Application of Biocatalysis in Synthetic Chemistry

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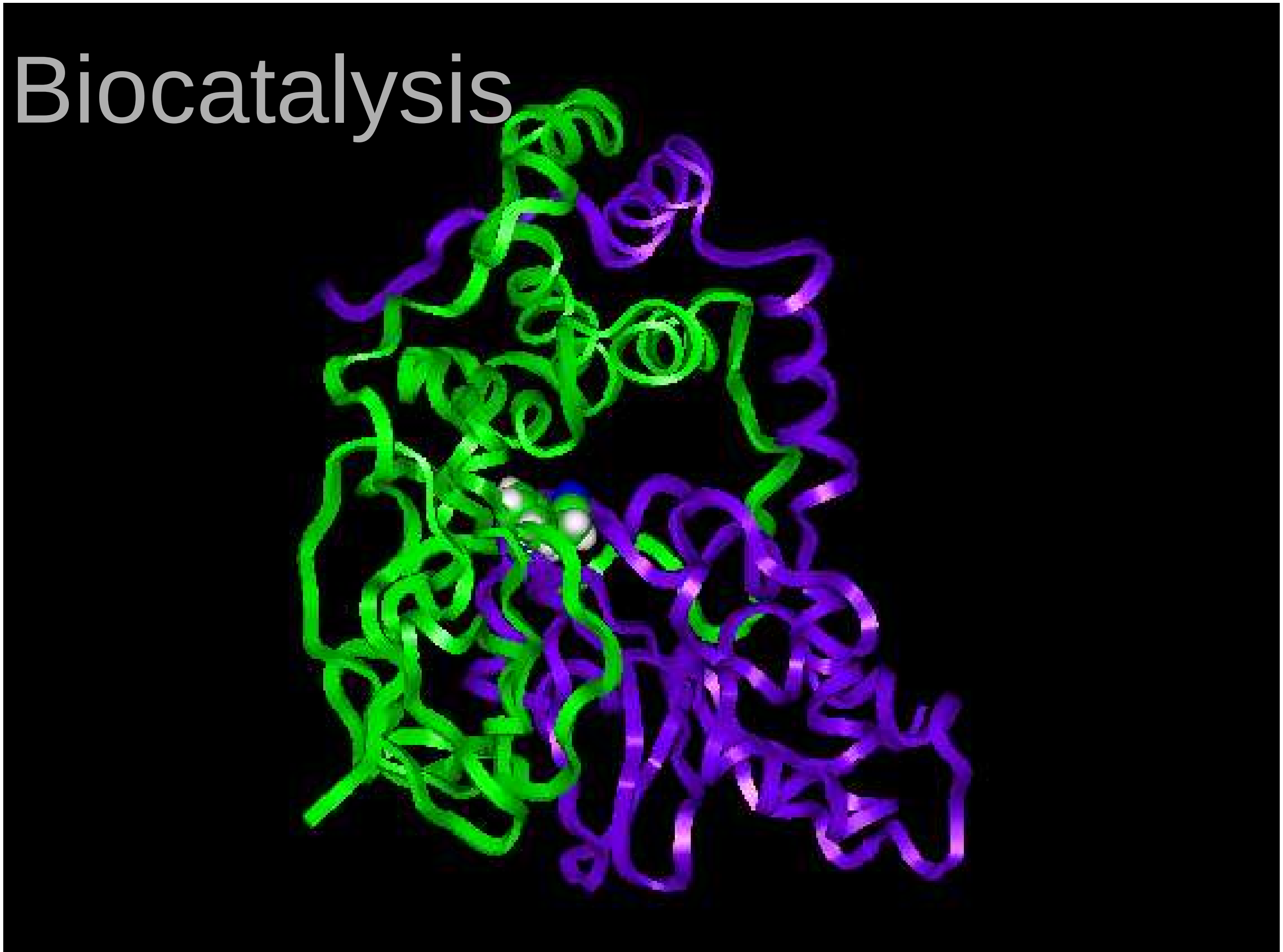
CATSA, 2006. **Mossel Bay, South Africa**

14-17 November 2006

Biocatalysis



CSIR
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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?

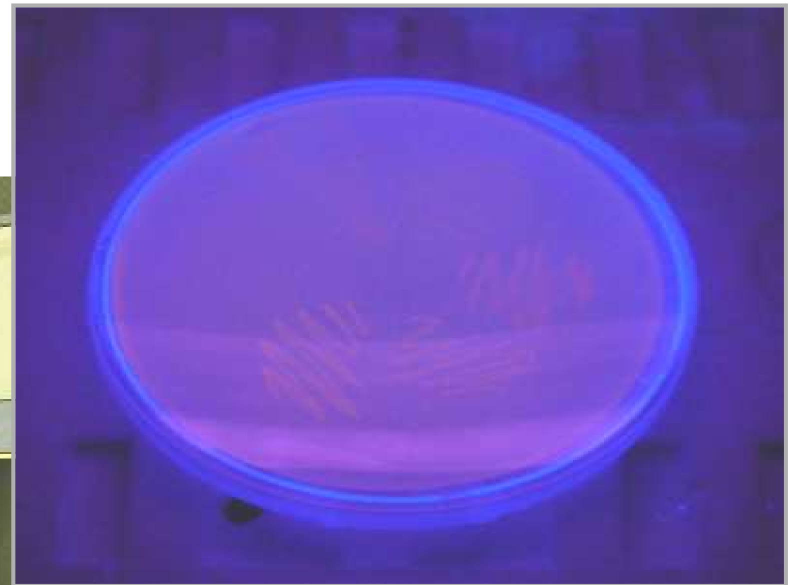
- Cargill,
- Codexis,
- Diversa,
- DSM,
- Degussa,
- Merck and Co Inc (USA)
- Merck (Europe)
- Pfizer
- BASF
- Lonza
- Du Pont
- Dow Chemicals
- Sigma-Aldrich, Fluka,
- Novartis.
- Numerous SMEs

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
- Phyolgeny, Bioinformatics
- Metagenomics
- Directed Molecular Evolution and Site Directed Mutation



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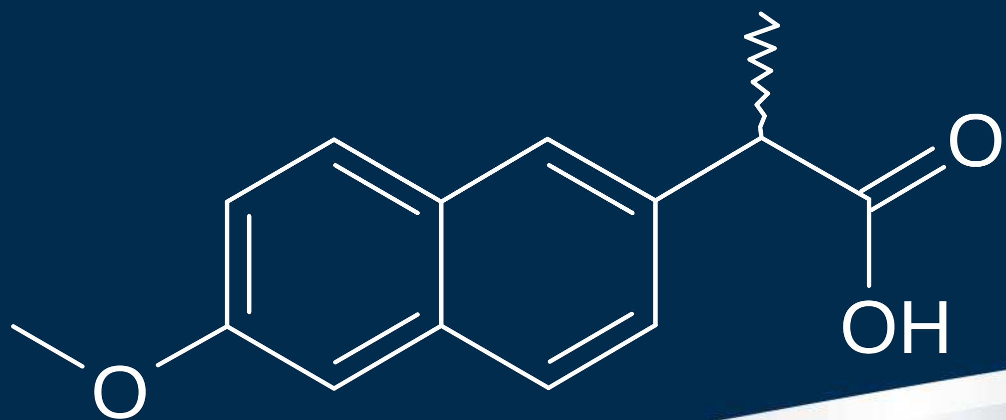
Biocatalysis at the CSIR

- 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



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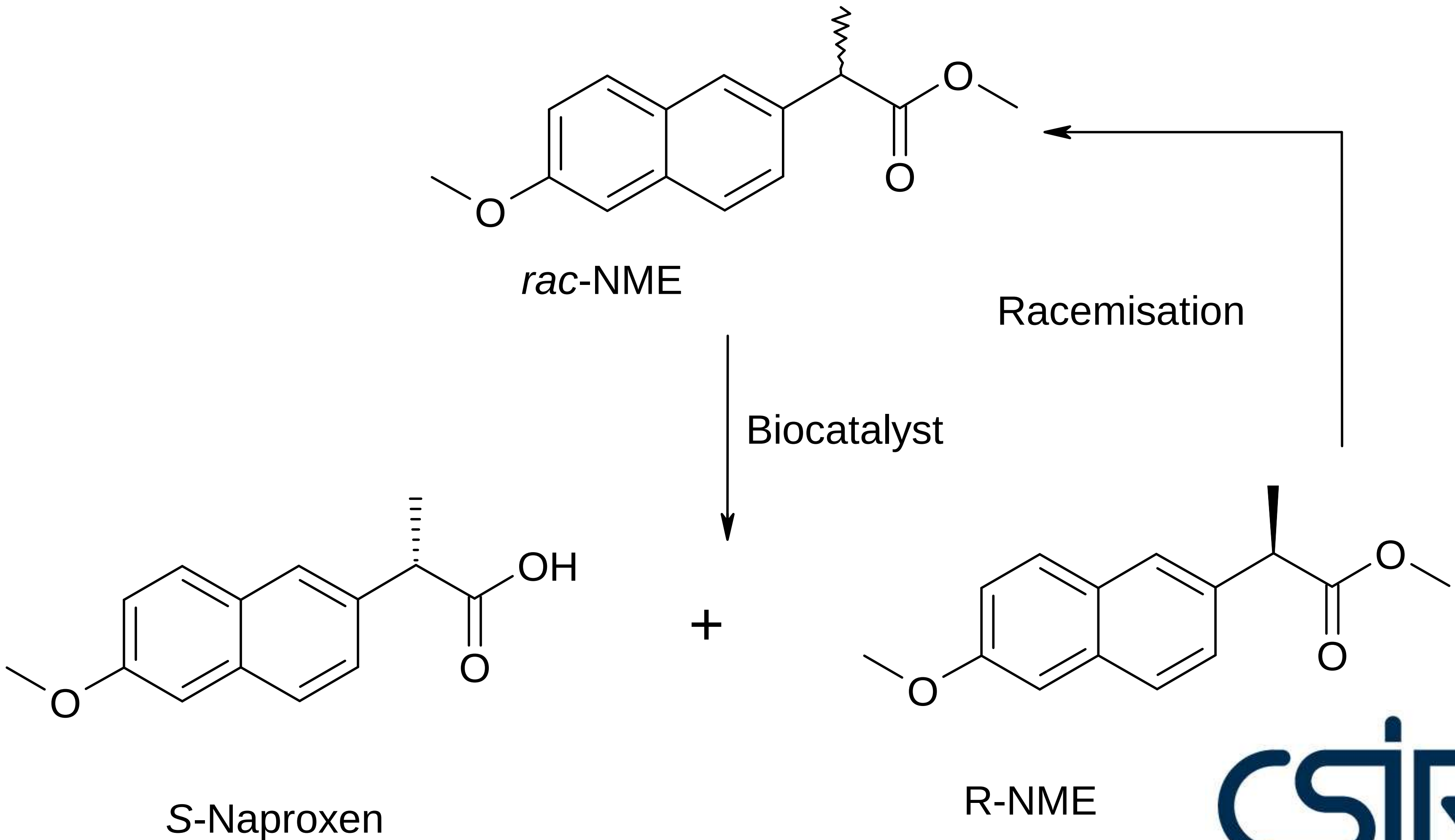
Naproxen

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



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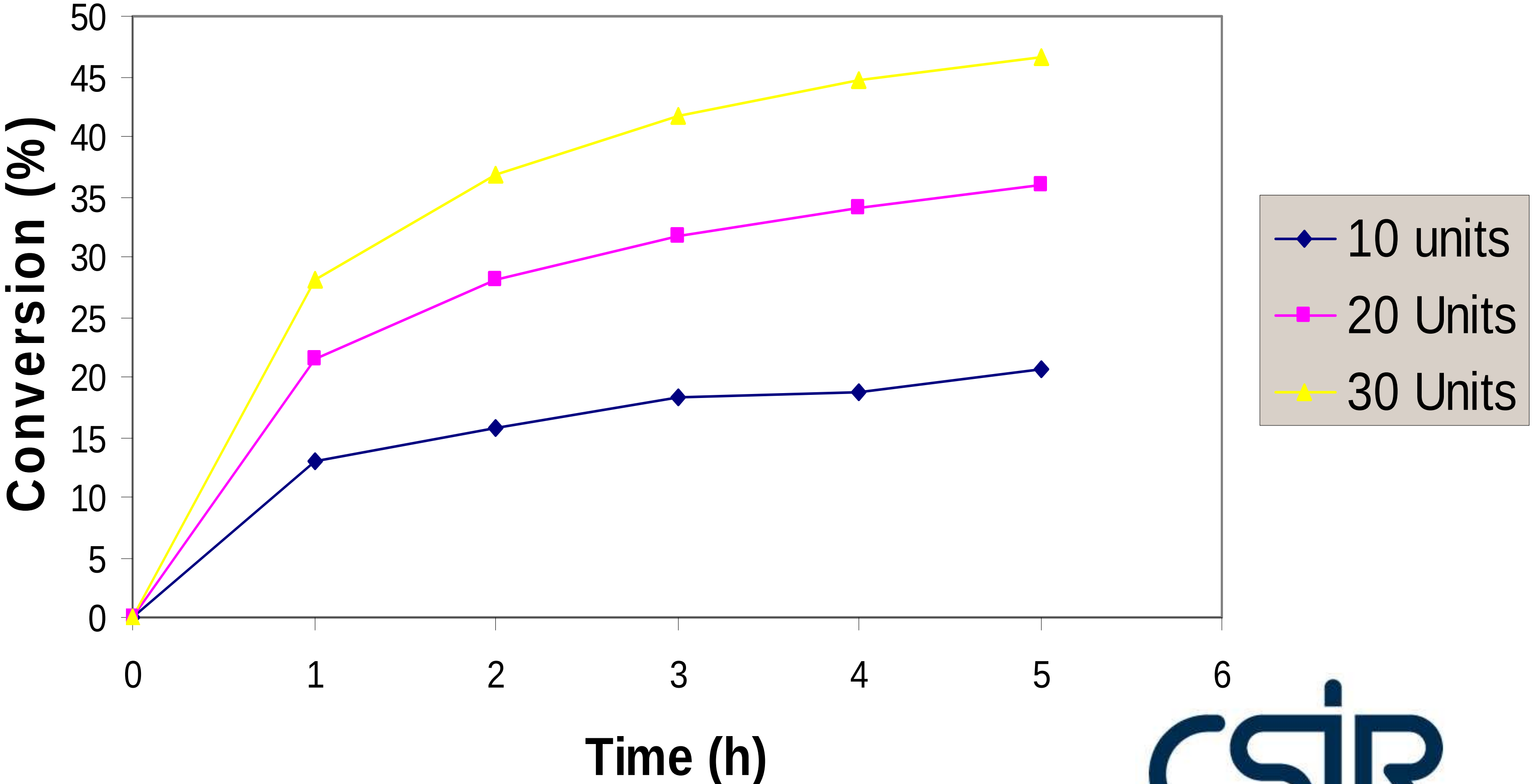
Naproxen

- 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, 4ºC)
- 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

Steenkamp L, and Brady D. (2003) Screening of commercial enzymes for the enantioselective hydrolysis of *R*,*S*-naproxen ester. Enzyme and Microbial Technology 32: 472-477

Brady D, Steenkamp L, Reddy S, Skein E, and Chaplin J. (2004) Optimisation of the enantioselective biocatalytic hydrolysis of naproxen ethyl ester using ChiroCLEC CR. Enzyme and Microbial Technology 34:283-291

Conversion of Naproxen Methyl Ester



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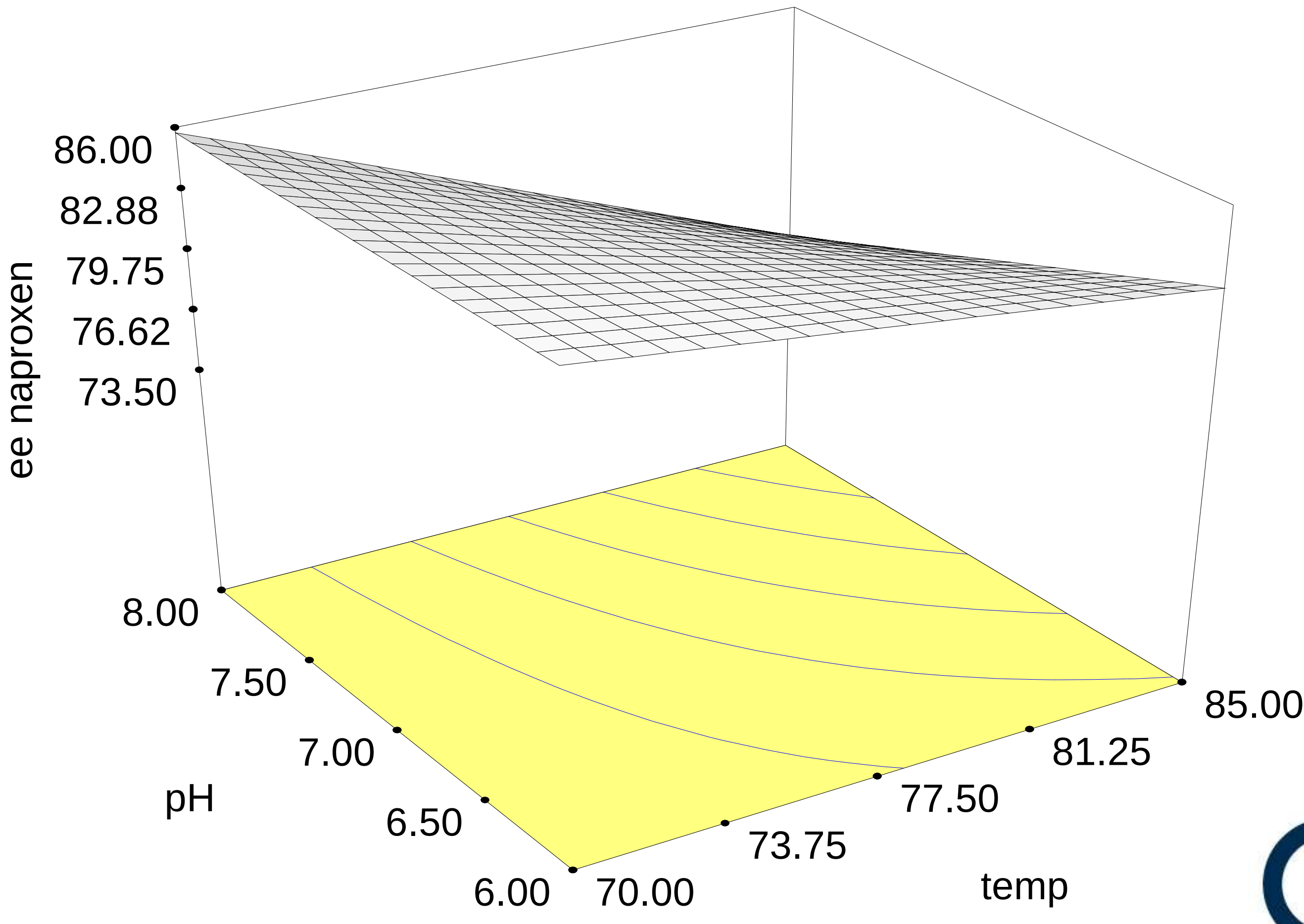
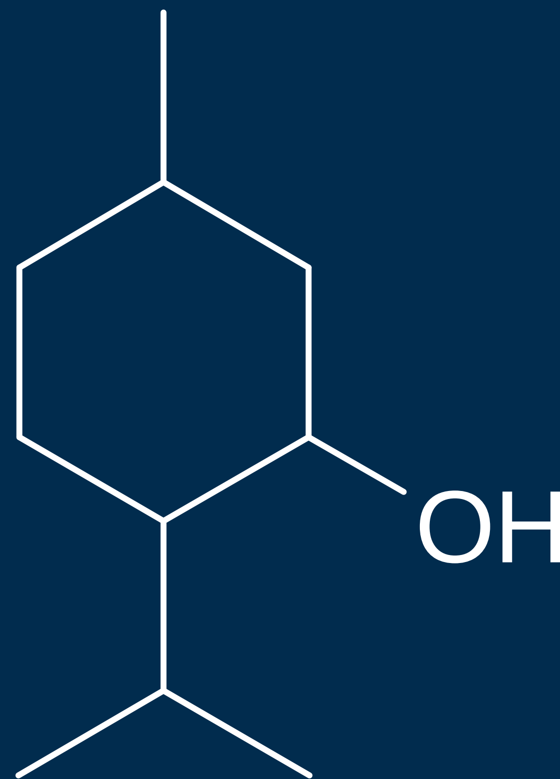
Thermostable lipase – ESL 001



Menthol



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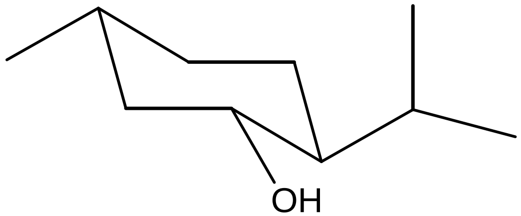
Menthol

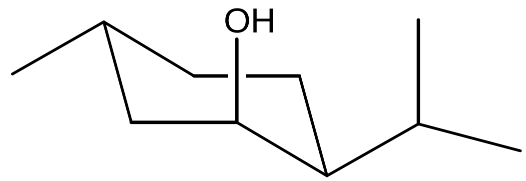
- Hydrogenation of **thymol** produces an **8 isomer mixture of 2-isopropyl-5-methylcyclohexanol**
- (+) and (–) enantiomers of menthol, isomenthol, neomenthol and neoisomenthol

- Chaplin, J.A., Gardiner, N.S., Mitra, R.K., Parkinson, C.J., Portwig, M., Dickson, M.D., Brady, D., Marais, S.F., Reddy, S., Process for preparing (-)-menthol and similar compounds, WO 0204384.
- Chaplin, J.A., Gardiner, N.S., Mitra, R.K., Parkinson, C.J., Portwig, M., Dickson, M.D., Brady, D., Marais, S.F., Reddy, S., Process for preparing (-)-menthol and similar compounds, WO 0236795.

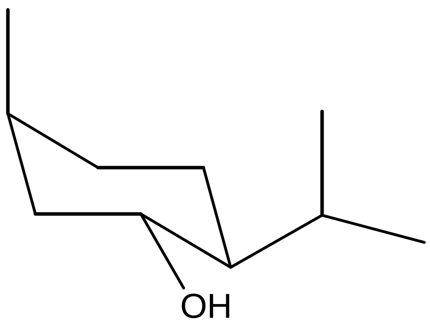
Menthol (continued)



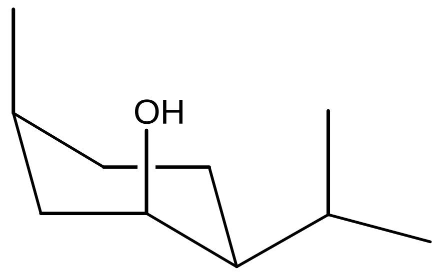
Menthol



Neomenthol



Isomenthol



Neoisomenthol

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

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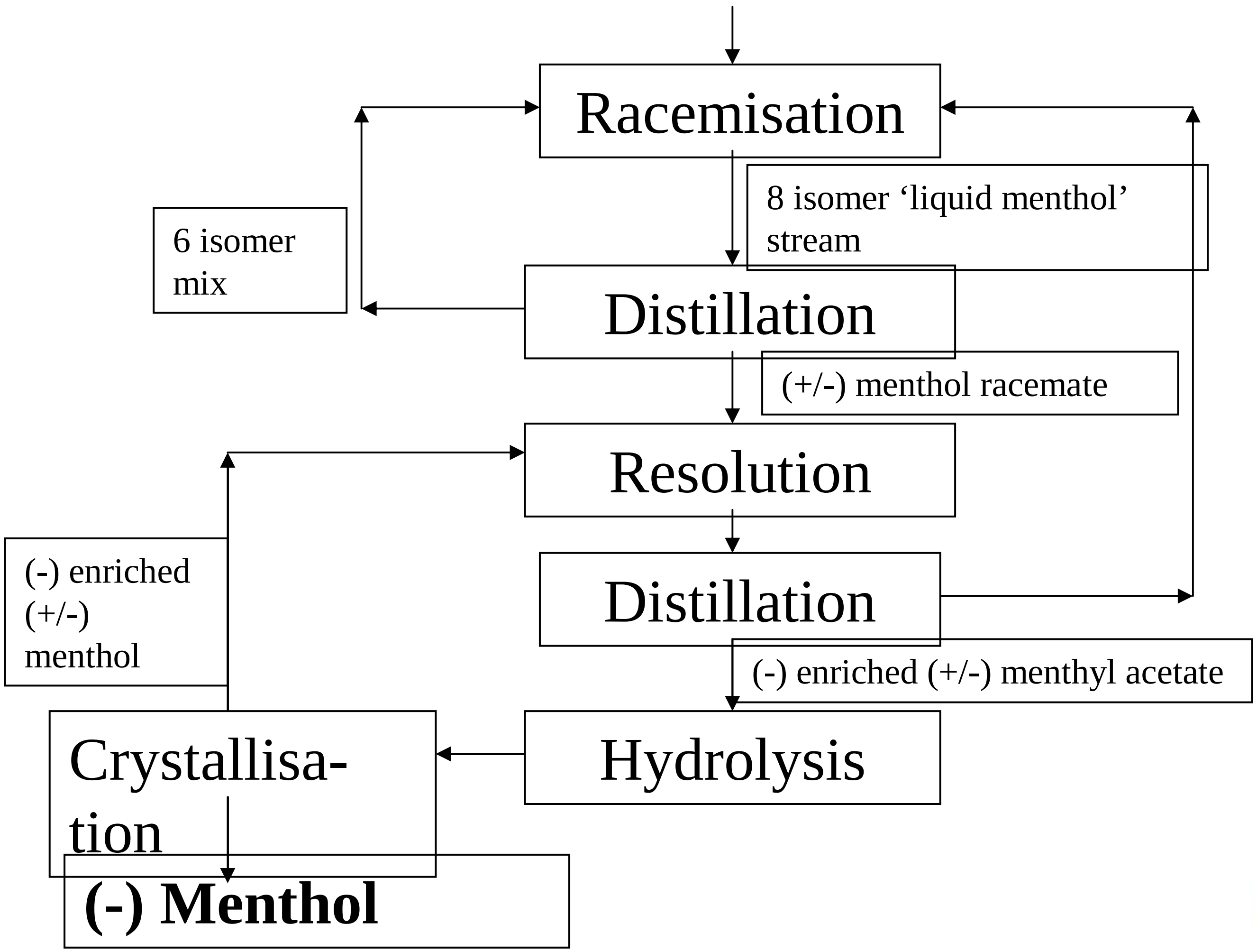
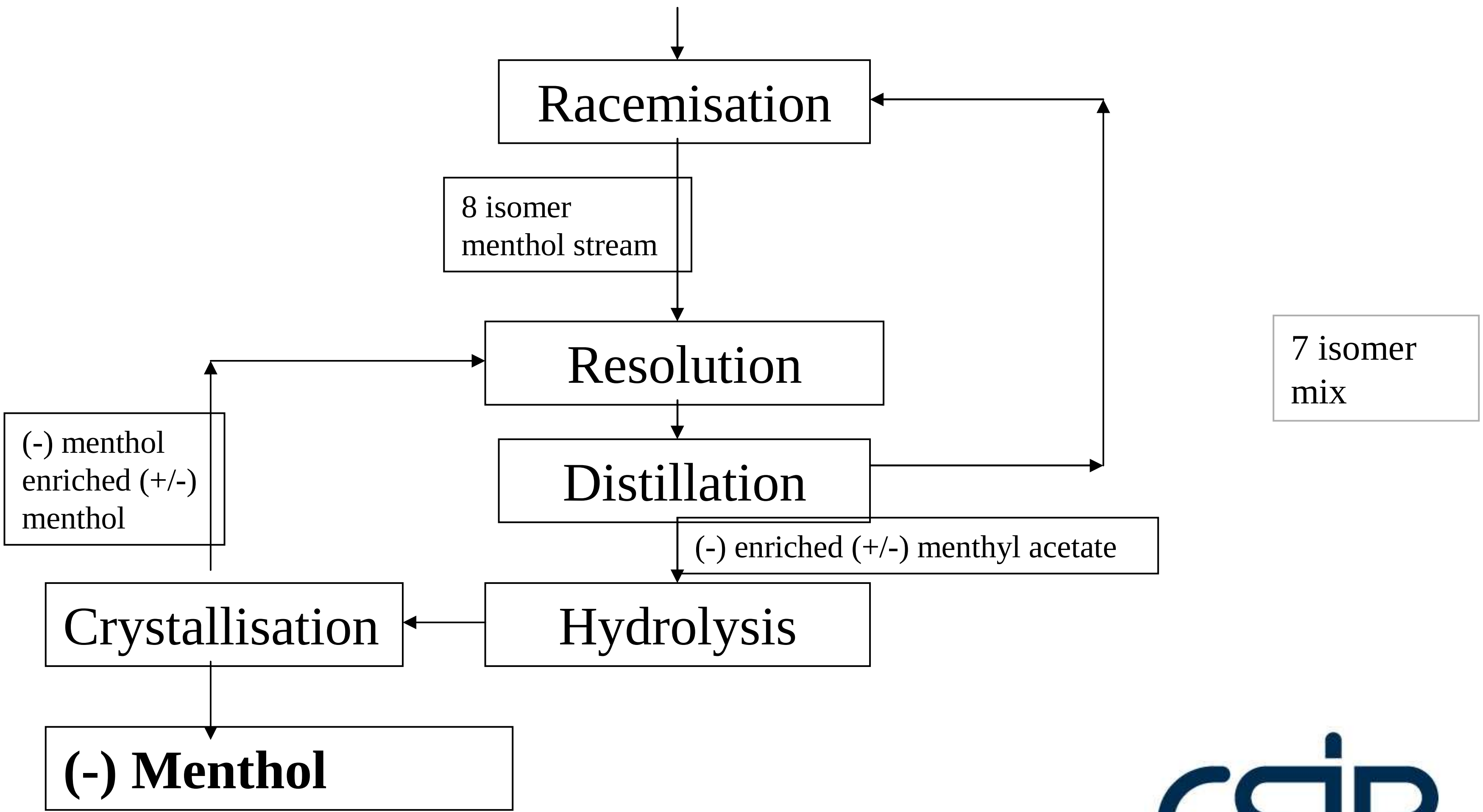
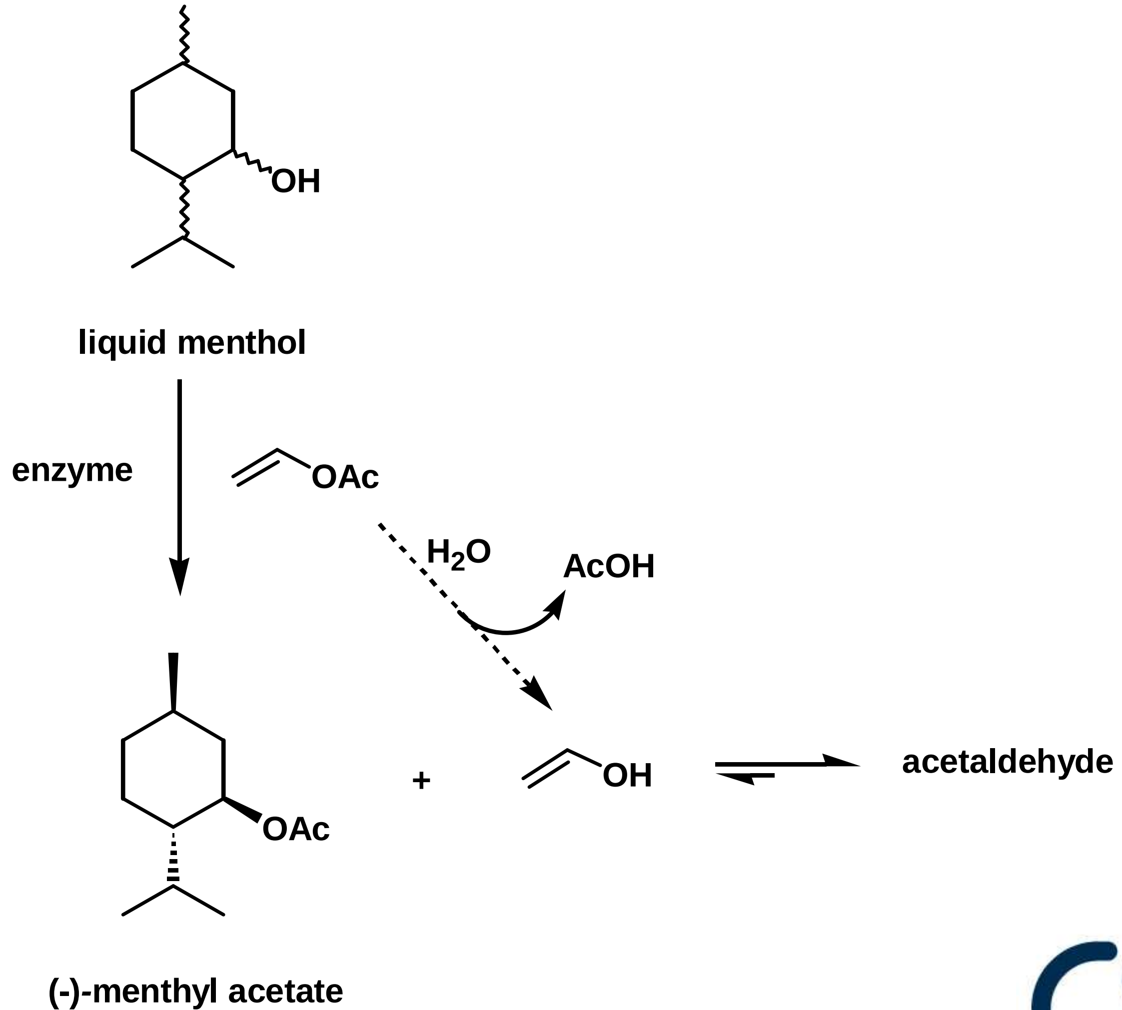
Original proposed resolution



Biocatalytic resolution



Enzymatic Resolution Reaction



+ other non-acylated menthol isomers

Reaction in Heptane !

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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 chemical acylation
  - Small molecule, facilitates diffusion to active site
  - Volatile donor facilitates recycle, volatile by-product of reaction allows facile removal during reaction

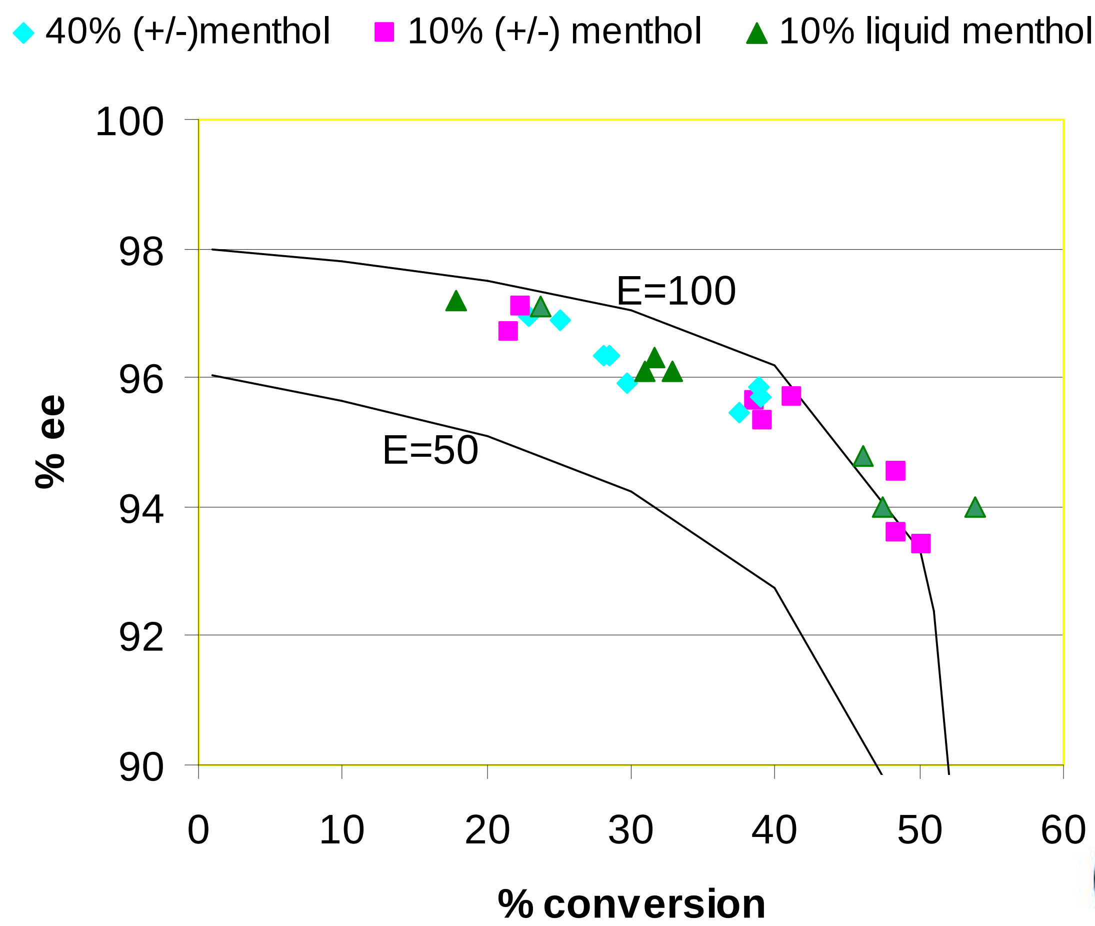
- Disadvantage of vinyl esters:
  - Acetaldehyde => toxicity to enzymes
  - Ester hydrolysis to form an acetate if water available

Effect of Isomenthol diastereomer content on enantio-efficiency of lab scale batch biocatalytic resolution

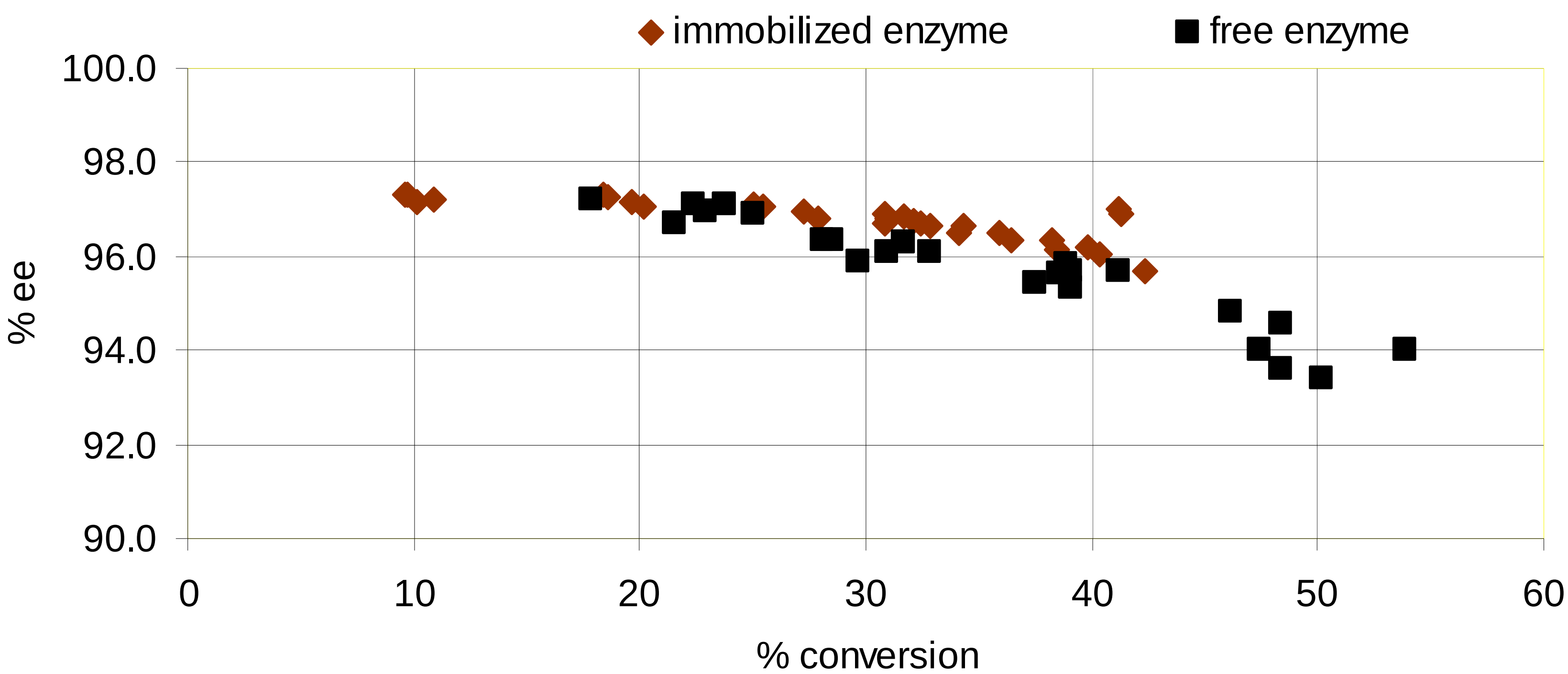
(+/-) menthol conc (% m/m)	Iso-menthol conc (% m/m)	Relative activity (adjusted for initial (–) menthol)	(+/-) menthol / iso-menthol ratio in feed	(-) Menthol acetate % ee	Ratio % of iso-menthol acetate to menthol acetate produced
40	0	1.00	-	96.7	0
50	2.8	1.09	20:1	96.8	<1
50	4.6	1.02	10:1	97.0	1
47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

Negligible reaction of neo or neo-iso observed by AK enzyme

Enantiomeric preference of biocatalyst in batch recycle with different menthol substrates



Lab Scale Stirred Batch Reactor



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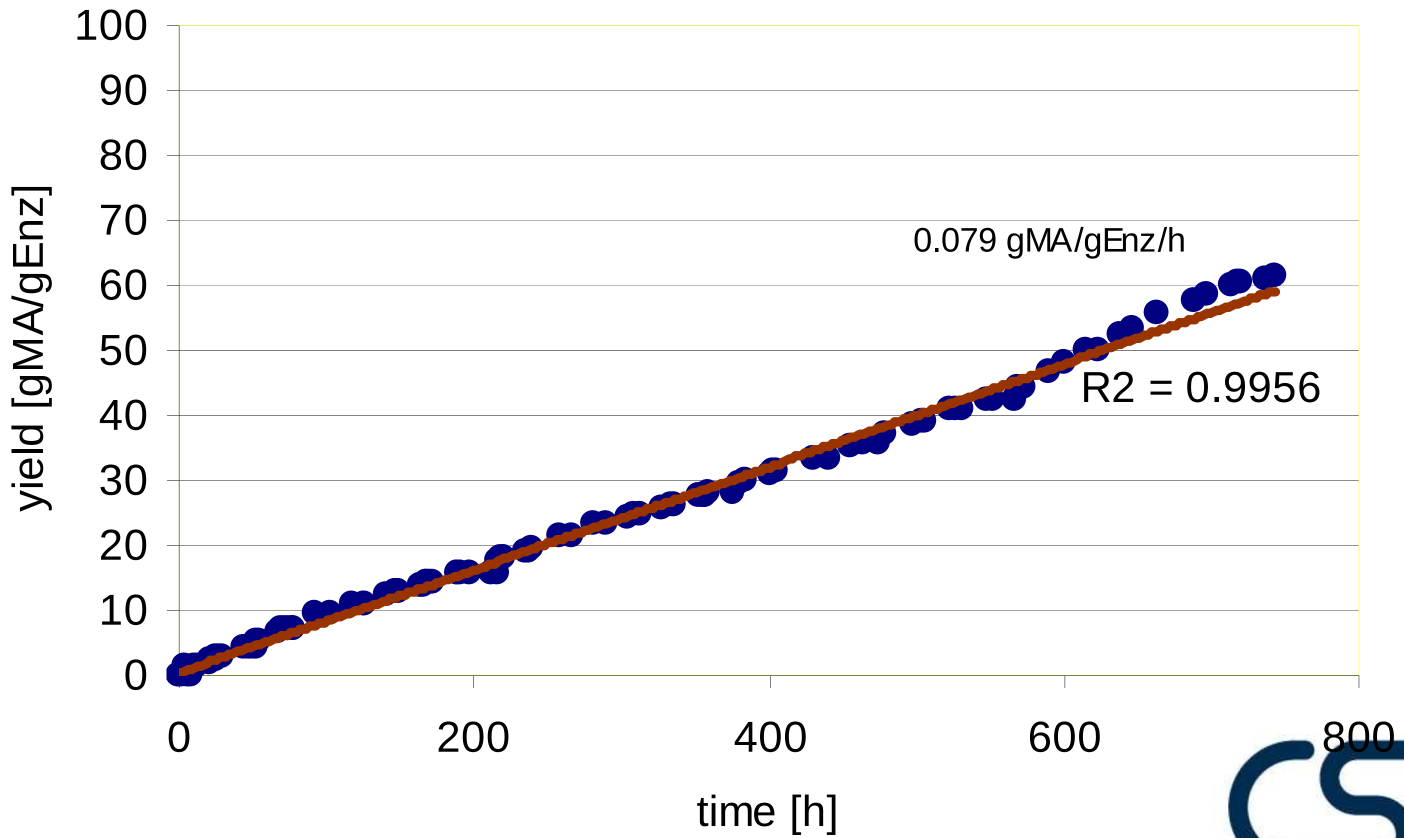
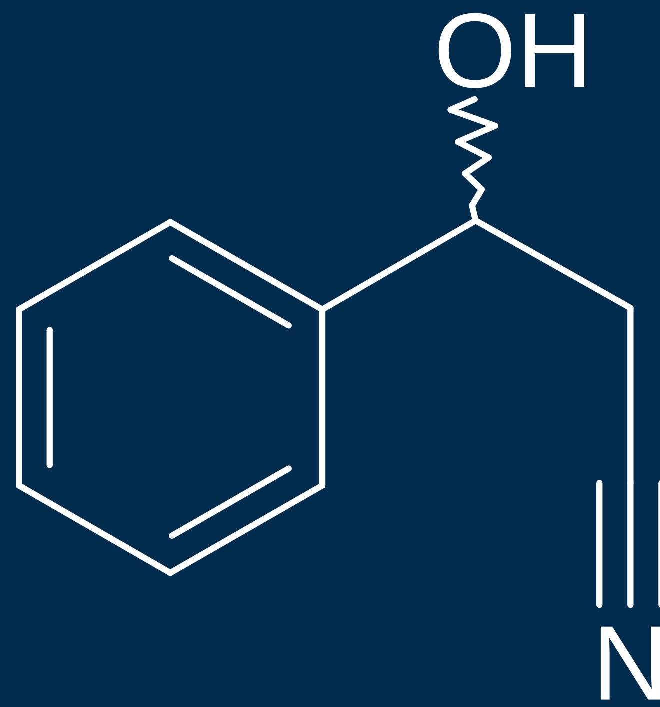
Productivity of Immobilised Lipase in Lab Scale CSTR on 65% m/m Liquid Menthols feedstream

(36% m/m (+/-) Menthol Content)



Nitriles



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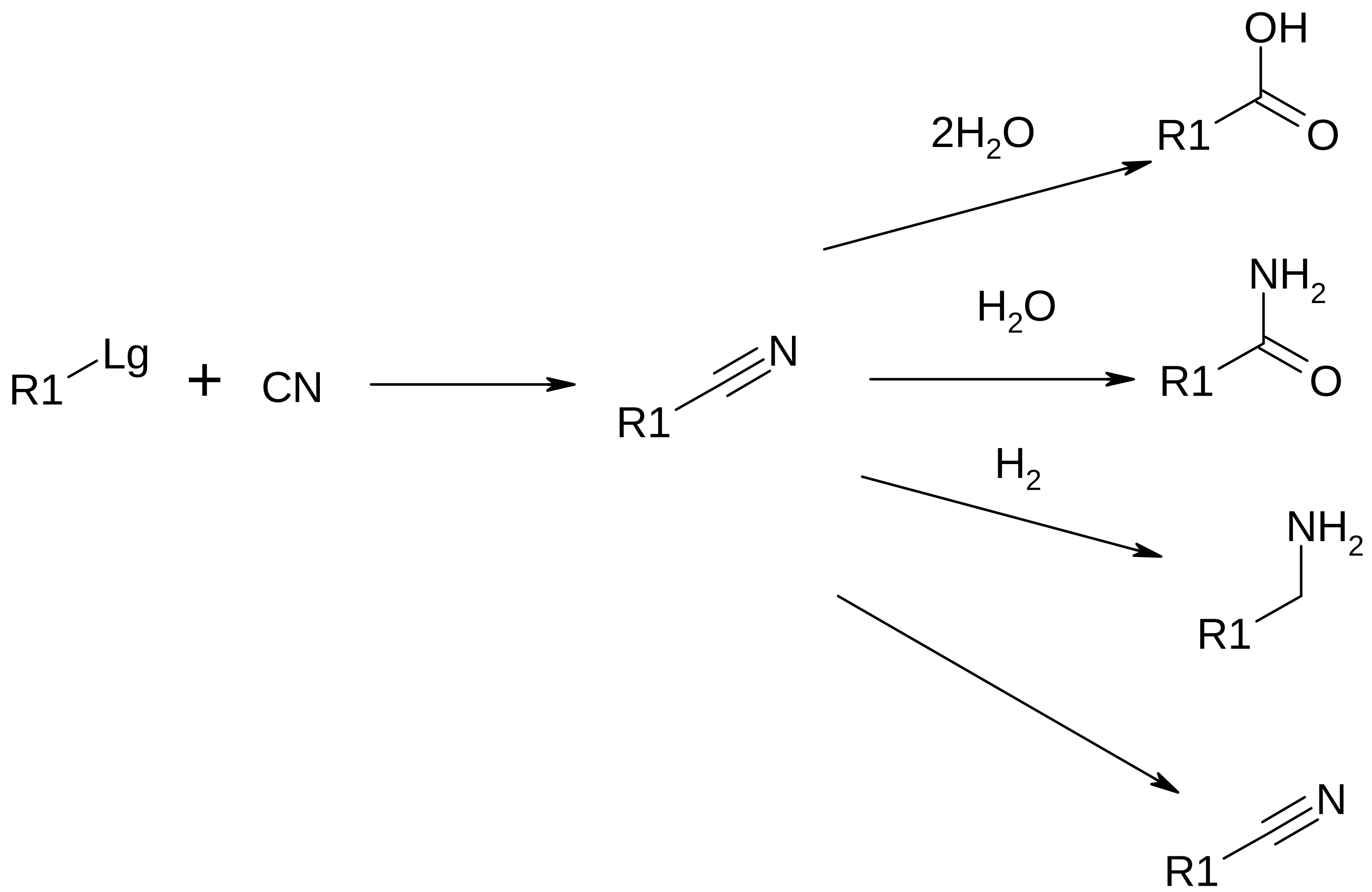
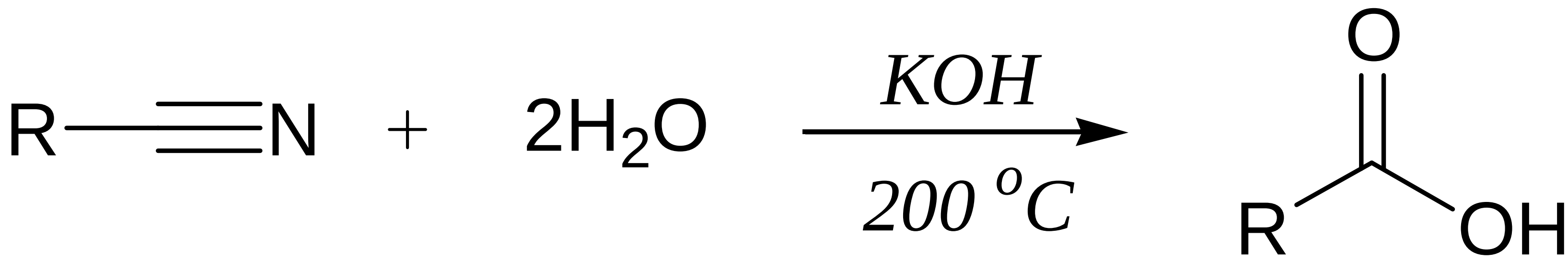
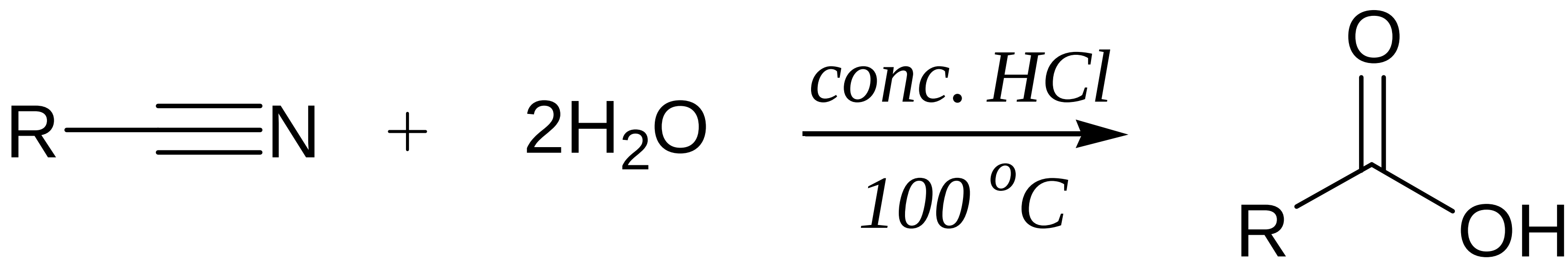
The versatility of the nitrile group

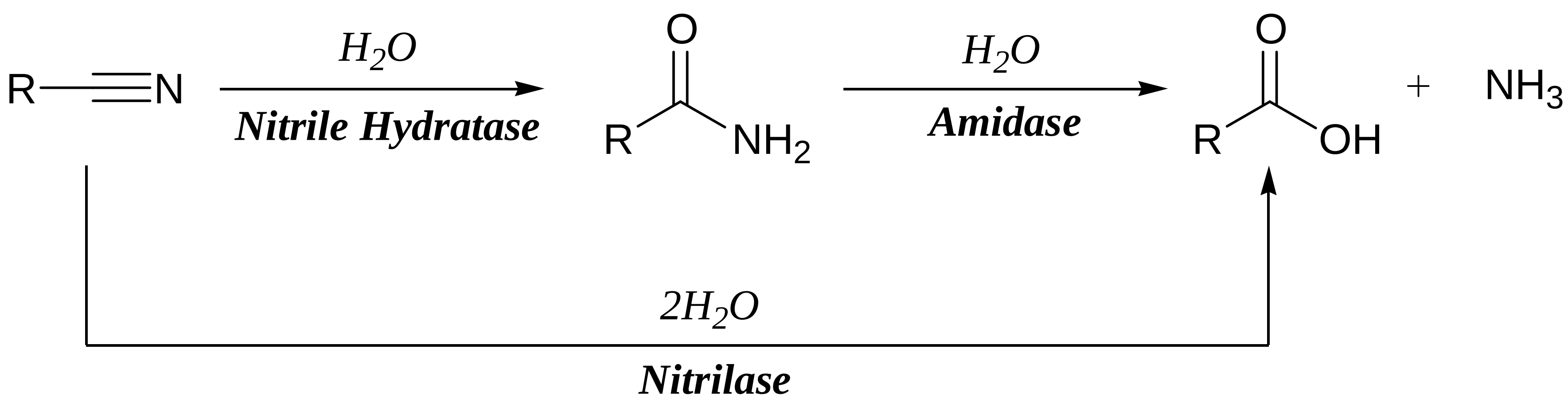


Chemical Nitrile Hydrolysis

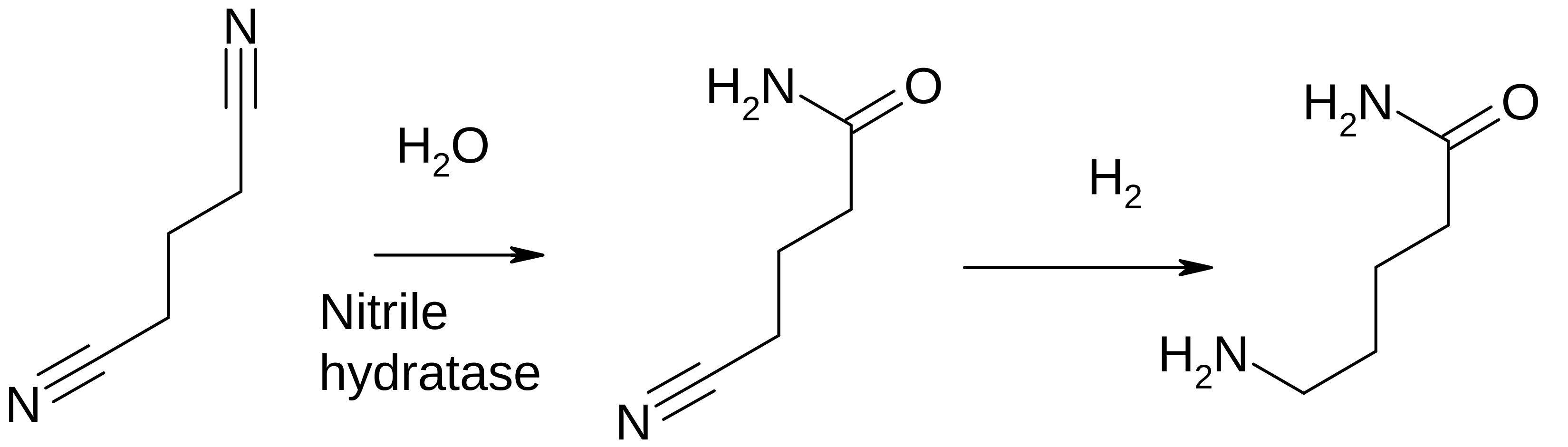




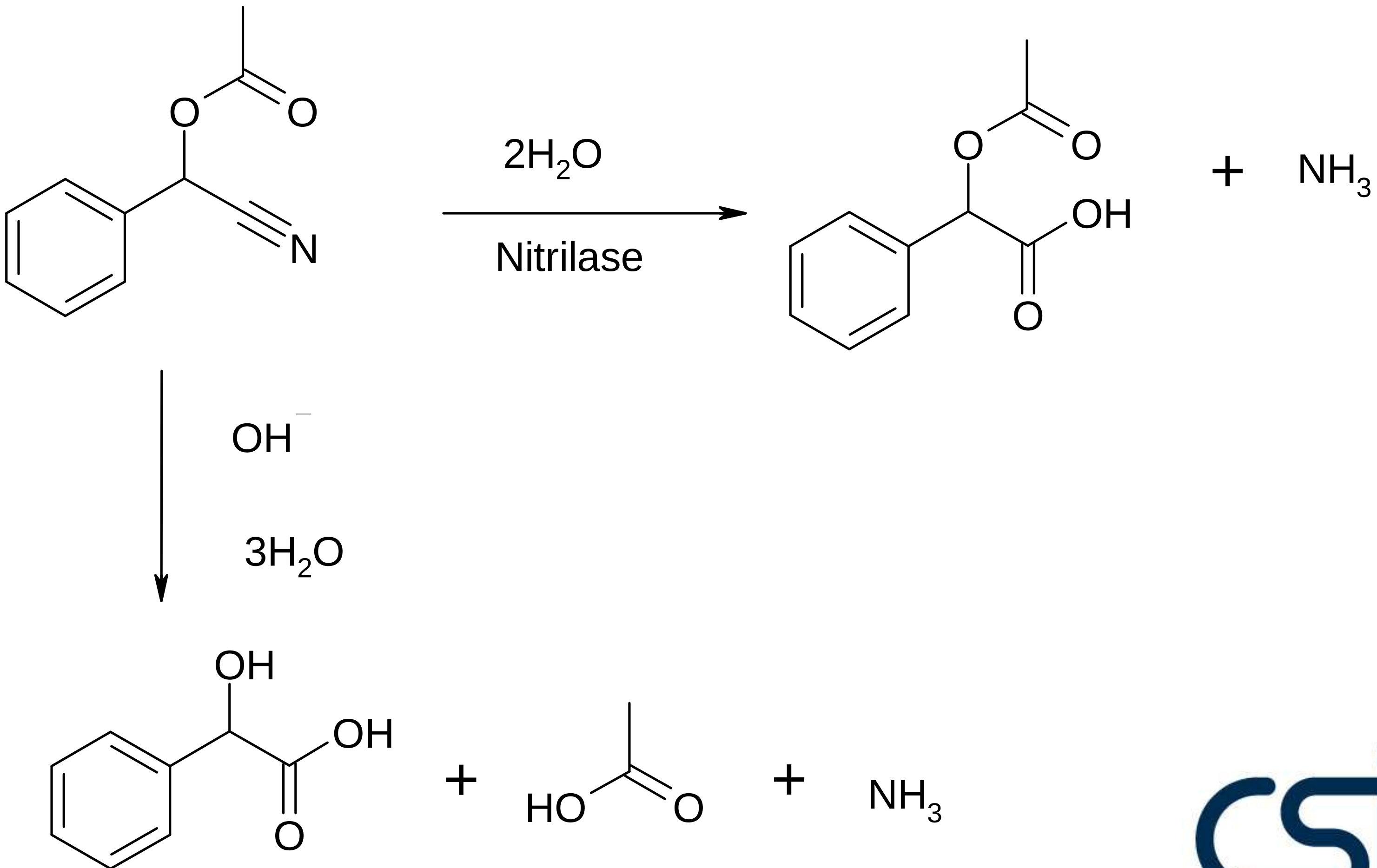
Nitrile Biocatalysis



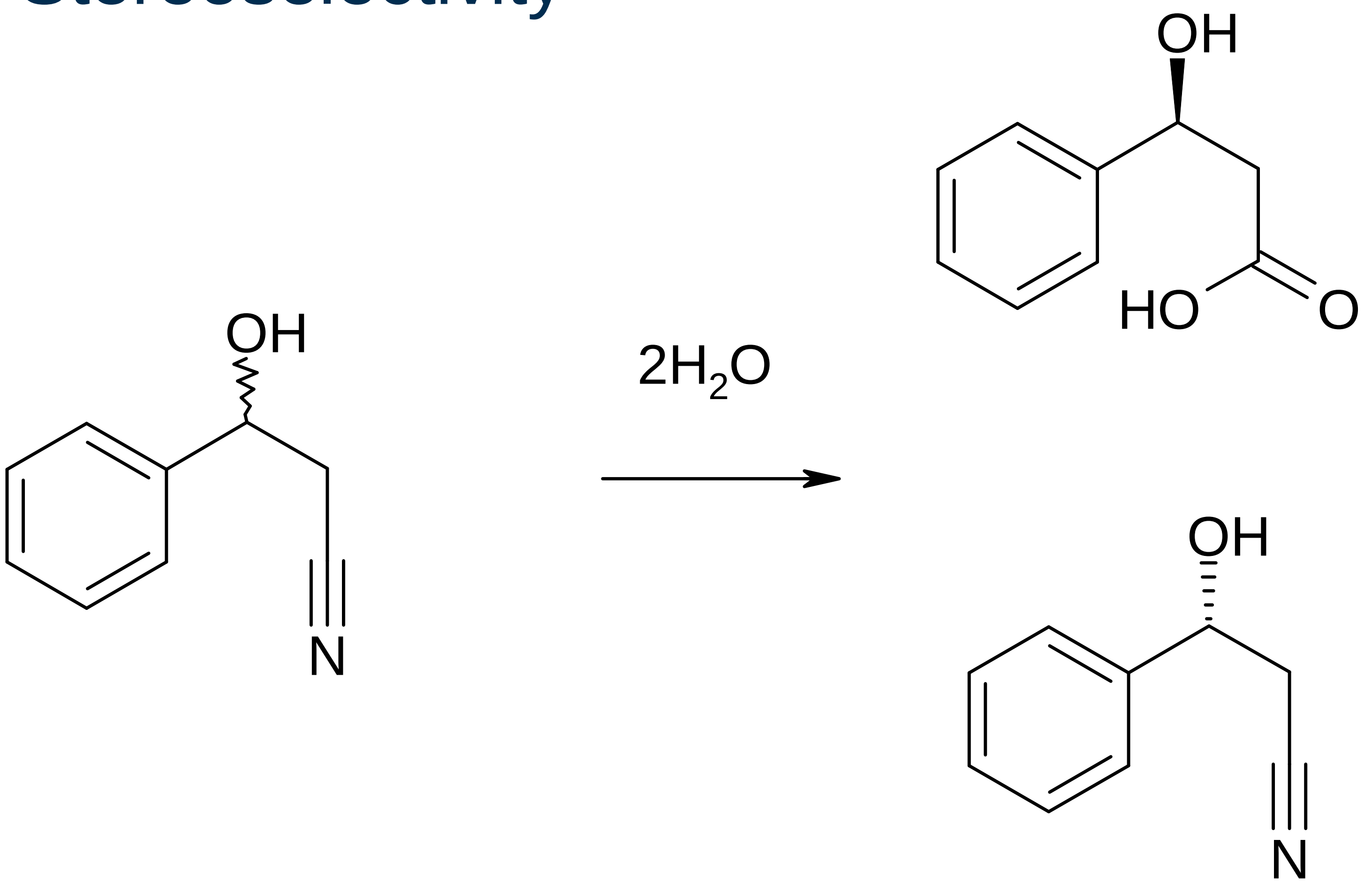
Regioselectivity



Chemoselectivity



Stereoselectivity



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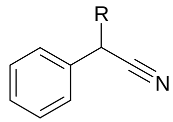
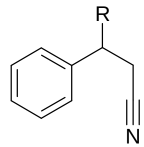
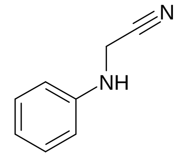


1 R = H
2 R = CH_3
3 R = NH_2
4 R = OH
5 R = CH_2CH_3



6 R = H
7 R = OH



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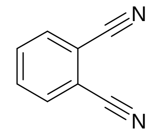

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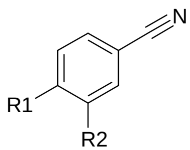
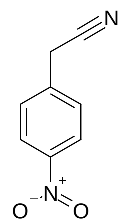


10 R1 = CH_3, R2 = H
11 R1 = OCH_3, R2 = OCH_3



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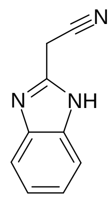

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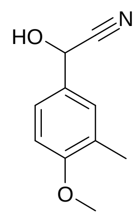

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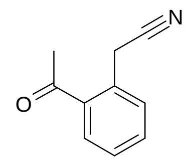

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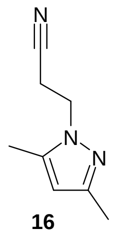

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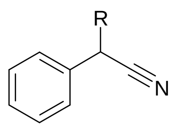
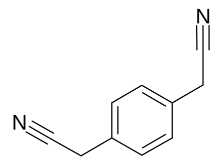


17 R = Br
18 R = C(O)OCH_2CH_3
19 R = OCH(O)Ph



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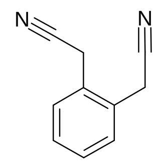

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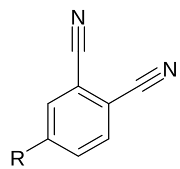
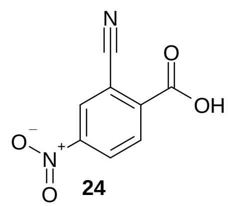


22 R = H
23 R = NO_2



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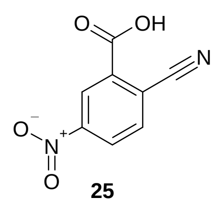

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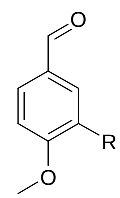
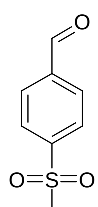


26 R = OCH_3
27 R = H



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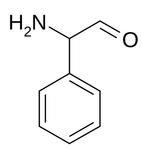

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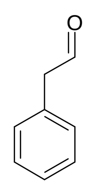

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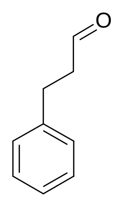

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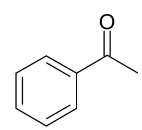

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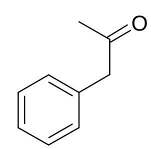

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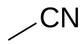

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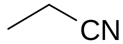

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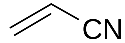

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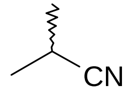

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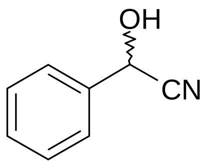

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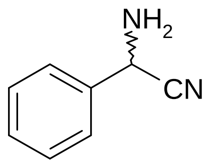

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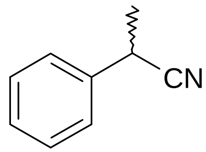

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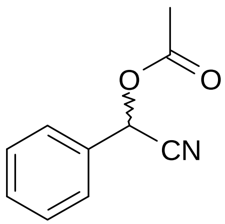

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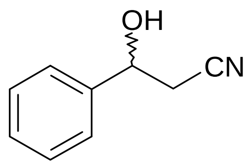

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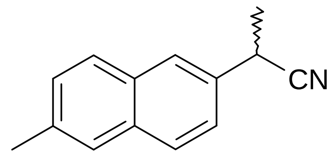

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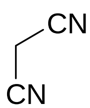

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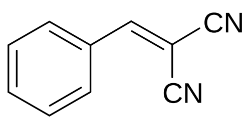

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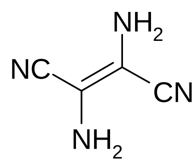

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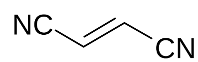

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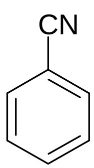

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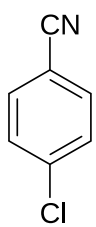

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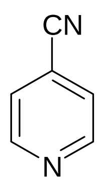

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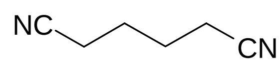

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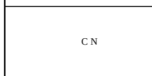
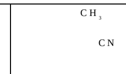
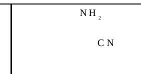
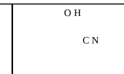
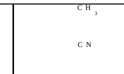
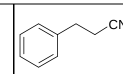
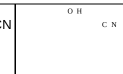
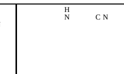
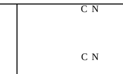
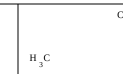
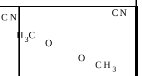
Biocatalyst Toolbox

	Compound										
Biocatalyst											
P. fluorescens nitrilase	100%	21%	89%	74%	1%	1%	0%	0%	11%	0%	0%
BioCatalytics nitrilase-1001	100%	0%	0%	0%	0%	319%	0%	639%	12%	23%	0%
BioCatalytics nitrilase-1004	100%	0%	0%	1%	0%	18%	0%	11%	3%	22%	2%
BioCatalytics nitrilase-1005	100%	0%	0%	0%	0%	64%	0%	128%	62%	128%	128%
BioCatalytics nitrilase-1006	100%	0%	14%	41%	0%	0%	0%	7%	3%	0%	0%
Arabidopsis thaliana nitrilase	100%	0%	0%	0%	0%	4011%	0%	332%	278%	0%	0%
Rhodococcus BCT-ABIs nitrile hydratase	100%	8%	5%	4%	10%	351%	167%	305%	118%	49%	37%
Rhodococcus BCT-ABFGs nitrile hydratase	100%	0%	0%	3%	0%	ND	9%	10%	0%	29%	11%
Rhodococcus DSMZ 44519 nitrile hydratase	100%	46%	12%	9%	2%	ND	76%	83%	29%	100%	43%
Rhodococcus NOVO SP361 nitrile hydratase	100%	15%	4%	4%	10%	ND	30%	7%	9%	27%	13%

ND Not determined

Brady D., Beeton A, Kgaje C, Zeevaart J, van Rantwijk F, Sheldon RA (2004) Characterisation of Nitrilase and Nitrile Hydratase Biocatalytic Systems. Applied Microbiology and Biotechnology. 64: 76-85.

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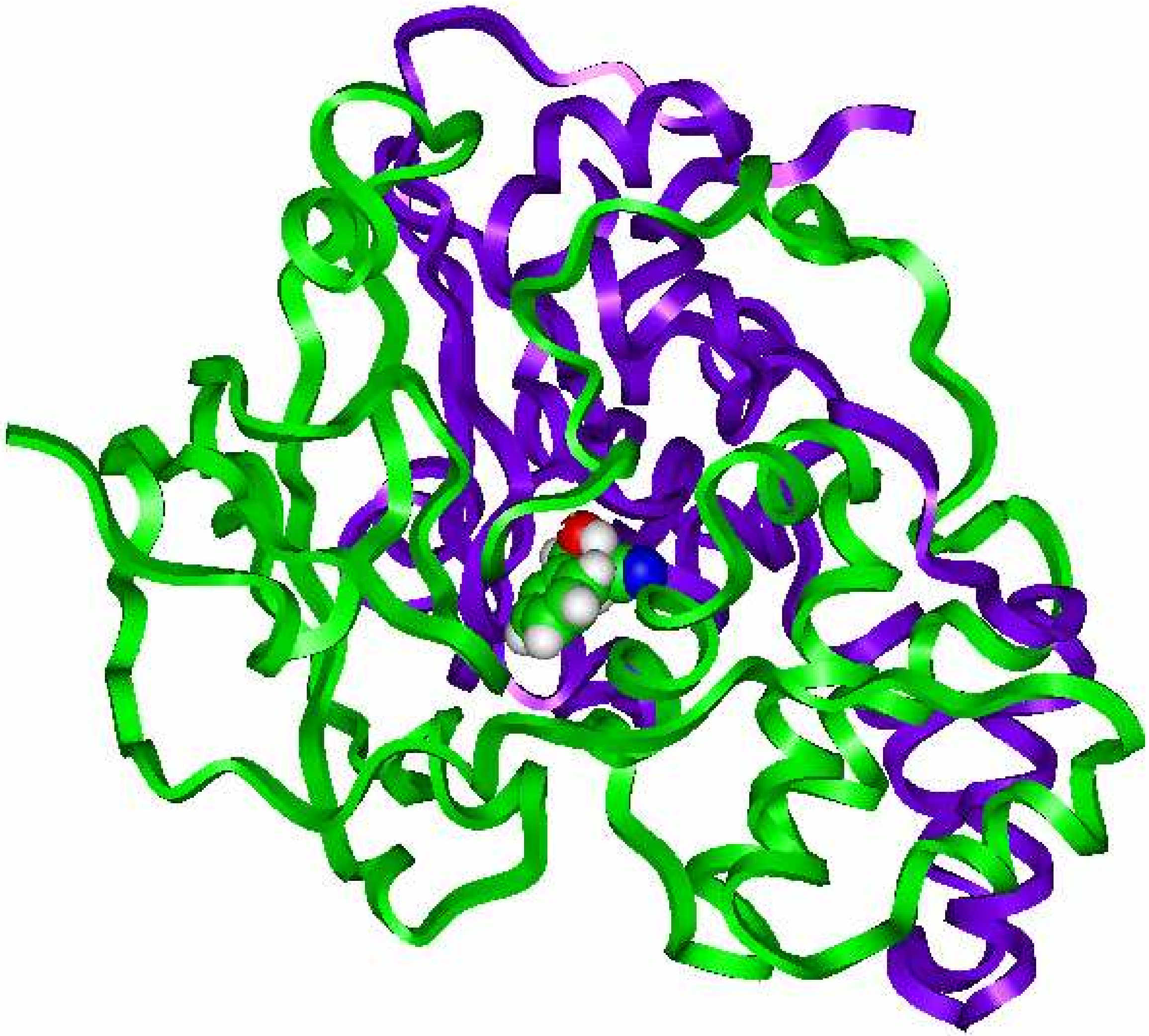
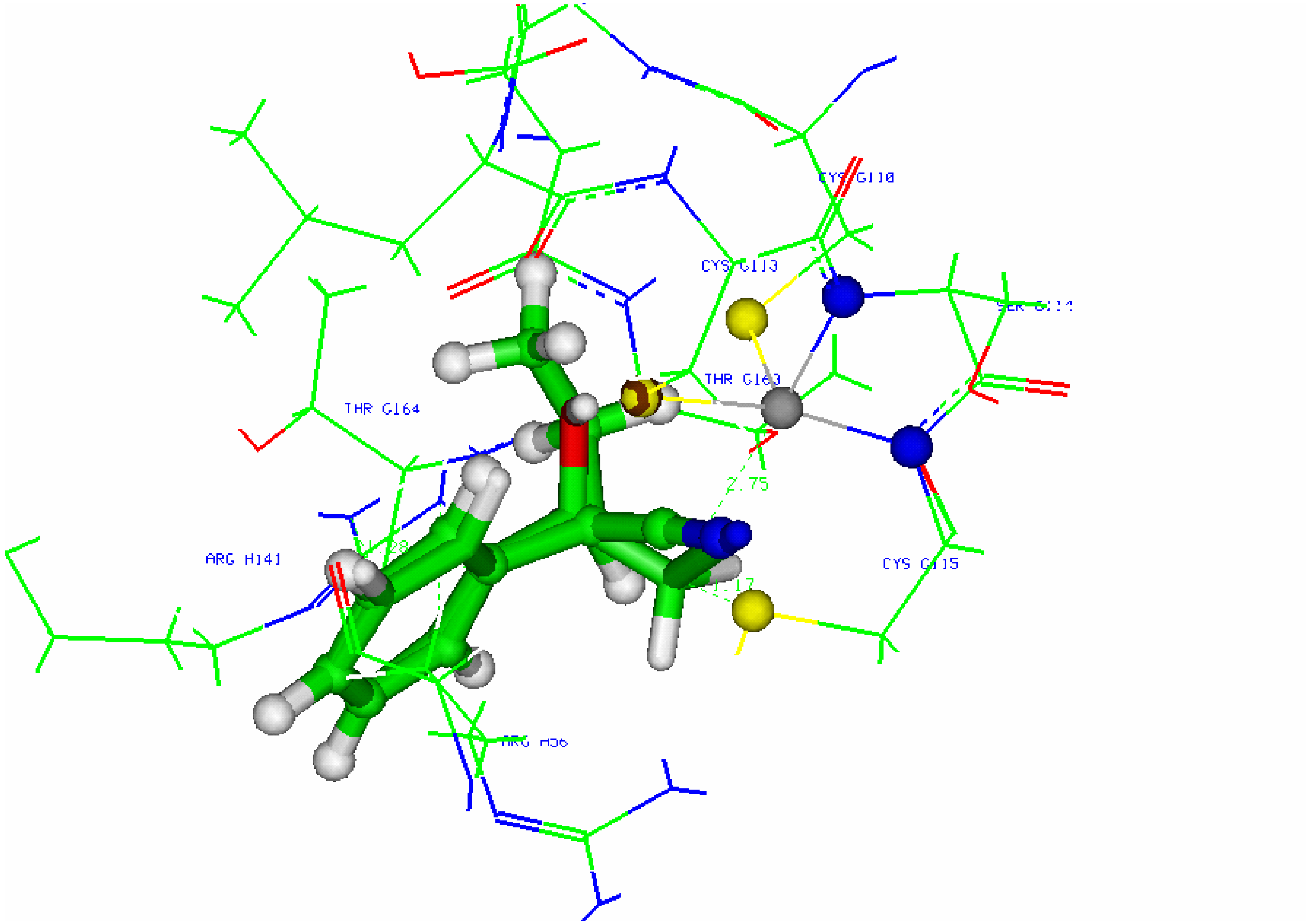
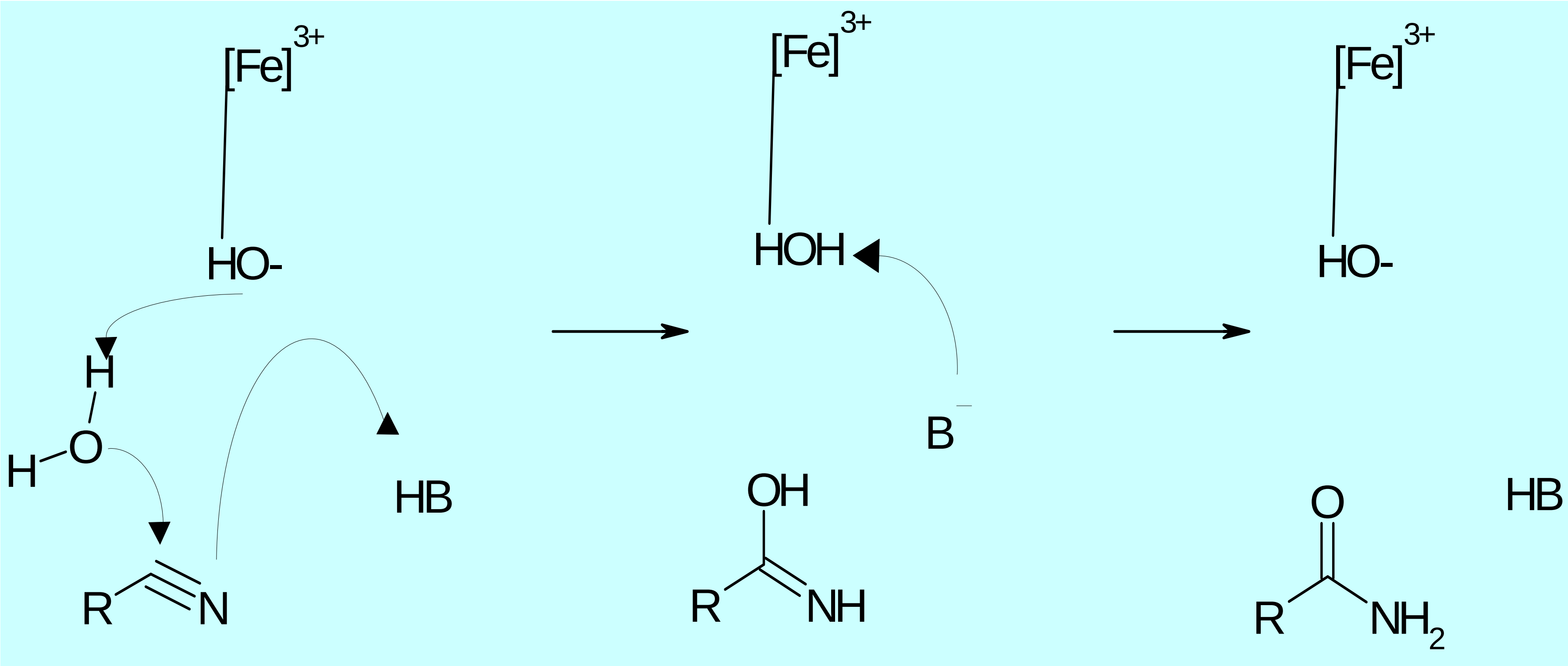
Ribbon model of the Rhodococcus sp R312 nitrilse hydratase with 3-hydroxy-3-phenylpropionitrile in the active site



Superimposition of 2-phenylbutyronitrile and 3-hydroxy-3-phenylpropionitrile in the active site of the nitrile hydratase

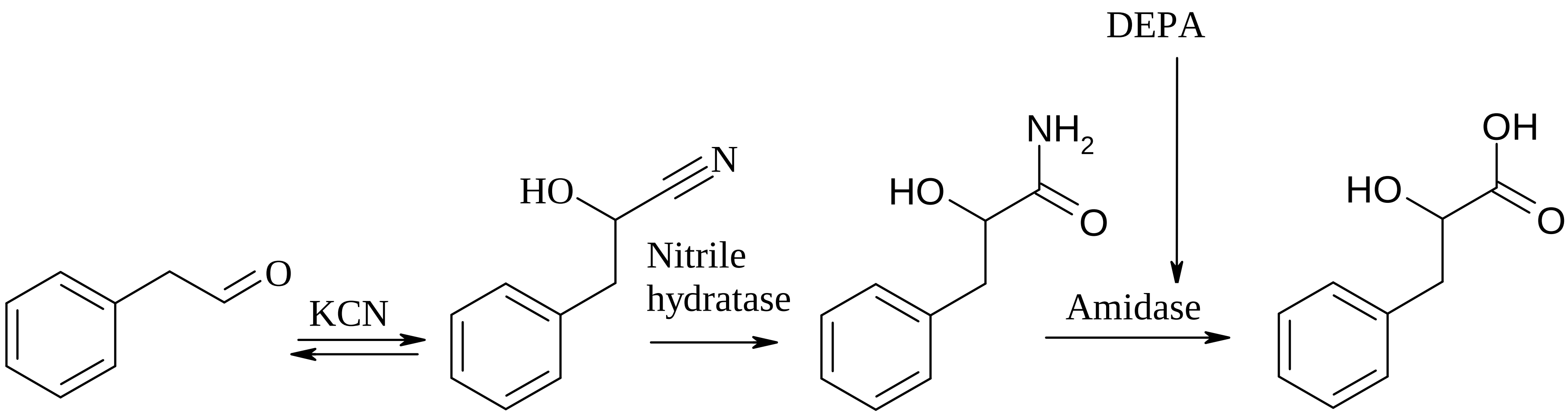


NITRILE HYDRATASE MECHANISM



Huang et al (1997), Structure 5:691-699.

DKR: Multi-step transformation



Phenyl-acetaldehyde

2-Hydroxy-3-phenyl-propionitrile

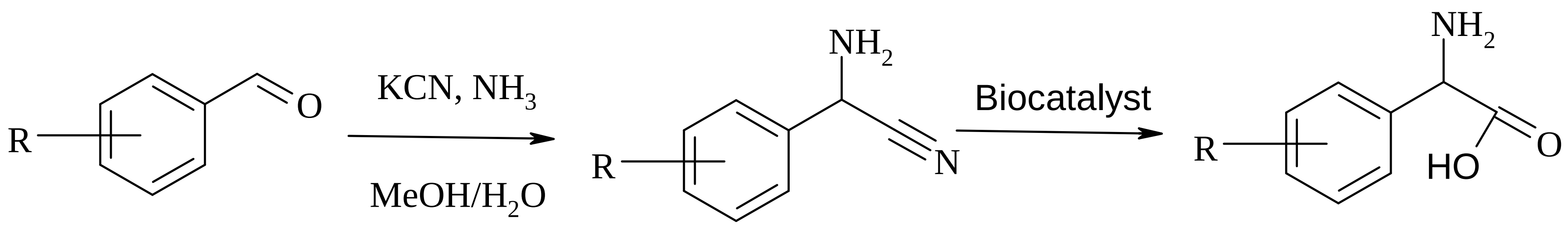
2-Hydroxy-3-phenyl-propionamide

2-Hydroxy-3-phenyl-propionic acid

Synthesis of carboxylic acids from aldehydes

Aldehyde compound incubated with cyanide and biocatalyst	Conversion to homologous α-hydroxy acid (%)
benzaldehyde	95
4-methylbenzaldehyde	51
4-hydroxybenzaldehyde	55
2-nitrobenzaldehyde	20
2-fluorobenzaldehyde	100
3-chlorobenzaldehyde	100
4-chlorobenzaldehyde	100
4-nitrobenzaldehyde	100
2-chlorobenzaldehyde	90
Vanillin	0
4- methoxy-benzaldehyde (p-anisaldehyde)	0
4-cyanobenzaldehyde	100
4-methylsulpnonyl-benzaldehyde	0
Trimethoxybenzaldehyde	0

Strecker Synthesis



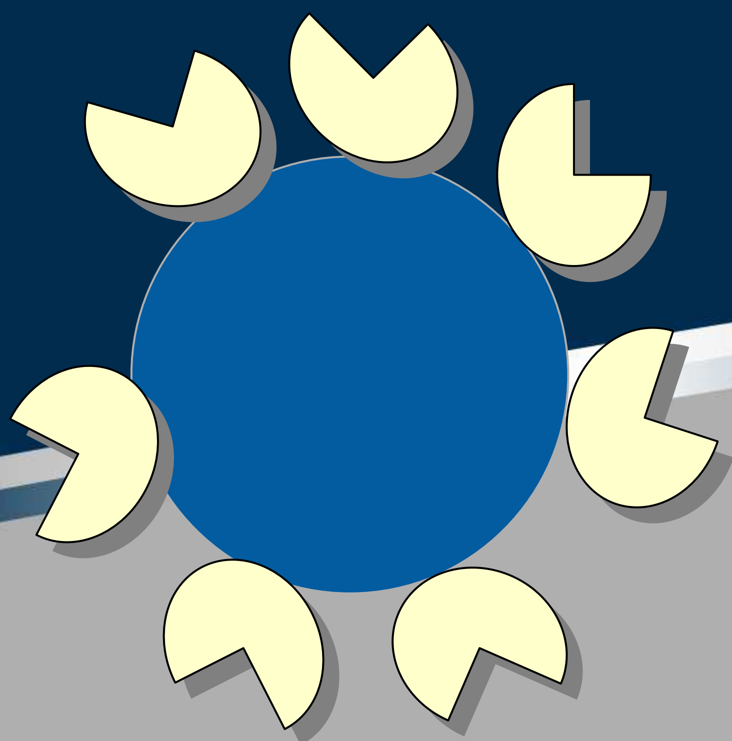
R = H, Cl, F, OH, NO_2, CH_3

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.

Brady D, Dube N, Petersen R. (2006) Green–chemistry, biocatalytic nitrile substrate specificity (S.African J. Sci, in print).

Immobilisation



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Immobilised Biocatalyst Preparation

Solubilisation of lipase powder in aqueous buffer (100 mM Pi)

↓

Slurry formation with celite (batch mode), pH 7

↓

Freeze drying

1 AK : 2 celite

↓

Milling of cake

↓

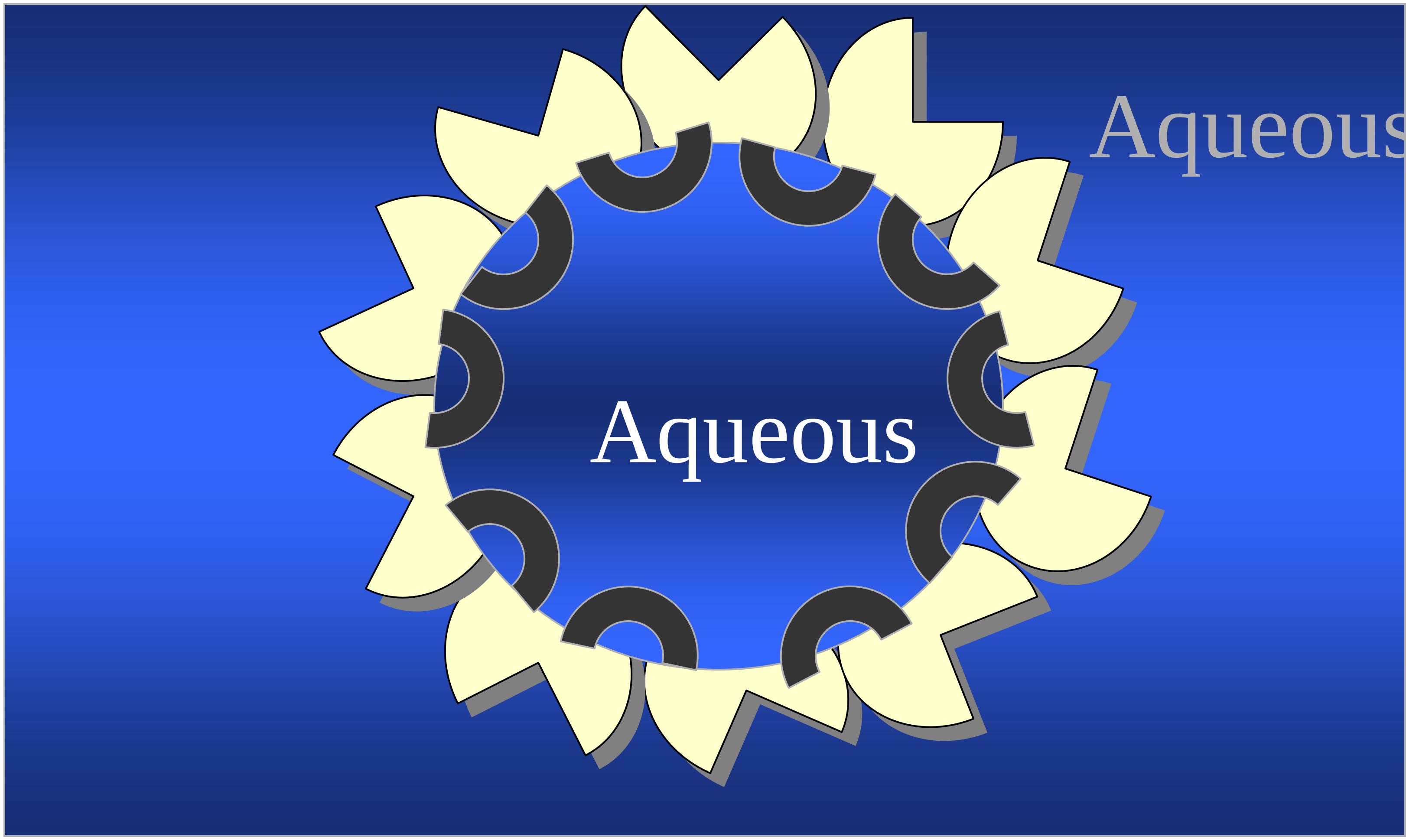
Size control (sieving/sedimentation)

↓

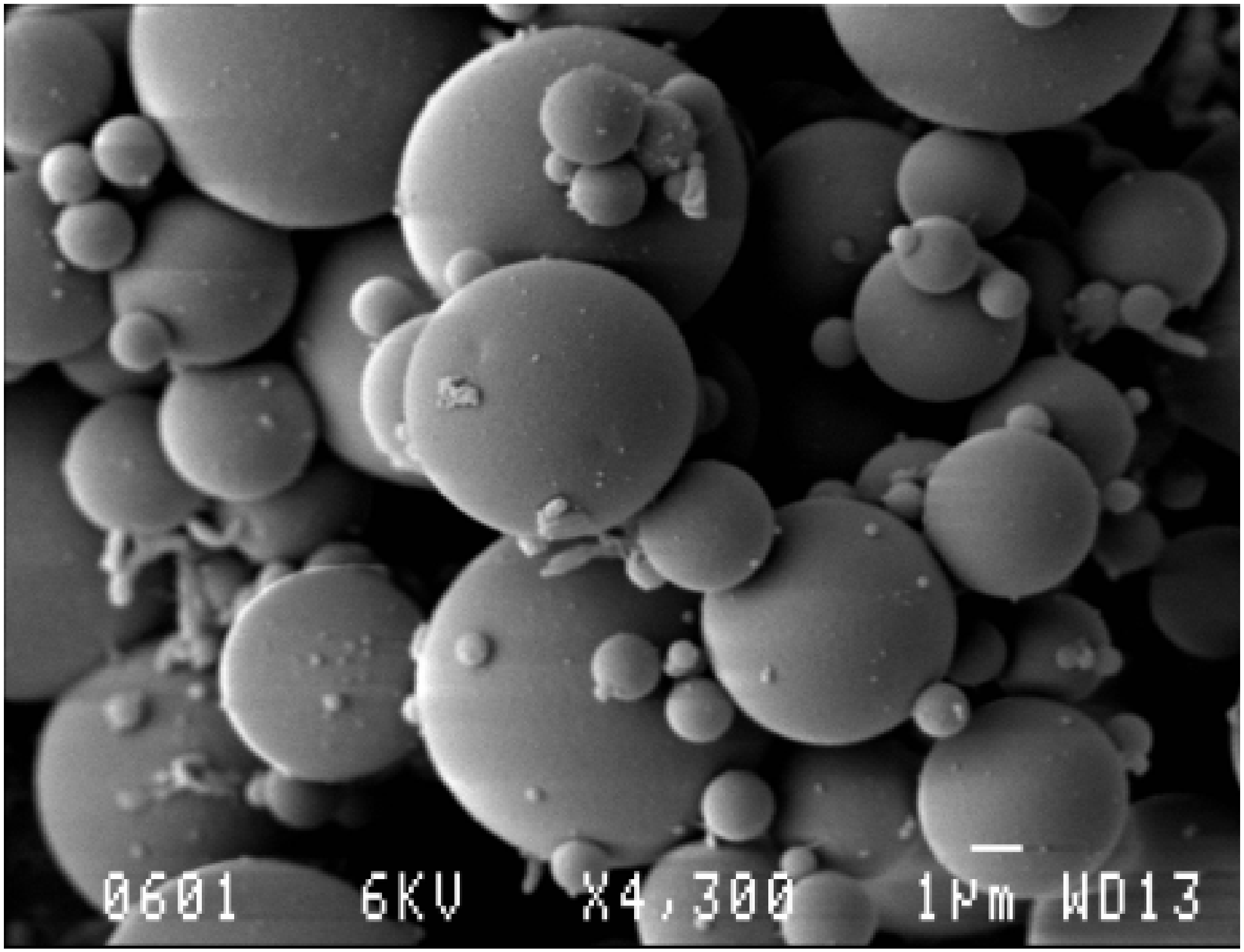
Storage under airtight conditions

Spherezyme

Moolman, S Brady D, Sewlall, AS Rolfes H, Jordaan J.   Stabilization of Enzymes WO 2005/080561



SEM:
Lipase/Albumin SphereZymes



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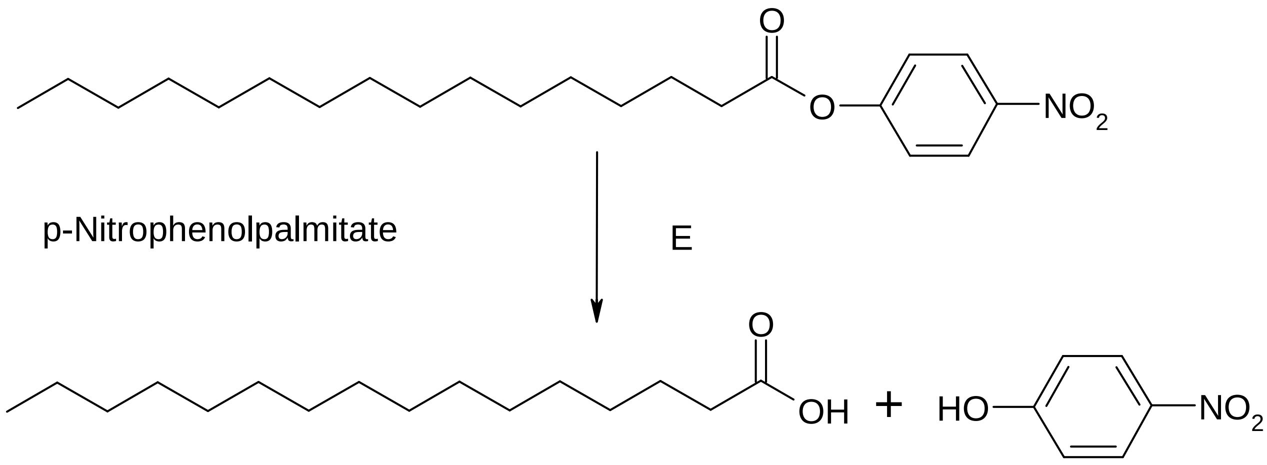
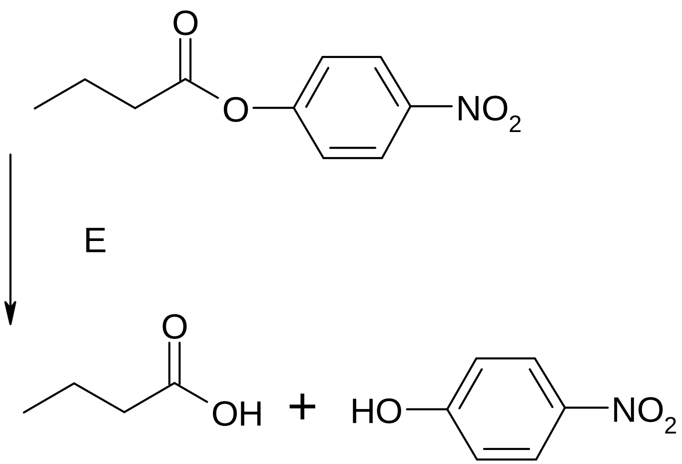
Standard substrates- 2 sizes



p-Nitrophenolpalmitate



p-Nitrophenolbutyrate

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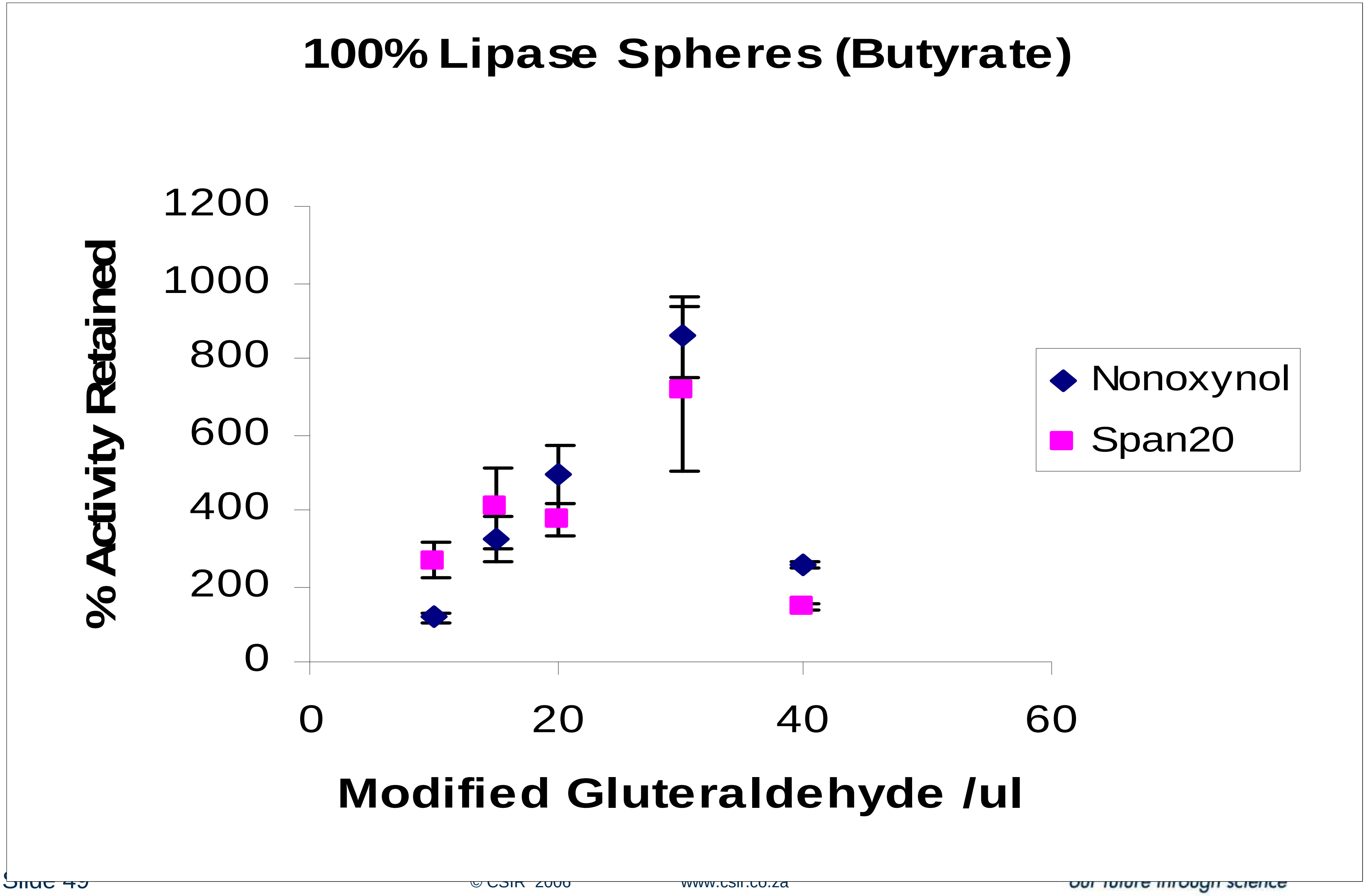
Hyperactivation



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