Industrial Biocatalysis: Considerations and practical requirements for the introduction of an industrial biocatalytic process

Joint Summer School
“Biocatalysis as a Key Enabling Technology”
CarbaZymes / MetaFluidics / ROBOX
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Biocatalysis @ InnoSyn

- InnoSyn is located on the Brightlands Chemelot Campus Geleen, The Netherlands, close to other SME and large industry companies.
- SME of ~48 employees.
- Core biocatalysis team of ~10 R&D colleagues.
**Why Biocatalysis?**

**Biocatalysis** is the use of enzymes as they are provided by nature and improved in the laboratory as catalysts for organic chemistry.

- **on the interface of Biology and Chemistry**
- **requiring the integration** of both disciplines **in one team**
- **Enzymes are valuable catalysts for organic synthesis as they are:**
  - generally **chemo-, regio- and often enantioselective** (quality)
  - generally working under **mild reaction conditions**: ambient temperature, pH and pressure (process costs)
  - **green chemistry**: often reduced environmental burden (e.g. less organic and inorganic waste streams)
Biocatalysis in Route Scouting

- Biocatalysis is typically embedded in a multi-step chemical process.
- Typically not just replacing a chemical reaction for a biocatalytic step.
- Enabling novel, shorter routes towards the target product.
- From alternative raw materials.

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A ➔ B ➔ C ➔ Z

X ➔ Y ➔ biocat
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Raw material ➔ product.
Example: Biocatalytic Routes to Pregabalin (Selection)

The diversity of enzyme reactions and their excellent selectivity enable the design of new routes on diverse starting materials thus further enabling the ideal choice of biocatalytic process.

Ene reductase: S. Debarge et al., OPRD (2013)
Regio- and Chemoselectivity

**Regioselectivity**
- discrimination of two identical chemical functionalities in one molecule
- e.g. oxidation of alcohol groups in 1,2-propanediol:

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HO-CH2-CH2-OH
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**Chemoselectivity**
- distinguishing similar chemical functionalities in one molecule
- e.g. specific methyltransferases for different atoms
The molecular basis for chirality

- Natural (proteinogenic) amino acids are L-stereoisomers.
- Proteins are intrinsically chiral: true for enzymes, but also for receptors (drug, taste, flavour, agrochemicals).

Different binding of (R)- vs. (S)-stereoisomer leads to different biological effect.
**Softennon® (thalidomide) case**

*Thalidomide molecule is chiral: racemic compound was marketed as sedative for pregnant women in 50-60-ies.*

*Only one form was the desired sedative; the other form caused fetal abnormalities* *

*Since the Softennon® drama: FDA / Pharma Industries investigate standardly the physiological effects of both enantiomers of a drug candidate.*

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*Simplified version, since the drug racemizes in the body*
Industrial Biocatalytic Process Requirements

- Biocatalyst identification
  - HTP-screening of commercial and proprietary enzyme platforms
  - Bio-IT: database and literature mining
- Enzyme production
  - Microbial hosts (bacterial, yeast and fungal)
  - Recombinant high cell-density production systems
- Biocatalytic processes development
- Enzyme engineering (directed evolution & structure guided design)
- Immobilisation and recycling of biocatalysts
- Integration of biocatalytic process steps into multi-step chemical process
  - Adaptation of existing processes
  - De novo route design
Biocatalysis workflow
Biocat selection & Strain construction

Screening of existing enzyme collection
- Biocatalytic screening
- Retest primary hits
- Biocatalytic Process Development

Search for new enzymes
- Literature / database screen
- Gene order & cloning
- Gene expression & characterization
- Gene identification & cloning
- Enrichments
Recombinant Biocatalyst Production

- Limited number of enzyme production organisms
- Higher activity per cell than natural isolates (specific activity)
- Higher cell density in less time as compared to isolates
- Higher volumetric productivity (activity / fermentation volume)
- Less interfering enzymatic activities (known background activities)

- Lower Biocat costs & shorter development times
Biocatalysis workflow
Biocat process development

**Process development**
Characterization & Optimization of process parameters incl. work-up on lab scale (10 ml – 1 l)

**Scalable enzyme production**
Enzyme production by scalable high cell density fermentation (10 l scale)

Pilot plant process (200 l scale)

Transfer to implementation
Fermentative Biocat Production

- Up to 20 L scale: in InnoSyn labs
- Larger scale fermentation capabilities at low costs:
  - e.g. 150 L, 1 m³, 25 m³, 65 m³
  - Through proven InnoSyn network of European manufacturers
  - Including down-stream-processing and formulation
Examples of Biocatalytic Processes on Industrial Scale

Highlighting typical industrial challenges and solutions
Challenges & Key Success Factors for Biocatalytic Production

1. Lowest cost biocatalyst production by fermentation

2. Highly efficient and productive enzymes
   a. High intrinsic activity → fast reaction with low biocat loading
   b. High operational stability → tolerance to high substrate & product concentrations → resistance to impurities

3. Biocatalytic reaction engineering to optimize variable and fixed costs → Optimal balance between biocat loading, yield and batch time

4. Enzyme engineering to optimize Biocat to process challenges → Rational design & random mutagenesis

5. Enzyme (purification,) immobilisation and recycling

6. Product recovery and integration into further process steps
Key Success Factors Biocatalytic Processes

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Example: Protease in DSM/Tosoh Aspartame process (JV Holland Sweetener Company)
HSC Aspartame Process

- Enzyme Thermolysin selects the right coupling position:
  - Thermolysin only accepts L-stereoisomer (L-PheOMe): stereoselectivity
  - Thermolysin couples only to \( \alpha \)-position: regioselectivity
- Out of four possible products, we only made L-\( \alpha \)-Asp-L-PheOMe (Aspartame)
  - Free of bitter side products
- Recycling of enzyme by Ultra-filtration
- At Holland Sweetener Company (DSM-Tosoh Joint Venture) in Geleen, NL
  - Run at several ktons per year for more than 10 years
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Example: Acylases in DSM β-lactam antibiotics processes
Semi Synthetic β-Lactam Processes
DSM-Sinochem Pharmaceuticals Joint Venture

13 chemical steps replaced by fermentation and two enzymatic steps

- world-wide β-lactam antibiotics (cephalosporins and penicillins) production
- Combining 1 fermentation and 2 biocatalytic steps
Cephalexin Production Key

Enzymatic Step

Acylase catalysed Peptide Coupling

- Activated phenyl-glycine derivative: amide (PGA) or methyl ester (PGM)
- Acyl-enzyme intermediate attacked by 7-ADCA (S) or H2O (H)
- PenG-acylase variant immobilized on DSM proprietary carrier
- Very low biocatalyst cost-price contribution
Biocatalyst Separation from Reaction Mix

“Stirred filtration” is an elegant method for efficient separation of two solids: biocatalyst and cephalexin (together with other reaction components)

WO 9323164 (Novo Nordisk/DSM, 1992)
Organic Process Research & Development (1998), 2, 128-133
Key Success Factors

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Example: Hydroxy-Nitrile Lyases in DSM processes
Hydroxynitrile Lyases (HNLs) - From the plant to the plant and back...

- HNL from the rubber tree *Hevea brasiliensis* produced in yeast *Pichia pastoris*
- as biocatalyst for the synthesis of (S)-cyanohydrins

More active pyrethroids leading to reduced chemical loading

Poechlauer et al. (2004) in (Blaser & Schmidt, Eds.) Asymmetric Catalysis on Industrial Scale, Wiley, pp. 151-164
(R)-HNL in *Pichia pastoris*

- (R)-HNL from almond tree *Prunus amygdalus*
- as biocatalyst for the synthesis of e.g. substituted (R)-mandelic acids
- Acid stability (pH 2.6) required to overcome competing chemical reaction:
  isoenzyme Pa HNL-5 cloned and overexpressed in *Pichia pastoris*

Activity of wild-type HNL too low for commercial application

Enzyme Engineering: Pa HNL-5 A111G

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mutation (Pa HNL-5-αMF)</th>
<th>Specific Activity [μmol/min.mg]</th>
</tr>
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<tbody>
<tr>
<td>Benzaldehyde</td>
<td>-</td>
<td>1450 ± 400</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>A111G</td>
<td>1150 ± 400</td>
</tr>
<tr>
<td>2-Chlorobenzaldehyde</td>
<td>-</td>
<td>67 ± 25</td>
</tr>
<tr>
<td>2-Chlorobenzaldehyde</td>
<td>A111G</td>
<td>409 ± 60</td>
</tr>
</tbody>
</table>

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Questions?