Sustainable Biocatalytic Synthesis of β-Hydroxyl-α-Amino Acids on an Industrial Scale
β-Hydroxyl-α-Amino Acids – Important Building Blocks

Vancomycin family antibiotics, e.g. Oritavancin, Teicoplanin

β-lactam antibiotics

Chloramphenicol
e.g. Typhus treatment of penicillin resistant strains

Traditional Synthesis of Chloramphenicol

1. Bromination with Br₂
2. Amination with Hexamine
3. Protection with Ac₂O
4. Aldol Addition with Formaldehyde
5. Reduction with Al(i-PrO)₃
6. Deprotection with HCl
7. Resolution
8. Amidation

More efficient and sustainable route is desired
The Benefits of Enzyme Catalysis

Shorter Synthetic Routes
- No need for protective group chemistry
- No need for prior substrate activation

Enabling use of Alternative Substrates
- Sugar Crops
- Starch Crops
- Bio Mass
- Waste
- Natural Gases
- New Feedstock
  - e.g. Bio based
  - or
  - Cheaper materials

Very Diverse Reaction Set
- Hydroxylation
- Enolate Reduction
- Transamination
- Alcohol Oxidation
- Ketone Reduction
- Aldehyde Oxidation
- Aldol Reaction
- Aldehyde Cyanation
- Decarboxylation
- Nitrile Hydrolysis

Extraordinary Selectivity
- Stereo selectivity
- Regio selectivity
- Chemo selectivity
  - By specific substrate binding

Mild Environment Friendly Reaction Conditions
- e.g. Ambient temperature
- No heavy metals

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Enzymatic Approach for Chloramphenicol

Problem:
Natural enzyme is industrially not applicable

Solution:
Enzyme engineering by directed evolution using Aldolase

Aldolase

% de 50%
10% conversion in 8 h
40 g/L substrate load

2 Steps

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Common Directed Evolution Approaches

1. DNA Libraries by Error Prone PCR
   - Theoretically full sequence space coverage
   - Library size $10^7$ to $10^9$ variants
   - Screening effort often ca. 100k variants
   - HPLC / GC screening not applicable
   - Loss of information
   - Subsequent site saturation recommended

2. Site Saturation Mutagenesis or DNA Shuffling
   - Smaller library sizes
   - HPLC / GC screening applicable
   - Comprehensive reaction insights
   - Superficial sequence space coverage
   - Easily stagnates at local maxima
Directed Evolution Using BioEngine®

- In silico hot spot identification covers full sequence space
- In silico enzyme activity screening (10,000 variants/day)
- In silico stability screening (1,000 variants/day)
- All screenings under real process conditions
- HPLC/GC analytics provide comprehensive reaction insights
- Only 4-5 weeks per round of evolution
- Online improvement of in silico methods
Optimization of Aldolase for Industrial Application using BioEngine®

Wildtype Enzyme:
- de 50%
- 10% conversion in 8 h
- 40 g/L substrate load

Industrial Enzyme:
- de >90%
- >80% conversion in 8 h
- 200 g/L substrate load

9 rounds of evolution

5 steps (60%) shorter route compared to chemical synthesis

Chloramphenicol
e.g. typhus treatment of penicillin resistant strains

Patent application No.: PCT/CN2018/086227

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Who is Enzymaster?

- Short Company Presentation
Enzymaster - Where Green Magic Happens

International consortium of founders

Enzymaster (Ningbo) Bio-Engineering Co., Ltd. in Ningbo (China):
Lab space: ca. 3000 m²
Fermentation pilot plant: up to 1000 L
Employees: ca. 100 (60% in R&D and Tech)

Enzymaster Deutschland GmbH in Düsseldorf (Germany):
Employees: 4

Shanghai:
SJTU π-Supercomputer account
Computational enzyme engineering
500,000 CPU hours/year
Enzymaster - History

- 2013
  - Enzymaster (Ningbo) Bio-Engineering Co., Ltd. established as sino-foreign Joint Venture

- 2014
  - Recipient of “Ningbo 3315 Programme” (Startup Team).
  - Angel-Round Financing completed.

- 2015
  - Scientific Advisory Board established.

- 2016
  - Technology commercialized at partner’s site (> 10 mt scale).

- 2017
  - Listed on Ningbo Equity Exchange Board.

- 2018
  - Headquarters with 1400 m² R&D space established.
  - Round-A Financing completed.
  - Awarded as HI-TECH enterprise.

- 2019
  - Moved R&D to new space with ca. 3000 m².
  - Enzymaster Deutschland GmbH established.

Setting up of BioEngine® platform

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Our Asset - Enzyme Directed Evolution

Panel Screening
- Project idea
- Computational enrichment of in-house enzyme libraries
- Enzyme screening to identify best enzyme
- In silico identification of hot spots

Directed Evolution
- In silico pre-screening
- In silico recombination
- Smart library creation by
  - Gene synthesis
  - Mutagenesis
- Recombinant enzyme library expression
- Use all collected data to improve computational models

Commercialization
- Industrial biocatalytic process
- Enzyme fermentation development
- Selection of most improved enzyme
- High throughput screening using HPLC, GC
- Use all collected data to improve computational models
Enzymaster - Service Offer

- Enzyme panel screening
- „Smart“ enzyme directed evolution
- Enzyme preparation by fermentation
- Enzyme catalytic process development
- Custom biocatalytic manufacturing

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Enzymasters’ Computational Resources

• In-house hardware:
  Graphics workstations
  Linux file server
  Linux GPU compute nodes

• SJTU π-Supercomputer account
  332 CPU Nodes 2x Xeon E5 (16 cores)
  20 fat Nodes 256GB RAM
  70 GPU Nodes (K20/40/80/P100)
  Usage of ~ 500,000 core hours/year

→ Extensive Lab & Computer Resources allow to perform up to 6 evolution projects in parallel
Enzymaster - Success Stories

Ketoreductase Industrial Enzyme:
• 24 h
• 99% conversion
• 400 g/L substrate load

Initial Enzyme:
• 48 h
• 57% conversion
• 60 g/L substrate load

50 MT scale Production of (R)-1,3-Butanediol

Industrial Enzyme:
• 24 h
• 99% conversion
• 400 g/L substrate load

The Information Presented here is the Property of Enzymaster (Ningbo) Bio-Engineering Co., Ltd.

Patent application No.: 201811537509.4
Yet to be published
### Key Reaction Types & Enzyme Classes

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Chemical Reaction</th>
<th>Enzyme Classes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transamination</td>
<td>$\text{R}_1\text{R}_2\text{CO} \rightarrow \text{R}_1\text{R}_2\text{NH}_2$</td>
<td>Aminotransferases</td>
</tr>
<tr>
<td>Alcohol Oxidation</td>
<td>$\text{R}_1\text{R}_2\text{OH} \rightarrow \text{R}_1\text{R}_2\text{CO}$</td>
<td>Ketoreductases/ADHs</td>
</tr>
<tr>
<td>Hydroxylation</td>
<td>$\text{R}_1\text{R}_2\text{OH} \rightarrow \text{R}_1\text{R}_2\text{OH}$</td>
<td>Aldolases</td>
</tr>
<tr>
<td>Ketone Reduction</td>
<td>$\text{R}_1\text{R}_2\text{CO} \rightarrow \text{R}_1\text{R}_2\text{OH}$</td>
<td>Esterases/Lipases</td>
</tr>
<tr>
<td>Aldehyde Oxidation</td>
<td>$\text{R}_1\text{R}_2\text{CO} \rightarrow \text{R}_1\text{R}_2\text{CO}_2$</td>
<td>Decarboxylases</td>
</tr>
<tr>
<td>Enoate Reduction</td>
<td>$\text{O} = \text{R}_1\text{R}_2\text{CO} \rightarrow \text{R}_1\text{R}_2\text{CO}_2$</td>
<td>Oxynitrilases</td>
</tr>
<tr>
<td>Aldehyde Cyanation</td>
<td>$\text{R}_1\text{R}_2\text{CO} \rightarrow \text{R}_1\text{R}_2\text{C}_2N$</td>
<td>Nitrilases</td>
</tr>
<tr>
<td>Carboligation</td>
<td>$\text{R}_1\text{R}_2\text{CO} + \text{R}_1\text{R}_2\text{O} \rightarrow \text{R}_1\text{R}_2\text{C}_2N$</td>
<td>TPP-dep. Lyases</td>
</tr>
<tr>
<td>Decarboxylation</td>
<td>$\text{R}_1\text{R}_2\text{CO}_2\text{OH} \rightarrow \text{R}_1\text{R}_2\text{CO}_2$</td>
<td></td>
</tr>
<tr>
<td>Nitrile Hydrolysis</td>
<td>$\text{R}_1\text{R}_2\text{C}_2N \rightarrow \text{R}_1\text{R}_2\text{CO}_2$</td>
<td></td>
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<tr>
<td>Imine Reduction</td>
<td>$\text{R}_1\text{R}_2\text{C}_2N \rightarrow \text{R}_1\text{R}_2\text{C}_2N$</td>
<td></td>
</tr>
</tbody>
</table>
Enzymes developed for own productions are demonstrating our industrial competence

<table>
<thead>
<tr>
<th>Building block and food supplement: β-Alanine</th>
<th>Chiral auxiliary: (R)-α-Phenylethylamine R-PEA</th>
<th>Chiral building block: Methyl(R)-3-hydroxybutyrate R-MHB</th>
<th>Chiral building block: (R)-1,3-Butanediol R-BDO</th>
<th>Chiral building block: L-tert-Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="β-Alanine Structure" /></td>
<td><img src="image" alt="PEA Structure" /></td>
<td><img src="image" alt="R-MHB Structure" /></td>
<td><img src="image" alt="R-BDO Structure" /></td>
<td><img src="image" alt="L-tert-Leucine Structure" /></td>
</tr>
<tr>
<td>Production Scale: 100 – 150 mt/month</td>
<td>Production Scale: 1000 mt</td>
<td>Production Scale: Up to 100 mt</td>
<td>Production Scale: Up to 50 mt</td>
<td>Production Scale: Up to 10 mt</td>
</tr>
<tr>
<td>Application: Pharma and Food</td>
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<td>Application: Pharma and Cosmetics</td>
<td>Application: Pharma</td>
</tr>
</tbody>
</table>
Enzymaster - Further Commercial Products

- **Ethyl (R)-3-Hydroxybutyrate (R-EHB)**
  - Molecular structure:
  - Food Supplement

- **(R)-3-Hydroxybutyric Acid, Sodium Salt (R-BHB)**
  - Molecular structure:
  - Food Supplement

- **Glutamine-S**
  - Molecular structure:
  - Food Supplement

- **(S)-α-Phenylethylamine**
  - Chiral auxiliary

- **(R)-1-[3,5-Bis(trifluoromethyl)phenyl]ethanol**
  - Molecular structure:
  - Aprepitant IM

- **L-Citrulline DL-Malate 2:1**
  - Molecular structure:
  - Food Supplement

- **L-Tyrosine**
  - Molecular structure:
  - Food Supplement

- **Tyramine**
  - Molecular structure:
  - Food Supplement

- **(R)-α-Naphtylethylamine**
  - Molecular structure:
  - Cinacalcet IM

- **(S)-2-Octanol**
  - Molecular structure:
  - Chiral building block
Green magic happens here

We aim to contribute to a better societal and environmental future

- Proprietary Technology
- Industrial Innovation
- Environmental Impact