Nature Versus Nurture: Developing Enzymes That Function Under Extreme Conditions

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Abstract

Many industrial processes used to produce chemicals and pharmaceuticals would benefit from enzymes that function under extreme conditions. Enzymes from extremophilic microorganisms have evolved to function in a variety of extreme environments, and bioprospecting for these microorganisms has led to the discovery of new enzymes with high tolerance to nonnatural conditions. However, bioprospecting is inherently limited by the diversity of enzymes evolved by nature. Protein engineering has also been successful in generating extremophilic enzymes by both rational mutagenesis and directed evolution, but screening for activity under extreme conditions can be difficult. This review examines the emerging synergy between bioprospecting and protein engineering in developing extremophilic enzymes. Specific topics include unnatural industrial conditions relevant to biocatalysis, biophysical properties of extremophilic enzymes, and industrially relevant extremophilic enzymes found either in nature or through protein engineering.

INTRODUCTION

T_{opt}: optimum activity temperature

Industrial reactions that produce chemicals and pharmaceuticals are often carried out under diverse conditions such as at high temperature and pressure, at nonneutral pH, and in nonaqueous environments. Increasing the process temperature, shifting pH, or using organic solvents can provide advantages by reducing the risk of contamination, decreasing viscosity, increasing substrate solubility, and increasing the rate of mass transfer. These conditions may also increase the rate of product formation and/or minimize undesired by-products. However, extreme reaction conditions are often unsuitable for enzymes, which are attractive for many industrial processes as faster and more environmentally friendly alternatives to traditional catalysts. Fortunately, microorganisms thrive in very diverse environments, and many have enzymes that can withstand extremes of temperature, pH, and salt concentration. Thus, discovering and/or engineering enzymes that function under unusual conditions is of growing importance as industrial processes and product demands continue to evolve.

Microorganisms have been discovered in environments such as ocean vents, arctic waters and soil, salt marshes, alkaline and acidic hot springs, and arid deserts. To survive in these environments, microorganisms must have enzymes that function under extreme conditions or employ mechanisms that temper the intracellular milieu from the external environment. Because extremophilic enzymes are often active under conditions similar to those under which the host microorganism resides, searching for microorganisms in extreme environments, or bioprospecting, is a powerful technique for discovering robust enzymes. In addition, enzymes found in one type of extreme environment (e.g., high temperature) are typically more tolerant of other extreme conditions (e.g., organic solvents); thus, naturally occurring robust enzymes can be used or evolved for use in a variety of harsh environments.

Although many industrially relevant enzymes have been isolated from organisms growing at high temperature, at high salt concentration, or in environments contaminated with organic solvents, there are significant challenges and limitations to bioprospecting for extremophilic enzymes. Harsh or extreme environments contain less diverse microbial communities, and extremophiles typically have smaller genomes that are specialized for the environment, which limits the number of synthetically interesting enzymes (1). Once discovered, enzymes from extremophiles can be challenging to use owing to limitations in recombinant expression in even the well-optimized expression host *Escherichia coli*. Prediction tools for determining the success of protein expression are lacking, and small-scale empirical screens are needed to check for proper expression (2). Expression of an extremophilic enzyme in a different host can even alter the enzyme's stability and activity. For example, recombinant expression of a thermophilic esterase from *Thermus thermophilus* HB27 in different yeast species resulted in a 30 to 40°C reduction in the optimum activity temperature (T_{opt}), most likely owing to protein misfolding (3–5).

Given these limitations of bioprospecting for extremophilic enzymes, much effort has been devoted to engineering well-characterized mesophilic enzymes for stability and tolerance to extreme conditions. Sequence and structural analysis of naturally occurring extremophilic enzymes can reveal the trends responsible for enhanced stability and activity. These trends may be used to rationally guide mutations in mesophilic enzymes to confer tolerance to a broad range of extreme conditions, some of which may not be found in nature. Modification of the hydrophobic, electrostatic, or covalent interactions within an enzyme can increase its stability and activity in certain conditions. Other protein engineering methods, such as error-prone polymerase chain reaction (PCR) or DNA shuffling, can also be used on naturally tolerant enzymes to increase their stability and activity even further.

Similar to bioprospecting, engineering proteins for stability and activity in extreme environments has challenges and drawbacks. Screening for enzyme activity under extreme conditions can be problematic and require additional steps if the expression host cannot tolerate the conditions. Ribosome display and screening techniques that rely on biotin-streptavidin/avidin binding are useful for selecting thermostable enzymes given that the ribosome from *Thermus aquaticus* and the biotin-streptavidin/avidin complex are thermostable, but neither screening technique can be used in organic solvents because the biomolecular machinery involved is not stable to denaturants (6, 7). Likewise, catalytic antibodies, which represent another type of protein engineering for biocatalysis, are produced under physiological conditions, thus limiting the range of suitable screening conditions and substrates (8). The assay itself is also subject to limitations. For example, an activity screen based on ionization of a colorimetric substrate analyzed by absorbance spectroscopy will not work properly in aprotic organic solvents. Ideally, separation of expression from screening is a more compatible approach for identification of extremophilic enzymes from a library of mutants. Cell-free protein expression, mRNA display, and DNA display allow for stepwise enzyme synthesis and screening and may prove successful for the identification of robust extremophilic enzymes designed via protein engineering.

Both bioprospecting and protein engineering are powerful tools for discovering new extremophilic enzymes that meet ever-evolving industrial needs. This review describes conditions, both natural and unnatural, that are relevant for industrial applications along with the biophysical characteristics that are known to render an enzyme stable and active under these conditions. **Figure 1** illustrates the three main enzyme classes discussed in this review and the conditions under which they are used. Naturally extremophilic and engineered enzymes with industrial or synthetic relevance are described, and exceptional examples of protein function under extreme conditions are presented. A key question that emerges is whether bioprospecting or protein engineering has

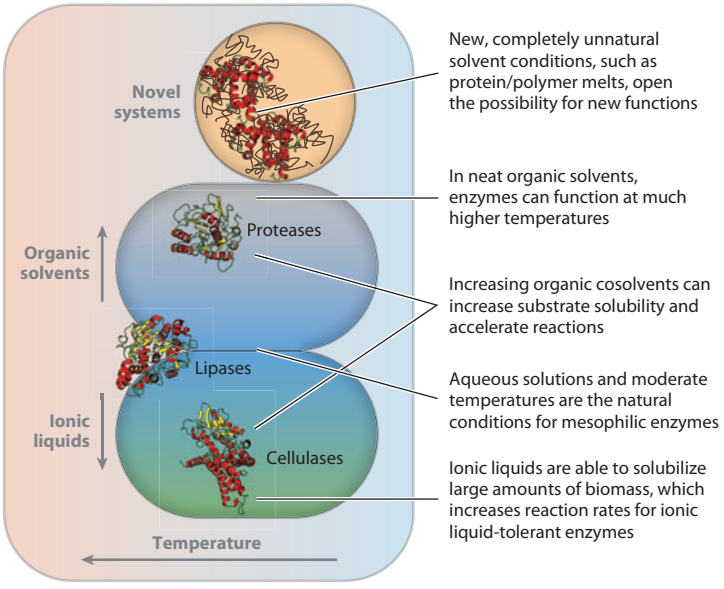


Figure 1

Extreme conditions covered in this review. Proteases, lipases, and cellulases are placed in the figure in the relevant conditions.

greater potential for success. The answer to this question is not obvious and may depend strongly on the conditions required, the desired enzymatic reaction, and the type of enzyme used.

EXTREME CONDITIONS RELEVANT TO INDUSTRIAL PROCESSES

Conditions Found in Nature

Microorganisms exist in very different environments, and their enzymes and proteins have adapted to extreme temperatures, pressures, alkalinity/acidity, and/or osmolarity. Many of these extreme conditions mimic those found in industrial processes that currently employ enzymes or stand to benefit from them. Nature, therefore, is an abundant source of enzymes and proteins tolerant to extreme conditions.

Extreme temperatures. Performing enzyme reactions at elevated temperatures has several potential advantages including higher substrate solubility, faster reaction rates, reduced risk of system contamination, lower solution viscosity, and increased solvent miscibility. However, there are many examples of enzymes used in processes that operate at lower temperatures as well, such as cold-active hydrolytic enzymes used in laundry detergents or for cleaning animal hides, proteases used for cleaning contact lenses, and pectinases for clarifying and extracting fruit juices (9). Mesophilic enzymes are less effective at higher or lower temperatures (i.e., above 40°C or below 25°C) owing to decreased enzyme stability, which leads to a shorter half-life and lower activity. Thermophilic and hyperthermophilic organisms, which thrive in a range of temperatures from 50 to 110°C, can be a source for thermostable enzymes, whereas psychrophiles produce a variety of enzymes that function well below 25°C. Although a wide range of enzymes can be found in thermophiles and psychrophiles, cloning and expression of these extremophilic enzymes can be problematic, and their useful temperature range can be prohibitively narrow. The latter problem has been addressed by protein engineering. For example, engineering a psychrophilic protease from *Bacillus* TA41 increased both its activity and stability at higher temperatures without altering its activity at lower temperatures (10). This result indicates that even though the enzyme was naturally optimized for a particular temperature, it was possible to custom tune the enzyme for improved activity over a broader range of temperatures (10).

Pressure, osmolarity, and alkalinity/acidity. Other extreme industrial conditions that mimic nature include high pressure, high osmolarity, and high (alkalinity) or low (acidity) pH. High pressure is used to limit evaporation, control reaction specificity, form gels or granules, or induce the transition of lipid phases (9). So-called piezophiles live in high-pressure environments such as the deep ocean and terrestrial subsurface. A comparison of orthologous proteins from the hyperthermophile *Pyrococcus furious* and the piezohyperthermophile *Pyrococcus absyii* revealed a positive correlation between piezophily and a higher proportion of smaller and/or more polar amino acids, which may allow for more efficient packing of the protein (11). In addition, factors contributing to thermal stability, such as hydrophobic interactions (12) and surface interactions (13), might also contribute to piezophily. However, a structural comparison of dihydrofolate reductases from the psychropiezophile *Moritella profunda* and *E. coli* did not reveal any structural adaptation to pressure (14). Piezophiles may thus represent a largely untapped source of enzymes that are naturally adapted to multiple, but not necessarily all, extremes.

Some processes found in the food, agriculture, petroleum, textile, and leather industries involve the production of solutions with complex mixtures of salts, organic solvents, and other compounds. The hypertonic water of salt lakes and marshes often mimics the high solute concentrations

of industrial solutions. Halophiles thrive under high salt conditions and regulate their osmotic pressure by adjusting the activity of ion channels and efflux pumps. Some halophiles even produce internal solutes (e.g., trehalose) to maintain osmotic pressure. In addition to these methods for controlling their intracellular environment, halophiles flourish by using proteins that are more stable in the presence of high salt compared with mesophilic proteins (15). Halophilic enzymes have great potential for biocatalysis in high-salt environments, such as in the desalination of wastewater; however, few halophilic enzymes are currently used in industry.

IL: ionic liquid

The halophilic enzyme nuclease H from *Micrococcus varians* subsp. *halophilus* degrades RNA at 60°C and 12% salt and is used industrially to produce the flavoring agent 5′-guanylic acid (16). Furthermore, α-amylase enzymes produced from the halophilic genus *Halomonas* could be used in starch processing. Natural proteases from halophiles are used in the food industry as well as in the production of detergents (17, 18).

Acidic and basic conditions are common in many industrial processes, including paper pulp bleaching, starch hydrolysis, and mining. Alkalophilic subtilisin-like serine proteases from the genus *Bacillus* are often used as additives in laundry detergents because of their activity and stability under basic conditions (19). Iron- and sulfur-oxidizing enzymes from acidophilic microorganisms such as *Acidithiobacillus ferrooxidans* could be used to treat areas contaminated with acid mine drainage (20). Hence, the discovery and application of enzymes from acidophiles and alkaliphiles are important to many industrial processes. Acidophiles and alkaliphiles live in pH conditions less than 4 and greater than 8, respectively. They are typically found in hydrothermal vents, acidic/basic lakes and hot springs, and acidic/basic sediments. Although acidophiles and alkaliphiles use proton pumps to maintain a neutral internal pH, their excreted enzymes must be functional in the harsh pH environments in which these organisms grow.

Unnatural Conditions

Although natural evolution has selected for enzyme activity in a wide range of extreme conditions found in nature, there is no selective pressure for the evolution of enzyme activity in unnatural conditions relevant to chemical synthesis, such as molecular organic solvents, ionic liquids (ILs), and other nonaqueous systems. Understanding the mechanism of enzyme function in these unnatural conditions will lead to better laboratory design of enzymes for industrial processes requiring such extremes.

Organic solvents. The most common of these unnatural conditions is presented by organic solvents [e.g., hexane, dimethylformamide (DMF), tetrahydrofuran, and alcohols]. Organic solvents span a large range of physicochemical properties, including dielectric constants, polarities, hydrogen bonding capacities, and boiling points (typically 50–190°C), which allows for effective optimization of reactions and system properties. Using organic solvents for enzymatic reactions can increase substrate solubility, alter reaction thermodynamics, and expose completely new reactivity, all of which can be useful in industrial processes (21, 22). Unfortunately, these benefits are often outweighed by the greatly reduced activity typically exhibited by enzymes in organic solvents, which limits the use of organic solvents in industrial-scale enzymatic processes.

Organic solvents can affect both enzyme structure and dynamics, ultimately leading to decreased activity and, depending on the solvent conditions, reduced stability. Protein motions play an important role in myriad protein functions, including catalysis and signaling (23); therefore, altered enzyme dynamics in organic solvents can have a detrimental effect on biocatalytic activity. Restricted motion has been strongly correlated with enzyme activity, or lack thereof, in organic solvents (24–26). Structural data available for proteins in organic solvents are limited when

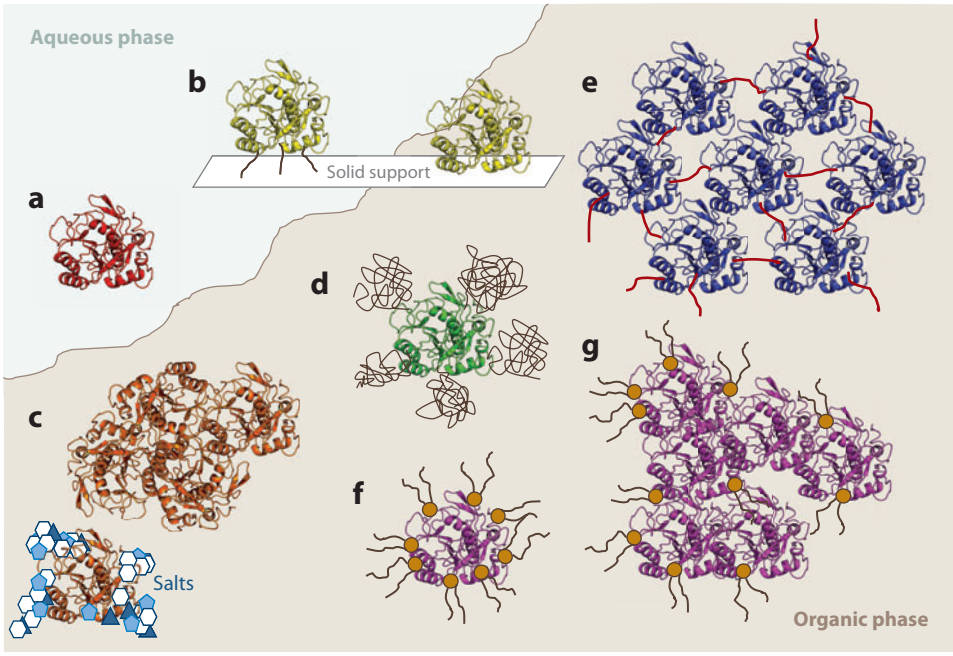


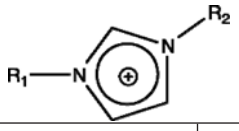
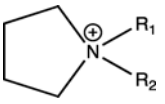
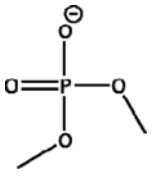
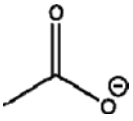
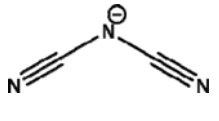
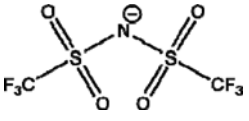
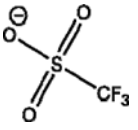
Figure 2

Preparation methods for enzymes in organic solvents: (*a*) native aqueous enzyme, (*b*) enzyme immobilized by covalent attachment or physisorption, (*c*) lyophilized enzyme powder with and without salts, (*d*) covalently modified solubilized enzyme, (*e*) cross-linked enzyme crystals, (*f*) surfactant-solubilized enzyme, and (*g*) directly solubilized lyophilized enzyme.

compared with the vast array of complete structures available for proteins in water. Overall or local changes in the secondary or tertiary structure of proteins in organic solvents are typically detected using Fourier transform infrared spectroscopy (27), circular dichroism (CD) (28), fluorescence, and electron spin resonance spectroscopy (29, 30), but there are few reports of data collected at the atomic level. NMR has the ability to provide higher-resolution information but to date has provided only bulk-average data (25), surface water properties (31), or single-site information (26). In one published high-resolution study, a crystal structure of the protease subtilisin Carlsberg formed in water and soaked in acetonitrile showed almost no structural change from the aqueous structure (32).

A second issue with the use of enzymes in organic solvents is that proteins are not readily soluble in organic solvents; thus, enzymes must be prepared and transferred in such a way as to retain residual water so that enzyme activity is preserved (33). The most common methods for preparing enzymes for use in organic solvents are shown in **Figure 2**. These preparations range from simple suspensions to more complex chemically modified formulations. Lyophilized powders of unmodified enzymes can be used as solid-phase catalysts. Other solid-phase preparation methods include salt activation, transition-state patterning (imprinting), colyophilization with protectants, and single-crystal or aggregate cross-linking (34–38). Chemical modifications can also be directed toward the enzyme surface via PEGylation or ion-pairing with charged surfactants so that the enzyme becomes soluble in organic solvents (39–42). Although guidelines for the use of particular enzymes in specific organic transformations have been compiled on the basis of reported enzyme activities, a general model for activation of enzymes in organic solvents has yet to be developed (43).

Table 1 Structures of some imidazolium- and pyrrolidinium-based ionic liquids (ILs) used with the enzymes discussed in this review

Cation					
R_1 =	$-CH_3$	$-CH_2CH_3$	$-CH_2OCH_3$	$-CH_2CH_3$	$-C_4H_9$
R_2 =	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
Acronym	[MMIM]	[EMIM]	[MOMMIM]	[EMIM]	[BMPyr]
Anion					
Acronym	[DMP]	[OAc]	[DCA]	[NTf_2]	[Tf]
Reference	(147)	(144)	(142)	(44)	(148)

Ionic liquids. ILs are an alternative to organic solvents as a nonaqueous reaction medium for certain enzymatic reactions. Commonly defined as organic salts with melting temperatures (T_m) less than 100°C, ILs have high viscosities (e.g., 50 to 40,000 cP for imidazolium-based ILs), are nonflammable, and have negligible vapor pressures. The solvent properties of ILs are tunable, given that specific ions in the cation-anion pair are interchangeable and alkyl substituents can be introduced in many cases (**Table 1**). As with organic solvents, ILs can be either water miscible or water immiscible. Use of ILs in enzymatic reactions can offer several advantages, including enhanced enzyme stability (44, 45), increased enantioselectivity (46), higher T_m (47), and increased yields of reaction products.

Although some ILs increase enzyme stability compared with organic solvents, reactions conducted with water-immiscible ILs can present problems for enzyme solubility and activity. Many of the preparation methods used for enzymes in organic solvents have been employed for ILs. Similar to organic solvents, high-resolution structural data are not available for proteins in ILs. Biophysical measurements that rely on spectroscopy in the UV range (i.e., CD or absorbance at 280 nm) are particularly difficult owing to the aromatic nature of most common ILs. Specific assays have been developed to obtain lower-resolution data on structural changes that occur in some ILs, thus providing a framework for engineering enzymes in these systems (48).

Other unnatural conditions. Recently a system for enzyme catalysis was developed using deep eutectic solvents (DESs), mixtures of a salt and a hydrogen bond donor (49, 50). Typical DES mixtures include choline chloride and urea or ethylammonium chloride and glycerol. These solvents are similar to ILs in their physical properties, low vapor pressure and nonflammability, but unlike ILs, the components are not completely ionized. DESs can be prepared from a simple mixture of two components, and they are less complex and expensive than ILs. To date, only a limited number of enzymatic reactions have been reported in DESs. One example transesterification reaction run in a DES with several different lipases gave initial reaction rates and conversions similar to the reactions in toluene (50).

As an interesting alternative to using proteins in ILs, ionic protein-polymer melts have recently been developed (51, 52). In this system, the protein is cationized with a diamine, then lyophilized

T_m: melting temperature

from a solution containing an anionic PEG surfactant. The resulting complex melts after annealing at 50°C and remains a liquid upon cooling to room temperature (cooling to −50°C caused resolidification). For example, Perriman et al. (52) prepared an ionic melt of myoglobin and demonstrated reversible binding of oxygen and carbon monoxide similar to that of the native protein. CD and UV-Vis spectroscopy indicated that the structure of the protein was not significantly perturbed in the protein melt. This system represents an extension of general enzyme-polymer encapsulation techniques in which the surface of the enzyme is modified to accept a functionalized polymer. This approach is often used to protect and stabilize enzymes in aqueous solutions or against organic solvents (53, 54).

STRUCTURAL PROPERTIES OF EXTREMOPHILIC PROTEINS

Structural differences between proteins from extremophiles and those from mesophiles are often subtle. This is not surprising given that the same amino acids and intramolecular forces are used in constructing proteins no matter in what environment an organism lives. A precise balance of hydrophobic, hydrostatic, and covalent interactions must be maintained for an enzyme to remain active under extreme conditions. In addition to maintaining stability, an enzyme must retain its active conformation, remain flexible enough to bind substrates and cofactors, and carry out its designated function. Hence, even though an enzyme may survive exposure to harsh conditions, it may exhibit little or no activity under those conditions. This distinction is important in industrial applications in which the operating conditions may vary; the conditions must enable the enzyme to remain active enough to meet the process requirements but not exceed the upper limit of the enzyme's stability. Here, we briefly describe how molecular interactions differ between extremophilic and mesophilic enzymes, and in some cases point out when stability and activity are enhanced or hindered. A more thorough discussion of biophysical characteristics is beyond the scope of this review and has been presented elsewhere (55–59).

Hydrophobic Interactions

Enzymes from thermophiles often have an increased number of hydrophobic residues, which results in a tighter packing of the core and reduced overall size of the protein (57). In an analysis comparing extremophilic and mesophilic proteins that were homologous and had solved crystal structures, 26 out of 35 thermophilic/hyperthermophilic enzymes had an increase in hydrophobic interactions at the oligomer-subunit interface in comparison with their mesophilic counterparts (60). In addition, the amino acid composition at the interface had more Phe and Ile residues and decreased numbers of Cys, Thr, and Asn (60, 61). This increase in hydrophobic interactions at the interface is thought to contribute to stability by tightening the entire quaternary structure (62).

Substitution of nonpolar amino acids for polar residues can contribute to increased hydrophobicity. Compared with mesophiles, thermophiles tend to decrease the amount of uncharged polar residues encoded in their genomes and to favor nonpolar amino acids (57, 61, 63–65). Reduced usage of uncharged polar residues prevents the deamidation of Asn and Gln by Ser and Thr that can occur at high temperatures (57, 61, 65, 66). In a genome comparison of 12 thermophiles and 6 mesophiles, the amounts of Val and Ile were statistically increased in thermophiles (66). In addition, a modeling study of the whole monomeric proteome from several thermophiles corroborated these observations, as it showed a positive correlation between the number of hydrophobic residues and the growth temperature of the organism from which the proteome was derived (67).

Increasing the number of aromatic side chains can also enhance stability in thermophilic enzymes through stacking interactions and by increasing the hydrophobicity of the core

(57, 61, 68). Mutational analysis of the aromatic cluster within the isocitrate dehydrogenase from *Thermotoga maritima* revealed a decrease in stability, underscoring the importance of aromatic residues in maintaining stability (69). This trend may have similar stabilizing effects on psychrophilic enzymes, as demonstrated by a large aromatic cluster within the isocitrate dehydrogenase from *Desulfotalea psychrophila* (68). However, mutational analysis of this enzyme and other psychrophiles is needed to confirm this trend.

In contrast to thermophilic enzymes, psychrophilic and alkaliphilic enzymes tend to have increased numbers of polar residues and reduces numbers of hydrophobic residues, which leads to an increase in hydrophilicity (57, 63, 70). For example, psychrophilic serine hydroxymethyltransferases contain more polar residues in the core, which prohibits the formation of stabilizing secondary structures and increases the backbone flexibility (71). In addition, the surface of this protein is more negatively charged, which allows for the development of stabilizing solvent interactions (71). In general, a decrease in hydrophobic residues in psychrophilic enzymes allows for more protein movement in cold environments that tend to rigidify proteins.

Electrostatic Interactions

Ion pairs or salt bridges are electrostatic interactions between positively and negatively charged side chains and are an important stabilizing factor for many extremophilic proteins. The primary sequences of thermophilic and psychrophilic proteins tend to have an increased number of charged residues that can be involved in ion pairs (55, 57). Hyperthermophilic proteins have increased numbers of Lys and Glu residues and tend to increase the number of Arg and Glu residues at subunit interfaces (60, 67). Several thermophilic proteins have increased numbers of positively charged residues not only in their interior but also on their solvent-exposed surface (61, 67). A charged surface has the ability to interact with the solvent, usually water, and provides stability on the surface, whereas charged residues internally can contribute to ion pairs and hydrogen bonding and stabilize the protein as a whole (61, 67).

Hydrogen bonds also can affect the stability of an enzyme under a given condition. An increased number of hydrogen bonds can enhance thermostability and has been shown to contribute to alkaline adaptation (70, 72–78). Inter- and intramolecular hydrogen bond networks can contribute to the rigidity of a protein and are critical for proper secondary structure formation (74–76). Maintenance of rigidity is especially important at high temperatures, at which protein motion can occur more freely and can promote denaturation and unfolding. Charged-neutral hydrogen bonds may be more favorable than ion pairs to stabilize thermophilic proteins owing to a lower desolvation penalty upon burial within the protein (79). A well-formed hydrogen bond network can result in a tightly bound, smaller protein that should be more stable at higher temperatures. Conversely, the number of hydrogen bonds generally decreases in psychrophilic enzymes, potentially owing to the need to maintain flexibility in a cold environment (80, 81). An exception to this trend was revealed by a study conducted on cold-adapted metalloproteases, which found that the psychrophilic enzymes had as many hydrogen bonds as their mesophilic counterparts and that the persistence of hydrogen bonds within the dynamic structure of the psychrophilic proteins contributed greatly to their flexibility (82). In this case, the authors suggested that hydrogen bonding within psychrophilic metalloproteases has been optimized to allow for flexibility.

Covalent Interactions

Disulfide bond formation is another important determinant of protein stability. Disulfide bonds increase the kinetic stability of a protein by increasing the activation energy barrier of

k_{cat}: turnover number or catalytic constant

unfolding (83). Disulfide bonds can also enhance stability by decreasing the entropy of the unfolded state (84). Previously it was believed that the reducing environment within the cytosol inhibited disulfide bond formation and that extremophilic proteins use few cysteine residues because they may be prone to oxidative degradation in the harsh environments where extremophiles thrive (83, 85). However, two recent reviews indicate that thermophiles use disulfide bonds to stabilize their proteins (83, 85). In 9 of the 25 archaeal and bacterial hyperthermophilic genomes analyzed, more than 10% of the intracellular cysteine residues were predicted to be involved in a disulfide bond (85).

A study of proteins from the hyperthermophile *Pyrobaculum aerophilum* indicated that disulfide bonds were widespread in whole-cell lysates, and 2D gel electrophoresis identified 16 different proteins containing disulfide bonds (86). The citrate synthase from *P. aerophilum* contains intramolecular disulfide bonds between cysteine residues on the same subunit, which contribute to protein catenation and render the protein chains inseparable even under denaturing conditions. Incubation of the enzyme *S*-adenosylhomocysteine hydrolase from the hyperthermophile *P. furiosus* at 80°C in 0.8 M dithiothreitol for 1 h resulted in a 63% loss of activity, which indicates that the Cys residues within this enzyme may be involved in disulfide linkages (87). Similar results were found in a study of purine-specific ribonucleoside hydrolase from the thermoacidophile *Sulfolobus solfataricus*, further proof that disulfide bonds may be an important structural mechanism for enzyme stability at high temperatures (88, 89). Interestingly, denaturation of the disulfide bonds of the psychrophilic α-amylase from *Pseudoaltermonas haloplanktis* by β-mercaptoethanol reduced activity more than stability (84).

ENZYMES THAT FUNCTION UNDER EXTREME CONDITIONS

A vast diversity of enzymes has been found in extremophiles, and many different enzymes have been the subjects of protein engineering. For the purposes of this review, we focus on a relatively small subset of representative enzymes of long-standing interest to industry: proteases, carboxylic acid hydrolases (lipases), and cellulases. **Table 2** provides a list of the enzymes discussed along with a description of their key features. Notably absent from this list are any enzymes that require coenzymes [e.g., NAD(H) or ATP], which impose their own limitations in addition to those of the enzyme.

Proteases

Proteases that are active at high temperature and in alkaline conditions are often used for peptide degradation and simple acid/base catalysis. The largest industrial use of alkaliphilic proteases is in laundry detergents as additives to help break down protein stains. In addition, proteases can be used to construct peptides in nonnatural conditions, such as in organic solvents. Because proteases have evolved specifically to bind peptides, they can also be used as catalysts for targeted modification of biomolecules.

Several proteases have been identified that are tolerant to high temperatures and have folds similar to those of the subtilisin family. Three natural subtilisin-like hyperthermophilic proteases, Tk-0076, Tk-1689 (also referred to as Tk-SP), and Tk-subtilisin, from the archaeon *Thermococcus kodakaraensis* have been identified, partially characterized, and recombinantly expressed. Both Tk-1689 and Tk-subtilisin are stable in the presence of surfactants (5–10%) and chaotropic salts, and they have T_{opt} values of 80 and 90°C, respectively (90). These proteases have comparable activity to mesophilic subtilisins, such as subtilisin E, at room temperature, but have ~30-fold higher catalytic constant, also known as a turnover number, (k_{cat}) values at 80°C. Both of these

Table 2 Key properties of enzymes discussed in the section Enzymes that Function under Extreme Conditions

Class	Enzyme	Source organism(s)	N/E[a]	Key properties or features	References
Proteases	Tk-1689	*Thermococcus kodakarensis*	N	T_{opt} 100°C, no bound Ca^{2+} ions	(90)
	Tk-subtilisin	*T. kodakarensis*	N	T_{opt} 90°C, seven bound Ca^{2+} ions	(90)
	Subtilisin E	*Bacillus subtilis*	E	Activity increased 470-fold in 60% dimethylformamide	(95–97)
			E	Functionally converted to Thermitase	(94)
	B-17N-1	*Bacillus* sp. *17N-1*	N	Remains active up to 70% acetonitrile and dimethylsulfoxide	(98)
	Subtilisin BPN′	*Bacillus amyloliquefaciens*	N	Active in neat organic solvents	(26)
	α-Chymotrypsin	*Bos taurus*	N	Active in neat organic solvents	(42, 101)
	Cubtilisin Carlsberg	*Bacillus licheniformis*	N	Active in neat organic solvents	(99)
	Protease	*B. licheniformis RSP-0937*	N	High transesterification activity in neat organic solvents	(100)
	PST-01	*Pseudomonas aeruginosa*	N	Isolated from organic solvent–tolerant host	(103)
			E	Increased k_{cat} tenfold	(105)
	PT-121	*P. aeruginosa*	N	Isolated from organic solvent–tolerant host, higher peptide synthesis activity	(108)
Esterases	Est	*Pyrobaculum calidifontis VA1*	N	T_{opt} 90°C, stable to >80% organic solvents	(113)
	AFEST	*Alicyclobacillus acidocaldarius*	N	T_{opt} 80°C	(114)
	Esterase	*Sulfolobus solfataricus P1*	N	T_{opt} 85°C	(115)
	Bovine serum albumin	*B. taurus*	N	Exhibits esterase activity up to 150°C	(116)
	Lipase B (CALB)	*Candida antarctica*	E	Increased half-life 27-fold at 70°C	(117)
			N	More stable to ILs than organic solvents	(128, 129)
	Lipase A	*B. subtilis*	E	Increased half-life >100-fold in 50% organic solvents	(124)
			E	Increased half-life ~450-fold at 55°C	(118)
			E	Increased half-life 270-fold at 55°C	(119)
	ρ-Nitrobenzyl esterase′	*B. subtilis*	E	Increased T_m 14°C and T_{opt} 10°C	(120)
	Lipase LST-03	*P. aeruginosa*	E	Increased half-life ~tenfold in 25% organic solvents	(121)
	Lipase (PCL)	*Pseudomonas cepacia*	N	See Table 4	(127)

(*Continued*)

Table 2 (*Continued*)

Class	Enzyme	Source organism(s)	N/E[a]	Key properties or features	References
Cellulases	11113132	*Humicola insolens*, *Chaetomium thermophilum*, *Hypocrea jecorina*[b]	E	Increased half-life >30-fold at 65°C	(136, 137)
	PA68	*Reticulitermes speratus*, *Nasutitermes takasagoensis*, *Coptotermes formosanus*, *Coptotermes acinaciformis*[b]	E	Increased T_{opt} 10°C	(138)
	WT–ChBD2	*Pyrococcus horikoshii*, *Pyrococcus furiosus*	E	Increased activity ~twofold at 50°C	(139)
	Cellulase mixture	*Trichoderma reesei*	N	Most common commercially available cellulases	(145,146)
	EBI-244	*Ignisphaera*-like species[c]	N	T_{opt} 109°C, T_m 113°C, most thermophilic cellulase reported	(147)
	CelA10	*Cellvibrio japonicus*[d]	N	Active up to 30% IL	(148)
			E	Activity increased ~threefold in 30% IL	(148)
	Cellulase	*Thermotoga maritima*	N	Retains 40% activity in 20% IL	(149)

[a]N, natural enzyme; E, engineered enzyme.
[b]The final enzyme was shuffled from these sources.
[c]16s rRNA accession number JF509453.
[d]83% similarity with cel5B sequence.

hyperthermophilic subtilisins are distinct from bacterial subtilisins, which typically bind one or two Ca^{2+} ions (91). Tk-subtilisin binds seven Ca^{2+} ions in the mature catalytic domain, and Tk-1689 has no calcium binding sites in the catalytic domain (but contains a separate β-jelly roll domain that binds two Ca^{2+} ions) (92, 93).

Mesophilic subtilisin E has been used as a scaffold for engineering protein function under extreme conditions. Rational design was used to make eight site-directed mutations to functionally convert subtilisin E into the more thermophilic subtilisin known as Thermitase (94). In addition to engineering for high-temperature activity, subtilisin E has been engineered to function in DMF (95–97). Several rounds of mutagenesis and screening produced a 12-mutation variant that was 470-fold more active than the wild type in 60% DMF (85). **Figure 3*a*** shows that the activities of wild-type and engineered subtilisin E decrease monotonically as the amount of solvent increases. The molecular basis for the benefit of the random mutations is currently under investigation (M.J. Liszka, personal communication). In contrast to subtilisin E, a halo-alkaliphilic protease isolated from *Bacillus* sp. 17N-1, protease-B-17N-1, remains active in 90% dimethylsulfoxide and 80% acetonitrile (98). **Figure 3*b*** shows the change in k_{cat}/K_M values for the protease-B-17N-1 as the amount of acetonitrile increases.

In neat organic solvents, many proteases catalyze transesterification reactions in place of hydrolysis. For example, the commercially available proteases subtilisin BPN′ (99), α-chymotrypsin (42), and subtilisin Carlsberg (26) catalyze the transesterification of *N*-acetyl-L-phenylalanine

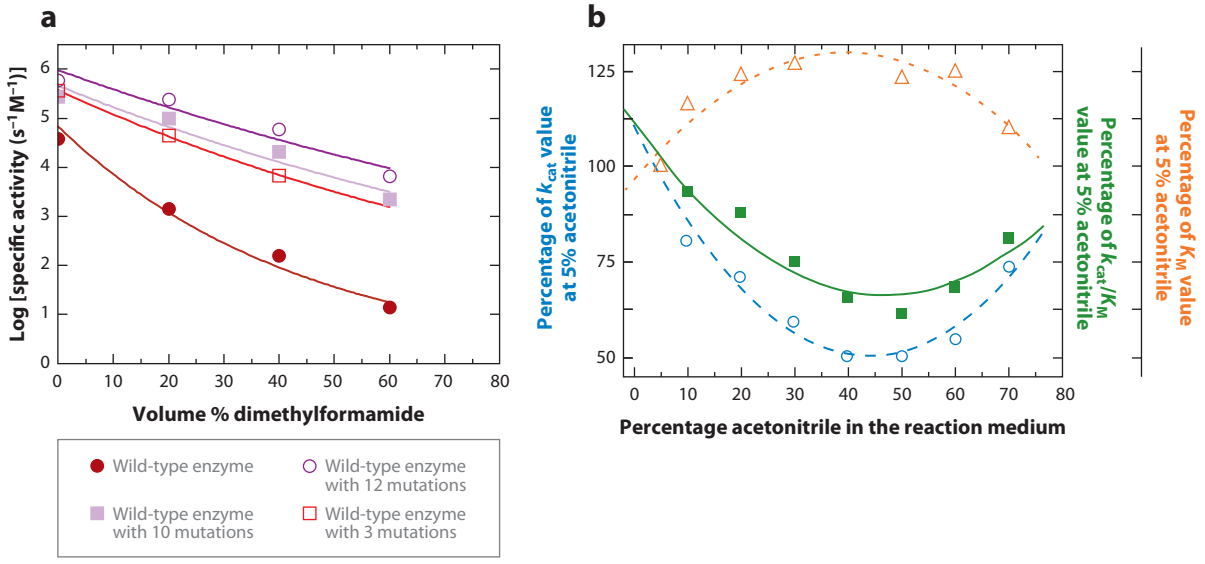


Figure 3

Activity trends of engineered and natural extreme proteases. (*a*) Engineered subtilisin E retains activity in the presence of organic solvents better than does the wild-type enzyme. (*b*) Protease B-17N-1 retains activity in solutions of greater than 70% acetonitrile. Adapted with permission from Reference 98, Taylor & Francis Group, http://www.informaworld.com.

ethyl ester (APEE) with 1-propanol in organic solvents. **Table 3** shows the activity for the APEE reaction of these commercially available mesophilic enzymes in different organic solvent preparations. The most active preparation of the mesophilic enzymes is a soluble preparation in which enzyme monomers are ion paired and extracted into an organic phase with a small amount of surfactant (42). In comparison with commercially available, mesophilic enzymes, the protease from the thermophilic bacterium *Bacillus licheniformis* RSP-0937 catalyzes the same reaction in nonpolar organic solvents at least 100-fold faster (100). The APEE transesterification reaction exhibits similar initial velocities throughout the range of water activities in both organic solvents and ILs (101). Protein engineering has yet to be applied in these systems owing to the difficulty of screening mutant libraries.

Table 3 Protease activities in organic solvents for transesterification of *N*-acetyl-L-phenylalanine ethyl ester and 1-propanol

Enzyme	Preparation	Solvent	k_{cat}/K_M (M^{-1} s^{-1})	Reference
Bacillus licheniformis RSP-09-37 protease	Suspended	Isooctane	230,000	(100)
Subtilisin BPN′	Solubilized	Octane	370	(99)
	Suspended	Octane	0.6	(99)
Chymotrypsin	Solubilized	Isooctane	3,020	(42)
	Suspended	Isooctane	1.26	(42)
Subtilisin Carlsberg	Solubilized	Isooctane	230–1,990	(26)
	Suspended	Isooctane	0.092	(25)

Proteases can also be used in a variety of aqueous/organic solvent mixtures for peptide synthesis. The type of protease used as well as the type and concentration of (co)solvent have the largest effect on activity and conversion in these systems. Proteases and other enzymes with increased tolerance to organic solvents have been identified from organisms that live in organic solvent–contaminated environments (102). The protease PST-01, from *Pseudomonas aeruginosa*, was one of the first reported solvent-tolerant proteases and can be overproduced from the native host to ~80 mg/liter of culture (103). The wild-type protease is stable to many polar organic solvents, but its peptide synthesis rate is low (104). However, the activity (k_{cat}) was improved tenfold by a single amino acid mutation of Y114S, made by comparison with a homologous thermophilic metalloprotease, Thermolysin (105). Interestingly, PST-01 was 50-fold faster in the synthesis of Cbz-Arg-Leu-NH_2 than Cbz-Asp-Phe-OMe (an aspartame precursor), which indicates that the substrate amino acid composition also significantly affects enzyme activity. In addition, protease PST-01 has been shown to retain activity longer in organic solvents than several other proteases, but kinetic parameters in the presence of organic solvents have not been reported (103, 106, 107). The protease from another *P. aeruginosa* strain, PT-121, exhibits similar stability as PST-01, with an aspartame precursor synthesis rate 4,500-fold faster than the best PST-01 mutant (108).

Carboxylic Ester Hydrolases

Lipases, or more generally carboxylic ester hydrolases, are among the most thoroughly studied and industrially important enzymes (109). They can perform nearly complete enantiomeric resolution of substrates containing hydroxyls, esters, carboxylic acids, and amines. In addition, lipases can transesterify triglycerides in a potentially less toxic process as compared with the use of strong base catalysts. Lipases used in pure (solvent-free) oils can perform transesterifications for biofuel production, which has been recently reviewed (110, 111).

Thermo- and hyperthermophilic ester hydrolases have been identified from various microorganisms. Several have been thoroughly characterized against canonical esterase substrates. The properties of hyperthermophilic carboxylic ester hydrolases have been reviewed recently (112). Three of the hyperthermophilic esterases described have similarly characterized kinetics and high esterase activity at high temperature (T_{opt} of 80–90°C) (113–115). These same three esterases were successfully cloned into *E. coli* for recombinant expression, achieving yields ranging from 0.6 to 17 mg/liter of culture.

Bovine serum albumin (BSA), although not a formal ester hydrolase, exhibits esterase activity above 60°C and up to 160°C (116). For activity at 150°C, 25-mM sodium dodecyl sulfate (SDS) was required to prevent aggregation, which indicates that the active species may be partially denatured or unstructured. BSA exhibited esterase activity but not lipase activity, as the enzyme did not hydrolyze triglycerides.

An additional esterase from *Pyrobaculum calidifontis* VA1 shows high stability to organic solvents, but its activity is severely reduced (47–99% lower) in the presence of misicible organic solvents, as shown in **Table 4** (113). This enzyme illustrates the point made earlier with regard to enzyme activity and stability under extreme conditions. Although an enzyme may be stable to high temperature or solvent incubation, the enzyme may not be active under the same conditions. This distinction is important in cases in which the enzyme is required to function, as opposed to only withstand its exposure, in the extreme environment.

In addition, the thermostability of mesophilic esterases has been increased using a variety of mutagenesis techniques. Two point mutations to *Candida antarctica* lipase B (CALB), found through directed evolution, increased the half-life at 70°C from 8 min to ~220 min (117). These two mutations also increased the activity at room temperature, but the effects on activity at higher

Table 4 Stability toward inactivation and relative activity in organic solvents of the hyperthermophilic esterase from *Pyrobaculum calidifontis*[a]

Organic solvent	% Residual activity[b]	% Aqueous activity[c]
Methanol	89	14
Ethanol	103	2.5
Isopropanol	94	1.0
Acetonitrile	116	2.9
Dimethyl sulfoxide	90	53
Dimethyl formamide	96	7.3

[a]Table adapted from Reference 113.
[b]As compared with aqueous incubation, after 1-h incubation in 80% solvent at 30°C.
[c]In 50% solvent.

temperatures were not reported (117). Several rounds of saturation mutagenesis of lipase A from *Bacillus subtilis*, based on crystal structure B-factors, increased the half-life at 55°C from <2 min to >900 min (118). In complementary work, error-prone PCR and site-directed mutagenesis of lipase A were used in combination to increase its stability at 55°C 270-fold while maintaining wild-type catalytic activity (119). In another example of engineering, the T_m of the ρ-nitrobenzyl esterase from *B. subtilis* was increased 14°C and the T_{opt} increased by 10°C using error-prone PCR and DNA shuffling (120). Although the specific activity of the enzyme initially decreased with an increase in the T_{opt}, further rounds of mutagenesis increased both the activity and the T_{opt}. Error-prone PCR, site-directed mutagenesis, and DNA shuffling are all highly useful protein engineering techniques that have led to an improvement in the function of esterases at high temperatures.

As a comparison, the reported activities (k_{cat}) of each of the esterases described above are shown in **Figure 4**. The activity (**Figure 4*a***) and stability (**Figure 4*b***) of each enzyme are plotted at the reported temperature. An enzyme activity for laboratory-scale organic synthesis of 0.1 mol of product would require a minimum k_{cat} value of approximately 1 s^{-1} (depending on the K_M value and the molecular mass of the enzyme). This value is also represented by the horizontal line in **Figure 4*a***. As seen in the figure, both engineered and natural enzymes cover a large range of activity and stability even at high temperatures. **Figure 4*c*** shows both the activity and the stability for each of the enzymes. For these thermophilic esterases, natural enzymes appear to exhibit higher stability and/or activity than engineered enzymes.

Native enzymes from organic solvent–tolerant microorganisms may have already evolved through natural selection for increased stability to solvents. For example, lipase LST-03, isolated from the solvent tolerant bacterium *P. aeruginosa*, is stable in the presence of 25% organic solvents at 30°C. Directed evolution was used to further stabilize the lipase three- to tenfold (121). Lipase LST-03 was successfully cloned into *E. coli* for recombinant expression; however, the stability and activity decreased above 40°C even without organic solvents (122). Stability to higher concentrations of organic solvents appears to be low, as activity assays for lipase LST-03 are terminated by addition of 40–66% (v/v) acetone, which causes precipitation of the enzyme (122, 123). In contrast, lipase A (whose thermostability was increased by B-factor saturation mutagenesis) also exhibited increased stability to organic solvents (124).

Natural and recombinant esterases and lipases are used in organic solvents to resolve chiral alcohols and carboxylic acids for organic synthesis. The (trans)esterification of chiral alcohols, carboxylic acids, and esters in anhydrous organic solvents has been reported for several decades (125). Highly selective resolution can be achieved by either achiral synthesis or dynamic kinetic resolution (126). In organic solvents, esterases can interact with previously unfavorable substrates,

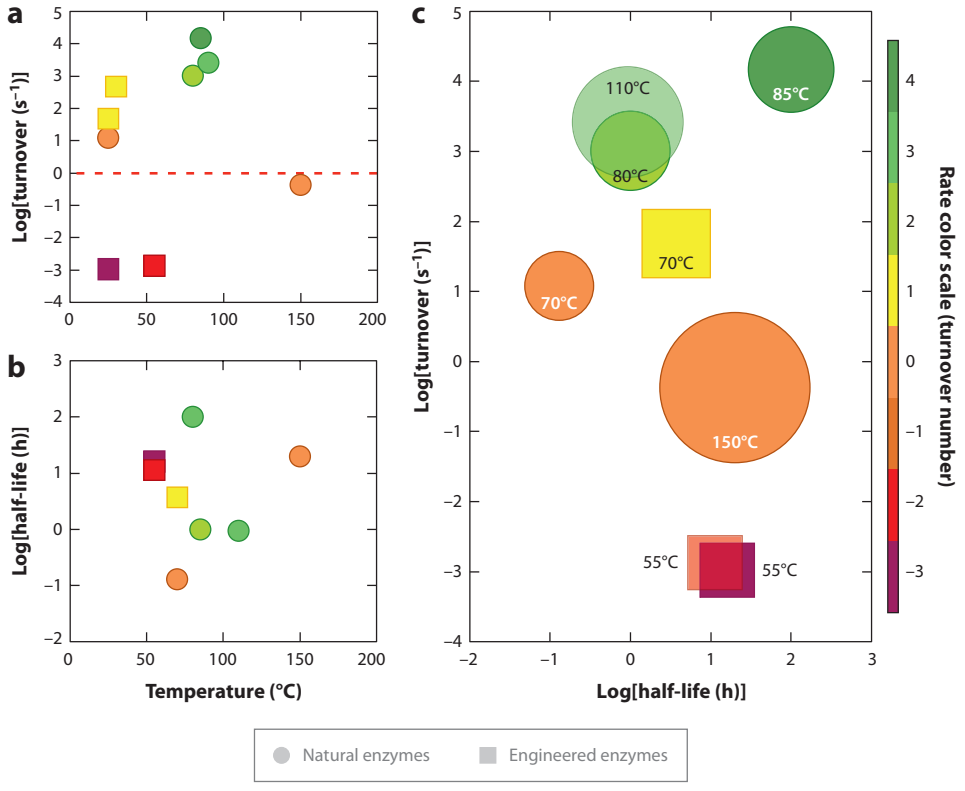


Figure 4

Activity and stability of natural and engineered thermophilic esterases. (*a*) Log of activity versus temperature. (*b*) Log of stability versus temperature. (*c*) Log of activity versus log of stability; size of symbol is scaled by temperature in degrees centigrade.

which opens the possibility of new reactivities. For example, the activity of *Pseudomonas cepacia* lipase correlates well with the polarity of both organic solvents and ILs (127) (see **Figure 5**).

Lipases function similarly in ILs as in organic solvents, but with an observed general increase in stability. For example, free or immobilized CALB is slightly more active and stable in imidazolium-based ILs compared with organic solvents (128, 129). Interestingly, a lipase lyophilized from a room-temperature IL solution exhibited increased activity and stability in organic solvents compared with suspended or salt-activated organic preparations. But, similar to organic solvents, specific interactions of the solvent with the enzyme ultimately dominate the stability and activity of the enzyme (130). The unique solubilization properties of ILs used in conjunction with organic solvents can increase the reaction yield of polar sugars and nonpolar fatty acid esters (131).

Cellulases

Research in biofuels production has recently refocused on using cellulases for the enzymatic hydrolysis of lignocellulosic plant materials. One of the main challenges with using these enzymes is low activity against solid substrates at lower temperatures. Cellulase activity is highly dependent on the physical and chemical properties of the substrate; therefore, caution must be used when making

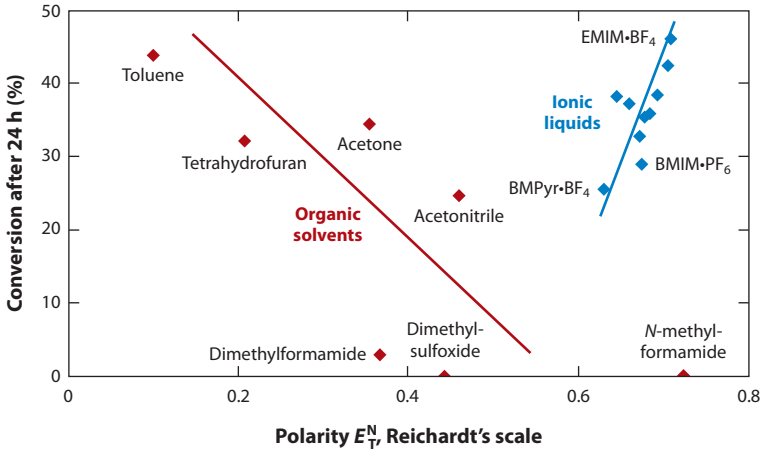


Figure 5

Pseudomonas cepacia lipase activity as a function of polarity for both organic solvents and ionic liquids (ILs). In organic solvents, enzyme activity decreases with increasing polarity, whereas in ILs enzyme activity increases with increasing polarity. Figure adapted with permission from Reference 127. Copyright © 2001 American Chemical Society.

comparisons between reported activities. Increasing the specific activity of cellulases is essential to reduce the time and amount of enzyme required to convert lignocellulose into fermentable sugars, and in turn to drive down costs and make biofuel production more economically viable (132).

The desire to find or engineer cellulases suitable for high temperatures stems from the reduced risk of contamination and the possibility of faster reaction rates afforded by higher process temperatures. As cellulose is degraded and glucose is produced, the risk of contamination by environmental microbes increases. Performing the reaction at a temperature of approximately 65°C would greatly reduce or eliminate the growth of common environmental contaminants. Thermophilic cellulases have been isolated from archaea, bacteria, and even fungi; however, there is evidence that the preferred substrates for extremophilic cellulases may be different from industrially relevant substrates (133, 134). For example, a thermophilic fungal endoglucanase from *Talaromyces emersonii* shows almost no activity on microcrystalline cellulose (Avicel) or carboxymethyl cellulose (CMC) (135).

Several protein engineering methods have been applied to increase the thermostability and thermoactivity of mesophilic cellulases in anticipation of higher process temperatures for biofuel production. For example, three fungal cellulases were shuffled using SCHEMA, a method of homologous protein shuffling, to result in a 33-fold increase in the half-life at 63°C while maintaining activity within the range of the parent enzymes (136). In further work, Arnold and coworkers (137) identified a single point mutation responsible for most of the increased thermostability and thus were able to increase both T_{opt} and the specific activity relative to the parent enzymes. Family shuffling of four termite cellulase genes increased the T_{opt} from 45 to 55°C but did not improve activity on crystalline cellulose (138). Taking another engineering approach, Kang et al. (139) fused a chitin-binding domain to a hyperthermophilic endoglucanase from *Pyrococcus horikoshii*, which doubled its activity at 85°C toward Avicel. Guidelines for engineering cellulases for specific conditions also can be developed from metagenomic analyses of biomass-degrading organisms that populate unique environments (140).

Performing cellulose hydrolysis in the presence of ILs would exploit the unique ability of ILs to dissolve lignocellulose and could also serve to inhibit growth of microorganisms in a manner similar to that of organic solvents (141). The solubility of glucose in ILs is similar to that in traditional organic solvents [with the exception of high solubility with dicyanamine anions (142)], but the advantage of ILs lies in the efficient disruption and dissolution of crystalline biomass. Pretreatment of cellulose with ILs has also been shown to increase cellulase binding to cellulose (143) and greatly enhance the rate of conversion to glucose (144). However, the hydrolytic activity and stability of *Trichoderma reesei* cellulases decrease with increasing IL concentration (145, 146), and a practical process for biomass degradation in the presence of ILs has yet to be developed.

Bioprospecting has recently uncovered cellulases with promising activity and stability in ILs, and protein engineering has been used to increase the activity of cellulases in the presence of ILs. Bioprospecting in thermal pools led to the discovery of the most hyperthermophilic cellulase to date from a previously uncharacterized archaeal species. The isolated cellulase, designated EBI-244, has a T_{opt} of 109°C and a T_m of 113°C (147). Interestingly, EBI-244 exhibits increased activity in 50% 1,3 dimethylimidazolium dimethyl phosphate ([MMIM][DMP]) below 74°C. The structure of EBI-244 has not been determined, but the catalytic domain displays homology to the glycosyl hydrolase family 5 (GH5). Two independent studies of cellulases annotated as members of the GH5 family have also shown resistance to inactivation in ILs. Pottkamper et al. (148) used metagenomic libraries created from temperate soil and aquatic samples to screen for cellulase activity in the presence of several ILs. The most active variant showed high similarity (83%) and identity (74%) with an endoglucanase from *Cellvibrio japonicas*. In another study, two cellulases from hyperthermophiles ($T_{opt} > 80$°C) were cloned and screened for activity in the presence of ILs (149). The most active enzyme retained 40% of its aqueous activity in 20% v/v IL; a mesophilic control enzyme lost all activity in 10% v/v IL. However, the specific activity of the best enzyme identified in this metagenomic study is approximately 100-fold lower than the activity of the hyperthermophilic cellulase from *T. maritima* even after one round of mutagenesis. **Figure 6** shows a structural comparison of the loop regions of *T. maritima* cellulase with a homologous mesophilic cellulase.

CONCLUSIONS AND FUTURE PROSPECTS

Clearly, both bioprospecting and protein engineering are useful sources of extremophilic enzymes, and the two strategies are complementary in many respects. Advocating one method over the other depends on whether the primary interest lies in understanding the design rules of nature or in finding the most expedient solution to the problem at hand. Nature has already solved the problem of function in extreme conditions for a host of natural environments relevant to industrial applications; however, the effort required to find an organism that has the right enzyme for the job can be much greater than the effort required to engineer the wrong enzyme into the right enzyme. Important considerations include whether a suitable host/enzyme combination is readily available, whether an adequate functional screen is at hand, and whether nature already supports life under the extreme conditions of the industrial process.

Much current research is focused on bridging the knowledge gap between how naturally evolved enzymes function under extreme conditions and how protein engineering can be used to transform mesophilic enzymes into more robust analogs. These efforts will greatly benefit from ongoing research directed toward a better understanding of the requirements for consistent and high-level expression of recombinant proteins. As our understanding of protein function in extreme environments improves, so will our ability to engineer proteins that can perform increasingly complex

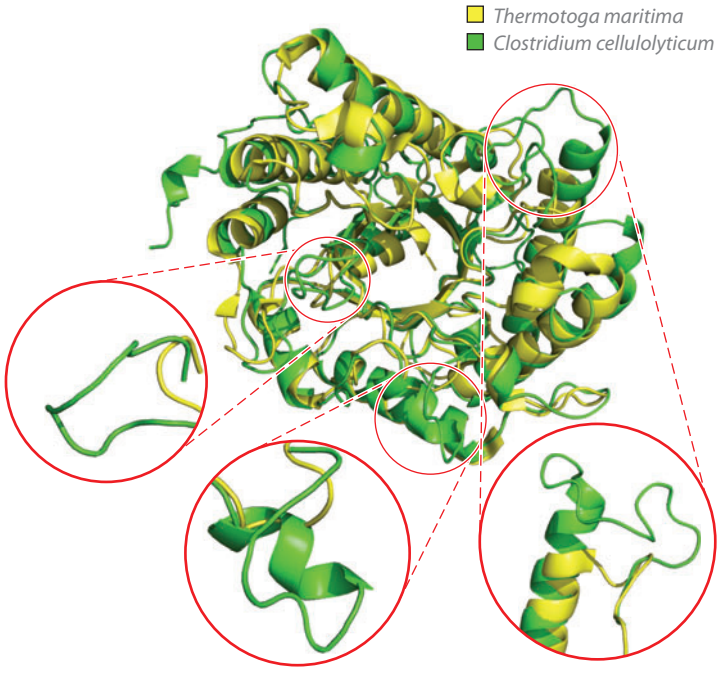


Figure 6

Comparison of glycosyl hydrolase family 5 cellulases from *Thermotoga maritima* (*yellow*) and *Clostridium cellulolyticum* (*green*). The crystal structures show smaller loop regions connecting secondary structural elements in the thermophilic cellulase, as has been observed more generally for thermophilic proteins (155, 156). These smaller loop regions may lead to fewer destabilizing interactions with ionic liquids (or high-temperature water) and a higher stability in mixed solvents. Insets show specific loop comparisons.

reactions under increasingly challenging conditions. In the meantime, laboratory evolution methods continue to improve and provide a practical, albeit empirical, bridge for the knowledge gap.

Looking forward, another exciting development in the engineering of enzymes for extreme conditions is the use of computational design methods to invent enzymes with completely new structures and functions. Advances in computational methods and computing power over the past few decades have begun to make this possible. In the past decade, protein structure and enzyme function not seen in nature have been designed de novo and expressed in bacterial hosts (150–152). Short of designing whole proteins, computer modeling of active-site structures and of small-molecule or macromolecular interactions will also improve our understanding of how extreme conditions affect enzyme catalysis (153, 154). These smaller design problems can contribute piecewise to the development of larger computational models of extremophilic enzymes. The combination of bioprospecting, protein engineering, and de novo design is creating unprecedented opportunities for utilizing enzymes in extreme environments of both natural and unnatural origin and is advancing the field along a path of development in which designing proteins for stability and activity under seemingly impossible conditions may someday become a reality.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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