differential scanning calorimetry	$\Delta G_u$	change in Gibbs free energy going from the folded to unfolded state
		$K_u$	concentration of unfolded species divided by the concentration of folded species
		$T_m$	temperature at which half of the species is in the unfolded state
		$C_{1/2}$	concentration of denaturant needed to unfold half of the species
kinetic (time scale on which a protein remains in the functional form before undergoing irreversible denaturation)	activity assays, sodium dodecyl sulfate resistance studies, proteolytic differential scanning calorimetry	$k_d$	overall rate constant for going from the native to the deactivated species
		$\tau_{1/2}$	time required for the activity to be reduced to half
		$T_{50}$	temperature at which the activity is reduced by half after defined time period
		$C_{50}$	concentration of denaturant at which the activity is irreversibly reduced by half after a defined time period
		$T_{opt}$	temperature leading to highest activity
		TTN	moles of product formed over the catalyst lifetime

<sup>a</sup>Definitions:  $\Delta G_u$ , free energy of unfolding;  $K_u$ , equilibrium constant;  $T_m$ , melting temperature;  $C_{1/2}$ , half-concentration;  $k_d$ , deactivation rate constant;  $\tau_{1/2}$ , half-life;  $T_{50}$ , temperature of half-inactivation;  $C_{50}$ , concentration of half-inactivation;  $T_{opt}$ , optimum temperature; TTN, total turnover number.

nonconventional reaction media has led to the development of many complementary methods, ranging from addition of simple stabilizing agents to highly sophisticated protein engineering approaches, to increase enzyme stability toward organic solvents. Interestingly, it has been shown that biocatalysts can be engineered to such a level that they work even in neat organic solvents. Ongoing improvements and a better understanding of biocatalysis in nonconventional reaction media are expected not only to increase the economic potential of existing enzymatic processes but also to allow new areas to be explored that have previously been precluded by the intrinsic instability of enzymes. Protein stability relates closely to the contemporary issues of protein science, protein–solvent interactions, and protein folding. Engineering of enzyme stability is therefore of both commercial and scientific interest.

### ■ FACTORS DETERMINING BIOCATALYST STABILITY

The main factor that has to be taken into account when performing biocatalysis in nonconventional media is water content. Lipases, proteases, and many other enzymes work efficiently in neat organic solvents. However, even in these cases, at least a few water molecules remain bound to the protein molecule. It is generally believed that fully dehydrated proteins are inactive.<sup>13</sup> Water acting as a lubricant promotes the conformational mobility required for optimal catalysis, whereas neat organic solvents lead to stronger intramolecular interactions. On the other hand, if the water content of an organic solvent exceeds a certain limit, the tendency of an enzyme to denature is increased because of higher conformational mobility. This observation explains why increasing the concentration of a water-miscible organic cosolvent in an aqueous medium generally decreases the enzyme activity. Most enzymes become almost totally inactive at an organic cosolvent concentration of 60–70% (v/v).

Conformational changes are the most common reason for enzyme deactivation in the presence of organic solvents.<sup>14–16</sup> In particular, hydrophilic solvents, which are known to penetrate into enzyme active sites, are capable of inducing secondary and tertiary structural changes.<sup>9,17</sup> Moreover, compared with hydrophobic solvents, hydrophilic solvents show a higher tendency to remove protein-bound water that is crucial for maintaining protein structure and function.<sup>9,13</sup> With regard to structural integrity, enzymes are much more tolerant of hydrophobic organic solvents than hydrophilic ones. Somewhat ironically, it is not contact with an hydrophobic organic solvent but prior dehydration that alters the enzyme structure and lowers catalytic activity in nearly anhydrous systems.<sup>18</sup> Since enzymes are usually insoluble in hydrophobic solvents, they are typically introduced into neat organic media as powders prepared by lyophilization.<sup>10,19</sup> Protein denaturation arising from dehydration is normally reversible upon rehydration in aqueous media. However, refolding in neat organic solvents is not trivial because of reduced structural mobility.<sup>20,21</sup>

### ■ METHODS FOR ASSESSING BIOCATALYST STABILITY

Protein stability can be defined as the energetics of the unfolding reactions from the native state to the fully unfolded state, which occurs via two basic pathways: unfolding can be either reversible or irreversible. Reversible unfolding of proteins, which is characterized by an equilibrium established between the native and unfolded (or partially unfolded) states, is termed thermodynamic or conformational protein stability.<sup>22</sup> Thermodynamic stability, which reflects a protein's ability to refold after being subjected to elevated temperature, extreme pH, or high organic solvent concentrations, can be represented by various parameters (Table 1), such as the Gibbs free energy of unfolding ( $\Delta G_u$ ), the unfolding equilibrium constant ( $K_u$ ),

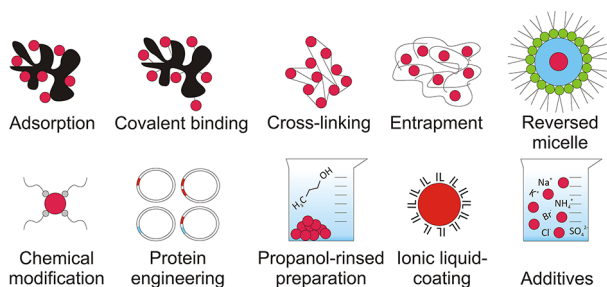
the melting temperature of the protein ( $T_m$ ), and the chemical equivalent half-concentration of denaturant ( $C_{1/2}$ ).<sup>23</sup> Typically, protein unfolding curves are obtained by either calorimetric or optical spectroscopy techniques (Table 1).

Irreversible unfolding frequently arises from protein aggregation, misfolding, chemical modification, or a lack of chaperons. In the simplest model, the irreversible unfolding reaction is characterized by a first-order rate constant of deactivation ( $k_d$ ) that can vary with temperature. On the basis of transition-state theory, the rate of irreversible denaturation can be related to the free-energy barrier ( $\Delta G^\ddagger$ ) that separates the native state of the protein from its nonfunctional forms (i.e., unfolded states and irreversibly denatured protein).<sup>24</sup> Since irreversible unfolding is kinetically driven, this is termed the kinetic stability.<sup>22</sup> In addition to  $k_d$ , the kinetic stability of proteins is often reported as the time required for the enzyme activity to be reduced by half ( $\tau_{1/2}$ ) following incubation under any conditions of interest.<sup>23</sup> Measurement of kinetic stability usually involves conducting activity assays under varying conditions. Other measures of biocatalyst kinetic stabilities are outlined in Table 1.

Irreversible alterations of proteins may readily take place under harsh extracellular conditions as well as in crowded intracellular environments. Even if these alterations occur from unfolded or partially unfolded states, they deplete the native and active state in a time-dependent manner. As a result, thermodynamic stability alone does not guarantee that a protein will remain active during the process. Typical process conditions required for biotechnological applications often result in irreversible denaturation. Therefore, kinetic control of operational stability is of considerable importance for successful biocatalysis implementation.<sup>24</sup> Despite this, the majority of studies dealing with the effects of solvent conditions on biocatalyst activity have focused on thermodynamic rather than kinetic stability.

## STRATEGIES FOR OBTAINING A STABLE BIOCATALYST

Strategies for obtaining biocatalysts that are stable in non-conventional reaction media may be grouped into three general categories: (i) isolation of novel enzymes that can function under extreme conditions, (ii) modification of enzyme structures to increase their resistance toward nonconventional media, and (iii) modification of the solvent environment to decrease its denaturing effect on enzymes (Figure 1). The following sections give brief overviews of these approaches with representative examples, accompanied by a summary of the advantages and limitations of individual strategies (Table 2). However, it should be noted that the examples given are not



**Figure 1.** Strategies for stabilization of enzymes toward organic solvents.

**Table 2.** Advantages and Disadvantages of Stabilization Strategies

strategy	advantages	disadvantages
intrinsically stable enzyme	applicability in a variety of harsh environments	laborious and time-consuming screening, possibly problematic recombinant expression, risk of altered stability and activity of enzymes produced in different hosts
immobilization	biocatalyst reuse, continuous mode of operation, in situ product recovery facilitated, definition of biocatalyst microenvironment, enhanced reaction rates, high volumetric productivities, generally applicable procedures, enzyme activity and enantioselectivity can be tailored	mostly empirical, high cost, mass transfer limitations, loss of enzyme activity, loss of the enzyme due to leakage, operational restraints, requirement for additional material and equipment, immobility of enzyme molecules inside carriers, particle erosion
chemical modification	increased solubility of the enzyme in organic solvents, decreased substrate diffusion limitations, nontoxic solvents, fast and simple procedure, knowledge of the enzyme structure not necessary, not limited to natural amino acids	differences between various batches; enzyme needs to be modified each time
genetic modification	enzymes tailored for a target application, "green" technology, no additional additives required	time-consuming, high cost, risk of improper protein folding, detailed knowledge of protein structure often required
propanol-rinsed enzyme preparation	drying of the enzyme without denaturation	only a few reported examples, risk of enzyme deactivation
ionic liquid coating	reduction of ionic liquid consumption, biocatalyst reuse, ionic liquid properties tailored for target application	only a few reported examples, uncertain toxicity and high price of ionic liquids, risk of enzyme deactivation
additives	simple procedure, enhancement of the enzyme flexibility, wide choice of additives available	complicated separation of additives from the product, incompatibility with the reaction system
microemulsions	the enzyme is present in the aqueous phase; increased solubility of substrates	risk of enzyme inactivation by the surfactant or large interface between the two phases

Table 3. Classification of Immobilization Methods

immobilization method	description	
immobilization onto an inert matrix	adsorption	formed by an adsorption of an enzyme onto a solid support based on van der Waals, electrostatic, and/or hydrophobic interactions
	covalent binding	formed by chemical bonds between the functional groups of an enzyme and those on the support
immobilization within an inert matrix	entrapment	formed by an incorporation of an enzyme within a gel or polymer
formation of water-insoluble particles	cross-linked enzyme crystal (CLEC)	formed by covalent bonds between enzyme crystals using a bifunctional agent
	cross-linked enzyme aggregate (CLEA)	formed by precipitation of the enzyme from aqueous buffer followed by covalent bonds between the resulting physical aggregates using a bifunctional agent
	protein-coated microcrystal (PCMC)	formed by dissolving an enzyme in a concentrated solution of a crystalline material followed by coprecipitation by addition of a water-miscible solvent

meant to be comprehensive but rather illustrative. Four excellent review articles on protein stabilization have recently been published by Bommarius and Paye,<sup>25</sup> Singh and co-workers,<sup>26</sup> Illanes and co-workers,<sup>2</sup> and Wijma and co-workers,<sup>27</sup> the last of which concerns use of computational approaches for engineering stable proteins.

**Isolation of Stable Biocatalysts.** Enzymes that are sufficiently robust under harsh reaction conditions can be isolated from living organisms by biodiversity prospecting. Microorganisms that can grow under extreme conditions, including high salinity, extreme pH, low or high temperature, and presence of organic solvents, have been shown to be an important source of valuable enzymes, so-called extremozymes.<sup>28</sup> A large collection of studies on the stability of extremozymes has revealed that nature employs many different structural strategies to obtain highly stable enzymes.<sup>29</sup> The improved hydration characteristics of extremozymes are probably the most critical for their stability because a main factor responsible for the loss of enzyme activity under extreme conditions is the loss of crucial water molecules.<sup>28</sup> A useful consequence of the hydration properties of extremozymes is the potential extension of their range of applications to nonaqueous environments.<sup>18</sup>

Organic solvent-tolerant bacteria are a relatively new group of extremophilic microorganisms with novel tolerance mechanisms that enable them to overcome the toxic and destructive effects of organic solvents. Several solvent-tolerant enzymes have been isolated from various strains of *Pseudomonas aeruginosa*.<sup>30–32</sup> Studies of the solvent-tolerant mechanism of protease from *P. aeruginosa* PST-01 isolated from soil containing a high concentration of several organic solvents preceded investigations of other organic solvent-tolerant enzymes.<sup>30</sup> Interestingly, the stability of this protease in solutions containing organic cosolvents was found to be higher than that in the absence of cosolvent. Ogino and co-workers reported that disulfide bonds and amino acid residues located at the protein surface are responsible for the solvent-stable nature of the enzyme.<sup>33,34</sup> Two disulfide bonds as well as a number of hydrophobic clusters were also identified at the surface of protease from *P. aeruginosa* PseA isolated from the soil by cyclohexane enrichment.<sup>31</sup> Gupta and co-workers proposed that these disulfide bonds and hydrophobic patches, covering 21% of the sequence, may possibly be responsible for the remarkable stability of the enzyme toward organic solvents. Exceptional tolerance toward elevated concentrations of organic solvents has also been observed for lipase from *P. aeruginosa* LST-03 grown in a medium containing olive oil as the carbon source and cyclohexane solvent.<sup>32</sup> Subsequently, residues potentially responsible for the lipase stability were

identified and mutated to further increase the enzyme stability in organic solvents. These mutations were found to induce various structural changes, including the formation of a salt bridge, hydrogen bonds, and improved packing of the hydrophobic core.

Another source of organic solvent-tolerant enzymes are halophilic and alkaliphilic organisms.<sup>35,36</sup> High tolerance toward organic solvents has been demonstrated for  $\alpha$ -amylase and alkaline phosphatase from *Haloarcula* sp. strain S-1 and *Streptomyces clavuligerus* strain Mit-1. Extracellular halophilic enzymes with high solvent tolerance can be used in industrial processes where high salt concentrations and hydrophobic organic solvents are present at the same time. Studies of organic solvent tolerance in the presence of high salt concentrations at alkaline pH offer the prospect of identification of biocatalysts that can function under harsh operational conditions.

Because of the difficulties related to isolation of extremophilic organisms, a more convenient approach is to clone genes from extremophilic organisms into suitable mesophilic hosts. Metagenome-based technologies have been successfully applied for the identification of novel biocatalysts within the past decade.<sup>37</sup> Recently, several studies describing the application of metagenomics of soil and marine microorganisms have led to the identification of organic solvent-stable lipases and esterases.<sup>38–40</sup> A major drawback of using a metagenome-based approach for biocatalyst discovery is that it does not always yield enzymes suitable for bioprocess reactions because housekeeping genes are much more abundant.<sup>41</sup> Nevertheless, the development of modern high-throughput screenings and the increasing availability of protein sequences in databases and bioinformatics tools have improved the chance of discovering novel biocatalysts by diversity prospecting.

**Modification of Biocatalysts. Enzyme Immobilization.** The use of immobilized entities instead of free forms represents the most common method for improving enzyme stability toward organic solvents. Reports on chemical immobilization of proteins and enzymes first appeared in the 1960s.<sup>42</sup> Since then, immobilized enzymes have been widely used in the processing of a variety of products. Stabilization has been attributed to the more rigid conformation of the immobilized biocatalyst, which prevents unfolding of the enzyme and malformation of its active site.<sup>43,44</sup> In addition to enhanced stability, enzyme immobilization enables efficient recovery of enzymes from the reaction environment as well as their use in continuous operations, thus reducing the enzyme and product costs significantly.<sup>2,26</sup> As a consequence, immobilization of enzymes is the most commonly employed strategy for stabilization of biocatalysts by the industry. Common methods of immobilization generally fall into three categories: (i) immobilization onto

an inert matrix, (ii) immobilization within an inert matrix, and (iii) formation of water-insoluble particles (Table 3).<sup>26</sup>

Physical adsorption of enzymes onto a supporting material is the simplest method of enzyme immobilization used to improve enzyme stability. A wide range of commercially available enzymes immobilized on solid supports have been examined for biocatalysis in organic solvents, some of which have already found industrial applications.<sup>4,7,45–47</sup> Probably the most well-known example of a physically adsorbed enzyme that exhibits excellent activity in neat organic solvents is *Candida antarctica* lipase B adsorbed on Lewatit ion-exchange resin (Novozym 435, Novozymes).<sup>48</sup> An industrial synthesis of polyol acrylates using commercially available Novozym 435 has been developed by BASF. This patented technology includes an enzymatic reaction lasting up to 3 days in neat *tert*-butanol, methyl *tert*-butyl ether, or acetone.<sup>5</sup> For example, the reaction of glycerol (12 g) and methyl acrylate (108 g) in acetone provided a crude product (81 g) containing 37% glycerol monoacrylate, 46% glycerol diacrylate, and 15% glycerol. Novozym 435 has also been used by Schering-Plough for the synthesis of an azole antifungal agent in hundred kilogram quantities with acetonitrile as the solvent.<sup>49</sup> Pfizer has patented the preparation of enantiomerically enriched 3-aminopentane-nitrile using various commercially available lipases immobilized on Accurel or Celite in methyl *tert*-butyl ether.<sup>7</sup> Chirazyme L-2 is another commercially available carrier-fixed lipase from *C. antarctica* B that is particularly suitable for reactions in organic solvents, where it displays high activity and stability. In addition, numerous examples of enzymes stabilized toward organic solvents by adsorption on the solid supports have been reported in the scientific literature. Ruiz and co-workers showed that the activity and stability of laccase from *Trametes versicolor* can be improved by adsorption on glass, glass powder, silica gel, and Nylon 66 membrane.<sup>50</sup> Significant improvements in the activity and storage stability were observed in diethyl ether and ethyl acetate, whereas the free enzyme exhibited no activity in these solvents. Lo and Ibrahim<sup>51</sup> demonstrated that adsorption on Amberlite XAD-7 and subsequent cross-linking with glutaraldehyde significantly stabilize lipase from *Pseudomonas* sp. AK. The stabilities of free and immobilized lipase were tested in hexane, heptane, and isooctane for 30 h at 32 °C. The free lipase exhibited less than 5% hydrolytic activity, whereas the immobilized lipase was found to be stable even after 30 h of incubation, maintaining 100% of the synthetic activity in all of the organic solvents tested. Co-immobilization of horse liver alcohol dehydrogenase and its cofactor NADH onto the surface of glass beads, which were then suspended in various water-immiscible organic solvents, was reported by Grunwald and co-workers.<sup>52</sup> Using this approach, the authors achieved reasonable operational stability. After six reuses over 3 weeks, the enzyme still exhibited more than one-third of its initial activity. An industrial-scale enzymatic conversion of (1 $\alpha$ ,2 $\beta$ ,3 $\alpha$ )-2-(benzyloxymethyl)cyclopent-4-ene-1,3-diol diacetate to the corresponding monoacetate, a key intermediate in the synthesis of entecavir (approved for treatment of hepatitis B viral infection), in 10% toluene was reported by researchers of the Bristol-Myers Squibb Pharmaceutical Research Institute. The asymmetric hydrolysis, catalyzed by lipase PS-30 from *Pseudomonas cepacia* immobilized on the hydrophobic resin Accurel polypropylene, provided the desired product (32.5 g) in 80% yield with 98% ee.<sup>6</sup>

High stabilization has also been achieved by multipoint covalent attachment, where the enzyme is linked to the

activated pre-existing support through several amino acid residues.<sup>53,54</sup> Wang and co-workers greatly enhanced the activity and stability of  $\alpha$ -chymotrypsin in organic solvents by covalent binding to a nanoporous silica glass.<sup>55</sup> The activity enhancements of immobilized  $\alpha$ -chymotrypsin observed in acetonitrile, isooctane, hexane, and methanol were 65-, 65-, 110- and 1000-fold, respectively, relative to the free enzyme. The improved properties of the enzyme were suggested to result because multipoint covalent attachment provided protection against structural denaturation of the enzyme as well as better contact with the reaction solution due to the large surface of nanoporous glass occupied by immobilized enzyme, since the free enzyme is insoluble in organic solvents.<sup>55</sup> Likewise, multipoint covalent attachment of chloroperoxidase from *Caldariomyces fumago* on the same material resulted in increased stability toward organic solvents. The stabilizing effect was found to be dependent on the pore size. The largest stabilization was observed with 200 Å sol-gel glass because of enzyme immobilization within the pores of the sol-gel.<sup>56</sup> Significant stabilization by covalent immobilization on metal-derivatized epoxy Sepabeads was achieved for NADP<sup>+</sup>-dependent alcohol dehydrogenase from the archaeon *Haloferax volcanii*. The immobilized preparation retained approximately 50% activity after incubation for 72 h in 30% DMSO or 30% methanol at 5 °C. However, its cofactor was not co-immobilized with the enzyme and had to be supplied to the reaction mixture.<sup>57</sup>

Enzyme entrapment is typically achieved using a polymer network, such as an organic polymer or sol-gel. Alcohol dehydrogenase from *Lactobacillus kefir* was entrapped with its cofactor NADPH + H<sup>+</sup> in polyvinyl alcohol gel beads. The immobilized preparation successfully transformed a number of hydrophobic ketones to the corresponding enantiomerically pure (*R*)-alcohols in the presence of pure hexane. The polyvinyl alcohol matrix protected both the enzyme and its cofactor from the deleterious effects of the organic solvent, but a slower diffusive movement of the cofactor in the gel than in water led to the need for a higher local concentration of the cofactor surrounding the enzyme.<sup>58</sup> Bruns and Tiller<sup>59</sup> reported the entrapment of horseradish peroxidase and chloroperoxidase in a nanostructured amphiphilic network consisting of poly(2-hydroxyethyl acrylate) and poly(dimethylsiloxane). Initially, the network was simply immersed into an aqueous solution of the enzyme. The hydrophilic polymer was then placed in *n*-heptane, causing the network to shrink, entrapping the protein in an enzyme-friendly environment. However, such an immobilization strategy carries the risk of enzyme deactivation, particularly by shrinking of the gel during the condensation and drying processes.<sup>60</sup> Interestingly, enzyme deactivation might be overcome by employing ionic liquids (ILs) as protective agents. Lee and co-workers demonstrated that the hydrolysis and esterification activities of lipase co-immobilized with a 1:1 molar ratio mixture of [C<sub>2</sub>MIM][BF<sub>4</sub>] and [C<sub>16</sub>MIM][Tf<sub>2</sub>N] were approximately 10-fold greater than in silica gel without ILs.<sup>61</sup> After 5 days incubation of immobilized lipase in *n*-hexane at 50 °C, 84% of the initial activity remained, whereas the residual activity of the lipase immobilized without ILs was 28%.

Increased resistance of enzymes toward organic solvents after the formation of water-insoluble particles such as cross-linked enzyme crystals (CLECs), cross-linked enzyme aggregates (CLEAs), and protein-coated microcrystals (PCMCs) is well-established (Table 3). Although both CLECs and CLEAs have been shown to dramatically enhance an enzyme's operational

stability toward heat, only a few reports have discussed the application of CLEAs in organic solvents.<sup>62–64</sup> For instance, CLEAs of tyrosinase have been shown to retain 75% and 62% of the original activity after incubation in pure 1,4-dioxane and acetone, respectively, whereas the residual activity of free tyrosinase was negligible at solvent concentrations of 40% (v/v) and higher.<sup>62</sup> More recently, Cui and co-workers reported significant stabilization of phenylalanine ammonia lyase in isopropanol by the formation of CLEAs.<sup>65</sup> The stabilizing effect was explained by the conformational rigidity of the enzyme as a consequence of the formation of multiple covalent linkages and a change in the properties of the reaction medium. Significant improvements in enzyme stability toward denaturation by organic solvents have been achieved by using PCMCs. A good example of cofactor-dependent enzyme stabilization against organic solvents by formation of PCMCs is the immobilization of various oxidoreductases reported by Kreiner and Parker.<sup>66</sup> Activation of crystals of horse liver alcohol dehydrogenase to catalyze benzyl alcohol oxidation in nearly anhydrous *tert*-butyl methyl ether and catalase to decompose hydrogen peroxide in nearly anhydrous *n*-propanol was found to be 50- and 25-fold, respectively. Moreover, because cofactor NAD<sup>+</sup> is insoluble in the precipitating organic solvent, it was coprecipitated with the enzyme and the carrier. This is particularly important because the insolubility of the cofactors can increase the turnover number of the cofactor.<sup>67</sup> Highly stable preparations of  $\alpha$ -chymotrypsin, subtilisin Carlsberg, and lipases have been prepared by cross-linking of PCMCs.<sup>68,69</sup> Cross-linked protein-coated microcrystals (CLPCMCs) were shown to exhibit even higher catalytic activity in the presence of organic solvents than simple PCMCs or CLEAs.<sup>69</sup> Interestingly, CLPCMCs of  $\alpha$ -chymotrypsin and subtilisin Carlsberg displayed higher catalytic activities in organic media than other preparations, even though their structure was significantly changed.<sup>69</sup> This paradox was not explained and requires further study.

**Chemical Modification.** Before the advent of molecular biology, one of the most widely used tools for improving stability of enzymes was the chemical modification of amino acid residues. Despite being overshadowed in recent years by protein engineering, chemical modification has remained a useful technique for enzyme stabilization. Among the chemicals used to modify proteins for resistance toward organic solvents, poly(ethylene glycol) (PEG) and its derivatives clearly predominate.

Surface modification of proteins by PEG was first described by Veronese and co-workers in 1985,<sup>70</sup> and one year later, PEGylation was applied to enzymes used in organic solvents.<sup>71</sup> Inada and co-workers have demonstrated that because of both the hydrophilic and hydrophobic properties of PEGs, modified enzymes become soluble in organic solvents such as benzene, toluene, and chlorinated hydrocarbons. Subsequently, various studies of enzymatic PEGylation reported enhanced properties of PEGylated enzymes in organic media, including increased stability, improved catalytic activity, and altered enantioselectivity.<sup>72–76</sup> Since the functional behavior of enzymes is usually directly linked to the protein structure, it is highly probable that these effects were due to an improvement in the structural properties of the enzymes.<sup>72</sup> However, only a few systematic studies have been performed to provide mechanistic insights into the increased enzymatic activities and stabilities upon PEGylation.

Castillo and co-workers have explored the effects of covalent modification of subtilisin Carlsberg with methoxy-PEG-succinimidyl propionate on the enzyme activity, enantioselectivity, and structural dynamics in 1,4-dioxane.<sup>72</sup> Comparison of the initial rates for the unmodified enzyme and the PEG-subtilisin conjugates with on average 1.1, 1.9, and 3.2 moles of PEG per mole of the protein revealed 30-, 57-, and 99-fold increases in enzymatic activity, respectively. However, the enzyme enantioselectivity decreased with increasing levels of PEGylation. On the basis of correlation analysis between the catalytic and structural dynamic parameters, the authors concluded that PEGylation increases the protein structural dynamics in 1,4-dioxane, which enhances the probability that the enzyme achieves a more active conformation. Improved flexibility was suggested as a possible explanation for the reduced enzyme enantioselectivity, since the three-dimensional space within the active site becomes less compact and more adaptable to binding of both enantiomers. PEGylation of the enzyme interestingly did not induce any additional structural changes. In contrast, Kwon and co-workers reported that the secondary structure of subtilisin non-covalently modified with poly(oxyethylene glycol) was changed compared with the native structure when dissolved in water-miscible organic solvents such as dimethyl sulfoxide, acetonitrile, and tetrahydrofuran.<sup>74</sup> Nevertheless, the activity of PEG-subtilisin in the presence of organic solvents was found to be approximately 10-fold higher than that of the unmodified enzyme.

**Propanol-Rinsed Enzyme Preparation.** Propanol-rinsed enzyme preparation (PREP) is a method for stabilizing enzymes for reactions in low-water media that is achieved by repeatedly rinsing the enzyme with dry *n*-propanol. Despite promising results achieved by PREP, only a few studies on this topic have been published to date. The PREP technique was first established by Partridge and co-workers, who prepared PREPs of silica-immobilized subtilisin Carlsberg and  $\alpha$ -chymotrypsin.<sup>77</sup> The PREPs of both enzymes were shown to exhibit 1000-fold higher activities than lyophilized powders in polar organic solvents such as acetonitrile and tetrahydrofuran. The higher activity of PREPs is believed to be due to the fact that unlike freeze-drying, removal of water associated with the protein by rinsing with propanol does not cause protein denaturation and leaves the majority of enzyme molecules active.<sup>77</sup> The PREP concept has also been adapted for enzymes in the free form. Preparation of so-called enzyme precipitated and rinsed with *n*-propanol (EPRP) involves a combination of three steps: (i) enzyme precipitation by alcohols, (ii) drying of the precipitate with *n*-propanol, and (iii) coprecipitation at high salt concentration.<sup>78</sup> With this methodology,  $\alpha$ -chymotrypsin and subtilisin have been shown to exhibit 57- and 116-fold greater transesterification activities in *n*-octane than their free forms.<sup>69</sup> However, cross-linking of EPRPs of  $\alpha$ -chymotrypsin and subtilisin led to decreased enzymatic activity.

**Ionic Liquid Coating.** ILs are organic salts composed of bulky asymmetric cations and weakly coordinating anions with melting points below 100 °C. Their other interesting properties are extremely low volatility, nonflammability, and thermal stability. They are considered to be good solvents for polar substrates, and their physicochemical properties are tunable by altering the ionic components. ILs can be used as cosolvents as well as enzyme-coating agents.<sup>79–81</sup> The IL-coated enzyme concept minimizes quantities of expensive and potentially toxic ILs. In recent years, IL coating has emerged as an efficient technique for the preparation of robust biocatalysts that can

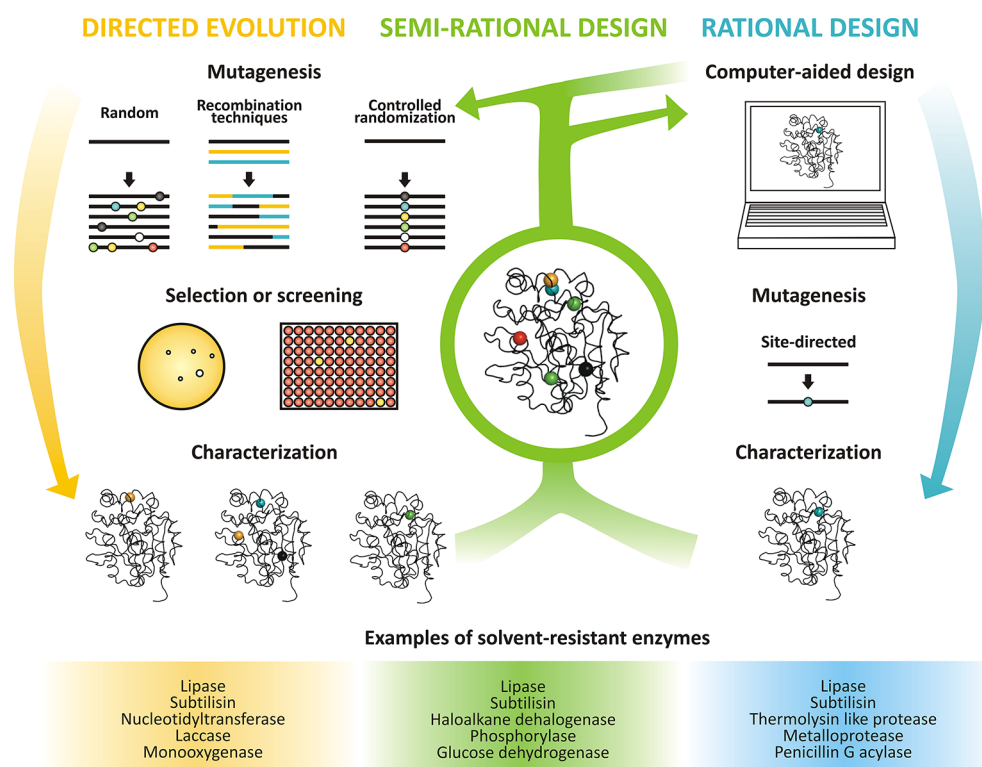
outperform previously reported coating strategies employing lipids or surfactants.<sup>81–83</sup>

The pioneering report of an IL-coated enzyme for biocatalysis in an organic solvent was published in 2002 by Lee and Kim,<sup>84</sup> who described the coating of *Burkholderia cepacia* lipase with [PPMIM][PF<sub>6</sub>]. In this case, enzyme powder was dispersed gently through the heated and melted IL. The mixture was subsequently cooled and cut into small pieces, which served as IL-coated biocatalysts. The coated enzyme was shown to catalyze the transesterification of different racemic secondary alcohols with vinyl acetate in neat toluene with reliable stability and markedly higher enantioselectivities than those observed for the free enzyme. However, the enzyme activity was not improved.<sup>84</sup> In contrast, enhanced activity was observed for the enzyme coated by IL during lyophilization. Promising results were also reported by Itoh and co-workers, who used the same enzyme with several types of imidazolium poly(oxyethylene) alkyl sulfate ILs as coating agents during lyophilization.<sup>85</sup> A remarkable acceleration from 500- to 1000-fold while maintaining excellent enantioselectivity was accomplished by using IL-coated lipase in diisopropyl ether. In an attempt to explain these effects, MALDI-TOF mass spectrometry was used to characterize the IL-coated enzymes. These experiments revealed that the coating agent binds to the enzyme and thus provides a microenvironment favorable for the reaction. Excellent stabilization of IL-supported immobilized *C. antarctica* lipase B has been investigated by Lozano and co-workers.<sup>86</sup> The lipase coated with [BTMA][Tf<sub>2</sub>N] or [TOMA][Tf<sub>2</sub>N] was found to be active in hexane even at very high temperature (95 °C). The ability of ILs to preserve secondary structure elements has been demonstrated by circular dichroism and fluorescence spectroscopy.<sup>86</sup>

**Genetic Modification.** Unlike extremozymes, most enzymes have evolved to be active within a defined set of standard conditions, close to those existing in mesophilic terrestrial organisms.<sup>23,87</sup> To overcome this limitation and develop enzymes that can work in the presence of organic solvents, tailor-made enzymes can be prepared from wild-type templates by protein engineering methods that use both the 20 canonical amino acids and unnatural amino acids as building blocks.<sup>2,26,88</sup> Genetic modifications can be divided into three main categories: (i) directed molecular evolution, (ii) rational design, and (iii) semirational design (Table 4). Directed molecular evolution involves the generation of vast molecular diversity at the level of a coding nucleic acid and subsequent identification of positive variants in the resulting library of mutated genes by a functional assay. In contrast, structure-based engineering focused on rational design enables the construction and characterization of a few mutants on the basis of knowledge of the enzyme structure and structure–function relationships (Figure 2).<sup>89–91</sup> The latest trend is to combine these two approaches and generate small, functionally rich mutant libraries using rationally preselected target sites and limited amino acid diversity in a so-called semirational design.<sup>92–94</sup> In general, both the stability and activity of an enzyme can be engineered to improve its performance in the presence of organic solvents. Because of the observed trade-off between activity and stability, simultaneous engineering during directed evolution offers several advantages for achieving a stable but active enzyme.<sup>95</sup> The structural features relevant for improvement of protein stability include: (i) surface regions,<sup>96–99</sup> (ii) the hydrophobic core,<sup>100,101</sup> (iii) access tunnels,<sup>101</sup> (iv) binding pockets,<sup>96,102–104</sup> (v) the interface of the enzyme's subunits,<sup>105</sup>

Table 4. Classification of Protein Engineering Methods

method	prerequisites	techniques	description
directed evolution	gene template, a functional assay for selection or screening of improved variants	random mutagenesis recombination techniques saturation mutagenesis site-directed mutagenesis	mutations are randomly generated alongside the whole gene with a preferred mutation rate; however, only one oligonucleotide in a codon can be changed per round fragments from different variants of the gene are combined into a novel DNA molecule; beneficial mutations can be combined, while deleterious ones are eliminated a degenerate oligonucleotide, which is randomized at a specific position, is incorporated within the coding sequence (e.g., at the hot spot identified by random mutagenesis) mutations are predicted computationally using enzyme structures, homology models, or sequence comparisons; a desired mutant is prepared using mutant oligonucleotides, which carry the desired mutation at certain position
rational design	knowledge of enzyme structure or a homology model, knowledge of structure–function relationships, computational design	saturation mutagenesis	a degenerate oligonucleotide randomized at a specific position is incorporated within the coding sequence according to computational design
semirational design	knowledge of enzyme structure or a homology model, knowledge of structure–function relationships, computational design, functional assay for selection or screening of improved variants or direct characterization of a number of mutants	saturation mutagenesis	



**Figure 2.** Protein engineering methods for stabilization of enzymes toward organic solvents. A comprehensive list of enzymes stabilized toward organic solvents is provided in Table S1 in the Supporting Information.

(vi) disulfide bridges,<sup>100,106</sup> (vii) surface charges,<sup>107–112</sup> and (viii) isolated charges.<sup>108,113</sup> Published studies demonstrate that representatives of different enzyme classes can be successfully tailored for improved performance in organic cosolvents. The enzymes engineered to date have mainly been cofactor-independent hydrolases (proteases, lipases, esterase, phospholipase, amidohydrolase, and haloalkane dehalogenase), but oxidoreductases (laccases, cytochromes, chloroperoxidase, and glucose 1-dehydrogenase), transferases, and lyases have also been engineered (Table S1 in the Supporting Information). The tailored enzymes have been predominantly monomeric with sizes of about 30 kDa. However, several studies focused on larger enzymes, such as laccase (80 kDa)<sup>114–116</sup> and cytochrome P450 2B1 (120 kDa).<sup>117</sup>

The first reports on the use of protein engineering to improve enzyme activity and stability in organic solvents were published by Arnold and co-workers in the 1990s.<sup>103,107,118–120</sup> Several rounds of random mutagenesis and screening yielded a variant of subtilisin E that hydrolyzed a peptide substrate 256-fold more efficiently than the wild-type enzyme in 60% (v/v) dimethylformamide.<sup>103,104</sup> The resulting mutant was almost as efficient a catalyst in 40% (v/v) dimethylformamide as the wild-type enzyme in pure buffer. Moreover, the set of mutations responsible for the enhanced catalytic activity in the presence of dimethylformamide did not compromise its kinetic stability. The kinetically controlled biosynthesis of poly(L-methionine) from L-methionine methyl ester in the presence of 70% (v/v) dimethylformamide led to the production of 15 mg of polymer. The detected half-lives of the wild-type and engineered enzymes were similar, as they retained 49% versus 43% of the initial activities after 460 h incubation in 70% (v/v) dimethylformamide. A combination of random mutagenesis and screening also proved to be successful in a study on p-

nitrobenzyl esterase engineered for the synthesis of the cephalosporin-derived antibiotic loracarbef carried out in the presence of dimethylformamide.<sup>119</sup> Four rounds of error-prone PCR followed by random pairwise gene recombination of two positive variants led to a 50–60-fold increase in total esterase activity of p-nitrobenzyl esterase in 25% (v/v) dimethylformamide. Random mutagenesis combined with efficient screening has subsequently been successfully applied to other enzymes,<sup>101,110,114,115,117,121–124</sup> including mammalian cytochrome P450 monooxygenase, which is naturally difficult to engineer for stability due to the fragile nature of its active site and heme cofactor.<sup>124</sup> A number of studies have reported that various enzymes such as lipase, sucrose phosphorylase, haloalkane dehalogenase, fructose biphosphate aldolase, protease, nucleotidyl transferase, and glucose 1-dehydrogenase that have been engineered for thermostability are also resistant toward organic solvents.<sup>97,98,101,105,106,125–127</sup> These observations led to the proposal of new focused strategies for the construction of enzymes that are stable in the presence of organic solvents.<sup>97,101,105</sup> Vazquez-Figueroa and co-workers demonstrated that thermostable variants of glucose-1-dehydrogenase developed via a structure-guided consensus concept also showed improved stability in solutions with high concentrations of water-miscible organic solvents.<sup>105,128</sup> The sequence of glucose 1-dehydrogenase from *Bacillus subtilis* strain 168 was aligned with 12 representative homologous sequences (amino acid sequence identity with glucose-1-dehydrogenase was 24.8–61.1%).<sup>128</sup> Using a consensus cutoff of 50%, 31 residues were identified as suitable for substitution. The number of residues was further reduced to 24 by consideration of (i) the distance from the cofactor binding site (>6 Å), (ii) the amino acid secondary structure propensity (helix stabilizers vs destabilizers), and (iii) existing hydrogen bonds and salt



bridges. Single mutants of glucose 1-dehydrogenase were generated by overlap extension, and only variants that resulted in at least a 50% increase in thermal stability (to template) were combined and further analyzed. The most stable 7-fold mutant showed more than 2500-fold increased resistance toward 20% (v/v) acetone, 20% (v/v) acetonitrile, and 20% (v/v) 1,4-dioxane at 25 °C based on determination of half-lives.<sup>105</sup> This result is valuable for industrial applications of oxidoreductases because glucose dehydrogenases are often used in regeneration of both NAD<sup>+</sup> and NADP<sup>+</sup>.

The B-FIT method, originally developed by Reetz and co-workers as a strategy for increasing protein thermostability, has also been successfully employed to stabilize enzymes toward organic solvents.<sup>97,129</sup> Iterative saturation mutagenesis was focused to six flexible sites previously selected as the residues with the highest B-factors in the X-ray structure.<sup>129,130</sup> Six small mutant libraries were constructed, and the best positive hit was identified and subsequently used as a template for iterative rounds of saturation mutagenesis in the remaining four flexible regions.<sup>129</sup> When tested in the presence of organic cosolvents, the best mutant of lipase A from *B. subtilis* exhibited an approximately 20-fold longer half-life in 50% (v/v) dimethyl sulfoxide and 50% (v/v) dimethylformamide and a more than 50-fold longer half-life in 50% (v/v) acetonitrile compared with the wild-type enzyme.<sup>97</sup> The activity of the same enzyme in the presence of dimethyl sulfoxide was recently modified by Yeddevali and co-workers.<sup>99</sup> They subjected all 91 amino acids of the loops to site saturation mutagenesis and identified six substitutions that led to 8-fold higher catalytic turnover of the lipase A in 60% (v/v) dimethyl sulfoxide. Interestingly, each approach identified different hot spots in lipase A, except Tyr139.

A semirational design strategy applicable for stabilization of a wide range of enzymes possessing buried active sites was introduced by Koudelakova and co-workers.<sup>101,131</sup> Iterative saturation mutagenesis of residues lining the access tunnel of the haloalkane dehalogenase DhaA, which connects the buried active site with the surrounding solvent, provided resistance toward dimethyl sulfoxide. Substitution of only four residues in the access tunnel extended the half-life of the enzyme by 4000-fold in 40% (v/v) dimethyl sulfoxide and doubled the half-concentration. Crystallographic analysis and molecular dynamics simulations revealed that the introduced substitutions improved the packing of the residues in the tunnel and prevented destabilization of the protein structure by organic solvent molecules entering the active site. The same mechanism of stabilization in organic solvents has also been described for other systems.<sup>33,117</sup>

Contrary to thermostability, enzyme stability in organic solvents has seldom been achieved by rational engineering because of a lack of understanding of the interactions between enzyme and solvent molecules. Further progress in understanding protein stability based on advances in structural biology, bioinformatics, and molecular modeling are necessary to improve the success rate of rational design for tailoring enzyme stability in organic solvents.<sup>92,132,133</sup> A few successful attempts to employ rational design of an enzyme's surface to increase its stability in polar organic cosolvents have been published.<sup>107,111,112,134</sup> In a pioneering study, Martinez and co-workers rationally engineered the surface of subtilisin E to improve its stability in the polar organic solvent dimethylformamide.<sup>107</sup> The final double mutant was 3.4 times more stable than the wild-type enzyme in 80% (v/v) dimethylformamide.

Yang and co-workers mutated two surface residues of penicillin G acylase from *Bacillus megaterium* and obtained a double mutant with a 2.5-fold longer half-life than the wild-type enzyme in 40% (v/v) dimethylformamide at 25 °C.<sup>111</sup> The authors selected basic nonconservative residues far from the active site, critical structure elements and interactions, and mutated them to alanines.<sup>111</sup> Park and co-workers used pre-existing knowledge of the importance of hydrogen bonds for enzyme stability in hydrophilic organic solvents.<sup>134</sup> They showed that the tolerance of *C. antarctica* lipase B toward 80% (v/v) methanol could be enhanced 1.5-fold by increasing the hydrogen-bonding interactions between surface side chains of the enzyme and water molecules. In a parallel study, sites in lipase B affected by methanol were identified by molecular dynamics.<sup>112</sup> Surface residues with high root-mean-square deviations in methanol were selected and redesigned using RosettaDesign. The most stable single-point mutant exhibited a 1.8-fold longer half-life than the wild-type enzyme in 80% (v/v) methanol at 30 °C.

**Modification of the Solvent Environment. Additives.** Among the various approaches used to increase the stability and activity of biocatalysts in organic solvents, the addition of agents such as inorganic salts, polyols, and sugars directly to the enzyme aqueous solution or prior to lyophilization represents one of the easiest techniques currently available. In fact, most of the enzyme formulations on the market today are stabilized using additives.<sup>8</sup> Association of biocatalysts with inert supports can greatly affect their stability by influencing the enzyme hydration level, dispersion in the medium, or entrapment of the enzyme in a more active conformation.<sup>10,135</sup> In general, the effects of additives on enzyme properties are still poorly understood because most additives are used in an empirical manner and only a few systematic studies have been published.<sup>136,137</sup>

The improvement of a biocatalyst for use in organic media by addition of salts was first reported by Khmel'nitski and co-workers, who found that the transesterification activity of subtilisin Carlsberg in hexane was dependent on the KCl content of the lyophilized preparation.<sup>138</sup> A preparation containing 98% (w/w) KCl had nearly 4000-fold higher catalytic efficiency in hexane than in the absence of KCl. Subsequently, several enzymes were reported to be activated for use in organic media by lyophilization in the presence of simple salts, implying that salt activation is a general phenomenon.<sup>9,139,140</sup> Spectroscopic methods have provided additional insight into the mechanism of salt activation. Eppler and co-workers focused on the role of water and its mobility on the protein dynamics in organic media. Deuterium spin relaxation was used to examine the motion of enzyme-bound water on subtilisin Carlsberg colyophilized with inorganic salts.<sup>136</sup> The results indicated that the time scale of motion for residual water molecules on subtilisin in hexane and acetone decreased by 10- and 100-fold, respectively, when the enzyme was colyophilized with 98% (w/w) CsF or KF. Thus, the dramatic activation of the biocatalyst preparations may be explained by the presence of highly mobile water that acts as a molecular lubricant and enhances the enzyme flexibility in neat organic solvents.

It has been shown that the addition of polyols and sugars to aqueous solutions of enzyme strengthens the hydrophobic interactions among nonpolar amino acid residues, leading to protein rigidification. The stabilization effect of these additives has also been attributed to their effect on water activity and decreased microbial contamination.<sup>8</sup> In early work, Dabulis and

Klibanov<sup>141</sup> reported that the activity of fungal proteases in anhydrous organic solvents was increased by over 60-fold through lyophilization of the enzyme in the presence of lyoprotectants such as sorbitol and PEG. Pazhang and co-workers employed glycerol, sorbitol, and trehalose to improve the stabilization of thermolysin in the presence of various water-miscible organic solvents.<sup>142</sup> They showed that trehalose has a significant stabilization effect on thermolysin in the presence of different concentrations of dimethylformamide, whereas glycerol and sorbitol are good enzyme stabilizers in the presence of *n*-propanol and isopropanol. The authors concluded that trehalose stabilizes thermolysin by preventing dimethylformamide from stripping essential water from protein molecules, whereas the protective effects of the other two polyols arise from their competition with *n*-propanol and isopropanol for binding to the hydrophobic sites of the studied protein.<sup>142</sup> Complex carbohydrates have also been used to improve enzyme performance in organic media.  $\alpha$ -Chymotrypsin and subtilisin Carlsberg lyophilized in the presence of methyl- $\beta$ -cyclodextrin ( $M\beta$ CD) displayed significantly higher activities in neat organic solvents than free enzymes.<sup>143,144</sup> Griebenow and co-workers investigated the effect of  $M\beta$ CD on the secondary structure of lyophilized subtilisin suspended in organic solvents.<sup>143</sup> They found that colyophilization with  $M\beta$ CD prevents structural changes of the enzyme caused by lyophilization, mainly with respect to the  $\alpha$ -helix content.

The use of crown ether is another generally applicable way to stabilize and activate proteins in nonaqueous environments.<sup>137,145–147</sup> For instance, significant activation of  $\alpha$ -chymotrypsin when colyophilized with 18-crown-6 was reported by van Unen and co-workers.<sup>137</sup> They proposed that the crown ether-enhanced enzyme activity is due to macrocyclic interactions between 18-crown-6 and the enzyme, which can lead to reduced formation of salt bridges and better refolding of the enzyme into active conformations.

**Surfactants.** By the introduction of amphiphilic surfactants into organic solvents, enzymes can be encapsulated in reverse micelles or water-in-oil microemulsions.<sup>12</sup> Microemulsions are thermodynamically stable mixtures of water, organic phase, and surfactant present at the organic–aqueous interface. Described as “nanobioreactors”, these systems have found wide application in biotransformations. One important advantage of microemulsions is that the enzyme is present in the aqueous phase while hydrophobic substrates can be dissolved in the water-immiscible organic phase. Enzyme activation and stabilization has been attributed to encapsulation of enzyme molecules into microdroplets.<sup>148–150</sup> For example, tyrosinase and glucose oxidase have been successfully adapted to an octane-based medium by entrapment in a system of reverse micelles of the surfactant dioctyl sodium sulfosuccinate (AOT) in octane.<sup>150</sup> However, wide application of enzyme-containing microemulsions is considerably hindered by the risk of enzyme inactivation caused by the use of high concentrations of surfactants, a large interface between the two phases, or vigorous agitation.<sup>151,152</sup>

One interesting development is water-in-IL microemulsion systems, where ILs are used as a substitute for the conventional organic solvent. When encapsulated in microemulsions composed of an anionic surfactant (AOT), a hydrophobic IL ([OMIM][Tf<sub>2</sub>N]), and 1-hexanol, horseradish peroxidase was found to be both more active and more stable than in a conventional microemulsion system composed of AOT/isooctane/water.<sup>153</sup> Similar findings have also been reported

for lipase from *Pseudomonas* sp. in water-in-IL microemulsions created by dissolving the anionic surfactant sodium bis(2-ethyl-1-hexyl)sulfosuccinate in hydrophobic [OMIM][Tf<sub>2</sub>N] containing 10% (v/v) 1-hexanol as a cosurfactant.<sup>148</sup> The results showed that lipase is much more active in water-in-IL microemulsions than in water-saturated IL or water-in-isooctane microemulsions.

**Combinatorial Approaches.** It has been demonstrated that a combination of various stabilization methods simultaneously or sequentially can lead to better stabilization.<sup>8</sup> It has recently been reported that the use of additives, protein engineering, and chemical modifications can lead to improvement of enzyme function after immobilization or better covalent attachment of the enzyme to a support.<sup>154</sup>

Grazu and co-workers achieved good stabilization of penicillin G acylase against various distorting agents by coupling protein engineering and multipoint covalent attachment. Six different variants of penicillin G acylase were constructed by introducing a cysteine residue into an area rich in lysine residues via site-directed mutagenesis. The highest stability against heat and organic cosolvents was exhibited by an immobilized mutant enzyme obtained by the introduction of a cysteine residue instead of a glutamine near the enzyme active site. This enzyme retained 90% of the initial activity after immobilization and was 30-fold more stable than the free enzyme in 60% (v/v) 1,4-dioxane.<sup>155</sup>

Significant stabilization of the same enzyme has also been achieved by coupling immobilization with chemical modification of the surface residues. Abian and co-workers reported co-immobilization of penicillin G acylase and polyethyleneimine on Sepabeads by covalent attachment followed by repetitive grafting of hydrophilic dextran–aldehyde polymer on the support and the enzyme.<sup>156</sup> The derivative with a hyperhydrophilic shell was able to withstand 90% (v/v) 1,4-dioxane for several days without significant loss of activity, whereas conventionally immobilized penicillin G acylase was readily inactivated under these conditions. Since this two-step strategy has also proven to be successful with a number of other enzymes, such as lipase, pig liver esterase, and  $\beta$ -galactosidase, the generation of hyperhydrophilic nanoenvironments may offer a general method for the stabilization of enzymes in the presence of organic cosolvents.<sup>157,158</sup>

The effect of different additives on the stability of soluble and covalently immobilized  $\alpha$ -chymotrypsin forms in various aqueous–organic solvent systems has been investigated by Laszlo and co-workers.<sup>159</sup> The largest increase in the stability was observed when the enzyme was immobilized on a silica-based support, which was associated with an enhancement of the activity by about 2-fold following addition of sorbitol or glucose. A combination of crown ether addition with cross-linking of enzyme crystals has been reported by van Unen and co-workers. Soaking of subtilisin Carlsberg crystals in a solution of 18-crown-6 resulted in up to 13-fold enhanced activity.<sup>145</sup>

## CONCLUSIONS AND PERSPECTIVES

**Start with Simple Stabilization Methods.** Universal and broadly applicable methods for enzyme stabilization toward organic solvents remain elusive. Less demanding techniques, such as the addition of stabilizing agents, PEGylation, or simple immobilization of enzyme by physical adsorption, are the current methods of first choice for enhancing enzyme stability in organic media. These simple approaches can be followed by more technically demanding immobilization methods such as

covalent binding to a support or formation of CLEAs, isolation of novel extremozymes, protein engineering, or a combination of several techniques. In particular, the simultaneous use of immobilization with chemical modification or protein engineering has created unprecedented stabilization of enzymes in nonconventional environments, opening the possibility of their use in various industrial applications.

**Look for Enzymes Showing Any Kind of Stability.** Temperature is the most studied deactivating factor, and many enzymes have been successfully stabilized toward temperature. As it is generally believed that enzymes with improved stability toward one denaturing factor are simultaneously more resistant to other denaturing factors, the screening of existing thermally stable variants is a logical first step in the search for biocatalysts that are stable in organic solvents. Another practical consequence is that screening or selection for thermostable variants can be utilized if high-throughput detection of improved variants in the presence of organic solvents is for some reason unfeasible. Likewise, enzymes found in one type of extreme environment are typically more tolerant to other extreme conditions. Thus, naturally occurring thermophiles and halophiles may also provide biocatalysts exhibiting high stability toward organic solvents. Ancient enzymes represent another prospective source of solvent-stable biocatalysts on the basis of the theory that the distant ancestors of current organisms were thermophiles and would have possessed proteins that were more thermostable than extant homologues. Moreover, thermostable enzymes obtained by protein engineering, isolated from organisms colonizing extreme environments, or inferred by phylogenetic reconstruction could be used as robust templates for mutagenesis since they can tolerate a larger number of substitutions.

**Learn Design Principles and Target Regions from Successfully Engineered Proteins.** Looking through the literature focused on the enzyme stabilization published over the last 10 years, one notices an overwhelming number of studies employing protein engineering techniques. It may seem surprising that regardless of recent advances in protein engineering, the majority of practically utilized biocatalysts are still being stabilized by additives, chemical modifications, and immobilizations. This apparent discrepancy may reflect the simplicity and reliability of the latter procedures in comparison with more sophisticated protein engineering techniques. Protein engineering still represents a time-consuming approach. Each enzyme has unique tertiary structure, and stabilization procedures that preserve the activity and enhance the stability can vary from protein to protein. In many cases, current protein engineering strategies for stabilization in organic solvents are based on empirical observations rather than detailed mechanistic understanding. Nevertheless, enzymes engineered for stability in organic solvents provide initial clues for focusing engineering efforts to: (i) surface regions, (ii) the hydrophobic core, (iii) access tunnels, (iv) binding pockets, (v) the interface of the enzyme's subunits, (vi) disulfide bridges, (vii) surface charges, and (viii) isolated charges. Reports of both successful and unsuccessful designs are equally important in this respect.

**Aim To Understand the Structural Basis of Stability and Protein–Solvent Interactions.** Although genetic strategies have not yet achieved the status of a first-line alternative for optimization of industrial biocatalysts, we anticipate that they may become the method of first choice in the future. Thanks to advances in recombinant DNA technology, computational methods, and robotic screening,

enzyme engineering is continuously becoming faster. Computer modeling tools (e.g., ROSETTA and FOLD-X) and web-based tools (e.g., HotSpot Wizard, 3DM, PoPMuSiC, PreTherMut, and Pro-Maya) allow the identification of sites suitable for mutagenesis. Wider application of these tools will provide smarter libraries containing a higher proportion of active enzyme variants with desired properties and help to reduce screening efforts. Deeper insight into the structural determinants of stability of naturally occurring solvent-stable enzymes and better understanding of protein–solvent interactions will lead to further improvement of *in silico* tools and more reliable predictions.

**Apply Emerging Materials for Stabilization by Immobilization.** The use of immobilized enzymes in industrial processes can provide enhanced stability and facilitate the separation of the enzymes from the products, significantly reducing the costs of operation. However, it is essential to select an appropriate carrier material in order to prepare an effective immobilized biocatalyst. The use of nanomaterials as enzyme carriers or entrapment agents is gaining a prominent place within immobilization methods. The principal advantage of nanostructured materials is the possibility to tune their pore diameter, hardness, hydrophobicity/hydrophilicity ratio, magnetic properties, or conductivity, enabling more precise control of enzyme immobilization. However, some of these innovations involve the use of materials that are substantially more expensive than the enzyme to be immobilized. The so-called IL-coated enzymes represent another emerging concept for enzyme immobilization. IL-coated enzymes exhibit better activities and stabilities and can be reused several times. In addition, IL coating allows the use of minimal quantities of expensive ILs. To date, however, only a few examples have been published where IL coating has been used for enzyme stabilization. Clearly, cheaper materials and more systematic studies are needed to utilize these novel immobilization concepts in large-scale applications.

**Replace Traditional Organic Solvents by Novel Alternatives.** Even though a great deal of research has been devoted to the development of biocatalysts that can function in the presence of organic solvents, the search for new reaction media to replace deleterious solvents remains important. In contrast to conventional organic solvents, ILs have virtually no vapor pressure, offering considerable promise for the development of cleaner processes. Since individual components of ILs directly influence physical properties that are crucial for solvent–enzyme interactions, it may be possible to optimize reaction media to meet the requirements of individual biocatalytic applications. Some ILs have been found to be better tolerated than organic solvents, by both isolated enzymes and whole cells. The use of whole cells in ILs is becoming a very promising field for bioconversions such as oxidations and reductions, which usually require the addition of cofactors. However, the following challenges must be resolved to enable the widespread use of catalytic processes in ILs: (i) the high cost, (ii) the presence of impurities, such as unreacted halides, (iii) the antibacterial activity and toxicity of some imidazolium and pyridinium ILs, and (iv) the undesirable tendency of the most commonly used anions ( $\text{PF}_6^-$  and  $\text{BF}_4^-$ ) to decomposed into hydrofluoric and phosphoric acid in water. On the contrary, emerging deep eutectic solvents (DESs) show many properties of ILs, including the potential to be tailored for specific applications, but are composed of biocompatible compounds that are nonhazardous to human health and the

environment. Biobased solvents derived from renewable sources represent another group of eco-efficient solvents that may replace traditional organic solvents in biocatalysis and organic chemistry. In particular, glycerol derivatives show some interesting properties, including good availability, easy derivatization, and tunable properties. An increasing amount of active research in these fields is expected in the coming years that is likely to result in the development of novel solvents for enzyme-based industrial processes.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Overview of protein engineering studies resulting in increased enzyme stability and activity in the presence of organic cosolvents. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

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## ■ ABBREVIATIONS

AOT, dioctyl sodium sulfosuccinate; [BTMA][Tf<sub>2</sub>N], butyltrimethylammonium bis(trifluoromethylsulfonyl)imide; CLEA, cross-linked enzyme aggregate; CLEC, cross-linked enzyme crystal; CLPCM, cross-linked protein-coated microcrystal; [C<sub>2</sub>MIM][BF<sub>4</sub>], 1-ethyl-3-methylimidazolium tetrafluoroborate; [C<sub>16</sub>MIM][Tf<sub>2</sub>N], 1-hexadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide; DES, deep eutectic solvent; EPRP, enzyme precipitated and rinsed with *n*-propanol; IL, ionic liquid; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; [OMIM][Tf<sub>2</sub>N], 1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide; PCMC, protein-coated microcrystal; PEG, poly(ethylene glycol); PREP, propanol-rinsed enzyme preparation; [PPMIM][PF<sub>6</sub>], 1-(3'-phenylpropyl)-3-methylimidazolium hexafluorophosphate; [TOMA][Tf<sub>2</sub>N], *N,N,N*-trioctylmethylammonium bis(trifluoromethylsulfonyl)imide.

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