Directed Evolution of Enzymes

Concept, Methods, and Selected Applications in Catalysis

MacMillan Group Meeting

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**Enzymes — Tailor-made Biocatalysts**

Evolutionary process leads to high substrate specificity

- High substrate selectivity = lacking generality

\[
\text{Lipase} \quad Pseudomonas aeruginosa \\
\text{H}_2\text{O} \quad \text{fast hydrolysis}
\]

\[
\text{Lipase} \quad Pseudomonas aeruginosa \\
\text{H}_2\text{O} \quad \text{slow hydrolysis}
\]

\[
\text{Lipase} \quad Pseudomonas aeruginosa \\
\text{H}_2\text{O} \quad \text{no hydrolysis}
\]

- Only a minor number of enzymes shows a desirable substrate promiscuity

Enzymes — Tailor-made Biocatalysts

Significant restrictions of natural enzymes

■ Substrate specificity: Limited tolerance to electronically or sterically modified substrates

■ Limited solvent variability: Water as almost exclusive solvent

■ Temperature: Lacking stability at elevated temperatures due to denaturation

■ Enantioselectivity: Satisfying values only for selected substrates
How to Expand the Synthetic Utility of Biocatalysts?
A closer look into the nature of enzymes

- Enzymes are proteins (polypeptides) possessing complex three-dimensional structures
  - e.g., molecular weight for *Pseudomonas aeruginosa* Lipase (PAL): ~29 kDa
  - specific substrate-protein interaction in catalytically active binding pocket
  - Limited degree of flexibility according to Koshland's "induced-fit" model

- Chemical modification to increase substrate range?
  - may require structural data
  - non-trivial endeavor to address selected sites
  - may be required for individually for every substrate/property
  - almost indefinite number of possible variations in protein space
How to Expand the Synthetic Utility of Biocatalysts

Learning from nature

One substrate–One enzyme principle is the result of an evolutionary process

Can we use an evolutionary approach to engineer enzymes on a lab scale?

*This requires*....

- accelerate the evolutionary process from \(\sim\) mio years to weeks
  - availability of suitable experimental techniques
    - establish a generally applicable concept
Directed Evolution

Definition

General blueprint for an evolutionary process of an enzyme

Phenotype

Starting protein (enzyme) → encoded by Starting DNA

Optimized protein (enzyme) → encoded by Optimized DNA

Selection or screening based on superior function

Library of mutant enzymes

Diversity creation by random mutation and/or replication

Gene expression

Library of mutant DNA

A Historical View on Directed Evolution

Timeline

1960 — 2000

1967

1967
M. Eigen reports a theory of evolution at the molecular level (Naturwissenschaften 1971, 58, 465)

1980's
rational mutagenesis approaches to engineer enzymes show only limited success

1986
Researchers at Synergen (Boulder/CO) succeed in the first directed evolution using an iterative rational mutagenesis approach (PNAS 1986, 83, 576)

1990
J. R. Knowles et al. report the first true random mutagenesis by using the full sequence space (PNAS 1990, 87, 696)

1993
F. H. Arnold et al. report the first iterative random mutagenesis and thus realize the evolutionary concept (PNAS 1993, 90, 217)

1997
M. T. Reetz & K.-E. Jaeger et al. use directed evolution to improve enantioselectivity of an enzymatic resolution (ACIEE 1997, 36, 2830)
Part I — An Introduction into Methods and Concepts

Flow diagram for a directed evolution process

Wild-type enzyme ← Gene (DNA) ↘ Random mutagenesis
Library of mutated genes ↓ Expression
Library of mutated enzymes ↓ Screening or selection
Positive Mutant

Starting with the Wild-type Enzyme: Limitation by Reaction Types
A selection of most frequent applications of enzymes

Typical enzyme-catalyzed transformations

\[
\begin{align*}
\text{Lipase/Esterase} & : \quad \text{R}^1\text{O} - \text{R}^2 + \text{H}_2\text{O} \rightarrow \text{R}^1\text{OH} + \text{HO} - \text{R}^2 \\
\text{Baeyer-Villigerase} & : \quad \text{R}^1\text{O} - \text{R}^2 + \text{H}_2\text{O} \rightarrow \text{R}^1\text{O} - \text{R}^2 \\
\text{Epoxide Hydrolase} & : \quad \text{R}^1\text{O} \rightarrow \text{R}^1\text{OH} - \text{OH} \\
\text{Aminotransferase} & : \quad \text{R}^1\text{O} - \text{R}^2 + \text{H}_2\text{O} \rightarrow \text{R}^1\text{NH}_2 - \text{NH}_2 \\
\text{Hydantoinase} & : \quad \text{R}^1\text{HN} - \text{NH} - \text{R}^2 \rightarrow \text{R}^1\text{NH} - \text{NH}_2 - \text{NH} \\
\text{Monoamine Oxidase} & : \quad \text{R}^1\text{NH}_2 - \text{NH}_2 + \text{H}_2\text{O} \rightarrow \text{R}^1\text{NH}_2 - \text{NH}_2 \\
\text{Aldolase} & : \quad \text{R}^1\text{OH} - \text{R}^2 + \text{H}_2\text{O} \rightarrow \text{R}^1\text{OH} - \text{R}^2 \\
\text{P450 Oxidase} & : \quad \text{R}^1\text{OH} - \text{R}^2 + \text{H}_2\text{O} \rightarrow \text{R}^1\text{OH} - \text{R}^2
\end{align*}
\]

**DNA Replication: Polymerase Chain Reaction (PCR)**

*Fully automated routine technique*

- A brief description of the basics

**Denaturation**

- 95 °C

**Annealing**

- (DNA Polymerase) 60 °C

**Elongation**

- 72 °C

- + Nucleosides

**Iterate**

(same for blue single strand)
Site-directed Mutagenesis
Non-recombinative methods

- Modified primers facilitate selective introduction of a single-point mutation

![Diagram showing the process of site-directed mutagenesis.](image-url)
Non-recombinative Mutagenesis Methods

More diversity

- **Saturation mutagenesis:** Randomize a selected position
  - Several mutated primers encoding all amino acids are used in a PCR-like process
  - Can be useful to optimize a selected position previously identified as a 'hot spot'

- **(Combinatorial) Cassette mutagenesis:** Randomize a selected region
  - Several mutated oligonucleotide sequences used to mutate a region previously identified
  - 'hot region' usually close to binding site
Non-recombinative Mutagenesis Methods

High-throughput methods

- *ep-PCR* ('error-prone'-PCR): A 'sloppy' PCR variant
  - Changing the experimental parameters (increased MgCl₂ concentration or addition of MnCl₂) leads to the incorporation of 2-3 'wrong' bases per replicated DNA strand
  - Takes advantage of the complete sequence space in a fully statistical process
  - Useful if no structural data available; can lead to the identification of hot spots

- Bacterial mutator strains: Using artificially enhanced natural mutation during replication
  - Natural mutation rate in *E. coli* ~0.0025/1000 base pairs in 30 generations
  - Caused by defects in repair mechanisms
  - Commercially available engineered bacterial strain XL1-Red causes 0.5/1000 base pair mutations
Recombinative Mutagenesis Methods

Rapid diversity creation

DNA shuffling: Cut & paste

- PCR-like recombination of small DNA fragments upon digestion to smaller oligomer units
- Recombinative method secures high mutation rate
- Particularly useful to amplify the desired properties of several mutated DNA strains in a second round

DNA shuffling

library of improved mutants

e.g. ep-PCR

selection of improved mutants

wild type
Part I — An Introduction into Methods and Concepts

Flow diagram for a directed evolution process

Wild-type enzyme → Gene (DNA) → Random mutagenesis → Library of mutated genes → Expression → Library of mutated enzymes → Screening or selection → Positive Mutant

Gene Expression

■ Mutated genes are introduced into bacterial hosts (E. coli) for amplification, transformed to the original bacteria which are cultivated

mutated Gene

E. coli

Amplification

Transfer to original bacteria

mutated Enzyme

Isolation

many

Cultivation

Transcription (DNA → RNA) followed by Translation (RNA → polypeptide)

■ (Automated) Picking of monoclonal colonies followed by testing for activity after isolation of the isolated enzyme

■ Active mutants can (but do not need to) be subjected to sequencing

Screening vs. Selection
Rapid identification of active mutants

Screening: In vitro evaluation of all mutants
- 1st law of directed evolution: "You get what you screen for"
- preferably using high-throughput assays on 96- or 384-microtiter plates (>1000 mutants per day)
- e.g., UV/Vis-based time-resolved enantioselectivity/activity assay for a lipase mutant

\[
\begin{align*}
\text{in well A} & & \text{Lipase mutant} & & \text{Oct} \text{Me} \text{O} \text{H} & & \text{Oct} \text{Me} \text{OH} & & \text{Nitrophenol} \\
\text{in well B} & & \text{Lipase mutant} & & \text{Oct} \text{Me} \text{O} \text{H} & & & & \text{no hydrolysis}
\end{align*}
\]

- other methods include: pH, fluorescence quenching, reporter enzyme systems, MS, NMR, IR, GC, HPLC

Selection: In vivo evaluation of all mutants, only active mutants are observed in assay

Part II — Selected Applications of Directed Enzyme Evolution

From solvent and temperature stability to enantioselective catalysis

- Early examples: Arnold's original report on the improvement of solvent stability

  [Image of protein structure]

  Subtilisin E mutant PC 3

  [Chemical reaction diagram]

  Relative activity: PC 3 mutant/wild type = 256/1

  - PC 3 mutant contains 10 point mutations
  - all beneficial mutations relatively close to binding site

- Applied approach: Random followed by site-directed mutagenesis

  [Diagram of mutagenesis process]

Arnold's Initial Approach to Directed Evolution

Engineering solvent stability

- Applied approach: random followed by site-directed mutagenesis

- Second step is less efficient because it does not take advantage of the mutants obtained in the initial mutation cycle, rather follows a manual procedure

An Improved Approach Using DNA Shuffling
Engineering the hydrolytic activity of a lipase

- para-Nitrobenzyl esterase: A problem from process research

\[
\begin{align*}
\text{Subtilisin mutants} & \quad \text{DMF/H}_2\text{O (25:75)} \\
& \quad 37^\circ\text{C}
\end{align*}
\]

- Model reaction suitable for high-throughput screening

\[
\begin{align*}
\text{Subtilisin mutants} & \quad \text{DMF/H}_2\text{O (25:75)} \\
& \quad 37^\circ\text{C}
\end{align*}
\]

An Improved Approach Using DNA Shuffling
Engineering the hydrolytic activity of a lipase

- Recombinative DNA shuffling with active mutants leads to new mutants showing additive effects

- Back shuffling versus wild-type eliminates inactive point mutations

An Improved Approach Using DNA Shuffling
Engineering the hydrolytic activity of a lipase

- Recombinative methods can accelerate directed evolution

- typical activity curve using non-recombinative methods

- typical activity curve using both methods

![Diagram showing activity over generation for random mutagenesis (ep-PCR) and DNA shuffling]
Directed Evolution of Enantioselective Enzymes

Introduction of the concept

- Hydrolytic kinetic resolution using a lipase

$$\text{Hex} \quad \text{O} \quad \text{Me} \quad \text{O} \quad \text{Me} \quad \text{H}_2\text{O} \quad \text{Hex} \quad \text{O} \quad \text{Me} \quad \text{H}_2\text{O} \quad \text{P. Aeruginosa}$$

- Applied concept: Iterative random mutagenesis based on ep-PCR

Wild type $\xrightarrow{\text{ep-PCR}}$ 1000 clones (1-2 mutations per enzyme) $\xrightarrow{\text{screening}}$ 1 mutant $\xrightarrow{\text{repeat 3 times}}$ active and selective mutant

Directed Evolution of Enantioselective Enzymes

Introduction of the concept

From almost unselective wild types to moderate selectivity

Enantioselective Enzymes: A Rational Approach

Hot spot identification

- Selective mutants showed frequent mutations at two amino acid positions 155 and 162

- Combinatorial cassette mutagenesis at one of the hot regions close to binding site (160-165)

- Rational selection of hot regions based on previously gained information and structural insight

Improved Efficiency by Focused Library Development
Reetz’ CASTing approach

- Hydrolytic kinetic resolution of epoxides: Epoxide hydrolase from *Aspergillus niger*

  ![Epoxide Hydrolase Reaction](image)

  $s = 4.6$ for wild type

- Combinatorial active site saturation test'

  ![Combinatorial Active Site Saturation Test](image)

  structural information $\rightarrow$ select promising positions close to binding pocket $\rightarrow$ conduct saturation mutagenesis simultaneously at two positions

  $\rightarrow$ select best mutant and repeat saturation mutagenesis for two other previously identified positions $\rightarrow$ iterate last step

- requires structural information (X-ray) as a starting point
- reduces number of mutant in screen
- considers cooperative effects because two positions are randomized at the same time

Improved Efficiency by Focused Library Development

Reetz’ CASTing approach

Additivity of positive mutations shows only minor dependency on the order

- Five iterations of CASTing furnish a catalyst with $s = 115$

- Result of sequence A→B→C→D→E→F comparable to result of sequence A→B→C→D→F→E*

- Rational input dramatically reduces the required number of surveyed mutants

* A-F are different pairs of amino acids selected for randomization

Improved Efficiency by Focused Library Development
Reetz' CASTing approach

• Experimental and theoretical data reveal many ways to make a 'good' enzyme

• For 5 iterations 5! = 120 pathways to obtain desired mutant are possible

• Analysis of all 120 pathways reveals that many follow an energetically favorable pathway

• \( s \sim \Delta G^\ddagger_{(R-S)} \implies \) every path having negative \( \Delta G^\ddagger \) is favorable

• 55 of 120 pathways (46%) are favorable
  \( \implies \) high probability to find an active mutant
  \( \implies \) if not, one single step backwards is implied

CASTing for... Substrate Tolerance
Rational approach to directed evolution

- Expanding substrate scope of lipase-catalyzed ester hydrolysis
  - Positions selected for iterative saturation mutagenesis selected based on X-ray data

\[
\text{Lipase}\quad P.\ aeruginosa
\xrightarrow{\text{H}_2\text{O}}
\begin{align*}
&\text{R} \quad \text{CO}_2\text{H} + \text{OH} \quad \text{C}_6\text{H}_4\text{NO}_2 \\
&\text{R} \quad \text{CO}_2\text{H} + \text{OH} \quad \text{C}_6\text{H}_4\text{NO}_2
\end{align*}
\]

CASTing for... Thermostability
Rational approach to directed evolution

■ X-ray structure data provide B values that quantify the flexibility of an atom ("smeering")

**Diagram:**
- Amino acid positions having highest B values are selected for saturation mutagenesis.
- Conduct saturation mutagenesis simultaneously at two of the selected positions.
- Screen for mutant encoding the enzyme with highest temperature stability.
- Iterate last two steps by randomizing other selected positions.

**Result:** Temperature range for the hydrolytic activity of a Lipase from *B. subtilis* can be extended from 50 °C to 80 °C.

The Number Problem in Saturation Mutagenesis
Consequences of statistical considerations

- Required 'oversampling' in activity screens

- In order to secure a high coverage of a generated library the actual number of enzyme variants to be screened is significantly higher than the number of mutants

The Number Problem in Saturation Mutagenesis
Consequences of statistical considerations

- Required 'oversampling' in activity screens

- In order to secure a high coverage of a generated library the actual number of enzyme variants to be screened is significantly higher than the number of mutants

Reducing the Amino Acid Space
Encoding only 12 amino acids greatly reduces the screening effort

A streamlined saturation mutagenesis approach based on encoding less amino acids

<table>
<thead>
<tr>
<th>Surveying 20 AA</th>
<th>Surveying 12 AA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of AA to be randomized</td>
<td>No. of mutants to be screened at 95% library coverage</td>
</tr>
<tr>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>3,066</td>
</tr>
<tr>
<td>3</td>
<td>98,163</td>
</tr>
<tr>
<td>4</td>
<td>3,141,251</td>
</tr>
<tr>
<td>5</td>
<td>100,520,093</td>
</tr>
</tbody>
</table>

Using a 'balanced mix of polar, nonpolar, aromatic, aliphatic, negatively, and positively charged amino acids while excluding most cases of structurally similar amino acids'

Reducing the Amino Acid Space
Application in hydrolytic kinetic resolution

- High quality of obtained library: From a non-binding substrate to an enantioselective process

```
\[
\begin{array}{c}
\text{Ph} \quad \text{O} \quad \text{Me} \\
\text{trans (rac)}
\end{array}
\quad \xrightarrow{\text{Hydrolase (A. niger)}}
\quad \begin{array}{c}
\text{Ph} \quad \text{OH} \quad \text{Me} \\
\text{trans (ent)}
\end{array}
\quad \begin{array}{c}
\text{Ph} \quad \text{OH} \\
\text{H}_2\text{O}
\end{array}
\]`

- Saturation mutagenesis of 3 positions based on 20 amino acids (15% coverage)

```
\[
wild \text{ type (does not accept substrate)} \quad \xrightarrow{\text{CASTing (sat. Mut.)}} \quad 5000 \text{ mutants} \quad \xrightarrow{\text{38 active}} \quad \text{best mutant:} \\
\text{TOF} = 17 \quad \text{s} = 101
\]`

- Saturation mutagenesis of 3 positions based on 12 amino acids (95% coverage)

```
\[
wild \text{ type (does not accept substrate)} \quad \xrightarrow{\text{CASTing (sat. Mut.)}} \quad 5000 \text{ mutants} \quad \xrightarrow{\text{511 active}} \quad \text{best mutant:} \\
\text{TOF} = 55 \quad \text{s} > 200
\]`
```

**Enantioselective Baeyer-Villiger Oxidation**

*An example of whole cell catalysis*

- Desymmetrization of cyclic ketones by engineered enzymes

\[
\begin{align*}
\text{Cyclohexanone monooxygenase} & \quad \text{Acinetobacter sp. NCIMB 9871} \\
\text{O}_2 & \quad \text{[catalyst]} \\
\text{OH} & \quad \text{[product]} \\
\end{align*}
\]

- Whole cells contain additional co-factor NADPH responsible for flavin co-factor regeneration

Enantioselective Baeyer-Villiger Oxidation

An example of whole cell catalysis

- Desymmetrization of cyclic ketones by engineered enzymes

\[
\text{Cyclohexanone monooxygenase} \\
\text{Acinetobacter sp. NCIMB 9871} \\
\text{O}_2
\]

Strategy: Iterative random mutagenesis by ep-PCR

1st round of ep-PCR

wild type (9% ee) → 10,000 mutants → 24 R-selective & 12 S-selective

selected mutant shows 1 single point mutation and 40% ee (R)

2nd round of ep-PCR

best mutant shows 4 single point mutations and 90% ee (R)

Enantioselective Baeyer-Villiger Oxidation
An example of whole cell catalysis

- Substrate promiscuity by generating a mutant library

Cyclohexanone monooxygenase mutants
Acinetobacter sp. NCIMB 9871

- Different mutants show different activity and selectivity for a range different substrates

Enantioselective Baeyer-Villiger Oxidation
Restricting the amino acid space

- A rational approach to generate an active catalyst

\[
\begin{align*}
&\text{phenylacetone monooxygenase} \\
&\text{mutants} \\
&T. fusca \\
&O_2 \\
\end{align*}
\]

- Saturation mutagenesis using a dramatically restricted amino acid space

wild type \((s = 1.2 \text{ for } S)\) \(\rightarrow\) selection of amino acids being used for saturation mutagenesis based on sequence comparison with related enzyme \(\rightarrow\) simultaneous saturation mutagenesis at 4 vicinal positions using reduced set of encoded amino acids \(\rightarrow\) 2587 mutants (@ 95% coverage) best mutant: \(s = 48 \ (R \text{ selective})\)

- > 3 mio. mutants needed if all 20 amino acids would have been considered
- no X-ray data available \(\rightarrow\) relevant positions and amino acids identified by comparison with sequence of Acinebacter active site that is known to be a Baeyer-Villigerase

Amine Oxidases: Deracemizations of Tertiary Amines
Application of mutator strains as an alternative to ep-PCR

- Directed evolution coupled in a tandem sequence

\[
\begin{align*}
\text{monoamine oxidase} \\
\text{mutants from} \\
\text{Aspergillus niger}
\end{align*}
\]

Strategy: Iterative random mutagenesis using bacterial mutator strains

- wild type (low activity) ➔ Iterative random mutagenesis using XL1 mutator strains ➔ screening indicates mutant bearing 5 point mutations as most active ➔ \( s = 134 \) (R selective)

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Remarkable regioselectivity for linear alkanes

- Directed evolution to develop enantio- and regioselective C–H hydroxylation

Cytochrome P450 BM-3 mutants from Bacillus megaterium

\[ \text{n-Alkyl} \rightarrow \text{Me} \rightarrow \text{Me} \rightarrow \text{OH} \rightarrow \text{OH} \rightarrow \text{Me} \]

+ NADPH
+ O₂, + H⁺
- NADP⁺
- H₂O

- natural substrates: Fatty acids (C-12 to C-18): e.g. myristic acid (54% ω-1, 25% ω-2, 20% ω-3)
- alkanes are as such toxic substrates limiting the activity of enzymes

- Strategy

wild type \[ \rightarrow \text{iterative random mutagenesis using ep-PCR and screening} \rightarrow \text{recombination of most active mutants} \rightarrow \text{iterative random mutagenesis using ep-PCR and screening} \rightarrow \text{tbc} \]

* a PCR-like variant of DNA shuffling

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Remarkable regioselectivity for linear alkanes

- Directed evolution to develop enantio- and regioselective C–H hydroxylation

- Strategy

  wild type ➔ iterative random mutagenesis using ep-PCR and screening ➔ recombination of most active mutants ("staggered extension process")* ➔ iterative random mutagenesis using ep-PCR and screening ➔ best mutant 139-3: ~40-fold increase in activity (vs wild type) ➔ recombination of most active mutants ("staggered extension process")* ➔ iterative random mutagenesis using ep-PCR and screening ➔ tbc

* a PCR-like variant of DNA shuffling

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Remarkable regioselectivity for linear alkanes

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<tr>
<td>Site-directed saturation mutagenesis</td>
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* a PCR-like variant of DNA shuffling

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Remarkable regioselectivity for linear alkanes

- Directed evolution to develop enantio- and regioselective C–H hydroxylation

Cytochrome P450 BM-3
mutant 1-12G
Bacillus megaterium

\[
\text{n-Hex} \quad \text{Me} \quad \rightarrow \quad \text{n-Hex} \quad \text{Me} \quad \text{OH}
\]

86% (52% ee)
(+ 5% 4-hydroxy)

- Switch from terminal hydroxylation preference (wild-type) to internal positions


Engineered P450 Enzymes: Enantioselective C–H Oxidation

Remarkable regioselectivity for linear alkanes

- Directed evolution to develop enantio- and regioselective C–H hydroxylation

\[
\text{Cytochrome P450 BM-3 mutant 1-12G}
\]

\[
\text{Bacillus megaterium}
\]

\[
\begin{align*}
\text{n-Hex} & \quad \text{Me} \\
\rightarrow & \\
\text{n-Hex} & \quad \text{Me} \\
\text{OH} & \\
\text{86\% (52\% ee)} & \\
\end{align*}
\]

\[
\begin{align*}
\text{n-Hex} & \quad \text{Me} \\
\rightarrow & \\
\text{n-Hex} & \quad \text{OH} \\
\text{3\%} & \\
\text{n-Hex} & \quad \text{Me} \\
\text{OH} & \\
\text{7\%} & \\
\text{(+ 5\% 4-hydroxy)} & \\
\end{align*}
\]

- Several linear alkanes (C-3 to C-9) as well as ethers are accepted
- Activity-based directed evolution, not ee-driven iterative process
  → screening for enantioselectivity could furnish highly enantioselective enzyme


Engineered P450 Enzymes: Enantioselective C–H Oxidation
Regio-, diastereo- and enantioselective hydroxylations of cyclopentane derivatives

- Same mutant library facilitates enantioselective hydroxylation of functionalized cycloalkanes

![Chemical Reaction Diagram]

Cytochrome P450 BM-3 mutants from *Bacillus megaterium* catalyze the oxidation of a substrate with the following conditions:

- $\text{NADPH} + \text{O}_2 + \text{H}^+$
- $-\text{NADP}^+
- -\text{H}_2\text{O}$

**Wild type**: 25% ee (R,R), 20% (80% 2-hydroxy), TON 2.6

**Mutant B**: 88% ee (S,S), 96:4 dr, 90%, TON 215

**Mutant 1-12G**: 88% ee (R,R), 98:2 dr, 95%, TON 213

Engineered P450 Enzymes: Enantioselective C–H Oxidation

Hydroxylation of aryl acetic acids

- Mutant library also facilitates enantioselective hydroxylation of aryl acetic acids


Degussa Synthesis of Enantiopure D-Amino Acids

An Industrial Scale Application of Engineered Enzymes: 'White Biotechnology'

- Triple enzymatic dynamic kinetic resolution process based on engineered enzymes

\[ \text{Hydantoin racemase} \]

\[
\begin{align*}
R & \quad \text{Hydantoinase} \\
\text{HN} & \quad \text{minimized substrate tolerance by genetic modifications} \\
\text{NH} & \\
\text{O} & \\
\text{NH} & \\
\text{O} & \\
\text{RN} & \\
\text{HN} & \quad \text{Hydantoinase} \\
\text{NH} & \\
\text{O} & \quad \text{minimized substrate tolerance by genetic modifications} \\
\text{NH} & \\
\text{O} & \\
\text{RN} & \\
\text{HN} & \\
\text{NH} & \\
\text{O} & \\
\text{RN} & \\
\end{align*}
\]

- *E. coli* host carries genetically modified hydantoinase and carbamoylase from
  *Arthrobacter crystallopoietes* DSM20117
- Degussa utilizes white biotechnology to produce a wide range of natural and unnatural AA's
  - e.g., D-aminobutyric acid, D-serine, D-methionine, D-tryptophan, D-phenylalanine

Summary — Streamlining Directed Evolution of Enzymes

Rational
Consideration of X-ray structural data or molecular modeling or comparison to related enzymes

Choice of a suitable enzyme displaying desired reactivity. Select property to be optimized

Random
several cycles of random mutagenesis (e.g. ep-PCR) followed by sequencing

Identification of 'hot spots' or 'hot region'

restricting the number of surveyed amino acids by rational input

iterative saturation mutagenesis at 'hot spots' or 'hot regions'

application of recombinative methods to increase diversity (e.g. DNA shuffling)

GOAL: Minimize number of surveyed mutants (= time, material), maximize the synthetic utility
Scope and Limitations of Directed Evolution of Enzymes

Scope

- Recent years show a considerable extension of substrate scope due to more sophisticated approaches
- Obtainable enantioselectivities are for most examples highly competitive
- Other important properties like temperature stability and solvent stability are adjustable
- So far, typical reactivity modes of enzymes have been explored, such as hydrolysis, oxidation, reduction

Advantages

- A routinely conducted tailor-made catalyst production for each substrate seems within reach
- One substrate — one catalyst; yet, protein space allows to develop specific catalysts for each substrate

Limitations

- Number of known synthetically interesting enzyme wild types appears limited
- Structural information is an urgent need but typically not available
- Reactivity modes of these enzymes cover only parts of the organic chemistry repertoire
Overcoming limitations in reaction space

- Design of hybrid organometallic/bioorganic catalysts and engineering them by directed evolution


- *De novo* design (*in silico followed by in vitro*) of artificial enzymes incorporating new reactivity patterns


- Expanding the reactivity scope by incorporation of new functionalized unnatural amino acids (*"Expanding the Genetic Code"*)

General References

General reviews: Directed evolution of enzymes focusing on enantioselective catalysis


Further reading on high-throughput screening Methods (not covered)


General References

- Short reviews & accounts


