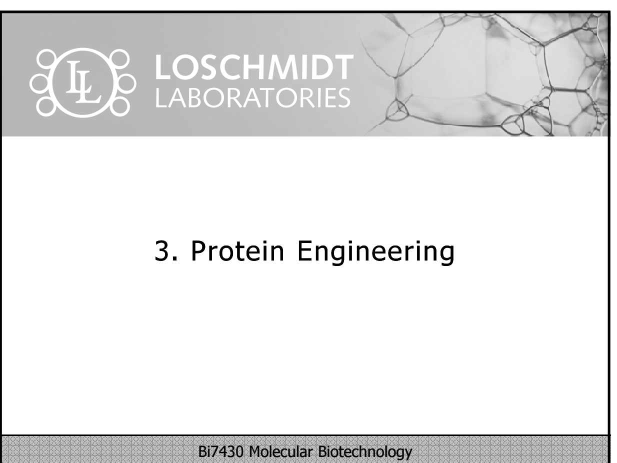
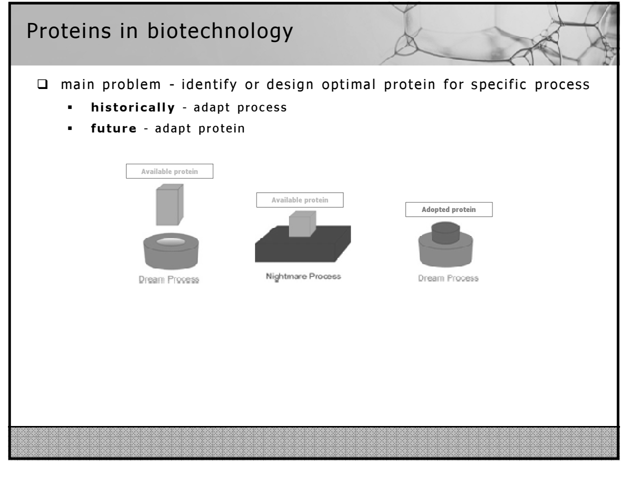


Outline

- Limitations of proteins in biotech processes
- Definition and aim of protein engineering
- Targeted properties of proteins
- Approaches in protein engineering
  - **Directed evolution**
  - **Rational design**



Proteins in biotechnology

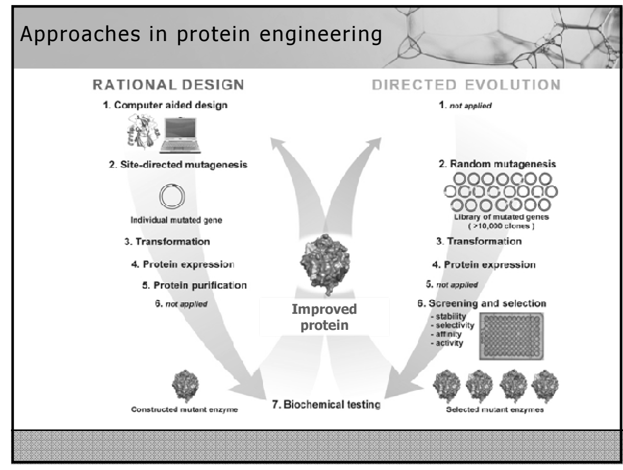
- **classical screening**
  - screening culture collections
  - polluted and extreme environment
- **environmental gene libraries**
  - metagenomic DNA
- **data-base mining**
  - genome sequencing projects
  - massive sequencing (Sargasso sea project)
  - numerous uncharacterised enzymes
- **protein engineering**

Protein engineering

- 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

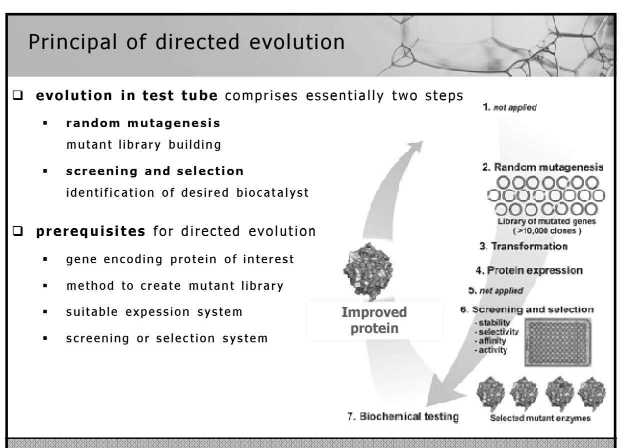
Targeted properties of proteins

- **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



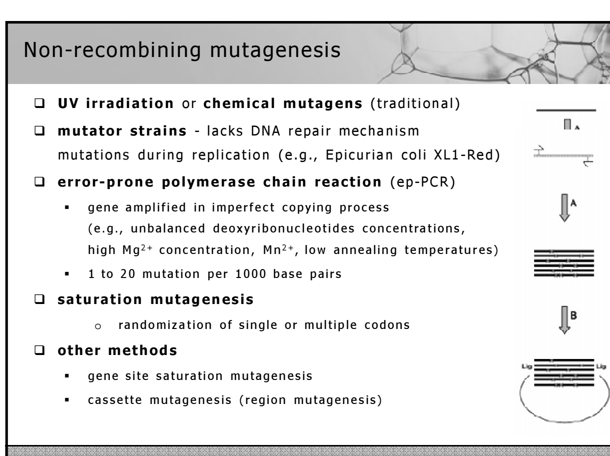
Directed evolution

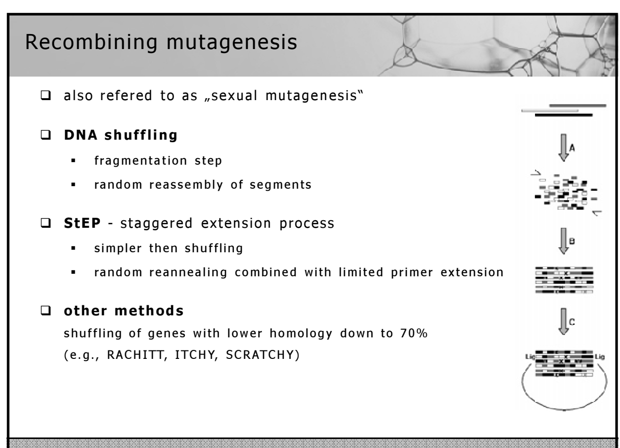
- directed evolution techniques emerged during mid-1990s
- **inspired by natural evolution**
- this form of "evolution" does not match what Darwin had envisioned
  - requires **outside intelligence**, not blind chance
  - does not create brand new species (macroevolution)
    only improvements (**molecular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



Methods to create mutant libraries

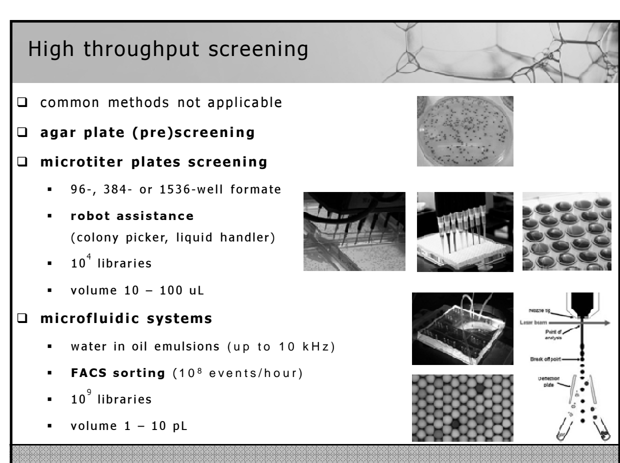
- technology to generate large diversity
  - **NON-RECOMBINING** (one parent gene -> variants with point mutations)
  - **RECOMBINING** (several parental homologous genes -> chimeras)

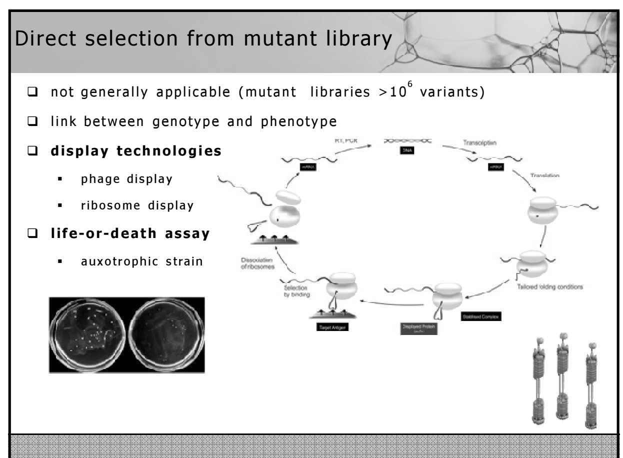




Screening and selection

- 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)

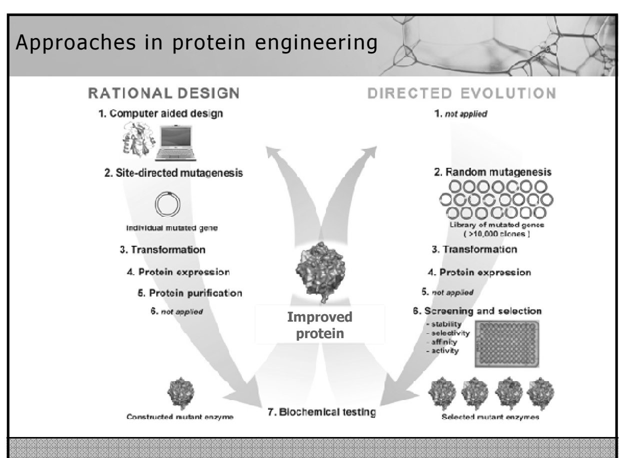




Example of Directed evolution

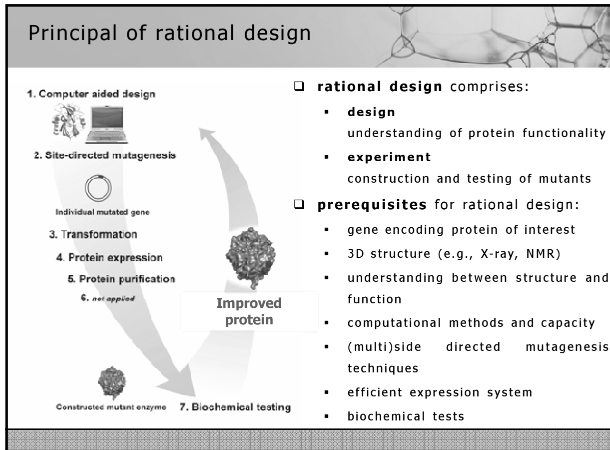
- **directed evolution of enantioselectivity**
  - lipase from *P. aeruginosa* (E-value improved from 1.1 into 51)
  - **spectrophotometric screening** of (*R*)- and (*S*)-nitrophenyl esters
  - the best mutant contains six amino acid substitutions
  - **40 000 variants screened**

Approaches in protein engineering

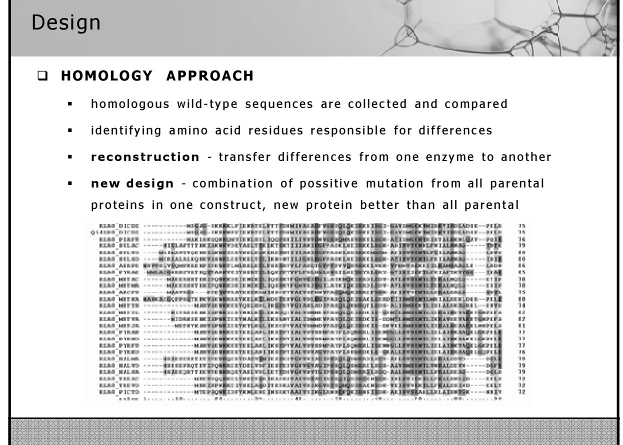


Rational design

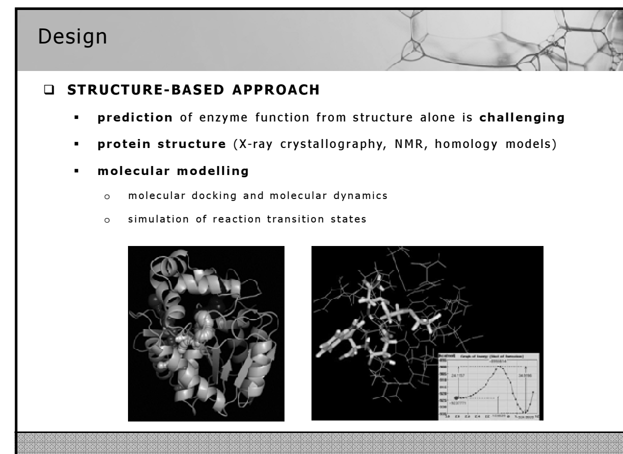
- emerged around 1980s as the original protein engineering approach
- combining theory (**knowledge based**) and experiment
- protein engineering cycle: „**structure-theory-design-mutation-purification-analysis**"
- **difficulty in prediction** of mutation effects on protein property



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)
understanding between structure and function
computational methods and capacity
(multi)side directed mutagenesis techniques
efficient expression system
biochemical tests



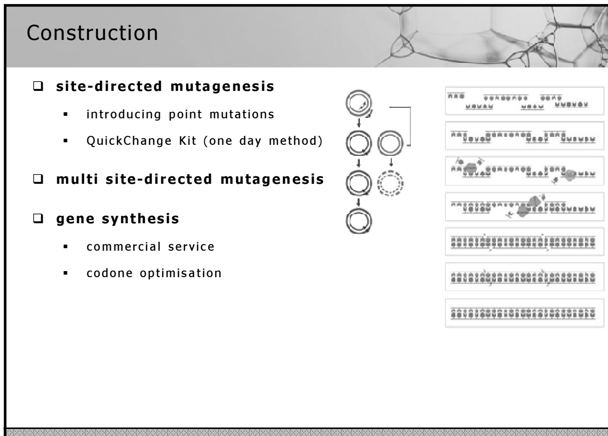
Design
HOMOLOGY APPROACH
homologous wild-type sequences are collected and compared
identifying amino acid residues responsible for differences
reconstruction - transfer differences from one enzyme to another
new design - combination of possitive mutation from all parental proteins in one construct, new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



Design
STRUCTURE-BASED APPROACH
prediction of enzyme function from structure alone is challenging
protein structure (X-ray crystallography, NMR, homology models)
molecular modelling
molecular docking and molecular dynamics
simulation of reaction transition states

Construction

- site-directed mutagenesis
  - introducing point mutations
  - QuickChange Kit (one day method)
- multi site-directed mutagenesis
- gene synthesis
  - commercial service
  - codone optimisation

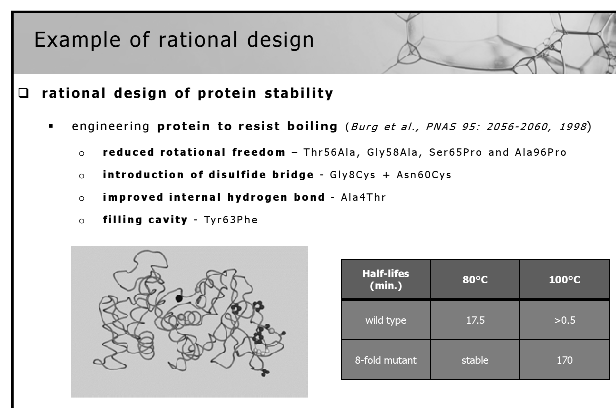


Example of rational design

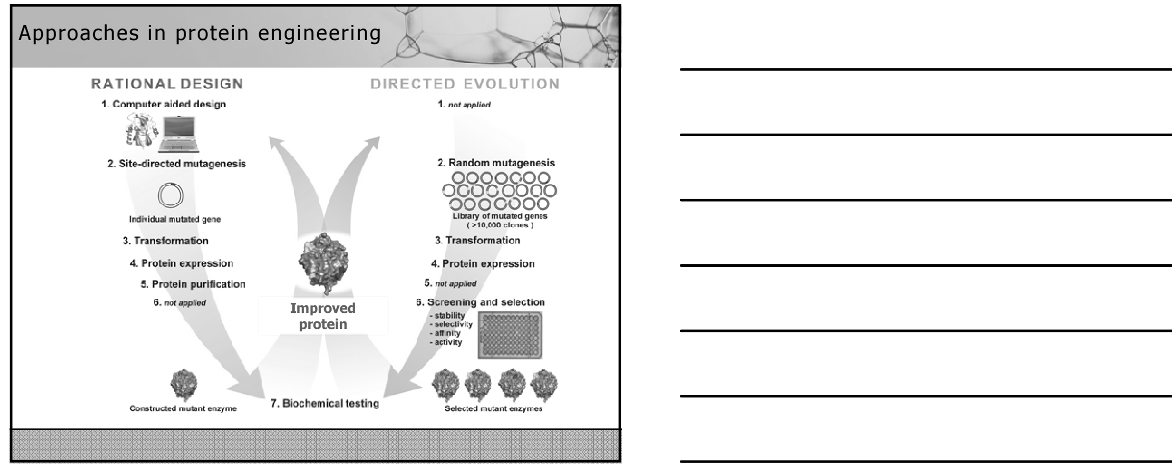
- **rational design of protein stability**
  - stability to high **temperature, extreme pH, oxygen stress, proteases** etc.
  - **stabilizing mutations** increase strength of weak interactions
    - salt bridges and H-bonds (*Eijsink et al., Biochem. J. 285: 625-628, 1992*)
    - S-S bonds (*Matsumura 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

- **rational design of protein stability**
  - engineering **protein to resist boiling** (*Burg et al., PNAS 95: 2056-2060, 1998*)
    - **reduced rotational freedom** – Thr56Ala, Gly58Ala, Ser65Pro and Ala96Pro
    - **introduction of disulfide bridge** - Gly8Cys + Asn60Cys
    - **improved internal hydrogen bond** - Ala4Thr
    - **filling cavity** - Tyr63Phe



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


Approaches in protein engineering
RATIONAL DESIGN
DIRECTED EVOLUTION
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 protein
7. Biochemical testing
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