Green Chemistry: highly selective biocatalytic hydrolysis of nitrile compounds

CSIR Conference

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The need for Green Chemistry

- On average 300 kg of chemicals are required to produce 1 Kg of a fine chemical or active pharmaceutical intermediate.
- This is due to the lack of specificity of most catalysts, requiring extensive use of protecting groups and additional reaction steps.
- Reaction yields are often low.
- The reactions often require extreme conditions of pressure and temperature, with special pressure reactors.
- Polluting waste levels are high, and solvent recycling costs add to the final product price.

Biocatalysis

- Selectivity:
  - Enantiomeric
  - Regioselectivity
  - Chemoselectivity

- Safety and environment:
  - Low pressure and temperature reactions (improved safety and CAPEX costs)
  - Less waste products (minimise or eliminate organic solvents)
  - Improved atom economy (less protecting groups and enantiomeric “ballast” leads to savings).

Nitrile Hydratase

- In 2000, sales in the chemical industry exceeded $1.7 trillion.
- $50 billion via biological routes (cellular biotransformations or biocatalysts)
- By 2015 this will have grown to approximately $250 billion.
- 15% of chiral technology is now achieved by biocatalysis, and is projected as 30% by 2009!

Biocatalysis in Application

- Lonza's Biotechnology microbial fermentation unit has 17 products (worth a $100 million) in its pharmaceutical and "nutraceutical" pipeline.
- Avecia: five of the firm's top 10 commercial pharmaceutical intermediates involves at least one biotransformation step.
- DSM Fine Chemicals: 30% of the more than 80 pharmaceuticals in development for third parties at DSM involve biocatalytic steps.

- Fluka’s products, 5% of are now made using biocatalysis,
- Dutch chemicals giant DSM uses 25 biocatalysis-based processes at large scale.
- Other companies involved in biotransformations include: Degussa and Direvo biotech (Germany)
- Daicel Chemical Industries (Japan)
- Dow Chemical, Diversa, and Du Pont (USA)

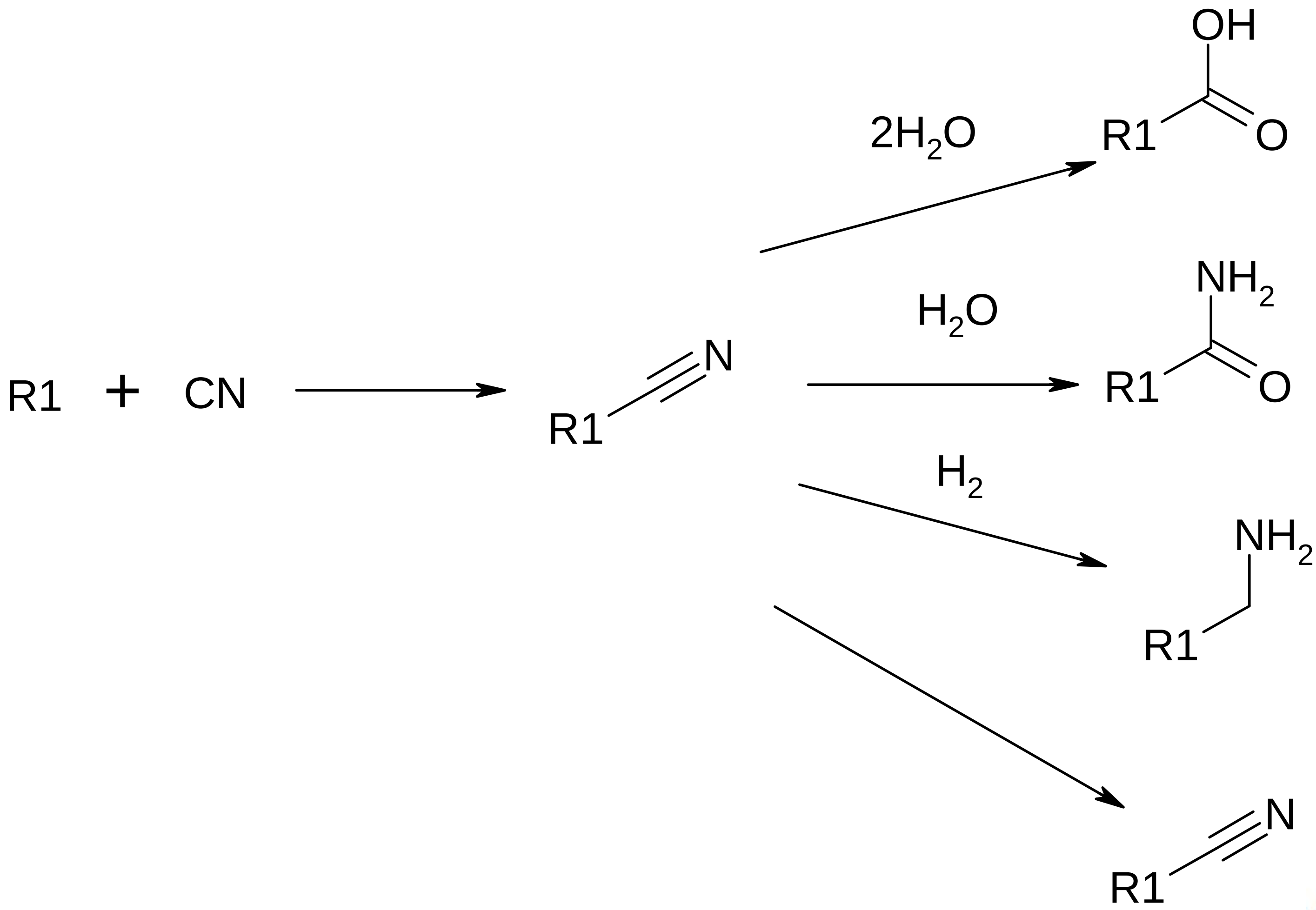
- Growing drug industry demand for enantiomerically pure compounds is the driver for pursuing biocatalytic technology.

- SA imports R14 billion p.a. speciality and fine chemicals.

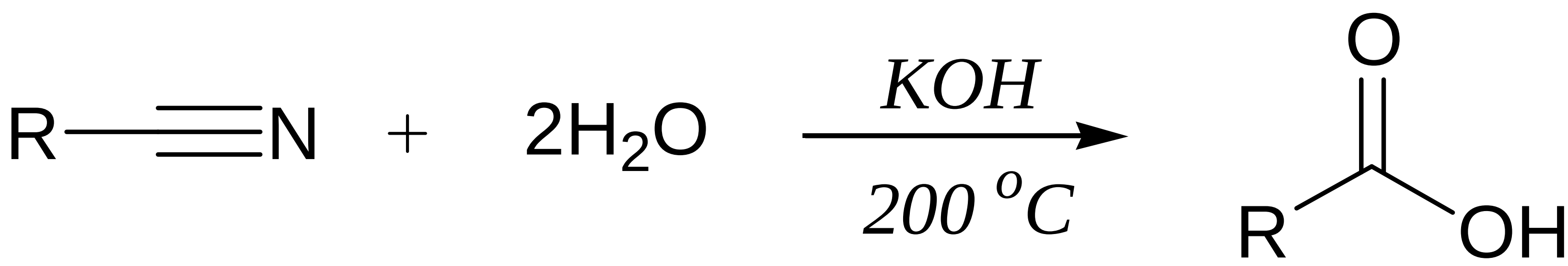
Biocatalysis at the CSIR

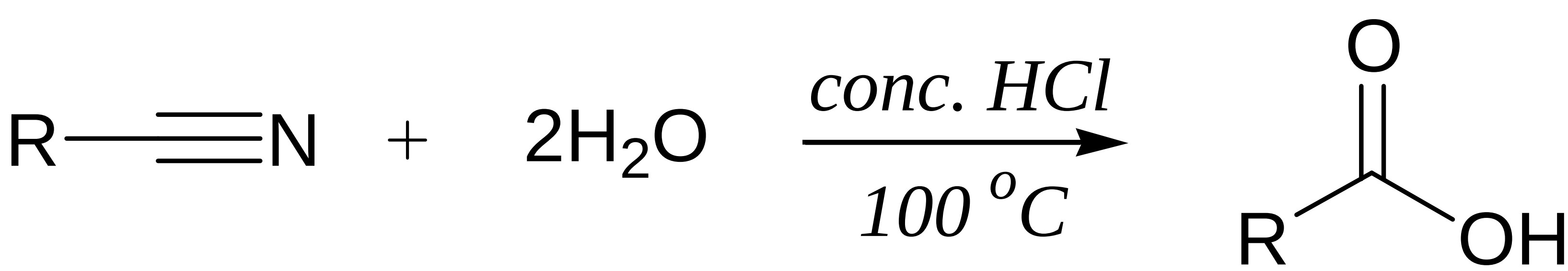
- The biocatalytic resolution of naproxen (as part of a commercial synthetic process).
- The biocatalytic resolution of menthol (as part of a commercial synthetic process).
- The epoxide technology platform has been progressed to the product stage.

The versatility of the nitrile group



Chemical Nitrile Hydrolysis





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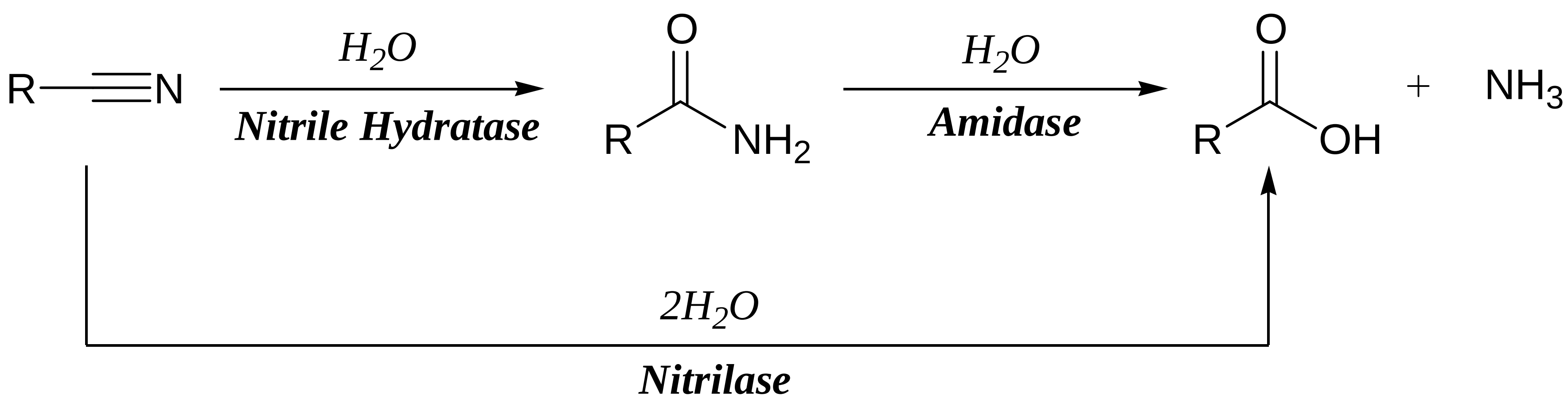
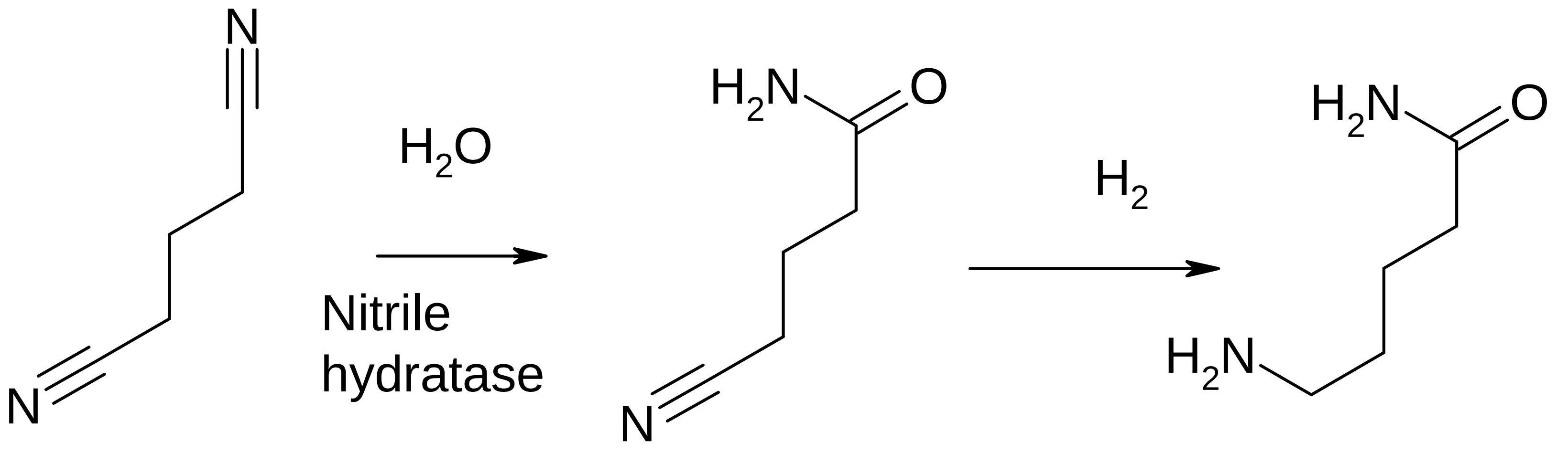
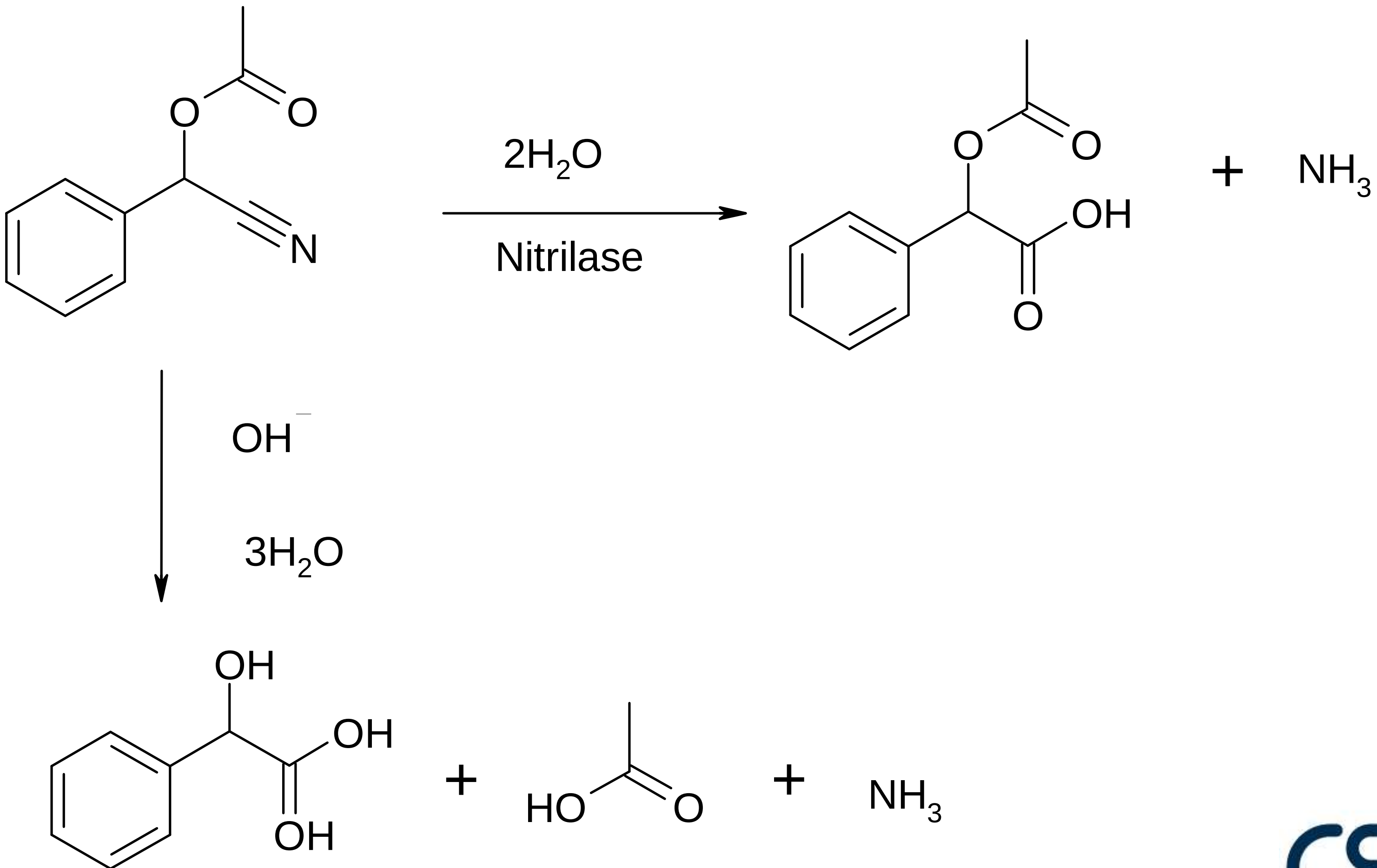
Nitrile Biocatalysis



Regioselectivity



Chemoselectivity



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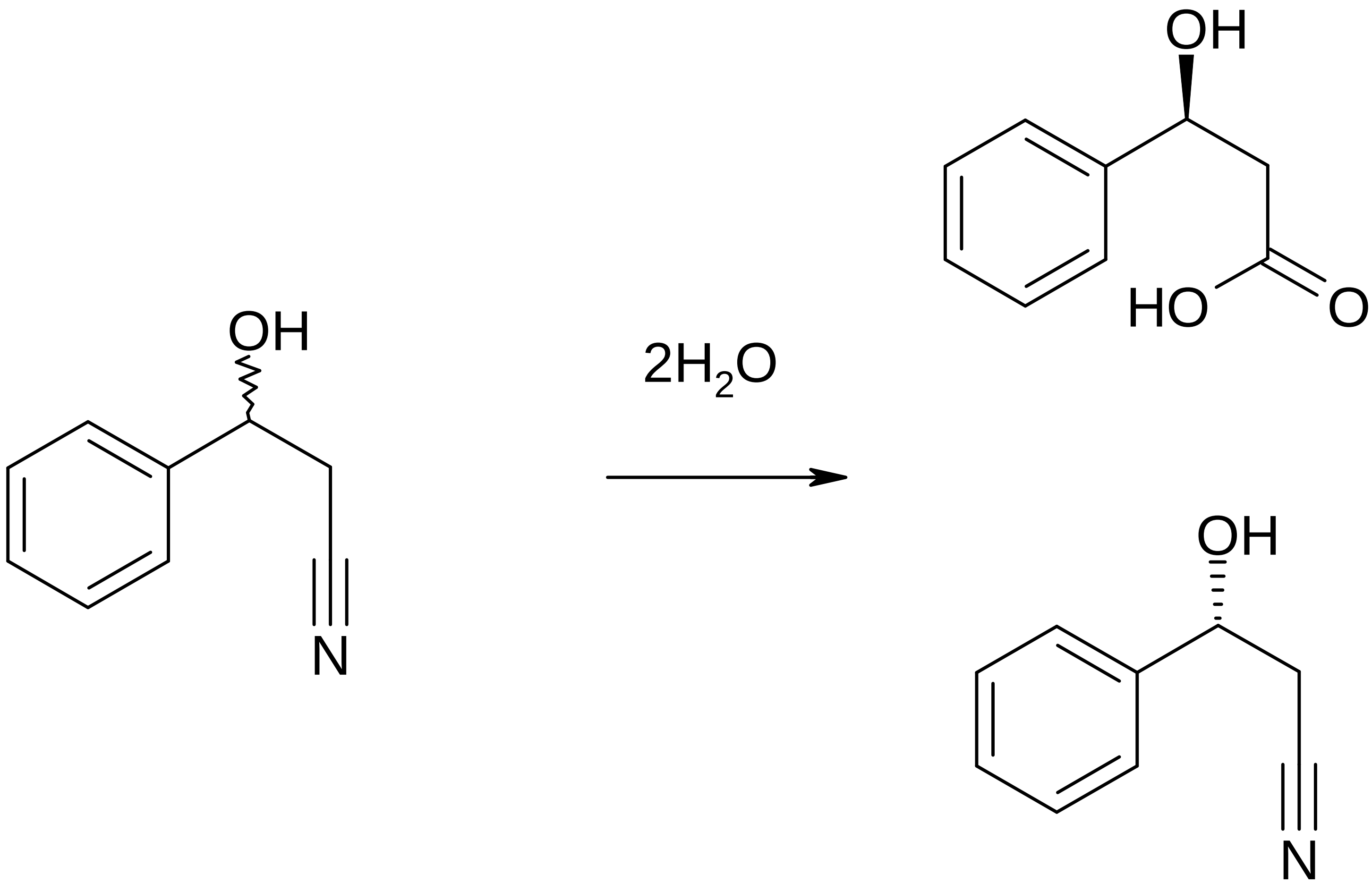
Stereoselectivity

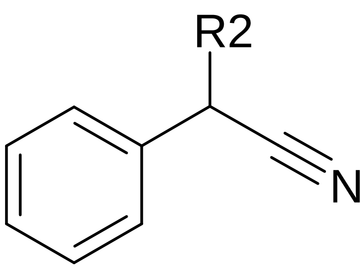


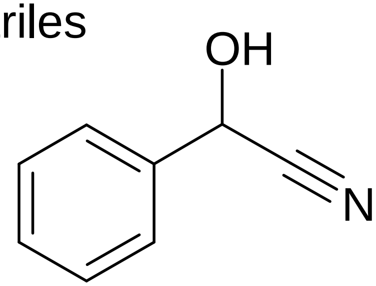
Problems with nitrile biocatalysts

- Availability of both nitrilases and nitrile hydratases
- Stability of nitrilases
- Enantioselectivity of nitrile hydratases

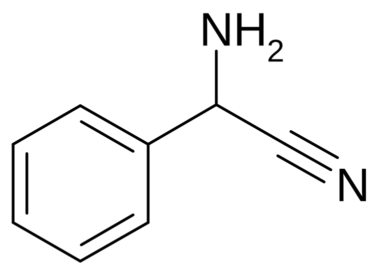
Alpha-subsituted Substrates

α-substituted phenyl propionitriles

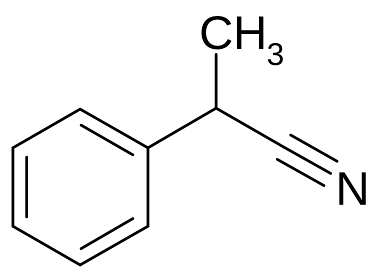




Hydroxy-phenyl-acetonitrile
(Mandelonitrile)

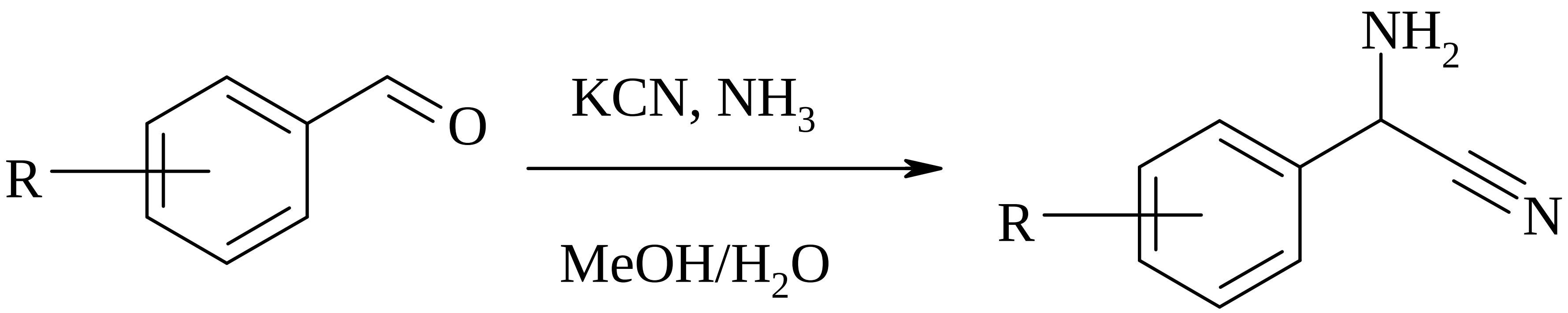


Amino-phenyl-acetonitrile
(2-phenylglycinonitrile)



2-Phenyl-propionitrile
(α-methyl-benzylcyanide)

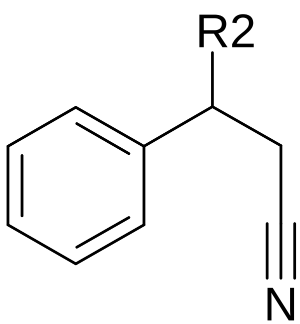
Strecker Synthesis

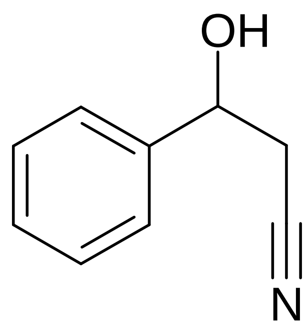


R = H, Cl, F, OH, NO_2, CH_3

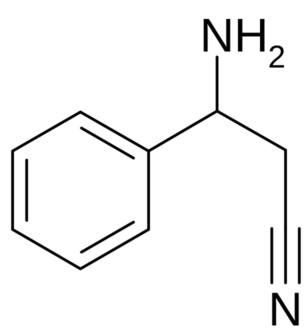
Beta-substituted substrates

β-substituted phenyl propionitriles





3-Hydroxy-3-phenyl-propionitrile



3-Amino-3-phenyl-propionitrile

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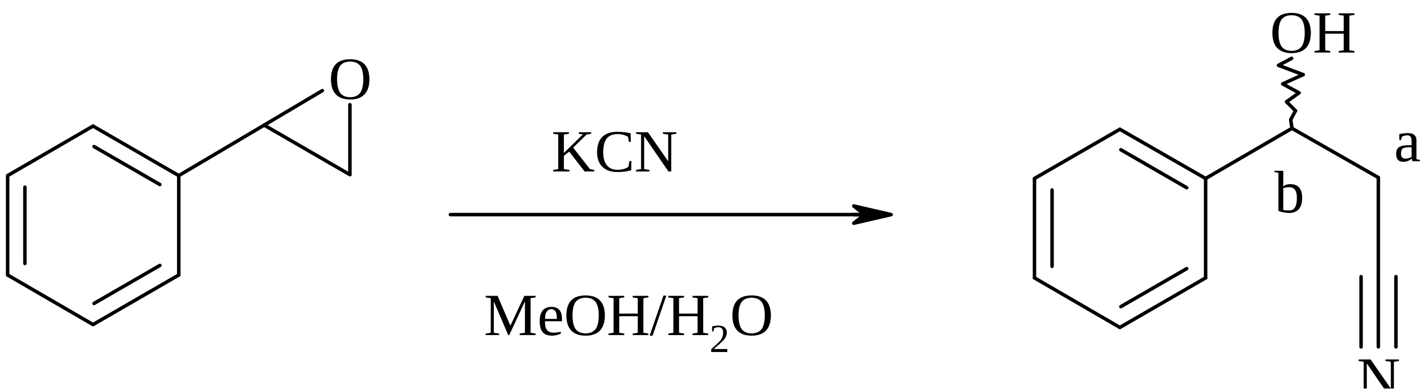
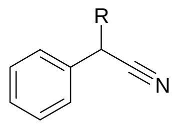
Beta-hydroxy substrates

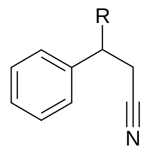


Styrene oxide

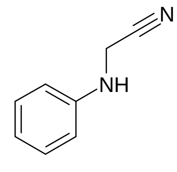
3-hydroxy-3-phenyl-propionitrile



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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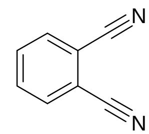

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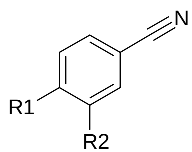
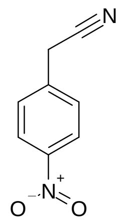


10 R1 = CH_3, R2 = H
11 R1 = OCH_3, R2 = OCH_3



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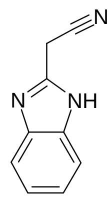

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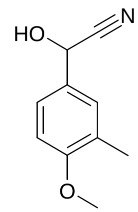

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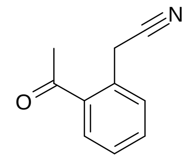

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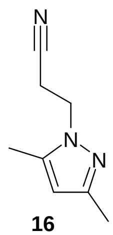

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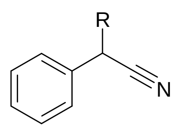
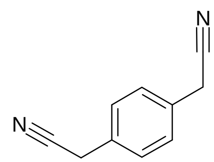


17 R = Br
18 R = C(O)OCH_2CH_3
19 R = OCH(O)Ph



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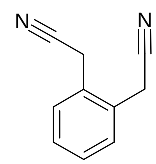

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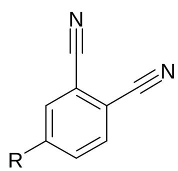
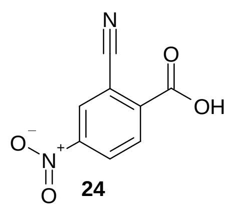


22 R = H
23 R = NO_2



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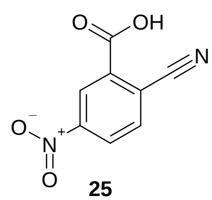

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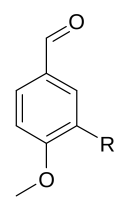
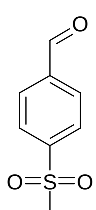


26 R = OCH_3
27 R = H



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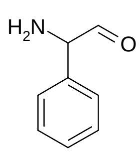

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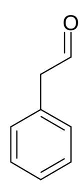

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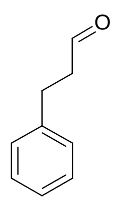

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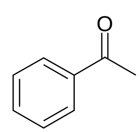

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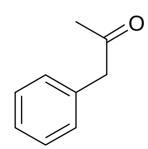

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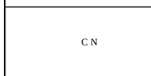
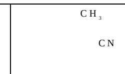
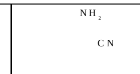
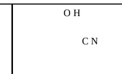
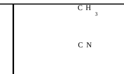
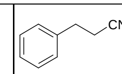
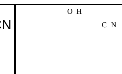
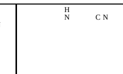
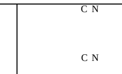
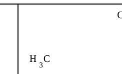
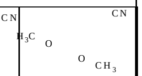
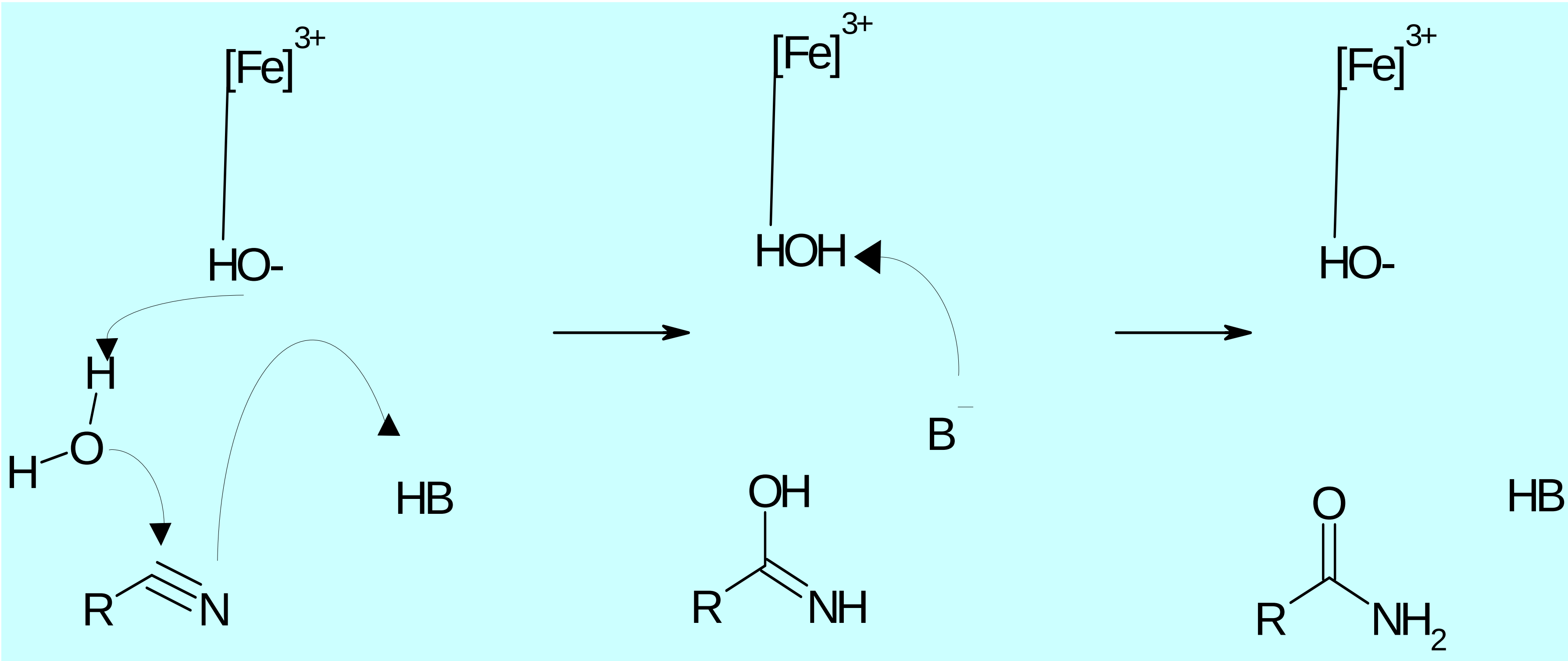
Commercial vs Isolated Organisms

Biocatalyst	Compound										
											
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

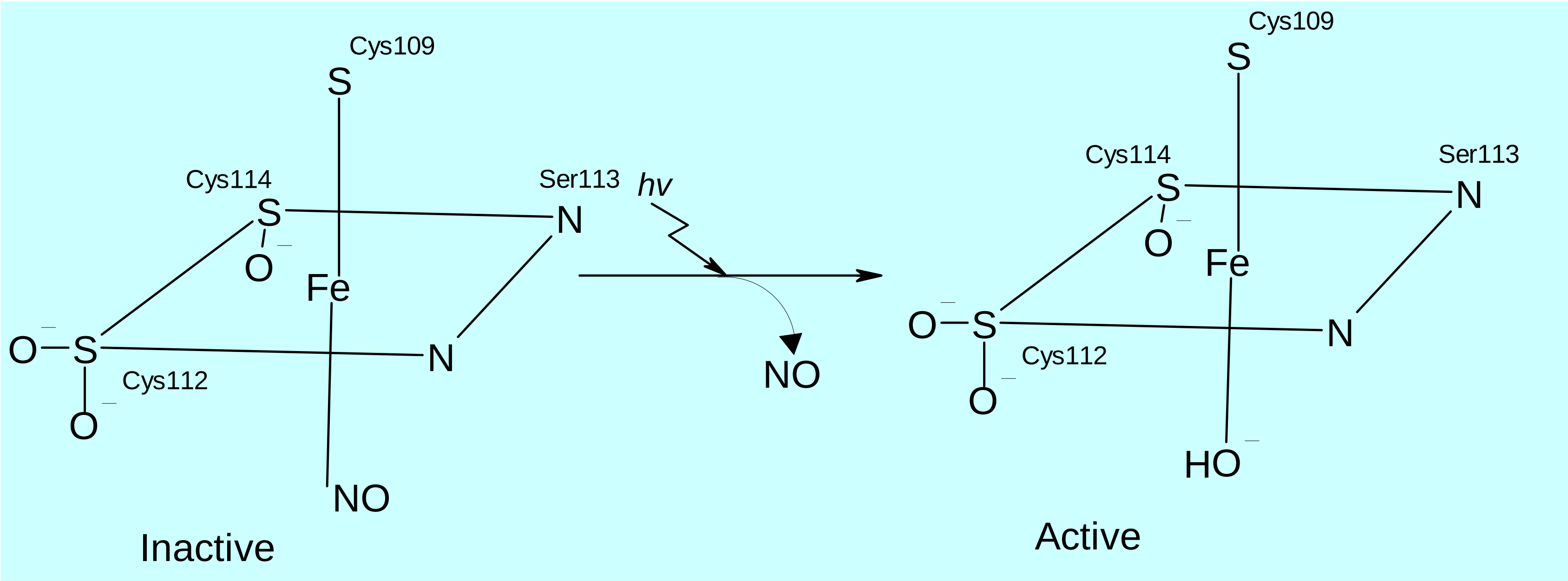
Brady D., Beeton A, Kgaje C, Zeevaart J, van Rantwijk F, Sheldon RA (2004) Characterisation of Nitrilase and Nitrile Hydratase Biocatalytic Systems. Applied Microbiology and Biotechnology. 64: 76-85.

NITRILE HYDRATASE MECHANISM



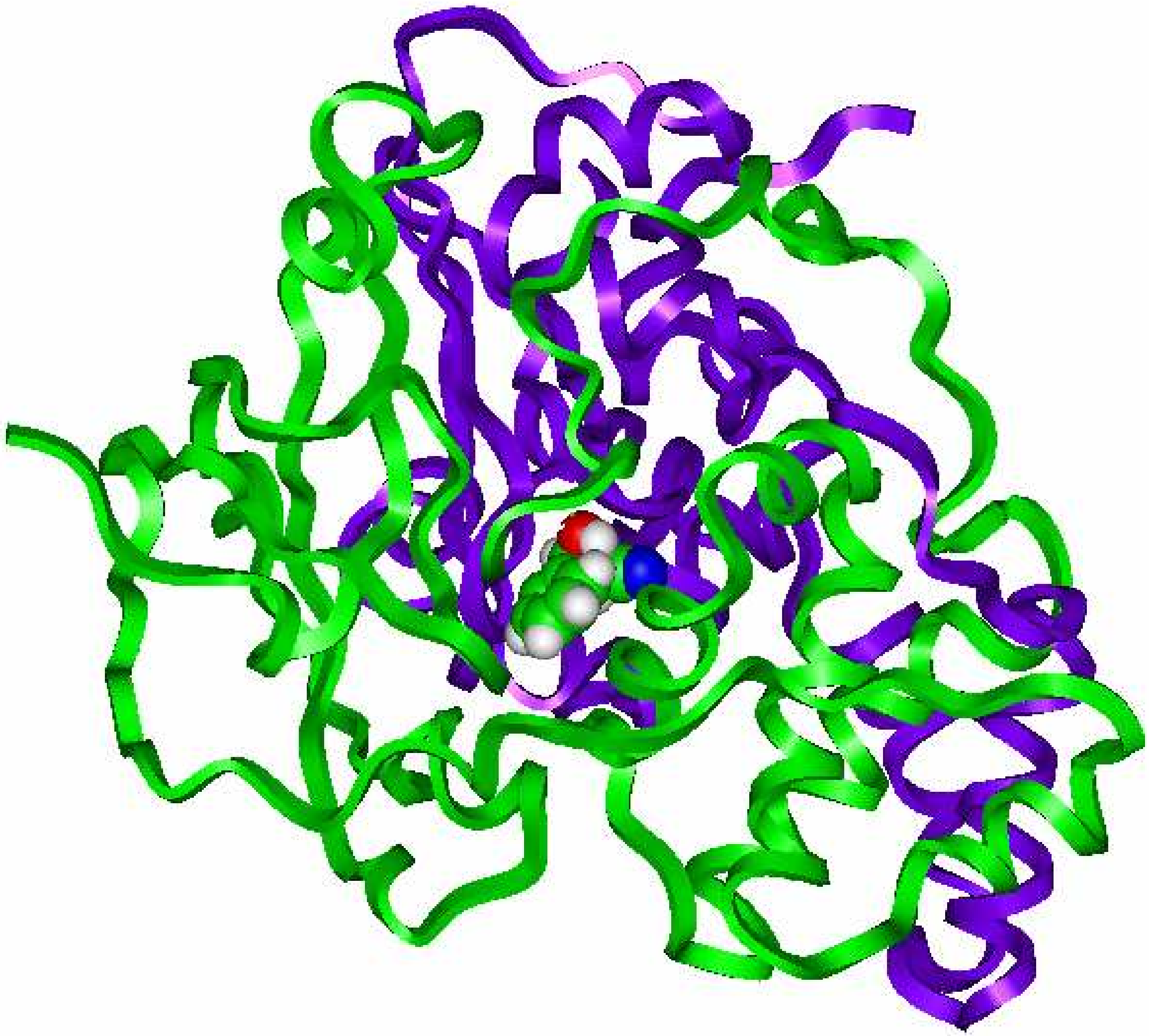
Huang et al (1997), Structure 5:691-699.

NITRILE HYDRATASE MECHANISM

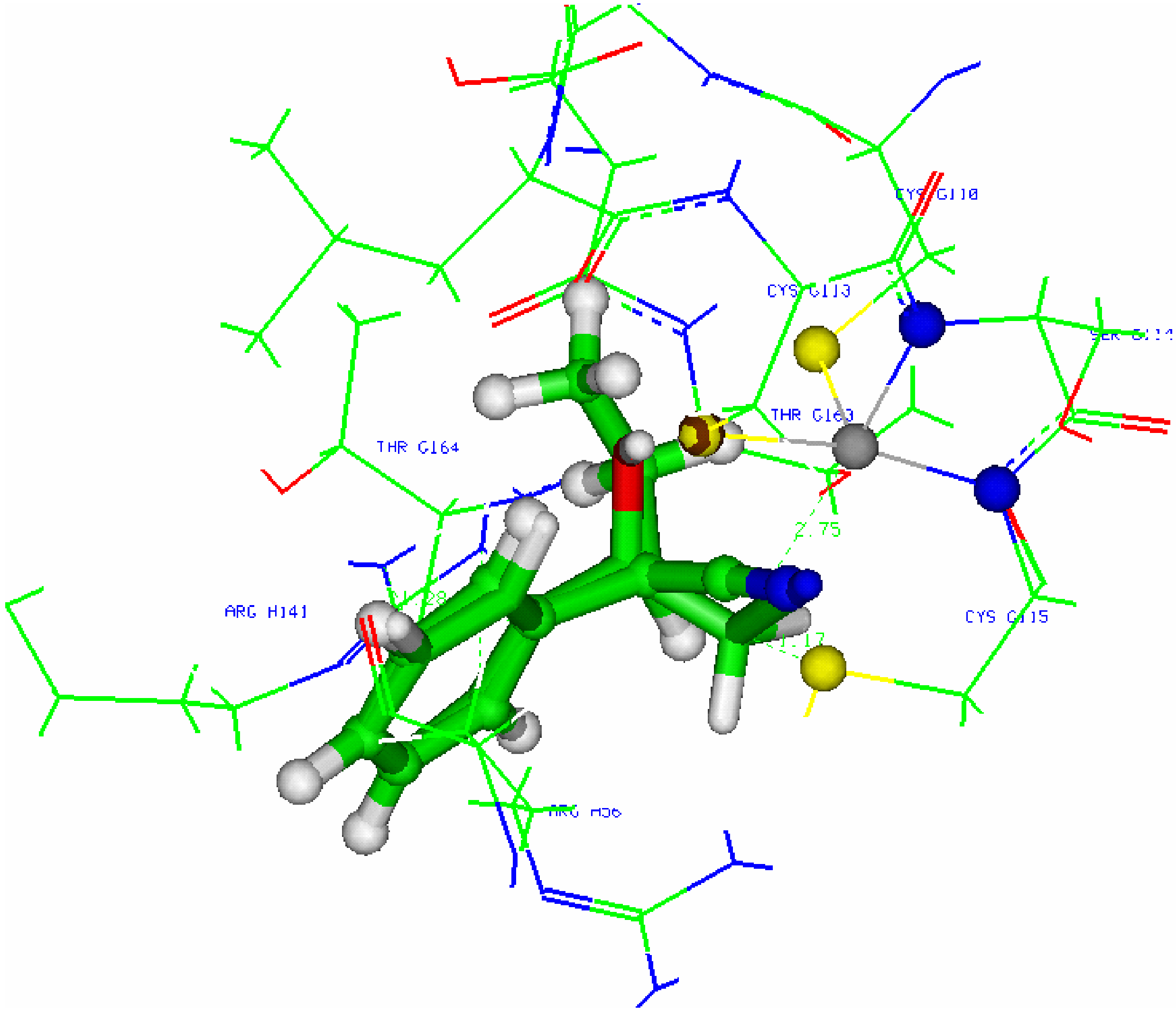


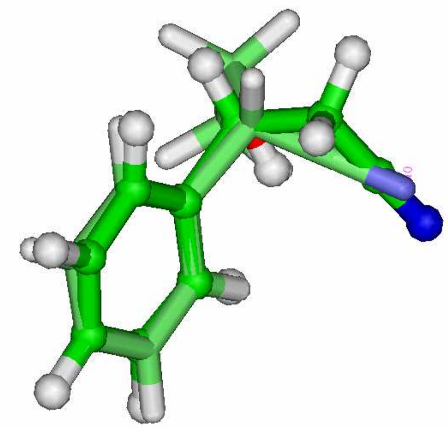
Endo et al (1999) TIBTECH 17:244-249.

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





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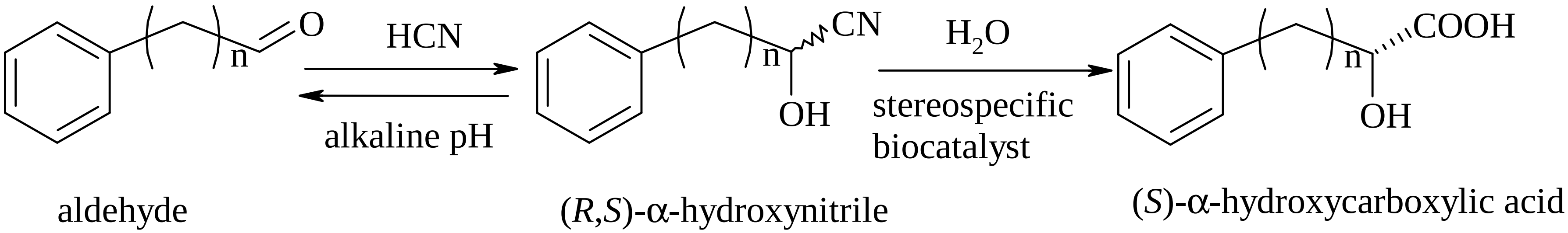
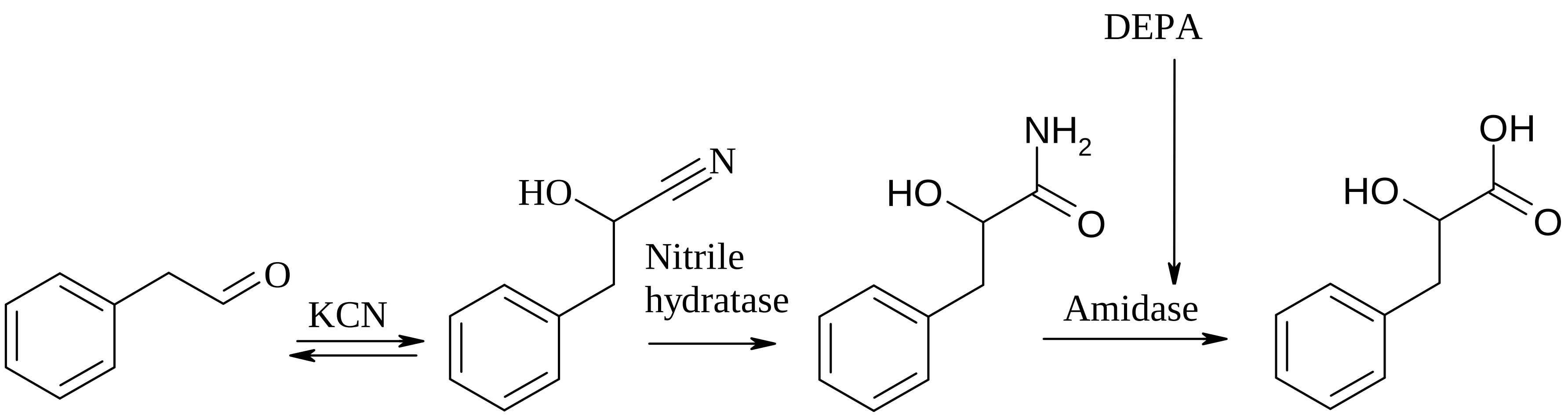
Two-step reaction



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

Multi-step transformation of phenyl-acetaldehyde to 2-hydroxy-3-phenylpropionic acid.



Phenyl-
acetaldehyde

2-Hydroxy-3-
phenyl-propionitrile

2-Hydroxy-3-
phenyl-propionamide

2-Hydroxy-3-phenyl-
propionic acid

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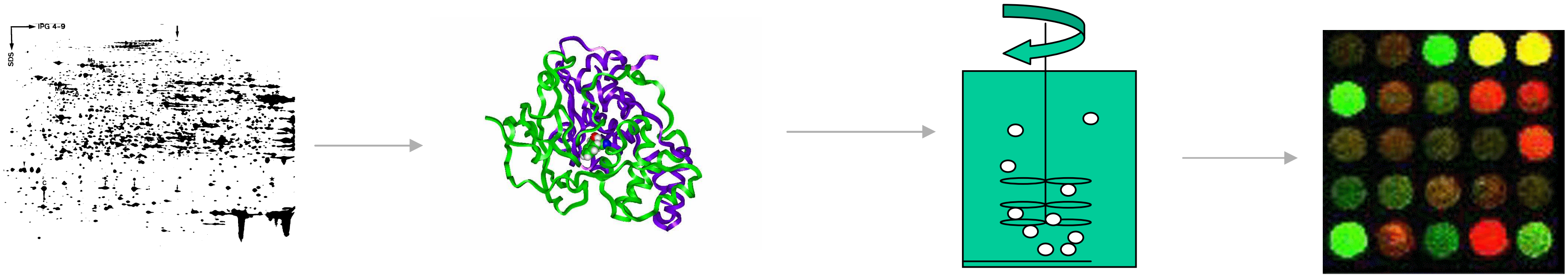
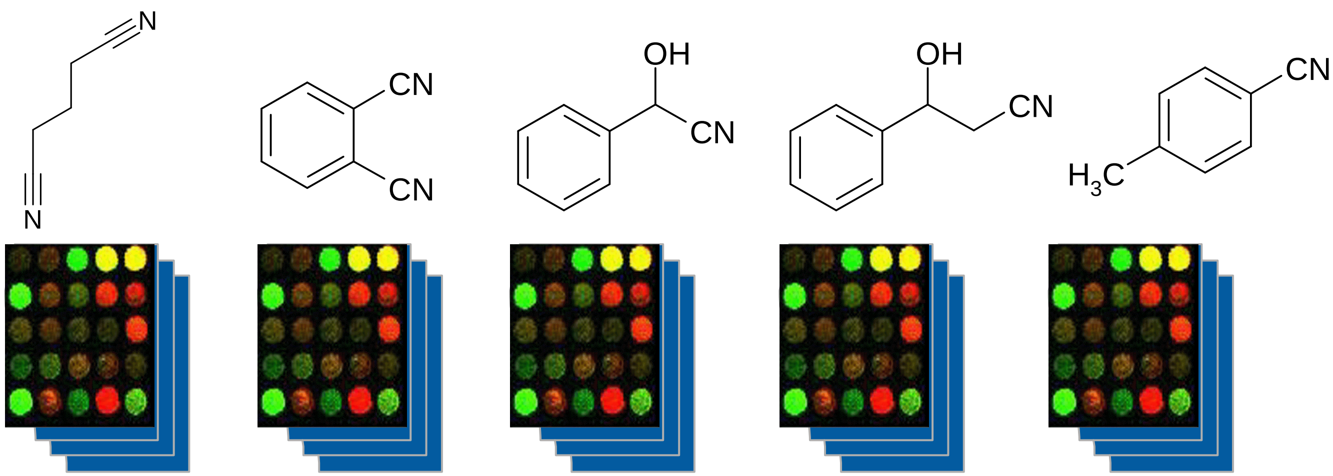
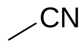
Biocatalysis tool boxes





Biocatalyst tool boxes



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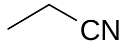

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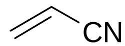

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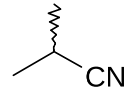

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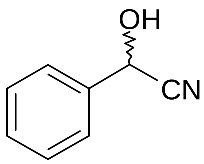

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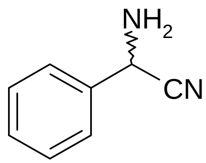

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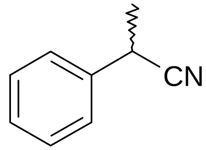

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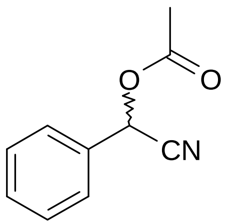

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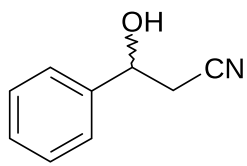

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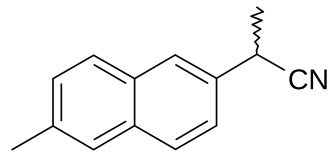

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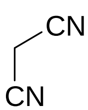

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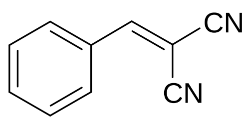

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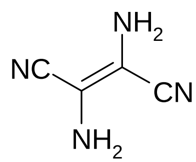

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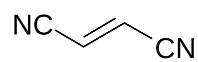

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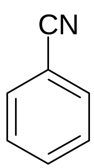

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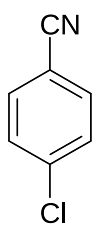

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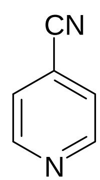

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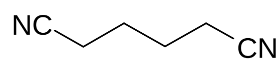

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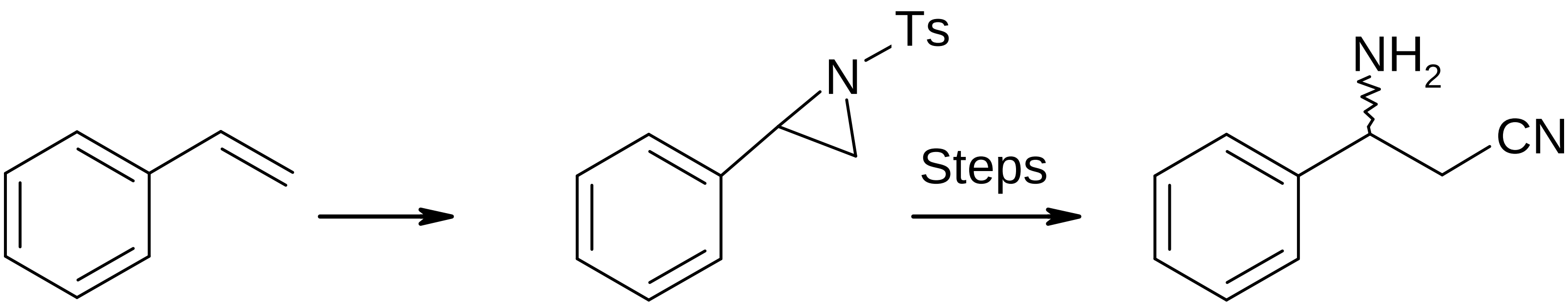
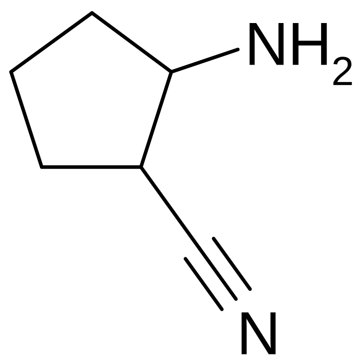
Beta-amino compounds





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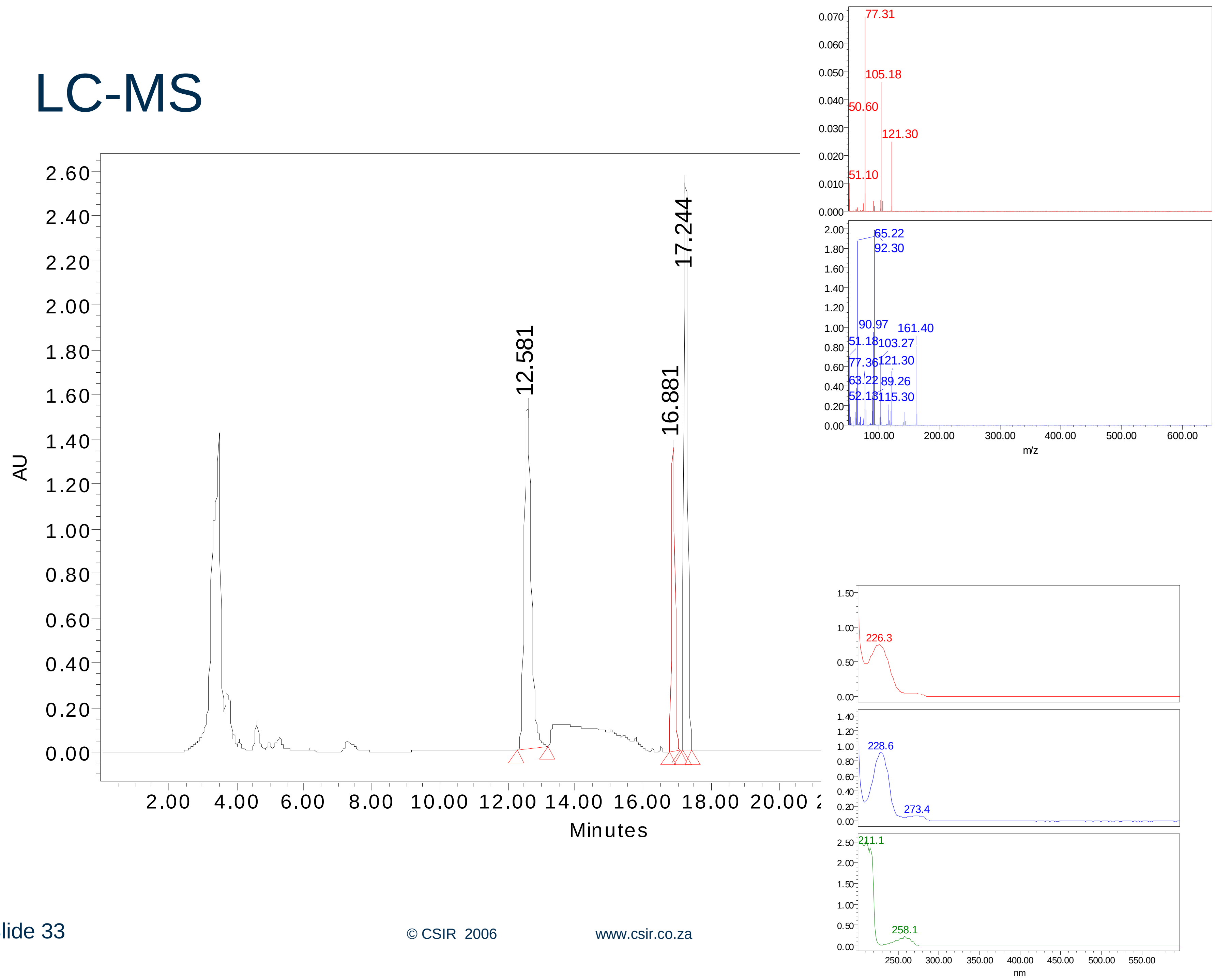
LC-MS



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Strain	**Substrate**																
	Acrylonitrile	Adipamide	Adiponitrile	Acetoxy-phenytl propinitrile	Acetoxy phenyl acetamide	α-methyl benzyl cyanide	Benzylidene malononitrile	Benzonitrile	Benzamide	4-cyanopyridine	Diamino malenitrile	Fumaronitrile	Isobutyronitrile	Malononitrile	Phenylglycinonitrile	Propionitrile	Propionamide
Aureobacterium	ND	ND	3	ND	ND	0	2	0	ND	0	ND	ND	ND	ND	4	3	ND
Alcaligenes faecalis 1	ND	ND	4	ND	ND	0	0	0	ND	ND	ND	ND	ND	ND	4	4	ND
A. faecalis ATCC 8750[11]	1	2	ND	ND	ND	ND	ND	ND	4	ND	1	1	1	0	ND	ND	3
Bacillus licheniformis 1	3	1	3	1	3	1	0	1	1	1	1	1	2	1	2	0	1
Bacillus licheniformis 2	2	1	3	1	1	0	0	0	1	0	1	0	1	1	3	3	1
Bacillus subtilis 1	4	3	3	0	4	3	0	0	4	2	0	0	3	0	4	4	4
Bacillus subtilis 2	3	2	3	2	4	0	0	0	4	0	1	0	2	1	4	4	3
Bacillus subtilis 3	1	1	3	1	1	0	0	3	1	3	1	1	1	1	2	4	1
Bacillus subtilis 4	3	3	3	0	0	0	0	1	1	2	0	0	2	0	1	3	0
Bacillus subtilis 5	2	1	4	0	0	0	0	1	2	1	0	0	2	0	4	5	0
Chryseomonas luteola	4	3	4	4	5	0	0	0	5	0	1	0	4	3	4	4	5
Microbacterium 1	4	4	4	3	1	0	2	0	3	0	1	0	4	1	4	4	5
Microbacterium 2	3	3	2	4	1	0	0	0	3	0	1	1	3	1	2	2	3
Pseudomonas diminuata	2	0	1	0	1	2	0	2	0	1	0	0		0	0	2	0
Pseudomonas alcaligenes 1	ND	ND	3	ND	ND	1	0	3	ND	1	ND	ND	ND	ND	1	3	ND
Pseudomonas alcaligenes 2	ND	ND	1	ND	ND	0	0	0	ND	0	ND	ND	ND	ND	3	3	ND
Pseudomonas alcaligenes 3	1	2	0	1	1	0	0	0	1	0	1	1	1	1	0	1	1
Pseudomonas alcaligenes 4	3	3	3	1	4	0	0	0	2	0	1	1	2	1	4	3	3

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Pseudomonas alcaligenes 2	ND	ND	1	ND	ND	0	0	0	ND	0	ND	ND	ND	ND	3	3	ND
Pseudomonas alcaligenes 3	1	2	0	1	1	0	0	0	1	0	1	1	1	1	0	1	1
Pseudomonas alcaligenes 4	3	3	3	1	4	0	0	0	2	0	1	1	2	1	4	3	3
Pseudomonas alcaligenes 5	2	1	1	1	3	1	0	1	1	0	1	1	2	1	1	2	2
Pseudomonas alcaligenes 6	1	1	3	1	1	1	0	1	0	1	1	1	1	1	1	3	1
Pseudomonas alcaligenes 7	2	ND	3	1	ND	1	0	0	ND	0	1	1	2	1	1	3	ND
Pseudomonas alcaligenes 8	ND	ND	2	ND	ND	0	0	0	ND	0	0	ND	ND	ND	2	2	ND
Pseudomonas alcaligenes 9	5	5	3	2	3	0	0	0	4	0	0	1	5	0	3	4	4
Pseudomonas alcaligenes 10	4	2	1	0	4	0	0	0	4	0	0	0	3	0	2	3	4
Pseudomonas alcaligenes 11	4	3	3	0	4	0	0	0	3	0	0	0	2	0	3	4	3
Pseudomonas alcaligenes 12	1	3	2	1	1	0	0	0	1	0	1	0	1	1	4	3	2
Pseudomonas alcaligenes 13	2	3	2	5	2	0	0	2	2	1	1	0	3	2	4	3	5
Pseudomonas alcaligenes 14	1	1	5	0	0	2	0	2	0	2	0	1	1	0	4	5	0
Pseudomonas alcaligenes 15	2	2	3	3	5	0	1	0	1	0	0	0	1	4	4	3	5
Pseudomonas alcaligenes 16	3	3	3	0	5	2	0	2	2	2	0	0	2	4	2	2	5
Pseudomonas maltophilia 1	3	3	3	0	3	2	0	2	2	2	0	0	3	0	1	3	0
Pseudomonas maltophilia 2	2	2	3	3	5	0	0	0	2	0	1	0	1	5	2	3	5
Rhodococcus 1	4	4	4	1	3	1	0	1	5	1	1	1	4	2	2	4	5
Rhodococcus 2	4	4	3	4	2	0	0	0	4	0	1	0	4	2	1	3	5
Rhodococcus 3	4	4	3	5	0	3	0	2	4	2	0	0	5	3	5	3	5
Rhodococcus 4 *(ls)*	5	5	4	3	4	0	0	1	5	1	0	0	5	4	4	5	5
Rhodococcus 5	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4	4	0
Unidentified 1	1	1	2	2	1	0	0	0	1	0	2	ND	1	1	4	2	1
Unidentified 2	1	1	2	0	0	0	0	0	1	0	0	0	1	0	1	2	1
Unidentified 3	1	1	3	4	2	0	0	0	1	1	1	0	1	3	2	2	5

Key: 5 - Exceptional; 4 - Strong; 3 – Good; 2 – Reasonable; 1 – Poor; 0 **-** no growth; ND – not determined

Species	Substrates													
	Acrylonitrile	Adipamide	Adiponitrile	Acetoxy-phenytl propinitrile	Acetoxy phenyl acetamide	Benzonitrile	Benzamide	Diamino malononitrile	Fumaronitrile	Isobutyronitrile	Malononitrile	Phenylglycinonitrile	Propionitrile	Propionamide
Candida famata	4	5	3	1	5	0	5	1	1	4	1	3	3	5
Candida guillermondii 1	5	5	3	1	5	0	5	1	1	4	1	3	3	5
Candida guillermondii 2	5	5	3	5	5	0	5	1	1	5	2	4	3	5
Candida haemulonii	5	5	3	1	5	0	5	2	2	4	5	3	3	5
Candida magnoliae 1	1	3	1	1	3	0	1	1	1	2	1	0	1	2
Candida magnoliae 2	2	2	1	1	4	0	3	0	1	4	1	1	0	4
Candida parapsilosis	5	4	3	1	5	0	5	2	2	3	3	3	3	5
Candida rugosa	2	4	3	2	4	0	4	0	1	4	2	3	3	5
Candida tenius	2	5	3	3	4	0	4	0	1	4	2	2	3	5
Candida tropicalis 1	3	2	0	1	2	0	1	1	1	3	1	0	0	3
Candida tropicalis 2	5	5	3	3	5	0	5	5	3	5	5	4	3	5
Cryptococcus humicola	5	5	3	1	5	0	5	1	1	5	5	3	3	5
Debaryomyces hansenii 1	3	3	3	1	3	0	2	1	1	1	2	3	3	2
Debaryomyces hansenii 2	5	5	3	3	5	0	4	1	1	5	2	3	3	4
Debaryomyces hansenii 3	5	4	3	2	5	0	5	1	1	5	1	3	3	5
Debaryomyces hansenii 4	5	5	3	2	5	0	5	3	3	4	3	3	3	5
Debaryomyces hansenii 5	5	5	4	4	5	0	2	1	1	5	3	3	3	5
Debaryomyces hansenii 6	3	2	2	3	5	0	5	1	1	2	3	2	3	3
Debaryomyces hansenii 7	3	4	2	1	5	0	4	1	1	3	1	2	1	4
Debaryomyces hansenii 8	4	5	3	1	5	0	5	1	1	4	1	3	2	5
Pichia guillermondii	5	4	4	3	5	0	5	1	1	5	2	3	3	5
Rhodotorula sp.	3	3	2	1	2	0	2	1	1	2	1	2	3	2
Trichosporon beigelii 1	5	5	2	1	5	0	5	1	1	5	1	3	3	5
Trichosporon beigelii 2	2	3	3	2	4	0	4	0	1	4	1	3	3	4
Trichosporon mucoides	2	5	2	4	4	0	4	0	1	5	5	3	3	5

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Table 4: NITRILE BIOCATALYSIS: Formation of carboxylic acid products

Biocatalyst	Substrate				
	O-acetoxy-phenyl-acetonitrile	**Benzo-nitrile**	**Chloro-benzo-nitrile**	**Naproxen nitrile**	**Phenyl-glycino-nitrile**
Nit 101 (commercial enzyme)	0%	55%	75%	0%	0%
Nit 105 (commercial enzyme)	0%	100%	100%	0%	0%
Nit 106 (commercial enzyme)	100%	100%	85%	0%	100%
P. alcaligenes	0%	0%	0%	0%	7.41%
R. rhodochrous Is	9.0%	100%	100%	100%	18.3%
Microbacterium	0%	0%	0%	39.8%	9.44%
A. faecalis	0%	0%	0%	2.22%	12.1%
C. tropicalis	0%	0%	6%	0%	11.3%
D. hansenii	0%	0%	0%	0%	5.0%

Comparison with other studies:

- Bacterial genera: *Rhodococcus*, *Pseudomonas,* *Bacillus* and *Alcaligenes*
- Yeast genera *Candida*, *Debaryomyces*, *Pichia*, *Rhodotorula*, *Trichosporon*
- Most nitrile biocatalysts have previously been found in these species.

Conclusion

- Brandão and co-workers (Appl. Environ. Microbiol. 69: 5754-5766, 2003) found that the nitrile hydratases produced from *Rhodococcus erythropolis* strains 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.

The Future

- Nitrile hydratase isolation and characterisation (Joni Fredericks)
- Nitrilase kinetics – new assay method (Dr Neeresh Rohitlall).
- Nitrile hdrolysing activity in yeasts (Dr Mapitso Molefe)
- Nitrilase structure and function (Prof. Trevor Sewell, UCT)
- Nitrile Biocatalysts application – biocatalytic reactions and immobilisation (COST group)

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- Godfrey Kupi
- Clement Stander
- Neil Wilde
- Dr Paul Steenkamp

TU Delft

- Dr Fred van Rantwijk
- Prof Roger Sheldon
- Dr Luuk van Laagen
- Dr Moira Bode

- **UCT**
- Prof T Sewell
- N Thuku

- **WITS**
- Prof H Dirr

The End

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