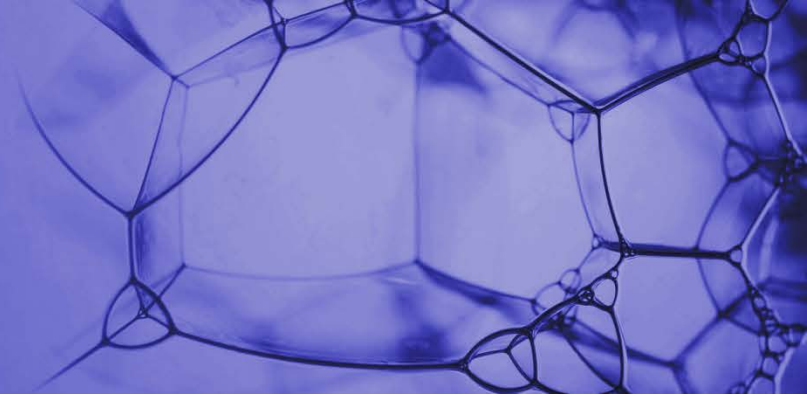


LOSCHMIDT
LABORATORIES



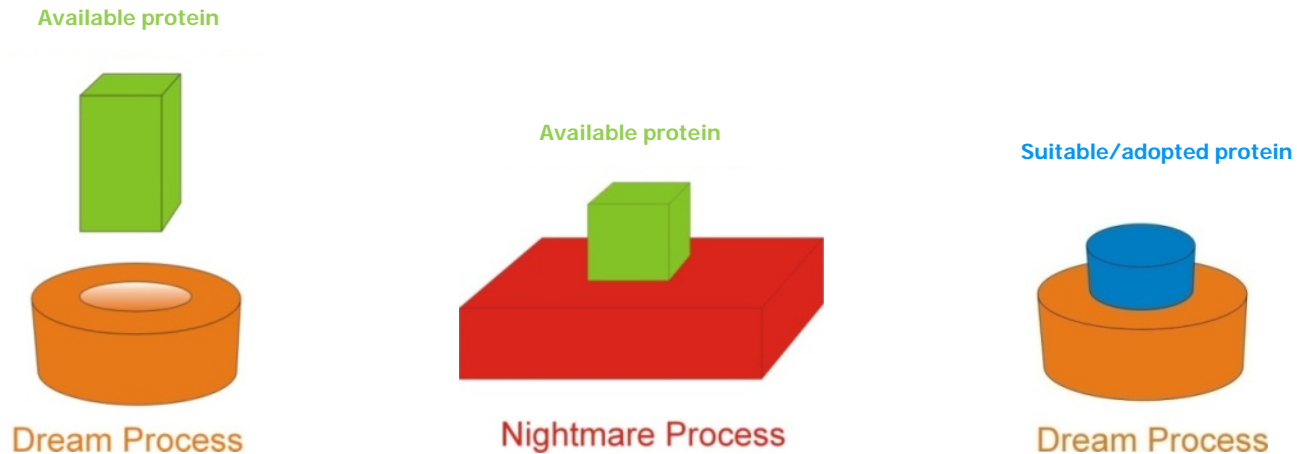
4. Protein Engineering

Outline

- ❑ Limitations of proteins in biotechnology processes
- ❑ Definition and aim of protein engineering
- ❑ Targeted properties of proteins
- ❑ Basic approaches in protein engineering
 - **DIRECTED EVOLUTION**
 - **RATIONAL DESIGN**
 - **SEMI-RATIONAL DESIGN**
- ❑ Examples

Proteins in biotechnology

- ❑ availability of optimal protein for specific process
- ❑ **traditional biotechnology** - adapt process
- ❑ **modern biotechnology** - adapt protein



Proteins in biotechnology

❑ classical screening

- screening culture collections
- polluted and extreme environment

❑ environmental gene libraries

- metagenomic DNA

❑ data-base mining

- gene databases
- genome sequencing projects
- numerous uncharacterised enzymes/proteins



Proteins in biotechnology



- ❑ the process of **constructing novel protein** molecules by design first principles or altering existing structure
- ❑ use of genetic manipulations to alter the coding sequence of a gene and thus **modify the properties of the protein**
- ❑ AIMS AND APPLICATIONS
 - **technological** - optimisation of the protein to be suitable in particular technology purpose
 - **scientific** - desire to understand what elements of proteins contribute to folding, stability and function

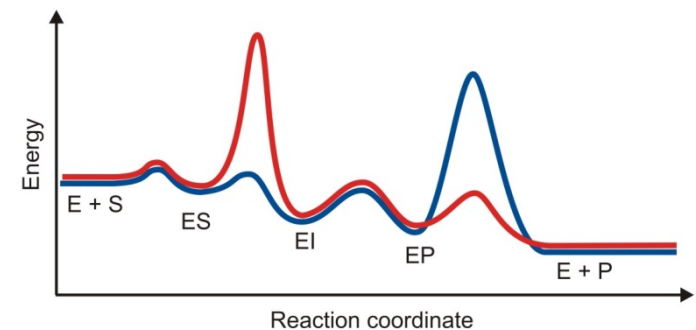
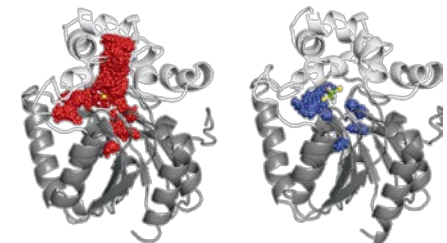
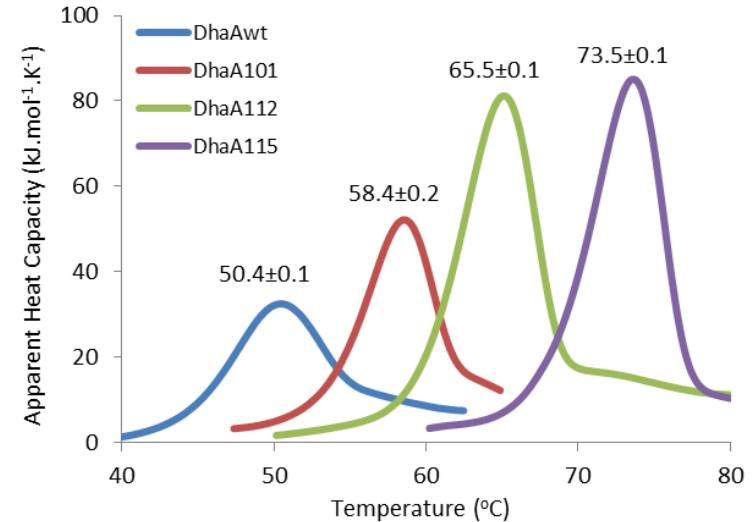
Targeted properties of proteins

□ structural properties of proteins

- stability (temperature, solvents)
- tolerance to pH, salt
- resistance to oxidative stress

□ functional properties of proteins

- reaction type
- substrate specificity and selectivity
- kinetic properties (e.g., K_m , k_{cat} , K_i)
- cofactor selectivity
- protein-protein or protein-DNA interactions



Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



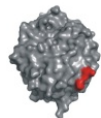
Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

6. *not applied*



Constructed mutant enzyme

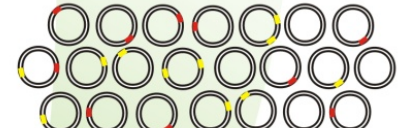
7. Biochemical testing

Improved
protein

DIRECTED EVOLUTION

1. *not applied*

2. Random mutagenesis



Library of mutated genes
(>10,000 clones)

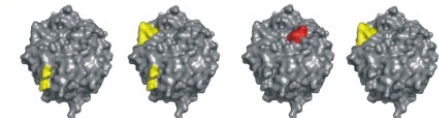
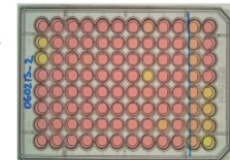
3. Transformation

4. Protein expression

5. *not applied*

6. Screening and selection

- stability
- selectivity
- affinity
- activity



Selected mutant enzymes

Directed evolution



- ❑ directed evolution techniques emerged during mid-1990s
- ❑ **inspired by natural evolution**
- ❑ this form of "evolution" does not match what Darwin had envisioned
 - requires **outside intelligence**, not blind chance
 - does not create brand new species, macroevolution, but only improvements of molecules, **molecular evolution**
 - does not take millions of years, but **happens rapidly**

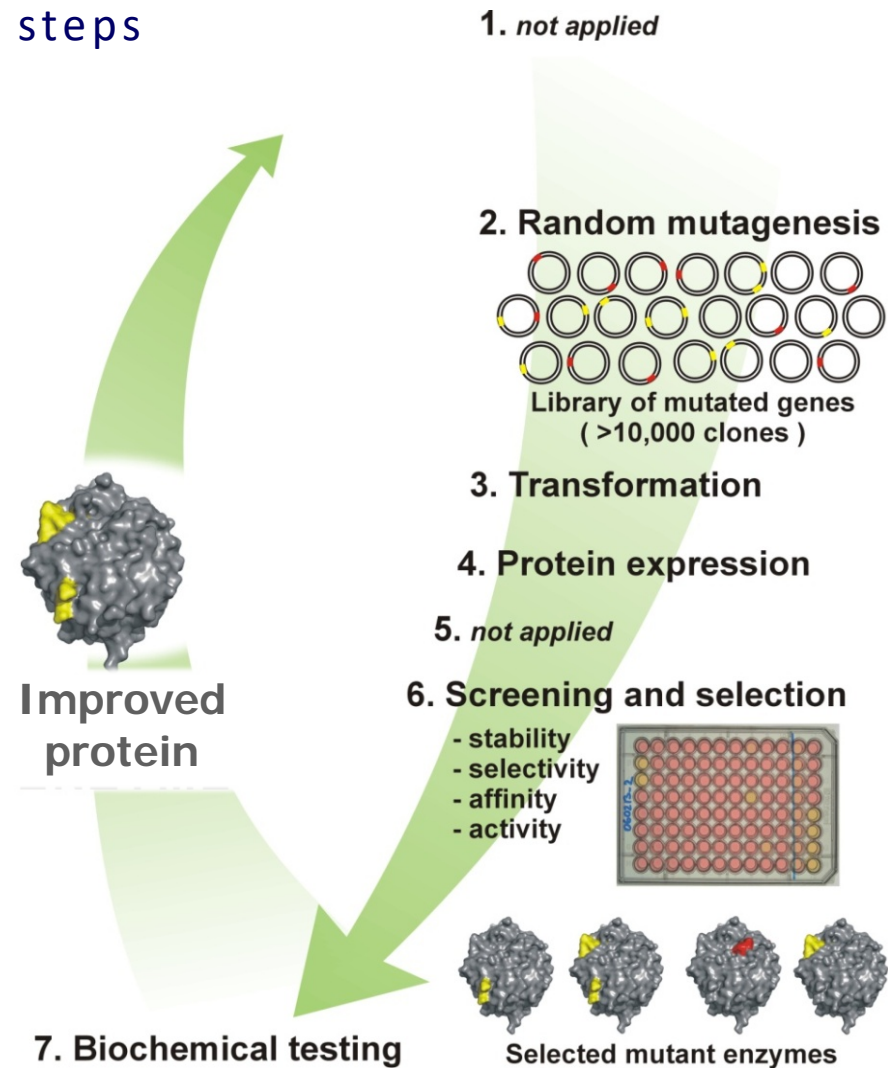
Directed evolution

❑ evolution in test tube comprises two steps

- **random mutagenesis**
mutant library building
- **screening and selection**
identification of desired biocatalyst

❑ prerequisites for directed evolution

- gene encoding protein of interest
- method to create mutant library
- suitable expression system
- screening or selection system

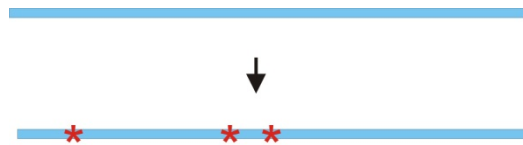


Methods to create mutant libraries

❑ technology to **generate large diversity**

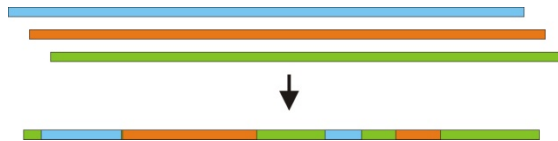
- **NON-RECOMBINING**

one parent gene -> variants with point mutations



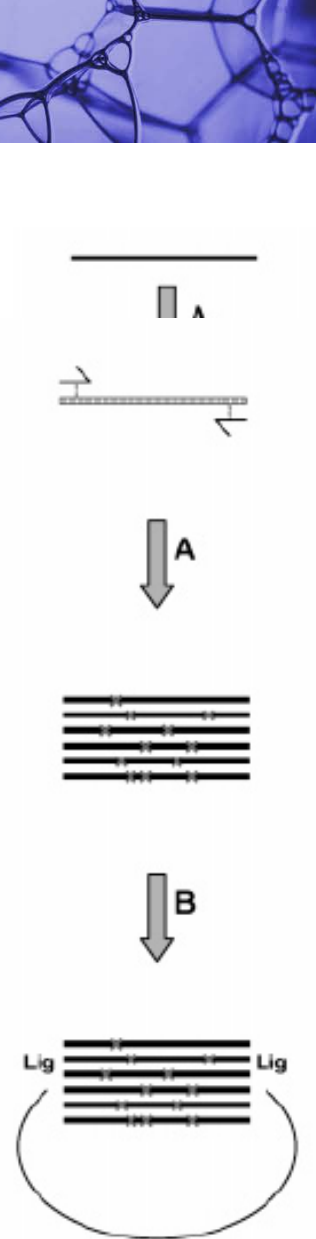
- **RECOMBINING**

several parental homologous genes -> chimeras



Non-recombining mutagenesis

- ❑ **UV irradiation or chemical mutagens** (traditional)
- ❑ **mutator strains** - lacks DNA repair mechanism
mutations during replication (e.g., *Epicurian coli* XL1-Red)
- ❑ **error-prone polymerase chain reaction (ep-PCR)**
 - gene amplified in imperfect copying process
(e.g., unbalanced deoxyribonucleotides concentrations,
high Mg^{2+} concentration, Mn^{2+} , low annealing temperatures)
 - 1 to 20 mutation per 1000 base pairs
- ❑ **saturation mutagenesis**
 - randomization of single or multiple codons
- ❑ **other methods**
 - gene site saturation mutagenesis
 - cassette mutagenesis (region mutagenesis)



Recombining mutagenesis

❑ also referred to as „sexual mutagenesis“

❑ DNA shuffling

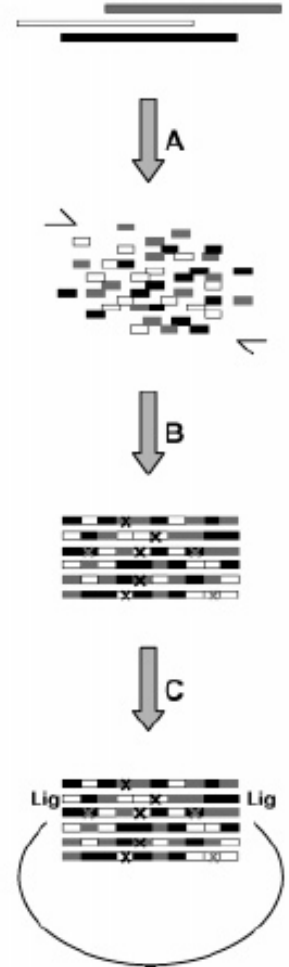
- fragmentation step
- random reassembly of segments

❑ StEP - staggered extension process

- simpler than shuffling
- random reannealing combined with limited primer extension

❑ other methods

shuffling of genes with lower homology down to 70%
(e.g., RACHITT, ITCHY, SCRATCHY)



Screening and selection

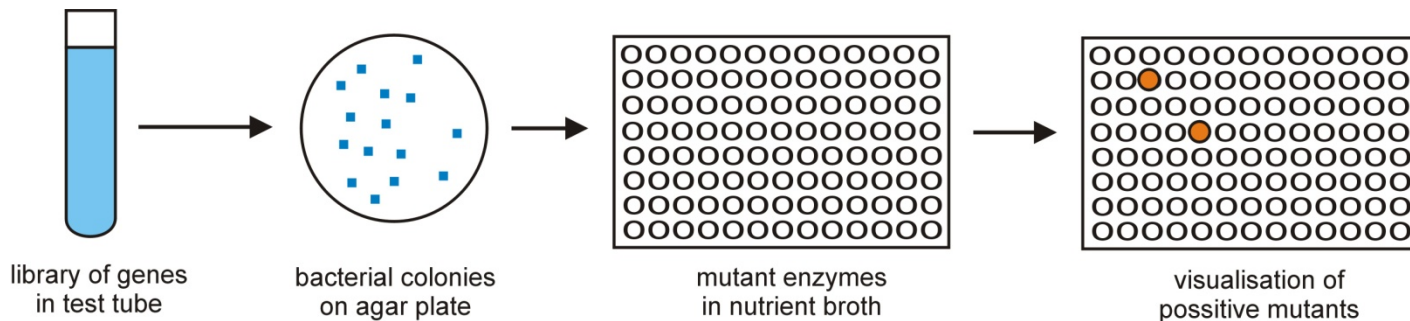
- ❑ most **critical step** of direct evolution
- ❑ isolation of positive mutants hiding in library

- **HIGH THROUGHPUT SCREENING**

individual assays of variants one by one

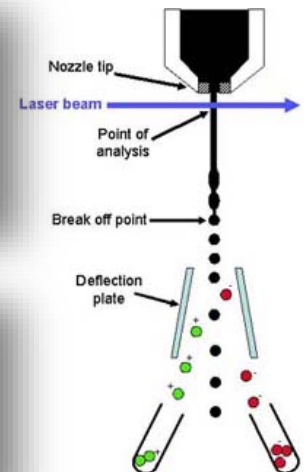
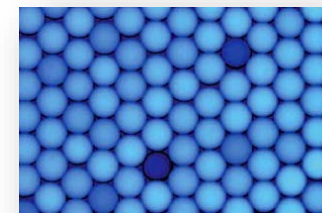
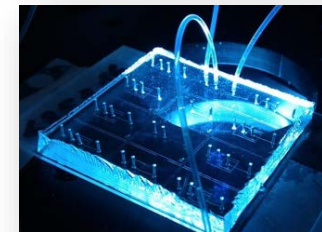
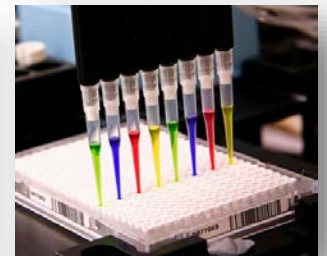
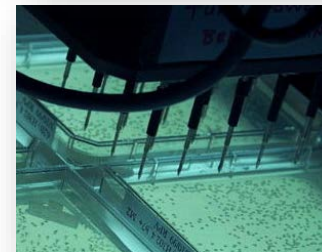
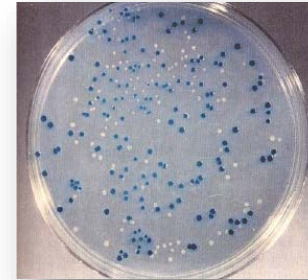
- **DIRECT SELECTION**

display techniques (link between genotype and phenotype)



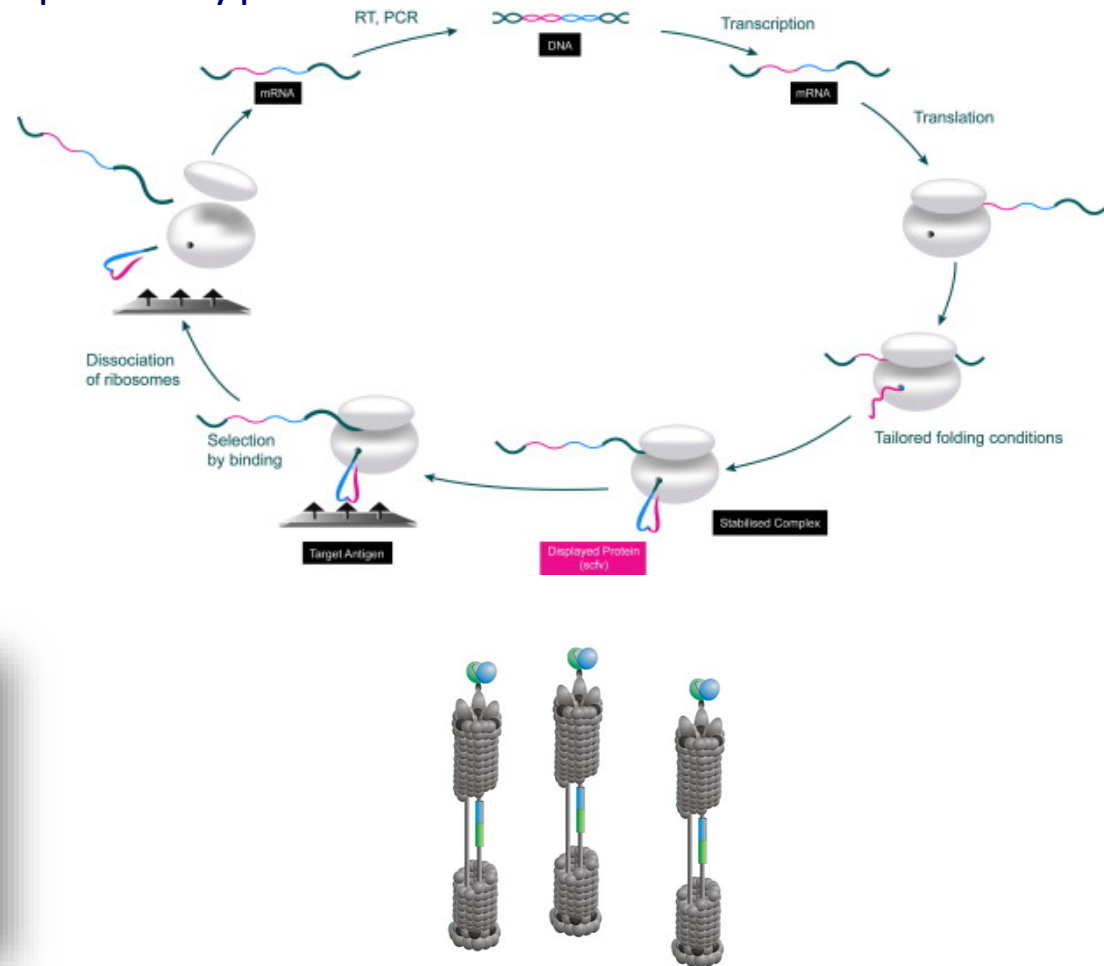
(Utra)High throughput screening

- ❑ common methods not applicable
- ❑ **agar plate (pre)screening**
- ❑ **microtiter plates screening**
 - 96-, 384- or 1536-well formate
 - robot assistance
(colony picker, liquid handler)
 - 10^4 libraries
 - volume 10 – 100 μ L
- ❑ **microfluidic systems**
 - water in oil emulsions (up to 10 kHz)
 - FACS sorting (10^8 events/hour)
 - 10^9 libraries
 - volume 1 – 10 pL



Direct selection

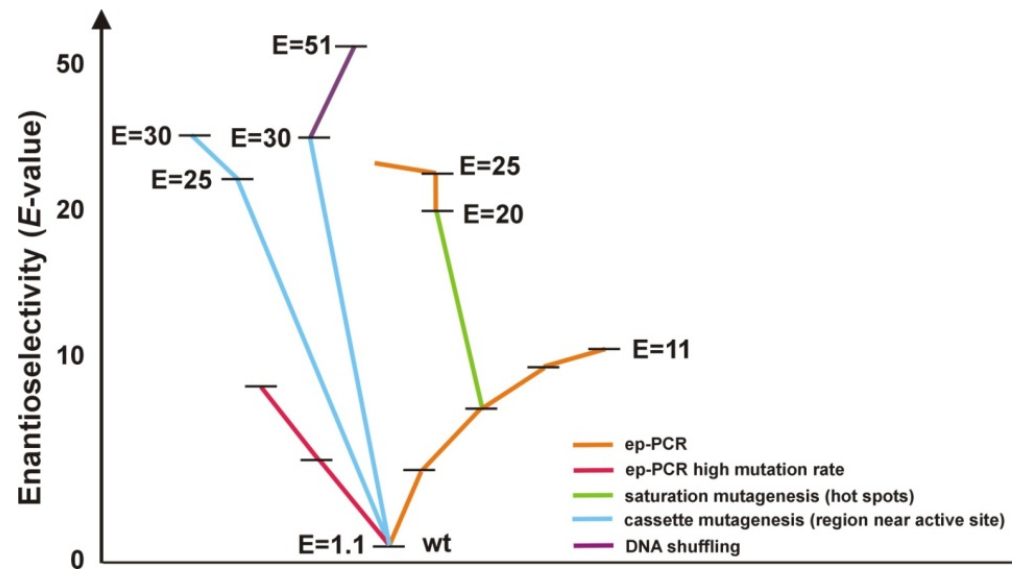
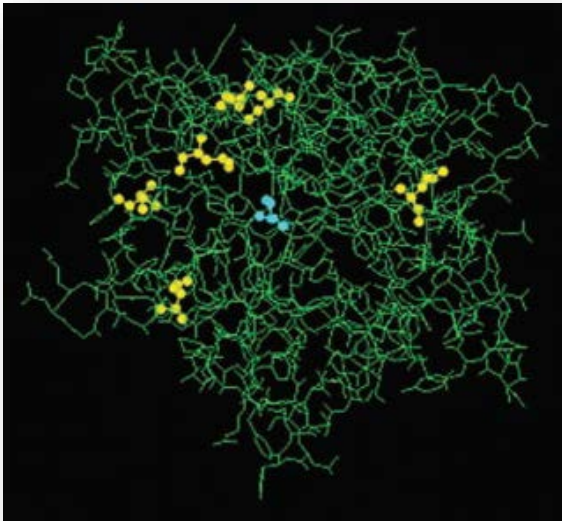
- ❑ not generally applicable (mutant libraries $>10^6$ variants)
- ❑ link between genotype and phenotype
- ❑ **display technologies**
 - ribosome display
 - phage display
- ❑ **life-or-death assay**
 - auxotrophic strain
 - toxicity based selection



Example of Directed evolution

□ directed evolution of **enantioselectivity**

- lipase from *P. aeruginosa* (E-value improved from 1.1 into 51)
- **spectrophotometric screening** of (*R*)- and (*S*)-nitrophenyl esters
- **40 000 variants** screened
- the best mutant contains six amino acid substitutions



Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



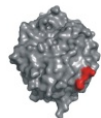
Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

6. *not applied*



Constructed mutant enzyme

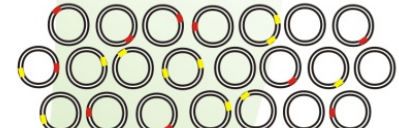
7. Biochemical testing

Improved
protein

DIRECTED EVOLUTION

1. *not applied*

2. Random mutagenesis



Library of mutated genes
(>10,000 clones)

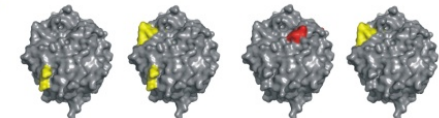
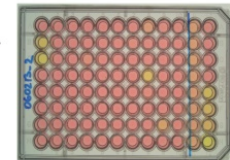
3. Transformation

4. Protein expression

5. *not applied*

6. Screening and selection

- stability
- selectivity
- affinity
- activity



Selected mutant enzymes

Rational design



- ❑ emerged around 1980s as the original protein engineering approach
- ❑ **knowledge based** - combining theory and experiment
- ❑ protein engineering cycle:
„structure-theory-design-mutation-purification-analysis“
- ❑ **difficulty in prediction** of mutation effects on protein property
- ❑ **de novo design**

Principal of rational design

1. Computer aided design



2. Site-directed mutagenesis



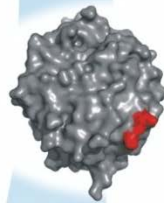
Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

6. *not applied*



Improved
protein



Constructed mutant enzyme

7. Biochemical testing

□ rational design comprises:

- **design** - understanding of protein functionality
- **experiment** - construction and testing of mutants

□ prerequisites for rational design:

- gene encoding protein of interest
- 3D structure (e.g., X-ray, NMR)
- structure-function relationship
- computational methods and capacity
- (multi)side directed mutagenesis techniques
- efficient expression system
- biochemical tests

Design

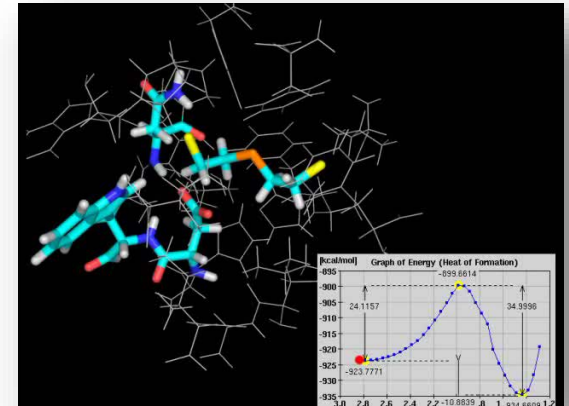
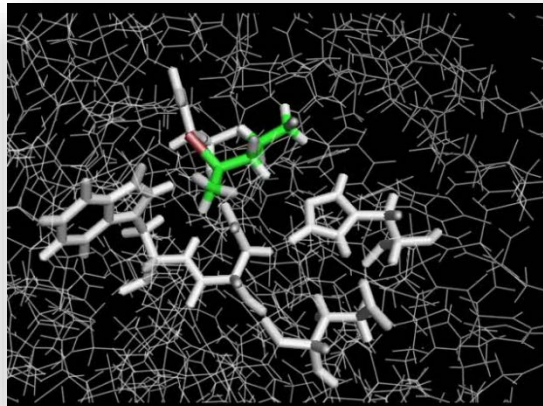
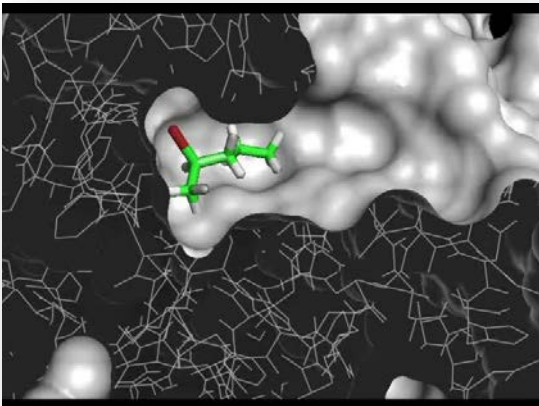
❑ HOMOLOGY APPROACH

- homologous wild-type sequences are collected and compared
- identifying amino acid residues responsible for differences
- **reconstruction** - transfer differences from one enzyme to another
- **new design** - combination of positive mutation from all parental proteins in one construct, new protein better than all parental

RLA0_DICDI	-----MSGAG-SKRRKLFIEKATKLTFTYDKMIVAEADFYGSSQLOKIRKSIRGI-GAVLMGKKTMIRKQVIRDLADSK--PELD	75
Q54LP0_DICDI	-----MSGAG-SKRRNVFIEKATKLTFTYDKMIVAEADFYGSSQLOKIRKSIRGI-GAVLMGKKTMIRKQVIRDLADSK--PELD	75
RLA0_PLAFB	-----MAKLSKQKKQMYIEKLSSLIQQYSKILVHVONVGNHMASVKKSLRGK-AEILMGKTRIRTALEKKNLAV--PQIE	76
RLA0_SULAC	-----MIGLAVTTTKIAKWKVDEVAELTSLKLTHTITIIANIEGFPADKLHEIRKKLRGK-ADIKVTKNLNFNIALKNAG----YDK	79
RLA0_SULTO	-----MRIMAVITQERKIAKWKIEEVKELEKLTREYHTIIIANIEGFPADKLHDIRKKMHGM-AEIKVTKNLTFGIAAKNAG----LDVS	80
RLA0_SULSO	-----MKRLALALKQRKVASWKLEEVKELELIKNSNTILIGNLEGFPADKLHEIRKKLRGK-AEIKVTKNLTFKIAAKNAG----IDIE	80
RLA0_AERPE	MSVVSIVGQMYKREKPIPEMKTLMRLRELESLFSKRHVYLFADITGPTTFVVGVRKKELWKK-YDMMVAKKRIILRAMKAAGLE--LDDN	86
RLA0_PYRAE	HMILAIGKRRYVRTROYFARKVKIVSEATELLQKYPYVFLFDLHGLSSRLHEKRYRLARY-GYIKIIRKPTLFKIAFTKVYGG--IPAE	85
RLA0_METAC	-----MAEERNHTEHIPQWKDEIENIKELIQSHKVFCHMGVIGILATKMKIRRDLDKV-AVLKVRNTLTERALNQLG----ETIP	78
RLA0_METMA	-----MAEERNHTEHIPQWKDEIENIKELIQSHKVFCHMGVIGILATKMKIRRDLDKV-AVLKVRNTLTERALNQLG----ESIP	78
RLA0_ARCFU	-----MAAVRGS--PPEYKVRAVEEIKRMISSEKVVVAIVSFRNVFAGDMKIRREFRGK-AEIKVVKNTLTERALDGLG----GDYL	75
RLA0_METKA	HAYKAKGQPPSGYEIPKYAENKRRREVKELELMDEYENYGLVDLEGIPAPOLQETRAKLRERDRIIRMSRNTLMRIALEEKLEDER--PELE	88
RLA0_METTH	-----MAHVAENKKKEVQELHDLIKGYEYVGIANLADIPAROLQKMRQTLRDS-ALIRMSKNTLISLAEKAGREL--ENVD	74
RLA0_METTL	-----MITAESENKIAIPWKIEEVNKLKLELLKNGQIVALVDMMYFAPAROLQEIIRDKIR-GTMLKMSRNTLTERALKEVALETGNPEFA	82
RLA0_METVA	-----MIDAKSENKIAIPWKIEEVNALKLELLKSANVIALIDHMEYFAPVLOEIRDKIR-DQMLKMSRNTLTERALKEVALETGNPEFA	82
RLA0_METJA	-----METKVAHVAPWKIEEVKTLKGLIKSKPYVAIVDMMYFAPAROLQEIIRDKIR-DKVKLRMSRNTLTERALKEAAELNHPKLA	81
RLA0_PYRAB	-----MAHVAENKKKEVEELANLIKSYVYIALVDVSSHPAYPLSQMRRLIRENGGLLRVSRNTLTERALKAAGELGKPELE	77
RLA0_PYRHO	-----MAHVAENKKKEVEELAKLIKSYVYIALVDVSSHPAYPLSQMRRLIRENGGLLRVSRNTLTERALKAAGELGKPELE	77
RLA0_PYRFU	-----MAHVAENKKKEVEELANLIKSYVYIALVDVSSHPAYPLSQMRRLIRENGGLLRVSRNTLTERALKAAGELGKPELE	77
RLA0_PYRKO	-----MAHVAENKKKEVEELANLIKSYVYIALVDVAGVPAYPLSKMRDLKIR-GKALLRVSRNTLTERALKAAGELGKPELE	76
RLA0_HALMA	MSAESERKTETIPENKQEEVDALIVEMIESYESYGVVNIAGIPEROLQDMRRDLHET-AELRVSRNTLTERALDDVD----DGLE	79
RLA0_HALVO	MSESEVRQTEVIPQWKREEVDELVDYFIESTESYGVVGVAGIPEROLQSMRRELHGS-AAVRMSRNTLVNRADEVN----DGFE	79
RLA0_HALSA	MSAEQRTEETEEPENKQEEVAELVDLLETYSYGVVNVVTGIPSKLODMRRGLHQQ-AALRMSRNTLLVRALEEAG----DGLD	79
RLA0_THEAC	-----MKESVQKKKELVNEITRIKASRVAIVDTAGIRTRQIODIRGKNRGK-INLKVIKNTLTFKALENLGD----EKLS	72
RLA0_THEVO	-----MRKINPKKKEIVSELADITKSKAYAIVDIKQVTRROMDIRAKHEDK-YKIKVVKNTLTFKALDSIND----EKLT	72
RLA0_PICTO	-----MTEPQWKIDFVKNLENTNSRKYAAIVSIKILRNHEFOKIRNSIRDK-ARIKVRARLLRLAIENTGK----NNIV	72
ruler	1.....10.....20.....30.....40.....50.....60.....70.....80.....90	

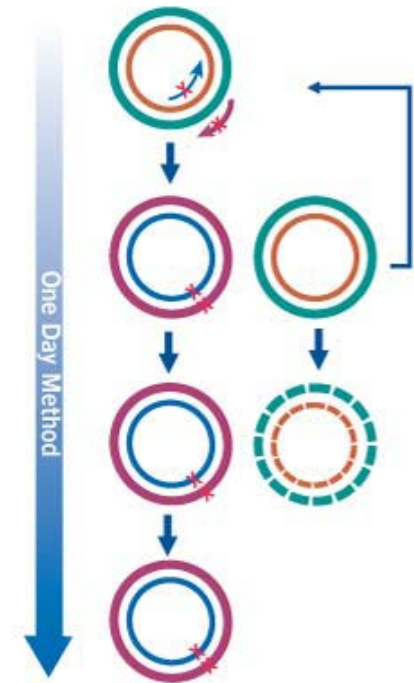
❑ STRUCTURE-BASED APPROACH

- **prediction** of enzyme function from structure alone is challenging
- **protein structure** (X-ray crystallography, NMR, homology models)
- **molecular modelling**
 - molecular docking
 - molecular dynamics
 - quantum mechanics/molecular mechanics (QM/MM)



Construction

- ❑ **site-directed mutagenesis**
 - introducing point mutations
- ❑ **multi site-directed mutagenesis**
- ❑ **gene synthesis**
 - commercial service
 - codone optimisation



GENEART
THE GENE OF YOUR CHOICE

GenScript
Make Research Easy

Example of rational design

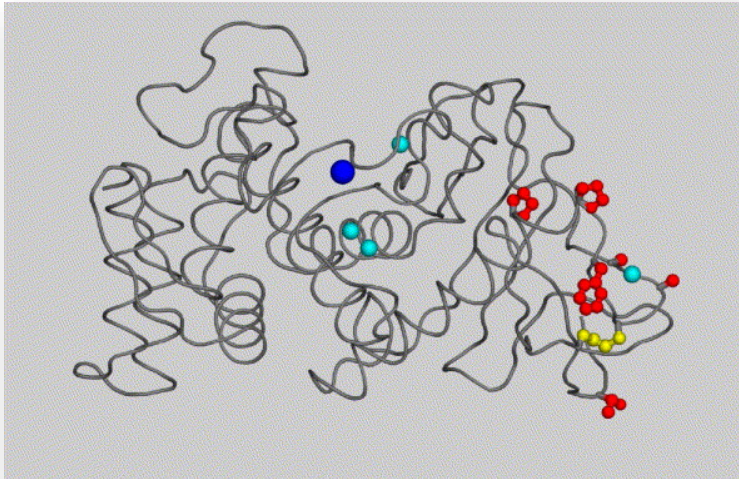
❑ rational design of protein **stability**

- stability to high temperature, extreme pH, proteases etc.
- **stabilizing mutations** increase strength of weak interactions
 - **salt bridges and H-bonds**
Eijsink et al., Biochem. J. 285: 625-628, 1992
 - **S-S bonds**
Matsumura et al., Nature 342: 291-293, 1989
 - **addition of prolines**
Watanabe et al., Eur. J. Biochem. 226: 277-283, 1994
 - **less glycines**
Margarit et al., Protein Eng. 5: 543-550, 1992
 - **oligomerisation**
Dalhus et al., J. Mol. Biol. 318: 707-721, 2002

Example of rational design

❑ engineering protein to resist boiling

- **reduced rotational freedom**
Ser65Pro, Ala96Pro
- **introduction of disulfide bridge**
Gly8Cys + Asn60Cys
- **improved internal hydrogen bond**
Ala4Thr
- **filling cavity**
Tyr63Phe



Half-lives (min.)	80°C	100°C
wild type	17.5	>0.5
8-fold mutant	stable	170

Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



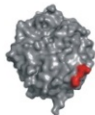
Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

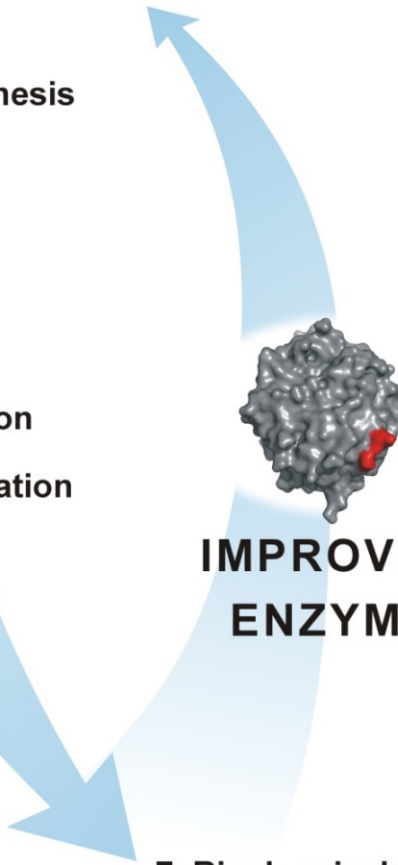
6. *not applied*



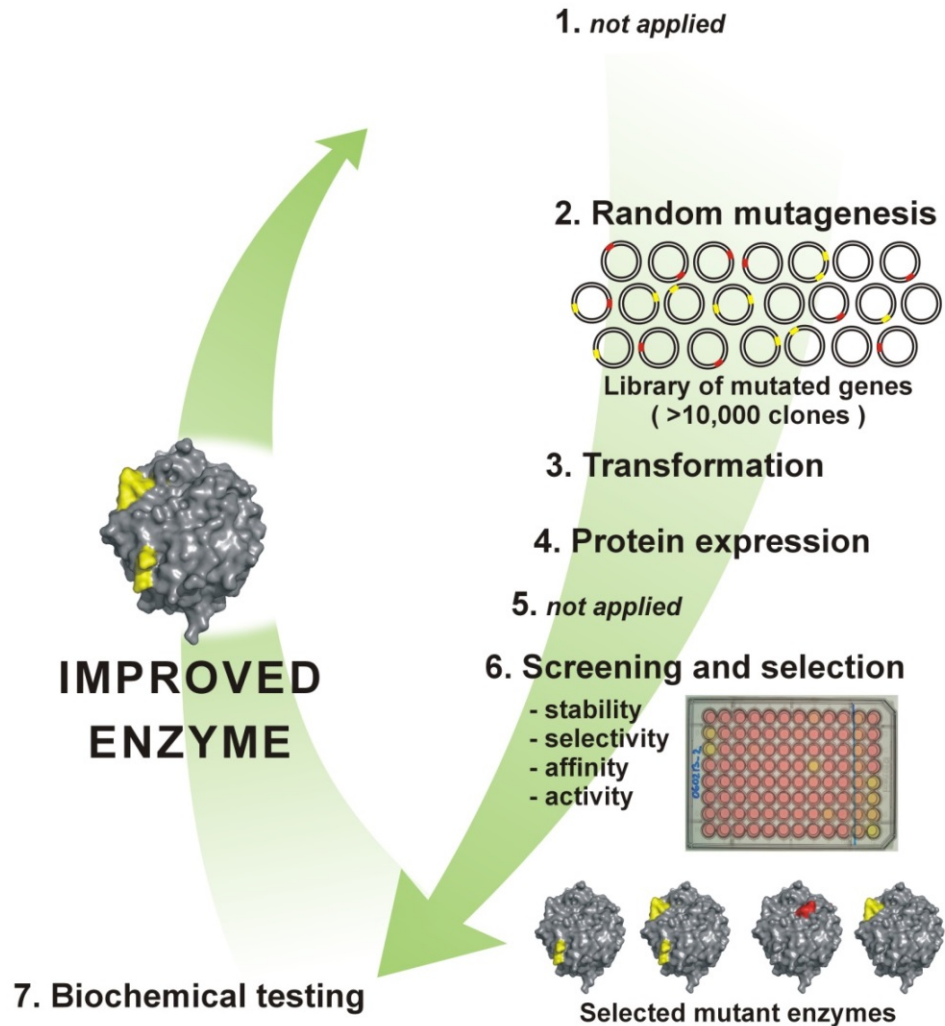
Constructed mutant enzyme

**IMPROVED
ENZYME**

7. Biochemical testing



DIRECTED EVOLUTION



Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



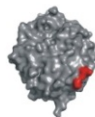
Individual mutated gene

3. Transformation

4. Protein expression

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6. *not applied*

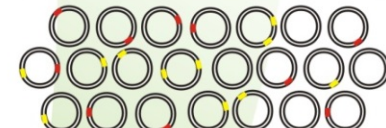


Constructed mutant enzyme

DIRECTED EVOLUTION

SEMIRATIONAL DESIGN

2. Random mutagenesis



Library of mutated genes
(>10,000 clones)

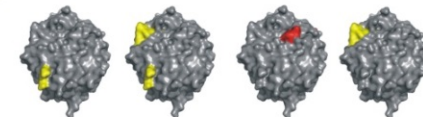
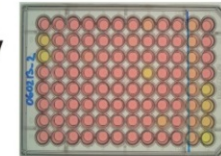
3. Transformation

4. Protein expression

5. *not applied*

6. Screening and selection

- stability
- selectivity
- affinity
- activity



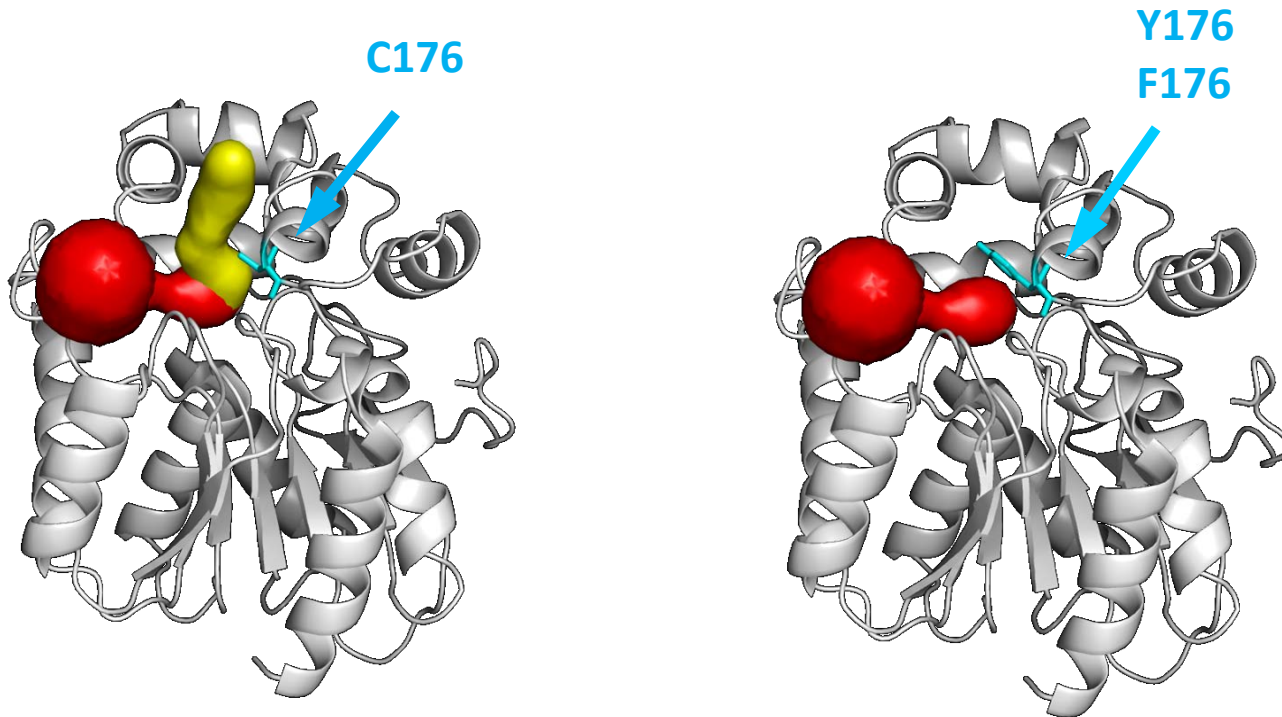
Selected mutant enzymes

7. Biochemical testing

IMPROVED
ENZYME

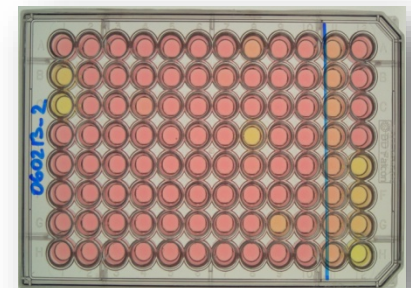
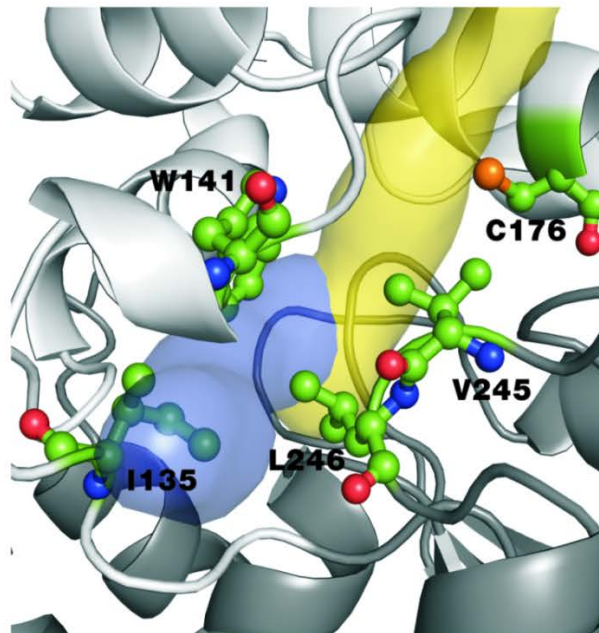
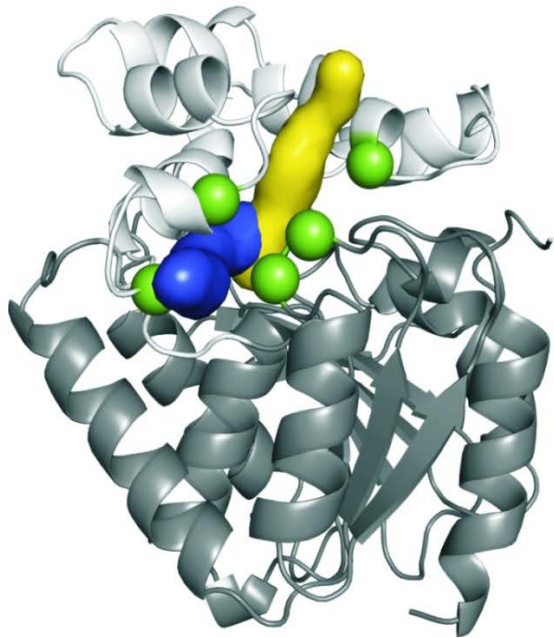
Example of rational design

- ❑ conversion of 1,2,3-trichloropropane by DhaA from *Rhodococcus erythropolis* Y2
- ❑ **DIRECTED EVOLUTION** - importance of access pathways



Example of rational design

- ❑ conversion of 1,2,3-trichloropropane by DhaA from *Rhodococcus erythropolis* Y2
- ❑ **DIRECTED EVOLUTION** - importance of access pathways
- ❑ **SEMI-RATIONAL DESIGN** - hot spots in access tunnels
- ❑ library of **5,300 clones** screened



Example of rational design

