Application of Biocatalysis in Synthetic Chemistry

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14-17 November 2006
What can Biocatalysis do?

Biocatalysis is an **enabling technology**

- Lower production costs:
  - Fewer Reaction Steps
  - Reduced material requirements
  - CAPEX costs reduced
- Reduces Environmental Pressure
- Improved safety.
Who is using Biocatalysis?

- Cargill,
- Codexis,
- Diversa,
- DSM,
- Degussa,
- Merck and Co Inc (USA)
- Merck (Europe)
- Pfizer
- BASF
- Lonza
- Du Pont
- Dow Chemicals
- Sigma-Aldrich, Fluka,
- Novartis.
- Numerous SMEs
Who is using Biocatalysis?

- Fluka: 5% of products are now made using biocatalysis.
- DSM uses 25 biocatalysis-based processes at large scale.
- 2005: 15% of chiral technology by biocatalysis
- 2009: 30%
- Biocatalysis is being applied to Pharma (> 50%), Food (25%), Cosmetics and Agro-food (25%).
Where can you get them from?

- Commercial Enzyme Preparations (> 90)
- Environmental microbial isolation and enrichment
- Culture collections, CSIR/CAMS 4000 organisms
- Phylogeny, Bioinformatics
- Metagenomics
- Directed Molecular Evolution and Site Directed Mutation
Biocatalysis at the CSIR

• The biocatalytic resolution of naproxen (as part of a commercial synthetic process).

• The biocatalytic resolution of menthol (as part of a commercial synthetic process).

• The epoxide biocatalysis technology platform has been progressed to the product stage.

• The nitrile biocatalysis, laccase, and nucleoside phosphorylases at research stage.
Naproxen
Naproxen

- Carboxyl esterase specific for the hydrolysis of S-naproxen methyl ester

\[ \text{S-Naproxen} \] + \[ \text{R-NME} \] \rightarrow \text{rac-NME} \]
Naproxen

- Sufficient biocatalyst for ton quantities of S-Naproxen - 20 000 L fermentation.
- Lysis of biomass to release over-expressed intracellular enzyme
- Stabilisation to give shelf stable biocatalyst (5 years, 4°C)
- Biotransformation: several hundred g/L rac-NME to give almost full conversion with >99% ee S-naproxen
- R-NME recycled
- **Total** technology was demonstrated at manufacturing scale and licensed to a major pharmaceutical company


Conversion of Naproxen Methyl Ester
Thermostable lipase – ESL 001
Menthol
Menthol

• Hydrogenation of thymol produces an 8 isomer mixture of 2-isopropyl-5-methylcyclohexanol

• (+) and (−) enantiomers of menthol, isomenthol, neomenthol and neoisomenthol


Menthol (continued)

- 60% m/m (+/-) menthol
- 27% m/m (+/-) neomenthol
- 11% m/m (+/-) iso-menthol
- 2% m/m (+/-) neoisomenthol
Original proposed resolution

1. Racemisation
   - 8 isomer ‘liquid menthol’ stream
   - (+/-) menthol racemate

2. Distillation
   - (-) enriched (+/-) menthol acetate

3. Resolution
   - (-) enriched (+/-) menthol acetate

4. Distillation
   - (+/-) menthol racemate

5. Distillation
   - 8 isomer ‘liquid menthol’ stream

6. Racemisation
   - 6 isomer mix

7. Crystallisation
   - (-) Menthol

8. Hydrolysis
Biocatalytic resolution

Racemisation

Resolution

Distillation

Hydrolysis

Crystallisation

(-) Menthol

8 isomer menthol stream

(-) enriched (+/-) menthol acetate

7 isomer mix

(-) menthol enriched (+/-) menthol

(-) enriched (+/-) menthyl acetate
Enzymatic Resolution Reaction

liquid menthol → enzyme → (-)-menthyl acetate + other non-acylated menthol isomers

OH

<table>
<thead>
<tr>
<th>OH</th>
<th>OAc</th>
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Acetaldehyde
Reaction in Heptane!
Enzymatic Resolution: Bulk Solvent

- **Heptane**:
  - High solvent hydrophobicity (log P = 4.2) facilitates lipase activity
  - Low health risk (accepted by FDA)
  - High flash point (reduced explosion risk)
  - Facile recoverability
Enzymatic Resolution: Acyl Donors

- **Vinyl acetate:**
  - Tautomerism of vinyl alcohol to acetaldehyde
  - Reduced background chemicalacylation
  - Small molecule, facilitates diffusion to active site
  - Volatile donor facilitates recycle, volatile by-product of reaction allows facile removal during reaction

- **Disadvantage of vinyl esters:**
  - Acetaldehyde => toxicity to enzymes
  - Ester hydrolysis to form an acetate if water available
Effect of Isomenthol diastereomer content on enantio-efficiency of lab scale batch biocatalytic resolution

<table>
<thead>
<tr>
<th>(+/-) menthol conc (% m/m)</th>
<th>Iso-menthol conc (% m/m)</th>
<th>Relative activity (adjusted for initial (-) menthol)</th>
<th>(+/-) menthol / iso-menthol ratio in feed</th>
<th>(-) Menthol acetate % ee</th>
<th>Ratio % of isomenthol acetate to menthol acetate produced</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>1.00</td>
<td>-</td>
<td>96.7</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>2.8</td>
<td>1.09</td>
<td>20:1</td>
<td>96.8</td>
<td>&lt;1</td>
</tr>
<tr>
<td>50</td>
<td>4.6</td>
<td>1.02</td>
<td>10:1</td>
<td>97.0</td>
<td>1</td>
</tr>
<tr>
<td>47</td>
<td>10.3</td>
<td>0.99</td>
<td>5:1</td>
<td>97.0</td>
<td>1</td>
</tr>
<tr>
<td>19.6</td>
<td>22.4</td>
<td>0.97</td>
<td>1:1</td>
<td>97.2</td>
<td>5</td>
</tr>
</tbody>
</table>

Negligible reaction of neo or neo-iso observed by AK enzyme
Enantiomeric preference of biocatalyst in batch recycle with different menthol substrates

- 40% (+/-)menthol
- 10% (+/-) menthol
- 10% liquid menthol

% ee vs. % conversion graph

- E=50
- E=100
Lab Scale Stirred Batch Reactor

% ee

% conversion

immobilized enzyme

free enzyme
Productivity of Immobilised Lipase in Lab Scale CSTR on 65% m/m Liquid Menthols feedstream
(36% m/m (+/-) Menthol Content)

\[ R^2 = 0.9956 \]

\[ 0.079 \text{ gMA/gEnz/h} \]
Nitriles
The versatility of the nitrile group

\[ R_1^{Lg} + CN \rightarrow R_1 \equiv N \]

- Reaction with \( H_2O \) to form \( R_1 \eq CO \)
- Reaction with \( H_2O \) further to form \( NH_2 \)
- Reaction with \( H_2 \) to form \( \equiv N \)

CSIR

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Chemical Nitrile Hydrolysis

\[ R\equiv N + 2H_2O \xrightarrow{KOH\ 200\ ^\circ C} R\equiv CH_2OH \]

\[ R\equiv N + 2H_2O \xrightarrow{\text{conc. HCl}\ 100\ ^\circ C} R\equiv CH_2OH \]
Nitrile Biocatalysis

\[
\begin{align*}
R&\equiv N \\
R&\equiv N
\end{align*}
\]

\[\xrightarrow{\text{Nitrile Hydratase}}\]

\[
\begin{align*}
R&\equiv N \\
R\equiv O &\equiv NH_2
\end{align*}
\]

\[\xrightarrow{\text{Amidase}}\]

\[
\begin{align*}
R&\equiv O \\
R\equiv OH &\equiv NH_3
\end{align*}
\]

\[\xrightarrow{\text{Nitrilase}}\]

\[
\begin{align*}
R&\equiv O \\
R\equiv OH &\equiv NH_3
\end{align*}
\]

This diagram illustrates the conversion of a nitrile group to an amide and then to an amine through the action of enzymes, specifically nitrile hydratase, amidase, and nitrilase.
Regioselectivity

\[
\begin{align*}
\text{Nitrile hydratase} & \rightarrow \text{H}_2\text{O} \\
\text{H}_2\text{N} & \rightarrow \text{H}_2\text{N} \\
\end{align*}
\]
Chemoselectivity

\[
\text{Nitrilase} \quad 2\text{H}_2\text{O} \quad \rightarrow \quad \text{phenylglycine} + \text{NH}_3
\]

\[
\text{phenylglycine} + \text{HO}_2\text{C} + \text{NH}_3
\]

\[
\text{OH}^- \quad \rightarrow \quad 3\text{H}_2\text{O}
\]
Stereoselectivity

\[
\begin{align*}
&\text{OH} \\
&\text{2H}_2\text{O} \\
&\text{OH}
\end{align*}
\]
1. $R = H$
2. $R = CH_3$
3. $R = NH_2$
4. $R = OH$
5. $R = CH_2CH_3$

6. $R = H$
7. $R = OH$
8. $R = NH$
9. $R = N$

10. $R_1 = CH_3$, $R_2 = H$
11. $R_1 = OCH_3$, $R_2 = OCH_3$

12. $R_1 = CH_3$, $R_2 = H$
13. $R_1 = OCH_3$, $R_2 = OCH_3$
14. $R = NO$
15. $R = OH$
16. $R = Br$
17. $R = COCH_2CH_3$
18. $R = C(O)O$Ph
19. $R = COCH_2CH_3$
20. $R = OH$
21. $R = CH_3$

22. $R = H$
23. $R = NO_2$
24. $R = OH$
25. $R = COCH_2CH_3$
26. $R = OCH_3$
27. $R = H$
28. $R = NO_2$
29. $R = COCH_2CH_3$
30. $R = OH$
31. $R = COCH_2CH_3$
32. $R = OH$
33. $R = COCH_2CH_3$
### Biocatalyst Toolbox

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cN cH</td>
</tr>
<tr>
<td><em>P. fluorescens</em> nitrilase</td>
<td>100% 21%</td>
</tr>
<tr>
<td>BioCatalytics nitrilase-1001</td>
<td>100% 0%</td>
</tr>
<tr>
<td>BioCatalytics nitrilase-1004</td>
<td>100% 0%</td>
</tr>
<tr>
<td>BioCatalytics nitrilase-1005</td>
<td>100% 0%</td>
</tr>
<tr>
<td>BioCatalytics nitrilase-1006</td>
<td>100% 0%</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> nitrilase</td>
<td>100% 0%</td>
</tr>
<tr>
<td><em>Rhodococcus</em> BCT-ABIs nitrile hydratase</td>
<td>100% 8%</td>
</tr>
<tr>
<td><em>Rhodococcus</em> BCT-ABFGs nitrile hydratase</td>
<td>100% 0%</td>
</tr>
<tr>
<td><em>Rhodococcus</em> DSMZ 44519 nitrile hydratase</td>
<td>100% 46%</td>
</tr>
<tr>
<td><em>Rhodococcus</em> NOVO SP361 nitrile hydratase</td>
<td>100% 15%</td>
</tr>
</tbody>
</table>

ND Not determined

---

Ribbon model of the Rhodococcus sp R312 nitrilase hydratase with 3-hydroxy-3-phenylpropionitrile in the active site
Superimposition of 2-phenylbutyronitrile and 3-hydroxy-3-phenylpropionitrile in the active site of the nitrile hydratase
NITRILE HYDRATASE MECHANISM

DKR: Multi-step transformation

Phenyl-acetaldehyde $\xrightarrow{\text{KCN}}$ 2-Hydroxy-3-phenyl-propionitrile $\xrightarrow{\text{Nitrile hydratase}}$ 2-Hydroxy-3-phenyl-propionamide $\xrightarrow{\text{Amidase}}$ 2-Hydroxy-3-phenyl-propionic acid

DEPA
## Synthesis of carboxylic acids from aldehydes

<table>
<thead>
<tr>
<th>Aldehyde compound incubated with cyanide and biocatalyst</th>
<th>Conversion to homologous α-hydroxy acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzaldehyde</td>
<td>95</td>
</tr>
<tr>
<td>4-methylbenzaldehyde</td>
<td>51</td>
</tr>
<tr>
<td>4-hydroxybenzaldehyde</td>
<td>55</td>
</tr>
<tr>
<td>2-nitrobenzaldehyde</td>
<td>20</td>
</tr>
<tr>
<td>2-fluorobenzaldehyde</td>
<td>100</td>
</tr>
<tr>
<td>3-chlorobenzaldehyde</td>
<td>100</td>
</tr>
<tr>
<td>4-chlorobenzaldehyde</td>
<td>100</td>
</tr>
<tr>
<td>4-nitrobenzaldehyde</td>
<td>100</td>
</tr>
<tr>
<td>2-chlorobenzaldehyde</td>
<td>90</td>
</tr>
<tr>
<td>Vanillin</td>
<td>0</td>
</tr>
<tr>
<td>4- methoxy-benzaldehyde (p-anisaldehyde)</td>
<td>0</td>
</tr>
<tr>
<td>4-cyanobenzaldehyde</td>
<td>100</td>
</tr>
<tr>
<td>4-methylsulphnylonyl-benzaldehyde</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoxybenzaldehyde</td>
<td>0</td>
</tr>
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</table>
Strecker Synthesis

R = H, Cl, F, OH, NO₂, CH₃
Conclusion

• Brandão and co-workers (Appl. Environ. Microbiol. 69: 5754-5766, 2003) found that the nitrile hydratases in microorganisms isolated from around the world had infra-species amino acid sequence differences, and this provided an explanation for the variability of nitrile substrate usage within the species.

• Our data supports this result - South African microbial diversity will include unique biocatalysts.

Immobilisation
Immobilised Biocatalyst Preparation

- Solubilisation of lipase powder in aqueous buffer (100 mM Pi)
- Slurry formation with celite (batch mode), pH 7
- Freeze drying
- Milling of cake
- Size control (sieving/sedimentation)
- Storage under airtight conditions

1 AK : 2 celite
Spherezyme
SEM:
Lipase/Albumin SphereZymes
Standard substrates - 2 sizes

p-Nitrophenolpalmitate

\[ \text{E} \downarrow \\
\text{p-Nitrophenolbutyrate} \]

\[ \text{E} \downarrow \\
\text{OH} + \text{HO} \]
Hyperactivation

100% Lipase Spheres (Butyrate)

% Activity Retained

Modified Gluteraldehyde /ul

Nonoxynol
Span20
<table>
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<tr>
<th>Name</th>
<th>Degree/Qualification</th>
<th>Name</th>
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<tbody>
<tr>
<td>Dr Justin Jordaan</td>
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<td>Ms Zimkhitha Sotenjwa (Btech)</td>
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<tr>
<td>Dr Fritha Hennessy</td>
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<td>Ms Nasreen Abrahams (Btech)</td>
</tr>
<tr>
<td>Dr Lucia Steenkamp</td>
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<td>Mr Tshwane Manchidi</td>
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<td>Dr Anu Idicula</td>
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<td>Ms Joni Frederick (MSc)</td>
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<td>Dr Konanani Rashamuse</td>
<td></td>
<td>Ms Banyatsi Mphela (BSc Hons)</td>
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<tr>
<td>Dr Phiyani Lebea</td>
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<td>Ms Letshego Molawa (MSc)</td>
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<tr>
<td>Mr Daniel Visser (MSc)</td>
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<td>Ms Salome Mathye (BSc Hons),</td>
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<tr>
<td>Mr Clinton Simpson (MSc)</td>
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<td>Ms Cherise Arumugam (Btech)</td>
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<td>Ms Varsha Chhiba (Btech)</td>
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<td>Dr Neeresh Rohitlall</td>
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<td>Mr Kgama Mathiba (Btech)</td>
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<td>Dr Dean Brady</td>
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<tr>
<td>Dr Mapitso Molefe</td>
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<td>Ms Thando Moutlana</td>
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<tr>
<td>Dr Sean Kirchmann</td>
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<tr>
<td>Dr Adri Botes</td>
<td></td>
<td>Mr Butana Mboniswa</td>
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<tr>
<td>Ms Shavani Reddy</td>
<td></td>
<td>Ms Sonia Rech</td>
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<tr>
<td>Ms Nosisa Dube</td>
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<td>Ms Alison Beeton</td>
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</table>
UCT (Prof Sue Harrison, Prof Steph Burton, Prof Trevor Sewell, Prof. Jonathan Blackburn)

Univ. Stellenboch (Prof. Emile van Zyl, Prof Eric Strauss)

UFS (Prof Derek Litthauer, Prof Martie Smit, Dr Ester van Heerden)

Wits (Prof Heni Dirr, Prof Charles de Koning, Prof Gustuv Bauer)

Rhodes Univ. (Prof. Rosie Dorrington, Dr Brett Pletscke)

TU Delft (Prof Roger Sheldon, Dr Fred vab Rantwijk, Dr Isabel Arends)

Academy of Science, Czech Republic (Dr Lumilla Martinkova, Dr Vlamir Krěn)

Dr Eric Mathur (Venter Institute)

AECI

DST

DSM

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GSK

Lifelab BRIC

Cargill

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