

Corner Engineering: A Directed Evolution Strategy for Enhancing Enzyme Resistance in Deep Eutectic Solvents

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Abstract:

As a green and cost-effective solvent, deep eutectic solvent (DES) shows promise in biocatalysis. However, enzyme activity in DESs is often inhibited, limiting their application. To address this, our project proposed a directed evolution strategy called Corner Engineering. Using *Bacillus subtilis* lipase A (BSLA) as a model, we thoroughly validated the effectiveness of the strategy and optimized it. Notably, the variant M137D/N138D displayed an impressive 3.0-fold increase in resistance compared to the WT in 95% (v/v) ChCl:ethylene glycol. To confirm the versatility and effectiveness of our optimized approach, we conducted validation experiments on *Bacillus subtilis* esterase (Bs2Est). In the 75% (v/v) ChCl:ethylene glycol, the resistance of these variants could reach up to 3.1-fold, thus affirming the broad applicability of our engineered enzyme strategy. The molecular investigations reveal that increased water molecules at substrate binding sites are the dominant determinant of elevated resistance, indicating a promising avenue for understanding enzyme-DES interactions.

Key words:

directed evolution, deep eutectic solvents, biocatalysis, corner engineering, molecular dynamics

Introduction:

Biocatalysis is a green and sustainable technology as far as the indicators and principles of green chemistry are concerned ¹. Some non-traditional reaction media, such as organic solvents (OSs) ², ionic liquids (ILs) ³, and supercritical fluids ⁴, have been extensively evaluated and utilized in biocatalytic reactions. In 2003, Abbott and colleagues first described deep eutectic solvents (DESs) ⁵ and in 2008, the subsequent demonstration of enzymatic reactions in these solvents by Kazlauskas and coworkers ⁶. DESs typically result from the combination of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) through concurrent heating and stirring until a liquid phase is achieved. Unlike conventional solvents, DESs exhibit low vapor pressure, are non-flammable, and frequently exhibit stability at elevated temperatures ⁷.

Indeed, the activity of enzymes in DESs can be limited, which significantly affects the efficiency of biocatalysis ⁸. Directed evolution and (semi-)rational design have frequently been employed to modify enzyme properties in unconventional environments ⁹. For instance, cellulases have been engineered for improved performance in ethanol ^{10,11}, lipases have been optimized for activity in 1,4-dioxane ¹², and laccases have been tailored for functionality in [C₂C₁Im][OAc] ¹³. However, to the best of our knowledge, very few studies reported successful efforts in tailoring enzyme stability in DESs. How to enhance the stability of enzymes in DESs remains a critical challenge. This limitation hampered the widespread adoption of DESs. Therefore, there is a pressing need for a simple and versatile strategy to achieve robust enzymes in DESs.

Charge engineering/modification was shown to be a promising approach to tailor the enzyme tolerance in ILs ^{14,15} and OSs ^{16,17}. What's more, the biological activity of enzymes is closely related to the secondary structure of proteins ¹⁸. The (semi-)rational design approach to tailor hotspot at a specific region, thereby altering the relevant protein's structure and even catalytic properties, presents a compelling avenue for reshaping the enzyme stability in DES. Herein, we developed Corner Engineering. In detail, charged amino acids were strategically introduced at two or three adjacent positions using carefully designed degenerate codons to achieve the efficient acquisition of beneficial variants exhibiting enhanced properties. Firstly, *Bacillus*

subtilis lipase A (BSLA) a well-studied enzyme was selected as the model to fully explore Corner Engineering. After screening five types of corner regions (Coil-Helix, Turn-Helix, Coil-Sheet, Helix-Sheet, Turn-Sheet) in BSLA, several beneficial substitutions were obtained. By analyzing the data gained from BSLA, we optimized the strategy and applied it to improve the resistance of *Bacillus subtilis* BHETase (Bs2Est) with reduced laborious work. Besides, we investigate the DES resistance profiles for the most improved variants. Molecular dynamics (MD) was utilized to explore the mechanisms behind the increased enzyme resistance in DESs. This contributed to a deeper insight into the enzyme's behavior and properties in unconventional solvents.

Results:

The DESs high-throughput screening system was established

A 96-well plate-based enzymatic activity assay method has been established, and the robustness and feasibility of this high-throughput screening system have been evaluated by measuring the enzyme activity of the WT throughout the entire 96-well plate and calculating its standard deviation¹⁹. The specific steps are shown in **Figure 1**, this method can be used to optimize enzyme performance, improve its adaptability in DESs, and provide a basis for further research.

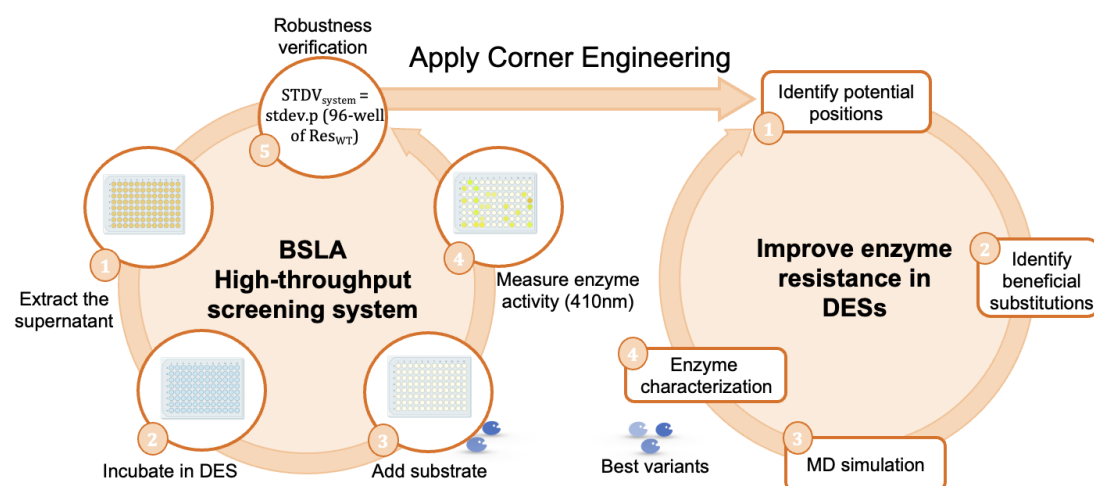


Figure 1. Specific experimental steps to establish a high-throughput screening system

All BSLA variants were screened in the presence of three DES solvents (Choline chloride:ethylene glycol, Choline chloride:acetamide, Tetrabutylphosphonium bromide:ethylene glycol). The concentration of 95% (v/v) ChCl:ethylene glycol, 30% (v/v) ChCl:acetamide, and 30% (v/v) TBPB:ethylene glycol were selected for the high-throughput screening system (**Figure 2**) with residual activity at 30–40% of the BSLA WT.

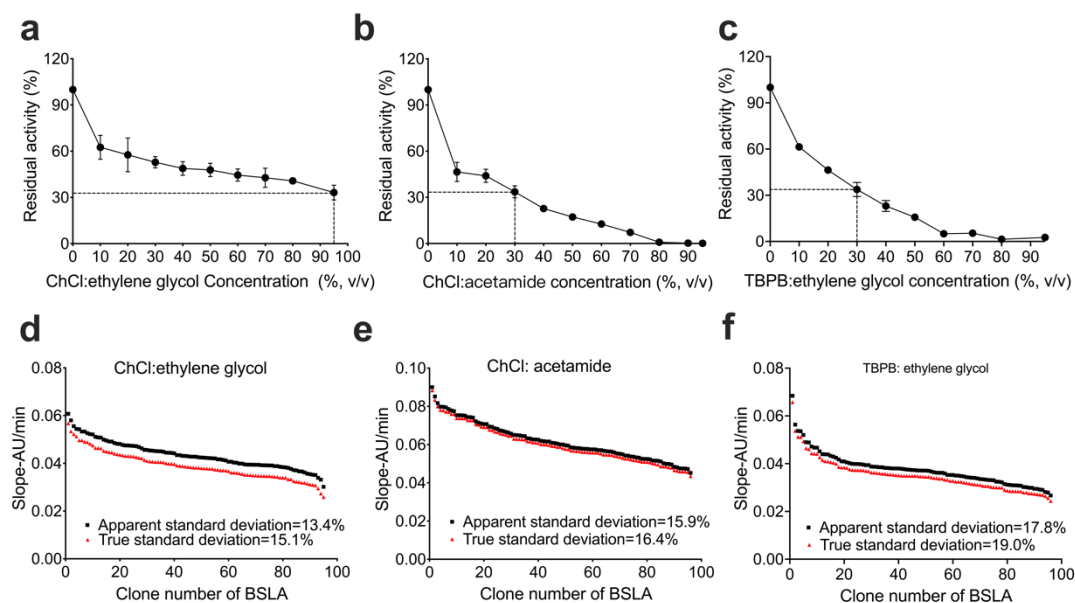


Figure 2. Screening system of BSLA in three DESs. Residual activity of BSLA WT in the DES of (a) ChCl:ethylene glycol, (b) ChCl:acetamide, and (c) TBPB:ethylene glycol. Slope of BSLA WT at (d) 95% (v/v) ChCl:ethylene glycol, (e) 30% (v/v) ChCl:acetamide, (f) 30% (v/v) TBPB:ethylene glycol, which used for the screening system to obtain residual activity of 30–40% of the BSLA WT. The standard deviation to evaluate the applicability of the 96-well MTP screening system for directed BSLA WT evolution. The apparent standard deviation was calculated without excluding the background, and the true standard deviation was calculated after deducting the background from the empty vector.

The effect of Corner Engineering was explored in BSLA

The secondary structure of a protein can be divided into Coil, Helix, Turn, Sheet. Firstly, we predict the secondary structure (**Figure 3a**) of BSLA using EMBI-EBI³³. The BSLA has a total of 25 corners (**Figure 3b**), which can be classified into 5 different types. Among them, there are 8 between Coil and Sheet, 9 between Coil and Helix, 5

After two rounds of screening, regarding the sequencing on the variants > 1.5-fold improvement towards at least two DESs, eight beneficial positions (thirteen variants) were identified in all three DESs. In detail, variant M137D/N138H demonstrated a 3.2-fold increase in resistance in the presence of ChCl:acetamide, and a 3.4-fold increase in resistance in the presence of ChCl:ethylene glycol. N88K/N89E showed a significant resistance increase in all three DESs (Figure 4). Additionally, certain variants, including S32H, T47K/N48K, M137/N138H, K88E/N89K, N89E, and Y161D/S162E/S163E, exhibited similar specific activity levels (1 to 1.5-fold) compared to the WT.

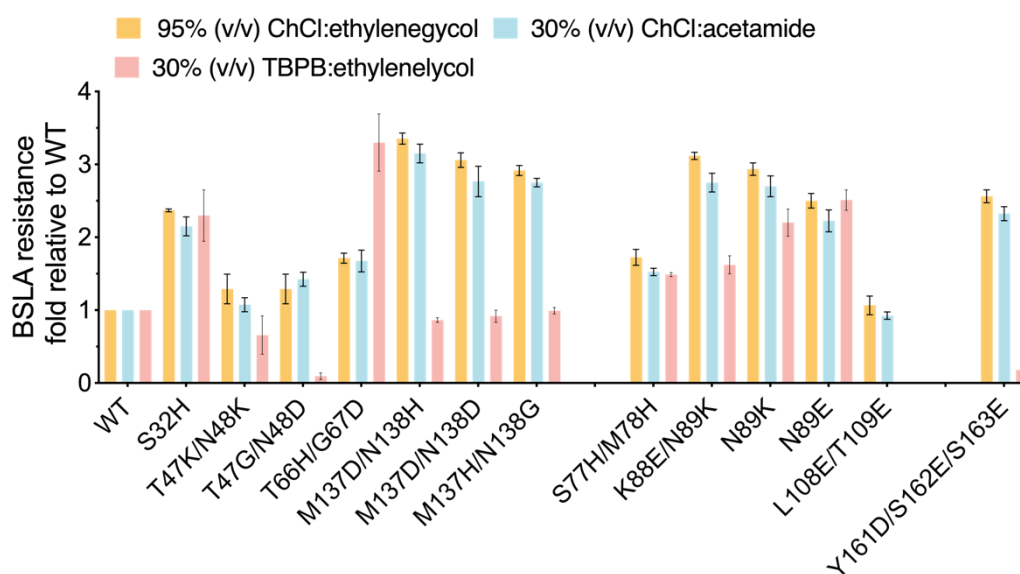


Figure 4. BSLA resistance folds relative to WT in three DESs.

The transferability of Corner Engineering was confirmed by investigating Bs2Est

To further validate the effectiveness of this strategy and improve the efficiency of engineering, the data from the screened BSLA mutants were analyzed. Among the 13 beneficial BSLA substitutions obtained (> 1.5-fold), more than half (54%, 7/13) were observed at Coil-Helix transition regions, signifying this region as a favorable location for acquiring enhanced variants. Therefore, we believe that targeting Coil-Helix as a hinge strategy can improve the stability of the enzyme in DES conditions with less effort. Bs2Est is used to validate the effectiveness of optimization strategies. The protein secondary structures of Bs2Est were obtained from EMBI-EBI (Figure S1). The adjacent amino acids at the Coil-Helix junction were identified as mutation sites (Figure 5a). Bs2Est libraries were screened in the presence of 75% (v/v) ChCl:ethylene glycol

(Figure S2). After the initial screening of 1848 variants, those demonstrating improved resistance compared to Bs2Est WT were rescreened with four replicates. Following the second round of screening, six beneficial variants were identified, with K205H displaying approximately 3.1-fold improved resistance in ChCl:ethylene glycol (Figure 5b).

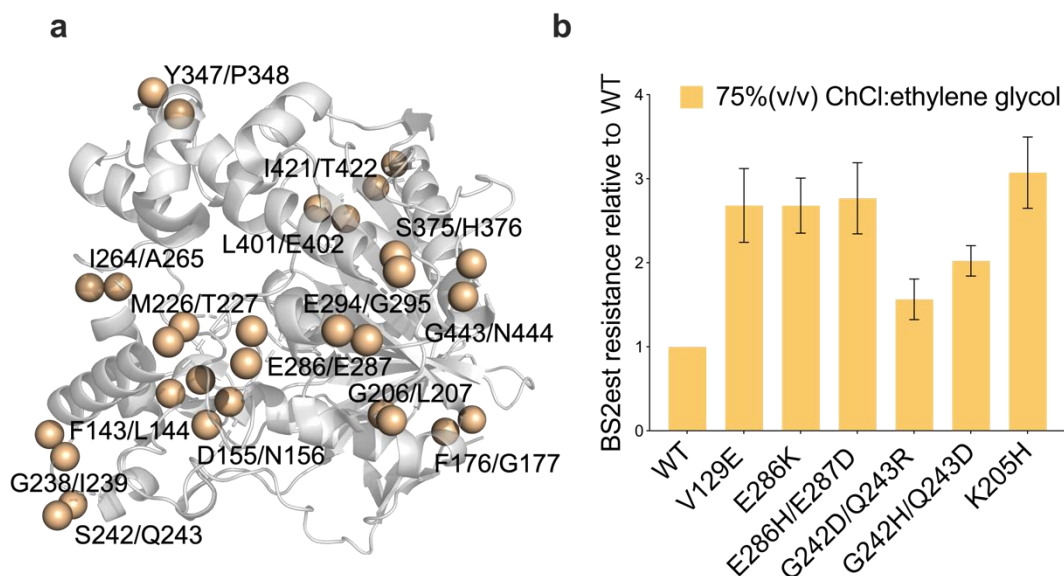


Figure 5. Optimized Corner Engineering in Bs2Est. (a) The three-dimensional structure of Bs2Est. The blue spheres represent amino acids at the junction of Coil and Helix, which are also useful potential sites for mutation. (b) Bs2Est resistance fold relative to WT.

Investigation of DES resistance profiles and kinetic characterization

To gain insights into the potential applications of the variants, we conducted enzymatic characterization. DES resistance profiles of the five BSLA variants (T66H/G67D, K88E/N89K, M137D/N138D, M137D/N138H, and Y161D/S162E/S163E) and BSLA WT were investigated at various ChCl:acetamide, ChCl:ethylene glycol, and TBPB:ethylene concentrations. As shown in Figure 6a-c, most variants shifted to higher residual activity under the entire range of concentrations of DES compared to WT. M137D/N138D showed 3.0-fold increased resistance relative to WT in 10% (v/v) ChCl:acetamide (Figure 6b). K88E/N89K, M137D/N138D, M137D/N138H have more than 40% residual activity at 0-30% (v/v) TBPB:ethylene glycol (Figure 6c). In addition, the kinetic characterization of purified BSLA variants (Figure S3) was studied more closely by investigating the specific activity at 30% (v/v) ChCl:acetamide, 30% (v/v)

TBPB:ethylene glycol, 95% (v/v) ChCl:ethylene glycol (Table S3). All variants had decreased K_M in 30% (v/v) ChCl:acetamide, which indicates that the mutation allowed the enzyme to increase its affinity for the substrate to some extent. K88E/N89K, M137D/N138H, and M137D/N138D showed significantly increased catalytic efficiency (K_{cat}/K_M) in the presence of three DESs (K889E/N89K up to a 10.0-fold increase relative to WT). In summary, by investigating the resistance and kinetic characterization of the enzyme in three DESs, we obtained two all-around BSLA variants (K88E/N89K, M137D/N138D) with significantly increased multiple biochemical characteristics (i.e., resistance and catalytic activity of the three DESs).

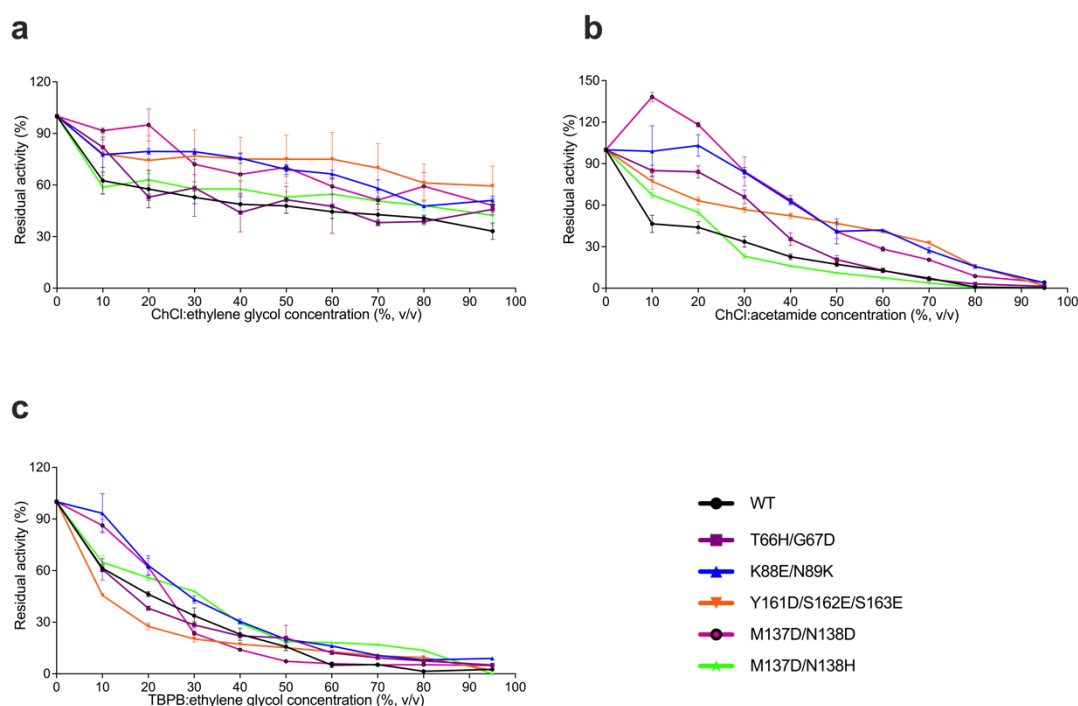


Figure 6. DES resistance profiles of the BSLA. Residual activity of BSLA variants in different concentrations of (a) ChCl:ethylene glycol (b) ChCl:acetamide (c) TBPB:ethylene glycol.

Three Bs2Est variants, E286K, E286H/E287D, and K205H, were selected for study due to their superior resistance performance in 75% ChCl:ethylene glycol compared to WT. Similar to the observations with BSLA, all these profiles were shifted to higher residual activities over nearly the entire range of the investigated DESs concentration (Figure 7a-c). These results suggested that the improvements were not limited to one DES employed. The “best” performance of these variants occurred in 30% (v/v) ChCl:ethylene glycol, showing that K205H and E286H/E287D had almost

2.5-fold and 1.9-fold higher DES resistance when compared to Bs2Est WT, respectively (Figure 7d). Notably, K205H showed 3.2-fold improved resistance in 15% TBPB:ethylene glycol. E286K showed increased K_{cat}/K_M in 75% ChCl:ethylene glycol (Table S4). These suggest that our strategy, with charged mutations introduced at the protein secondary structure somewhat improves the substrate affinity of the enzyme.

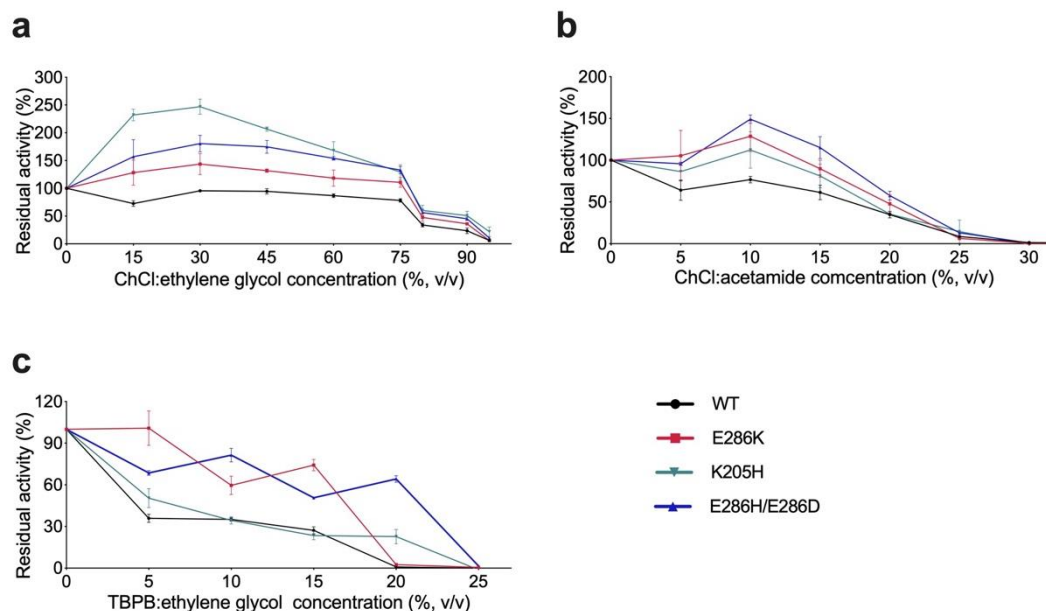


Figure 7. Characterization of Bs2Est variants in DESs. Residual activity of BSLA variants in different concentrations of (a) ChCl:acetamide (b) ChCl:ethylene glycol (c) TBPB:ethylene glycol.

Computational analysis revealed the molecular mechanism of improved DES resistance of variants

In order to explore the mechanism of BSLA tolerance to DES, we performed MD simulations to analyze the possible causes. The simulations involved proteins including WT and five variants (T66H/G67D, K88E/N89K, M137D/N138H, M137D/N138D and Y161D/S162E/S163E), and contained three DES components: 30% (v/v) ChCl:acetamide, 30% (v/v) TBPB:ethylene glycol and 95% (v/v) ChCl:ethylene glycol. As a result of the overall structural analysis (Figure 8), a slight increase in the time-averaged RMSD values (Figure 8a) for the last 40 ns of the four BSLA variants compared to the WT, whereas the change in the internal hydrogen bonding (Figure 8b) is less than 4. In all simulation systems, the localized regions of RMSF values (Figure 8c) for five BSLA variants displayed varying degrees of increased ($> 1 \text{ \AA}$), which

indicated that BSLA variants exhibited higher flexibility compared to WT, thereby affirming the improved stability of BSLA.

Consequently, water and DES molecules in the substrate binding site as well as around substitution sites (Figure 8d and 8e). Interestingly, an increase in the number of water molecules (almost 1-6) in the substrate binding site in all three DES systems (Figure 8d), whereas the number of DES molecules remained almost constant (Figure 8e). It interpreted the experimental observation that the resistance of four variants increased further in 30% (v/v) ChCl:acetamide, 30% (v/v) TBPB:ethylene glycol and 95% (v/v) ChCl:ethylene glycol. This finding corresponded well with the generally recognized perception that water molecules were necessary for enzymes to preserve their catalytic activity and that the degree of enzyme activity generally depended on the content of bound waters. In summary, we discovered that the resistance of BSLA to DESs essentially determined by two factors: (i) localized regions of improved flexibility; and (ii) an increase of water molecules combined in substrate binding site.

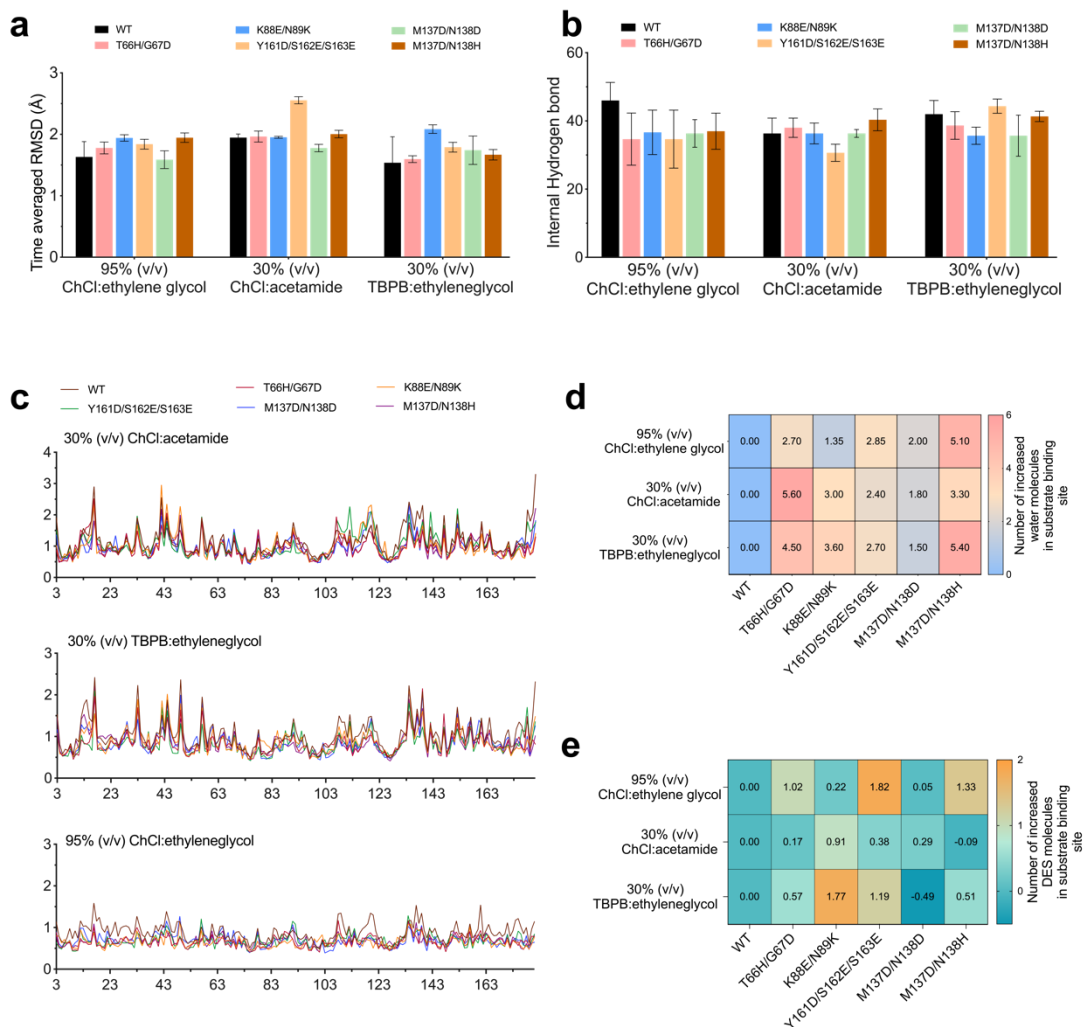


Figure 8. Overall and local structural change of BSLA variants in cosolvents. (a) Time-averaged RMSD of backbone and (b) internal Hydrogen bond determined from the last 40 ns of simulations under 30% (v/v) ChCl:acetamide, 30% (v/v) TBPB:ethylene glycol, and 95% (v/v) ChCl:ethylene glycol. Error bars show the standard deviation from three independent MD runs. (c) RMSF of BSLA variants residues in 30% (v/v) ChCl:acetamide, 30% (v/v) TBPB:ethylene glycol, and 95% (v/v) ChCl:ethylene glycol determined from the last 40ns of MD simulations. The data averaged from three independent runs. The dashed line indicates areas of significantly increased flexibility. (d) The number of water molecules was defined as hydration level. The heatmaps indicated the number of increased water molecules in the substrate binding site averaged over the last 40 ns of MD trajectories. (e) The number of DES molecules was defined as hydration level. The heatmaps indicated the number of increased DES molecules in the substrate binding site averaged over the last 40 ns of MD trajectories.

Discussion:

In the last few decades, directed evolution has emerged as one of the most efficient and practical methods to improve enzyme resistance. Corner Engineering proposes two complementary rational design principles to enhance DESs: (1) surface charge engineering and (2) altering enzyme secondary structure. Both of these design principles can be achieved by using different gene mutagenesis techniques (SDM/SSM). Significantly, we have discovered that the electrostatic interaction between enzymes and water is of paramount importance in enhancing resistance to resistance through surface charge manipulation. This phenomenon is closely linked to the restoration of vital water molecules at specific substitution sites.

With less experimental effort, we are able to quickly and accurately screen BSLA mutant library with 25 corners among 171 amino acids and find out 8 beneficial corners (13 substitutions) with up to 3.4-fold increase resistance in DES. Later analysis reveals that variant M137D/N138H displayed a remarkable 3.2-fold resistance when exposed to ChCl:acetamide and an even more substantial 3.4-fold increase in resistance in ChCl:ethylene glycol. Conversely, N88K/N89E exhibited a notable resistance augmentation across all three DESs. Furthermore, we analyzed the data of BSLA to optimize the strategy finding that the region Coil-Helix is preferable for constructing a smart mutant library with less experiment efforts. Then, Bs2Est was used to verify our findings. After screening 21 corners among 462 amino acids with up to 3.1-fold increased resistance in DES, which verified that the optimization of Corner Engineering.

The investigation of DES resistance profiles and kinetic characterization showed the excellent performance of this engineered enzyme toward DESs. The molecular investigations reveal that increased water molecules at substrate binding sites are the dominant determinant of elevated resistance, indicating a promising avenue for understanding enzyme-DES interactions. Coupling Corner Engineering with obtained molecular insights, the enzyme-DES interaction patterns are illuminated and it fosters the rational design of more DES-resistant and thermostable enzymes.

Through the enhancement of enzyme resistance, we open up new horizons for the utilization of enzymes in catalytic reactions. This improvement not only boosts

product purity and yield but also enhances the overall adaptability and efficiency of industrial production processes. The concept of Corner Engineering holds the promise of being a guiding template for the redesign of additional enzymes, facilitating their efficient functioning in DESs. The success of this strategy may provide a reference for re-engineering other enzymes (e.g. cellulases, hemicelluloses, laccase in biomass degradation, and esterase in plastics degradation) to efficiently catalyze DES-related reactions. This will further promote the widespread use of biocatalysis in the emerging bio-based economy, reducing the use of hazardous organic solvents and reducing the environmental impact of industrial processes, in line with green chemistry and sustainable development. This will facilitate the development of new industrial catalysis technologies and drive industrial production in a more environmentally friendly, sustainable, and efficient direction.

Methods:

Materials

All chemicals were of analytical grade or higher and were purchased from MACKLIN and Sigma. Enzymes (Polymerases, DpnI) and reaction buffers were purchased from NEB, Solarbio, and Takara. Plasmid extraction and polymerase chain reaction (PCR) purification were purchased from Axygen and Solarbio.

Strain and plasmids

The template for all PCRs was the plasmid pET22b (+)- BSLA-WT and pET22b (+)- Bs2Est -WT. *Escherichia coli* DH5 α was used as a cloning host. *Escherichia coli* BL21 was used for the expression and mutation library generation of BSLA and Bs2Est respectively.

Site saturation mutagenesis on the whole BSLA and Bs2Est gene sequence

Mutant libraries were generated using degenerated codons. The two degenerated codons SRC (H, R, D, and G) and RAA (E and K) were mixed in a ratio of 1:1 to cover

the charge amino acid (H, R, D, E, and K). Primers are shown in Table S1-2. For site-saturated mutagenesis (SSM), the template plasmid was stepwise mutated by PCR according to the QuikChange mutagenesis method. The PCR product was digested with DpnI (20 U, 37 °C, overnight) and transformed into *Escherichia coli* BL21(DE3).

BSLA and Bs2Est expression and activity assay

Following the PCR product transformation, individual colonies were cultured on LB_{Amp} agar plates. For each amino acid position library, we selected 88 BSLA clones and cultured them in a 96-well microtiter plate (MTP)²⁰. Four BSLA WT clones and four empty vector clones were also included as controls within the same MTP. After the expression, the BSLA fraction was separated from the cells through centrifugation (at 4 °C, 4000 rpm, for 20 minutes), and the resulting cell culture supernatant containing BSLA was transferred to new 96-well MTPs for subsequent analysis. The resistance of the BSLA variant was defined as the ratio of activities in the presence and absence of DES. The DES concentration resulting in a residual BSLA WT activity of 30%-40% was selected for the experiment. To measure the activity of BSLA, *p*-nitrophenyl butyrate (*p*NPB) was utilized as the substrate. The assay's activity was determined by adding TEA buffer (95 µL) to the supernatant (5 µL) and a freshly prepared substrate solution (100 µL) containing *p*NPB (0.5 mM) and acetonitrile [10% (v/v)] to each well. The release of *p*-nitrophenolate was recorded by measuring A410 at room temperature over an 8-minute interval using a microtiter plate reader (Biotek Synergy H1/Synergy2).

Optimization of screening conditions in DESs

30% (v/v) ChCl:acetamide, 95% ChCl:ethylene glycol, and 30% (v/v) TBPB:ethylene glycol were used in this work to screen the generated BSLA library. 75% ChCl:ethylene glycol was used in this work to screen the generated Bs2Est library. In order to determine the concentration of the three DESs for library screening, the solvents were supplemented with TEA buffer to obtain DESs with different concentrations. Concentrations leading to a residual activity between 30-40% of the WT were chosen to screen the libraries.

Enzyme purification and kinetics characterization

BSLA and Bs2Est were purified using Ni-IDA 6FF Sefinose(TM) Resin Kit and then concentrated by centrifugal ultrafiltration (10 kDa and 30kDa MWCO; Merck Millipore) after flask expression. The correct peak fractions (~35 kDa) were analyzed by SDS-Page (5 % stacking gel and 12 % separating gel) and pooled together. The protein concentration was measured by the BCA Protein Quantification Kit (Vazyme, China). The concentration of pNPB ranged from 0.002 to 4 mM, and the purified BSLA concentration was fixed at 1 μ M (20 μ g/mL). Plates were stirred briefly, and the absorbance changes were measured continuously by the Tecan Infinite MP200 plate reader (410 nm) in TEA buffer (50 mM triethanolamine, pH 7.4) and three DESs, respectively. Kinetics were determined by fitting the calculated reaction rates to the Michaelis–Menten equation using the software Origin Pro 8.6, as reported previously.

Molecular dynamics (MD) simulations

Molecular dynamics (MD) simulations of BSLA WT and variants were performed using the GROMACS 2022 software package and GROMOS96(54a7) force field in various DESs. The crystal structure of the BSLA chain with PDB ID 1I6W was obtained from Protein Data Bank (3-181 amino acids). The DES models were obtained from ATB (<https://atb.uq.edu.au>) with the optimized parameters set matching the GROMOS96(54a7) force field. Protein protonation was performed using the gmx self-contained tool gmx pdb2gmx, and the protein molecules were placed in a cubic box (12 Å from the edge of the box). Depending on the DES concentration, a corresponding number of ~140 choline cation ions, ~140 chloride anion ions and ~280 acetamide molecules in 30% (v/v) ChCl:acetamide system, ~56 tetrabutylphosphonium cation ions, ~56 bromine anion ions and ~112 ethylene glycol molecules in 30% (v/v) TBPB:ethylene glycol system, ~424 choline cation ions, ~424 chloride anion ions and ~848 ethylene glycols molecules in 95% (v/v) ChCl:ethylene glycol system were added to the box. Afterwards, the rest system was filled with SPCE water model. The charge of the system was neutralized with Na⁺ and Cl⁻, and the net charge was zero in all setup systems.

References

- (1) Sheldon, R. A.; Woodley, J. M. Role of Biocatalysis in Sustainable Chemistry. *Chem. Rev.* **2018**, *118* (2), 801–838. <https://doi.org/10.1021/acs.chemrev.7b00203>.
- (2) Cui, H.; Vedder, M.; Zhang, L.; Jaeger, K.-E.; Schwaneberg, U.; Davari, M. D. Polar Substitutions on the Surface of a Lipase Substantially Improve Tolerance in Organic Solvents. *ChemSusChem* **2022**, *15* (9), e202102551. <https://doi.org/10.1002/cssc.202102551>.
- (3) Xu, P.; Liang, S.; Zong, M.-H.; Lou, W.-Y. Ionic Liquids for Regulating Biocatalytic Process: Achievements and Perspectives. *Biotechnol. Adv.* **2021**, *51*, 107702. <https://doi.org/10.1016/j.biotechadv.2021.107702>.
- (4) Hobbs, H. R.; Thomas, N. R. Biocatalysis in Supercritical Fluids, in Fluorous Solvents, and under Solvent-Free Conditions. *Chem. Rev.* **2007**, *107* (6), 2786–2820. <https://doi.org/10.1021/cr0683820>.
- (5) Abbott, A. P.; Capper, G.; Davies, D. L.; Rasheed, R. K.; Tambyrajah, V. Novel Solvent Properties of Choline Chloride/Urea Mixtures. *Chem. Commun.* **2003**, No. 1, 70–71. <https://doi.org/10.1039/b210714g>.
- (6) Gorke, J. T.; Srienc, F.; Kazlauskas, R. J. Hydrolase-Catalyzed Biotransformations in Deep Eutectic Solvents. *Chem. Commun.* **2008**, No. 10, 1235. <https://doi.org/10.1039/b716317g>.
- (7) Domínguez De María, P.; Maugeri, Z. Ionic Liquids in Biotransformations: From Proof-of-Concept to Emerging Deep-Eutectic-Solvents. *Curr. Opin. Chem. Eng.* **2011**, *15* (2), 220–225. <https://doi.org/10.1016/j.cbpa.2010.11.008>.
- (8) Khodaverdian, S.; Dabirmanesh, B.; Heydari, A.; Dashtban-moghadam, E.; Khajeh, K.; Ghazi, F. Activity, Stability and Structure of Laccase in Betaine Based Natural Deep Eutectic Solvents. *Int. J. Biol. Macromol.* **2018**, *107*, 2574–2579. <https://doi.org/10.1016/j.ijbiomac.2017.10.144>.
- (9) Zhang, X.; Peng, Y.; Zhao, J.; Li, Q.; Yu, X.; Acevedo-Rocha, C. G.; Li, A. Bacterial Cytochrome P450-Catalyzed Regio- and Stereoselective Steroid Hydroxylation Enabled by Directed Evolution and Rational Design. *Bioresour. Bioprocess.* **2020**, *7* (1), 2. <https://doi.org/10.1186/s40643-019-0290-4>.
- (10) Wang, M.; Cui, H.; Gu, C.; Li, A.; Qiao, J.; Schwaneberg, U.; Zhang, L.; Wei, J.; Li, X.; Huang, H. Engineering All-Round Cellulase for Bioethanol Production.

ACS Synth. Biol. **2023**, *12* (7), 2187–2197.

<https://doi.org/10.1021/acssynbio.3c00289>.

- (11) Qiao, J.; Sheng, Y.; Wang, M.; Li, A.; Li, X.; Huang, H. Evolving Robust and Interpretable Enzymes for the Bioethanol Industry. *Angew. Chem. Int. Ed.* **2023**, *62* (12), e202300320. <https://doi.org/10.1002/anie.202300320>.
- (12) Cui, H.; Eltoukhy, L.; Zhang, L.; Markel, U.; Jaeger, K.; Davari, M. D.; Schwaneberg, U. Less Unfavorable Salt Bridges on the Enzyme Surface Result in More Organic Cosolvent Resistance. *Angew. Chem. Int. Ed.* **2021**, *60* (20), 11448–11456. <https://doi.org/10.1002/anie.202101642>.
- (13) Stevens, J. C.; Shi, J. Modifying Surface Charges of a Thermophilic Laccase Toward Improving Activity and Stability in Ionic Liquid. *Front. Bioeng. Biotechnol.* **2022**, *10*, 880795. <https://doi.org/10.3389/fbioe.2022.880795>.
- (14) Nordwald, E. M.; Armstrong, G. S.; Kaar, J. L. NMR-Guided Rational Engineering of an Ionic-Liquid-Tolerant Lipase. *ACS Catal.* **2014**, *4* (11), 4057–4064. <https://doi.org/10.1021/cs500978x>.
- (15) Nordwald, E. M.; Kaar, J. L. Stabilization of Enzymes in Ionic Liquids via Modification of Enzyme Charge. *Biotechnol. Bioeng.* **2013**, *110* (9), 2352–2360. <https://doi.org/10.1002/bit.24910>.
- (16) Cui, H.; Zhang, L.; Eltoukhy, L.; Jiang, Q.; Korkunç, S. K.; Jaeger, K.-E.; Schwaneberg, U.; Davari, M. D. Enzyme Hydration Determines Resistance in Organic Cosolvents. *ACS Catal.* **2020**, *10* (24), 14847–14856. <https://doi.org/10.1021/acscatal.0c03233>.
- (17) Cui, H.; Jaeger, K.; Davari, M. D.; Schwaneberg, U. CompassR Yields Highly Organic-Solvent-Tolerant Enzymes through Recombination of Compatible Substitutions. *Chem. Eur. J.* **2021**, *27* (8), 2789–2797. <https://doi.org/10.1002/chem.202004471>.
- (18) Yang, L.; Gao, Z.; Cao, Y.; Xing, R.; Zhang, X. Effect of PblI on the Secondary Structure and Biological Activity of Trypsin. *ChemBioChem* **2005**, *6* (7), 1191–1195. <https://doi.org/10.1002/cbic.200400267>.
- (19) Frauenkron-Machedjou, V. J.; Fulton, A.; Zhao, J.; Weber, L.; Jaeger, K.-E.; Schwaneberg, U.; Zhu, L. Exploring the Full Natural Diversity of Single Amino Acid Exchange Reveals That 40–60% of BSLA Positions Improve Organic Solvents Resistance. *Bioresour. Bioprocess.* **2018**, *5* (1), 2. <https://doi.org/10.1186/s40643-017-0188-y>.

(20) Reetz, M. T.; Bocola, M.; Carballeira, J. D.; Zha, D.; Vogel, A. Expanding the Range of Substrate Acceptance of Enzymes: Combinatorial Active-Site Saturation Test. *Angew. Chem.* **2005**, *117* (27), 4264–4268.
<https://doi.org/10.1002/ange.200500767>.