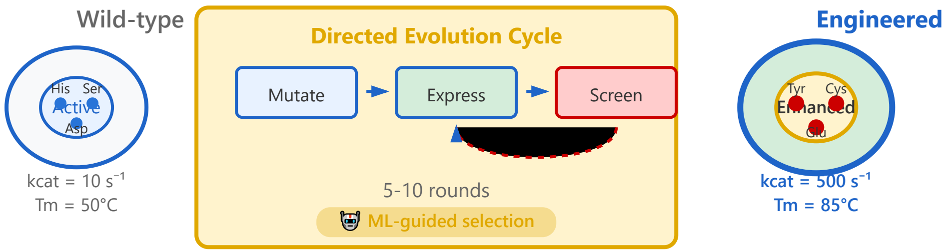
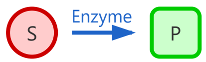
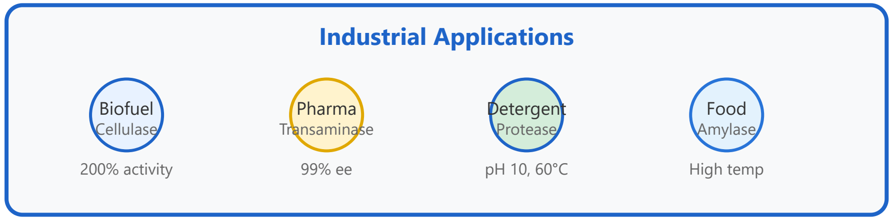
Enzyme Engineering

Directed Evolution & Rational Design



Catalytic Reaction





Activity improvement
kcat/Km optimization

Substrate specificity
Promiscuity engineering

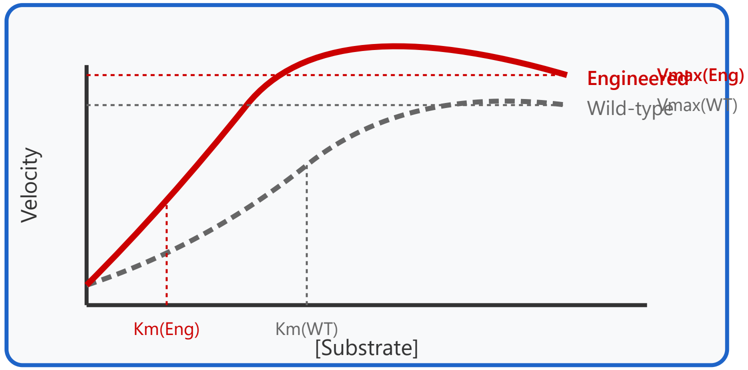
Thermostability
High temperature operation

Solvent tolerance
Organic solvent resistance

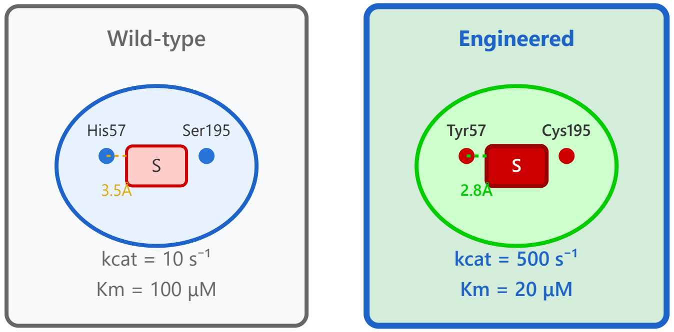
Directed evolution
Iterative improvement cycles

1. Activity Improvement (kcat/Km Optimization)

Catalytic Efficiency Enhancement



Active Site Optimization



Objective

Enhance the catalytic efficiency (kcat/Km) of enzymes to increase reaction rates and substrate binding affinity. This is crucial for industrial processes requiring high throughput.

Key Strategies

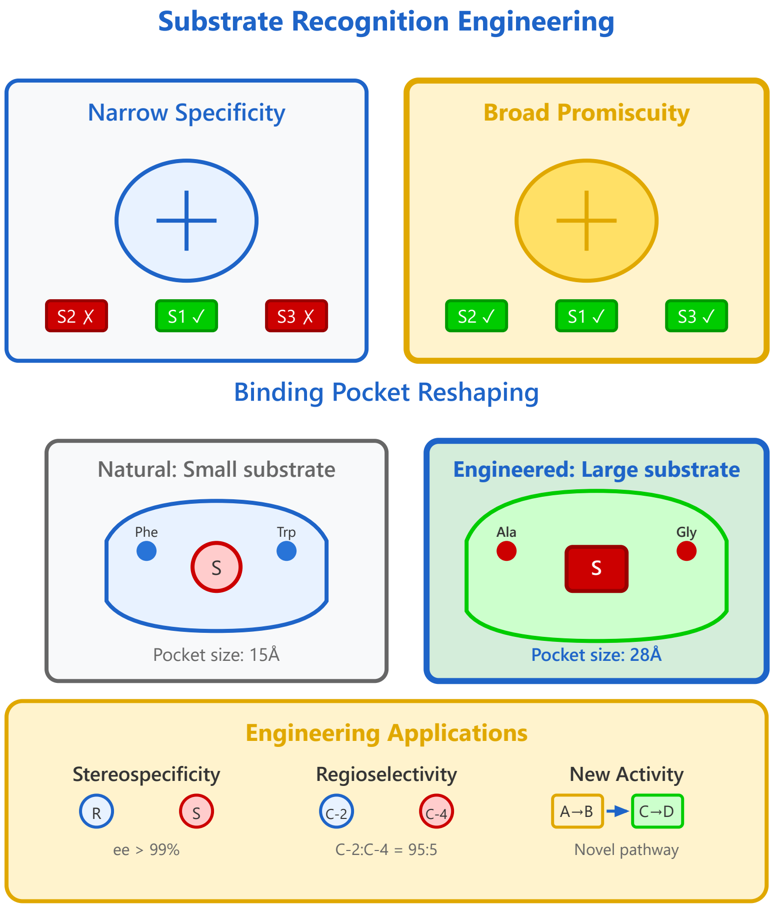
- **Transition state stabilization:** Modify active site residues to better stabilize the transition state
- **Substrate binding optimization:** Engineer binding pocket geometry for improved substrate fit
- **Product release enhancement:** Reduce product inhibition by facilitating product dissociation
- **Catalytic triad engineering:** Optimize spatial arrangement and pKa of catalytic residues

Case Study: Subtilisin Protease

Wild-type: kcat/Km = 1.0×10^5 $M^{-1}s^{-1}$
Engineered (N62D/G166D): kcat/Km = 5.2×10^6 $M^{-1}s^{-1}$
Improvement: 52-fold increase in catalytic efficiency through rational design of the oxyanion hole and substrate binding pocket.

Impact: Activity improvements of 10-1000 fold are achievable through directed evolution combined with computational design, enabling reduced enzyme loading and lower production costs.

2. Substrate Specificity & Promiscuity Engineering



Objective

Modify substrate binding specificity to either narrow selectivity for a single substrate or broaden promiscuity to accept multiple substrates. This enables enzymes to process non-natural substrates or improve stereoselectivity.

Engineering Approaches

- **Binding pocket reshaping:** Alter size and geometry through mutations (e.g., Phe→Ala for pocket enlargement)
- **Electrostatic tuning:** Change charge distribution to favor specific substrate classes
- **Hydrophobic interactions:** Engineer aromatic residues for π-stacking with substrates
- **Gatekeeper residue modification:** Control substrate entry and selectivity

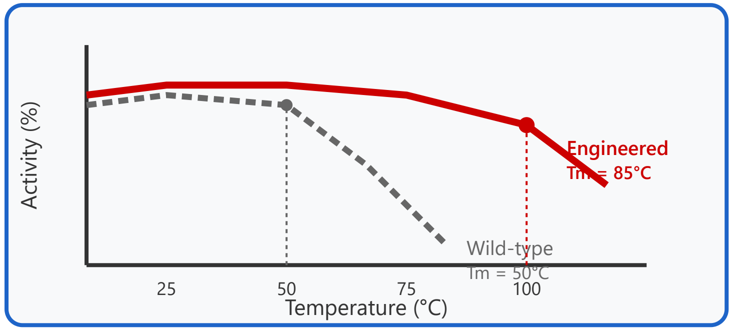
Case Study: P450 BM3 Hydroxylase

Wild-type: Hydroxylates C12-C16 fatty acids
Engineered (9 mutations): Accepts propane and alkanes (C2-C8)
Achievement: Complete substrate scope inversion - from long-chain to short-chain hydrocarbons, enabling production of valuable chemical intermediates.

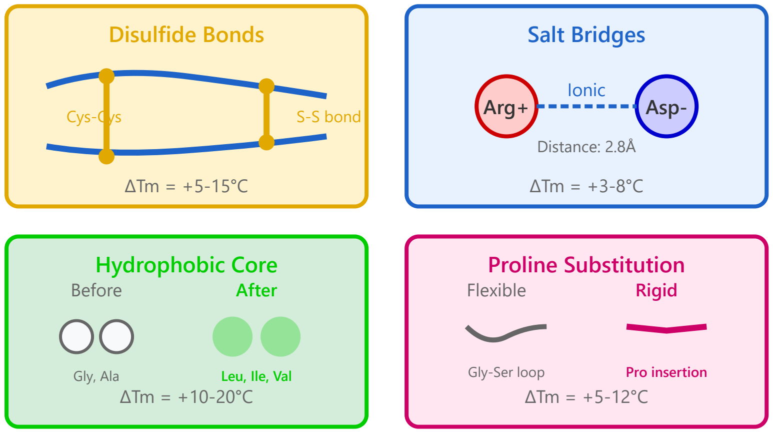
Applications: Pharmaceutical synthesis (>99% ee), biocatalytic cascades, plastic degradation, and production of non-natural amino acids and chemicals.

3. Thermostability Engineering

Thermal Stability Enhancement



Molecular Stabilization Mechanisms



Objective

Increase enzyme thermal stability (Tm) to enable operation at elevated temperatures, which improves reaction rates, reduces contamination risks, and extends enzyme shelf life in industrial processes.

Stabilization Strategies

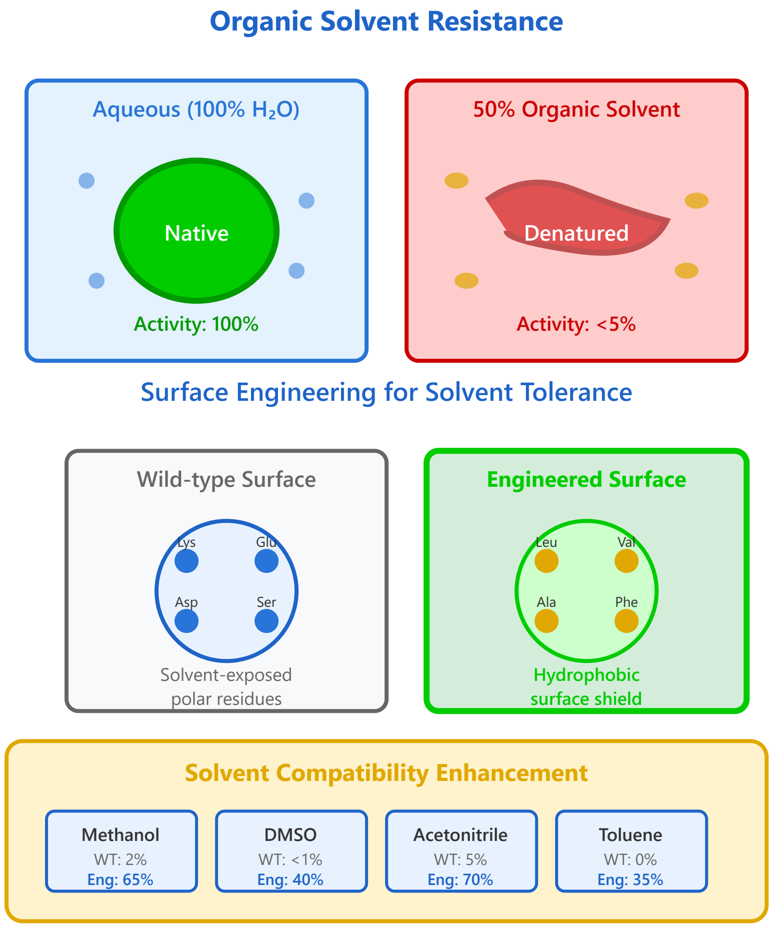
- **Disulfide bonds:** Introduce Cys-Cys bridges to constrain structure (5-15°C increase)
- **Salt bridges:** Engineer ionic interactions between charged residues (3-8°C increase)
- **Hydrophobic core packing:** Replace small residues (Gly, Ala) with bulky hydrophobic ones (Leu, Ile, Val) for tighter packing (10-20°C increase)
- **Proline substitution:** Reduce loop flexibility by inserting proline (5-12°C increase)
- **N/C-terminal modifications:** Add stabilizing residues or tags
- **Glycosylation:** Attach sugar moieties for protection

Case Study: Bacillus α-Amylase

Wild-type Tm: 55°C (half-life: 15 min at 90°C)
Engineered (15 mutations): Tm = 95°C (half-life: 7 hours at 90°C)
Key mutations: Introduction of 3 disulfide bonds, 5 salt bridges, and 7 core packing improvements. Now used in high-temperature starch processing.

Industrial Impact: Thermostable enzymes enable higher process temperatures (70-100°C), reducing viscosity, increasing mass transfer, and preventing microbial contamination without sterilization.

4. Solvent Tolerance Engineering



Objective

Engineer enzymes to maintain activity and stability in organic solvents, enabling reactions with hydrophobic substrates and products that are poorly soluble in water.

Engineering Strategies

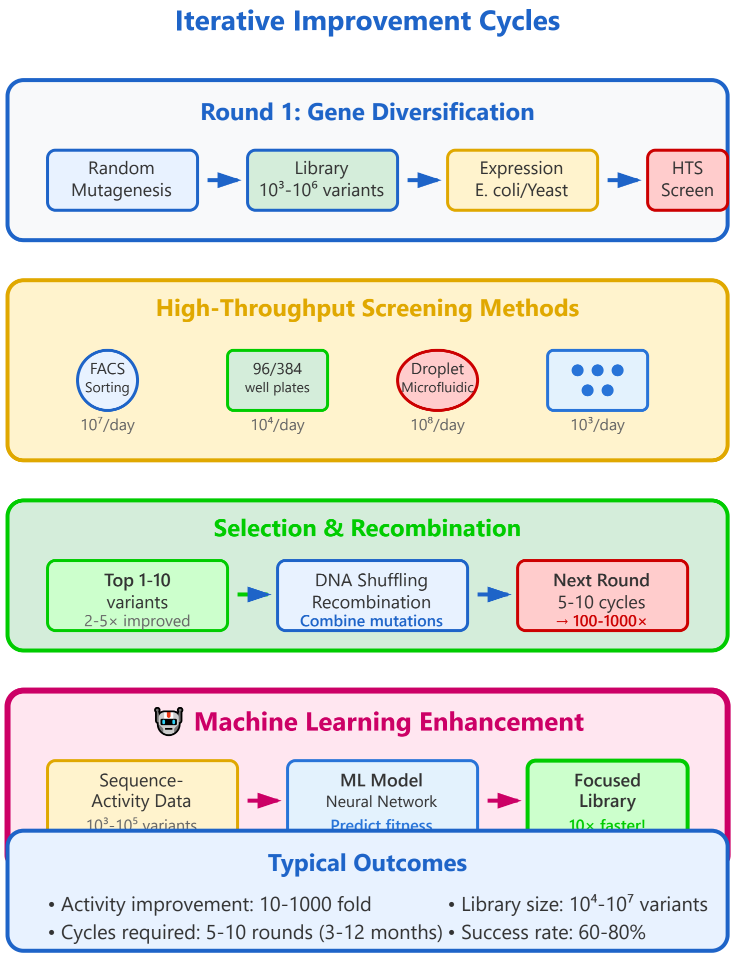
- **Surface hydrophobicity:** Replace charged surface residues (Lys, Glu, Asp) with hydrophobic ones (Leu, Val, Ala, Phe)
- **Core stabilization:** Strengthen hydrophobic core to resist solvent penetration
- **Removal of water-binding sites:** Eliminate surface pockets that trap destabilizing water molecules
- **Increased rigidity:** Reduce conformational flexibility through proline substitutions and disulfide bonds
- **Active site protection:** Shield catalytic residues from solvent deactivation

Case Study: Candida antarctica Lipase B (CALB)

Wild-type: 30% activity in 30% methanol
Engineered (K26L/D223A/E226V): 85% activity in 70% methanol
Application: Used in biodiesel production and synthesis of pharmaceutical esters in high solvent concentrations, dramatically improving product yields.

Industrial Benefits: Enables biphasic reactions, increases substrate/product solubility, reduces water activity for reversing hydrolysis, and facilitates downstream product recovery.

5. Directed Evolution Strategy



Objective

Use iterative rounds of random mutagenesis, recombination, and selection to evolve enzymes with desired properties without requiring detailed structural knowledge. This approach mimics natural evolution but accelerated 1000-fold.

Key Components

- **Mutagenesis methods:** Error-prone PCR (0.1-1% mutation rate), DNA shuffling, saturation mutagenesis
- **Library construction:** Generate 10^3-10^7 variants with diverse mutations
- **High-throughput screening:** FACS (10^7/day), microfluidics (10^8/day), or plate-based assays (10^4/day)
- **Selection criteria:** Activity, stability, specificity, or multiple properties simultaneously
- **Recombination:** DNA shuffling to combine beneficial mutations from different variants
- **ML-guidance:** Machine learning models predict promising variants, reducing screening by 10-fold

Nobel Prize Example: Frances Arnold's P450 Evolution

Goal: Evolve P450 for propane hydroxylation (non-natural activity)
Starting: 0% activity on propane
After 5 rounds: 300,000 turnovers with 98% selectivity
Impact: Enabled sustainable biocatalytic production of valuable chemicals from cheap alkanes, awarded 2018 Nobel Prize in Chemistry.

Advantages: No structural knowledge required, can optimize multiple properties simultaneously, discovers unexpected beneficial mutations, and integrates easily with computational design and ML prediction.