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# The state-of-the-art strategies of protein engineering for enzyme stabilization

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Research review paper

# The state-of-the-art strategies of protein engineering for enzyme stabilization

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ABSTRACT

Enzymes generated by natural recruitment and protein engineering have greatly contribute in various sets of applications. However, their insufficient stability is a bottleneck that limit the rapid development of biocatalysis. Novel approaches based on precise and global structural dissection, advanced gene manipulation, and combination with the multidisciplinary techniques open a new horizon to generate stable enzymes efficiently. Here, we comprehensively introduced emerging advances of protein engineering strategies for enzyme stabilization. Then, we highlighted practical cases to show importance of enzyme stabilization in pharmaceutical and industrial applications. Combining computational enzyme design with molecular evolution will hold considerable promise in this field.

# Content

# Introduction

- To date, comprehensive studies revealed that the mechanisms that affect enzyme stability are complicated due to the multiple interactions including the hydrophobic bonds, hydrogen bonds, salt bridges, charge on the protein surface, disulfide bonds and metal ions.

- These interactions result from a combination of amino acid and enzyme composition.

- Therefor, there is no uniform rule to guide strategies for its improvement.

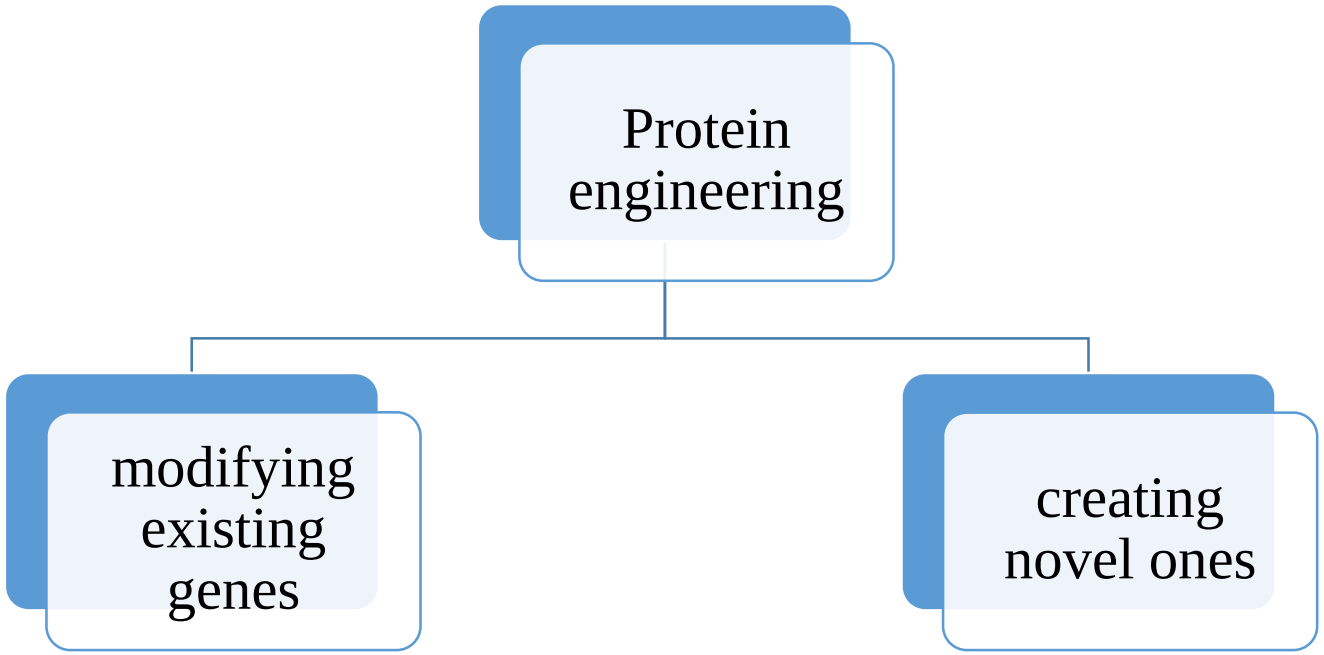
# What Protein Engineering Is?

DEFINITION | AIM | APLICATION

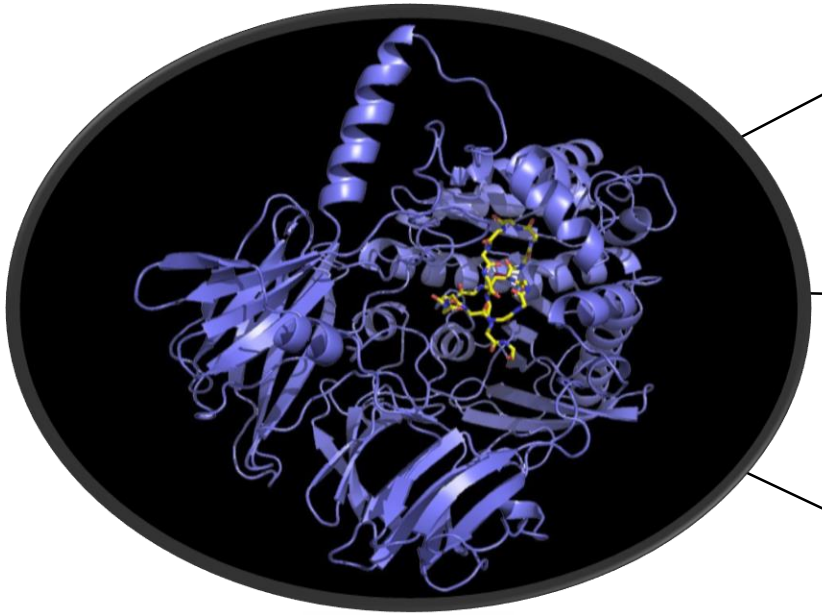
# Protein Engineering

**Definition:**

- It is a  techniques for the design and construction of proteins with the desired properties.
- **It** can be done through modifying existing genes or creating novel ones, either by **site targeted** or **mutated library screening** for enzymes that meet specific requirements



# Aim of Protein Engineering

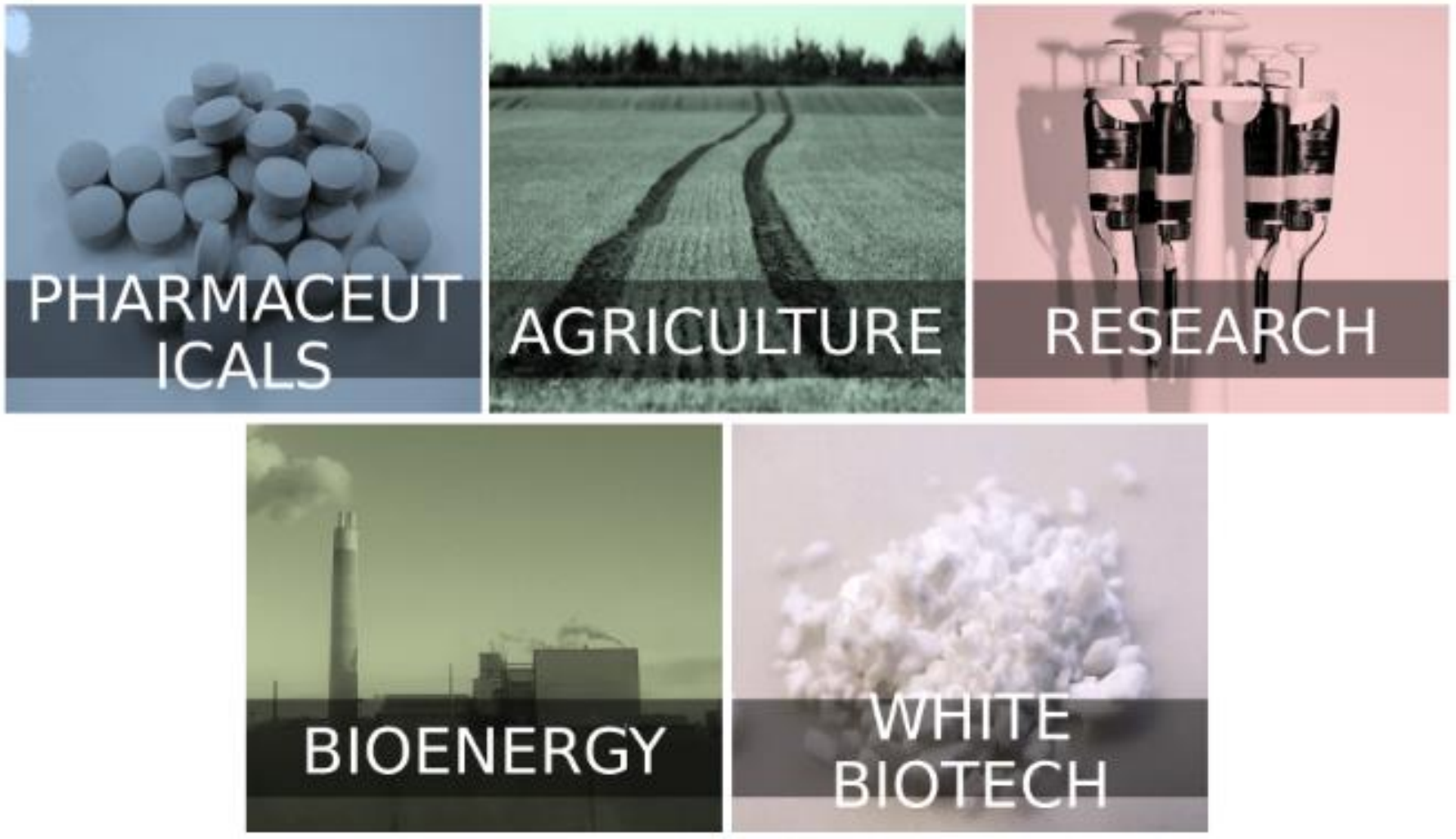


STABILITY — Temperature, denaturant, solvent, pH, Salt.

ACTIVITY — Catalysis, Specificity..

solubility — solvent, pH, Salt.

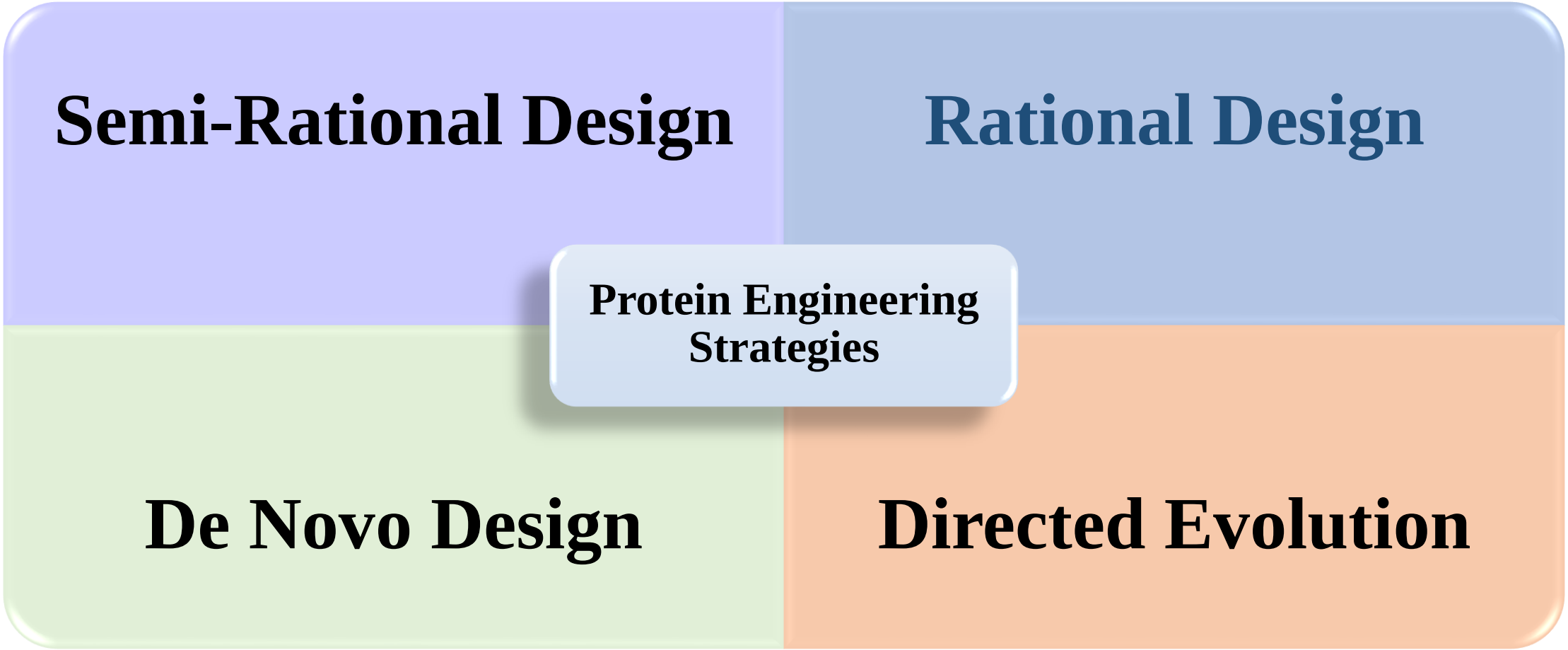
# Application of Protein Engineering



# Protein Engineering Strategies

RATIONAL I SEMI-RATIONAL I DIRECTED EVOLUTION I DE NOVO DESIGN

# Protein engineering Strategies



# Rational Design

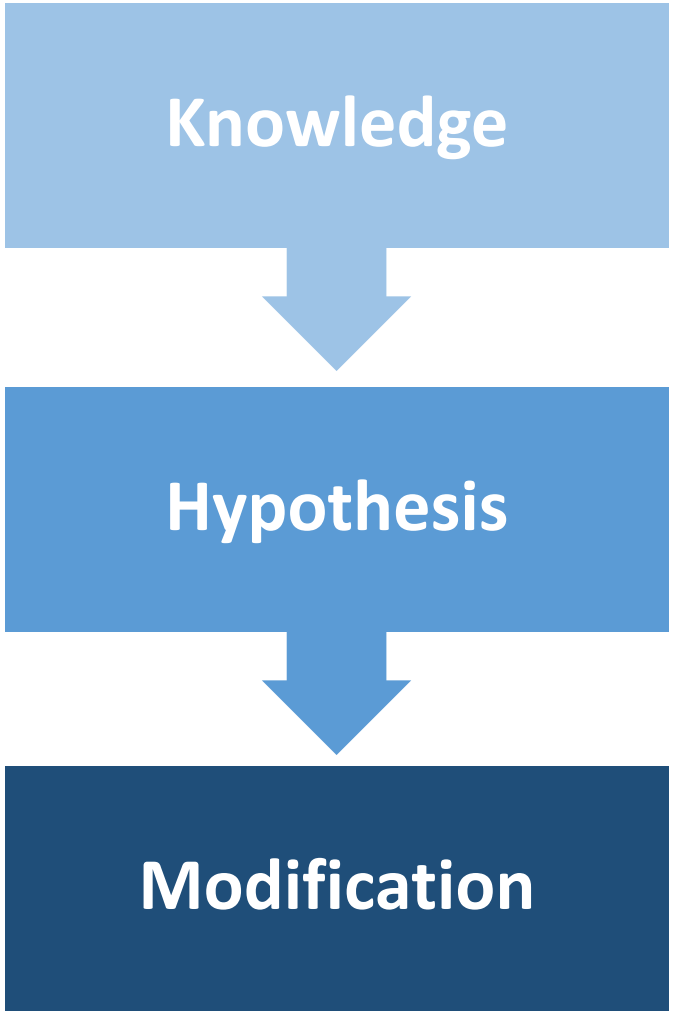
METHODS I EXAMPLE I LIMITATION

# Rational Design

- The strategy is mainly completed *in silico*.

- **Requires:**
  1. Amino acid sequence,
  2. 3D structure,
  3. Catalytic mechanisms of the protein (structure-function relationship).

- Use site-directed mutagenesis (substitution, insertion, or deletion) approach!

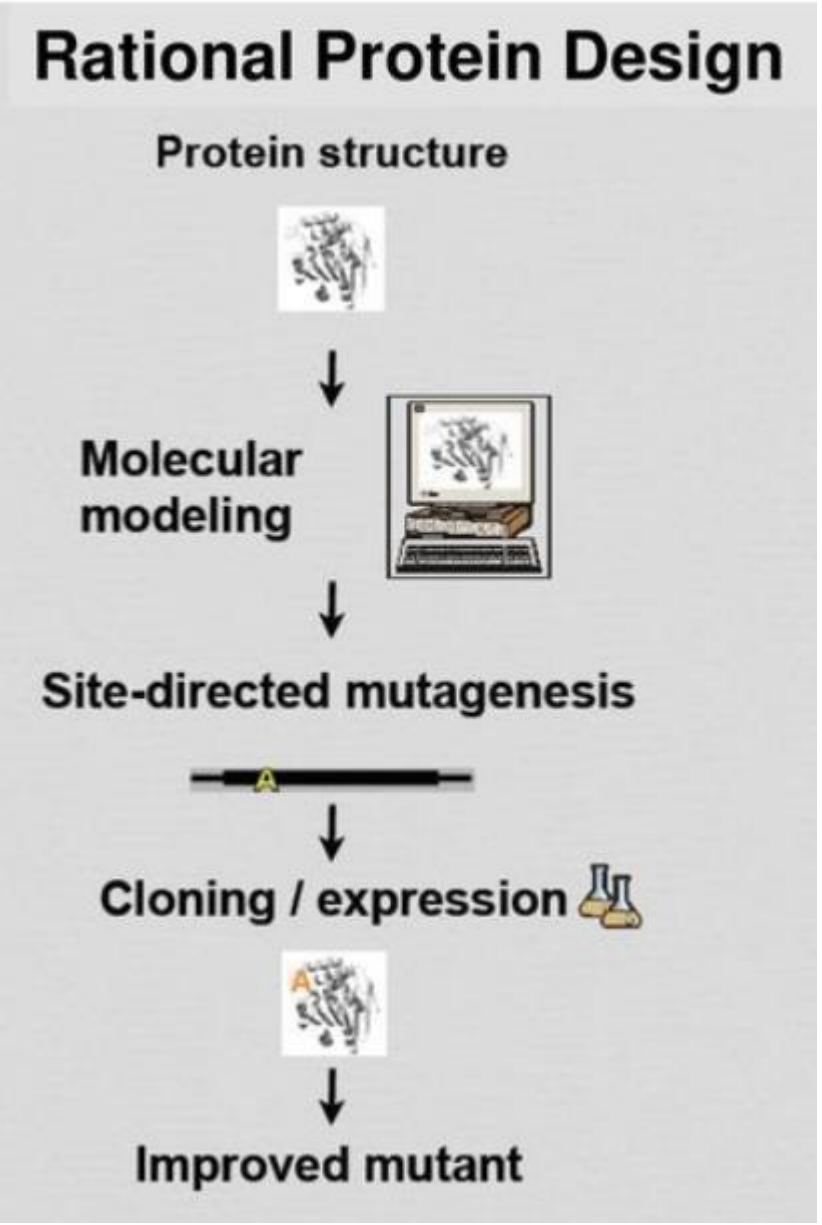


# Rational Design

- As yet, 144,464 **protein structures** have been identified and are now available in the PDB database, thus providing a solid basis for rational design.

- Algorithms that integrate **structural**, **thermodynamic**, and **functional data** are used to predict parameters for enzyme stabilization and to localize potential "hotspots".

- Thereafter, variants can undergo accurate site-directed mutagenesis (substitution, insertion, or deletion) in the next iteration.



# Pros and Cons of Rational Design

## Advantage

- Rational design for enzyme stabilization involves rapid identification of residues or structural motifs associated with enzyme stability to restrict the number of mutations required.

## Limitation

- Requires deep understanding of structural-functional Info. for starting protein and changes.
- Requires more solid data which are necessary needed for support.

# Rational Design

- Presently, widely used methods for stability-enhancing rational design emphasize

  - homology comparison,
  - disulfide bond introduction,
  - salt bridges addition,
  - Modeling.

With the continuous improvement of **crystal elucidation** techniques and development of **bioinformatics tools**, rational design has advanced from homology comparison to the current customization of design strategies according to differences in structure. There are several practical tools for structure prediction, including Swiss-Model, Rosetta

# Case study using Rational Design

OPEN ACCESS Freely available online

PLOS ONE

# Protein Thermal Stability Enhancement by Designing Salt Bridges: A Combined Computational and Experimental Study

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## Abstract

Protein thermal stability is an important factor considered in medical and industrial applications. Many structural characteristics related to protein thermal stability have been elucidated, and increasing salt bridges is considered as one of the most efficient strategies to increase protein thermal stability. However, the accurate simulation of salt bridges remains difficult. In this study, a novel method for salt-bridge design was proposed based on the statistical analysis of 10,556 surface salt bridges on 6,493 X-ray protein structures. These salt bridges were first categorized based on pairing residues, secondary structure locations, and Cα–Cα distances. Pairing preferences generalized from statistical analysis were used to construct a salt-bridge pair index and utilized in a weighted electrostatic attraction model to find the effective pairings for designing salt bridges. The model was also coupled with B-factor, weighted contact number, relative solvent accessibility, and conservation prescreening to determine the residues appropriate for the thermal adaptive design of salt bridges. According to our method, eight putative salt-bridges were designed on a mesophilic β-glucosidase and 24 variants were constructed to verify the predictions. Six putative salt-bridges leaded to the increase of the enzyme thermal stability. A significant increase in melting temperature of 8.8, 4.8, 3.7, 1.3, 1.2, and 0.7°C of the putative salt-bridges N437K–D49, E96R–D28, E96K–D28, S440K–E70, T231K–D388, and Q277E–D282 was detected, respectively. Reversing the polarity of T231K–D388 to T231D–D388K resulted in a further increase in melting temperatures by 3.6°C, which may be caused by the transformation of an intra-subunit electrostatic interaction into an inter-subunit one depending on the local environment. The combination of the thermostable variants (N437K, E96R, T231D and D388K) generated a melting temperature increase of 15.7°C. Thus, this study demonstrated a novel method for the thermal adaptive design of salt bridges through inference of suitable positions and substitutions.

# Mesophilic β-Glucosidase from *Bacillus polymyxa*

**AIM**

**Improve β-Glucosidase thermostability**
salt-bridge design was proposed based on the statistical analysis

**ENGINEERING**

- In total, the data set consisted of 32,096 salt bridges from 6,493 X-ray protein structures used To analyze the specific geometry of salt bridges on the protein surface.
- Site-directed mutagenesis approach.

**OUTCOME**

- Six putative salt-bridges leaded to the increase of the enzyme thermal stability.
- A significant increase in melting temp. of 8.8, 4.8, 3.7, 1.3, 1.2, and 0.7ºC of the putative salt-bridges N437K–D49, E96R–D28, E96K–D28, S440K–E70, T231K–D388, and Q277E–D282 was detected, respectively.

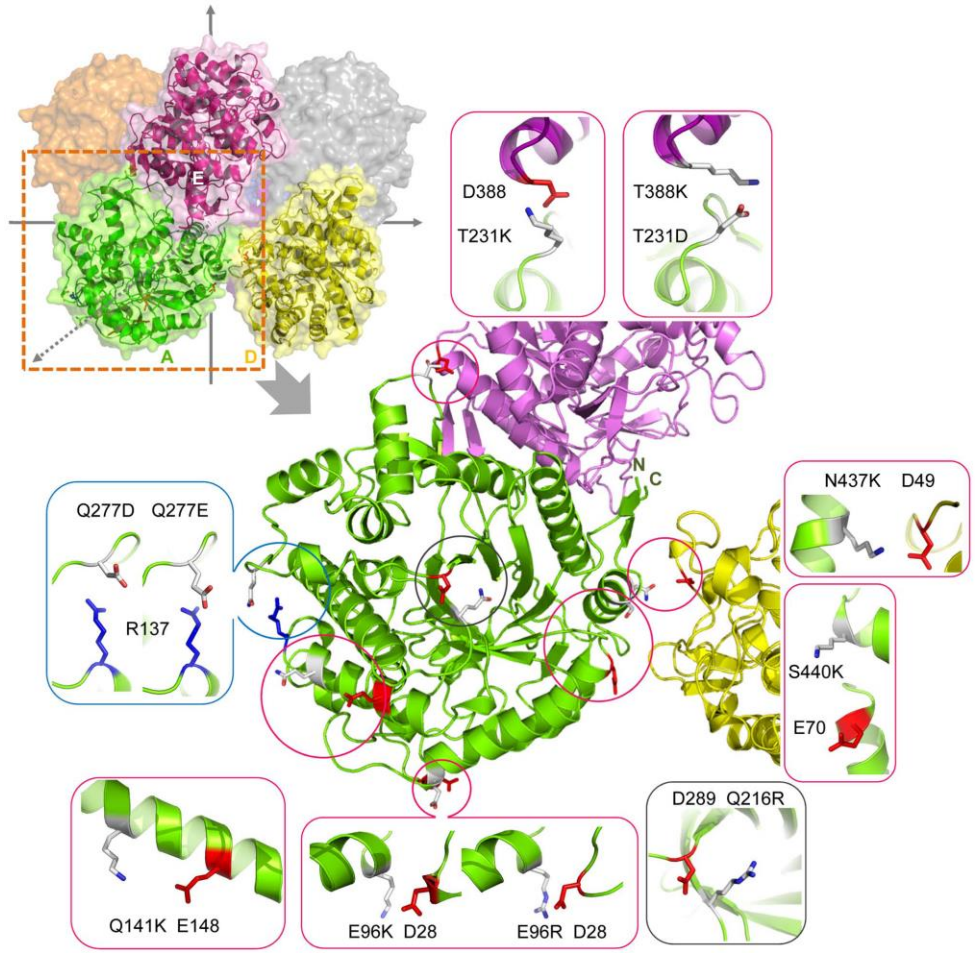



Figure. Locations of predicted pairs in the octameric structure of β-Glucosidase .

# Directed Evolution

METHODS I LIMITATION I COMPARSION

# Directed evolution

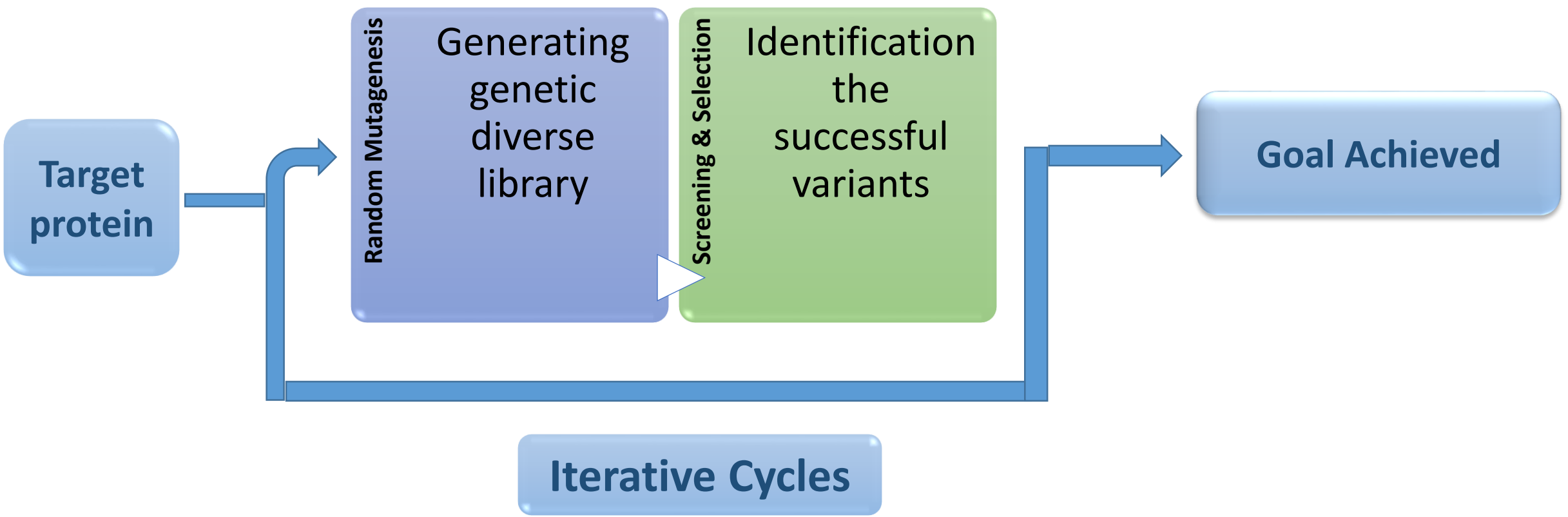
⟶ mimics natural evolution process *in vitro*.

- ❖ NO structural info. required.
- ❖ NO protein understanding required.

- Experimental control over
  - Mutation rate
  - Environment
  - Selection pressure

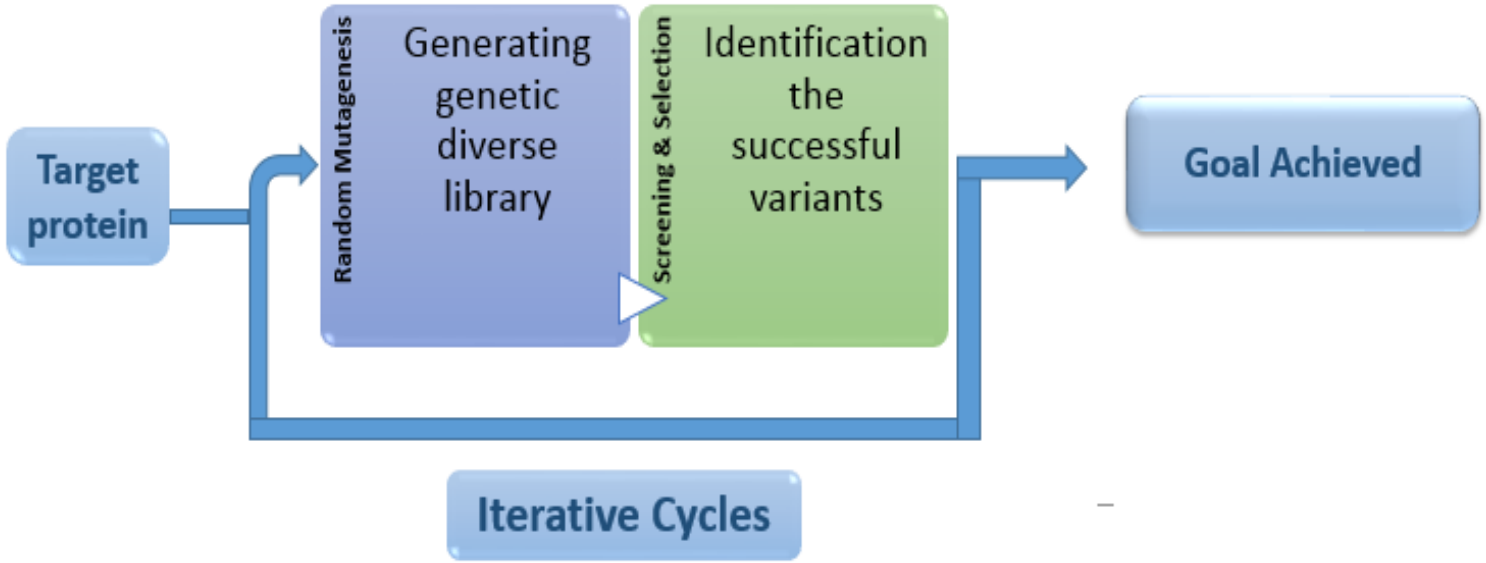
# Directed evolution

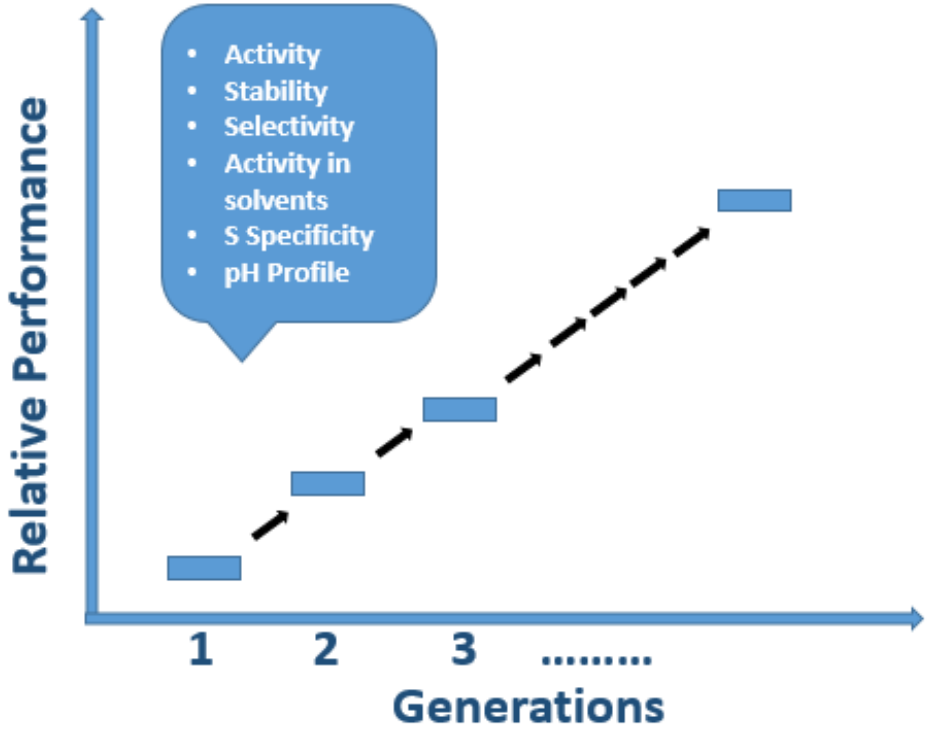
## General procedure:



# Directed evolution

## General procedure:



# Directed evolution

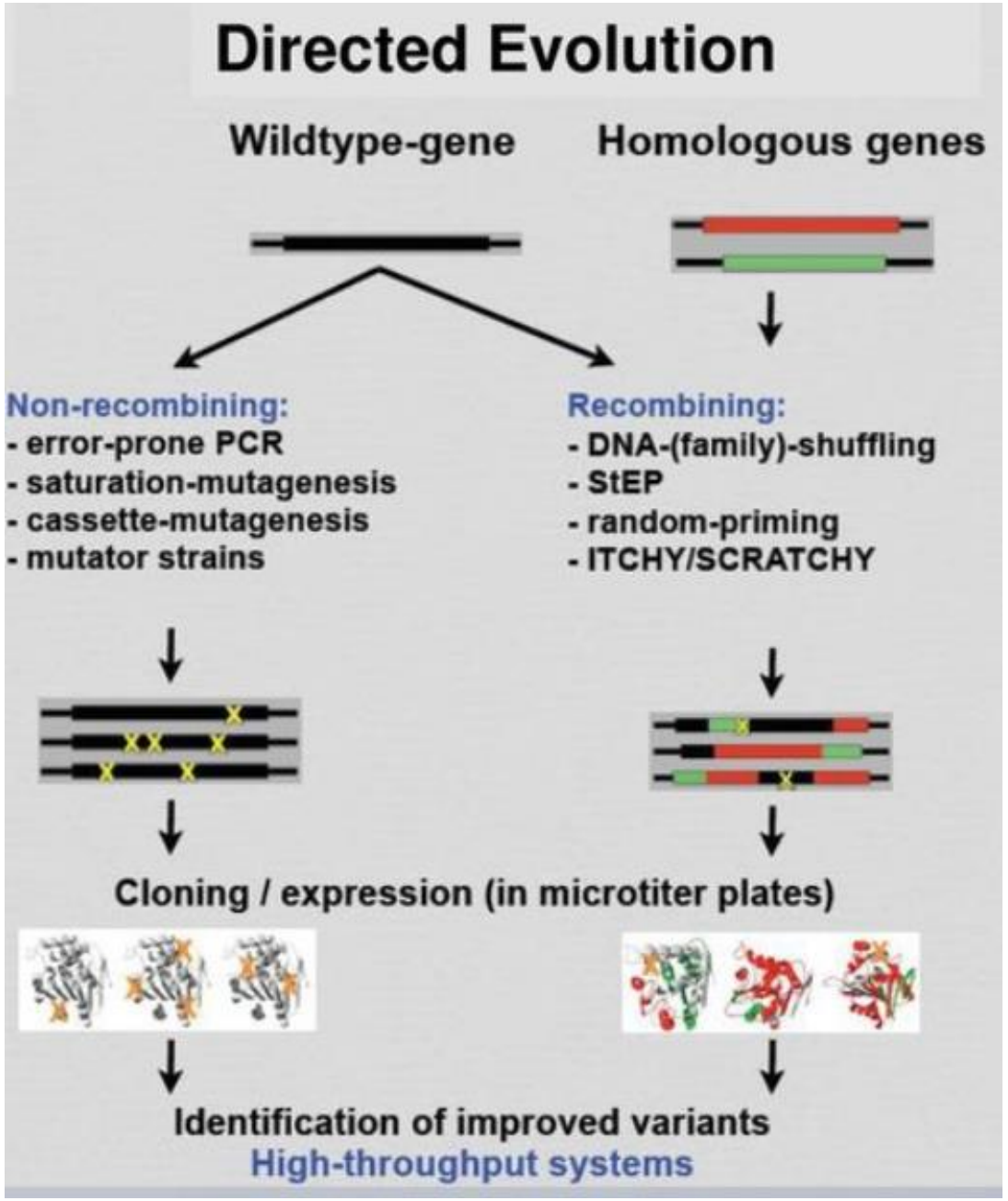
## Generating genetic diverse library

- Point mutations-non-recombinant approaches
  - Error prone PCR,

  (Random mutations)

- Shuffling-recombinant approaches
  - Requires similar genes

  (Can use multiple genes)



# Pros and Cons of Directed evolution

## Advantage

- Simple concepts
- Requires No knowledge of protein
  - (or of mutations)

## Limitation

- large numbers of mutants must be screened in tandem.
- The entire workflow is time- and labor-consuming.
- requirement a robust or high throughput screening methodology.

## RATIONAL DESIGN

1. Computer aided design

2. Site-directed mutagenesis

Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

6. *not applied*

Constructed mutant enzyme

## DIRECTED EVOLUTION

1. *not applied*

2. Random mutagenesis

Library of mutated genes
( >10,000 clones )

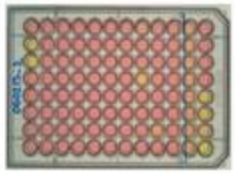
3. Transformation

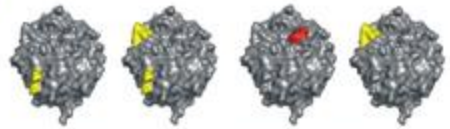
4. Protein expression

5. *not applied*

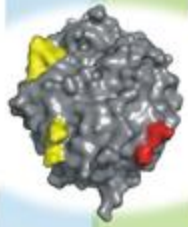
6. Screening and selection

- stability
- selectivity
- affinity
- activity





Selected mutant enzymes



IMPROVED ENZYME

7. Biochemical testing

# Semi-Rational Design

INTEGRATED STRATEGIES

# Semi-Rational Design

- This approach combines the advantages of **rational** & **directed evolution protein design.**

Initial protein designs or modifications done rationally

Engineered protein then evolved to optimise function

# Semi-Rational Design

- Semi-Rational Design create smaller, smarter libraries and enhancing the efficiency of the evolution process.

# *De Novo* Design

CHALLENGING APPROACH

# *De novo* design

**A highly challenging approach, offering the broadest possibility for new structure**

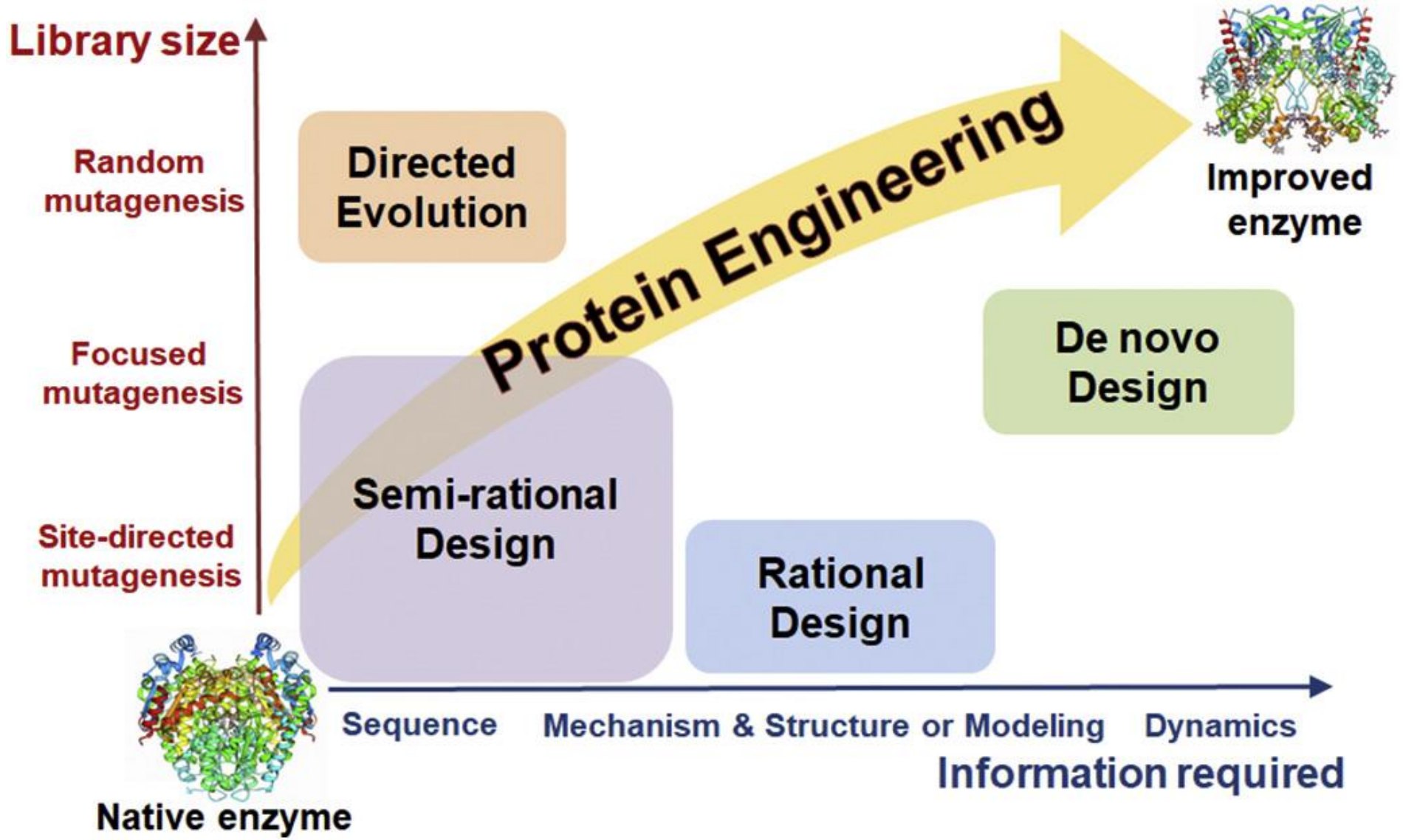
- This approach identify the specific amino acid residues required for lowering the activation energy, and for stabilizing the transition state.

- The strategy is based on silico modeling of the **active site** by using **quantum-mechanic simulations.**

# *De novo* design

A remarkable study reporting a de novo design used Rosetta to accurately create disulfide and cyclized constrained peptides that were exceptionally stable to thermal and chemical denaturation (Bhardwaj et al., 2016).

# Protein engineering Strategies



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- Bhardwaj, G., et al (2016) Accurate de novo design of hyperstable constrained peptides. *Nature* 538 (7625), 329–335.