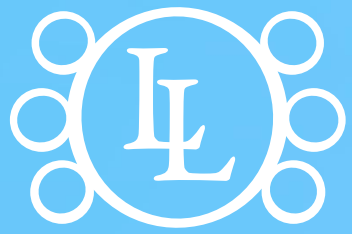

LOSCHMIDT LABORATORIES

PROTEIN ENGINEERING

BIOTECHNOLOGY, ENZYME APPLICATIONS, PROTEIN ENGINEERING APPROACHES

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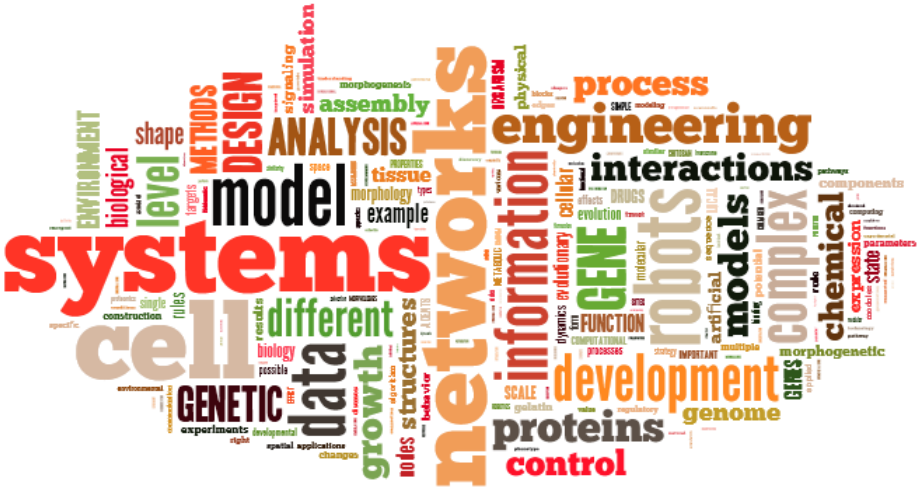
Department of Experimental Biology
Masaryk University, Brno

Outline

- Biotechnology
- Enzymes in technologies
- Enzyme applications
- Enzyme advantages and disadvantages
- Common targets of protein engineering
- Enzymes with desired properties
- Protein engineering, strategies of protein engineering
- Examples of protein engineering

Biotechnology

- any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use

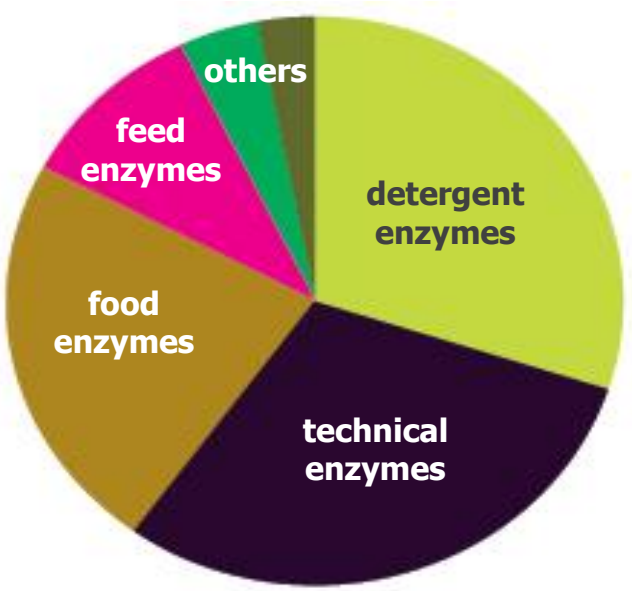


Enzymes

- mostly proteins, RNA (ribozyme)
- catalysis of chemical reactions
- lowering of activation energy = increasing of reaction rate
- non toxic substances
- catalysis under mild conditions
- high efficiency, easy regulation
- high specificity (functional specificity, substrate specificity)
- requirement of cofactors (oxidoreductases, transferases)

Enzymes in technologies

- to manufacture both bulk and high added-value **products**
  - food and animal feed
  - fine chemicals
  - pharmaceuticals
- to provide **services**
  - housework
  - industry
  - environmental technologies
  - analytical purposes
  - diagnostics

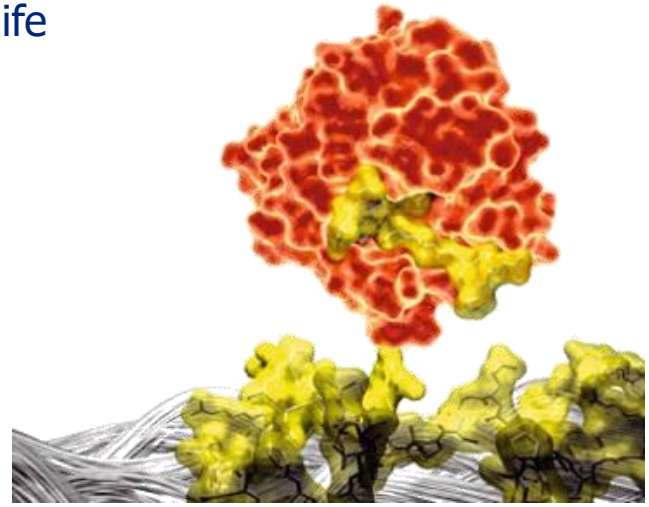


Distribution of sales in **Novozymes**

Detergent industry

- **laundry** and **dishwashing** detergents
- proteases, lipases, amylases, cellulases
- enzymes reduce the environmental load of detergents products

  - save energy and CO_2 emissions by enabling lower wash temperatures
  - have no negative environmental impact on sewage treatment process
  - do not present a risk to aquatic life



Computer simulation:
A laundry detergent enzyme (red) attacks the soil (yellow) on a textile fiber (gray).

Food processing

- improvement of **bread** quality (alpha-amylases)
- production of **sugars** from starch (amylases)
- fruit **juice** and **wine** manufacture (pectinases, cellulases, amylases)
- **brewing** industry (enzymes from barley)
- **milk** industry (chymosin, beta-galactosidases, lactases)
- **meat** tenderizers (papain, bromelain)

Paper industry

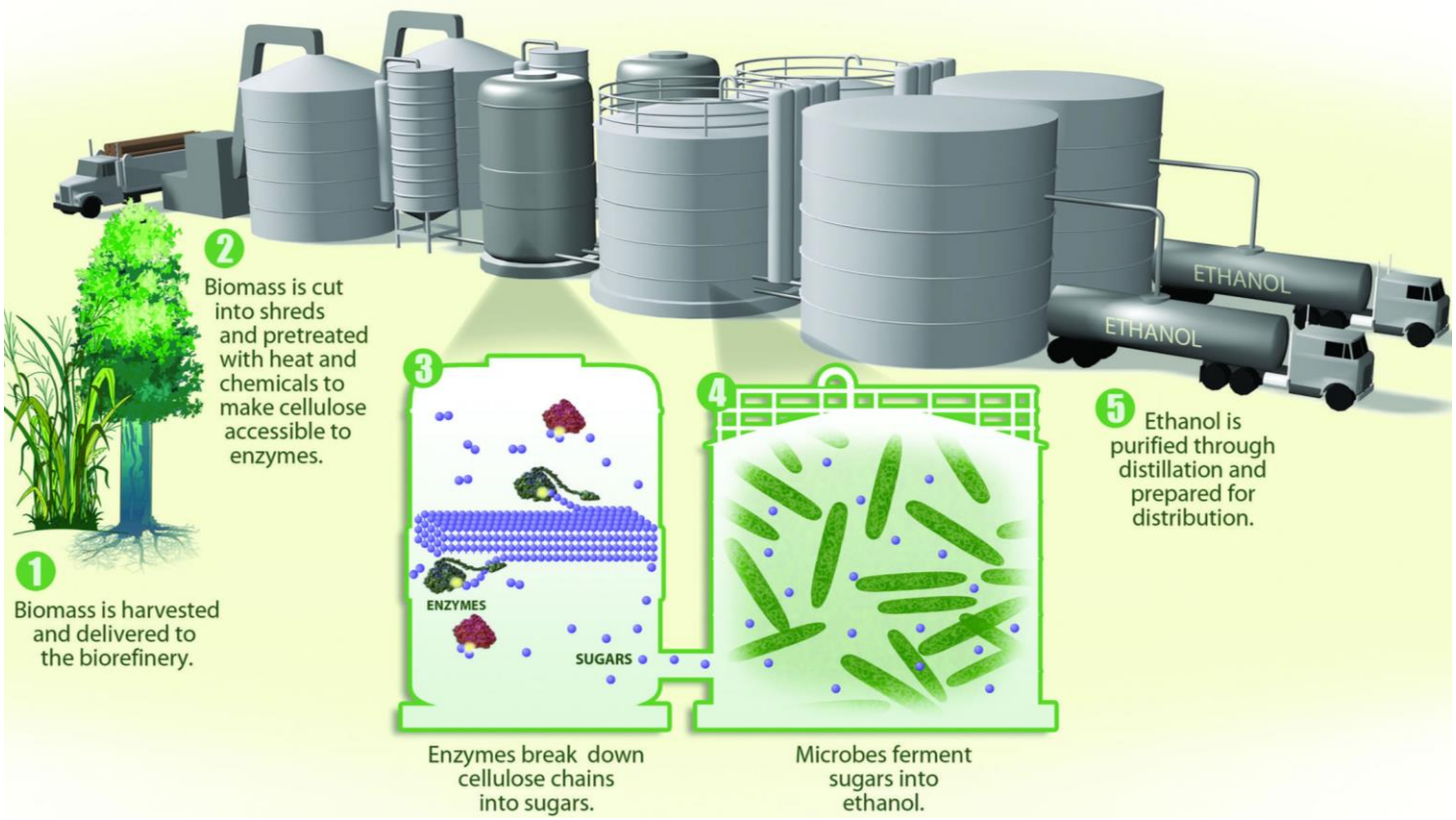
- **xylanases** reduce bleach required for decolorizing
- **cellulases** smooth fibers and enhance water drainage
- **amylases** degrade starch to lower viscosity sugars
- **lipases** reduce pitch

Textile industry

- **cellulases** are used in denim washing for a stone-washed look
- **amylases** are used for desizing of textile fibers
- **catalases** are used for bleach clean-up
- **laccases** are used as bleaching agents

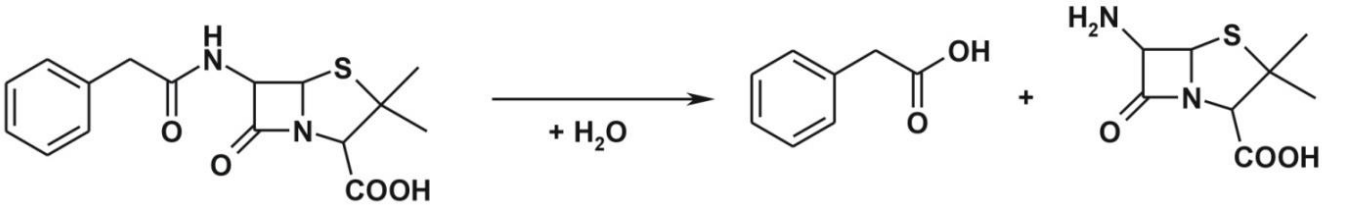
Biofuel industry

- bacterial and fungal **cellulases** break down cellulose into sugars that can be fermented



Synthesis of chemicals and pharmaceuticals

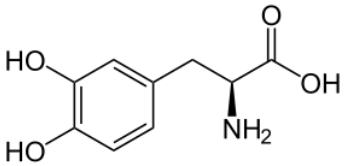
- kiloton-scale production of **acrylamide** (nitrile hydratase)
- synthesis of 6-APA – precursor of antibiotics (penicilin amidase)



penicillin G — 6-amino penicillanic acid

- synthesis of **single enantiomers** – precursors of drugs (lipases)

dopa



(*R*)- causes granulocytopenia
(*S*)- anti-parkinson

Specialty enzymes

- clinical **analytical** applications
  - glucose biosensor (glucose oxidase)
  - alkaline phosphatase and peroxidase immunoassays
- **flavor** production
  - production of glutamates used in food flavouring (glutamases)
- **personal care** products
  - contact lens cleaning (lipase, proteinase)
  - in toothpaste to convert glucose to H_2O_2 (glucose oxidase)
- **DNA** technology
  - restriction enzymes (restriction endonuclease)
  - DNA-modifying enzymes (ligase)

Bioremediation

- use of microorganisms or their enzymes to return the **environment** altered by contaminants to its original condition
- examples of biodegradation **enzymes and pollutants**

  - monooxygenases – alkane, steroids, fatty acid and aromatic compounds
  - dioxygenases – phenolic and aromatic compounds
  - peroxidases – lignin and other phenolic compounds
  - lipases – organic pollutants such as oil spill
  - cellulases – cellulosic substances
  - haloalkane dehalogenases – halogenated hydrocarbons
  - proteases – proteins

Advantages of enzymes

- high catalytic **efficiency**
- high degree of **selectivity**
- **compatibility** of each other
- **reusability**
- **sustainability**
  - produced from biomass
  - easily biodegradable
  - non-toxic and non-flammable
  - less byproducts and wastes
  - operate at mild conditions

Disadvantages of enzymes

- generally **less stable**
- insufficient **activity**
- insufficient **selectivity**
- **cofactor** requirement
- **allergies**

Common targets of protein engineering

- enzyme **stability**
- enzyme **activity**
- enzyme **substrate specificity**
- enzyme **enantioselectivity**

Enzyme stability

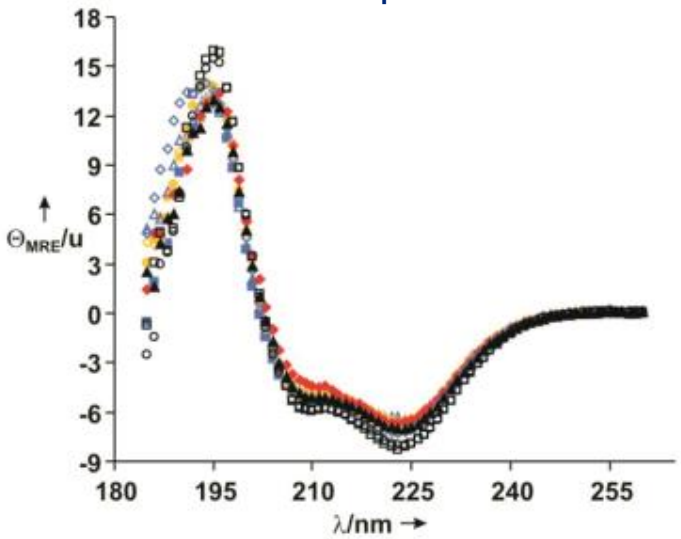
- thermodynamic x kinetic stability

Definitions of various stability parameters.

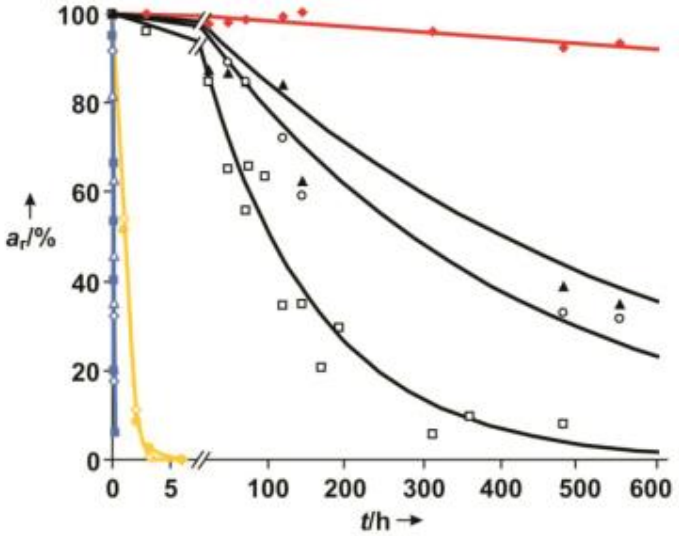
Measure	Symbol	Type of stability	Definition
Free energy of unfolding	ΔG_u	Thermodynamic	Change in Gibbs free energy going from the folded to unfolded state
Melting temperature	T_m	Thermodynamic	The temperature at which half of the protein is in the unfolded state
Unfolding equilibrium constant	K_u	Thermodynamic	The concentration of unfolded species divided by the concentration of folded species
Half-concentration	$C_{1/2}$	Thermodynamic	The concentration of denaturant needed to unfold half of the protein (chemical equivalent of T_m)
Observed deactivation rate constant	$k_{d,obs}$	Kinetic	Overall rate constant for going from native to deactivation species
Half-life	$\tau_{1/2}$	Kinetic	Time required for residual activity to be reduced to half
Temperature of half-inactivation	T_{50}	Kinetic	Temperature of incubation to reduce residual activity by half during a defined time period
Optimum temperature	T_{opt}	Kinetic	Temperature leading to highest activity
Total turnover number	TTN	Kinetic	Moles of product produced over the lifetime of the catalyst

Enzyme stability

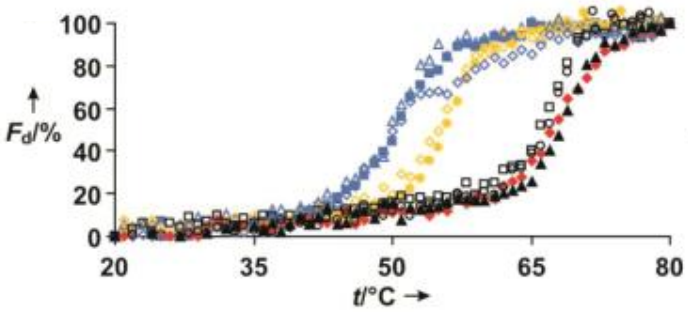
CD spectra



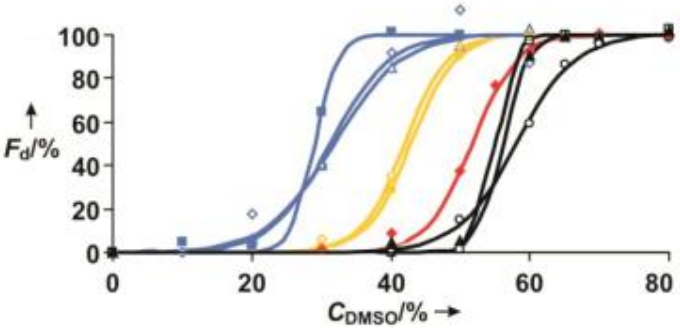
Half-life



Melting temperature

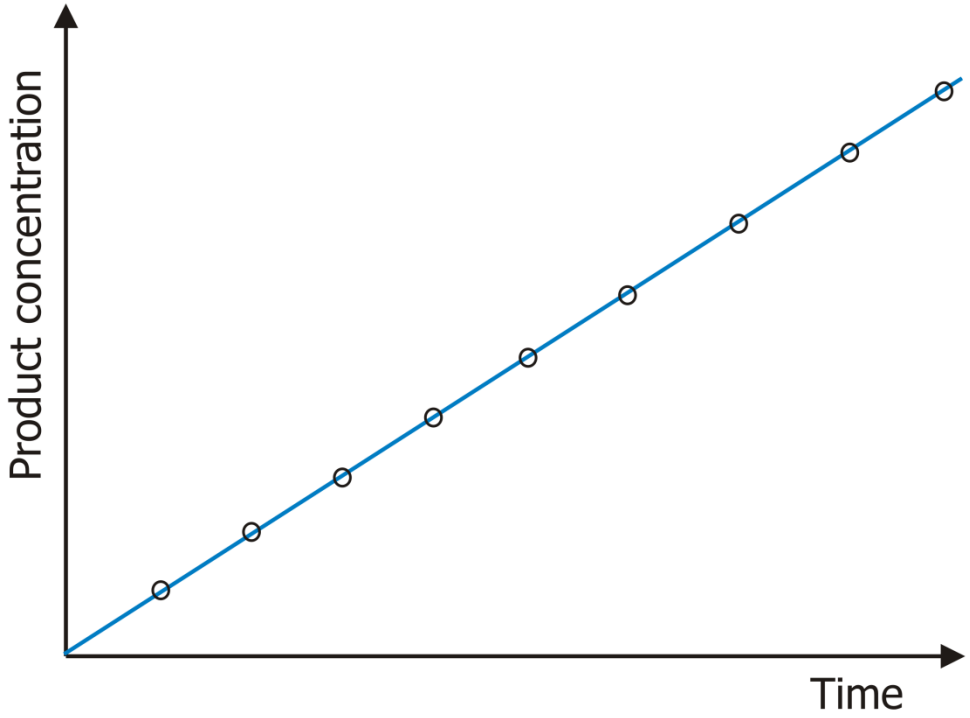


Half-concentration



Enzyme activity

- enzyme property measured by the increase in reaction rate
- reaction rate – concentration of product produced per time



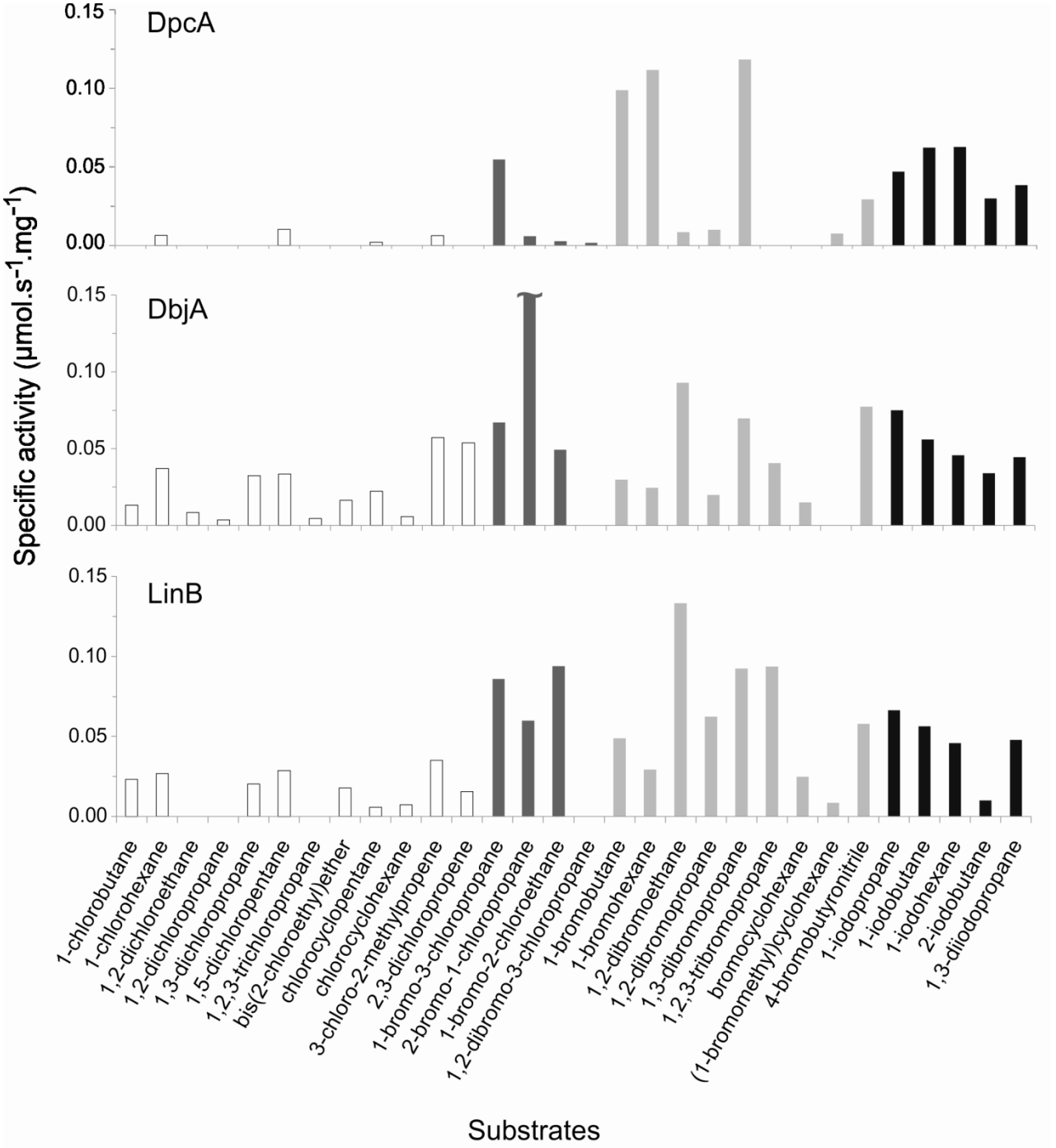
Enzyme activity

Units of enzyme activity

- **SI unit, katal (kat)** – amount of enzyme that catalyzes conversion of 1 mole of substrate per second ($mol.s^{-1}$)
- **activity unit (U)** – amount of enzyme that catalyzes conversion of 1μmol of substrate per minute ($\mu mol.min^{-1}$), 1U = 16.67 nkat
- **specific activity** – activity of enzyme per milligram of total protein ($\mu mol.s^{-1}.mg^{-1}$)

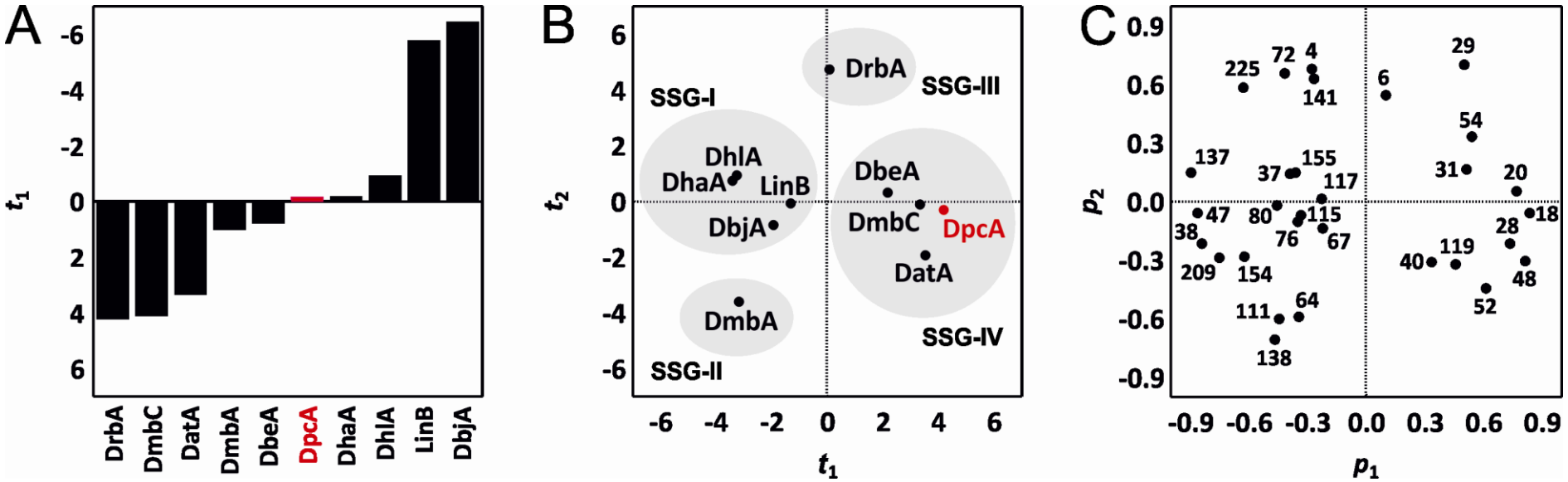
Enzyme substrate specificity

- definition of substrate specificity – **discrimination between** several **substrates competing for** enzyme **active site**
- commonly used meaning – enzyme activity with alternative substrate in the absence of specific (native) substrate
- enzyme activity measured towards a broad range of substrates under similar conditions
- fingerprint of enzyme ability to convert various substrates
- usually compared for different enzymes
- quantitative comparison of enzyme data – statistical analysis


DpcA
DbjA
LinB
0.15
0.10
0.05
0.00
Specific activity (µmol.s-1.mg-1)
1-chlorobutane
1-chlorohexane
1,2-dichloroethane
1,2-dichloropropane
1,3-dichloropropane
1,5-dichloropentane
1,2,3-trichloropropane
bis(2-chloroethyl)ether
chlorocyclopentane
chlorocyclohexane
3-chloro-2-methylpropene
2,3-dichloropropene
1-bromo-3-chloropropane
2-bromo-1-chloropropane
1-bromo-2-chloroethane
1,2-dibromo-3-chloropropane
1-bromobutane
1-bromohexane
1,2-dibromoethane
1,2-dibromopropane
1,3-dibromopropane
1,2,3-tribromopropane
bromocyclohexane
(1-bromomethyl)cyclohexane
4-bromobutyronitrile
1-iodopropane
1-iodobutane
1-iodohexane
2-iodobutane
1,3-diiodopropane
Substrates

Enzyme substrate specificity

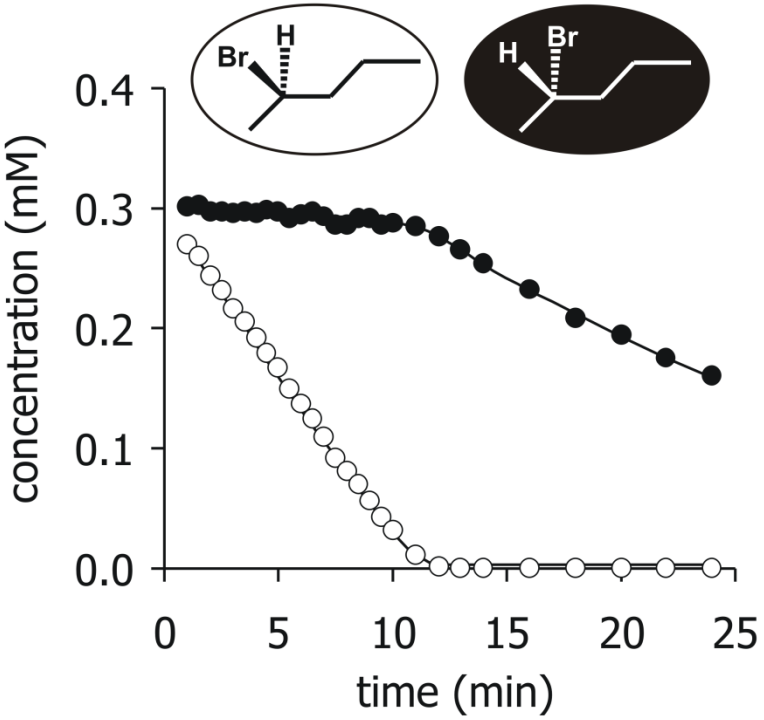
- statistical analysis of substrate specificity data – sorting of enzymes according to their preference to different substrates
- identification of unique SSGs within one enzyme family



SSG – substrate specificity group

Enzyme enantioselectivity

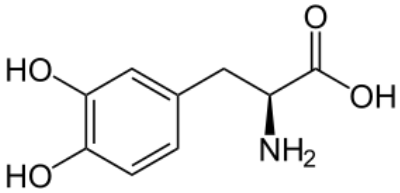
- discrimination between enantiomeric substrates or products
- preferential conversion of one enantiomer of chiral substrate



Enzyme enantioselectivity

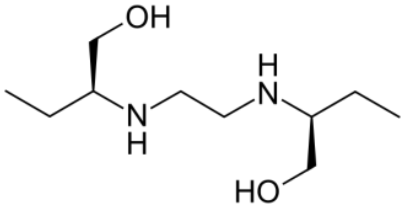
- discrimination between enantiomeric substrates or products
- preferential conversion of one enantiomer of chiral substrate

dopa



(*R*)- causes granulocytopenia
(*S*)- anti-parkinson

ethambutol



(*R*,*R*)- causes blindness
(*S*,*S*)- tuberculostatic

Enzyme enantioselectivity

- characterized by **enantiomeric ratio *E***

$$E = \frac{k_{cat}^{(R)}/K_m^{(R)}}{k_{cat}^{(S)}/K_m^{(S)}}$$

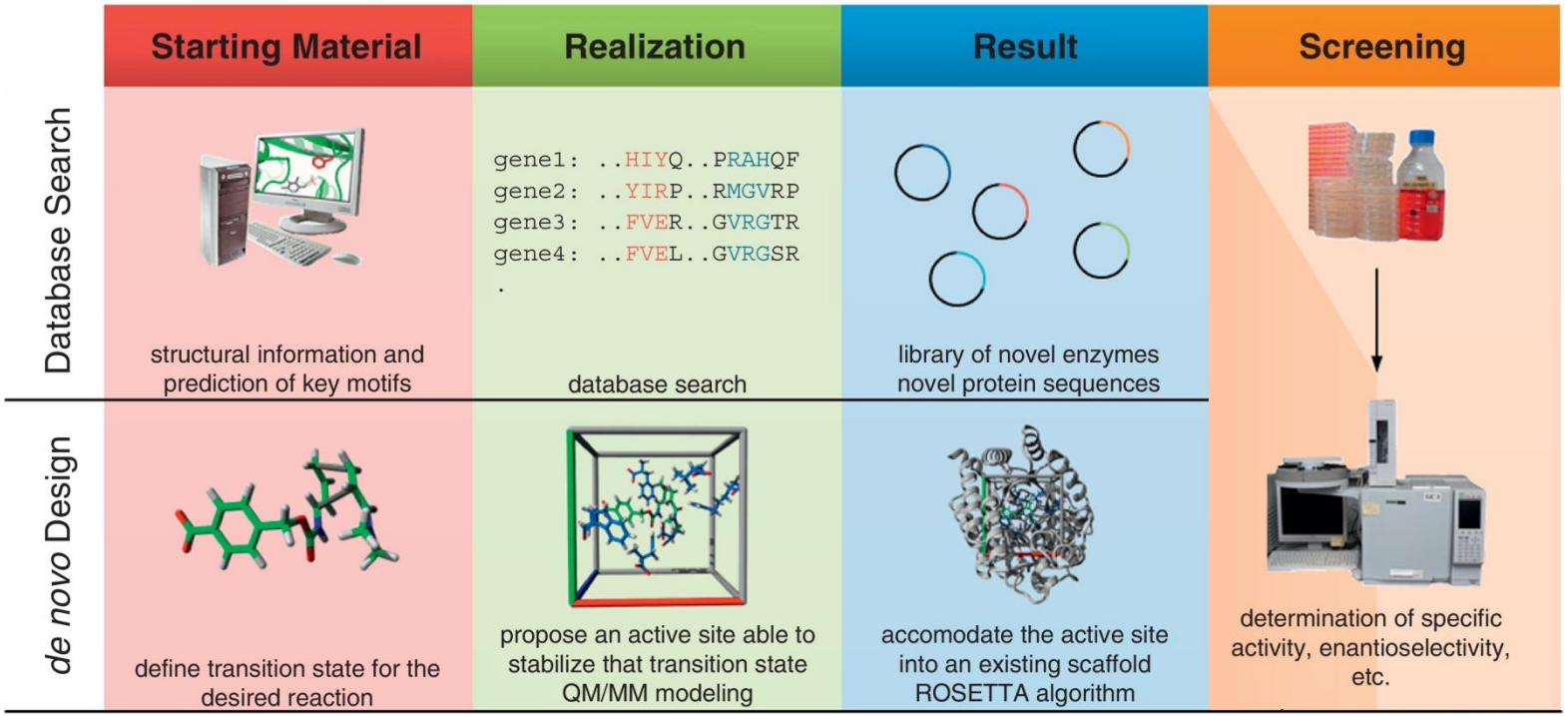
- *E* – description how the enzyme discriminates between the enantiomers of a substance under given reaction conditions
- *E* – intrinsic property of a given system consisting of the enzyme, its substrate and the environment
- *E* – strongly affected by the environment (T, pH, solvents, …)

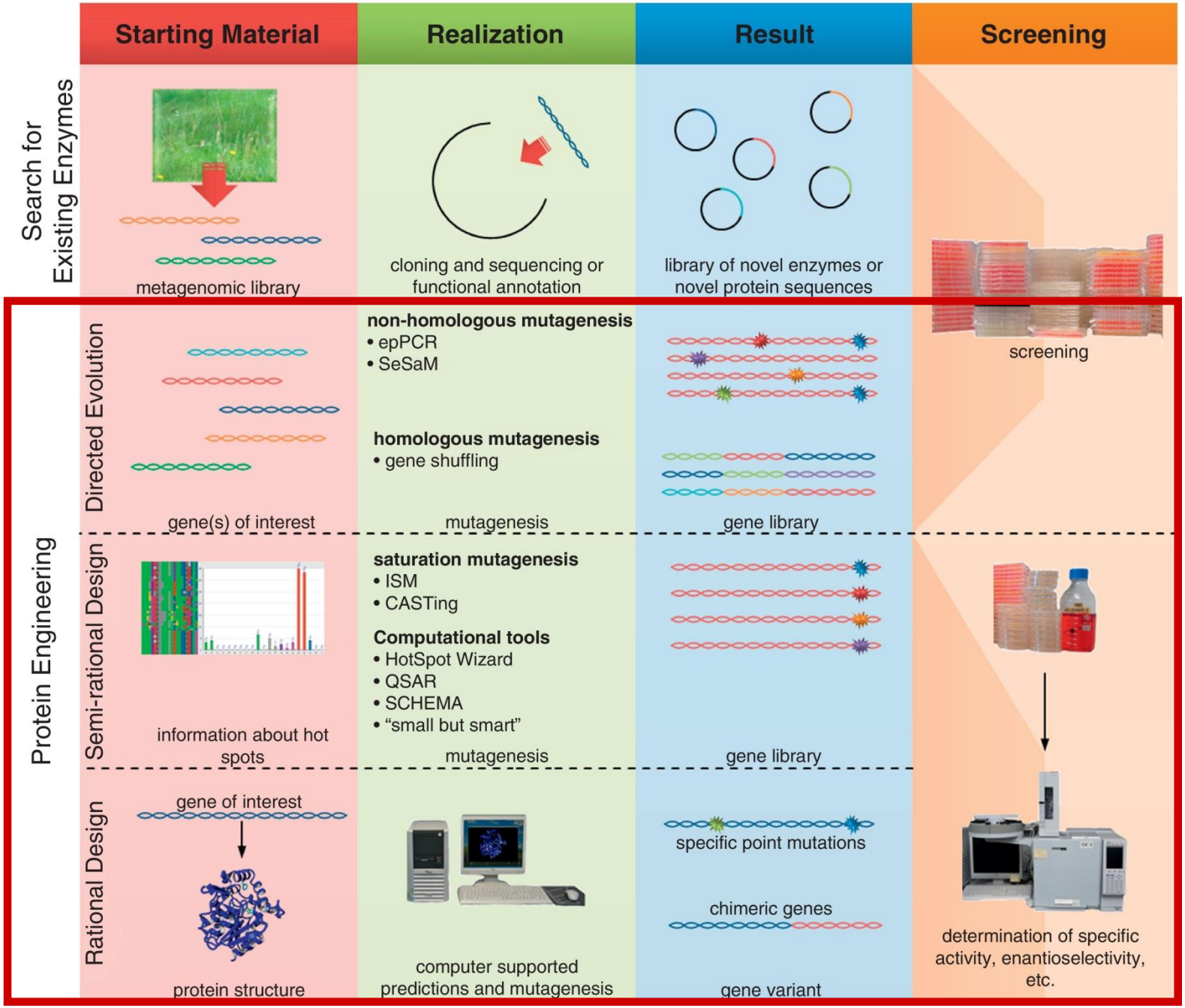
Process design criteria

- higher activity at process conditions
- increased process stability
- increased thermostability to run at higher temperatures
- stability to organic solvents
- absence of substrate and/or product inhibition
- increased selectivity (enantio-, regio-, chemo-)
- accept new substrate
- catalyse new reactions

Enzymes with desired properties

- concepts used to identify/create enzymes with desired properties




Starting Material
Realization
Result
Screening
Search for Existing Enzymes
metagenomic library
cloning and sequencing or functional annotation
library of novel enzymes or novel protein sequences
screening
Protein Engineering
Directed Evolution
non-homologous mutagenesis
• epPCR
• SeSaM
homologous mutagenesis
• gene shuffling
gene(s) of interest
mutagenesis
gene library
Semi-rational Design
saturation mutagenesis
• ISM
• CASTing
Computational tools
• HotSpot Wizard
• QSAR
• SCHEMA
• "small but smart"
information about hot spots
mutagenesis
gene library
Rational Design
gene of interest
specific point mutations
chimeric genes
protein structure
computer supported predictions and mutagenesis
gene variant
determination of specific activity, enantioselectivity, etc.

Protein engineering

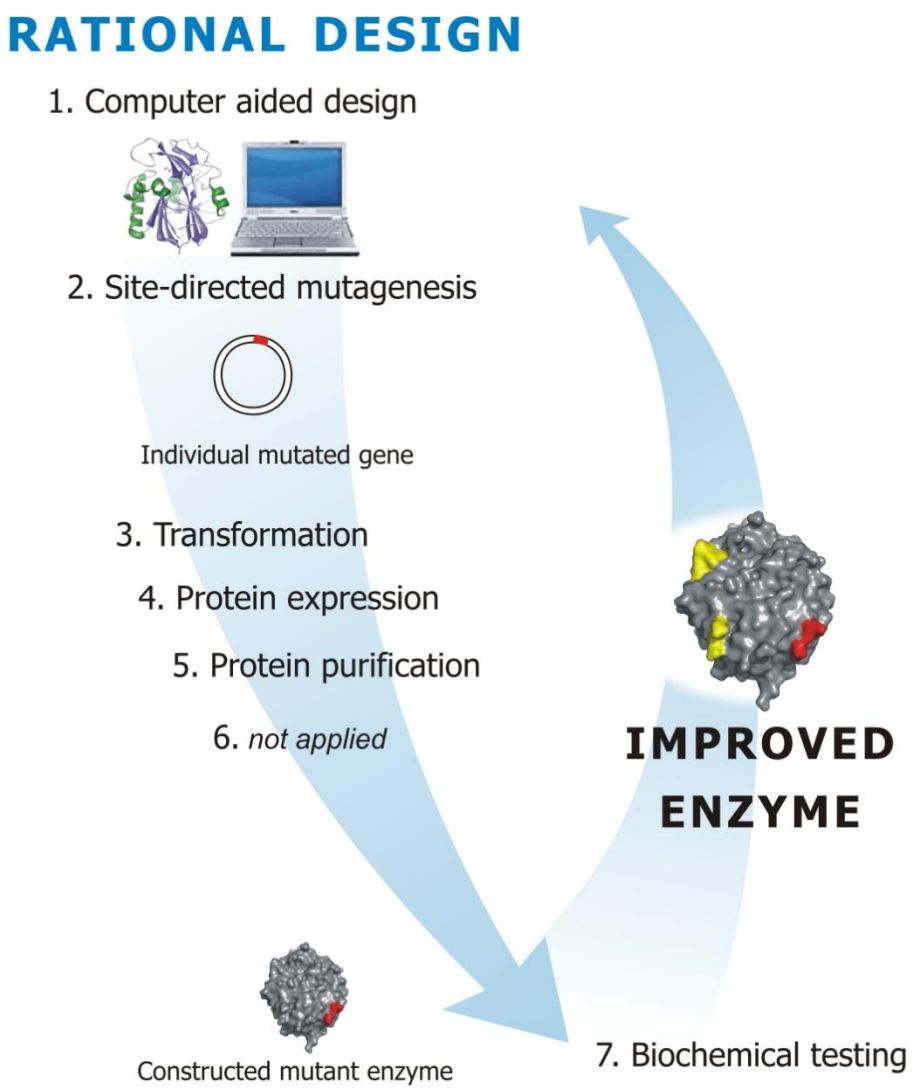
- altering the structure of existing protein to improve its properties
- overcome limitations of natural enzymes as catalysts
- basic understanding of how enzymes fuction and have evolved
- already point to many industrial successes

- "*in the past, an enzyme-based process was designed around the limitations of the enzymes; today, the enzyme is engineered to fit the process specifications*"

Protein engineering

- three main approaches of protein engineering
  - **rational design**
  - **directed evolution**
  - **semi-rational design**

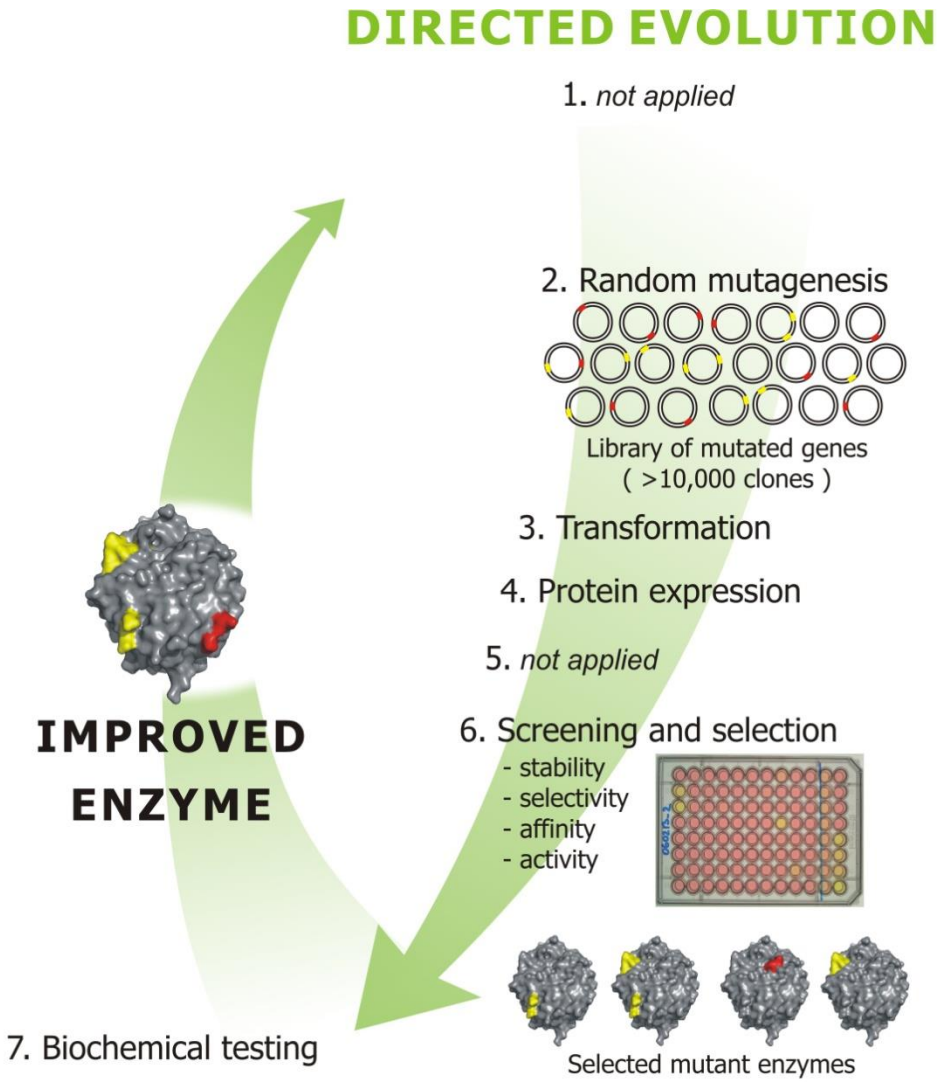
Rational design



Rational design

- **site-specific changes on the target enzyme**
- few amino-acid substitutions that are predicted to elicit desired improvements of enzyme function
- **based on** detailed **knowledge of protein structure, function and catalytic mechanism**
- relatively simple characterization of constructed variants
- factor limiting general application of rational design – complexity of structure-fuction relationship in enzymes

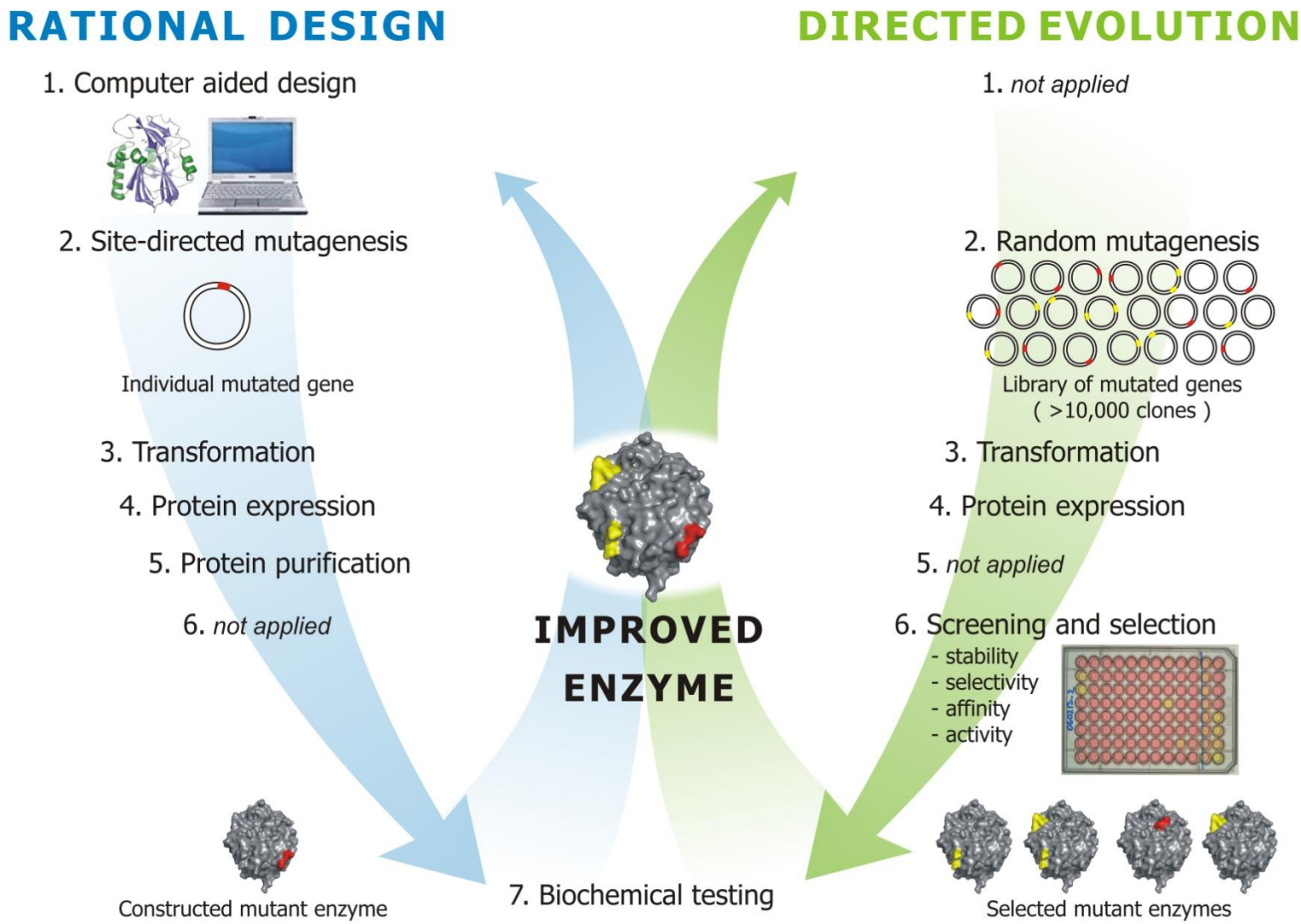
Directed evolution



Directed evolution

- **large numbers of mutants randomly generated**
- mimicking natural evolution processes
- evolution without knowledge of enzyme structure and function
- **identification of** functionally **improved variants required powerful screening or selection**
- limitation – necessity of developing a high-throughput screening

Semi-rational design



Semi-rational design

- also called focused directed evolution
- based on knowledge of structure and fuction of target enzyme
- **combine** advantages of **rational and random approaches**
- **selection of promising target sites**
- **creation of** small focused "**smart**" **libraries**
- elimination the need of high-throughput screening
- increase likelihood of beneficially modifying property

Protein engineering approaches

Comparison of protein engineering approaches

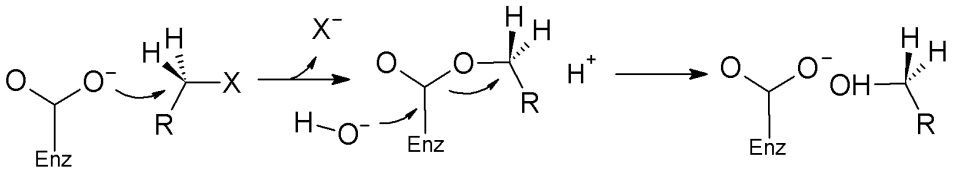
	Rational design	Directed evolution	Semi-rational design
high-throughput screening/selection	not essential	essential	advantageous but not essential
structural and/or functional information	both essential	neither essential	either is sufficient
sequence space exploration	low	high, random	moderate, targeted
probability to obtain synergistic mutations	moderate	low	high

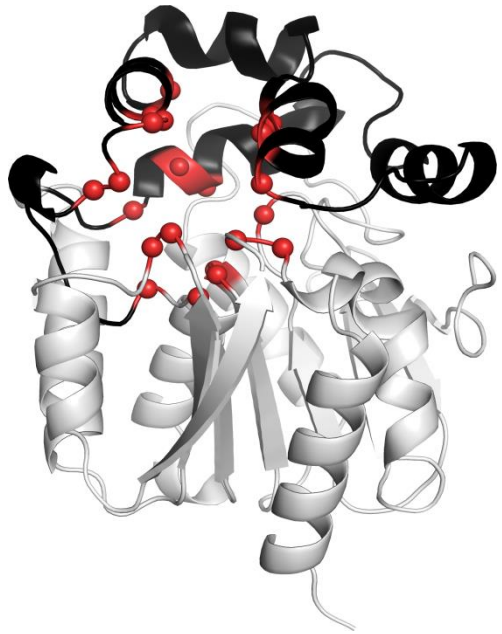
Process design criteria

- **higher activity at process conditions**
- **increased process stability**
- **increased thermostability** to run at higher temperatures
- **stability to organic solvents**
- absence of substrate and/or product inhibition
- increased selectivity (enantio-, regio-, chemo-)
- accept new substrate
- catalyse new reactions

Target enzyme family

- **haloalkane dehalogenases** (HLDs)
- microbial enzymes – α/β hydrolases[1]
- hydrolytic cleavage of C-X bond

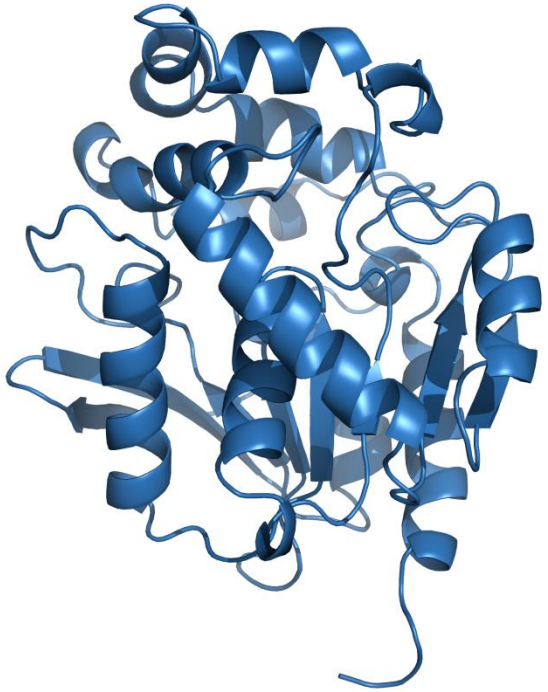




- broad substrate specificity
- high enantioselectivity
- potential applications: biodegradation, biosensing, biosynthesis

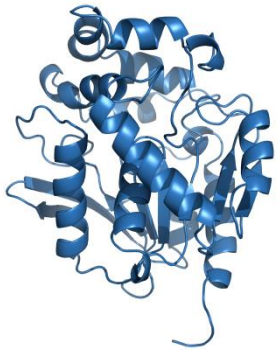
[1]Ollis, D.L. et al. : *Protein Eng*. 5, 197-211 (1992)

Studied HLD



DhaA
from *Rhodococcus rhodochrous*

Directed evolution

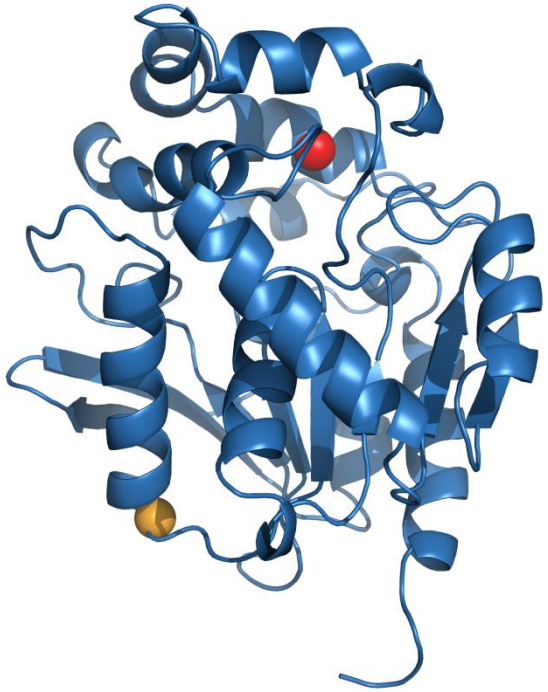


DhaA

epPCR

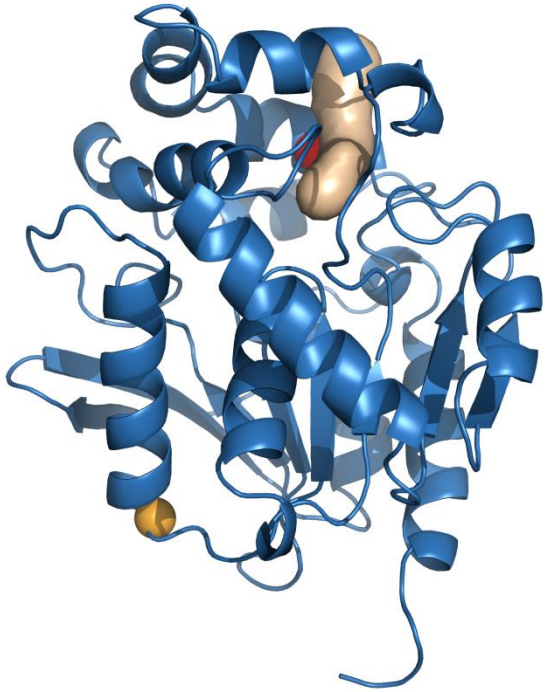
4 positive hits

Mutant resistant to DMSO

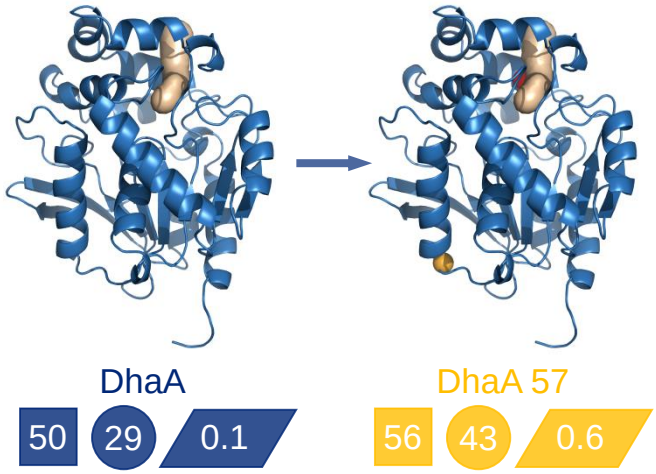


DhaA 57

Mutant resistant to DMSO



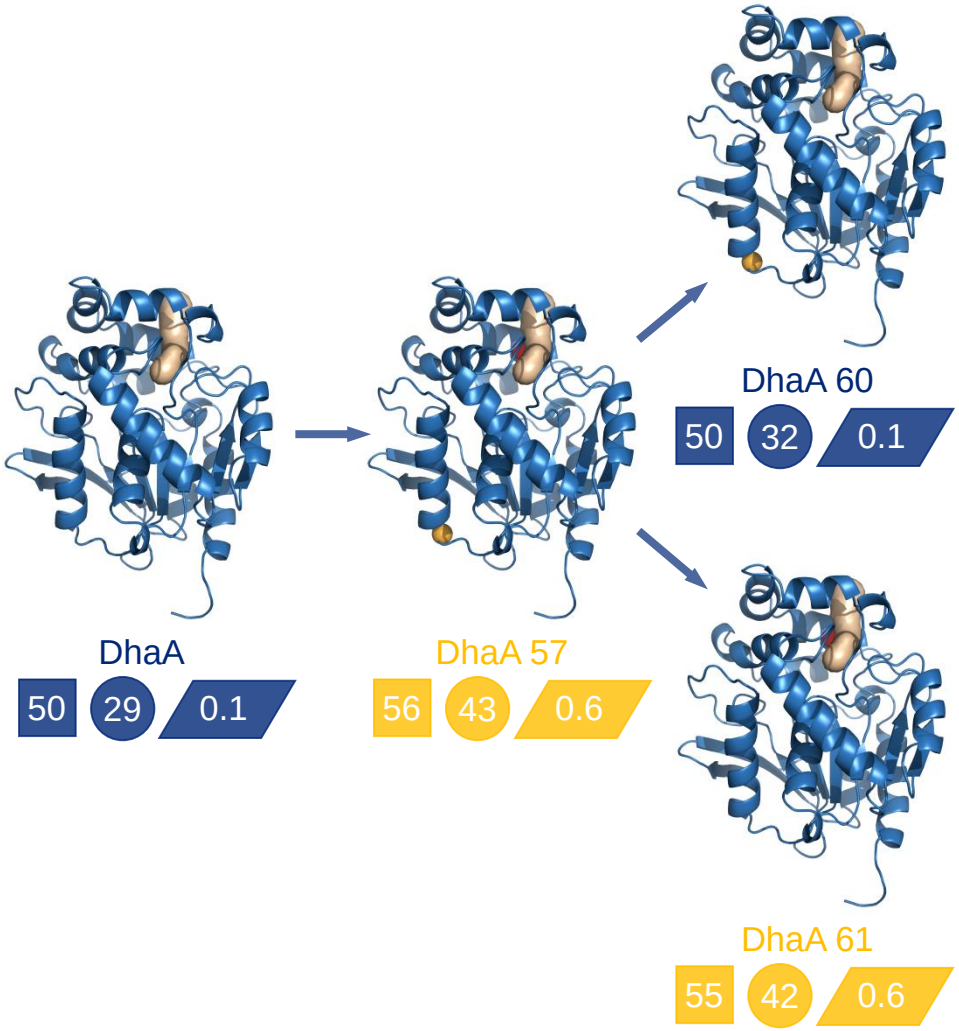
DhaA 57



melting temperature in buffer (°C)

half-concentration of DMSO (%)

half-life in 40% DMSO at 37 °C (h)



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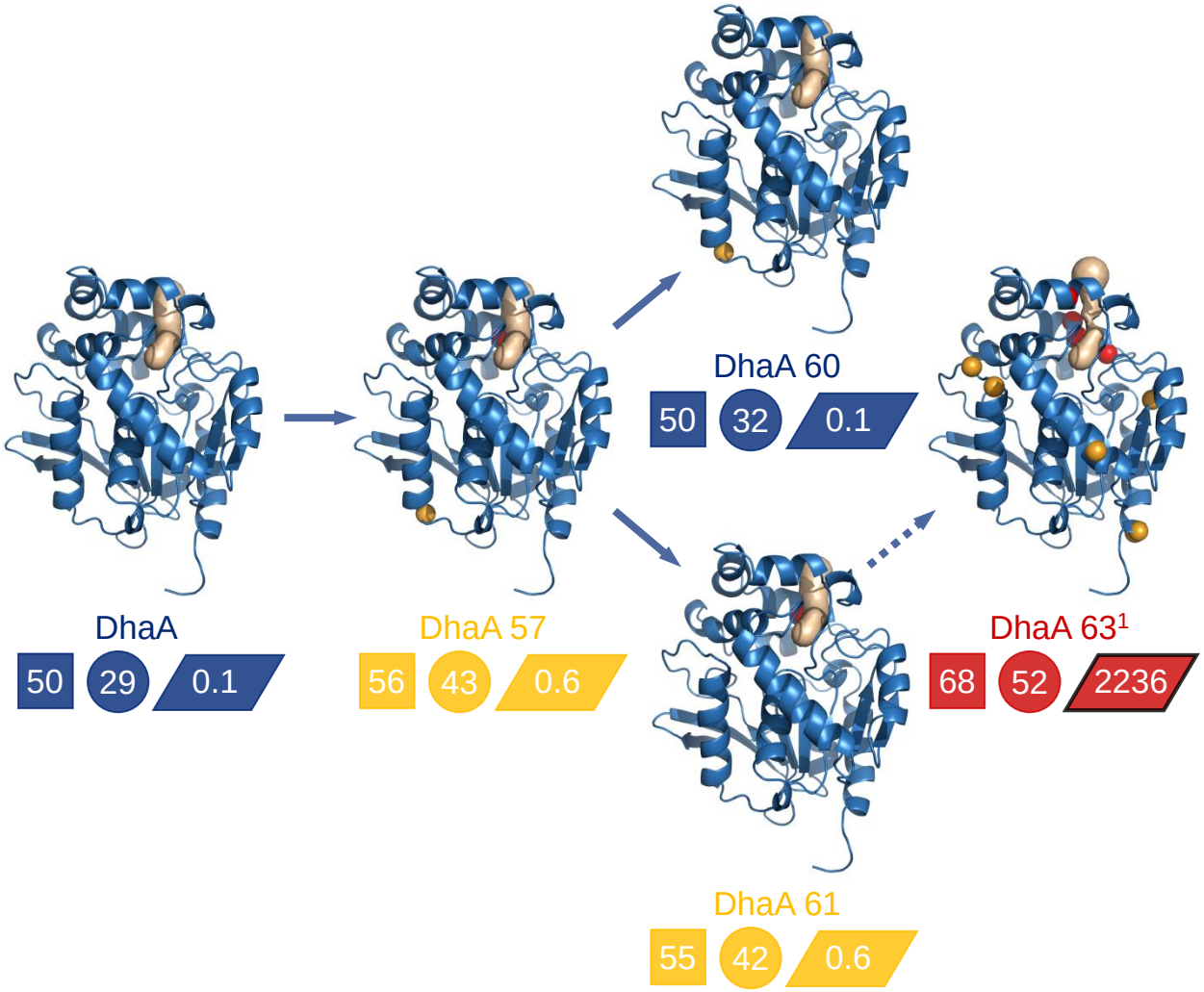
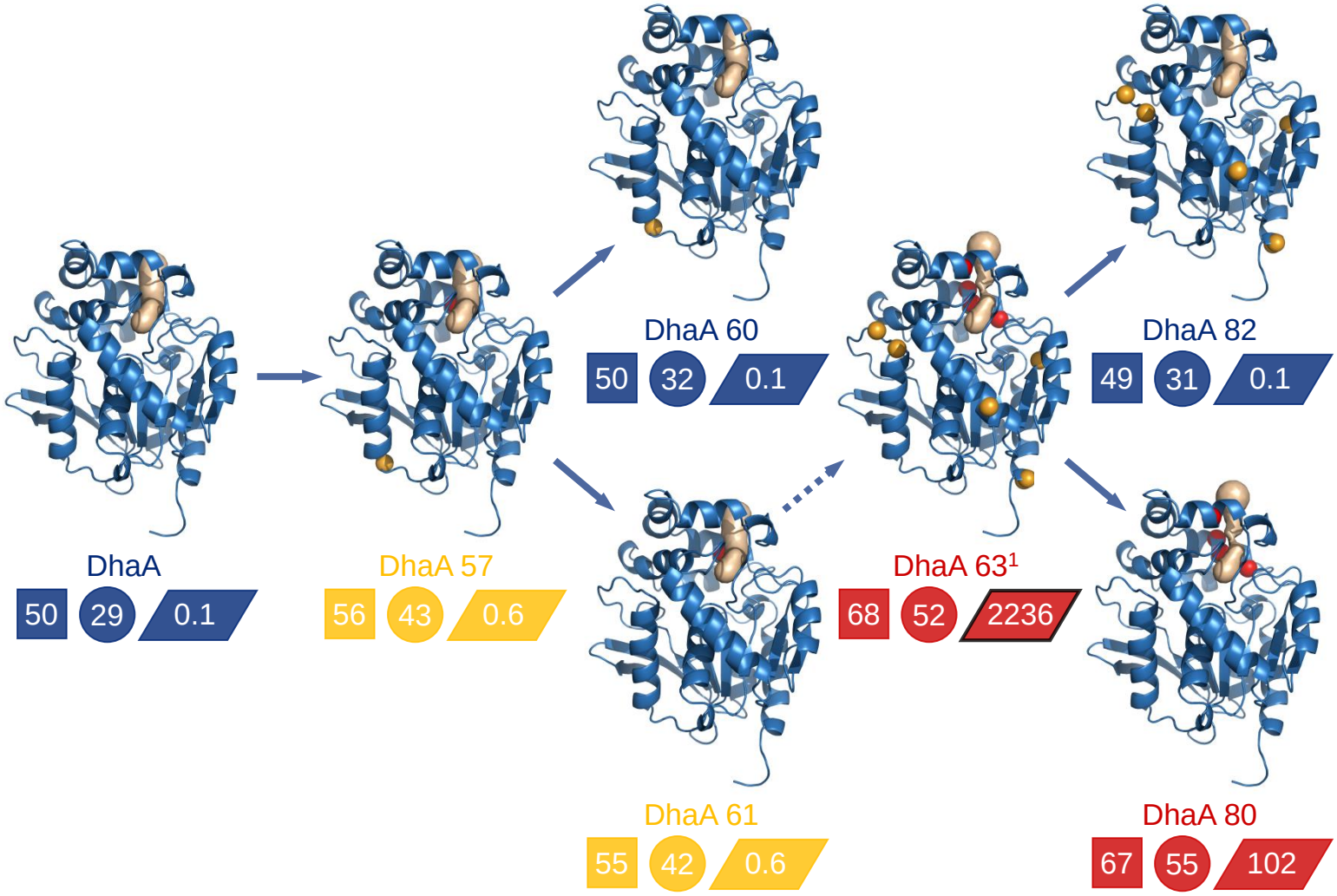


melting temperature in buffer (°C)

half-concentration of DMSO (%)

half-life in 40% DMSO at 37 °C (h)

[1]Gray, K.A. et al.: *Adv*. *Synth*. *Catal*. 343, 607-617 (2001)

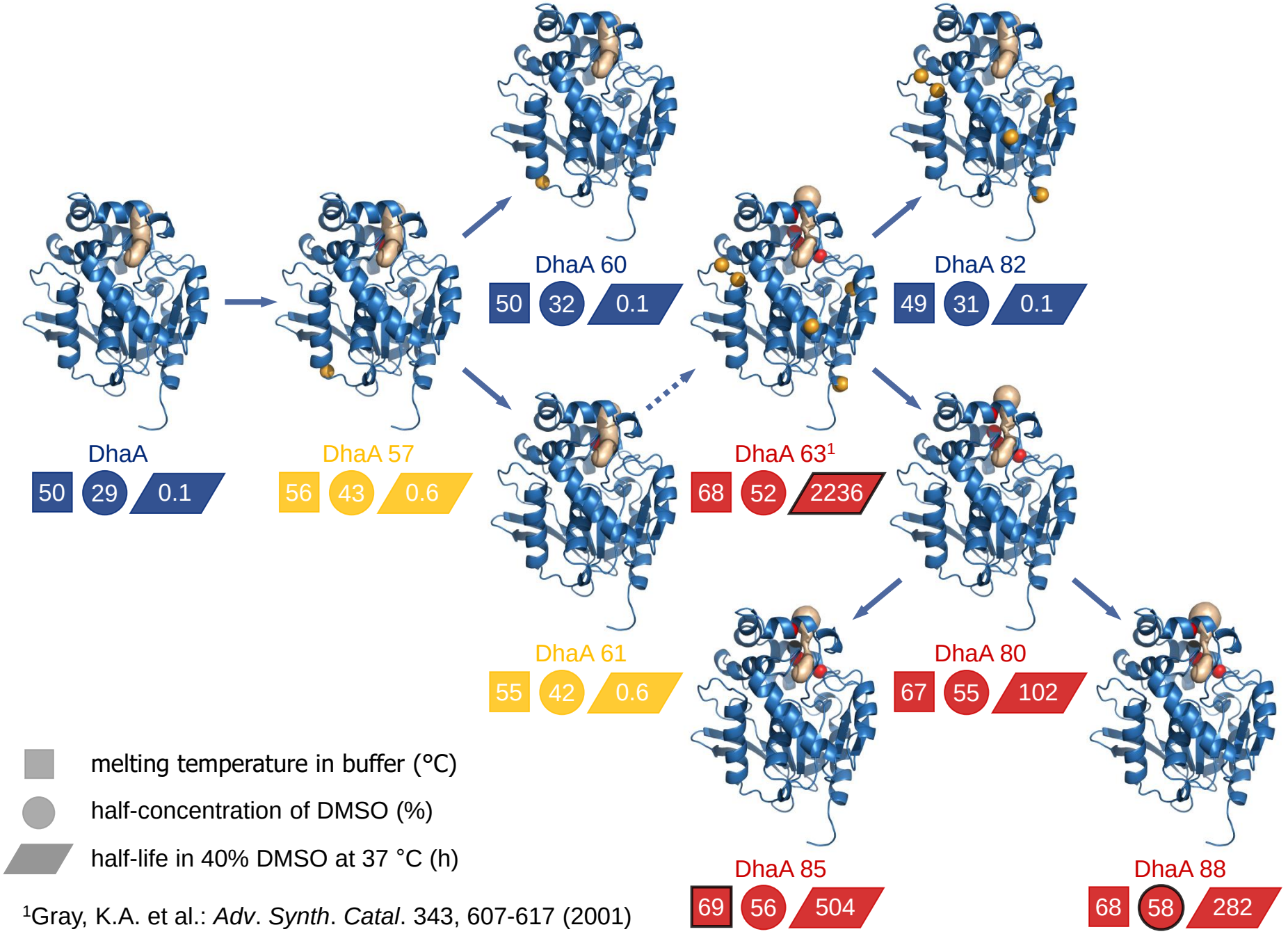


melting temperature in buffer (°C)

half-concentration of DMSO (%)

half-life in 40% DMSO at 37 °C (h)

[1]Gray, K.A. et al.: *Adv*. *Synth*. *Catal*. 343, 607-617 (2001)



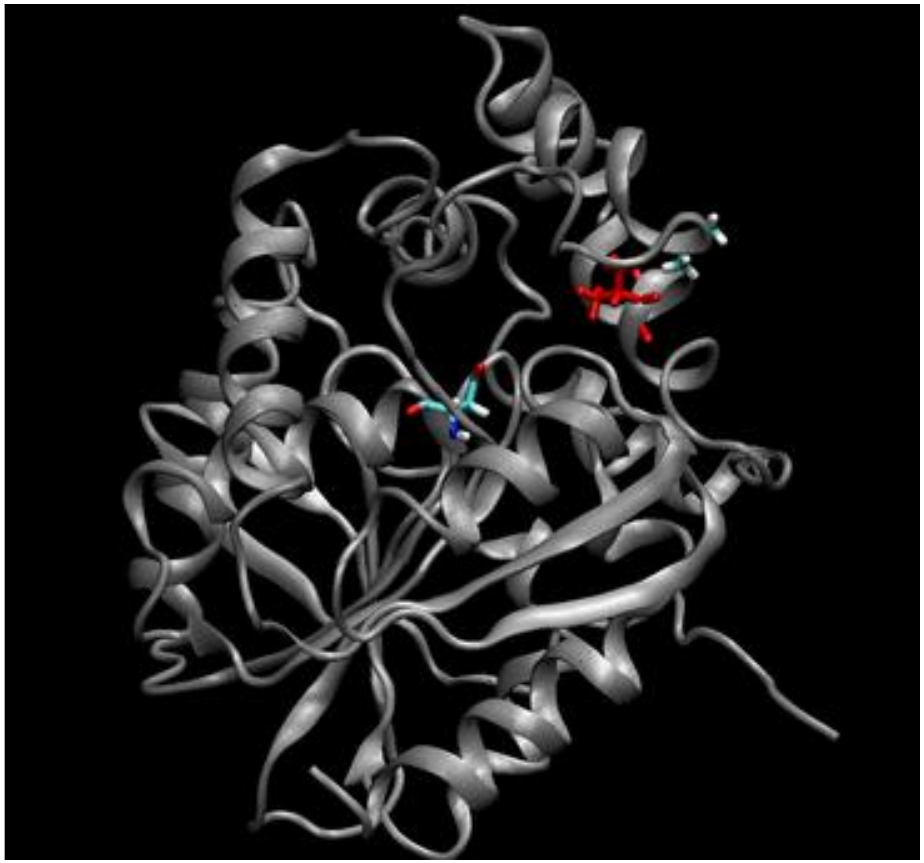
[1]Gray, K.A. et al.: *Adv. Synth. Catal.* 343, 607-617 (2001)

Mutant resistant to DMSO

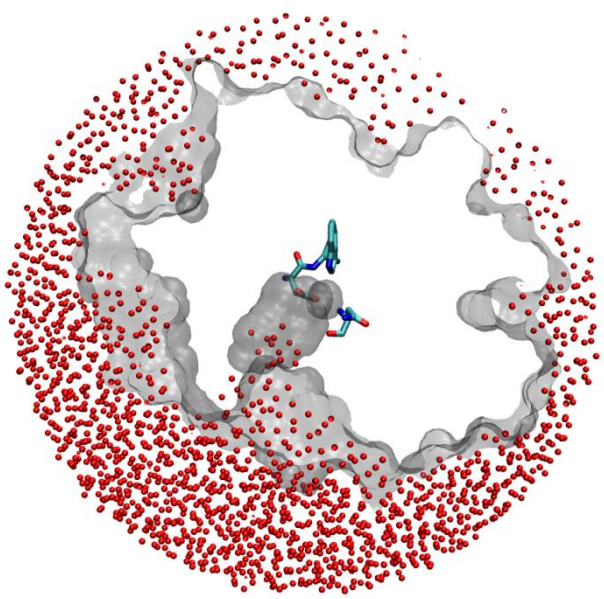
DhaA wt

Mutant resistant to DMSO

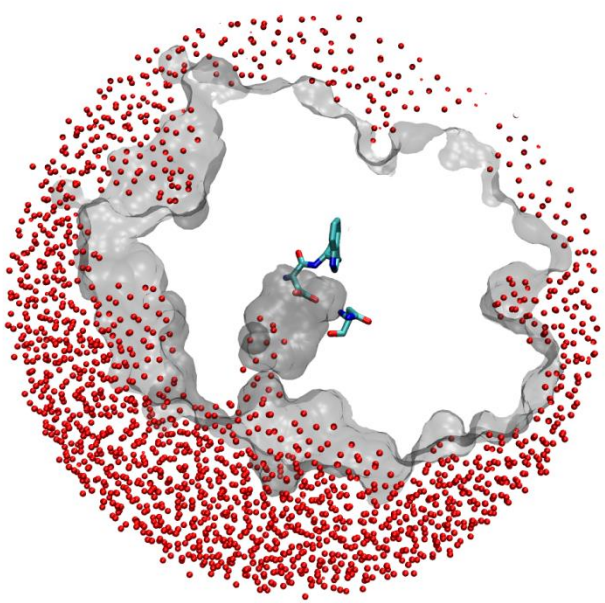
DhaA 57



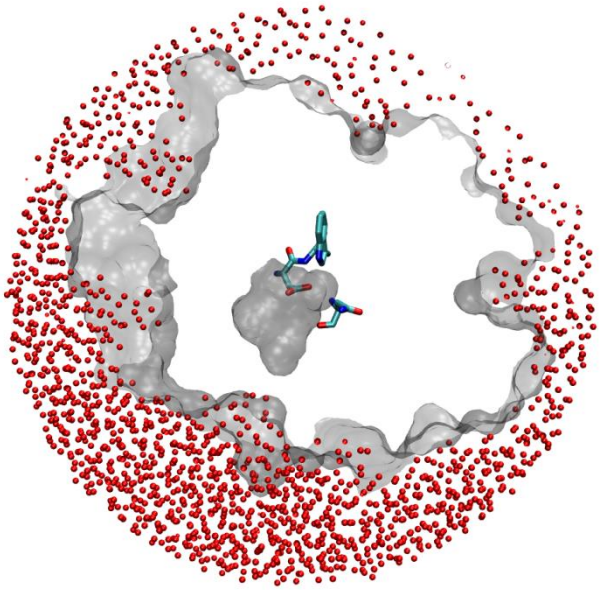
Mutant resistant to DMSO



DhaA wt



DhaA 57



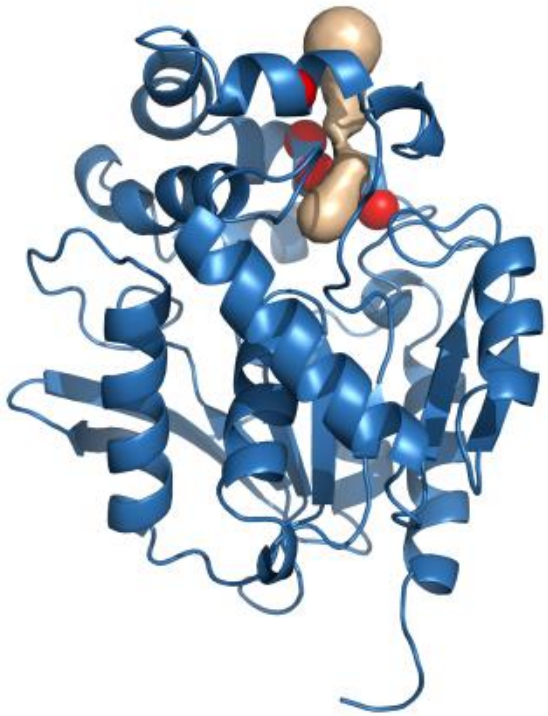
DhaA 80

Conclusion I

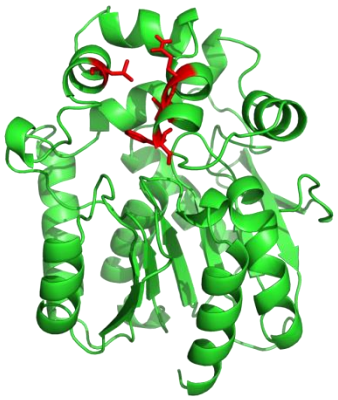
- **resistance** towards organic cosolvents **correlates with thermostability**
- mutations lining access tunnel modulate **occupancy** of active site by solvent and can **stabilize** protein
- robust catalysts (DhaA80) were developed:
  4 point mutations, T_m ↑ **19 °C**, $\tau_{1/2}$ (40 % DMSO) **min** → **days**
- **engineering of access tunnels represents novel strategy for engineering of robust catalysts**

DhaA80

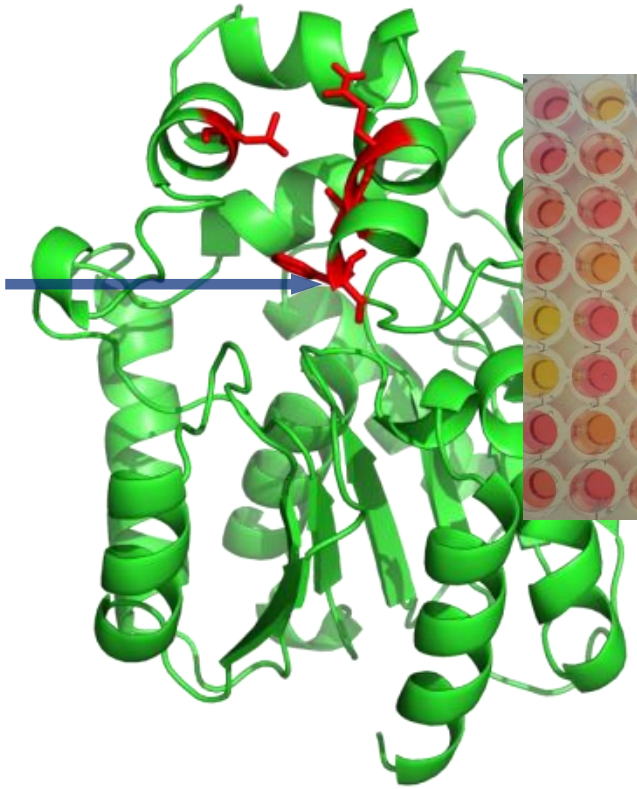
- 4 point mutations: T148L, G171Q, A172V and C176F
- $T_{1/2}$ (40 % DMSO) **improved 4000-fold**
- ΔT_m = **16 °C**
- **very low activity** in buffer

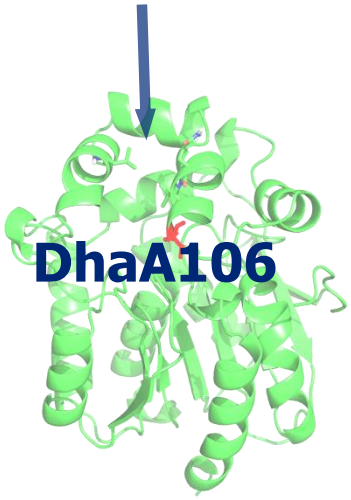


Saturation mutagenesis



DhaA80



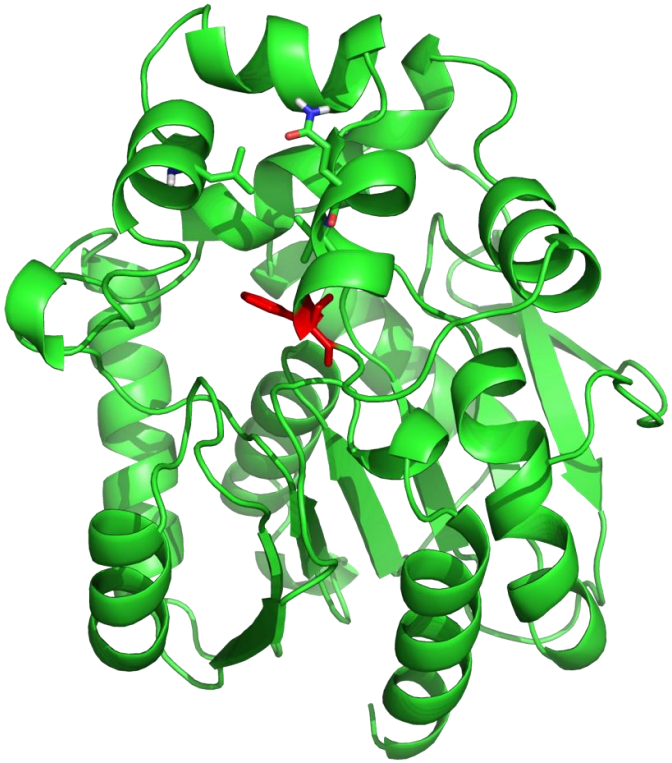


Sequencing
+ characterization
of selected hits

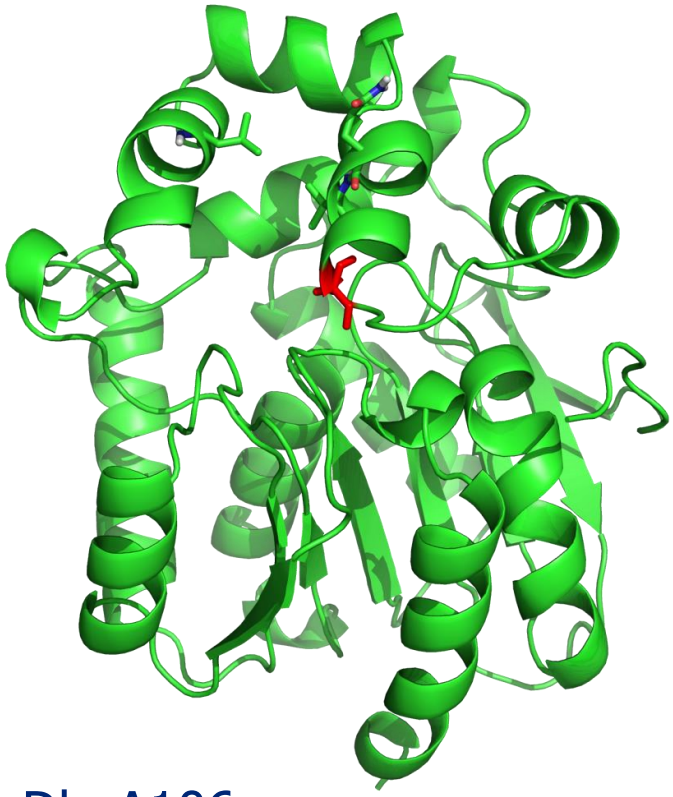
DhaA80
positions 172, 176

DhaA106

- T148L, G171Q, A172V and F176G



DhaA80

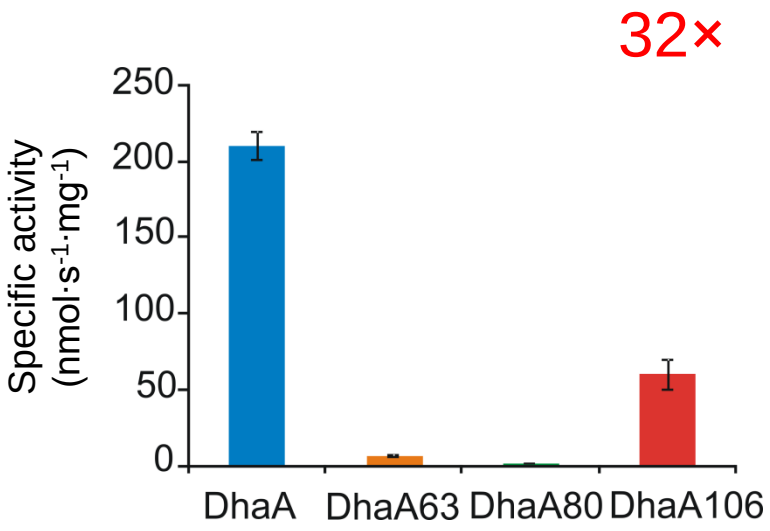


DhaA106

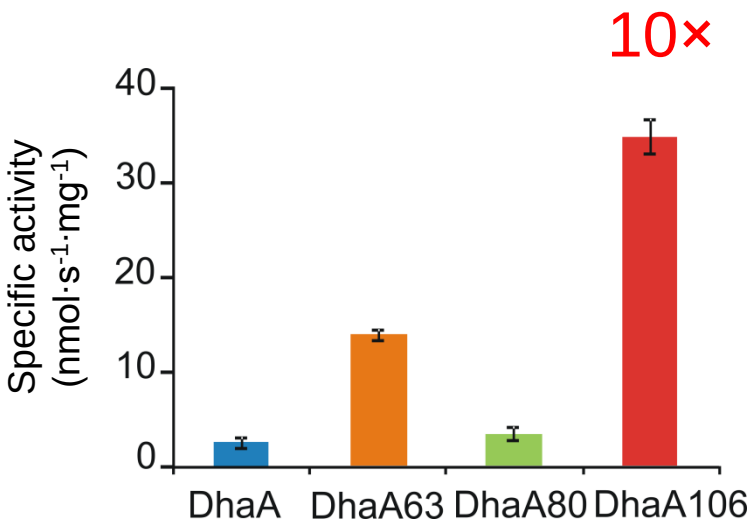
Stability and specific activity[1]

Variant	DhaAwt	DhaA63	DhaA80	DhaA106
T_m (°C)	50.4 ± 0.3	68.3 ± 0.3	66.8 ± 0.2	62.7 ± 0.1

Aqueous environment



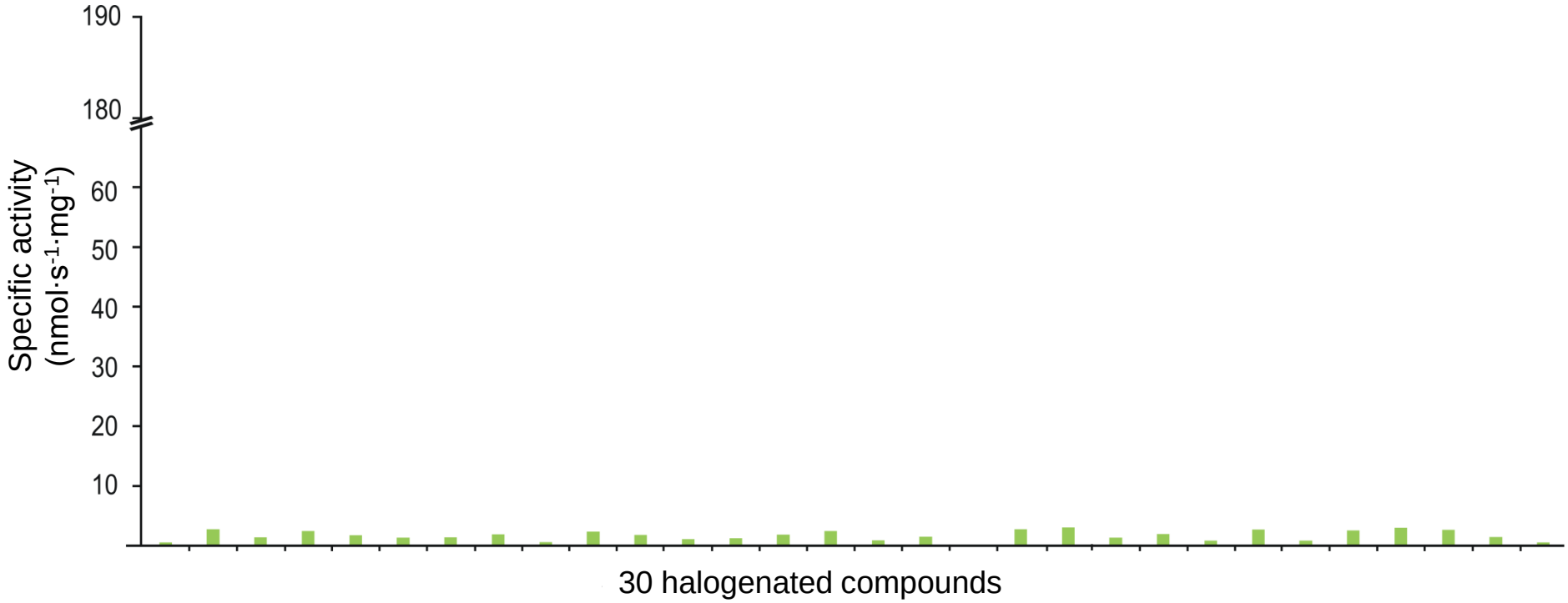
40% DMSO



[1]Measured with 1,2-dibromoethane

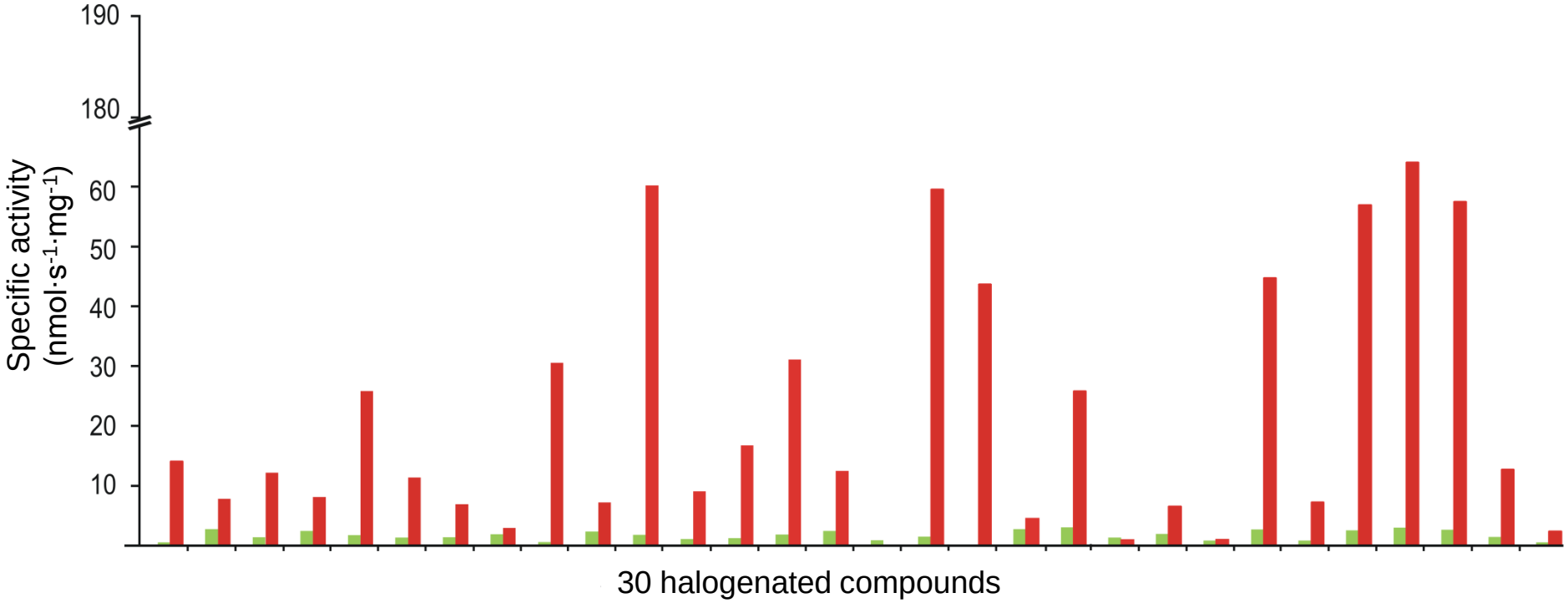
Substrate specificity

DhaA80



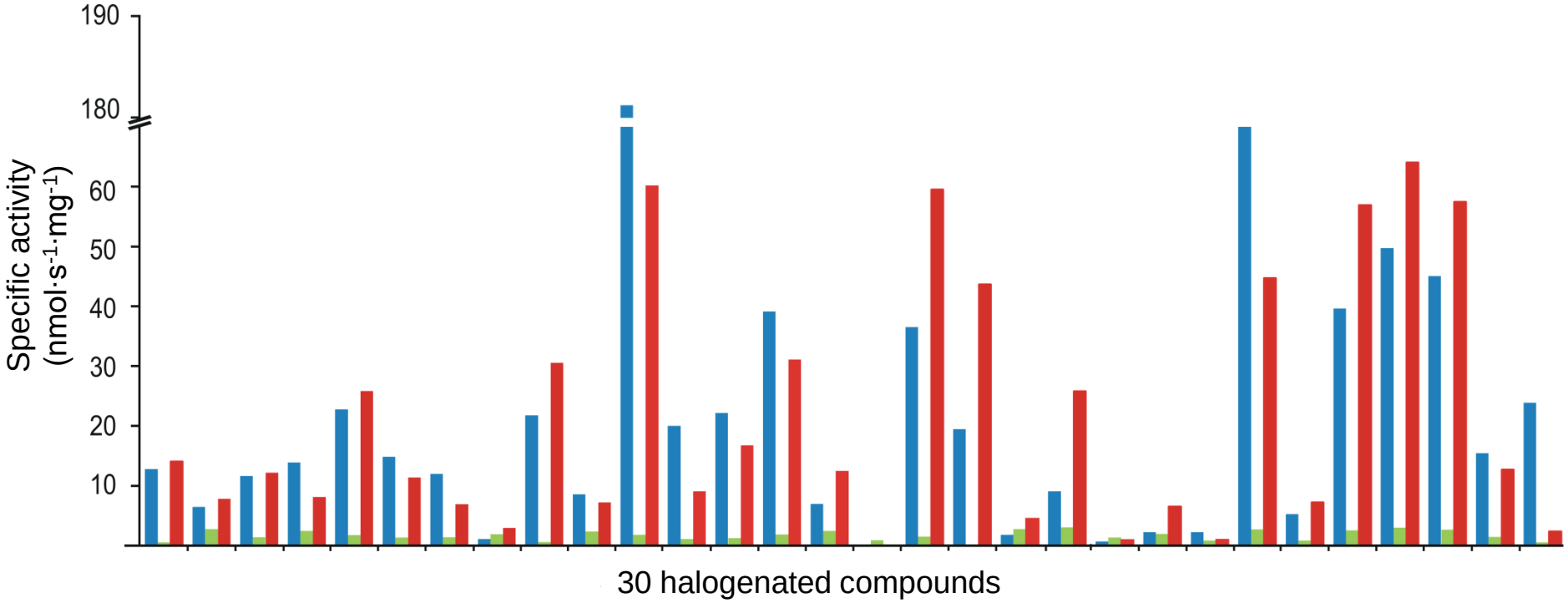
Substrate specificity

DhaA80 **DhaA106**

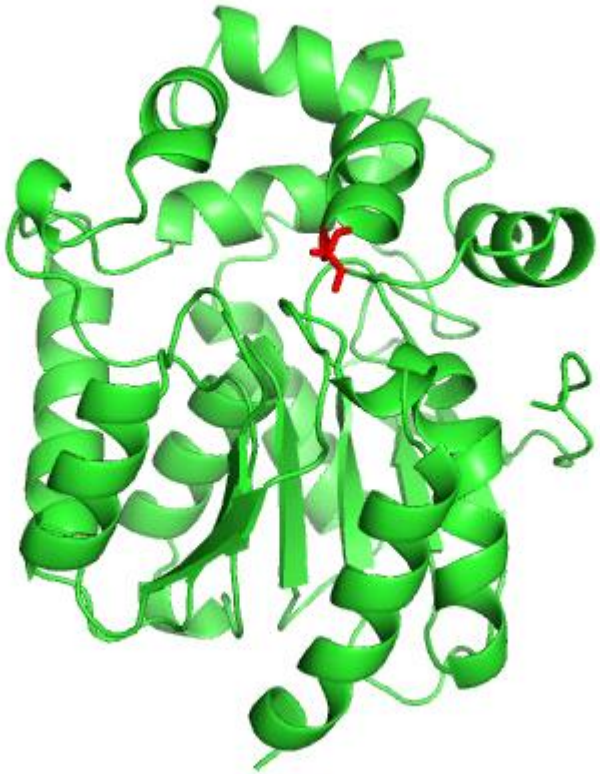


Substrate specificity

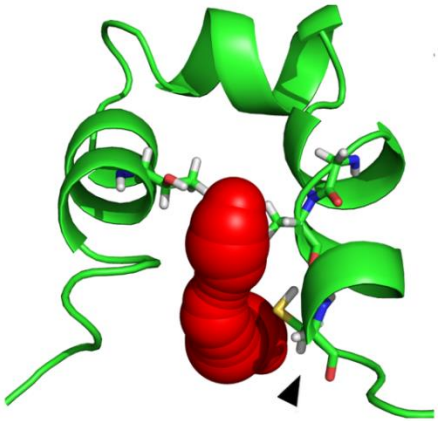
DhaA80 **DhaA106** **DhaAwt**

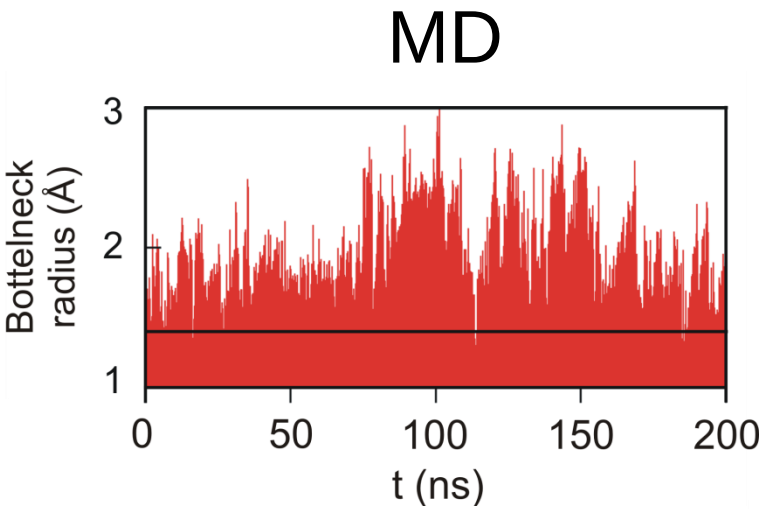


Structure and molecular modeling



DhaAwt





92 %

Conclusion II

- enzyme catalytic performance enhanced by fine-tuning the geometry and flexibility of its access tunnel
- **tunnel residues are good targets to balance activity-stability trade-off of enzymes**

Helpful references

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