Green Chemistry: Highly Selective Biocatalytic Hydrolysis of Nitrile Compounds.

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Abstract

The application of highly substrate-specific catalysts, such as biocatalysts, can reduce the number of synthetic steps required to generate organic compounds. This results in improved efficiencies and reduced waste. For the synthesis of amides and carboxylic acids, nitrile hydrolysing biocatalysts can be used. Hence we screened for nitrile biocatalysis in microorganisms isolated in South Africa.

A wide range of bacteria and yeast cultures were enriched on nitriles as the sole source of nitrogen and evaluated for their substrate profiles. The substrates included aliphatic and aromatic nitriles, as well as structurally related amides. Small-scale liquid reactions were monitored using high pressure liquid chromatography and gas chromatography, combined with mass spectroscopy.

The microbial biocatalysts demonstrated a wide variation of activities between genera, within genera, and even within species. The range of substrates transformed and the inter- and intra-species differences in specificity of the individual biocatalysts, suggests that it is possible to provide multiple catalytic or bioremediation agents for the fine chemicals industry.

Introduction

Despite its previously poor reputation, chemistry is now leading the shift towards more benign technologies which reduce the impact of industry on the environment. This trend is reflected in the collective term, 'Green Chemistry'[1]. One of the leading technologies in this area is the use of enzymes and whole microorganisms as biological catalysts. Biocatalysis can be used in aqueous solutions at near ambient temperatures and moderate pH, thereby reducing environmental impact, minimising the cost of equipment and improving reaction safety[2]. A further benefit is that biocatalysts can synthesise complex chemicals selectively[3]. This avoids the additional reaction steps necessary to protect labile compounds during synthesis using non-selective catalysts. Wide application of biocatalysts requires that they're robust, commercially available and specific, yet sufficiently general to be applicable to most classes of compound.

Hydrolytic enzymes, which do not require co-factors and are often highly robust, have been the most successful application in synthetic catalysis to date. This applies particularly to lipases, proteases and amylases as they possess both the necessary attributes and are already widely used in the detergent, food, and beverage industries[4,5]. Following the commercial adoption of these biocatalysts, the race is on to develop and apply the next generation of biocatalysts, including the nitrile hydrolysing enzymes represented by nitrilases and nitrile hydratases.

The addition of a nitrile group to an organic compound is often relatively easy[6-9] and facilitates the subsequent addition of a carbon atom during chemical synthesis, since the nitrile group may be converted to an amine, amide, or carboxylic acid to provide the desired functional group. Traditionally, nitrile hydrolysis involves strongly alkaline conditions; subsequent neutralisation produces a polluting, salt stream. The use of strong acid or alkali is not chemospecific and can eliminate labile groups on the same molecule. Nor is it enantio- and regio-selective. Enzymes, on the other hand, can provide chemo-, regio-, or enantio-selectivity. Selective hydrolysis of the nitrile by nitrile hydratases can generate amides. Carboxylic acids can be synthesised from nitriles using either nitrilases or a combination of nitrile hydratase and amidase (Fig. 1). Moreover, selective hydrolysis of a single nitrile group in a dinitrile molecule permits the incorporation of the unreacted nitrile into the final product or further functionalisation, such as reduction to an amino group. Hence, these enzymes can be used to provide access to specific complex nitrile, amine, amide and carboxyl compounds, representing a large proportion of the functional groups found in fine chemicals and active pharmaceuticals.

Nitrile biocatalytic technology has already been implemented by Mitsubishi Rayon Corp, in the synthesis of acrylamide from acrylonitrile, and by Lonza in the synthesis of nicotinamide. Companies, such as Du Pont, Pfizer, DSM and Dow, are researching and developing additional processes. Such commercialised biocatalytic processes include L-aspartic acid, ephidrine and α-aspartame[3]. Commercial sources of nitrilases and nitrile hydratases are, however, limited[6]. Moreover, most of the enzymes isolated tend to be thermally labile, have a limited range of substrates, and are generally intolerant of high reactant concentrations. An additional problem is that the enzymes may be unstable in the organic solvents most suitable for nitrile solubility while, conversely, only low conversion rates in aqueous solutions are achievable due to the insolubility of aromatic and aliphatic nitriles in water[6]. The available enzymes generally also display low enantiomeric specificity. New sources of efficient nitrile biocatalysts are, therefore, of considerable interest. This paper reports on our investigations into the nitrile substrate range of nitrile biocatalytic bacteria and yeast.

Methods and Materials

In the present study bacteria and yeast were isolated by means of selective growth on nitrile substrates. Selected organisms were then grown on media containing other nitriles to evaluate their catalytic range. Finally, a few organisms representative of the most commonly isolated genera were cultured, nitrile degrading activity induced and the resultant whole cell biocatalysis subsequently quantitatively evaluated in bench-scale reactions. Analysis of the reaction mixtures was performed using either high pressure liquid chromatography (HPLC) or gas chromatography (GC), depending on the volatility of the compounds. In some cases the analyses were also performed with mass spectrometers in order to confirm the identity of the reactants. The synthesis of certain commercially unavailable compounds was also attempted.

Isolation of microorganisms

Approximately 150 bacterial strains and 28 different strains of yeasts were isolated from industrial and natural environmental sites in South Africa. The bacteria were enriched on a selective, defined medium at pH 7.0[10], with 10 mmol/l succinate as carbon source and a nitrile as the sole nitrogen source, usually 0.75 mmol/l 3-hydroxy-3-phenylpropionitrile.[9] Yeast were selected through growth on phenylglycinonitrile. Only nitrile hydrolysing

organisms are able to release the nitrogen required for growth from the 3-hydroxy-3-phenylpropionitrile or phenylglycinonitrile.

The taxonomy of the biocatalytic bacterial isolates was determined based on morphology (API diagnostic strips, bioMérieux, France) or on 16s rRNA sequences using BLAST. The isolate *Rhodococcus rhodochrous* 4 has been lodged with the American Type Culture Collections as *Rhodococcus rhodochrous* ATCC BAA-870.

Secondary screening of isolated cultures against different nitriles

In order to evaluate the catalytic versatility of the biocatalysts, the specificity and substrate range of the nitrile degrading cultures, isolated as described above, was determined by screening on a defined agar medium. The medium contained nitrile and amide substrates (5.0 mmol/l), namely, aromatic nitriles, aliphatic nitriles or structurally related amides (Fig. 2). Glucose (10 mmol/l) was included as a carbon source. The plates were incubated at 30°C and observed daily for growth, which was subjectively evaluated as none, poor, moderate, good or exceptional (Table 1 and 2). *Alcaligenes faecalis* ATCC (catalogue number 8750) was included in the set as a positive control.

Biomass production

Species from each of the bacterial genera represented in Table 1 and 2 were selected for further studies. These included *Pseudomonas alcaligenes, Rhodococcus rhodochrous, Alcaligenes faecalis,* and *Microbacterium* and two yeast strains, *Candida tropicalis* and *Debaromyces hansenii.* Since biomass was low when grown on defined minimal medium, a rich complex medium followed by induction in a defined medium[11] was used to produce high biomass. For this purpose, the organisms were routinely cultivated at pH 7.0 and 30°C with shaking at 200 rpm in 50 ml of rich liquid media, consisting of (in g/l): bactopeptone (5.0), yeast extract (5.0), NaCl (0.1), $FeSO_4$ (0.05), $MgSO_4.7H_2O$ (0.02) and KH_2PO_4 (0.2), glucose (50 mmol/l) and 5.0 mmol/l benzonitrile, proprionitrile or isobutyronitrile. The cultures were then harvested in the early exponential phase by centrifugation at 3000 rpm x 15 minutes (Sorval Benchtop Centrifuge), washed three times in 50 mmol/l phosphate buffer, pH 7.0 and transferred into induction medium (100 ml, pH 7.2) containing: KH_2PO_4 (2.0 g/l), NaCl (1.0 g/l), $MgSO_4.7H_2O$ (0.2 g/l), $FeSO_4.7H_2SO_4$ (0.03 g/l) and 20 mmol/l benzonitrile as inducer in 1 l flasks. The flasks were shaken at 200 rpm at 30°C for 20 hours and harvested.

All cell suspensions were washed in three volumes of 7.5 ml 50 mmol/l phosphate buffer and resuspended in 7.5 ml of buffer. From each of the suspensions, 0.75 ml aliquots were transferred to 1.5 ml Snaplock Eppendorf tubes and rapidly frozen in liquid nitrogen to be stored at –80°C for later use[12] while 2.0 ml was used for biomass determination.

Biomass determination

Two ml of cell suspension was transferred to labelled, pre-weighed Eppendorf tubes, reweighed, centrifuged at 12000 rpm for 5 minutes (Biofuge Pico Microfuge, Heraeus Instruments) and then decanted. The pellets were dried at 105°C for 48 hrs. Subsequently, samples were cooled in a desiccator and weighed again, the difference providing the dry mass of the biomass.

Biocatalyst assays

For these assays, frozen biomass was rapidly thawed at 30ºC and resuspended in buffer. A cell suspension (0.75 ml) of each organism, diluted to an absorbance of 1 (representing approximately 10 mg dry mass), in 0.75 ml buffer was added to a 1.5 ml Eppendorf tube containing 75 µl of 100 mmol/l stock substrate and 675 µl of 50 mmol/l potassium phosphate buffer at pH 7.0. The reaction mixture (total volume 1.5 ml) was incubated at the lower temperature of 30°C in an incubator shaker (200 rpm) for 1 - 2 hr. All tubes were then centrifuged at 12000 rpm (Biofuge Pico Microfuge, Heraeus Instruments) for 5 minutes. Reactions were monitored for the decrease of substrate and the formation of the structurally related carboxylic acid by means of HPLC or GC (see below).

Samples for analysis by HPLC were prepared by filtering through 0.22 µm filters (Cameo 25SS PES, Osmonics) and diluting in the mobile phase. Processing of the biocatalytic reactions for analysis by GC involved acidification of the reaction mixtures to below pH 4 to ensure subsequent extraction of the carboxylic acids into organic solvent. The mixtures were then saturated with sodium chloride and extracted four times into ethyl acetate. The organic phase extracts were pipetted out, pooled, transferred into vials, and analysed by GC. The efficiency of the extractions was evaluated using 5 mmol/l propionitrile, propionic acid, and propionamide as reference standards in the reaction matrix, with 0.75 ml of phosphate buffer in place of the enzyme solution.

High Pressure Liquid Chromatography (HPLC)

Substrates and products were routinely analysed using a Chromolith (Merck) speedrod (RP18e) C18 column, eluted isocratically by 20% acetonitrile, 80% H_2O and 0.1% trifluroacetic acid (TFA). The eluent was monitored using a Waters Alliance 2690 HPLC, Photo Diode Array detector (PDA Waters 996), and a Waters Integrity System Thermabeam Mass Detector.

Alternatively, a Phenomenex Luna C18 (2) 150 x 4.6 mm column (Separations, SA) was used for the separation and analysis of aryl aliphatic nitriles and their respective acids and amides, by means of gradient elution. The mobile phase was 95% of 16 mmol/l KH_2PO_4 buffer and 5% 80:20 (v/v) acetonitrile to tetrahydrofuran (THF). During elution the ratio was changed to 80% of organic mixture to 20% buffer. The elution of aromatic compounds was monitored at 215 nm in all HPLC methods.

Gas Liquid Chromatography (GC)

A Hewlett Packard Series II 5890 GC with a Stabilwax-DA, (30 m x 0.25 mm, 0.25 um) column (Restek Corporation, USA, supplied locally by Chromspec) and a flame ionisation detector, was used for identification and quantification of aliphatic mononitriles and their respective products. Commercial standards were purchased from Sigma-Aldrich and Fluka.

Where combined gas liquid chromatography-mass spectrometry (GCMS) was used, authentic standards, adiponitrile (108.14 g/mol mol wt), adipamide (144.18 g/mol) and adipic acid (146.14 g/mol), from Sigma-Aldrich were selected as representative analytical reference standards. The column, zebron zb-624 (Phenomenex), was chosen for its intermediate polarity status suitable for both polar and non-polar compounds. Samples

were eluted between 0 – 260°C (the temperature range for the column used). The mass detection range selected for the nitriles, amides and carboxylic acids was between 25 – 259 g/mol using helium gas as the mobile phase.

We also explored the use of near infrared (Foss 6500 NIR System), as a means of real-time analysis of the nitrile biocatalysis reactions while the method of Banerjee and coworkers[13] was assessed as a means of high throughput screening of reactions.

Preparation of (±)-3-hydroxy-3-phenylpropionitrile.

The direct enantio- and regio-selective formation of *β*-hydroxy nitriles by reaction of 1,2-epoxides with potassium cyanide in the presence of metal salts, was adapted for the preparation of (±)-3-hydroxy-3-phenylpropionitrile[14] (compound **9,** Fig. 3). The metal salts used to facilitate the ring-opening reaction of styrene oxide (compound **19**, Fig. 3) were $LiClO_4$, $Mg(ClO_4)_2$, and NH_4Cl with yields of 77%, 84% and 92%, respectively. A white suspension of (±)-styrene oxide (41.64 mmol), KCN (208.19 mmol) and NH_4Cl (91.60 mmol.) in MeOH-H_2O (71 ml : 9 ml) was heated at reflux for 21 h. The reaction mixture changed from white to a dark brown colour upon heating. The reaction mixture was cooled to room temperature and concentrated *in vacuo* to yield a dark brown residue, which was taken up in 100 ml ethyl acetate (EtOAc), and washed with 3x 100 ml distilled water. The aqueous washings were combined and extracted with EtOAc (200 ml). The organic extracts were combined, dried over $MgSO_4$, and concentrated *in vacuo* to yield dark brown oil. Purification by column chromatography on silica gel with 10–30% EtOAc-hexane mixtures yielded (±)-3-hydroxy-3-phenylpropionitrile, as determined by TLC, in the form of an orange oil (4.1267 g, 67%) (Fig. 3). This modification provides an improvement on the yield obtained by previous methods. [9,15]

Attempted preparation of N-(*p*-toluenesulfonyl)-2-phenylaziridine.

We had envisaged synthesising (±)-3-amino-3-phenylpropionitrile **22** from the nucleophilic ring-opening reaction of the aziridine with cyanide anion (Fig. 4). The aziridine was prepared from styrene **20** by the method of Sudalai and co-workers[16] using anhydrous chloramine-T. We were unable to generate **22,** however. Other research groups have also experienced difficulty in generating the aliphatic β-amino compound with traditional methods (N. Klempier, personal communication) and have therefore concentrated on cyclic β-amino nitrile compounds and protected β-amino nitrile compounds (e.g. the Tosylated intermediate **21** in Fig. 4) as substrates for nitrile hydrolysing biocatalysts.[17-19]

Results

Of the 150 bacteria isolated from the environment in the primary screen, approximately 40 morphologically distinct environmental isolates grew on 3-hydroxy-3-phenylpropionitrile and were used in the secondary screen for substrate specificity (listed in Table 1). Some nitrile biocatalysts came from the prokaryote genera *Microbacterium*, *Aureobacterium* and *Alcaligenes*, but most commonly from the genera *Rhodococci*, *Pseudomonas*, and *Bacillus* (Table 1), with the *Rhodococci* having activity against the broadest range of substrates. Most strains showed stronger growth on the nitrile substrates than the *Alcaligenes faecalis* strain ATCC 8750 included as a positive control. Yeasts came mostly from the *Candida*,

Debaryomyes, *Trichosporon* and *Pichia* genera and, amongst these, the genera *Debaryomyes* and *Candida* were particularly well represented (Table 2).

Interestingly, a few strains of bacteria showed consistent preference for aliphatic nitriles. Four strains of yeasts showed specificity for α-substituted aryl aliphatic mononitriles (chiral compounds) and some bacteria were observed to display the same substrate preference. Most organisms grew well on adiponitrile (a long chain aliphatic dinitrile); two yeast cultures and a few bacterial strains demonstrated a high specificity for this substrate.

The cultures were also screened for growth on amides. Since the nitrile hydratase system involves associated amidases, the expression of amidases in microorganisms could also suggest the presence of an associated nitrile hydratase, which are often genetically linked to an amidase.[17] Most bacterial and yeasts strains demonstrated good growth on amides. The amide specificity of a few bacterial strains was studied, demonstrating differing capacities for the hydrolysis of structurally related nitrile and amide compounds.

A comparative, quantitative biocatalytic study (Table 3) was carried out on representative organisms (see Tables 1 and 2) against commercial nitrilases available in experimental quantities from Biocatalytics Inc. USA. These experiments further demonstrated the broad substrate range of the *R. rhodochrous* 4. Hence *Rhodococci* are excellent candidates for any nitrile biocatalysis toolbox. As *Rhodococci* generally express nitrile hydratases, they can provide either carboxylic acid derivatives or amides, although the latter may require genetic or chemical disruption of the amidase activity.[9]

Discussion

The nitrile hydrolysing microorganisms reported in this study represent the same genera as nitrile biocatalysts discovered elsewhere, indicating a relatively uniform global distribution of these microbes. Among the bacteria, the genera *Rhodococcus*[12, 20-26], *Pseudomonas*[27], *Bacillus*[11] and *Alcaligenes*[28] have previously been discovered and studied. Again, as in this study, the nitrile hydrolysing yeasts associated with a Brazilian gold mine discovered by Rezende and co-workers were from the genera *Candida*, *Debaryomyces*, *Pichia*, *Rhodotorula*, *Cryptococcus*, as well as *Aureobasidium*, *Geotrichum*, *Tremella* and *Hanseniaspora*. During the liquid extraction of gold, the simplest nitrile, cyanide, is applied[29], which would select for cyanide or nitrile degrading organisms.

It should not be concluded, however, that because of this global similarity, it is of no value to source organisms from different geographical locations. Brandão and co-workers[20] found that the nitrile hydratases produced from *Rhodococcus* strains isolated from around the world had intra-species amino acid sequence differences, which provided an explanation for the variability of nitrile substrate usage within the species. Since small changes in amino acid sequence can significantly change the activity of an enzyme[30] this implies that unique biocatalysts may be found at any locality.

The use of a rich medium allowed us to increase the biomass to approximately 28 g/l levels, similar to those reported for the wild type organism used by Mitsubishi Rayon in the acrylamide process. However, increased biomass was achieved at the expense of specific activity.

We used LC and GC (both with and without linked MS) to determine conversion of the aromatic and aliphatic compounds, respectively. These methods were specific, reliable, and quantitative. Colorimetric methods were not so successful. The pH indicator method, which follows the decrease in pH caused by organisms releasing carboxylic acids from nitriles and amides, was insensitive and prone to high background readings, especially in the yeasts which tend to generate metabolic acids. In the hope of achieving a real-time kinetic assay in which the conversion of substrate can be observed during the progress of the reaction, we explored the use of near infrared spectroscopy, but unfortunately the substrate and product signals were obscured by those of the aqueous medium at the concentrations of the reactions. Dadd and co-workers[31] used the mid-infrared range to monitor nitrile biocatalysis, and found that the technique was only applicable at high substrate and product concentrations. Hence we are currently exploring other spectroscopic analytical methods as well as investigating a novel colorimetric method utilizing an enzymatically activated intermediate.

It was demonstrated in the present study that *Rhodococcus rhodochrous* 4 has a broad nitrile degrading profile (Table 3). The capacity of the biocatalyst to transform α-substituted compounds was confirmed with isobutryonitrile, but the structurally related α-methylbenylcyanide was not a good substrate for growth, and was only weakly converted in this and a previous study[9]. Phenylglycinonitrile was converted to phenylglycine, although some of the substrate spontaneously hydrolysed to benzaldehyde, ammonia and cyanide. The aliphatic dinitriles, adiponitrile and malononitrile, were both converted, but fumaronitrile, benzylidenemalononitrile, and diaminomaleonitrile were not.

An interesting observation was the conversion of *O*-acetoxyphenylacetonitrile. Similar to the biocatalytic capacity for mono-nitrile hydrolysis of dinitriles that we and others have shown, this demonstrates the ability of enzymes to specifically transform functional groups in the presence of other labile groups. In accordance with the results of Stolz *et al*[32], the conversion of naproxen nitrile was achieved by a *Rhodococcus* sp., yielding the non-steroidal anti-inflammatory drug, naproxen.

Of all the substrates, benzylidenemalononitrile appears to have been the least easily converted by the bacteria, and the yeasts did not appear to grow on benzonitrile. The structurally related amides were generally easier to transform than the nitriles.

Conclusions

These results highlighted the substrate specificity of the nitrile biocatalysts and emphasized the need for a multi-component toolbox of nitrile biocatalysts to provide a broad range of solutions to synthetic problems[9,33].

Biocatalysts are typically selective, allowing for specific chemical transformations without the need for wasteful use of protective groups and circuitous synthetic routes. Future work will involve further evaluation of the chemical- and enantioselectivity of the yeast cultures for specific substrates, and purification of the enzymes from *Rhodococcus rhodochrous* 4 in order to characterise the activities of the isolated enzymes. Discovery of enantioselective nitrile hydratases would be of immense interest as these enzymes, unlike the amidases and nitrilases, are typically non-enantioselective[2]

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Figure Legends

Figure 1: Nitrile hydrolysis: chemical and biocatalytic pathways via nitrilase or the sequential action of nitrile hydratase/amidase systems.

Figure 2: Nitriles used in this study: acetonitrile (1), proprionitrile (2), acrylonitrile (3), isobutyronitrile (4), mandelonitrile (5), 2-phenylglycinonitrile (6), α-methyl benzyl cyanide (7), *O*-acetylphenyl acetonitrile (8), 3-hydroxy-3-phenylpropionitrile (9), naproxen nitrile (10), malononitrile (11), benzylidene malononitrile (12), diaminomaleonitrile (13), fumaronitrile (14), benzonitrile (15), 4-chlorobenzonitrile (16), 4-cyanopyridine (17) adiponitrile (18).

Figure 3: Synthesis of 3-hydroxy-3-phenylpropionitrile

Figure 4: The proposed synthetic route for of 3-amino-3-phenylpropionitrile.

Table Headings

Table 1: Growth of bacterial cultures on various nitriles. Subjective growth ratings were: 5 - Exceptional; 4 - Strong; 3 – Good; 2 – Moderate; 1 – Poor; 0 - no growth; ND – not determined.

Table 2: Growth of yeast cultures on various nitriles. Subjective growth ratings were: 5 - Exceptional; 4 - Strong; 3 – Good; 2 – Moderate; 1 – Poor; 0 - no growth; ND – not determined.

Table 3: Comparative activity profile of microorganims with purified nitrilases as determined by substrate conversion.

Table 1:

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	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
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 sp. 1	4	4	4	1	3	1	0	1	5	1	1	1	4	2	2	4	5
Rhodococcus sp. 2	4	4	3	4	2	0	0	0	4	0	1	0	4	2	1	3	5
Rhodococcus sp. 3	4	4	3	5	0	3	0	2	4	2	0	0	5	3	5	3	5
Rhodococcus sp. 4	5	5	4	3	4	0	0	1	5	1	0	0	5	4	4	5	5
Rhodococcus sp. 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

Table 2:

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
Trichosporon sp. 1	5	5	3	1	5	0	5	1	1	5	1	3	3	5
Trichosporon sp. 2	4	4	3	2	4	0	4	1	1	5	1	2	3	4
Unidentified	4	4	3	1	5	0	4	1	1	2	1	2	3	3

Table 3:

Biocatalyst	**Substrate Conversion to the Carboxylic Product**					
	O-acetoxy-phenyl-aceto-nitrile	**3-hydroxy-3-phenyl propionitrile**	**Benzonitrile**	**4-Chloro-benzo-nitrile**	**Naproxen nitrile**	**Phenyl-glycino-nitrile**
Nit 101 (enzyme)	0%	0%	55%	75%	0%	0%
Nit 105 (enzyme)	0%	0%	100%	100%	0%	0%
Nit 106 (enzyme)	100%	0%	100%	85%	0%	100%
P. alcaligenes 1	0%	ND	0%	0%	0%	7.41%
R. rhodochrous 4	9.0%	100%	100%	100%	100%	18.3%
Microbacterium 1	0%	ND	0%	0%	39.8%	9.44%
A. faecalis 1	0%	ND	0%	0%	2.22%	12.1%
C. tropicalis 2	0%	ND	0%	0%	0%	11.3%
D. hansenii 5	0%	ND	0%	0%	0%	5.0%

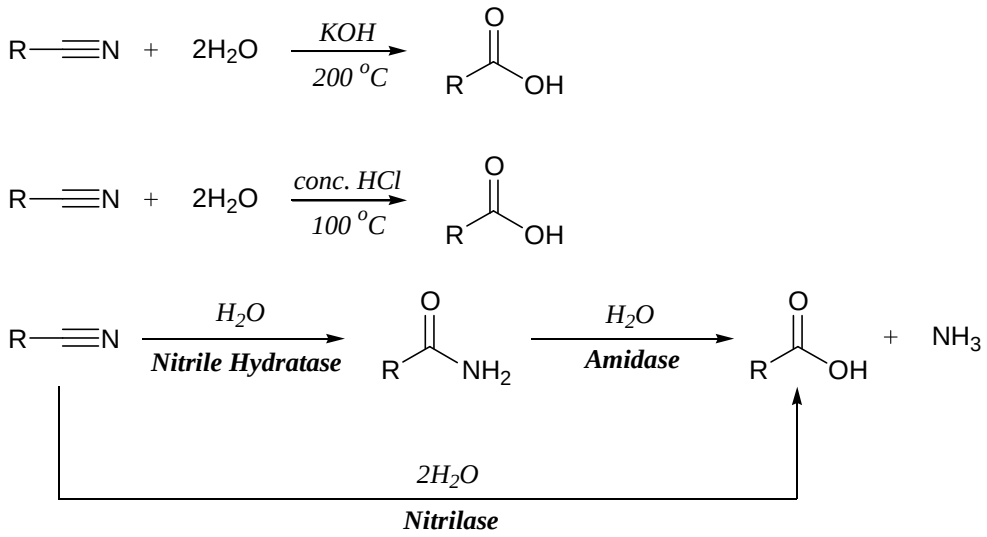
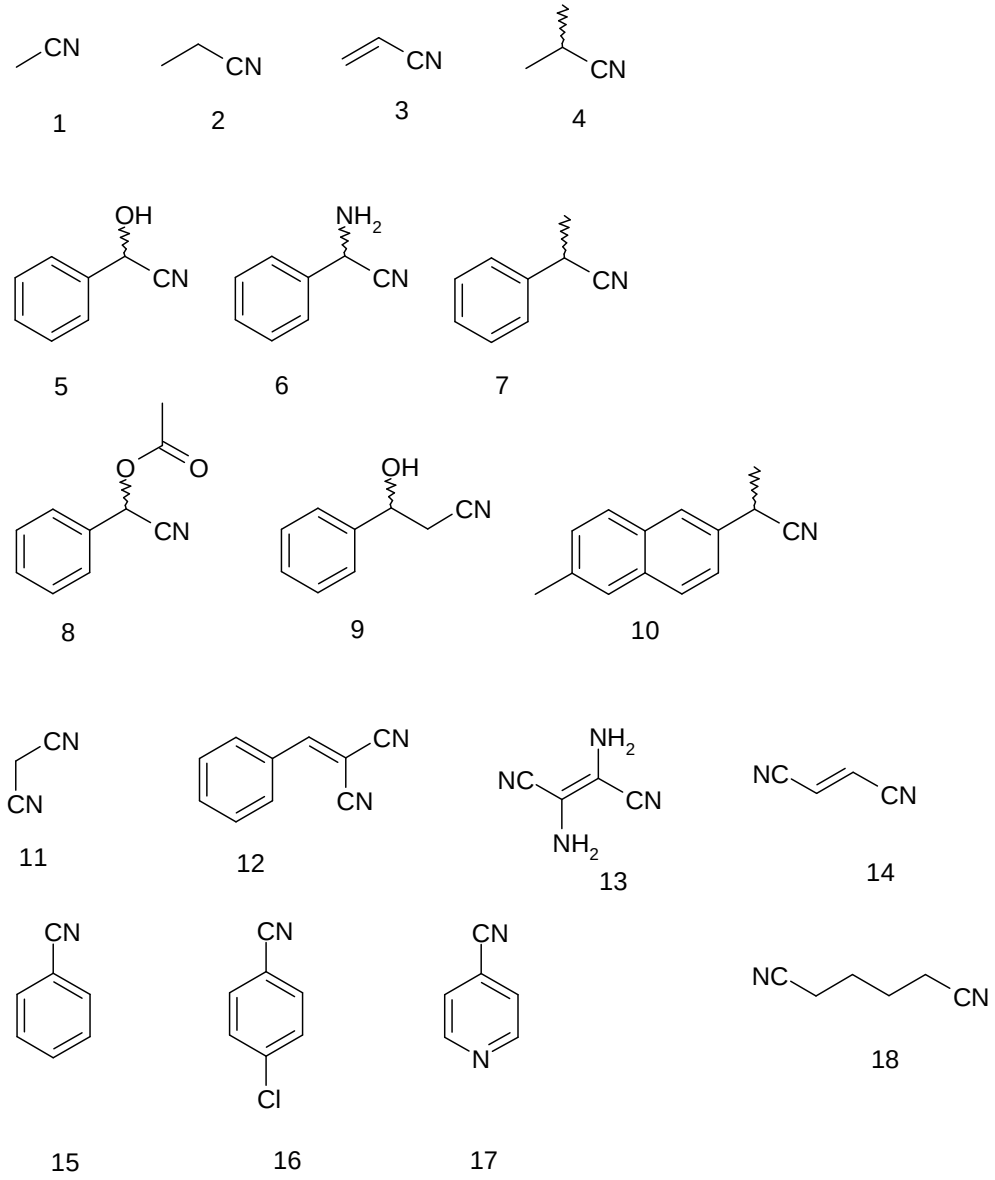

R—≡N + 2H2O
KOH
200 °C
O
R
OH
R—≡N + 2H2O
conc. HCl
100 °C
O
R
OH
R—≡N
H2O
Nitrile Hydratase
O
R
NH2
H2O
Amidase
O
R
OH
+ NH3
2H2O
Nitrilase

Figure 1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CN
2
CN
3
CN
4
OH
CN
5
NH2
CN
6
CN
7
O
O
CN
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CN
9
CN
10
CN
CN
11
CN
CN
12
NH2
NC
CN
NH2
13
NC
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14
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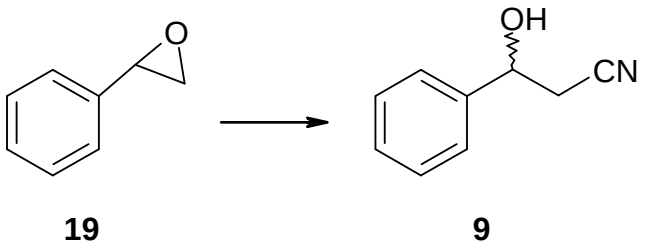
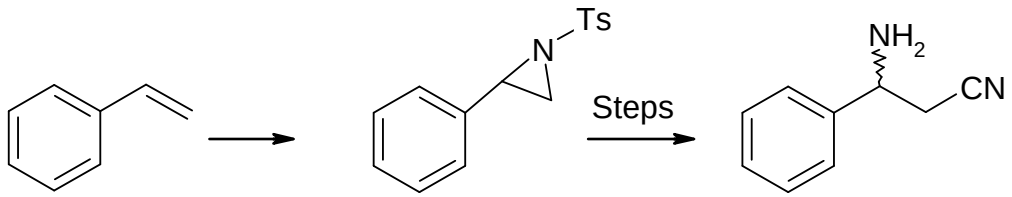
Cl
16
CN
N
17
NC
CN
18

Figure 2.



19 **9**

Figure 3



20 **21** **22**

Figure 4