Directed Evolution of Stereoselective Biocatalysts

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CHEM575 Literature Seminar

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Importance of Stereoselective Synthesis
Catalytic Approaches

Small Molecules
• High substrate scope
• Stability/Solubility
• Synthetic accessibility
• Decades of development

Enzymes
• Poor solubility/stability
• Greener chemistry
• Tremendous complexity
• Excellent stereoselectivity
How can we get better enzymatic catalysts?

- Cloning natural enzymes
- De novo design
- Rational modification
- Directed evolution
Directed Evolution

- Random Mutagenesis
- Transformation & Expression
- Selection / Screening
- Cloned DNA
Mutagenesis Methods

Error Prone PCR (epPCR)

Primer
Cloned DNA

Taq
MnCl₂

Site Saturation Mutagenesis

Randomized Primers

DNA Shuffling

The Sorting Problem

Large Libraries:
Good for diversity, Bad for sorting

**Screening**
- Brute Force
- Time/Labor/Resource Intensive
- Simple
- General

**Selection**
- Efficient
- Scalable
- Not Simple
- Not General

Solutions to the Sorting Problem
Selection

Chorismate Mutase

Libraries expressed in cells lacking Chorismate Mutase

Solutions to the Sorting Problem

Screening

- Involves analysis of reaction products
- Throughput is key!

Successful examples
Baeyer Villiger Oxidation

Mechanism

Chiral Products

Enzymatic Baeyer Villiger Oxidation

Cyclohexanone Monooxygenase (CHMO)

Directed Evolution of Baeyer Villigerases

- **Mutagenesis Strategy**
  - epPCR – 10,000 in round 1
  - 2,000 in round 2

- **Screening**
  - Chiral GC – 800 variants/day

- Libraries expressed in E. coli.

- Screen reaction run with whole cells

Directed Evolution of Baeyer Villigerases

![Chemical structure](image)

First round hits

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Amino acid exchanges</th>
<th>Favored enantiomer of 3</th>
<th>e.r.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>–</td>
<td>R</td>
<td>55:45</td>
</tr>
<tr>
<td>1-C2-B7</td>
<td>F432Y, K500R</td>
<td>R</td>
<td>67:33</td>
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<tr>
<td>1-F1-F5</td>
<td>L143F</td>
<td>R</td>
<td>70:30</td>
</tr>
<tr>
<td>1-E12-B5</td>
<td>F432I</td>
<td>R</td>
<td>75:25</td>
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<tr>
<td>1-H7-F4</td>
<td>L426P, A541V</td>
<td>R</td>
<td>77:23</td>
</tr>
<tr>
<td>1-H3-C9</td>
<td>L220Q, P428S, T433A</td>
<td>S</td>
<td>59:41</td>
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<tr>
<td>1-F4-B9</td>
<td>D41N, F505Y</td>
<td>S</td>
<td>73:27</td>
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<td>1-K6-G2</td>
<td>K78E, F432S</td>
<td>S</td>
<td>89:11</td>
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<tr>
<td>1-K2-F5</td>
<td>F432S</td>
<td>S</td>
<td>90:10</td>
</tr>
</tbody>
</table>

*e.r. = enantiomeric ratio


## Results of Directed Evolution

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mutant</th>
<th>Selectivity</th>
<th>e.r.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improved R Variant</td>
<td>w.t.</td>
<td>R</td>
<td>55:45</td>
</tr>
<tr>
<td>Substrate Scope</td>
<td>2-D19-E6</td>
<td>R</td>
<td>95:5</td>
</tr>
<tr>
<td></td>
<td>1-K2-F5</td>
<td>S</td>
<td>90:10</td>
</tr>
<tr>
<td></td>
<td>1-K2-F5</td>
<td>S</td>
<td>&gt;99:1</td>
</tr>
<tr>
<td></td>
<td>1-K2-F5</td>
<td>S</td>
<td>&gt;98:2</td>
</tr>
<tr>
<td></td>
<td>1-K2-F5</td>
<td>S</td>
<td>&gt;98:2</td>
</tr>
</tbody>
</table>

Conclusions from Reetz Study

How is this study significant?

• Unselective enzyme made synthetically useful
• Significant reversal of stereoselectivity
• Simplistic mutagenic strategy
• Substrate scope
• Prior knowledge requirements
• Superiority to other approaches
N-acetylneuraminic lyase (NAL)

(Aldolase)

Wild type NAL (S:R) = 74:26

Sialic acids

Directed Evolution of NAL

- **Mutagenesis Strategy**
  - epPCR – 2500/round
  - site-saturation mutagenesis
  - semi-rational design

- **Screening**

  \[
  \text{MeCO}_2^- + \text{Pr}_2\text{N} \overset{\text{OH}}{\text{OH}} \overset{\text{OH}}{\text{O}} \text{Pr}_2\text{N} \overset{\text{AcHN}}{\text{OH}} \overset{\text{OH}}{\text{CO}_2^-} + \text{Pr}_2\text{N} \overset{\text{OH}}{\text{OH}} \overset{\text{OH}}{\text{O}} \text{Pr}_2\text{N} \overset{\text{AcHN}}{\text{OH}} \overset{\text{OH}}{\text{CO}_2^-} \]

  \[
  \text{MeCO}_2^- \overset{\text{OH}}{\text{OH}} \overset{\text{OH}}{\text{O}} \text{Pr}_2\text{N} \overset{\text{AcHN}}{\text{OH}} \overset{\text{OH}}{\text{CO}_2^-} + \text{Pr}_2\text{N} \overset{\text{OH}}{\text{OH}} \overset{\text{OH}}{\text{O}} \text{Pr}_2\text{N} \overset{\text{AcHN}}{\text{OH}} \overset{\text{OH}}{\text{CO}_2^-} \]

  \[
  \text{NADH} \overset{\text{lactate dehydrogenase}}{\longrightarrow} \text{MeCO}_2^- + \text{NAD}^+ \]

Structural Considerations

4S-selective
Green

4R-selective
Red

Directed Evolution of NAL

Best
4S-Selective

66% Yield
d.r. >98:2

Best
4R-Selective

70% Yield
d.r. >98:2

Structural Considerations

4R-selective
E192N
T167V
S208V

4S-selective
E192N
T167G

Substrate analog

Evolution of an Enantioselective Aldolase

deoxy-D-ribose 5-phosphate aldolase (DERA)

\[ \text{Me} + \text{OH} \text{OPo}_3^2 \rightarrow \text{OH} \text{OPo}_3^2 \]

D-Glyceraldehyde 3-phosphate

2-Deoxy-D-ribose 5-phosphate

Proposed Application

\[ \text{Cl} + \text{Me} \rightarrow \text{Cl} \text{OH} \]

Cl Lys

- e.r. > 99.9:0.1
- Low activity
- Limited substrate scope
- Substrate Inhibition

Directed Evolution of an Enantioselective Aldolase

• Goals
  Improve the activity of DERA
  Decrease substrate inhibition

• Mutagenesis Strategy
  epPCR – 3,000 clones per round
  DNA Shuffling of best hits

• Screening
  Target reaction run in cell free extract
  Activity determined by GC
  Throughput: 300 samples/day

Results of Directed Evolution

<table>
<thead>
<tr>
<th>Mutagenic Method</th>
<th>Rounds</th>
<th>Improved Hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>epPCR</td>
<td>3</td>
<td>63</td>
</tr>
<tr>
<td>DNA Shuffling</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Site Saturation Mutagenesis</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Best Hits Displayed:
- Increased activity
- Reduced substrate inhibition

DE of an Enantioselective Aldolase

Atorvastatin (Lipitor)

- Statin
- Inhibits HMG-CoA-Reductase
- Marketed by Pfizer
- 2006 Sales: $12.9 billion

Single-Enantiomer Synthesis

Conclusions

Directed Evolution stands as an underutilized, yet potentially general and powerful way to access stereoselective catalysts

Benefits

- Exceptional catalyst stereoselectivity
- Methodological complementarity to transition metals
- Strategic generality
- Green chemistry

Limitations

- Current enzymatic scope/availability
- Overhead

Future Directions
Acknowledgements

- Professor Silverman
- Professor Burke
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