Chiral Biotransformations Of Cyclic Nitrile Compounds

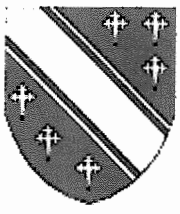
A Thesis for the Degree of Doctor of Philosophy

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SCIENCE LI
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I dedicate this thesis to the memory of my father Derek

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Abbreviations

Å	Angstroms
a.a.	Amino acid
ATR	Attenuated Total Reflection
Bp	Base pairs
° C	Degrees centigrade
^{13}C	Carbon 13 radioactive labelling
CO_2H/COOH	Acid group
CCE	Chiral Capillary Electrophoresis
CTAB	Hexadecyltrimethyl ammonium bromide
Da	Daltons
dd H_2O	Deionised water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylene-diamine-tetra-acetic acid
e.e	Enantiomeric excess
FTIR	Fourier Transform Infrared Spectroscopy
g	Grams
g	Acceleration of gravity
GC	Gas Chromatography
GCMS	Gas Chromatography/Mass Spectroscopy
h	Hours

Chiral biotransformations of cyclic nitrile compounds: abbreviations

^1H	Hydrogen radioactive labelling
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HPLC	High pressure liquid Chromatography
IR	Infrared
Kda	Kilo Daltons
kg	Kilogram
K_m	Michaelis constant
l	Litres
LB	Luria-Bertani broth
LCMS	Liquid Chromatography/Mass Spectroscopy
M9	Minimal media
MHz	Mega hertz
min	Minutes
M/Mm	Molar/milliMolar
ml	Millilitres
mm	Millimetres
mRNA	Messenger ribonucleic acid
N	Molar
NCIMB	National Collections of Industrial and Marine Bacteria
NB	Nutrient broth
Nhase, N/H,	Nitrile hydratase
Nit	Nitrilase
NMR	Nuclear Magnetic Resonance Spectroscopy
NSAID	Non steroidal anti-inflammatory drugs

OD	Optical density
Ph	Phenyl
PCR	Polymerase Chain Reaction
ppm	Parts per million (mg/l)
R_f	Retardation factor
RNase	Ribonuclease
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TBE	Tris borate EDTA buffer
TE	Tris EDTA
TES	Tris EDTA saline
Tm	DNA melting temperature
TLC	Thin layer Chromatography
TSB	Tripticase soy broth
UTP	Uracil triphosphate
µl	Microlitres
µm	Micrometers
UV	Ultraviolet
V_{max}	Maximum velocity
v/v	Volume for volume
w/w	Weight for weight

Chiral biotransformations of cyclic nitrile compounds: abbreviations

Enzymes

Nitrile hydratase	EC	4.2.1.84
Amidase	EC	3.5.1.4
Nitrilase	EC	3.5.5.1

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Abstract

The aim of this thesis was to improve the understanding of how bacteria (in particular *Rhodococcus rhodochrous* LL100-21) use enzymatic reactions to biotransform nitrile compounds. Particular emphasis was given to some compounds of potential interest to the pharmaceutical industry.

Due to the difficulty in selectively converting one cyano group of a polynitrile by conventional chemical techniques, regioselectivity of *Rhodococcus rhodochrous* LL100-21 was investigated using (cyanomethyl) benzonitrile compounds. Depending on the induction/growth conditions and the position of ring substitution of the substrate, different products were obtained following the biotransformation. For example, bacteria grown/induced on propionitrile or benzonitrile converted the substrate 2-(cyanomethyl) benzonitrile to 2-(cyanophenyl) acetic acid- with a 64 % yield compared to the starting substrate. Conversely, bacteria grown on acetonitrile converted the same substrate to a mixture of amide intermediates but at very low yield. Furthermore, the aliphatic cyano group of 2-(cyanomethyl) benzonitrile was preferentially hydrolysed. In the case of 3- and 4-(cyanomethyl) benzonitrile, the aromatic cyano group was converted to the acid thereby suggesting ring substitution was an important factor in the biotransformation.

Initial rate studies were undertaken in order to greater understand the biotransformations. The results suggested that steric hindrance influenced the reaction. Furthermore, when there was a cyano or bromo group in the *ortho* position of the aromatic ring it was found that there was an in increase in the initial rate of hydrolysis, suggesting these groups also affect the enzymatic reaction.

In addition, PCR and Southern blot techniques were used to investigate the presence of nitrile hydrolysing enzymes in a range of bacteria.

Mid infrared spectroscopy was carried out to study the enzyme kinetics involved in nitrile biotransformations permitting, for the first time, nitrile biotransformations to be monitored in real-time. This opens up React IR MP as a useful technique for studying a wide range of biocatalytic processes in real-time.

Bioconversion of prochiral nitriles was also undertaken using *Rhodococcus rhodochrous* LL100-21. The results suggested that the bacterium could biotransform these nitriles and, with further chemical rearrangement, could yield novel compounds of interest to the pharmaceutical industry.

Finally, the biotransformation of benzonitrile was undertaken in the presence of hydroxylamine (NH_2OH), which is more nucleophilic than water. Interestingly, when bacteria were grown on benzonitrile (to induce the nitrilase enzyme) the hydroxylamine competed with the water and the novel product, benzohydroxamic acid, was formed. Furthermore, the abiotic control afforded another product benzamide oxime.

Publications

1: Dadd, M., Claridge, T., Walton, R., Pettman, A. & Knowles, C. (1999). Biotransformation of the dinitrile (cyanomethyl) benzonitrile by the soil bacterium *Rhodococcus rhodochrous* LL100-21. *International Union of Microbiological Societies, IXth International Congress Of Bacteriology and Applied Microbiology, Sydney, Australia.* , Page 51.

2: Dadd, M., Claridge, T., Walton, R., Pettman, A. & Knowles, C. (2000). Regioselective biotransformation of the dinitrile compounds 2-, 3- and 4-(cyanomethyl) benzonitrile by the soil bacterium *Rhodococcus rhodochrous* LL100-21. *Accepted by Enzyme and Microbial Technology,* November, 2000.

3: Dadd, M., Sharp, D., Pettman, A. & Knowles, C. (2000). Real-time monitoring of nitrile biotransformations by mid-infrared spectroscopy. *Journal of Microbiological Methods* **41**, 69-75.

4: Dadd, M, Claridge, T, Pettman, A & Knowles, C. (2000). *Rhodococcus rhodochrous* LL100-21 catalyzes the biotransformation of benzonitrile in the presence of hydroxylamine to produce benzohydroxamic acid. *Accepted by Biotechnology Letters, November, 2000.*

Chapter 1: Introduction to enzymatic biotransformations

1.0 General introduction

This thesis concerns the regioselective, enantioselective and stereoselective biotransformations of nitrile compounds. The introduction covers nitrile biotransformations and includes chemical and biological nitrile hydrolysis, nitrile hydrolysing enzymes, and the regio-/stereoselective capabilities of the nitrile hydrolysing enzymes. Mid infrared spectroscopy is also discussed as it was found to be a useful technique for the real-time monitoring of nitrile biotransformations. In this study the technique was used to follow enzymatic hydrolysis of mononitriles, but it has many other envisaged biotechnological applications such as the monitoring of stereoselective biotransformations (for stereo-control of reactions), regioselectivity and secondary metabolites.

1.1 Introduction to nitrile groups

Nitriles are organic compounds whose common feature is a carbon nitrogen (C≡N) triple bond as a functional group. Characteristics of this type of bond include a high electron density that is extremely polar due to the electronegativity of the nitrogen. A high dipole moment enables intermolecular association and consequently a higher than predicted boiling point. Furthermore, carbon nitrogen triple bonds confer a high dielectric constant that explains the varied solubility of simple nitriles in water (Organic Chemistry, Morrison and Boyd, 6th Edition, 1992).

1.2 Chemical synthesis of nitriles

Nitriles can be synthesised chemically in a number of different ways. For aliphatic nitriles the most common method of synthesis uses alkyl chloride and sodium cyanide in dimethylsulfoxide (DMSO) at 140-150°C (Organic Chemistry, Morrison and Boyd, 6th Edition, 1992)

Aromatic nitriles on the other hand, can be synthesised chemically using an aromatic amine, sodium nitrite (in aqueous HCl) and copper cyanide (0-5°C). Other methods available for the production of aromatic nitriles are the Strecker (Strecker, 1850) and the Sandmeyer reactions (Sandmeyer, 1884). In plants and microorganisms nitriles are derived from amino acids (Thimann and Mahadevan, 1964a). In such cases the carbon nitrogen triple bond can be hydrolysed in either acidic or basic conditions to yield an amide or carboxylic acid and ammonia.

1.2.1 Acid catalysed nitrile hydrolysis

Acid catalysed nitrile hydrolysis occurs firstly by protonation of the nitrile nitrogen followed by nucleophilic addition of water to yield a sp^2 hybridised hydroxy imine. Rearrangement of this intermediate gives the amide; further hydrolysis produces the acid.

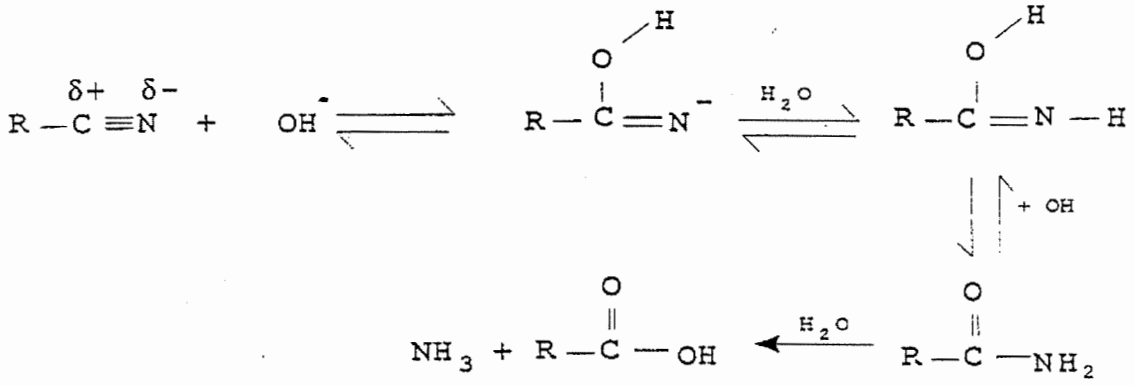


Figure 1.1: Reaction mechanism for alkaline catalysed nitrile hydrolysis

1.2.2 Alkaline catalysed nitrile hydrolysis

The mechanism of alkaline hydrolysis involves nucleophilic addition of a hydroxy anion to give the hydroxy imine as an intermediate that is converted to the amide. Under severe conditions (KOH, 200°C) there is rapid hydrolysis to the acid.

To reduce the demand for energy and to increase the reaction rate, metal ions have been used as catalysts. The products (amides or acids) can be used directly or as precursors of many industrial products, for example non-steroidal inflammatory drugs (NSAID) (Organic Chemistry, Morrison and Boyd, 6th Edition, 1992). The reaction mechanism is shown in figure 1.1

1.3 Enzymes (general)

Enzymes are proteins that act as highly efficient and specific biological catalysts. Rates of conversion of substrate to product are faster than non-enzymatic reactions even when a low concentration of enzyme is used.

In comparison to chemical catalysis reaction conditions of those carried out by enzymes are mild. Lowering of the activation energy by the enzyme enables the reaction to be carried out at pH 5-8, 20-40 °C and at atmospheric pressure rather than the high pH's and pressures required for chemical catalysis. Furthermore, application of enzymes reduces side products that result from decomposition, racemisation` and rearrangement.

1.3.1 Chemospecificity and stereospecificity

Enzymes normally catalyse only one type of reaction, on a well-defined group of substrates that are chemically and structurally related, often enabling good regio and/or chemoselectivity. The three dimensional and asymmetric environments can enable an enzyme to display selectivity with respect to different stereochemical configurations of the substrate. Therefore, enzymes are capable of resolving racemates by transforming exclusively or preferentially one of the available enantiomers. This is called kinetic resolution and in an ideal situation would yield 50% of one isomer product from a racemate.

Another use for enzymes is asymmetric synthesis, which results in products with chiral centres. This occurs when an enzyme recognises the spatial orientation of only one pair of identical substituents in the prochiral substrate. The reaction then proceeds stereospecifically giving one enantiomer (Fischer, 1898).

1.3.2 Regiospecificity

Certain residues are needed to keep the active site of enzymes in a three dimensional conformation therefore, enabling it to display great regioselectivity with respect to the position on the substrate where the reaction takes place.

The substrate is 'guided' into the correct orientation within the active site by additional amino acid residues which are found at the boundary of the active site itself (reviewed by Bashir *et al.*, 1995; Sweers and Wong, 1986).

1.4 Introduction to nitrile hydrolysing enzymes

Enzyme-catalysed hydrolysis of nitriles has been shown to proceed by two distinct routes. In the first of these a nitrilase enzyme (Harper, 1976; Harper, 1977; Kobayashi and Shimizu, 1994) directly hydrolyses the nitrile to the corresponding carboxylic acid and ammonia, using two molecules of water to catalyse the reaction. Alternatively, a nitrile hydratase hydrates the nitrile to the corresponding amide followed by the activity of an amidase, converting the amide to the acid and ammonia, using one molecule of water for each step. The first molecule in each case is added to the C≡N triple bond (Asano *et al.*, 1982; Asano *et al.*, 1980). These mechanisms can be seen in Figure 1.2.

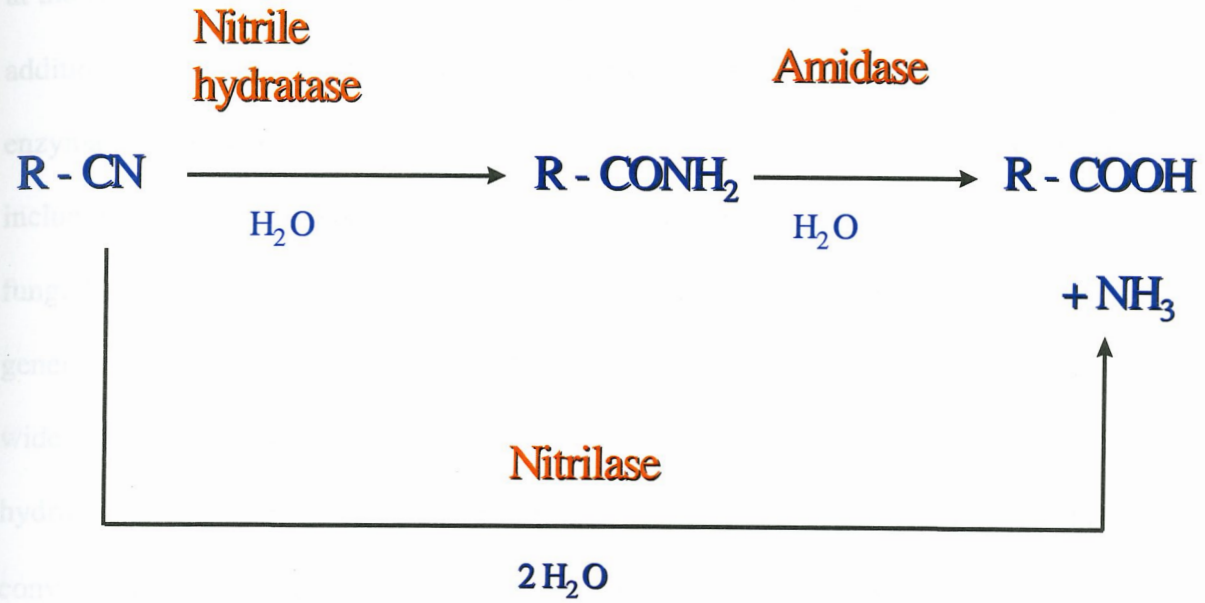


Figure 1.2: Mechanism for the hydrolysis of nitriles showing the two distinct enzymatic pathways involved. These are the one step nitrilase pathway which hydrates the nitrile directly to the corresponding carboxylic acid and the two step pathway which firstly hydrates the nitrile to the corresponding amide via a nitrile hydratase and then to the carboxylic acid via an amidase enzyme.

1.5 Nitrile hydratase enzymes

Nitrile hydratases are soluble bacterial metalloenzymes that catalyse the hydration of nitriles to amides (Nagasawa and Yamada, 1989). They contain a non-heme iron (Sugiura *et al.*, 1987) or a non-corrinoid cobalt (Brenann *et al.*, 1996) atom at the catalytic site and compose of two subunits (α and β) (Kobayashi *et al*, 1991) in addition to the metal ion. Most microorganisms isolated to date that have this enzymatic activity belong to the order *Actinomycetales*. However, many other bacteria including *Micrococcus* spp. *Pseudomonas*, *Bacillus* spp. *Agrobacterium* and some fungi (*Ascomycetes*) can grow on acetonitrile as a sole source of nitrogen. These genera are usually found in soil where the hydrolytic enzymes enable them to utilise a wide range of naturally occurring polymers and metabolites (Bunch, 1998). Nitrile hydrolysing enzymes are rare in plants and most literature has focused on the conversion of β-cyanoalanine to its amide derivative asparagine (Castric *et al.*, 1972).

The first observation of nitrile hydratase was by Mimura *et al.*, (1969) who showed that, during growth of *Corynebacterium nitrophilus* on acetonitrile-containing medium, a temporary accumulation of an amide was observed. Nitrile hydratase was further described in studies of microbial degradation of toxic cyano-group-containing compound such as acrylonitrile and acetonitrile which are utilised in the chemical industry (Asano *et al.*, 1980).

Industrial acrylamide production has been performed with nitrile hydratase from *Rhodococcus* sp. N 774 as the first generation catalyst, then with nitrile hydratase from *Pseudomonas chlororaphis* B23, currently it is being produced from *Rhodococcus rhodochrous* J1 (Kobayashi *et al.*, 1992a).

The former two of these enzymes contain iron as a co-factor whereas the latter contains cobalt. This manufacturing process is the first successful example of a bioconversion process for the production of commodity chemicals. Other studies have been carried out using *Nocardia rhodochrous* LL100-21 (Digeronimo and Antonie, 1976).

Early studies on nitrile hydratase suggested it contains a putative pyrrolquinnoline quinone PQQ as carbonyl cofactor with a non-heme iron as the prosthetic group. The hydration stage is believed to involve complexation of the nitrile nitrogen to the transition metal followed by hydration mediated by PQQ. However, other research groups who have worked in this area have doubted the presence of PQQ as a co-factor (Brennan *et al.*, 1994).

The nitrile hydratases from *Rhodococcus* R312 and *P. chlororaphis* were the first non-heme iron enzymes that have been demonstrated to contain a low spin ferric active site (Sugiura *et al.*, 1987 and 1988). The active ferric centre of the enzyme had been proposed to contain an unusual octahedral ligand of two cysteine thiolates, a hydroxide, and three N-donor ligands (derived from histidine imidazoles) (Jin *et al.*, 1993, Doan *et al.*, 1996). Three-dimensional analysis of this enzyme revealed that the former ligands are the cysteine in the Cys-X-Y-Cys-Z-Cys motif (Komeda *et al.*, 1996).

The nitrile hydratases from *Rhodococcus* N-774 (Nakajima *et al.*, 1987, Ikehata *et al.*, 1989) and *Rhodococcus* R312 (Mayaux *et al.*, 1990), have identical amino acid sequences to the *Rhodococcus* N-771 nitrile hydratase (Noguchi *et al.*, 1996, Honda *et al.*, 1992a).

These nitrile hydratases show unique reactivity to light. Inactive nitrile hydratase is activated by light irradiation whereas absence of light results in the transformation of the active form to the inactive form (see figure 1.3) upon aerobic incubation (Nagamune *et al.*, 1990a and 1990b).

Subsequent studies showed the chromophore responsible for the photoactivation is an iron complex (Honda *et al.*, 1994) on the α subunit, with light irradiation inducing a conformational change of the subunit (Tsujimura *et al.*, 1996). An endogenous NO is bound to the non-heme iron in the inactive nitrile hydratase and its photodissociation activates the enzyme. The reassociation of exogenous NO with the iron atom on the other hand, inactivates the enzyme (Odaka *et al.*, 1997 and 1996, Noguchi *et al.*, 1995).

The crystal structure of nitrile hydratase has been deduced and has been found to contain a 'claw setting' of oxygen atoms which interact with the NO. This feature gives the Fe-type nitrile hydratases their photoreactivity (Nagashima *et al.*, 1998).

In the inactive enzyme, nitric oxide (produced by NO synthase in the cell) is bound to the non-heme ferric iron at the catalytic centre, itself stabilised by another claw-like structure formed by two post-transitionally modified cysteines and a serine. The inactive nitrile hydratase is activated by the photo-induced release of the nitric oxide (Murakami *et al.*, 2000; Endo *et al.*, 1999). The proposed molecular mechanism can be seen in figure 1.3 and examples of microorganisms that possess nitrile hydratase activity are described in table 1.1.

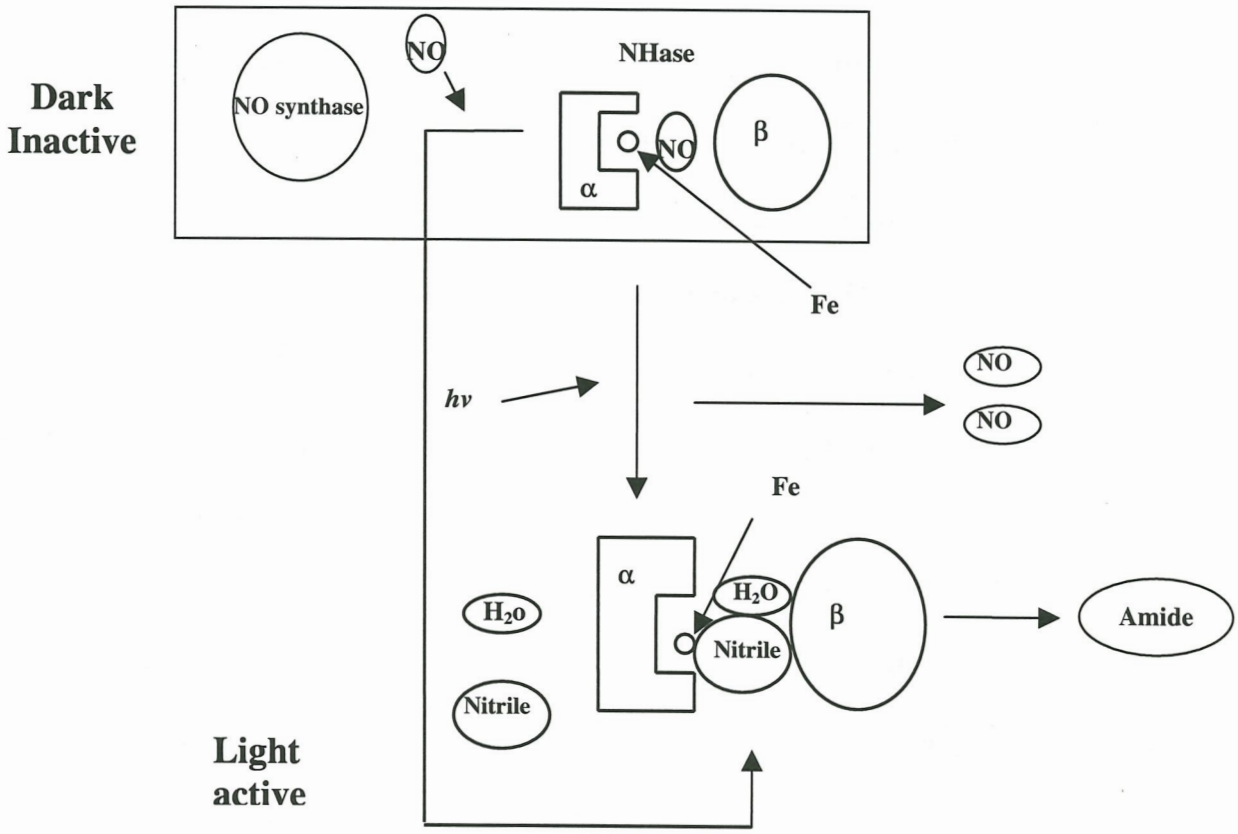


Figure 1.3: Proposed molecular mechanism for the inactivation and activation of the nitrile hydratase enzyme. Photodissociation and association of nitric oxide, which is produced by NO synthase regulates the activity of the enzyme.

Table 1.1: Microorganisms reported to possess nitrile hydratase activity.

Microorganism	Reference
Agrobacterium sp.	(Martikova *et al.*, 1995)
Agrobacterium tumefaciens D3	(Bauer *et al.*, 1994)
Arthrobacter sp. J1	(Asano *et al.*, 1982)
Arthrobacter sp. IPCB-3	(Ramakrishna and Dessai., 1982)
Ascomycetes	(VanderWalt *et al.*, 1993)
Bacillus sp.	(Arnaud *et al.*, 1976 b)
Brevibacterium R312	(Arnaud *et al.*, 1976 a)
Corynebacterium pseudodipthteriticum ZBB-41	(Li *et al.*, 1992)
Corynebacterium sp. C5	(Tani *et al.*, 1989)
Corynebacterium nitrophilus	(Amarant *et al.*, 1989)
Myrothecium verrucaria	(Maier-Greiner *et al.*, 1991)
Rhodococcus rhodochrous LL100-21	(Digeronimo and Antonie., 1976)
Pseudomonas sp.	(Robinson and Hook., 1964)
Pseudomonas chloraphis 102	(Weiqiang *et al.*, 1989)
Pseudomonas chloraphis B23	(Nagasawa *et al.*, 1987)
Pseudomonas (Group III) NCIMB 10477	(Firmin and Gray., 1976)

Table 1.1 continued

Microorganism	Reference
Rhodococcus equi TG 328	(Gilligan *et al.*, 1993)
Rhodococcus sp. N-771	(Noguchi *et al.*, 1995), (Ikehata *et al.*, 1989)
Rhodococcus sp. C311	(Layh *et al.*, 1994)
Rhodococcus A 3270	(Blakey *et al.*, 1995)
Rhodococcus rhodochrous J1 (L-form)	(Kobayashi *et al.*, 1989)
Rhodococcus rhodochrous J1 (H-form)	(Nagasawa *et al.*, 1991)
Rhodococcus erythropolis JCM 2892 and JCM 6823	(Duran *et al.*, 1993)
Rhodococcus butanica ATCC 21197	(Kakeya *et al.*, 1991a)
Rhodococcus sp.	(Cohen *et al.*, 1990)

1.6 *Amidase enzymes*

These enzymes form the second step of the two step pathway, deaminating amides produced from nitriles by the action of the nitrile hydratase enzymes, to their carboxylic acids and ammonia. This reaction is highly substrate specific see, for example, Nawaz *et al.*, 1996. As a general rule amidases have higher activity, are more stable than nitrile hydratases and do not have associated metal ions or co-factors.

Studies of the bacterium *Rhodococcus* sp. (Nawaz *et al.*, 1994) revealed iron was present in the amidase, a novel feature of the enzyme in this bacterium that differentiates it from other amidases. The role of the iron in the amide hydrolysis was unclear but the inhibition of the amidase activity by specific iron chelators suggested a crucial role of iron in the active site of the enzyme. The amidase was inhibited by thiol blocking agents which also suggested the S-H groups are involved at the active site or have a function in the tertiary structure of the enzyme. Further to these studies, the amidase from *Klebsiella pneumoniae* NCTR1 was investigated (Nawaz *et al.*, 1996). This enzyme was capable of degrading acrylamide and aliphatic amides. The enzyme was a monomer with a molecular weight of 62,000 Da. Using Inductively Coupled Plasma Atomic Emission Spectroscopy analysis it was found that the native amidase contained 0.37 mol of cobalt and 0.33 mol iron per mol of native enzyme. In *K. pneumoniae* the metal ions are thought to be involved at the active site as the removal of the metal ions by chelators abolished activity, again suggesting that in constitutive amidases the iron has a crucial role at the active site. The amidase enzyme from *K. pneumoniae* did not lose activity at temperatures above 50° C. Therefore, the amidase from this bacterium appears to be the first thermostable amidase reported.

The amidase enzyme of *Pseudomonas chlororaphis* B23 has a broad range of activity with aliphatic aromatic and unsaturated amides (Cistanik *et al.*, 1995). It also exhibits enantioselectivity for aromatic amides such as (S)-2-phenylpropionamide (enantiomeric excess (e.e) = 100%), which has also been reported in other bacterial amidases such as *Rhodococcus sp.* (Mayaux *et al.*, 1991), *Brevibacterium* R312 (Mayaux *et al.*, 1990) and *Rhodococcus rhodochrous* J1 (Kobayashi *et al.*, 1991). These enzymes are all homodimers which are similar in size, have no metal ions, and with the exception of *P. chlororaphis*, are all sensitive to thiol reagents.

The amidase enzyme from *Brevibacterium* R312, which was first isolated by Arnaud *et al.* (1976a), was further characterised and cloned by Mayaux *et al.* (1990). The enzyme is active on several racemic 2-aryl or 2-aryloxy propionamides. It was also found to be a stereospecific enzyme and is significantly homologous to several amidases from different microorganisms.

The structural gene encoding the stereospecific amidase is *amd*A, which is located immediately upstream of the gene encoding the nitrile hydratase. This suggests that both enzymes are co-ordinately expressed in the cell. In comparison to the structure of the nitrile hydratase of *Rhodococcus* species, (Ikehata *et al.*, 1989), it is suggested that the enzyme from the two microbes *Rhodococcus* N-774 and *Brevibacterium* R312 are probably identical or very closely related, with only one nucleotide difference in more than 1.55kb (Mayaux *et al.*, 1990).

From these observations, it is possible that nitrile hydratase can be expressed from a polycistroic mRNA initiated upstream from the *amd*A since no sequence that corresponds to a termination sequence has been discovered in the intercistronic region between the two genes.

Furthermore, Ikehata *et al.* (1989) showed that in *Escherichia coli* nitrile hydratase can be expressed from a promoter inserted at the *sph* 1 site located at the *amd*A locus. The results indicated that the cell has to coregulate at least under some conditions, the gene expression of both enzymes. Examples of the amidases described to date are detailed in table 1.2.

Table 1.2: Examples of microorganisms possessing amidase activity

Microorganism	Reference
***Arthrobacter* sp. J1**	**(Asano *et al.*, 1982a)**
***Brevibacterium* R312**	**(Jallageas *et al.*, 1978)**
***Corynebacterium* sp. C5**	**(Tani *et al.*, 1989)**
***Klebsiella pneumoniae* NCTR 1**	**(Nawaz *et al.*, 1996)**
***Pseudomonas* sp. GD 1211**	**(Kagayama and Ohe., 1990)**
***Pseudomonas chlororaphis* B23**	**(Cistanik *et al.*, 1995)**
***Rhodococcus* sp. N774**	**(Hashimoto *et al.*, 1991)**
***Rhodococcus erythropolis* MP50**	**(Knackmuss *et al.*, 1996)**

1.7 Nitrilase enzymes

Although distribution of nitrilases appears to be much wider than nitrile hydratases they have been studied in much less detail. This is because much of the early work on nitrile hydrolysing enzymes concentrated on commercial production of acrylamide by a nitrile hydratase. Moreover, purification of nitrilases can be problematic. However, nitrilases can have useful biotechnological potential and could prove to be more important in nitrile metabolism than nitrile hydratases (Kobayashi and Shimizu, 1994).

The first nitrilases to be partially purified and characterised were obtained from barley leaves (Thimann and Mahadevan, 1964a). The enzyme hydrolysed 3-indolyacetonitrile to the corresponding carboxylic acid with no evidence of an intermediate. Properties indicated that the enzyme involved was similar in many respects to those found later in microorganisms. The enzyme was active with 2-, 3- and 4- cyanopyridine plus a range of substituted benzonitriles, and could also hydrolyse several aliphatic nitriles (Thimann and Mahadevan, 1964b).

The first bacterium isolated that possessed a nitrilase enzyme hydrolysed the naturally occurring nitrile ricinine (N-methyl-3-cyano-4-methoxy-2-pyridene) to the corresponding carboxylic acid. However, stability of the enzyme was a problem, (Robinson and Hook, 1964).

The first report of nitrilase activity in *Rhodococcus* species was made by (Harper, 1977). He and co-workers observed benzonitrile metabolism in *Rhodococcus rhodochrous* NCIMB 11216. However, there was no utilisation of benzamide as a substrate on benzonitrile grown cells, nor was there any by-product.

From this evidence the authors concluded that a single nitrilase enzyme was present.

The purified enzyme showed a time-dependent activation in the presence of benzonitrile that was a function of enzyme concentration temperature and pH. The process involved the aggregation of 47000 Da subunits into a 12-subunit aggregate, which had an optimal activity at pH 8.0. Concentrations as low as 5 nM of the thiol reagents *p*-hydroxymercuribenzoate and phenylmercuric acetate abolished the activity of the enzyme. The nitrilase was specific for nitrile groups directly attached to a benzene ring. *Ortho*-substituted benzonitriles could not act as substrates (except when fluorine was used) whereas *meta*- and *para*-substituted benzonitriles could be hydrolysed. Benzamide could not be utilised for growth and the purified nitrilase did not produce benzamide as an intermediate (Harper, 1977).

Using *Rhodococcus rhodochrous* NCIMB 11216, Gradley and Knowles, (1994) showed that this bacterium was capable of asymmetric hydrolysis of R-(-), S-(+)-2-methylbutyronitrile and other chiral nitriles. Using benzonitrile, propionitrile or propionamide as a sole source of carbon and nitrogen it was possible to induce the nitrilase activity. Only when the cells were grown on propionitrile or propionamide was amidase activity observed. In cell free extracts prepared from propionitrile grown cells no activation of the nitrilase was seen.

When R-(-), S-(+)-2-methylbutyronitrile was used as a substrate for cell-free extracts a kinetically resolved transformation of the two isomers was seen. The S isomer was hydrolysed before the R isomer. Whole cells had the same specificity but non-selectivity metabolised the acid products. A range of C-2 substituted substrates can be stereoselectively transformed using whole cells.

The best kinetic resolution was observed with (+/-)-2-methylhexanitrile (2-methylhexanenitrile), where the (+) isomer was initially favoured (Gradley *et al.*, 1994).

Using *R. rhodochrous* NCIMB 11216 Hoyle *et al.* (1998) investigated the possibility that different nitrile-hydrolysing enzymes were produced under different nitrile growth conditions (propionitrile and benzonitrile). Nitrilase activity in whole cell suspensions from either bacteria grown on propionitrile or benzonitrile were capable of biotransforming a wide range of nitriles. The propionitrile-induced nitrile degrading activity hydrolysed 3-cyanobenzoate and both the nitrile groups in 1,3-dicyanobenzoate. In contrast, the benzonitrile induced activity hydrolysed only one of the nitrile groups of 2,3-dicyanobenzoate, but did not affect 3-cyanobenzoate. Both nitrilases biotransformed 2-(cyanomethyl) benzonitrile to produce 2-(cyanophenyl) acetic acid. The nitrilases were purified and the N-terminus of each enzyme sequenced. SDS-PAGE analysis identified a subunit molecular weight of 45.8 kDa for each nitrilase. N-terminus sequences showed significant similarity with other sequenced nitrilases and, with the exception of a single amino acid, were identical with each other. Other workers have also reported multiple nitrilases in plants (Bartel and Fink, 1994) (Bestwick *et al.*, 1993) and microorganisms (Bandyopadhyay *et al.*, 1986).

Bhalla *et al.* (1992) purified and determined the NH_2 terminus of *R. rhodochrous* PA 34, which is involved in production of α-amino acids from amino nitriles. The sequence was subsequently used to design oligonucleotide probes for a Southern hybridisation, and the resulting probe hybridised with a 9.5 kb fragment from *R. rhodochrous*. Further nucleotide sequence analysis revealed that the nitrilase gene codes for a putative polypeptide of 380 amino acids.

NH_2 terminal and sequence homology has been found in the nitrilases of many bacteria that include *Alcaligenes faecalis* JM3, *A. faecalis* ATCC 8750, *Klebsiella pneumoniae* and *Acinetobacter* AK226.

Nitrilases that have been reported to hydrolyse aromatic and aliphatic nitrile are shown in table 1.3, and examples of microorganisms that possess a nitrilase enzyme are shown in table 1.4.

1.8 Hydrolysis of nitriles (stereoselectivity and regioselectivity)

1.8.1 Stereoselective biotransformations

This is potentially the most important biotechnological application of nitrile hydrolysing enzymes. The general area of stereoselective biotransformations has been covered extensively for the enantioselective production of natural products such as precursors of pharmaceutical agents and amino acids, for example Holland (1998) or Bunch (1998).

Using a putative *Acinetobacter* sp.it was shown that it was possible to produce up to 87% of the L-isomer of alanine from α amino propionitrile. Interconversion of the two isomers of amino-propionitrile aided high yields of the L-amino acids (Macadam and Knowles, 1984). In comparison, Arnaud *et al.* (1980) isolated a *Brevibacterium* sp. which possessed a non-specific nitrile hydratase and stereoselective amidase. These produced L-amino acids and the intermediate D-amides from racemic amino nitriles. The D-amides were then recycled by a chemical racemisation process to give 50% yields of the L-isomer. *R. rhodochrous* PA-34 was also studied (Bhalla *et al.*, 1992). It contains a broad specificity nitrilase and can stereoselectively hydrolyse racemic amino nitriles to L-amino acids (except D-alanine). More derivatives of amino acids have also been stereospecifically produced (Choi and Goo, 1986). They hydrolysed α-aminophenylacetonitrile from *Aspergillis fumarigeins* to yield S-α-hydroxyphenylglycine. More interestingly, it is the other enantiomer, R-α-hydroxyphenylglycine which is the raw material for the antibiotics amoxycillin, ampicillin and cephalosporin, (Choi and Goo, 1986).

Table 1.3: Nitrilases that have been reported to hydrolyse aromatic and aliphatic nitriles.

Microorganism	Hydrolysis of aromatic or heterocyclic nitriles	Hydrolysis of aliphatic nitriles	Reference
Rhodococcus rhodochrous NCIMB 11216	YES	YES	(Gradley and Knowles., 1994a)
Acinetobacter sp. AK226	YES	YES	(Yamamoto and Komatsu., 1991)
Rhodococcus PA 34	YES	YES	(Bhalla *et al.*, 1992)
Rhodococcus rhodochrous sp.	NO	YES	(Kobayashi *et al.*,1991)
Alcaligenes faecalis ATCC 8750	NO	YES	(Yamamoto *et al.*, 1991b)
Alcaligenes faecalis JM3	NO	YES	(Kobayashi *et al.*, 1992)

Table 1.4: Examples of microorganisms that possess a nitrilase enzyme

Microorganism	Reference
Acinebacter sp. AK 226	(Yamamoto and Komatsu., 1991)
Arthrobacter sp. J1 (A)	(Bandyopadhyay *et al.*, 1986)
Arthrobacter sp. J1 (B)	(Bandyopadhyay *et al.*, 1986)
Alcaligenes faecalis JM3	(Kobayashi *et al.*, 1992b)
Alcaligenes faecalis ATCC 8750	(Yamamoto *et al.*, 1991)
Klebsiella pneumoniae	(McBride *et al.*, 1986)
Rhodococcus rhodochrous 39484	(Stevenson *et al.*, 1990)
Rhodococcus rhodochrous NCIMB 11215	(Harper, 1985)
Rhodococcus rhodochrous J1	(Kobayashi *et al.*, 1988) (Kobayashi *et al.*, 1989) (Nagasawa *et al.*, 1988)
Rhodococcus rhodochrous PA 34	(Bhalla *et al.*, 1992) (Bhalla *et al.*, 1996)
Rhodococcus rhodochrous K22	(Kobayashi *et al.*, 1992b)
Rhodococcus rhodochrous M8	(Sinolitskii *et al.*, 1997)

1.8.1.2 Mandelonitriles

Another example of a stereoselective biotransformation is the optical resolving agent R-(-) mandelic acid which can be produced from racemic mandelonitrile (figure 1.4) by resting cells of *Alcaligenes faecalis* ATCC 8750 (Yamamoto *et al.*, 1991). The nitrilase from this bacterium is selective for R-(-) mandelic acid (figure 1.4).

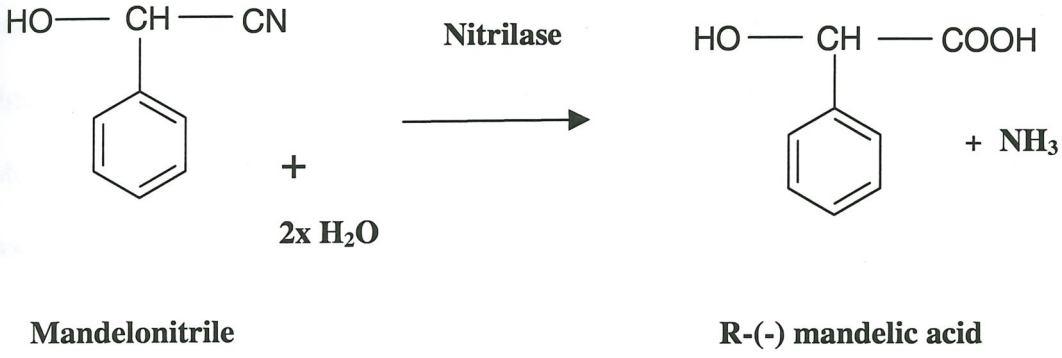


Figure 1.4: Schematic diagram of mandelonitrile (racemate) and its hydrolysis to mandelic acid. The bacterium *A. faecalis* selectively forms R-(-) mandelic acid.

1.8.1.3 Arylnitriles

Most work on enantioselective reactions has in the past been focused on arylpropionitriles. 2-Arylpropionic acids are used as pharmaceutical compounds, one example are profens, non-steroidal anti-inflammatory drugs (NSAID), which are used as racemic compounds.

Using enzymes present in *Rhodococcus* sp. ATCC 21197, Kakeya *et al.* (1991a) performed a kinetic resolution of α-arylpropionitriles. These compounds containing sterically bulky electron withdrawing and electron donating groups.

In all cases R-amides of high enantiomeric excess were produced by the enzymatic conversion, as well as S-carboxylic acids. The authors also suggested that this bacterium contained nitrilase and nitrile hydratase enzymes. They also showed that an amidase was present that preferentially converted S-amides to the corresponding S-carboxylic acid. These results indicate that stereoselective conversions are possible using microbial nitrile hydratases, amidases and nitrilases. Enantioselective hydrolysis of nitriles by pseudomonads has also been observed by Masutomo *et al.* (1995). Transformation was the result of a nitrile hydratase having poor enantioselectivity and an amidase that only used the S-isomer of the amide.

1.8.1.4 Ibuprofen Naproxen and ketoprofen nitriles

Production of S-(+) ibuprofen from racemic 2-(4-isobutylphenyl) propionitrile was achieved by (Yamamoto *et al.* (1990) with 95 % enantiomeric excess. This reaction is ideal from an industrial point of view, as the remaining 50 % of the substrate can be recycled (figure 1.5).

Further discovery of bacteria capable of stereoselective biotransformations was achieved by Layh *et al.* (1992). They isolated bacteria from soil which could grow with racemic naproxen nitrile (2-(6-methoxy-2-napthyl) propionitrile) as a source of nitrogen and succinonitrile as a carbon source. These organisms, *Rhodococcus erythropolis* MP50 and C311, converted naproxen nitrile via naproxen amide to naproxen through different enantiomers to give S-naproxen enantiomeric excess (e.e.) >99% with a conversion rate of up to 80 % of the theoretical value.

R. erythropolis MP50 has also been used to metabolise ketoprofen nitrile (2-(3-benzophenyl) propionitrile) with no inhibition of the enzyme by ketoprofen amide or ketoprofen. In a scale up experiment the bacteria produced S-ketoprofen for 9 hours with an enantiomeric excess (e.e.) of >97% (Layh *et al.*, 1994).

Racemic ketoprofen amide has also been enantioselectively hydrolysed using the amidase enzyme from *Comamonas acidovorans* (Yamamoto *et al.*, 1996).

Given the broad substrate specificity of the enzymes involved the use of stereospecific chemicals is likely to find more extensive applications in the future.

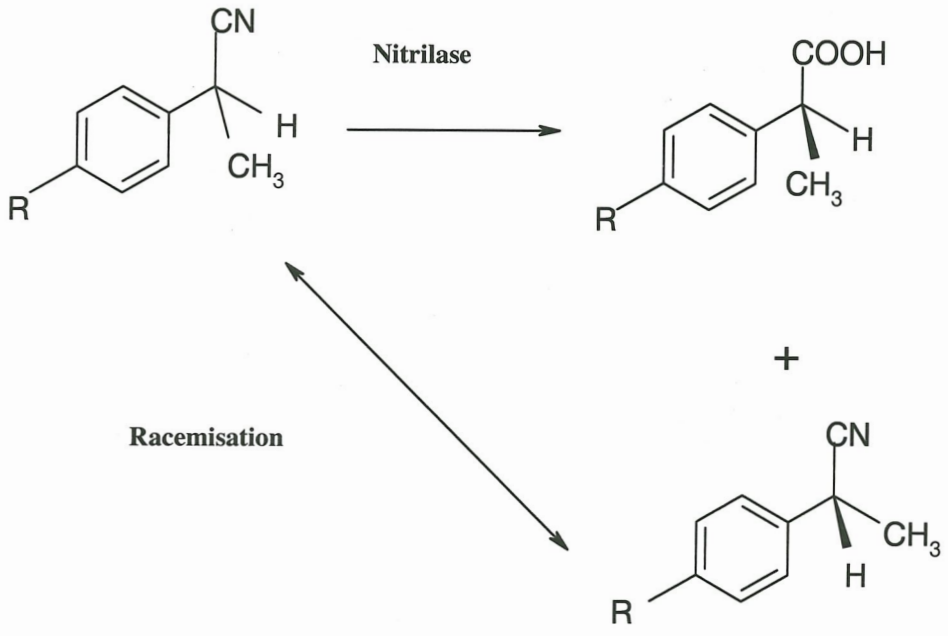


Figure 1.5: Production of S-(+) ibuprofen from racemic 2-(4-isobutylphenyl) propionitrile.

R= $(CH_3)_2$ $CHCH_2$

1.8.2 Nitriles and regioselectivity

1.8.2.1 Symmetrical and non-symmetrical aromatic dinitriles

Investigations have shown that it is possible for microbes to transform one or both nitrile groups of dinitriles. The first studies were by Kobayashi *et al.* (1988) using purified nitrilase from *R. rhodochrous* J1. Isophthalonitrile and terephthalonitrile were hydrolysed to give 3- and 4- cyano benzoic acids respectively, indicating that a nitrilase was the catalyst for mono-hydrolysis of these symmetrical aromatic dinitriles, as there was no evidence of the diacid (figure 1.6).

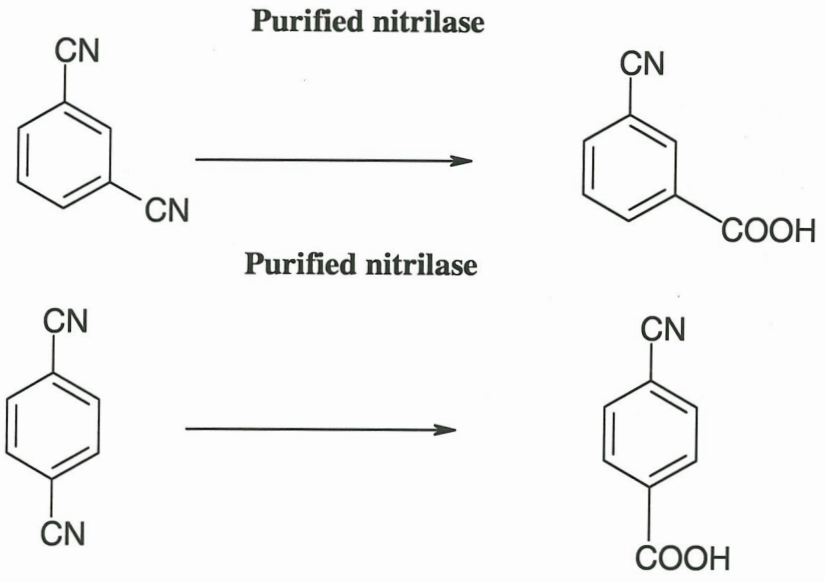
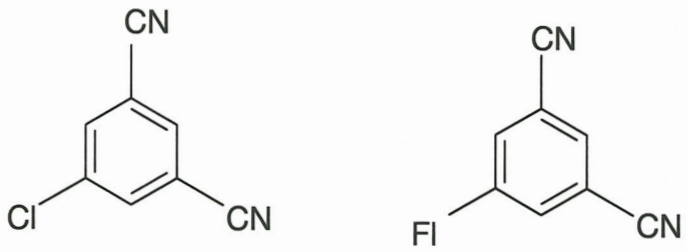


Figure 1.6: Biotransformation of isophthalonitrile (a) and terephthalonitrile (b) using the purified nitrilase from *Rhodococcus rhodochrous* J1.

Regioselective transformation of non-symmetrical dinitriles by nitrile hydrolysis was shown in 1994 by Crosby *et al* using an immobilised *Rhodococcus* sp. Usually isophtalonitrile was converted to the cyano-acid; however, by changing the incubation conditions the intermediate amide could be produced, but at lower yields.

Crosby *et al.* (1994) also showed when terephthalonitrile, a symmetrical dinitrile, was used as a substrate, only one nitrile group was selectively transformed. On the other hand, when the corresponding diamide was used as a substrate for the amidase, both amide groups were converted to the acid.

Finally these workers showed that with aromatic fluoro-dinitriles selective production of the corresponding monoamide could be achieved. The fluoro derivative of 5-chloroisophthalonitrile (figure 1.7) was regioselectivly biotransformed. Only the group at position 1 in the molecule was hydrolysed, and in the case of fluorinated aromatic dinitriles, where one cyano group is *ortho* substituted with respect to an amide group single amide isomers of the *ortho* cyanide group could be obtained. It was unclear from these results if the selectivity was due to electronic and/or steric factors, but suggestions were made that the *ortho* amino group may assist in the protonation step required in the conversion of a nitrile to a corresponding amide.



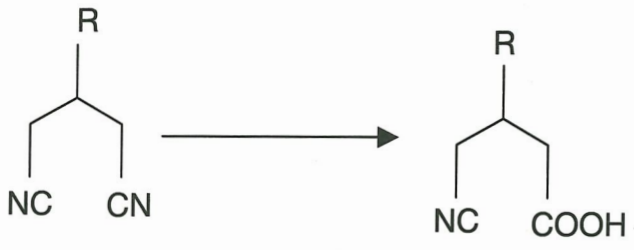
(a) (b)

Figure 1.7: Structural representation of 5-chloroisophthalonitrile (a) and 5-fluoroisophthalonitrile (b).

Further to these studies, Novo Industri of Denmark have developed an immobilised whole cell catalyst of *R. rhodochrous* sp 361 which has been used selectively to hydrolyse isophthalonitrile to the cyano carboxylic acid with a 91% yield. In this case the diacid was obtained by esterification of the carboxylic acid, which was re-exposed to the catalyst. If isophthalamide was used as a substrate the diacid was produced suggesting monohydrolysis only occurs in the biotransformation of nitrile to amide (Cohen *et al.*, 1990).

1.8.2.2 Glutaronitriles

The hydrolysis of prochiral β-substituted aliphatic glutaronitriles also has been reported (figure 1.8). For example, (Kakeya, *et al.*, 1991b) used the enzyme systems from *R. butanica* ATCC 2119 and found that this system preferentially hydrolysed the pro (S) cyano group of the dinitriles resulting in the formation of the corresponding cyano carboxylic acid.



R= CH_2Ph, OCH_2Ph, OCOH

Figure 1.8: Biotransformation of prochiral β-substituted glutaronitriles using *Rhodococcus butanica* ATCC 2119 as the biocatalyst.

Contrary to the observations by Kakeya *et al.* (1991b) the presence of an aromatic ring in the substrate is not an absolute requirement for enantioselectivity to be observed. *R. butanica* ATCC 21197 has been used previously in studies of β-substituted glutaronitriles. This study hydrolysed prochiral butylmethyl malononitrile to the amide and then the carboxylic acid (Yokoyama, *et al.*, 1993). These products can be used to produce α-methyl and α-amino acids.

Further studies of the hydrolysis of substituted glutaronitriles ($NC(CH_2)_3CN$) using *Brevibacterium* R312 with R groups OCOPh and OCH_2Ph have been carried out by Kerridge and Parratt (1995). (S) mono acids were obtained at 70 % yield and (e.e.) enantiomeric excess of 99 %. In agreement with results of other workers there was no evidence for the formation of the corresponding amide. This evidence was provided using mutant strains of *Brevibacterium* R312 which showed the two-step enzyme system was used. In accordance with this strain A4 blocked the amidase step and strain 19 the nitrile hydratase.

Again using glutaronitriles (substituted in position 3), (Beard *et al.*, 1993) used immobilised *R. rhodochrous* SP 361 for biotransformation to the corresponding cyano carboxylic acid. There was an enantiomeric excess (e.e) of the (S) configuration of the product with benzyl and benzene giving values of 84 % and 83 % respectively. The model suggested for the biotransformation of prochiral dinitriles is shown in figure 1.9.

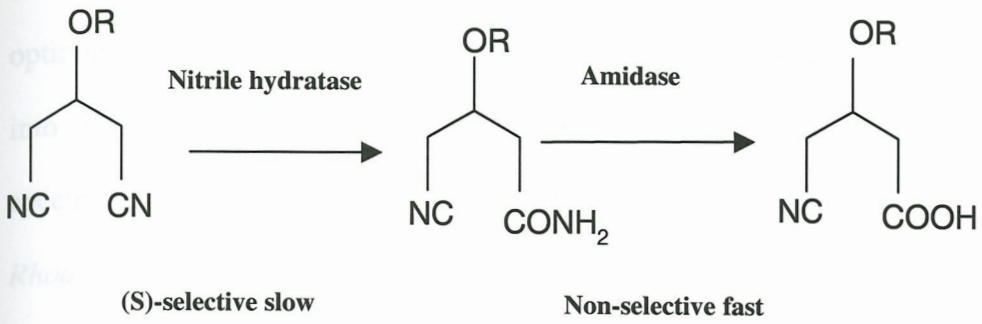


Figure 1.9: Model for the hydrolysis of prochiral dinitriles.

The bacterium *Rhodococcus rhodochrous* AJ270 was isolated from soil and grew well on 32 out of 36 aliphatic, aromatic and hetero-aromatic nitriles at high concentration (0.25 – 0.38 M). It had the ability to catalyse the monohydrolysis of malononitrile ($NCCH_2CN$) and adiponitrile ($NCCH_2(CH_2)_3CN$) as well as the dihydrolysis of succinonitrile ($NC(CH_2)_2CN$) and glutaronitrile ($NC(CH_2)_3CN$), (Blakey *et al.*, 1995).

1.8.2.3 Recent dinitrile studies

As a prelude to studying the hydrolysis of prochiral dinitriles Meth-Cohn and Wang (1997a and 1997c) made a broad study of the effect of structure on the regioselectivity and hydrolysis of aliphatic dinitriles using *R. rhodochrous* AJ270.

They observed that aliphatic dinitriles undergo regioselective hydrolysis to give monoacids with up to four methylenes between the nitrile functions (2-3 optimum). They then carried out an in-depth study of the biotransformation of nitriles into amides or acids and concluded that *Rhodococcus* AJ270 tolerates a broad spectrum of nitrile substrates. Both nitrile hydratase and amidase activities involved in *Rhodococcus* AJ270 are mainly influenced by steric and co-ordinating factors, in particular the presence of α-or *ortho*-substituents. Effective nitrile hydrolysis was also dependent on a molecular diameter of the substrate of no more than *ca.* 7 Ångstroms (Meth-Cohn and Wang, 1997b).

More recently, Wang and Meth-Cohn (2000) have studied enantioselective biotransformations of racemic α-substituted phenylacetonitriles (figure 1.10) and phenylacetamides using *Rhodococcus* AJ270.

The nitrile hydratase involved generally had a broad substrate spectrum against phenylacetonitriles irrespective of the electronic nature of the α-substituent while the amidase was very sensitive to both electronic and steric factors of the substituent amides. The overall enantioselectivity of nitrile hydrolysis was mainly determined by the combination of selectivities of nitrile hydratase and of amidase, with the latter being a major contributor. The amidase had a high (S)-enantiocontrol against amides while the nitrile hydratase exhibited low (R)-selectivity against nitriles (Meth-Cohn and Wang, 2000).

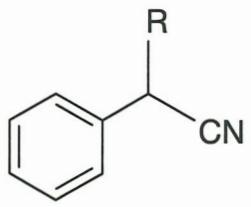


Figure 1.10: Structural representation of a α-substituted phenylacetonitrile.

1.9 Mid infrared spectroscopy

1.9.1 React IR MP monitoring

The React IR MP spectrophotometer is a modular reaction analysis system that was designed for real-time analysis and monitoring of chemical reactions and processes (figure 1.11). It is based on Fourier Transform Infrared (FTIR) technology (Harrick, 1979). The React IR MP produces specific information about the identity and quantity of chemical species present in a process or chemical reaction. The system uses the mid infrared region of the absorption spectra and provides a characteristic spectral fingerprint of the reaction investigated (Doak and Phillips, 1999). Reaction profiles can be generated that reflect the changes in concentrations of components of a reaction as a function of time. This analytical method was originally designed for use by process chemists, engineers and analysts. For example, during chemical manufacture the relative concentrations of key species can be measured and used to determine if a reaction is operating within a given specification. Similarly, in chemical engineering research and development it is useful to determine the effect of reaction variables in real-time on overall process performance.

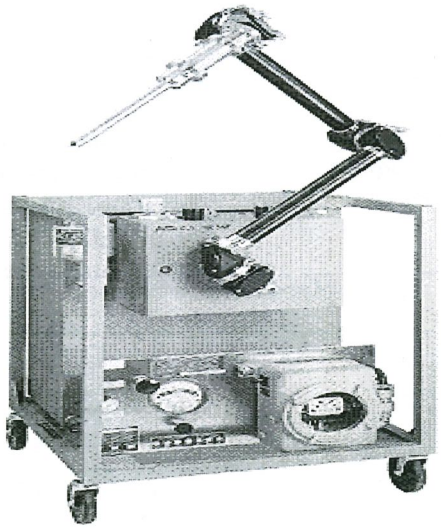


Figure 1.11: Representation of a React IR MP modular system with silicon Comp probe attached.

1.9.2 React IR probes

FTIR probes (figure 1.12) use the principle of internal reflection (Attenuated Total Reflection or ATR) to permit analysis of complex, dynamic processes (Doyle and Jennings, 1990; LeBlond *et al.*, 1998; Milosevic *et al.*, 1995; Sheridan and Rein; 1991). They make use of optical materials such as silicon and diamond as ATR elements. These are chemically resistant and can therefore be applied to a diverse range of reaction conditions. The probes directly measure changes in absorption spectra and can be used to identify the reactants, intermediates, by-products and products of chemical reactions.

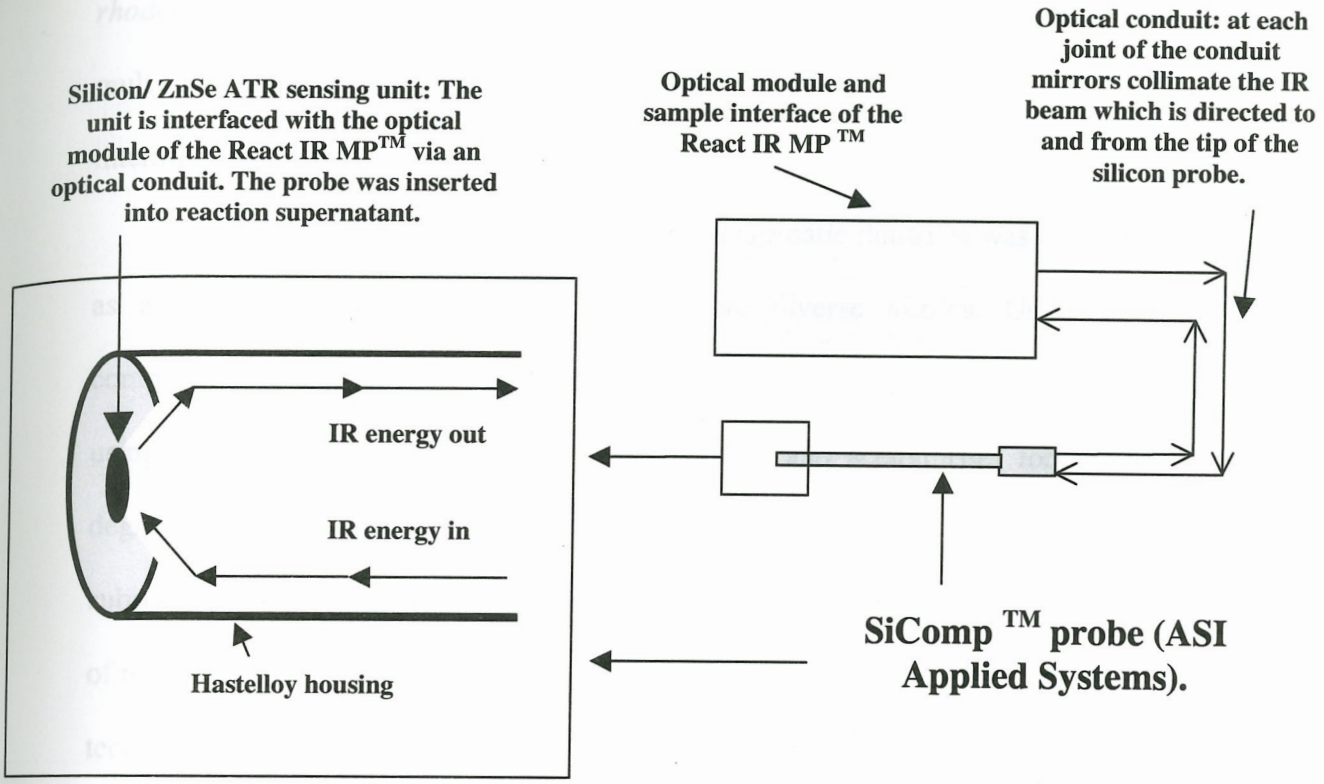


Figure 1.12: Schematic drawing of a ReactIR MP ™ module with a silicon probe attached. Infrared radiation is reflected into the chemically resistant ATR disk (e.g. diamond or silicon) which is in contact with the reaction mixture. Individual spectra of the reaction mixture are taken every 2 minutes and the data are shown on a computer screen as a 3D plot by the React IR™ software.

1.10 Aims of the project

The overall aim of the project was to use the bacterium *Rhodococcus rhodochrous* LL100-21 (or other isolated bacteria) to biotransform a range of novel multifunctional nitriles. These nitriles were of interest as potential pharmaceutical intermediates and precursors for many different drugs.

Regioselectivity of the bacterium towards aromatic dinitriles was to be studied as a preliminary to the investigation of more diverse nitriles. Using dinitrile compounds (cyanomethyl) benzonitriles, the aim was to study the biotransformations using different inducers (propionitrile, benzonitrile and acetonitrile) for the nitrile degrading enzyme systems. Aromatic ring substitutions of these dinitriles and their subsequent biotransformation were also to be studied. The intermediates and products of these biotransformations were to be identified using a range of analytical chemistry techniques, for example HPLC, NMR and mass spectroscopy.

Furthermore, chiral and prochiral derivatives were to be synthesised to investigate both enantioselectivity and stereoselectivity of the bacterium towards these substrates.

Prochiral substrates were chemically synthesised and utilising the nitrile hydratase and nitrilase enzymes from *R. rhodochrous* LL100-21 it was hoped that chiral centres could be generated within the compounds. The chiral products could then be subject to further chemical rearrangements to form compounds of possible interest to the pharmaceutical industry. Each prochiral nitrile was to be investigated using different induction conditions and analysed using a variety of analytical chemistry techniques.

Chiral biotransformations of cyclic nitrile compounds: chapter 1

The enzymatic pathways and enzyme kinetics of *R. rhodochrous* LL100-21 were to be studied using the substrates mentioned. Furthermore, these conventional biochemical and microbiological techniques were to be complimented using molecular biology techniques such as PCR, RT-PCR or Southern hybridisation.

An additional objective was to study the addition to the medium of other compounds such as solvents or hydroxylamine to establish whether novel biotransformations of the nitrile could occur. It was hoped that the addition of these compounds would compete with the conventional enzymatic hydrolysis and afford novel new products from the biotransformation. Initially it was envisaged that the study would use whole cell suspensions of the bacterium *R. rhodochrous* LL100-21 that had been grown on benzonitrile to induce the one step nitrilase pathway. This type of synthesis has not been documented within the scientific literature and therefore was of interest as an alternative method for synthesis of commodity chemicals.

Rhodococcus rhodochrous LL100-21 was chosen as its growth and maintenance has been described in the literature (Digeronimo and Antonie, 1976) and it is able to biotransform a range of nitriles (Collins and Knowles, 1983 and Linton and Knowles, 1986). It was also readily available within the laboratory culture collection.

Mid infrared spectroscopy is a useful technique to monitor chemical reactions in real-time. Therefore, part of the project has been designated to investigate its potential. Using nitrile biotransformations as a model system the nitrile hydratase and nitrilase enzymatic pathways from *R. rhodochrous* LL100-21 could be monitored using React IR MP. The potential of the technique to monitor stereoselectivity of racemates by this bacterium was also to be investigated.

Chapter 2: Materials and Methods

2.1 General biotransformations and chemical analysis

2.1.1 Growth conditions and bacteria

Rhodococcus rhodochrous LL100-21 obtained from the Knowles laboratory culture collection and was maintained on nutrient agar plates stored at 4 °C (Digeronimo and Antonie, 1976). The organism was grown in minimal medium consisting of M-9 salts (Miller, 1972) with ammonium salts omitted, plus 1 ml trace metals 1^{-1} (Bauchop and Elsden, 1960; appendix 4) and the following carbon and nitrogen sources: benzonitrile (10 mM), propionitrile (10 mM or 20 mM) or acetonitrile (10 mM or 20 mM). To inoculate experimental flasks (either 250 ml flasks containing 50 ml media or 500 ml medium in 2 litre flasks) a 2 % (v/v) inoculum (unless otherwise stated) of a 24 h stationary phase starter culture grown on the same carbon and nitrogen source as the experimental cultures was used. Cells were incubated on an orbital shaker at 200 rpm and 30 °C. To monitor growth, samples were taken (1 ml) at desired intervals and the absorbance recorded at 600 nm.

2.1.2 Harvesting of the cells

The bacteria were harvested at the mid exponential phase of growth (OD_{600} 0.4-0.8) by centrifugation at 23,000 g for 10 min at 4 °C. The bacteria were washed twice with 100 mM potassium phosphate buffer (pH 7.3) and re-suspended in fresh buffer to 1 % of the original culture volume.

2.1.3 Dry weight determination

Aliquots of resting cell suspensions were filtered through Whatman 0.2 μM cellulose acetate filters. The filters had been pre-dried at 90°C for 24 h and weighed. The filters plus the cells were dried at 90 °C for a further 24 h and re-weighed. They were then dried for further periods until a constant weight was achieved.

2.1.4 Biotransformation of substrates

Nitriles (10 mM or 50 mM) were added to cell suspensions harvested at OD_{600} 0.4-0.8, and concentrated 10 fold in phosphate buffer (pH 7.3). Lowly water-soluble substrates were added from a stock solution in methanol to give a final concentration of 100 mM in the suspension medium. The suspensions were incubated at 30 °C in an orbital shaker at 200 rpm. Samples (1 ml) of the reaction mixture were removed at intervals, and the reaction halted by centrifugation at 13,000 g for 2 min at 4°C. The supernatant was removed and assayed for biotransformation activity by the ammonia released.

The sample (40 • l) was added to 1 ml phenol reagent, containing 0.106 M phenol and 0.170 M $Na_2Fe(CN)_5NO$. Samples were mixed prior to the addition of 1 ml of hypochlorite reagent containing 0.11 mM NaOCl and 0.125 M NaOH. The reaction mixture was incubated at 37 °C for 15 minutes before the absorbance was measured at 570 nm against a blank containing no ammonia (Fawcett and Scott, 1960). The rate of hydrolysis was expressed as μmol ammonia released / minute / mg dry weight cells. All experiments were carried out in duplicate and the mean activity calculated. Remaining supernatants were stored at -20 °C before analysis.

2.1.5 Product recovery from the biotransformations

The reaction was halted by centrifugation at 13,000 g for 15 min at 4 °C, before the supernatant was removed, and the pH adjusted to 10.5 by the addition of 5 M NaOH. Diethyl ether was added (1:1 v/v) to the supernatant and after mixing for 5 min, the two phases were allowed to separate and the solvent phase discarded. This procedure was repeated three times. The aqueous phase was then acidified to pH 2.5 with 5 M HCl to protonate the organic acids in the medium. Diethyl ether was added (1:1 v/v) and mixed for 5 min, and the two phases allowed to separate. The solvent phase containing the extracted organic compounds was retained. This was repeated three times. The pooled extracts were dried over anhydrous magnesium sulphate before rotary evaporation at 45 °C.

2.1.6 Modified extraction

A modified extraction procedure was carried out. The methods used are detailed in section 2.1.5, but 100 ml of the aqueous phase was extracted with a mixture of 90 ml diethyl ether and 10 ml methanol (1:1 v/v).

2.1.7 High Performance Liquid Chromatography (HPLC)

Analysis of reference compounds and products were carried out using a Milton Roy 4000 HPLC with a C18, Nucleosil ODS 5 μ, 25 cm x 4.6 mm Jones Chromatography column fitted with a 3 cm x 4.6 mm Nucleosil ODS 5 μ guard column. The parameters used for the detection of the compounds were a wavelength of 254 nm, flow rate of 1.5ml/min, a mobile phase of between 25-35 % acetonitrile and 0.2 % phosphoric acid in distilled water.

Water and phosphoric acid were pre-filtered through Whatman 0.2 μm cellulose acetate filters before addition of acetonitrile. The mobile phase was degassed by helium for 15 min. Injections of 20 μl 1:2 dilutions of the supernatant were used and standard curves were constructed for available compounds.

2.1.8 Nuclear Magnetic Resonance (NMR) Spectroscopy techniques

^{1}H NMR, ^{13}C NMR, 2D ^{1}H-^{13}C heteronuclear multiple quantum correlation (HMQC:/one bond correlation) and 2D ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC:/multiple bond correlation) experiments were carried out on Bruker DPX250 (250 MHz) and AMX500 (500 MHz) NMR spectrometers at 25 °C. Samples of products and reference samples (100 mg) were dissolved in dimethylsulfoxide, DMSO-d6.

2.1.9 Fourier transmission infra red (FTIR) spectroscopy

Samples were placed in a Nicoet Magna 550 Infrared spectrophotometer with a Kbr disc and analysed for characteristic bond appearances and disappearances.

2.1.10 Mass spectroscopy

Spectra of molecular ions and fragmentation patterns were obtained using a Trio-1000 TSP- mass spectrophotometer

2.1.11 Thin Layer Chromatography (TLC)

Thin layer chromatography of substrates and products were carried out using 20 x 20 cm, 0.25 mm silica gel 60 F_{254} plates (Merck, Germany). Due to the different polarities, two mobile phases were used depending on the substrate or product used. These were either (5:1 v/v) ethyl acetate: hexane or (2:1 v/v) ethyl acetate: hexane. The plates were stained with potassium permanganate solution: 3 g $KMnO_4$, 18g Na_2CO_3 and 300 ml ddH_2O, and then dried with a Black and Decker hot air applicator. The plates were observed at 254 nm with UV light.

2.1.12 Chiral Capillary Electrophoresis (CCE)

Chiral Capillary Electrophoresis was carried out by Pfizer Inc; Sandwich, UK.

2.2 React IR and mid-infrared spectroscopy

2.2.1 Growth and harvesting of bacteria

Methods for maintenance and growth of the bacteria are described in sections 2.1.1 and 2.1.2. Bacteria were harvested in the mid exponential phase of growth (OD_{600} 0.4-0.8) and resuspended (10-fold concentration) in phosphate buffer (pH 7.3).

2.2.2 Biotransformation of substrates and monitoring of the reactions

Reactions were monitored using a React-IR MP FTIR based process monitoring system (ASI, Applied Systems, Mettler Toledo, Annapolis, Maryland, USA) with a dry nitrogen purge and ambient temperature water-cooling. Attached to this was a diamond or silicon probe (diameter = 5/8 16 mm, length = 370 mm).

Each spectrum acquired was generated by averaging 128 scans in the spectral range 650 cm^{-1} to 4000 cm^{-1}.

A spectrum was acquired every 2 min at a resolution of 8 cm^{-1}. Reactions were monitored over a period of 3-24 hours depending on substrate and inducer. A spectrum of phosphate buffer pH 7.3, containing bacteria was taken before the reaction and was used as the experimental background.

The reaction was initiated after 5 scans (time 0) by addition of the nitriles to 50 ml suspensions of bacteria suspended in phosphate buffer pH 7.3 (nitrile concentration of 250 mM unless otherwise stated). Biotransformations were carried out at 30-32 °C in a heated water bath. Both the reaction mixture and water bath were stirred at 200 rpm using magnetic stirrers.

2.3 Molecular biology techniques

2.3.1 Cell harvesting and bacterial genomic DNA extraction

Rhodococcus sp. and *Rhodococcus rhodochrous* LL100-21 were grown overnight in LB (Luria-Bertani broth, Appendix 4 (2)) medium in 250 ml flasks at 30°C and 200 rpm. Cell cultures were then pelleted at 4°C in a Beckman J2-21 centrifuge at 17500 g for 15 minutes.

Genomic DNA was extracted using a modified CTAB procedure (Bramwell *et al*; 1995). Bacterial cell cultures (50 ml) were harvested and frozen at -70°C. Following rapid thawing, the cell pellet was resuspended in 750 µl TE pH 8.0 (10 mM Tris.Cl (pH 8.0) (Sambrook *et al.*, 1989). Lysis buffer (200 µl) (Appendix 4 (3)) was added to the resuspended bacteria and incubated at 55° C for 30 min in a water bath (with occasional inversion). To each tube, 100 µl of pre-warmed 5 M NaCl and 80 µl of pre-warmed CTAB solution was added and the tubes incubated at 65 °C for 10 min.

DNA was extracted by isoamylalcohol: chloroform: (1:24 v/v). Genomic DNA was precipitated by addition of $1/10^{th}$ volume 3 M sodium acetate solution (pH 5.2), followed by a 2-fold excess of chilled 100 % ethanol. DNA was collected by microcentrifugation (full speed) and the resultant DNA was washed in 70 % ethanol before resuspending in 100 µl TE (pH 8.0, containing 1 µl of a 10 mg/ml RNase A solution).

2.3.2 Alternative DNA extraction procedure

Pseudomonas fluorescens genomic DNA was extracted by the chromosomal DNA preparation described by Page and Sockett (1999).

Bacteria were grown overnight at 30 °C in 200 ml LB (Luria-Bertani broth) using 500 ml flasks. Cell cultures were pelleted at 4 °C for 10 min and 4300 rpm in a Jouan BR4I centrifuge. Cells were then washed with TES pH 8.0 (10 mM Tris.Cl (pH 8.0), 1m M EDTA (pH 8.0), 100 mM NaCl), recentrifuged at 4°C for 10 min at 4300 rpm and the cell pellet resuspended in 15 ml TES. Lysis buffer (0.15 % w/v) (Appendix 4 (4)) was added to the cell suspension and incubated in a water bath at 37 °C for 1-3 hours.

Following lysis, Tris-saturated phenol (20 ml) was added to each tube and the volume made up to 50 ml by addition of TE (pH 8.0), (10 mM Tris.Cl (pH 8.0), plus 1 mM EDTA (pH 8.0)). The tubes were then mixed gently for 10 min and centrifuged at room temperature for 45 min.

The aqueous layers were pooled in a 250 ml sterile chilled beaker and the genomic DNA precipitated by addition of 10 ml of 3 M sodium acetate (pH 5.0-5.2), followed by a 2-fold excess of chilled 100 % ethanol.

DNA was collected by 'spooling' onto a glass rod. The resultant DNA was washed with 70 % ethanol and allowed to air dry for 15-30 min.

Finally, the DNA was resuspended in 2 ml of TE (pH 8.0, containing 20 µl of a 10 mg/ml RNase A solution). This was stored at 4°C before quantification and usage.

2.3.3 DNA electrophoresis

Electrophoresis of DNA was carried out using a horizontal gel apparatus (Bio-Rad, Hemel Hempstead, UK) with an agarose gel concentration of 0.7 %. Gels consisted of agarose dissolved in Tris borate EDTA (TBE) buffer (Sambrook *et al*; 1989) and were run in TBE. Loading buffer was mixed with samples and gels were run at 80 V for 45 min to 1 hour. HyperLadder I DNA size marker (Bioline, London, UK) was used for estimation of the sizes of the DNA fragments and their quantification. Fragments were visualised by staining in TBE containing 0.2 µg/ml ethidium bromide for 45 min and observed under UV light using a Gel Doc 1000 system (Bio-Rad).

2.3.4 Sequence acquisition and analysis for Southern blot analysis

Bacterial primers were designed by Dr Simon Baker, (OCEB, Department of Engineering Science, Oxford University) and the 5' digoxigenin-11-d UTP (DIG) labelled primers were synthesised by Life Technologies, UK.

Other primers used were synthesised by Sigma Genosys, UK and were fluorescein labelled using Gene Image ^{TM} random primer labelling module (Amersham International plc) according to the manufactures instructions.

2.3.4 DNA restriction digests and quantification

Genomic DNA was quantified using standard spectrophotometric methods as described by Sambrook *et al.* (1989). Restriction digests contained the appropriate restriction enzyme, for example *pst* I (New England Biolabs, Beverly, MA, USA) at between 1 and 10 units per reaction (depending on the quantity of DNA to be digested). Suitable buffer, and BSA (where required) were added and reactions were made up to a total volume of 20 µl with ddH_2O. Digests were performed at 37°C for 2 to 24 hours and were observed using the DNA electrophoresis (section 2.3.2).

2.3.5 Southern blot analysis

Southern blot analysis was carried out using a DIG detection kit (Roche molecular biochemicals, UK) or fluoroscein detection kit (Amersham International plc). Procedures were followed according to the manufacturer instructions, which were modified from standard methods described by Southern (1975).

2.3.6 Polymerase Chain Reaction (PCR).

The standard PCR mixture (50 µl) consisted of 3 µl of each respective primer (100 pm/µl), 200 µM of each deoxyribonucleotide triphosphate (dNTP), 25 mM $MgCl_2$, 5 µl 10 x PCR buffer and 2.5 U (1µl) Taq polymerase (ABgene, Epsom), made with filter sterilised ddH_2O.

All primers were synthesised by Life Technologies UK and made up to 100 pmol/µl with ddH_2O. PCR reactions were carried out with the following amplification conditions:

Denaturation	95 °C- 3 min	
Denaturation	95 °C- 1 min	X 10
Annealing	Tm (x)- 1 min	
Extension	72 °C- 1 min	
Denaturation	95 °C- 30 sec	X 30
Annealing	Tm (x)- 30 sec	
Extension	72 °C- 1 min	

Each reaction was carried out using an annealing temperature (Tm (x)) of 40 °C, 45 °C, 50 °C. A PTC-225 DNA Engine Tetrad (MJ Research, Waltham, MA, USA) was used for all PCR cycling. PCR products were electrophoresed on 0.7 % TBE agarose gels as described in section 2.3.2.

2.4 Chemicals

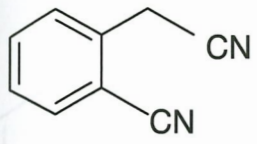
Chemicals were of reagent grade and obtained from either Sigma Ltd, Lancaster Ltd, or synthesised at Pfizer Inc.

CHAPTER 3: Biotransformation of (cyanomethyl) benzonitrile compounds

3.1 Biotransformation of 2-(cyanomethyl) benzonitrile

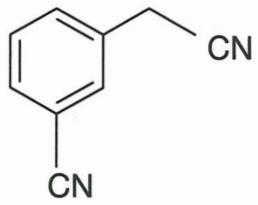
3.1.1 Introduction

2-(Cyanomethyl) benzonitrile and associated compounds are dinitriles with an aliphatic side chain of $-CH_2CN$, plus an aromatic nitrile group directly attached to the benzene ring. The compounds and the products of the biotransformations carried out in the following studies are shown in figure 3.1. Cyanomethyl benzonitrile compounds were studied since it is difficult to selectively convert one cyano group of a polynitrile by conventional chemical techniques (Bengis-Garber and Gutman, 1989). As a consequence there is considerable potential for biocatalysis to selectively hydrolyse one cyano group in nitriles containing two or more cyano groups. Once hydrolysed, these compounds could be utilised as pharmaceutical 'tools' or as intermediates for active pharmaceutical compounds. Therefore, cyanomethyl benzonitrile compounds were studied as a prelude to investigating the biotransformation of more complex di- and trinitriles.



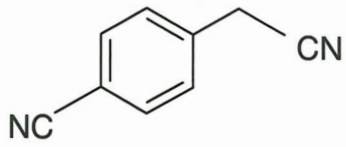
(a)

2-(cyanomethyl) benzonitrile



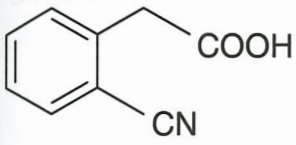
(b)

3-(cyanomethyl) benzonitrile



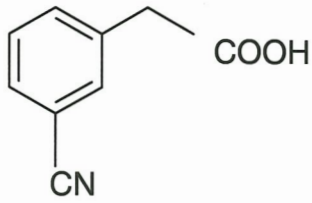
(c)

4-(cyanomethyl) benzonitrile



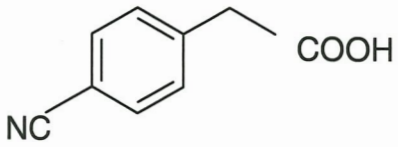
(d)

2-(cyanophenyl) acetic acid



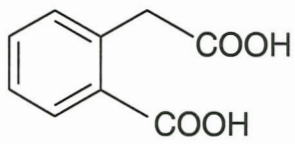
(e)

3-(cyanophenyl) acetic acid



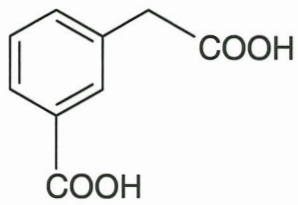
(f)

4-(cyanophenyl) acetic acid



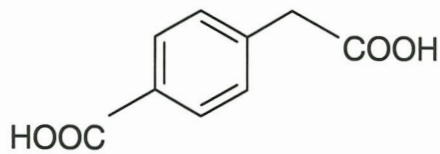
(g)

2-(carboxyphenyl) acetic acid



(h)

3-(carboxyphenyl) acetic acid

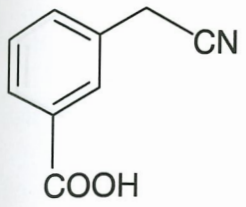


(i)

4-(carboxyphenyl) acetic acid

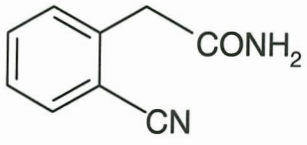
Figure 3.1: Structural representation of cyanomethyl benzonitrile compound and associated compounds.

Figure 3.1continued



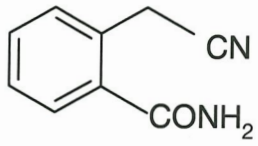
(j)

3-(cyanomethyl) benzoic acid



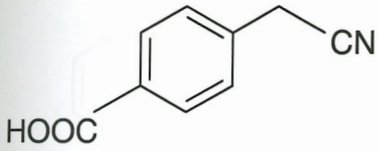
(k)

2-(cyanophenyl) acetamide



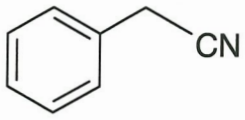
(l)

2-(cyanomethyl) benzamide



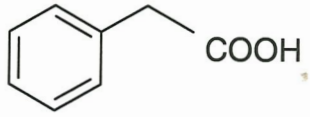
(m)

4-(cyanomethyl) benzoic acid



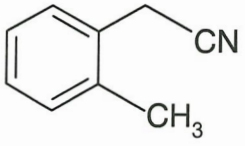
(n)

Phenylacetonitrile



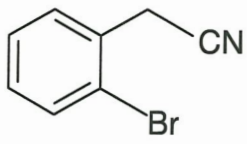
(o)

Phenylacetic acid



(p)

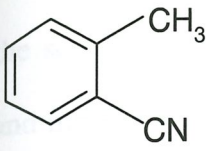
2-(methylbenzyl) cyanide



(q)

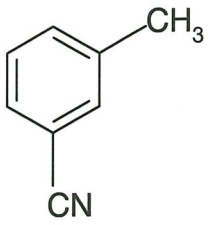
2-(bromophenyl) acetonitrile

Figure 3.1continued



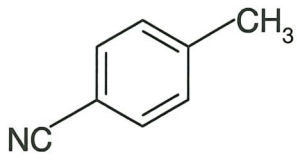
(r)

2-methyl benzonitrile



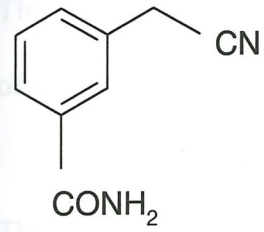
(s)

3-methyl benzonitrile



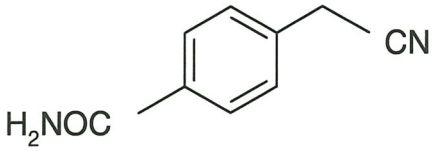
(t)

4-methyl benzonitrile



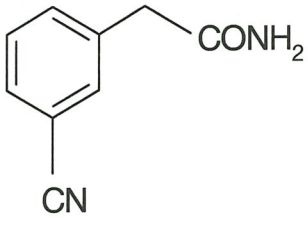
(u)

3-(cyanomethyl) benzamide



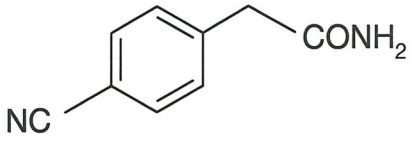
(v)

4-(cyanomethyl) benzamide



(w)

3-(cyanophenyl) acetamide



(x)

4-(cyanophenyl) acetamide

3.1.2 Biotransformation by propionitrile grown cells

Rhodococcus rhodochrous LL100-21 was grown on propionitrile (10mM) as the sole source of carbon and nitrogen to induce both the nitrile hydratase/amidase and nitrilase enzyme systems (Linton and Knowles, 1986). The cells were harvested in mid exponential phase (OD_{600} 0.4-0.8) and concentrated 10 fold in potassium phosphate buffer (100 mM, pH 7.3) at 4 °C. The biotransformation of 10 mM 2-(cyanomethyl) benzonitrile (**a**) was monitored by the rate of appearance of ammonia in the suspension medium. Within 25 h the concentration of ammonia detected approached 10 mM, i.e. 50 % of the potential maximum. This was equivalent to hydrolysis of only one of the nitrile groups. The specific activity by propionitrile grown cells was calculated for the biotransformation (0.02 μmols/min/mg dry wt). This was 40 % of the rate of benzonitrile hydrolysis (data not shown). In the absence of added nitrile no ammonia production was observed.

A larger scale biotransformation was carried out using 5 L of bacterial culture. The bacteria were harvested in early exponential phase, washed and resuspended in 500 ml of potassium phosphate buffer (100 mM) pH 7.0 (10 fold concentration). The reaction was carried out using 50 mM 2-(cyanomethyl) benzonitrile (**a**), which was introduced into the flask from a methanol stock over a 4-hour period. A white/cream powder was recovered (diethyl ether solvent extraction and rotary evaporation) with a 64 % yield compared to the starting substrate.

The following analytical chemistry techniques were carried out to identify the unknown compound.

3.1.2.1 High performance liquid chromatography (HPLC)

HPLC analysis of the recovered products, and for reference purposes, of 2-(cyanomethyl) benzonitrile (**a**) and 2-(carboxyphenyl) acetic acid (**g**) was carried out (2-(cyanophenyl) acetic acid (**d**) was not commercially available). The data recorded suggested that the recovered product was an intermediate of the dinitrile and diacid as it had a retention time between these two reference compounds. Only one product was observed, suggesting that there was only a single product from the biotransformation. HPLC analysis of the time course of the reaction showed the concomitant disappearance of the nitrile and appearance of the product in the supernatant. No other products were recorded in the supernatant.

3.1.2.2 Thin layer chromatography (TLC)

Rf values for the unknown compound (0.81), 2-(cyanomethyl) benzonitrile (**a**) (0.91) and 2-(carboxyphenyl) acetic acid (**g**) (0.12) were obtained using 5:1 v/v ethyl acetate:hexane. The Rf value of the reaction product obtained was between the values for the reference compounds, again suggesting the recovered product was an intermediate.

3.1.2.3 Nuclear magnetic resonance spectroscopy (NMR)

3.1.2.3.1 ^{1}H NMR

To investigate which of the nitrile functional groups was hydrolysed during the biotransformation, ^{1}H NMR was carried out using the recovered product. Again 2-(cyanomethyl) benzonitrile (**a**) and 2-(carboxyphenyl) acetic acid (**g**) were used as references for the identification of the unknown product. A comparison of the spectra of these compounds showed that there was a chemical shift difference of 0.45ppm

between the CH_2 groups of the product and 2-(cyanomethyl) benzonitrile (**a**) (figure 3.2). This suggested that the aliphatic cyano functional group had been hydrolysed.

No chemical shift in the spectrum was seen in the aromatic region, indicating that the aromatic cyano group remained unchanged. Differences in ^{13}C chemical shifts were constant with these data.

To further confirm the data, and to unequivocally determine the structure of the compound 2D NMR techniques were carried out.

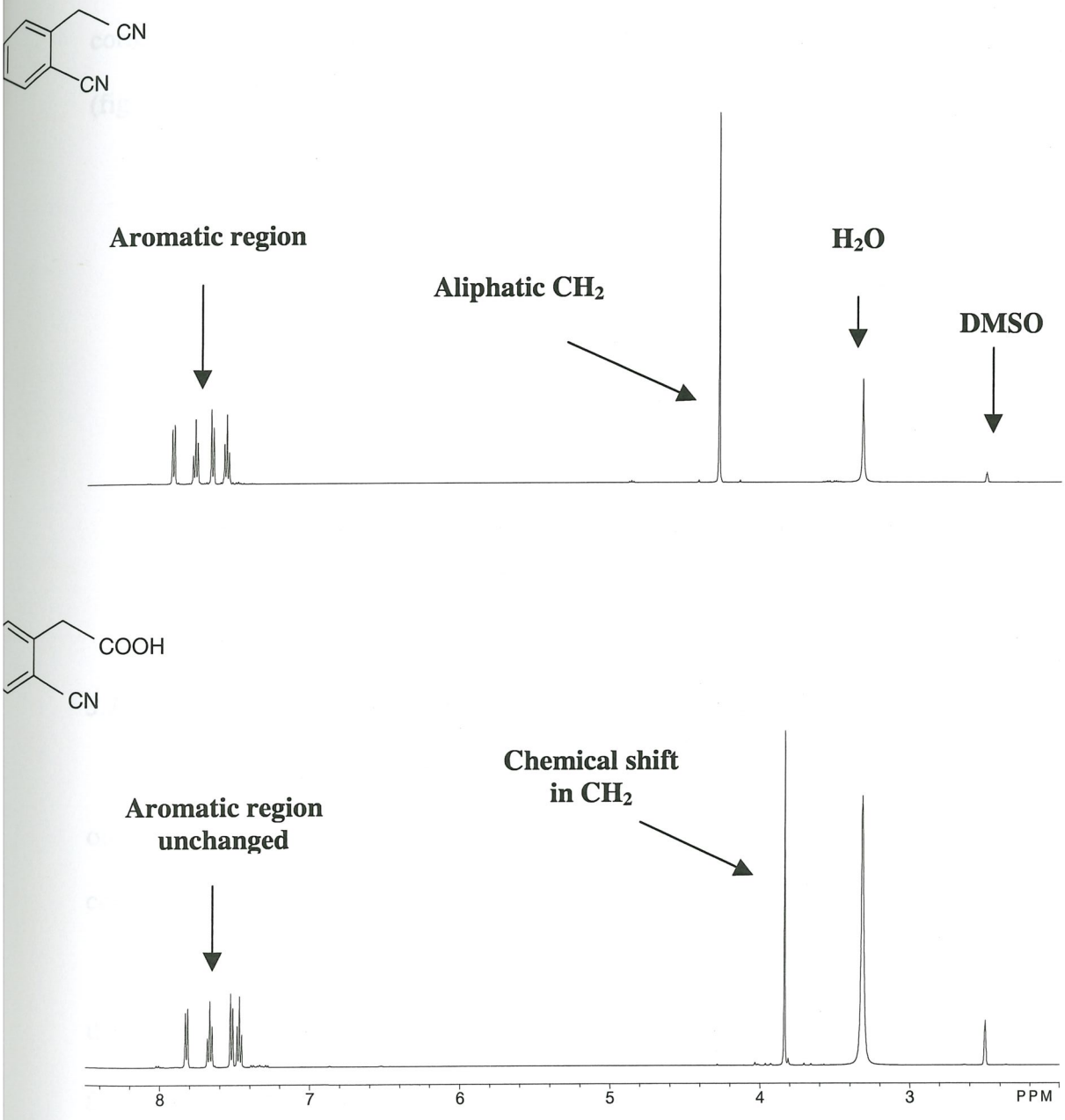


Figure 3.2: ^{1}H NMR spectra of 2-(cyanomethyl) benzonitrile (a) and the biotransformation product 2-(carboxyphenyl) acetic acid (g). A chemical shift difference was seen in the aliphatic CH_2 but no change was seen in the aromatic region.

3.1.2.3.2 Heteronuclear multiple quantum correlation HMQC

The spectrum obtained for the recovered compound showed 2 x triplets (coupled to 2H) and 2x doublets (coupled to 1H) indicating an aromatic ring was present. Although the signal for CH_2 was obscured by the signal from the H_2O correlation of H-C bonds within the structure gave a structural skeleton of the product (figure 3.3).

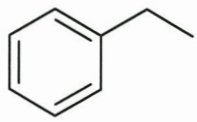


Figure 3.3: Structural skeleton of the compound represented by HMQC

3.1.2.3.3 Heteronuclear multiple bond correlation (HMBC)

To further quantify the results of the HMQC experiment HMBC was carried out. This experiment establishes H-C correlation over more than one bond (long range correlations of 2 to 3 bonds).

A correlation between the CH_2 on the aliphatic chain and other bonds within the molecule was observed. A strong cross peak with the CH_2 showed that a CO_2H group was located two bond lengths away. From this it was clear that there was a correlation between the CH_2 group and a CO_2H group but not to a cyano group on the aliphatic chain. This confirmed other data which indicated hydrolysis of the aliphatic cyano group to the acid and not the aromatic group.

Other observations suggested that the CH_2 group did not correlate to a cyano group. A small signal correlated a bond within the aromatic ring to a cyano group less than three bond lengths away. This proved beyond doubt that the structure of the recovered product was that of the intermediate 2-(cyanophenyl) acetic acid (**d**) (figure 3.4).

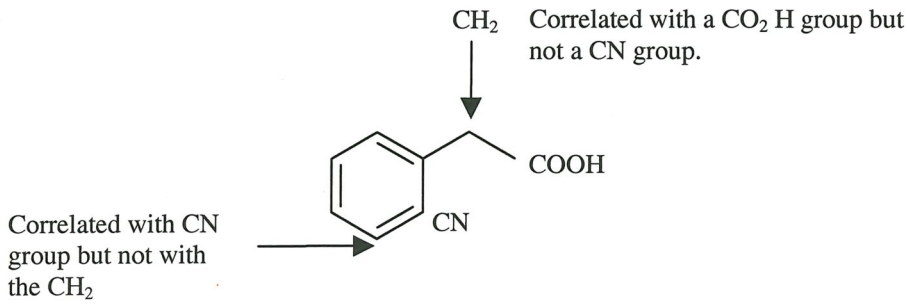


Figure 3.4: Structure and correlation made using heteronuclear multiple bond correlation (HMBC).

3.1.2.4 Fourier transmission infra red spectroscopy (FTIR)

Spectra of 2-(cyanomethyl) benzonitrile (**a**) (reference) and the recovered product were run. The reference compound showed two main peaks at 2240 cm^{-1} and 2220 cm^{-1}, which were correlated with the aliphatic cyano group and the aromatic cyano group of the known compound respectively.

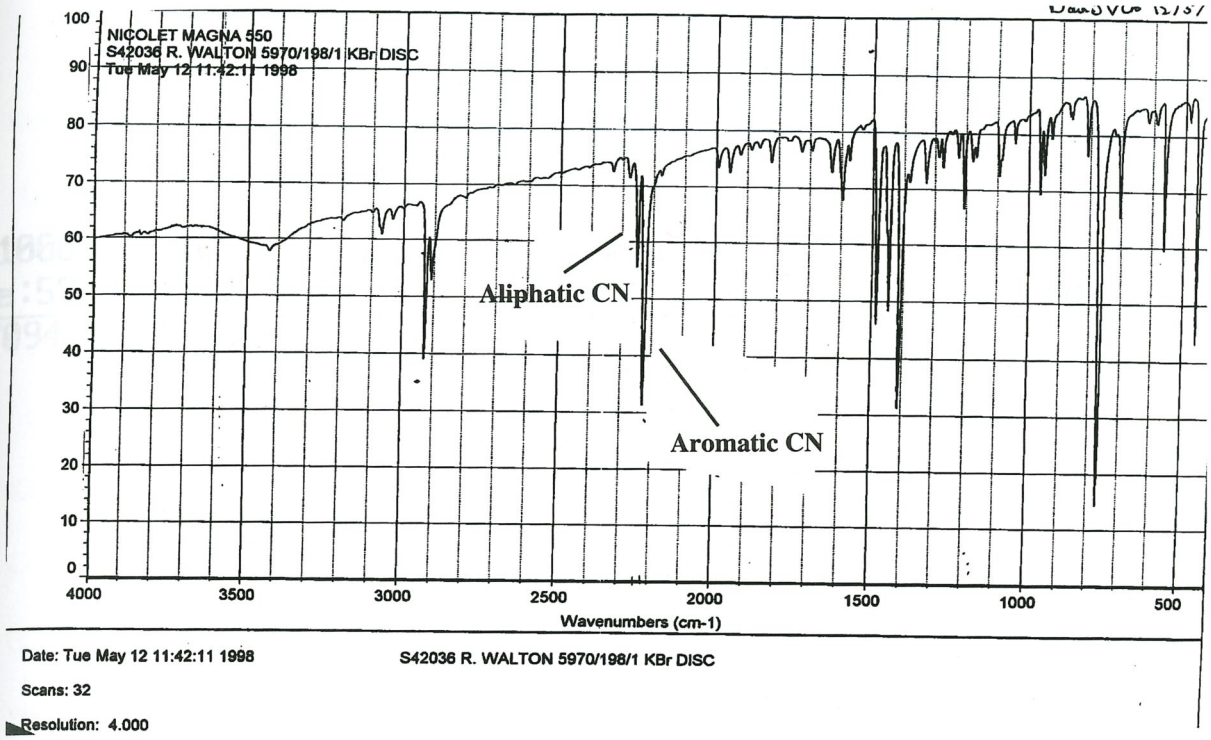
The spectrum of the recovered compound showed only one of these peaks, which was present at 2220 cm^{-1}, therefore indicating that the aliphatic cyano group had been hydrolysed (figure 3.5).

Also, an acid peak was seen at 1700 cm^{-1} which further confirmed the aliphatic cyano group had been converted to an acid group.

Hence, these data confirm the product was the mononitrile/monoacid, 2-(cyanophenyl) acetic acid **(d).**

3.1.2.5 Mass spectrometry

From the spectra obtained (figure 3.6) the products of the biotransformation (using propionitrile and benzonitrile grown cells) had a molecular ion value of 161 ($179 + NH_4^+$). This was correct for the theoretical mass data using the periodic table, therefore confirming the molecular weight of the compound 2-(cyanophenyl) acetic acid **(d)**. The fragmentation pattern for the compound could not be determined therefore no structural data are available for the products using this technique.



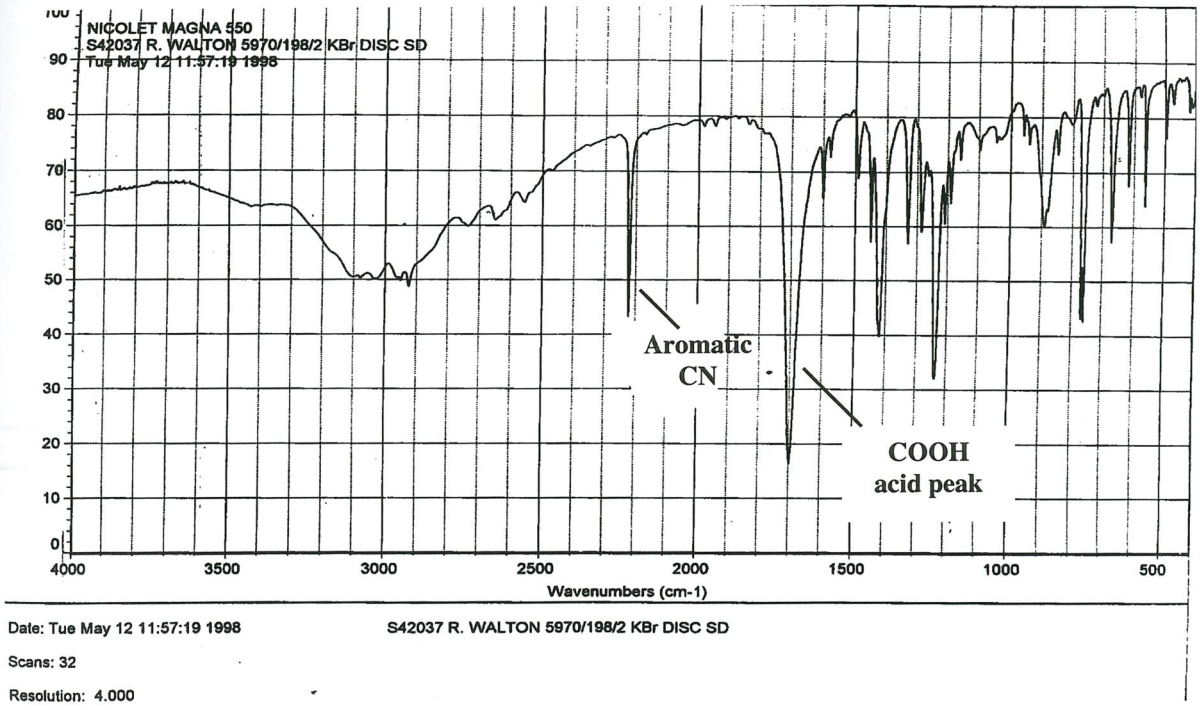


Figure 3.5: Comparison of FTIR spectra of 2-(cyanomethyl) benzonitrile (a) and 2-(cyanophenyl) acetic acid (g).

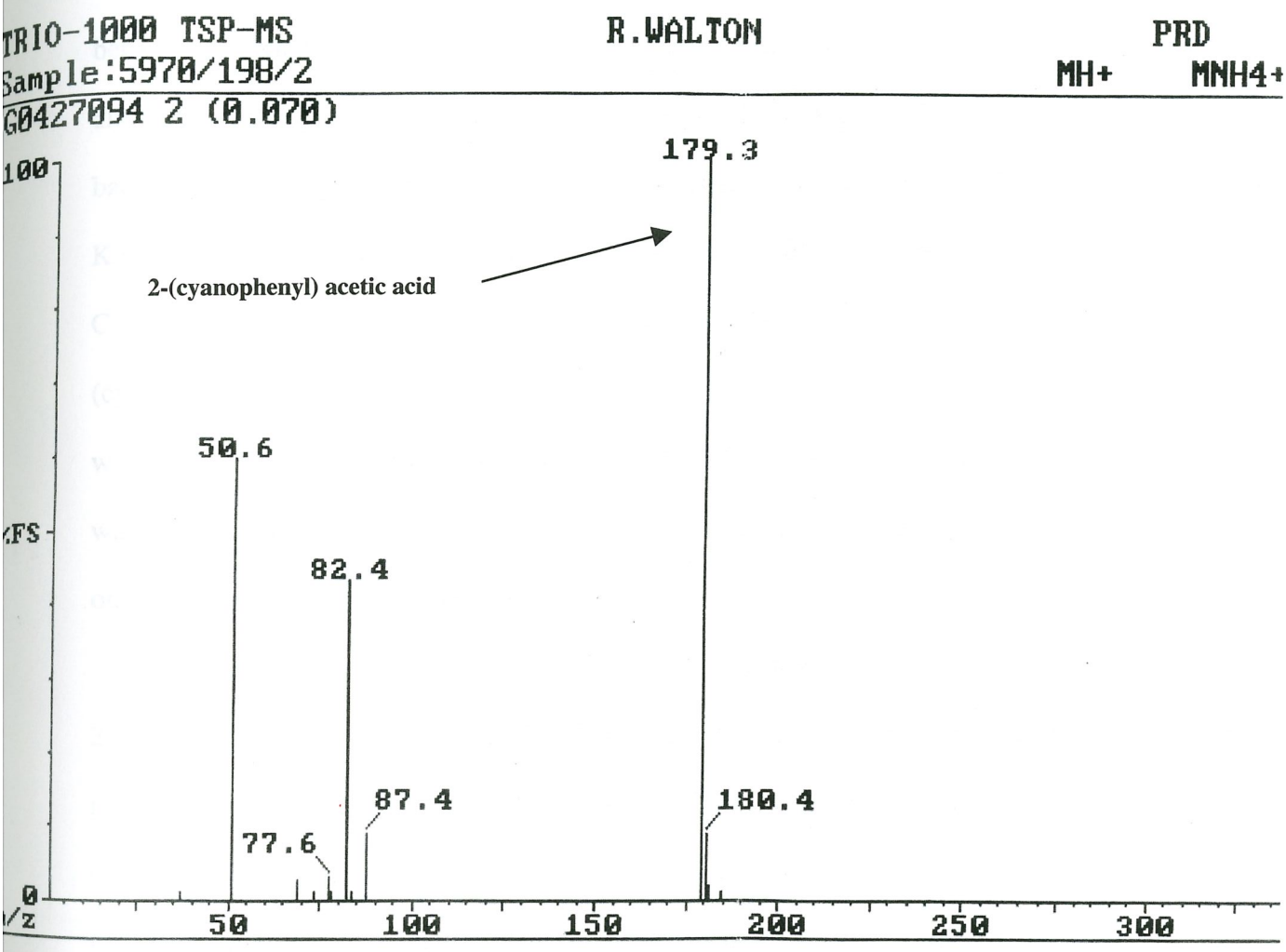


Figure 3.6: Mass spectra of the recovered product 2-cyanophenyl acetic acid (d). This indicates a molecular ion value of 161 (179 + NH^+_4).

3.1.3 Biotransformation by benzonitrile grown cells

Growth of *Rhodococcus rhodochrous* LL100-21 on propionitrile induces both the nitrile hydratase and the nitrilase pathways (Dadd, unpublished), section 3.8.1; (Linton and Knowles, 1986) yet conversion of the dinitrile, 2-(cyanomethyl) benzonitrile **(a),** by propionitrile grown bacteria resulted in conversion of only the aliphatic nitrile group to the corresponding carboxylic acid group. However, use of bacteria grown on benzonitrile (which induces only the nitrilase enzyme, (Collins and Knowles, 1983)) might indicate if the nitrilase was involved in the bioconversion. Consequently, benzonitrile grown cells were used to test the biotransformation of 2-(cyanomethyl) benzonitrile **(a)** (10 mM). The initial rate of utilisation of the dinitrile was 8.3 % the rate of utilisation of benzonitrile, and the maximal yield of ammonia was nearly 10 mM, which is 50 % the potential maximum. This again suggested that only one cyano group was hydrolysed.

A larger scale biotransformation (50 mM substrate) was carried out using 2-(cyanomethyl) benzonitrile **(a).** A white/cream powder was recovered from the reaction, with a 56 % recovery yield. All the analytical chemistry techniques carried out for propionitrile grown cells (see previous sections) were repeated for benzonitrile grown cells, giving identical data, and it was concluded that the same product had been recovered, i.e. 2-(cyanophenyl) acetic acid **(d).**

3.1.4 Biotransformation by acetonitrile grown cells

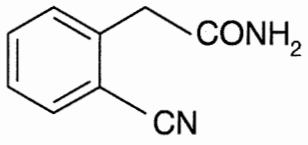
All biotransformations carried out with acetonitrile grown cells (which induces only the nitrile hydratase/amidase enzymes; Collins and Knowles, 1983) produced no detectable amounts of ammonia.

This indicated there was no biotransformation from the dinitrile to give the possible mononitrile/monoacid products or the diacid. However, there could be biotransformation of the dinitrile to one or both of the mononitrile/monoamide or the diamide derivatives without release of ammonia. Therefore, a larger scale biotransformation and product recovery was carried out using acetonitrile grown cells. All procedures were similar to those for propionitrile and benzonitrile grown cells. A white/brown powder was recovered, but only at 3 % yield compared to the starting substrate. HPLC analysis of the time course of the reaction indicated that only a small amount of the substrate had been biotransformed and only one significant product was formed. On subsequent comparison to 2-(cyanomethyl) benzonitrile **(a)**, 2-(cyanophenyl) acetic acid **(d)** and 2-(carboxyphenyl) acetic acid **(g)**, two previously unobserved peaks were revealed indicating formation of two new compounds.

^{1}H NMR and ^{13}C NMR were carried out and compared to the spectra of the dinitrile, propionitrile grown product and diacid. New peaks were seen corresponding to a mixture of two compounds, which suggested the formation of both possible mononitrile/monoamide derivatives. HMQC and HMBC analyses were carried out to the conclusion that the new products were the mononitrile/monoamides 2-(cyanophenyl) acetamide **(k)** and 2-(cyanomethyl) benzamide **(l)** (figure 3.7)

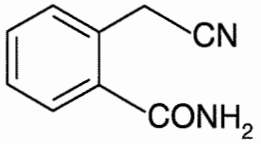
As mentioned earlier, the overall yield of these products were low (about 3%). HPLC data and previous work suggested that the major product was 2-(cyanophenyl) acetamide **(k).**

Presumably, access to the enzyme (as it is within the intact cells), steric hindrance, or electronic effects may have affected the ability to hydrolyse the substrate. Alternatively the enzyme(s) had little activity on this substrate.



(k)

2-(cyanophenyl) acetamide



(l)

2-(cyanomethyl) benzamide

Figure 3.7: Structural representation of the two mononitriles/monoamides recovered from the biotransformation supernatant. Bacteria had been grown on acetonitrile to induce the two-step nitrile hydratase/amidase pathway.

3.1.5 Abiotic control

To indicate if any abiotic influences occurred during the larger scale biotransformations an abiotic control was carried out in which *R. rhodochrous* LL100-21 was omitted from the reaction mixture. After the recovery steps had been carried out, no product was detected and hence it was concluded that the biotransformation was carried out solely by the bacterium.

3.1.6 Modified extraction procedure

Due to the increased polarity of the putative diacid a modified extraction procedure was used. Using the biotransformation of 2-(cyanomethyl) benzonitrile **(a)** supernatants from propionitrile-, benzonitrile- and acetonitrile-grown bacteria (100ml) were extracted using diethyl ether (90ml) and methanol (10ml). The final solvent phase was then dried using magnesium sulphate and rotary evaporation. This extraction procedure yielded a small amount of yellow liquid that was subsequently analysed by HPLC and ^{1}H NMR.

HPLC and ^{1}H NMR spectra revealed the starting substrate 2-(cyanomethyl) benzonitrile **(a).** These data confirmed previous results in which the diacid was also not formed and therefore was not detected during the biotransformation.

3.2 Biotransformation of 3-(cyanomethyl) benzonitrile

3.2.1 Introduction

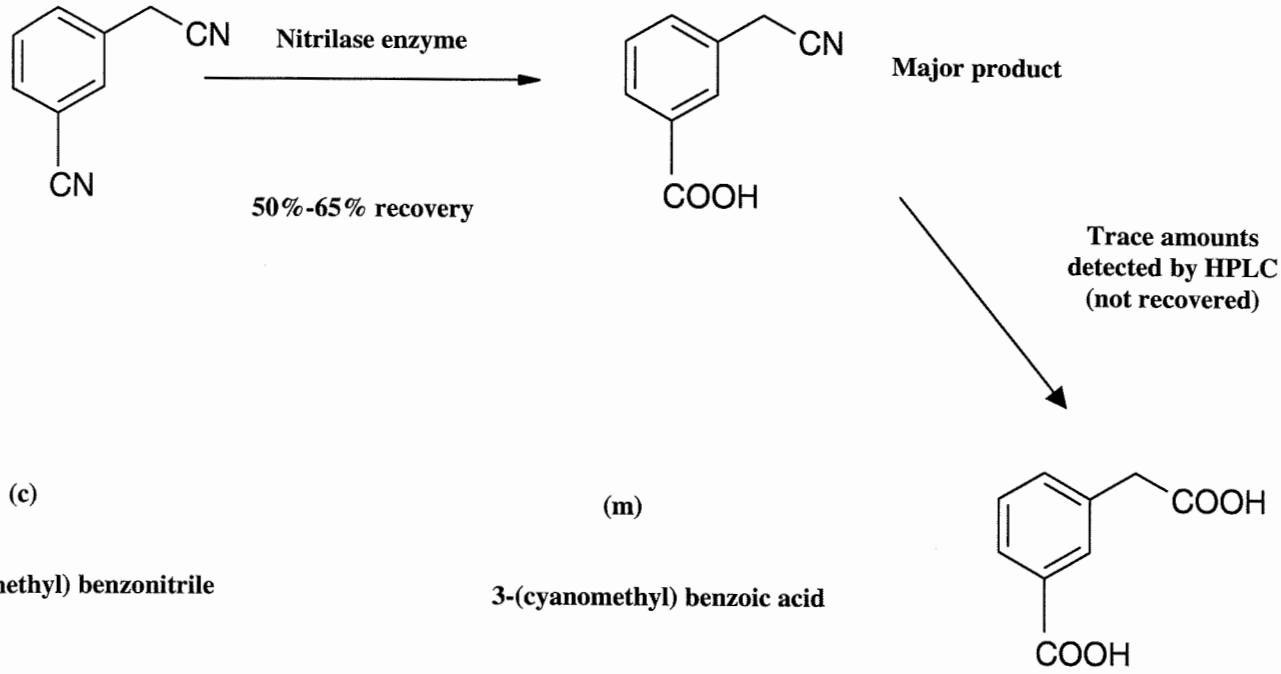
The potential biotransformation of the *meta* substituted compound 3-(cyanomethyl) benzonitrile **(b)** (10 mM) was studied to investigate the effects of the position of ring substitution on the biotransformation. Smaller scale biotransformations were carried out using benzonitrile- and propionitrile-grown cells (10mM) in phosphate buffer (100mM, pH 7.3). Bacteria were concentrated 10 fold and reactions were carried out in phosphate buffer (100mM. pH 7.3) at 30°C. As with 2-(cyanomethyl) benzonitrile **(a),** it was found that about 10mM of ammonia was produced. This was stiochometrically equivalent to one cyano group being hydrolysed in both cases. Therefore, larger scale biotransformations were carried out to enable analysis of the reaction products.

Using HPLC, ^{1}HNMR and HMBQ the following results were obtained:

3.2.2 Propionitrile and benzonitrile grown cells

Bacteria grown on propionitrile and benzonitrile biotransformed the compound 3-(cyanomethyl) benzonitrile **(b)** to the corresponding mononitrile/monoacid 3-(cyanomethyl) benzoic acid **(j).** There was a recovery of 56-60% compared to the starting substrate. In contrast to 2-(cyanomethyl) benzonitrile **(a),** the aromatic cyano group was readily hydrolysed in preference to the aliphatic cyano group during a 5-hour incubation period. A scheme of this reaction can be seen in figure 3.8.

These results were quantified using HPLC, ^{1}H NMR ^{13}C NMR and HMQC. Using HPLC with appropriate reference compounds, low concentrations (1-3 %) yield) of the diacid were seen to form (3-4 hours) but then disappeared. No other intermediates were detected during the time course of the experiment.



(c)

-(cyanomethyl) benzonitrile

(m)

3-(cyanomethyl) benzoic acid

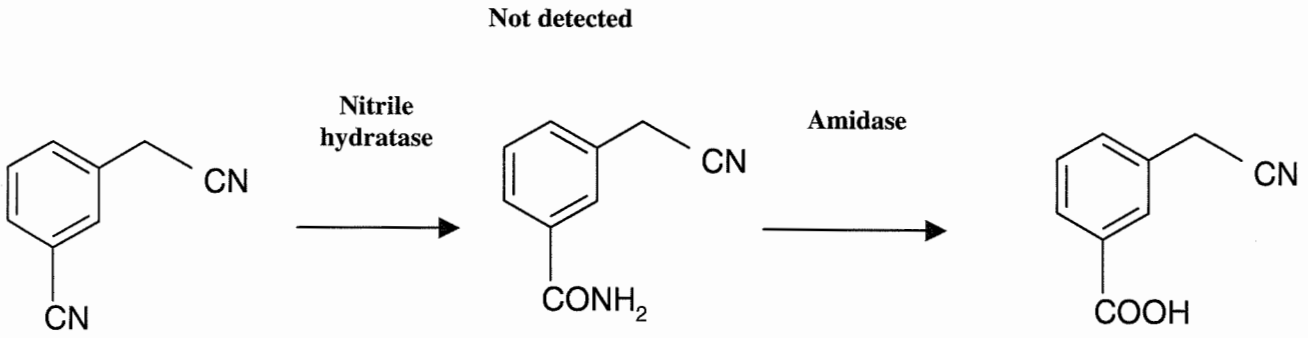
(i)

3-(carboxyphenyl) acetic acid

Figure 3.8: Biotransformation of 3-(cyanomethyl) benzonitrile (50 mM) using *Rhodococcus rhodochrous* LL100-21 that were grown on benzonitrile and propionitrile (10 mM). Bacteria were concentrated 10 fold and the reaction was carried out in phosphate buffer (100 mM, pH 7.3) at 30°C.

3.3.3 Acetonitrile grown cells

In previous experiments it was shown that, following growth on acetonitrile, the biotransformation of 2-(cyanomethyl) benzonitrile **(a)** produced a mixture of the two amide derivatives in very low yield, with no indication of any other products being formed e.g. no ammonia release. A small-scale biotransformation of 3-(cyanomethyl) benzonitrile **(b)** produced 10mM ammonia suggesting one cyano group had been hydrolysed. Using ^{1}H NMR, the dominant product from the biotransformation was identified as 3-(cyanomethyl) benzoic acid **(j)**. This indicated that the aromatic cyano group was converted to the corresponding acid, although it was formed in low yield (1%). Since no amide intermediate was detected by HPLC it suggests that the amidase activity was more rapid than the nitrile hydratase activity (figure 3.9).



(b) **(u)** **(j)**

3-(cyanomethyl) benzonitrile **3-(cyanomethyl) benzamide** **3-(cyanomethyl) benzoic acid**

Figure 3.9: Biotransformation of 3-(cyanomethyl) benzonitrile using *Rhodococcus rhodochrous* LL100-21 grown on acetonitrile. Bacteria were concentrated 10 fold and the reaction carried out in phosphate buffer (100mM, pH 7.3) at 30°C. The major product identified was 3-(cyanomethyl) benzoic acid (J) which suggested the nitrile hydratase enzyme had converted the substrate to the mono nitrile/mono amide. This was immediately and rapidly converted to the mono nitrile/mono acid by the amidase enzyme.

3.4 Biotransformation of 4-(cyanomethyl) benzonitrile

3.4.1 Introduction

Biotransformation of the *para* substituted compound 4-(cyanomethyl) benzonitrile **(c)** was also studied to investigate the effect of ring substitution on the biotransformation. Smaller scale biotransformations were carried out using benzonitrile and propionitrile grown cells (10mM). Reactions were carried out in phosphate buffer (100mM, pH 7.3) at 30°C. As with 2 and 3-(cyanomethyl) benzonitrile **(a)** and **(b),** it was found that 10mM of ammonia was produced from 10 mM substrate, equivalent to one cyano group being hydrolysed. Again larger scale biotransformations were carried out to enable product recovery.

3.4.2 Propionitrile and benzonitrile grown cells

Using HPLC analysis one major and one minor product (possibly the diacid) was formed over the course of the reaction. Product purification and recovery was carried out and after analysis by ^{1}H NMR and HMQC the major product was identified as 4-(cyanomethyl) benzoic acid **(m).**

Recovery of the product was 50%-65% that of the starting substrate. Biotransformation of 3 and 4-(cyanomethyl) benzonitrile **(b) and (c)** showed the aromatic cyano group was hydrolysed in preference to the aliphatic cyano group, unlike 2-(cyanomethyl) benzonitrile **(a).** This indicated that the aromatic cyano group in the *ortho* position of 2-(cyanomethyl) benzonitrile **(a)** could play an important role in determining the biotransformation. A scheme of the reaction is given in figure 3.10.

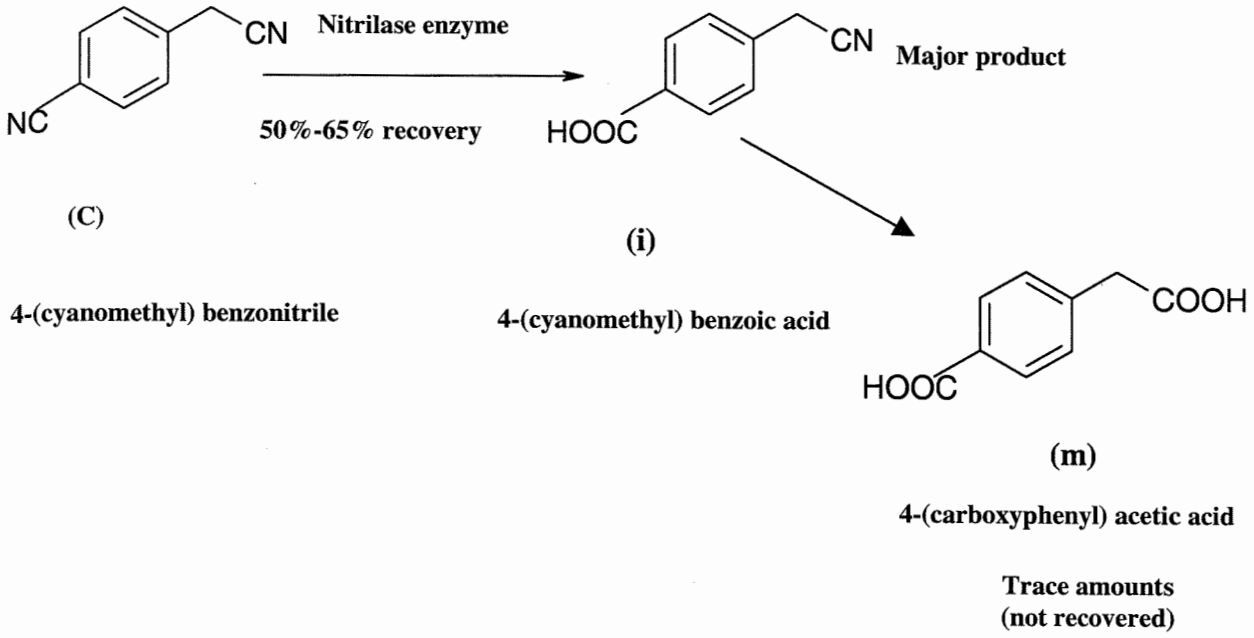


Figure 3.10: Biotransformation of 4-(cyanomethyl) benzonitrile using *Rhodococcus rhodochrous* LL100-21 that had been grown on benzonitrile and propionitrile (10mM). Reactions were carried out using suspensions of bacteria which were concentrated 10 fold in phosphate buffer (100mM, pH 7.3) at 30°C.

3.4.3 Acetonitrile grown bacteria

Previous studies (section 3.1.4 and 3.3.3) had suggested that *R. rhodochrous* grown on acetonitrile (inducing the two step pathway) showed no regiospecificity towards cyanomethyl benzonitrile compounds. Therefore, the biotransformation of 4-(cyanomethyl) benzonitrile (**c**) was carried out to investigate effects when the bacteria had previously been grown on acetonitrile. A small-scale biotransformation produced 10 mM ammonia suggesting one cyano group had been hydrolysed.

When a larger scale biotransformation and product recovery was carried out HPLC and ^{1}HNMR spectra suggested the major product recovered (only 1 %) was 4-(cyanophenyl) acetic acid (**f**).

A scheme of the reaction can be seen in figure 3.11. Results confirm that the nitrile hydratase and amidase enzyme show no regiospecificity towards cyanomethyl benzonitrile compounds when bacteria are grown on acetonitrile.

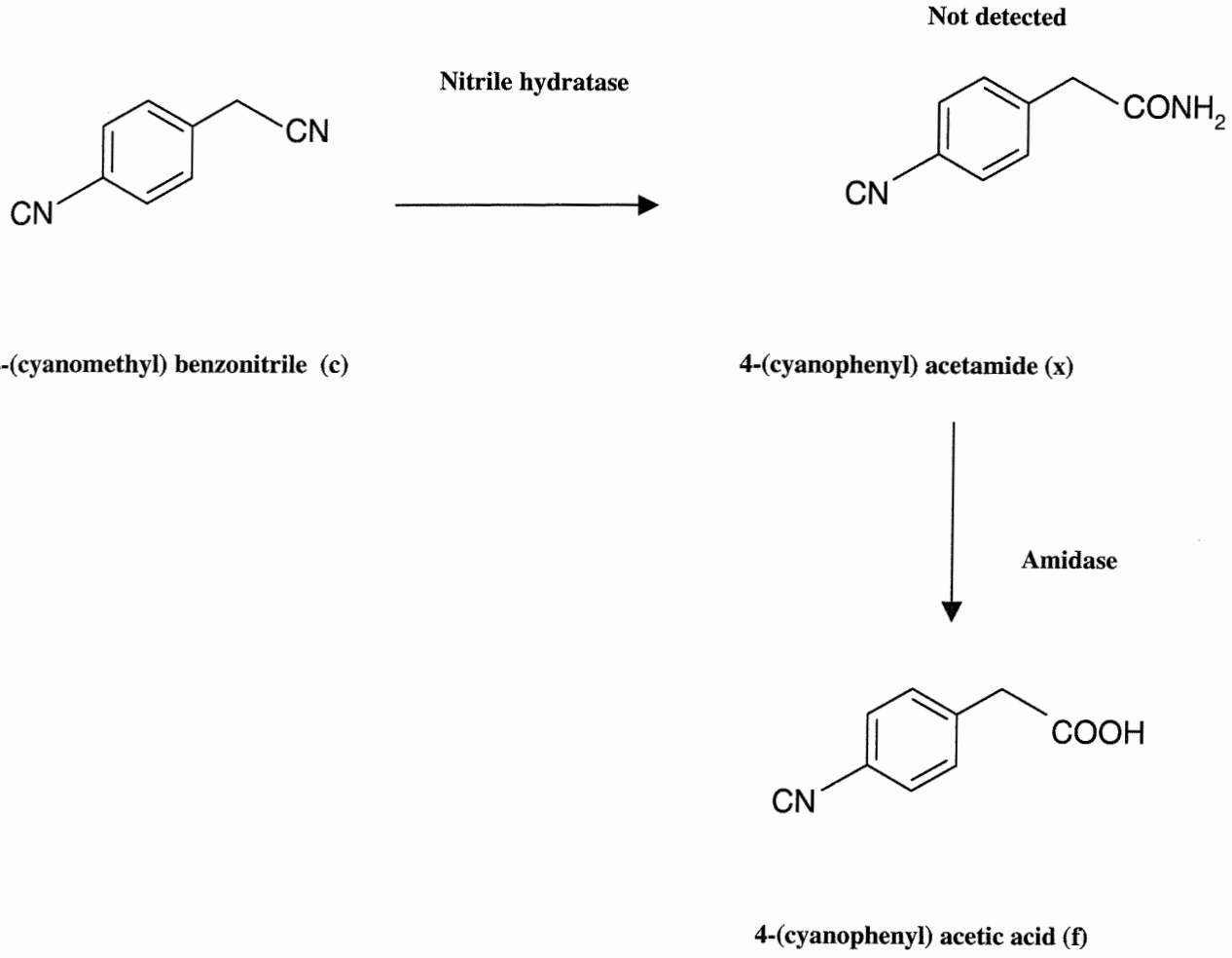


Figure 3.11: Biotransformation of 4-(cyanomethyl) benzonitrile using suspensions of bacteria (harvested OD_{600} 0.4-0.8, concentrated 10 fold) that had been grown on acetonitrile (10mM). Reactions were carried out in phosphate buffer (100mM, pH 7.3) at 30°C. Results suggest acetonitrile grown bacteria show no regioselectivity towards the substrate confirming previous results.

3.5 Biotransformation of 2-, 3- and 4-methyl benzonitrile

As a qualitative comparison to the previous study, 2-, 3- and 4-methyl benzonitrile **(r)**, **(s)** and **(t)** were used to investigate steric hindrance and/or electronic effects on product formation using a suspension of bacteria that had been grown on either propionitrile or benzonitrile (10mM). Reactions were carried out in phosphate buffer (100mM, pH 7.3) at 30°C. Both 3- and 4-methyl benzonitrile were hydrolysed to release ammonia following growth on propionitrile and benzonitrile, but there was no hydrolysis of 2-methyl benzonitrile (figure 3.12). These data complement results obtained with 2-, 3- and 4-(cyanomethyl) benzonitrile **(a)**, **(b)** and **(c)**.

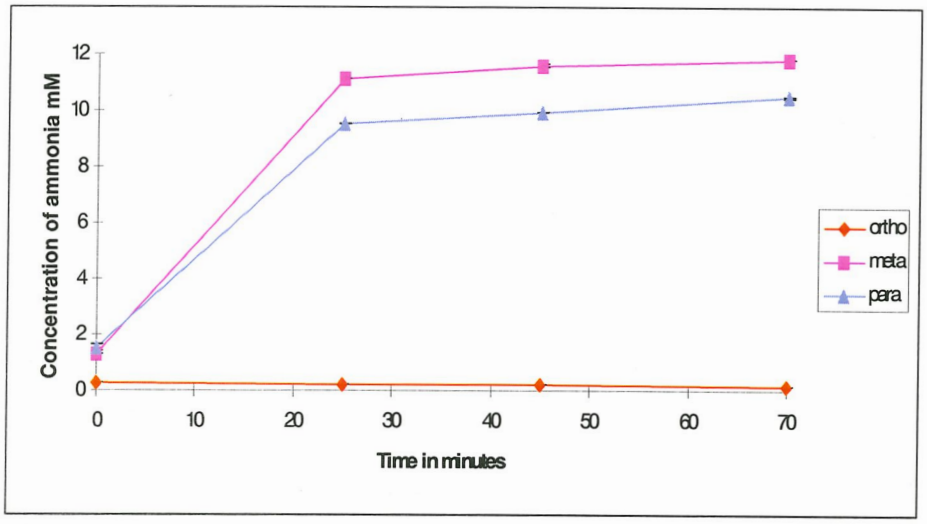


Figure 3.12: Biotransformation of 2-, 3- and 4-methyl benzonitrile (r) (s) (t) (10mM). 3-and 4- methyl benzonitrile were biotransformed, releasing approximately 10mM ammonia. In contrast 2-methyl benzonitrile produced only negligible amounts of ammonia therefore suggesting steric hindrance was a factor in the biotransformation. These data verify previous results using 2-, 3- and 4-(cyanomethyl) benzonitrile.

Ortho = 2-, Meta = 3- , Para = 4-

3.6 Biotransformation of phenylacetonitrile

As an introduction to initial rate studies, the biotransformation of phenylacetonitrile (10 mM) (**n**) was undertaken. Following the growth of bacteria on benzonitrile or propionitrile, HPLC analysis (using the appropriate reference compounds) was carried out during the course of the reaction. Phenylacetonitrile (**n**) (10 mM) was biotransformed to phenylacetic acid (**o**) in both cases. Furthermore, the substrate was fully converted within 22 h, with stoichiometric production of ammonia (figure 3.13).

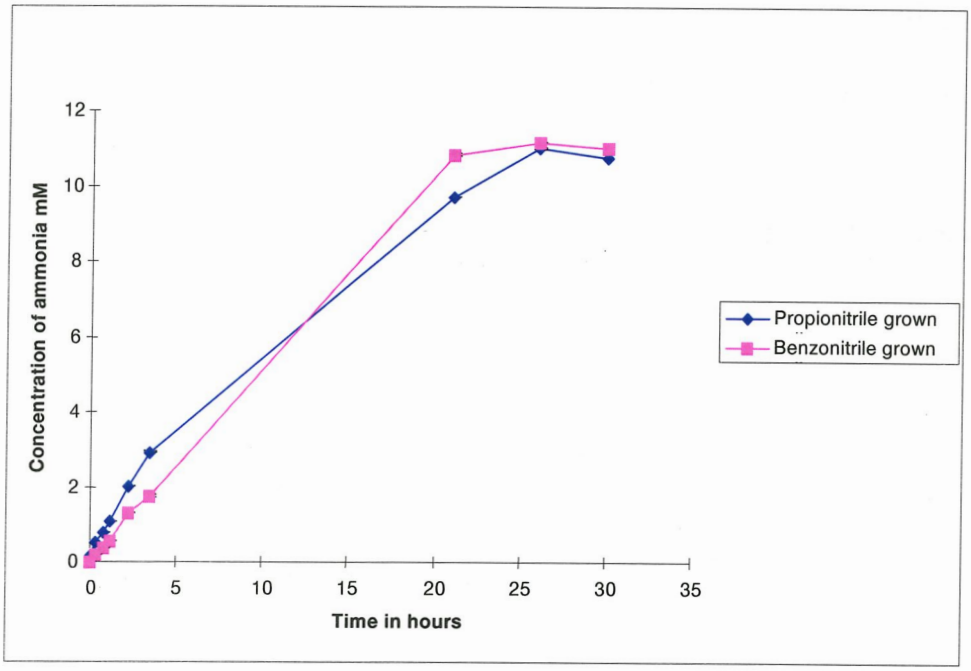


Figure 3.13: Biotransformation of phenylacetonitrile (10mM). Full conversion of substrate to product was seen in approximately 22 h by the production of ammonia in the supernatant. Bacteria were grown to the same concentration of cells on propionitrile and benzonitrile and were resuspended in phosphate buffer (100mM, pH 7.3). Reactions were carried out at 30°C. Error bars are a range of duplicate samples.

3.7 Initial rate studies

To investigate the electronic effects of ring substitution initial rates of utilisation of the following compounds were compared: 2-(cyanomethyl) benzonitrile **(a)**, 2- (bromophenyl) acetonitrile **(q),** 2-(methylbenzyl) cyanide **(p)** and phenylacetonitrile **(n).** Bacterial suspensions were grown on benzonitrile (10mM) to induce the one step nitrilase pathway.

Initial rates were calculated for 10 minutes as μ mols ammonia released/minute/mg dry weight bacteria (table 3.1). The initial rate of 2-(cyanomethyl) benzonitrile **(a)** hydrolysis was 7 times faster than phenylacetonitrile, indicating the presence of the aromatic cyano group increases the initial rate of hydrolysis. In addition, reactivity was found to be CN>Br>CH_3>H) indicating that the presence of a cyano or bromo group in the *ortho* position affects the initial rate of the reaction, possibly by interaction with the active site of the enzyme.

Table 3.1: Comparison of initial rates of hydrolysis of (cyanomethyl) benzonitrile compounds by the production of ammonia in the supernatant. Bacteria were grown on benzonitrile (10mM) and resuspended in phosphate buffer (100mM, pH 7.3). Reactions were carried out at 30°C. Results are given as mean ± range of triplicate samples.

Substrate	Initial rate: μ mols ammonia released/minute/mg dry weight bacteria
2-(cyanomethyl) benzonitrile	0.093 ± 0.005
2-(bromophenyl) acetonitrile	0.077 ± 0.005
phenylacetonitrile	0.014 ± 0.005
2-(methylbenzyl) cyanide	0.040 ± 0.005

Initial rates of substrate conversion using 3- and 4-(cyanomethyl) benzonitrile were calculated (table 3.2) for bacteria that had been grown on propionitrile (10mM). The results showed similar rates of utilisation of both substrates suggesting steric hindrance played no part during these bioconversions. These data were not comparable to the previous substrates as a different group was hydrolysed (the aromatic cyano group) during the biotransformation.

Table 3.2: Comparison of the initial rates for the biotransformation of 3-(cyanomethyl) benzonitrile (b) and 4-(cyanomethyl) benzonitrile (c) by *Rhodococcus rhodochrous* LL100-21 that had been grown on propionitrile (10mM). Rates were similar, in both cases the aromatic cyano group was preferentially hydrolysed. Results are given as mean ± range of triplicate samples

Substrate	Rate μ mols ammonia/minute /mg dry weight bacteria
3-(cyanomethyl) benzonitrile	4.55 ± 0.005
4-(cyanomethyl) benzonitrile	4.33 ± 0.005

3.8 Growth of Rhodococcus rhodochrous LL100-21 on aliphatic and aromatic mononitriles

To investigate growth on aliphatic /aromatic nitriles and enzyme induction *R. rhodochrous* LL100-21 was grown on benzonitrile (10 mM), acetonitrile (20 mM) or propionitrile (20 mM) (sole source of carbon and nitrogen) or on succinic acid (20 mM) with ammonium chloride (10 mM).

Growth was recorded over a period of 28 hours by monitoring optical density at 600 nm (figure 3.14). As a negative control a flask containing no nitrile was used; no growth was recorded in this flask. Specific growth rates and doubling times were calculated from growth curves (converted to log scale) and are shown in table 3.3.

R. rhodochrous LL100-21 grew well with benzonitrile, propionitrile or acetonitrile as a sole source of carbon and nitrogen. Propionitrile-, benzonitrile- and acetonitrile-grown cells had similar specific growth rates (μ) and doubling times (t_d) (table 3.3).

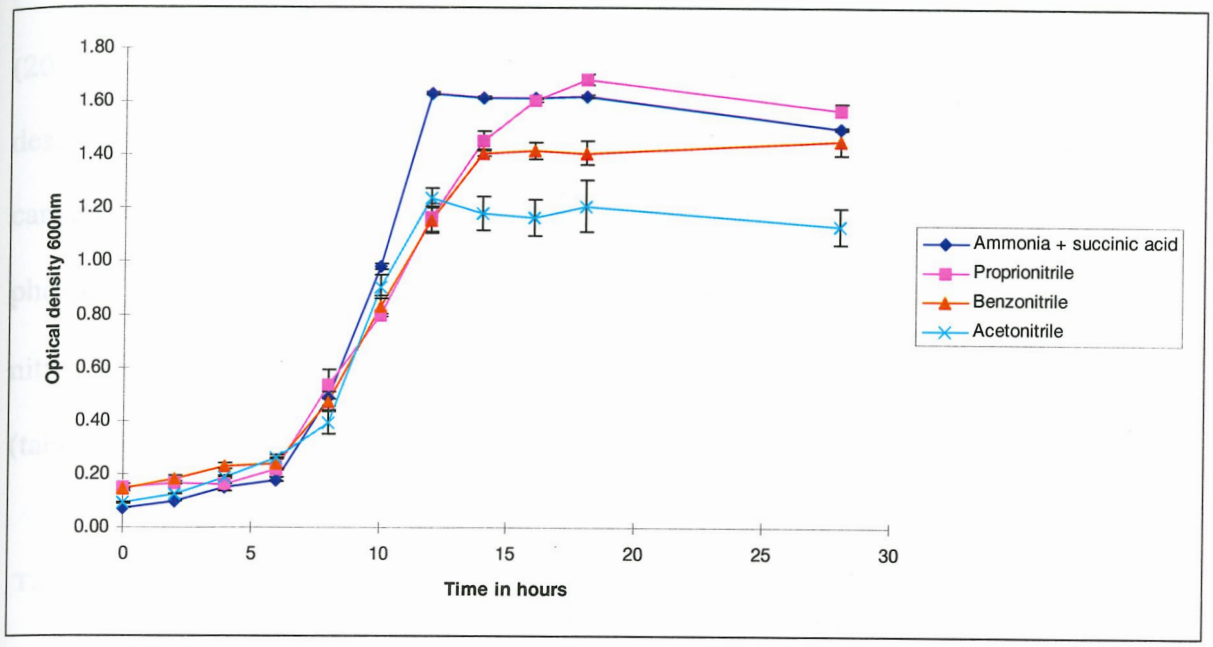


Figure 3.14: Growth curves for the bacterium *Rhodococcus rhodochrous* LL100-21 grown on various aliphatic and aromatic nitriles. Error bars are standard error and were calculated using triplicate samples.

Table 3.3: Specific growth rates and doubling times of the soil bacterium *Rhodococcus rhodochrous* LL100-21 when grown on the carbon and nitrogen sources indicated. Ammonia and succinic acid gave the highest specific growth rate indicating that they were the preferred substrates for growth. Results are given as ± range of triplicate measurements.

Growth medium	Specific growth rate μ (h^{-1})	Doubling time t_d (hours)
Ammonia and succinic acid	0.3 ± 0.005	2.4 ± 0.005
Benzonitrile	0.2 ± 0.005	4.6 ± 0.005
Propionitrile	0.2 ± 0.005	4.5 ± 0.005
Acetonitrile	0.2 ± 0.005	3.3 ± 0.005

3.8.1 Enzyme induction by benzonitrile, propionitrile and acetonitrile

R. rhodochrous LL100-21 was grown on benzonitrile (10 mM), propionitrile (20 mM) or acetonitrile (20 mM). Cells were harvested in early exponential phase as described in the Materials and Methods (section 2.1.2). Biotransformations were carried out with the three nitriles (10 mM in each case) as substrates in potassium phosphate buffer (100 mM), at pH 7.0 and 30 °C and the specific activity of each nitrile was calculated as μ mols ammonia produced /minute/mg dry weight of cells (table 3.4).

Table 3.4: Initial rates of hydrolysis of nitriles by suspensions of harvested bacteria. Values were calculated as μmols of ammonia/min/mg dry wt bacteria.

Growth substrate	Biotransformation substrate: Acetonitrile	Propionitrile	Benzonitrile
Acetonitrile	**0.253**	**0.057**	**<0.01**
Propionitrile	**0.550**	**0.500**	**0.241**
Benzonitrile	**<0.01**	**<0.01**	**0.233**

Table 3.4 suggests that growth on benzonitrile which induces the nitrilase (an enzyme which hydrolyses aromatic nitriles directly to the corresponding carboxylic acid) results in bacteria that are able to preferentially hydrolyse aromatic nitriles.

Acetonitrile induces the nitrile hydratase/amidase enzymes (Collins and Knowles, 1983), and therefore preferentially hydrolyses aliphatic nitriles (acetonitrile and propionitrile) to the amide and then the carboxylic acid (table 3.4).

Propionitrile grown bacteria hydrolysed both aliphatic and aromatic mononitriles suggesting both the nitrile hydratase/amidase and the nitrilase enzymes were induced during growth.

3.9 Growth of Rhodococcus rhodochrous LL100-21 on 2-(cyanomethyl) benzonitrile, 2-(cyanophenyl) acetic acid and 2-(carboxyphenyl) acetic acid

2-(Cyanomethyl) benzonitrile **(a)** could be used by *R. rhodochrous* LL100-21 as a biotransformation substrate. This has been clearly shown earlier in this chapter (section 3.1) by its biotransformation to the corresponding monoacid/mononitrile or amide depending on the nitrile used as an inducer during growth.

Growth experiments were carried out using M9 minimal media to test a range of other nitriles as substrates for growth. Substrates were used as the sole carbon and/or nitrogen source and a (5 %) inoculum of 24 hour stationary phase *R. rhodochrous* LL100-21 that had been previously grown on propionitrile was utilised. The ability to grow was monitored by following the optical density at 600 nm.

No growth was seen on the 2-(cyanomethyl) benzonitrile **(a)** when supplemented with excess carbon in the form of succinic acid i.e, it did not act as a nitrogen source for growth. Therefore, it was concluded 2-(cyanomethyl) benzonitrile **(a)** acts as a substrate for the enzymes but not as growth substrate. When 2-(cyanophenyl) acetic acid **(d)** (sole C+N and N source) was used as a growth substrate for the bacteria no growth was observed. Therefore, it was concluded that the bacteria were either unable to metabolise the ring carbon further or the compound was toxic to the bacteria at the concentrations used.

A control experiment was performed using ammonium chloride (Nitrogen source) with and without 2-(carboxyphenyl) acetic acid (**g**) and no growth was recorded. Consequently, it was concluded that this was again either an inhibitor of the enzymes or toxic to the bacteria (table 3.5).

Table 3.5: Putative growth substrates for the bacterium *R. rhodochrous* LL100-21. No growth was seen on any of the combinations.

GROWTH SUBSTRATE	GROWTH OBSERVED
2-(cyanomethyl) benzonitrile (10 mM) as a sole carbon and nitrogen source	No growth
2-(cyanomethyl) benzonitrile (2 mM) + 50 mM succinic acid as a carbon source	No growth
2-(cyanophenyl) acetic acid (10 mM) (sole carbon and nitrogen source)	No growth
2- (cyanophenyl) acetic acid (10 mM) + 50 mM succinic acid (carbon source)	No growth
2-(carboxyphenyl) acetic acid (10 mM) + 2 mM NH_4Cl	No growth

3.10 Determination of the enzyme kinetics of the nitrilase enzyme of Rhodococcus rhodochrous LL100-21 with 2-(cyanomethyl) benzonitrile as the substrate

Data presented earlier in this chapter indicated that *R. rhodochrous* LL100-21 was unable to grow on, but could transform, the substrate 2-(cyanomethyl) benzonitrile **(a).** Therefore, experiments were designed to investigate the enzyme kinetics of the nitrilase enzyme with this substrate. Bacterial suspensions were used (10 fold concentration of harvested cells (OD_{600} 0.4-0.8)) that had been grown on benzonitrile (10mM) and reactions were carried out in phosphate buffer (100mM, pH 7.3) at 30°C.

Various concentrations (0.5 mM-30 mM) of 2-(cyanomethyl) benzonitrile **(a)** were biotransformed. The optimal concentrations were found to be between 0.5 mM and 3 mM. The initial rate for each concentration of substrate (2-(cyanomethyl) benzonitrile) was plotted as shown in figure 3.15.

The graph has a sigmoidal slope, which suggests a diffusion-limited reaction as the nitrilase enzyme of *R. rhodochrous* LL100-21 is located in the cell. Simple Michaelis Menton parameters were therefore impossible to calculate.

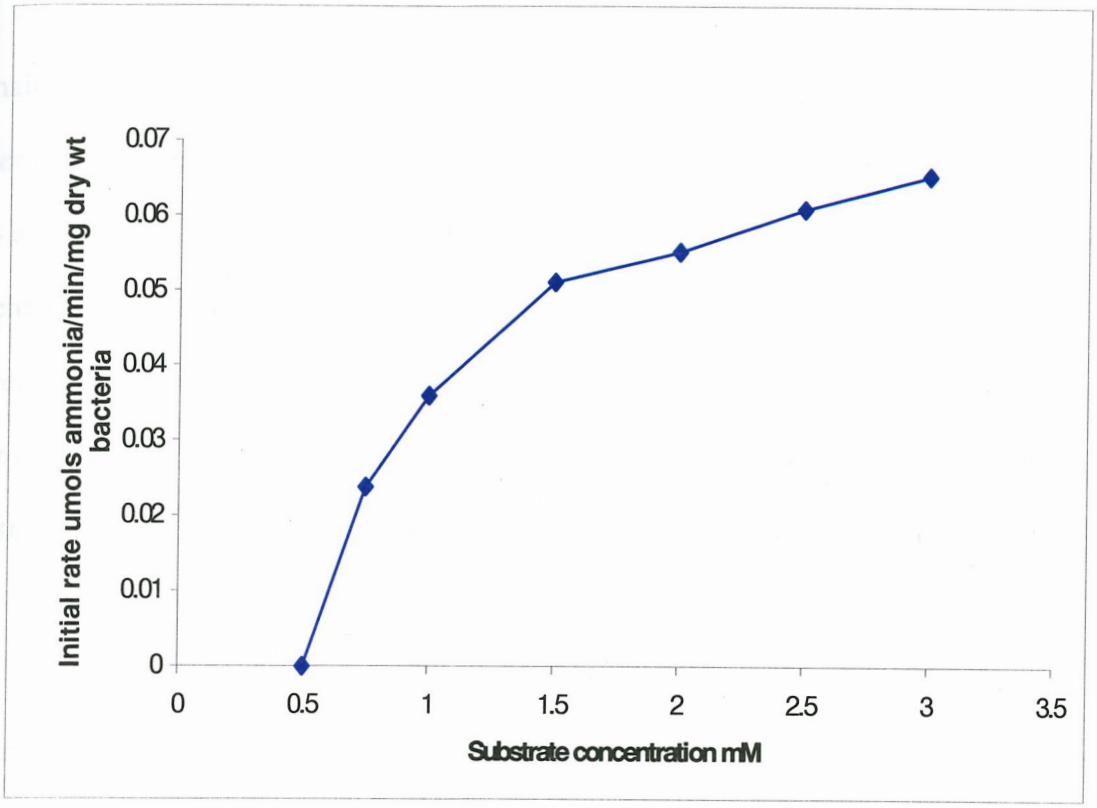


Figure 3.15: Initial rate verses substrate concentration for the nitrilase enzyme towards the substrate 2-(cyanomethyl) benzonitrile (a). Optimal concentrations were found to be between 0.5mM and 3mM.

3.11 Conclusions

3.11.1 Biotransformation of (cyanomethyl) benzonitrile compounds

The hydrolysis of the dinitriles 2-, 3- and 4-(cyanomethyl) benzonitrile **(a)** **(b)** and **(c)** by benzonitrile-, propionitrile- and acetonitrile-induced cells, clearly illustrated that regioselective hydrolysis of nitriles is possible using intact cells of *R. rhodochrous* LL100-21.

Bacteria that had been grown on benzonitrile and propionitrile converted 2-(cyanomethyl) benzonitrile **(a)** to the corresponding mononitrile/monoacid 2-(cyanophenyl) acetic acid **(d)** with a 56-64% recovery.

One possible explanation for this biotransformation is that the aliphatic side chain of the compound prevented the hydrolysis of the aromatic cyano group due to steric hindrance. These data were confirmed by carrying out the biotransformation of 3- and 4-(cyanomethyl) benzonitrile **(b)** and **(c)** using bacteria that had been grown on benzonitrile and propionitrile. In both cases it was the aromatic cyano group that was preferentially hydrolysed to the products 3- and 4-(cyanomethyl) benzoic acid **(j)** and **(m).** In these cases the aliphatic cyano group was not hydrolysed, suggesting that the nitrilase enzyme can only hydrolyse the group when there is a cyano group (or other substituent) in the *ortho* position.

It has been suggested that the nitrilase in *R. rhodochrous* NCIMB 11216 was specific for nitrile groups attached to the benzene ring and aliphatic nitriles were not hydrolysed, (Harper, 1977). In contrast, Hoyle *et al.* (1998) found the biotransformation of dinitriles gave the lowest activity toward the aromatic nitrile and further suggested that different inducers lead to distinct nitrilase enzymes.

The presence of the aromatic cyano group, even though not hydrolysed, could also affect the rate of the reaction. Therefore, initial rate studies were carried out to investigate the effects of different functional groups in the *ortho* position. The results indicated the presence of certain groups substituted in the aromatic ring increased the initial rate of hydrolysis. These groups (cyano and bromo) were found to be the most reactive ($CN>Br>CH_3>H$) and this reactivity decreased with the addition of a methyl group or with no ring substitution. Furthermore, the initial rate of 2-(cyanomethyl) benzonitrile **(a)** hydrolysis was 7 times faster than that of phenylacetonitrile, suggesting the cyano group could play an important part in the reaction.

These *ortho* substituted groups could interact with amino acids or metals near or within the active site of the enzyme and aid positioning of the aliphatic nitrile, thus giving greater stabilisation to the enzyme substrate complex. Furthermore, cyano and bromo groups are Lewis bases and therefore could interact with these metallo groups (if present) within the active site.

The effect of ring substitution on the rate of hydrolysis of the aliphatic nitrile group appeared not to explain results, since any electronic effect would require transmission through the SP^3 methylene carbon atom. Rathermore, the results suggest that steric hindrance prevented the hydrolysis of this aromatic cyano group.

Growth of the bacteria on acetonitrile afforded different products during the biotransformation of 2-, 3- or 4-(cyanomethyl) benzonitrile. Acetonitrile grown cells induce the nitrile hydratase/amidase enzymes. The nitrile hydratase enzyme was formed but was unable to act effectively and without regioselectivity towards the substrates. All biotransformations by acetonitrile grown bacteria yielded only small amounts of product after purification and recovery. This further suggested that the nitrile hydratase enzyme showed little affinity with respect to 2-, 3- and 4-(cyanomethyl) benzonitrile **(a)** **(b)** and **(c)** as in each case different biotransformation products were recovered.

These results show that regiospecificity towards (cyanomethyl) benzonitrile compounds can be achieved by the bacterium *R. rhodochrous* LL100-21. Regiospecificity depended on the growth substrate/inducer of the bacteria and more importantly the position of substitution in the aromatic ring.

3.11.2 Growth and induction studies

To investigate growth and enzyme induction using mononitriles, *R. rhodochrous* LL100-21 was grown on propionitrile, benzonitrile and acetonitrile. Each of these substrates were subsequently biotransformed by suspensions of bacteria in phosphate buffer. The bacteria grew well on benzonitrile, propionitrile or acetonitrile as a sole source of carbon and nitrogen. Propionitrile-, benzonitrile- and acetonitrile-grown cells had similar specific growth rates (μ).

Benzonitrile is an aromatic mononitrile, which induces preferentially the nitrilase enzyme (Linton and Knowles, 1986) which hydrolyses aromatic nitriles directly to the corresponding carboxylic acid. This was shown during this study as benzonitrile induced cells would only hydrolyse the benzonitrile as a substrate.

Furthermore, the results showed that acetonitrile induces the nitrile hydratase/amidase enzymes and therefore preferentially hydrolyses aliphatic nitriles to the amide and then the corresponding carboxylic acid. Propionitrile is an inducer of both the above enzyme systems (also documented by Linton and Knowles, 1986). Similar results also have previously been obtained with benzonitrile induced cells (Harper (1976) and Harper (1977)) and for propionitrile induced cells (Gradley *et al.*, 1994).

An exception to this general rule has been documented by other workers (Gradley *et al.*, 1994) using *Rhodococcus rhodochrous* NCIMB 11216, which possesses an inducible enantioselective nitrilase that is capable of biotransforming both aliphatic and aromatic nitriles.

Further to these experiments, growth studies were carried out using 2-(cyanomethyl) benzonitrile (**a**). The results showed that the substrate did not act as a source of nitrogen for growth. Further experiments concluded that 2-(cyanomethyl) benzonitrile (**a**) acts as a substrate for the enzyme but not as a growth substrate. 2-(carboxyphenyl) acetic acid (**g**) was not utilised by the bacteria indicating the substrate either could not enter the cell, was not metabolised further or was toxic to the bacterial cells.

Future work could investigate enzyme induction by growth of the bacteria on succinate and ammonia, harvesting /washing the bacteria, and then testing whether the biotransformation of 2-(cyanomethyl) benzonitrile (**a**) would occur. Zero or low enzyme activity would confirm the substrate was not an inducer. Furthermore, investigation at the genetic level could reveal the presence of these nitrile hydrolysing enzymes in a range of bacteria.

This potentially could be achieved relatively easily by using the Southern blot technique, PCR (c.f. chapter 5) or RT-PCR to investigate specific mRNA species within the bacteria under various induction conditions.

3.11.3 Kinetic studies using a nitrilase with 2-(cyanomethyl) benzonitrile

Previous data within this chapter suggested that *R. rhodochrous* LL100-21 was unable to grow on, but could transform, the substrate 2-(cyanomethyl) benzonitrile (**a**). Therefore, experiments were carried out to investigate enzyme kinetics of the nitrilase enzyme with this substrate. Optimal concentrations were found and the initial rate for each concentration of substrate 2-(cyanomethyl) benzonitrile was plotted. The resulting curve was sigmoidal. Therefore, simple Michaelis Menton kinetics could not be calculated.

These data suggested the reaction was a diffusion limited reaction as the nitrilase enzyme is located in the cell. Future studies could therefore concentrate on the specific nature of these results and elucidate the exact type of kinetics that occur.

Chapter 4: Real-time monitoring of nitrile biotransformations by mid infrared spectroscopy

4.1 Introduction

In this study, mid infrared spectroscopy was used to study the enzyme kinetics involved in nitrile biocatalysis using whole cell suspensions of the bacterium *Rhodococcus rhodochrous* LL100-21. The reactions were followed by React IR MP using either a diamond or a silicon probe. Mid infrared spectroscopy is a novel technique and has potentially useful applications in biocatalysis.

4.2 Initial study using a diamond Comp probe

R. rhodochrous was grown on acetonitrile (10mM) to induce the two step nitrile hydratase/amidase pathway (Collins and Knowles, 1983). Acetonitrile (250mM) was then biotransformed using a suspension of bacteria in phosphate buffer (pH 7.3) at 30°C. The reaction was monitored using React IR MP and a diamond mid infrared probe. The resulting spectra showed the biotransformation product, acetamide, 1667.5 cm^{-1}/1598.0 cm^{-1} (amide I and II bonds) was converted to acetic acid 1553.5 cm^{-1}/1416.6 cm^{-1}. Acetic acid was observed as two peaks because the final pH (7.95) of the supernatant caused the carboxylate ion to be formed. The diamond probe has a natural absorbency band in the triple and cumulated double bond region 1900-2200 cm^{-1}, and therefore disappearance of the nitrile was impossible to follow. Further experiments were designed to use a silicon probe to illustrate the nitrile disappearance and overcome the problem of the diamond probe cut off region.

Benzonitrile (10mM) and 2-(cyanomethyl) benzonitrile (10mM) were biotransformed using a suspension of bacteria that had been grown on benzonitrile (10mM) (to induce the one step nitrilase pathway). However, the insolubility of these nitriles in the aqueous phase made spectra obtained impossible to follow.

Consequently, for subsequent experiments water solubility profiles were carried out to determine which of a range of nitriles were suitable for further study. Only those nitriles sufficiently water soluble to permit detection by the React IR MP were selected for further investigation.

4.3 Biotransformation of acetonitrile

4.3.1 Biotransformation of acetonitrile by bacteria grown on acetonitrile

Bacteria were grown on acetonitrile (10 mM) to induce the two-step nitrile hydratase/amidase pathway (Collins and Knowles, 1983). Figure 4.1 illustrates the initial conversion of acetonitrile at 2262.0 cm^{-1} to acetamide at 1667.5cm^{-1} /1598.0 cm^{-1} and then more slowly to acetic acid at 1553.5 cm^{-1}/1416.6 cm^{-1} by suspensions of harvested bacteria in phosphate buffer (pH 7.3). Transient formation of the characteristic amide I and amide II bonds was detected. Two other peaks were subsequently detected, and corresponded to the formation of the final product, i.e. the carboxylate ion of acetic acid. The acetonitrile peak (2262cm^{-1}; figure 4.1) was very small and was explained by either the low extinction coefficient of acetonitrile or rapid utilisation of the substrate.

A profile generated from these data (figure 4.2) illustrates both steps of the biotransformation. There was initial conversion of the nitrile into the amide by the nitrile hydratase enzyme, followed by a slower formation of the carboxylic acid from the amide by the amidase enzyme.

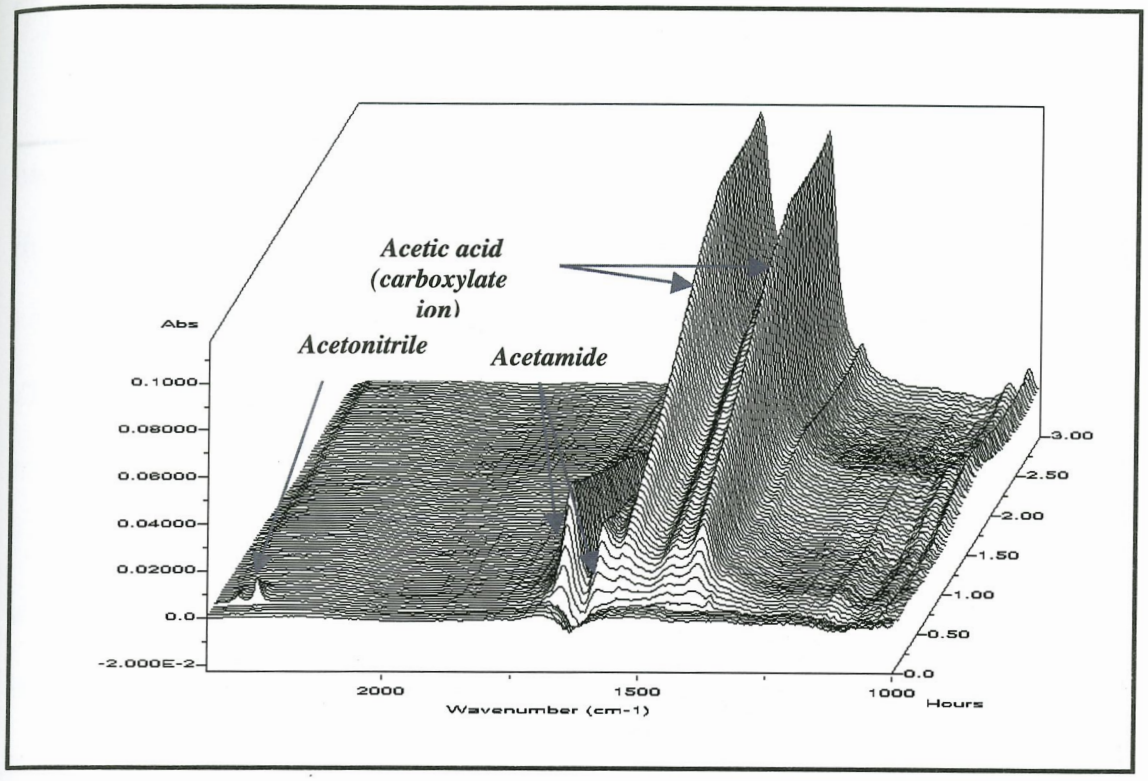


Figure 4.1: Real-time 3D spectrum of acetonitrile (250mM) biotransformation by suspensions of *Rhodococcus rhodochrous* LL100-21 that had been grown on acetonitrile (10mM). Suspensions were harvested (OD_{600} 0.4-0.8) and concentrated 10 fold. Reactions were carried out at 30 °C in phosphate buffer (pH 7.3). The two-step nitrile hydrolysing pathway was induced, which can be seen to convert the nitrile to amide and then to the corresponding carboxylic acid. Monitoring was achieved using a React IR MP with silicon mid infrared probe attached.

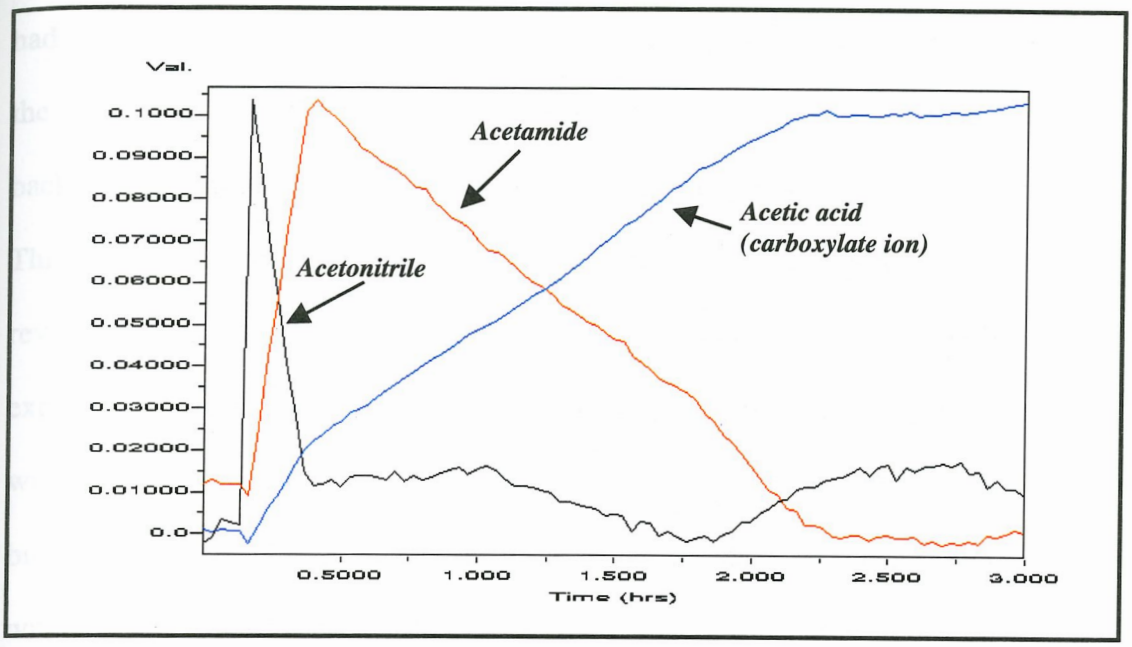


Figure 4.2: Real time profile generated from the spectral data (figure 4.1) of the biotransformation of acetonitrile (250 mM) by suspensions of *Rhodococcus rhodochrous* LL100-21 that had been grown on acetonitrile. The nitrile was converted to the amide via the nitrile hydratase enzyme and then to the carboxylic acid by the amidase within a 2.5 hour period.

4.3.2 Biotransformation of acetonitrile by bacteria grown on benzonitrile

Acetonitrile (250 mM) was biotransformed using suspensions of bacteria that had been grown on benzonitrile (10mM). Unknown peaks were seen to appear over the time course of the reaction. Therefore, spectra results were compared to a background of water vapour and it was concluded the probe had been contaminated. This made the interpretation of the biotransformation difficult. Initial analysis revealed the one-step nitrilase pathway could be hydrolysing the substrate but further experiments need to be carried out. The experiment was repeated using nitrogen dried with silica gel, but once again a spectrum of water vapour obscured the nitrile biotransformation. Therefore, future experiments will have to consider this problem within the experimental set-up of the probe.

4.3.3 Biotransformation of acetonitrile by bacteria grown on propionitrile

R. rhodochrous LL100-21 was grown on propionitrile to induce both the aliphatic and aromatic enzyme systems (Linton and Knowles, 1986). The cells were harvested in mid exponential phase and concentrated 10 fold in potassium phosphate buffer (100 mM, pH 7.3) at 4 °C.

The biotransformation of acetonitrile (250 mM) was monitored using React IR MP and a silicon probe. The final spectrum showed rapid hydrolysis of acetonitrile 2262.0 cm^{-1} and the formation of acetamide 1667.5cm^{-1}/1598.0 cm^{-1} (both amide I and amide II bonds, followed by formation of acetic acid 1553.5 cm^{-1}/1416.6 cm^{-1} (carboxylate ion) (figure 4.3). Figure 4.4 illustrates both steps of this reaction, clearly showing hydrolysis of acetonitrile by the nitrile hydratase and amidase enzymes.

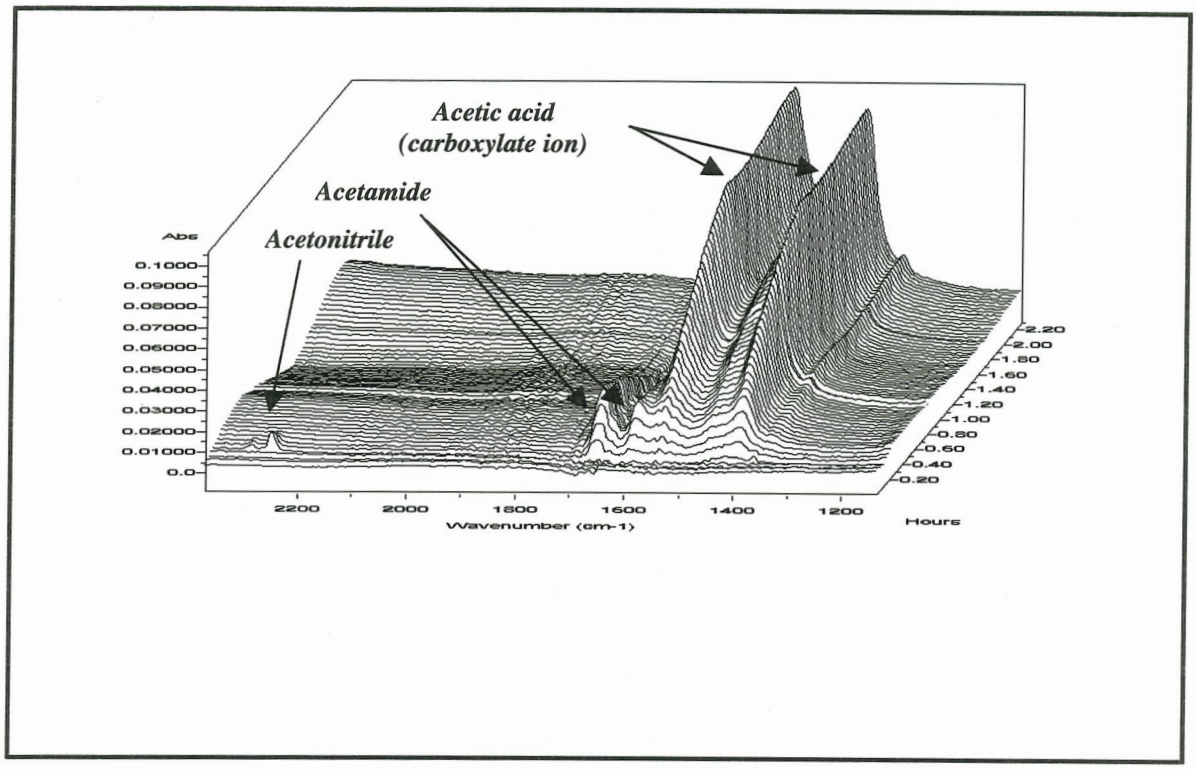


Figure 4.3: Biotransformation of acetonitrile (250 mM). The reaction was carried out at 30°C with a suspension of bacteria (OD_{600} 0.4-0.8/ 10-fold concentration) that had been grown on propionitrile (10mM). The spectrum shows the two-step pathway of nitrile biodegradation (nitrile hydratase and amidase enzymes).

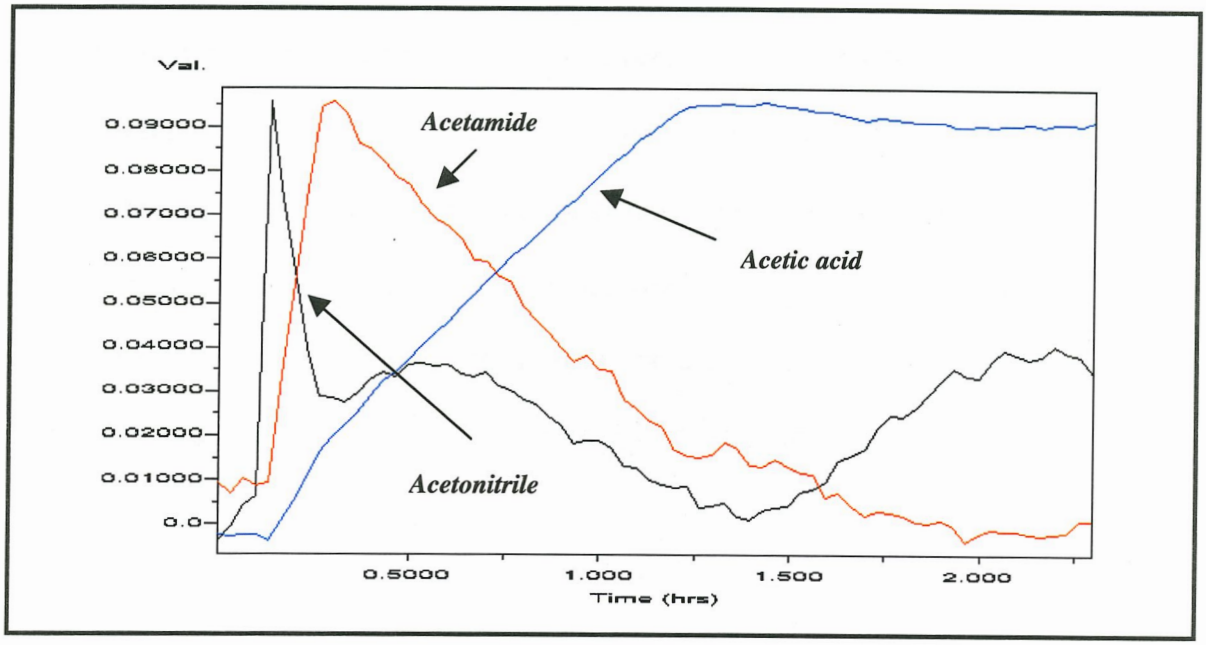


Figure 4.4: Real-time reaction profile generated from figure 4.4 showing the biotransformation of acetonitrile (250 mM) by bacteria that had been grown on propionitrile (10mM). Acetonitrile was hydrolysed within 1.5 hours by conversion to acetamide by a nitrile hydratase enzyme and then to acetic acid (carboxylate ion) by an amidase.

4.4 Biotransformation of benzonitrile

4.4.1 Biotransformation of benzonitrile by bacteria grown on acetonitrile

Bacteria were grown on acetonitrile (10mM) to induce the two step nitrile-degrading pathway (Collins and Knowles, 1983). The cells were concentrated 10 fold in phosphate buffer (pH 7.3) at 4°C, and the biotransformation of benzonitrile (250mM) was carried out at 30°C. The reaction was monitored using React IR MP using a silicon probe. From the resulting 3D spectrum (not shown) it was impossible to determine the stages of the reaction, as again the probe became contaminated with water vapour. The experiment was repeated twice and each time similar results were observed.

4.4.2 Biotransformation of benzonitrile by bacteria grown on benzonitrile

This is an example of the one step nitrilase pathway, again monitored in real time. Benzonitrile has three characteristic peaks at 2231.0 cm^{-1}, 1489.8 cm^{-1} and 1447.5 cm^{-1}, and was stoichiometrically degraded over a 6-hour period (Figure 4.5).

The benzonitrile appeared to be converted directly to benzoic acid (carboxylate ion) at 1540.1 cm^{-1} and 1389.6 cm^{-1} as no benzamide peak(s) were detected. The small benzonitrile peaks observed (figure 4.5) could be due to either the low extinction coefficient of benzonitrile compared to the products, or the low solubility of benzonitrile in the aqueous phase.

A real time profile of this data was produced and clearly illustrates only two species, benzonitrile and benzoic acid, were present in the medium during the reaction with no sign of a benzamide signal (figure 4.6).

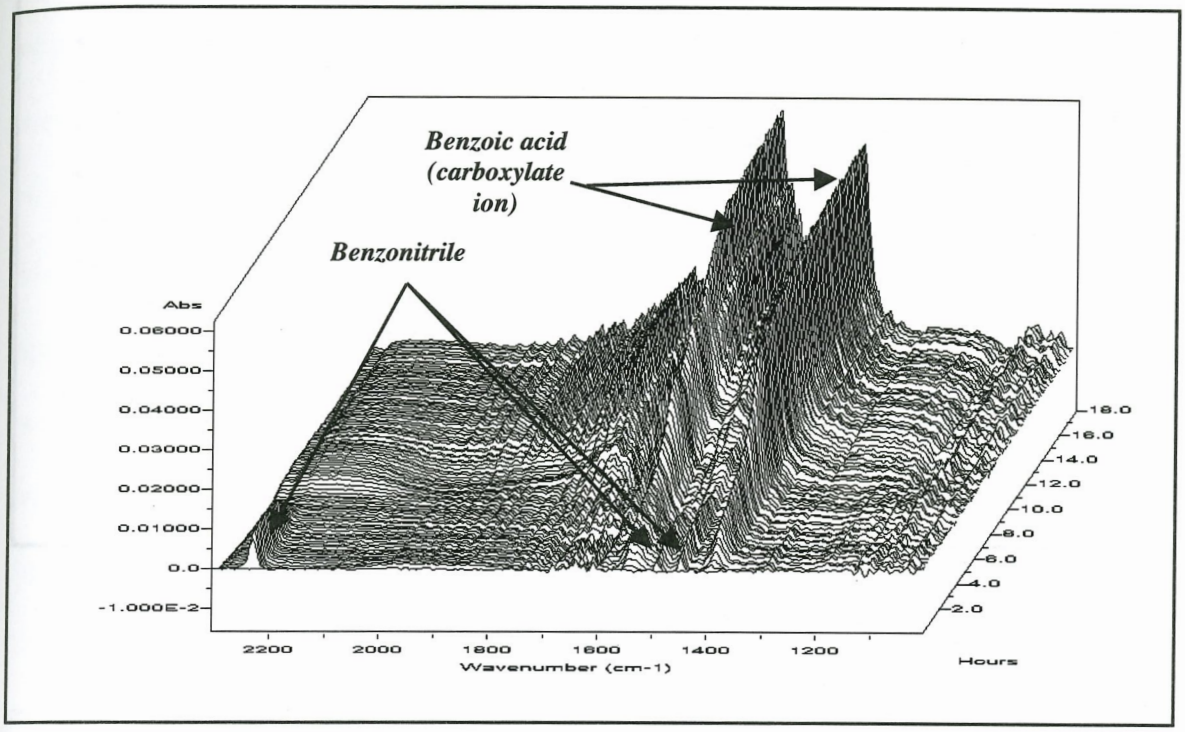


Figure 4.5: Real time 3D spectrum of the biotransformation of benzonitrile (250mM) by suspensions of *Rhodococcus rhodochrous* LL100-21 (OD_{600} 0.4-0.8 /concentrated 10 fold) that had been grown on benzonitrile (10mM). Other peaks within the spectrum were caused by water vapour contamination within the probe.

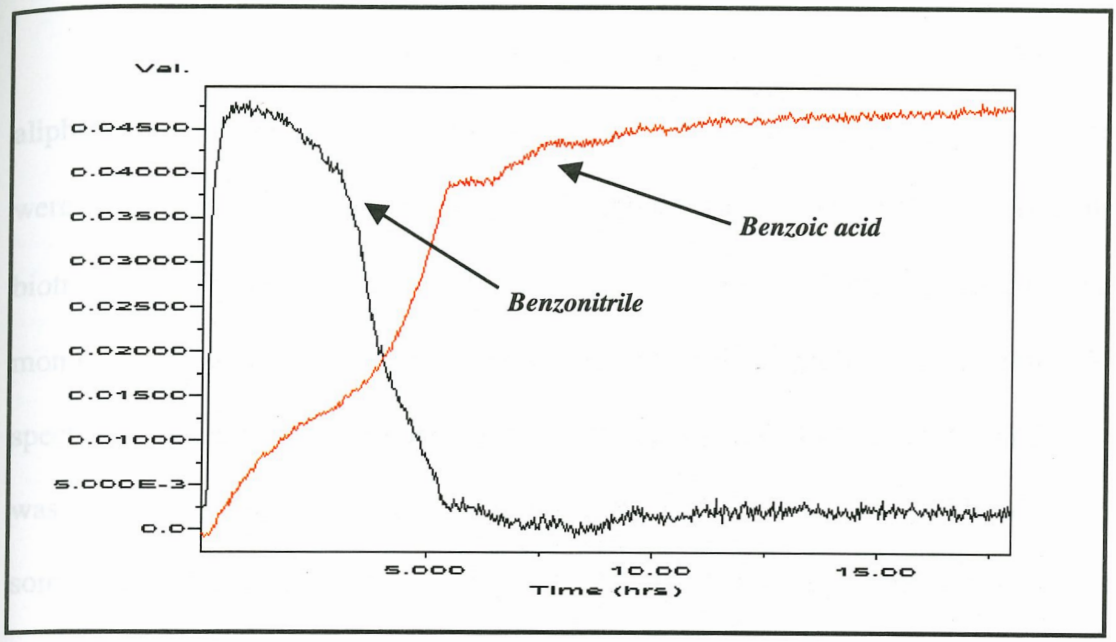


Figure 4.6: Real time profile generated from figure 4.6 showing conversion of the benzonitrile (250mM) directly to benzoic acid within a 6 hour period.

4.4.3 Biotransformation by bacteria grown on propionitrile

R. rhodochrous LL100-21 was grown on propionitrile to induce both the aliphatic and aromatic enzyme systems (Linton and Knowles, 1986). The bacteria were harvested, concentrated at 4°C in phosphate buffer (pH 7.3) and the biotransformation of benzonitrile (250mM) was carried out at 30°C. The reaction was monitored by mid infrared spectroscopy using a silicon probe. The resulting 3D spectrum (figure 4.7) showed that benzonitrile (2231.0 cm^{-1}/1489.8 cm^{-1}/1447.5 cm^{-1}) was converted directly to benzoic acid (1540.1 cm^{-1}/1389.6 cm^{-1}). The data are somewhat confusing: Figure 4.7 suggests that there was an initial lag period of approximately 4 hours. Conversely, figure 4.8 shows benzoic acid production from the start of the reaction. This lag could be due to the relatively low solubility of the benzonitrile in the aqueous phase, (Stephenson, 1994). This low solubility is illustrated by the increase of benzonitrile detected during the first two hours (figure 4.8). Although both enzyme pathways are induced, the results suggest that only the nitrilase enzyme biotransformed the substrate.

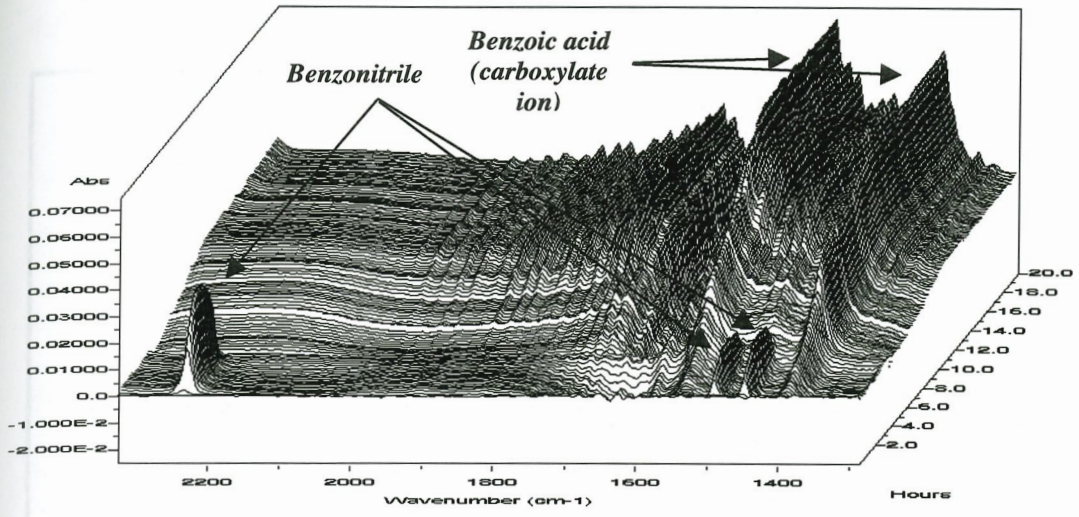


Figure 4.7: Real-time 3D spectrum of benzonitrile (250 mM) biotransformation by suspensions bacteria that had been grown on propionitrile (10mM). This growth substrate induced both the one and two step nitrile hydrolysing pathways. The spectrum suggests only the one step nitrilase pathway was active in hydrolysis of the substrate, as it was converted directly to benzoic acid. Furthermore, an apparent lag period was seen (4 hours) which could be due to the low solubility of the benzonitrile. Other peaks are due to water contamination in the probe.

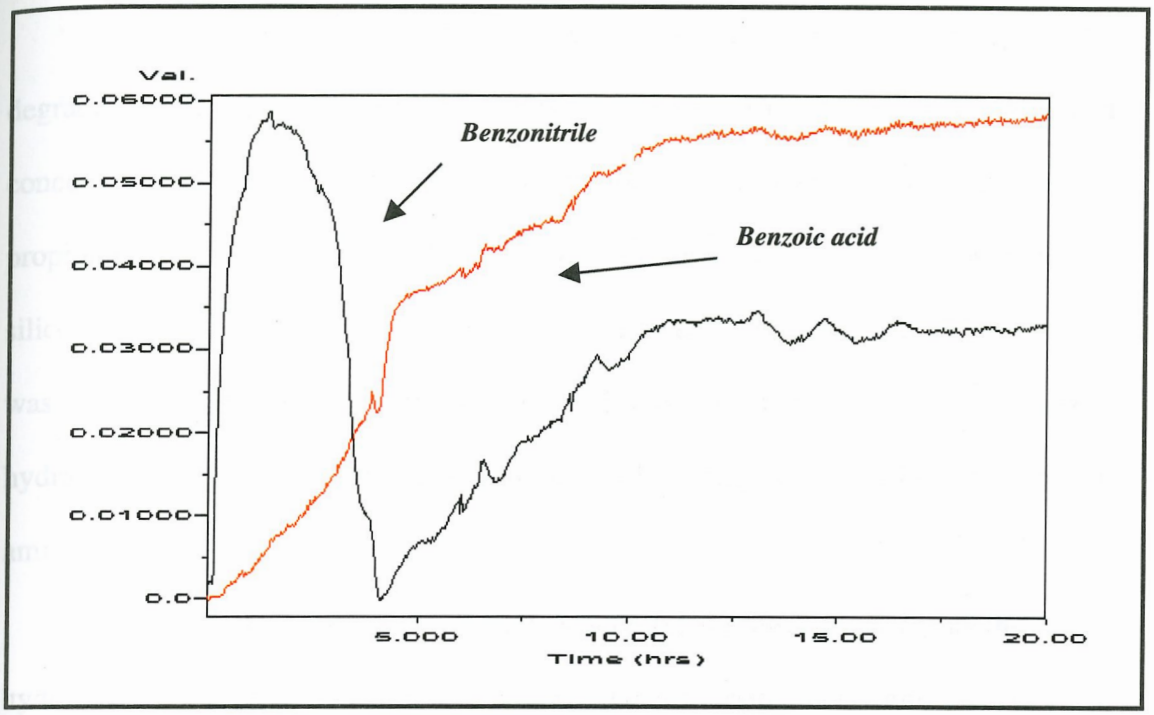


Figure 4.8: Real-time reaction profile generated from the data in figure 4.7 showing conversion of benzonitrile (250mM) to benzoic acid by the nitrilase enzyme. Benzoic acid was produced from time zero. The absorbance of benzonitrile is seen to increase in the first 2 hours due to its insolubility in the aqueous phase.

4.5 Biotransformation of propionitrile

4.5.1 Biotransformation by bacteria grown on acetonitrile

Bacteria were grown on acetonitrile (10mM) to induce the two-step nitrile-degrading pathway (Collins and Knowles, 1983). Cells were harvested and concentrated 10 fold in phosphate buffer (pH 7.3). The biotransformation of propionitrile (250mM) was performed and monitored using React IR MP with a silicon probe. The 3D spectrum (figure 4.9) shows that propionitrile (2254.2 cm^{-1}) was rapidly converted to propionamide (1636.6 cm^{-1}/1652.0 cm^{-1}) by a nitrile hydratase enzyme, and then to propionic acid (1462.9 cm^{-1}/1544.0 cm^{-1}) by an amidase.

The reaction profile figure 4.10 produced from these data suggests the nitrile hydratase conversion is much more rapid than the conversion by the amidase enzyme.

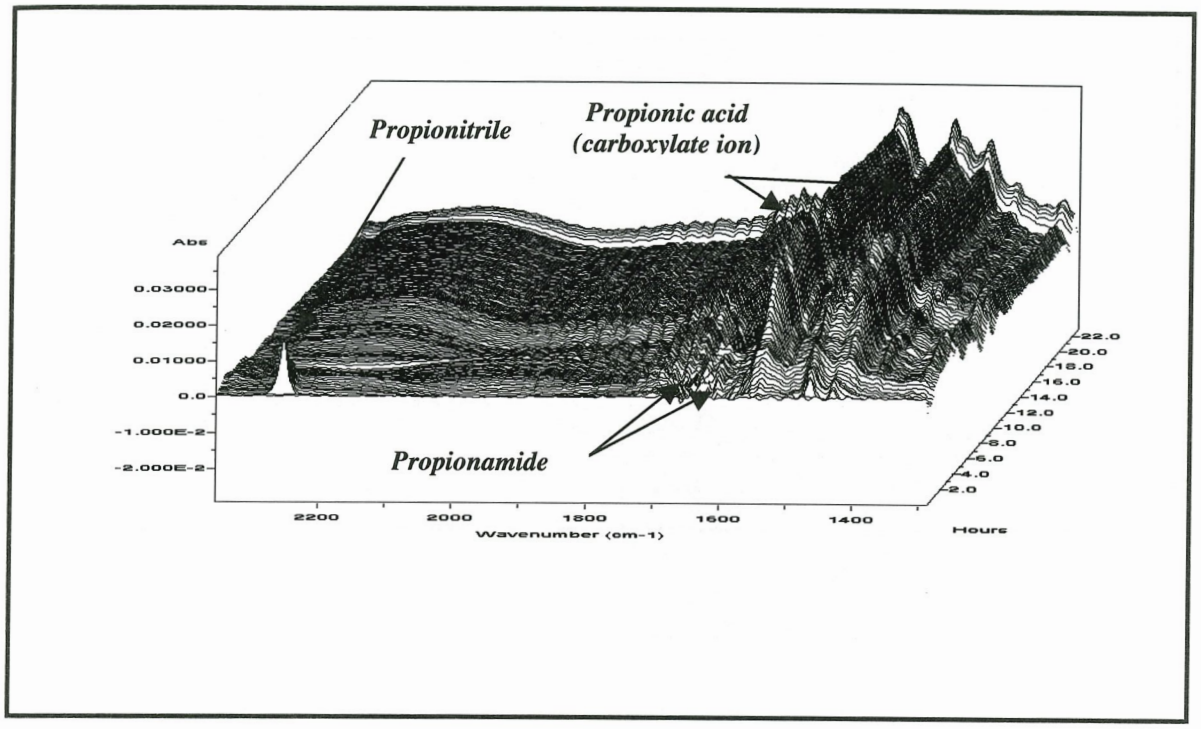


Figure 4.9: Real-time 3D spectra of the biotransformation of propionitrile (250 mM) by suspensions of bacteria (OD_{600} 0.4-0.8/concentrated 10 fold) that had been grown on acetonitrile (10mM). Propionitrile was converted via the amide intermediate by the nitrile hydratase enzyme and then to the acid by the amidase enzyme.

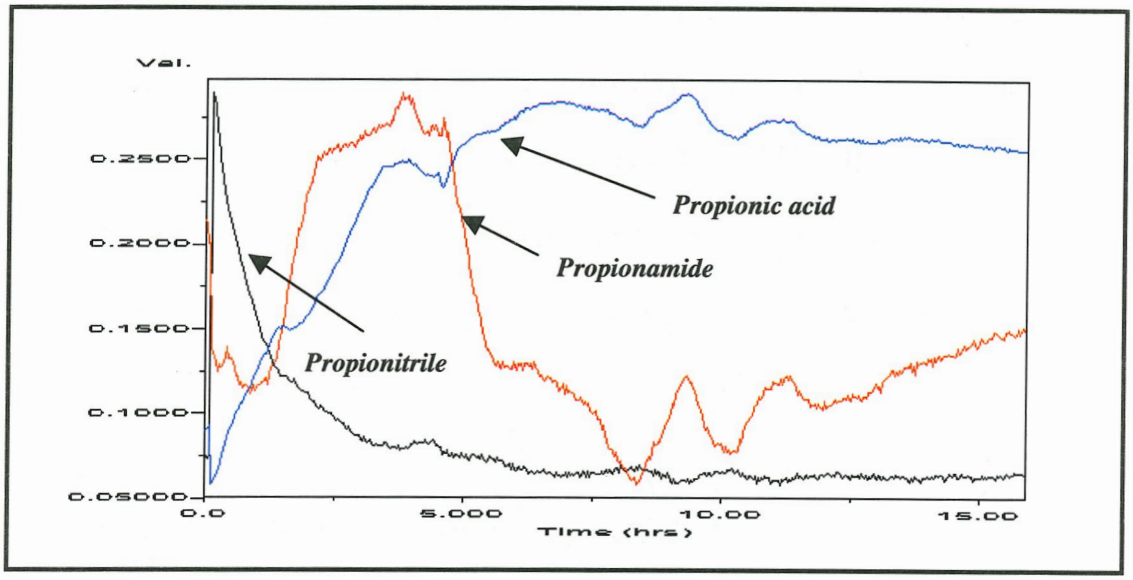


Figure 4.10: Real-time profile of the reaction carried out in figure 4.9 using bacteria grown on acetonitrile (10mM). Propionitrile (250 mM) was hydrolysed to propionamide and then to propionic acid by the two-step nitrile degrading pathway.

4.5.2 Biotransformation by bacteria grown on benzonitrile

R. rhodochrous LL100-21 was grown on benzonitrile (10mM) to induce the one step nitrilase pathway (Collins and Knowles, 1986). Bacteria were harvested and concentrated 10 fold and the biotransformation of propionitrile (250mM) was carried out in phosphate buffer (pH 7.3, 30°C). Unfortunately, the resulting spectrum was contaminated with water vapour and any reaction was therefore impossible to determine.

4.5.3 Biotransformation using bacteria grown on propionitrile

Bacteria were grown on propionitrile (10mM) to induce both the one and two step nitrile degrading pathways (Linton and Knowles, 1983). Figure 4.11 illustrates the conversion of the propionitrile (2254.2 cm^{-1}) to propionic acid (carboxylate ion) (1462.9 cm^{-1}/1544.0 cm^{-1}). No intermediate compounds e.g propionamide were detected suggesting a nitrilase and not the nitrile hydratase/amidase enzymatic pathway had converted the substrate. However, it is also possible that the conversion of propionamide to propionic acid was rapid enough to prevent detection of the amide. Figure 4.12 illustrates the profile for the reaction.

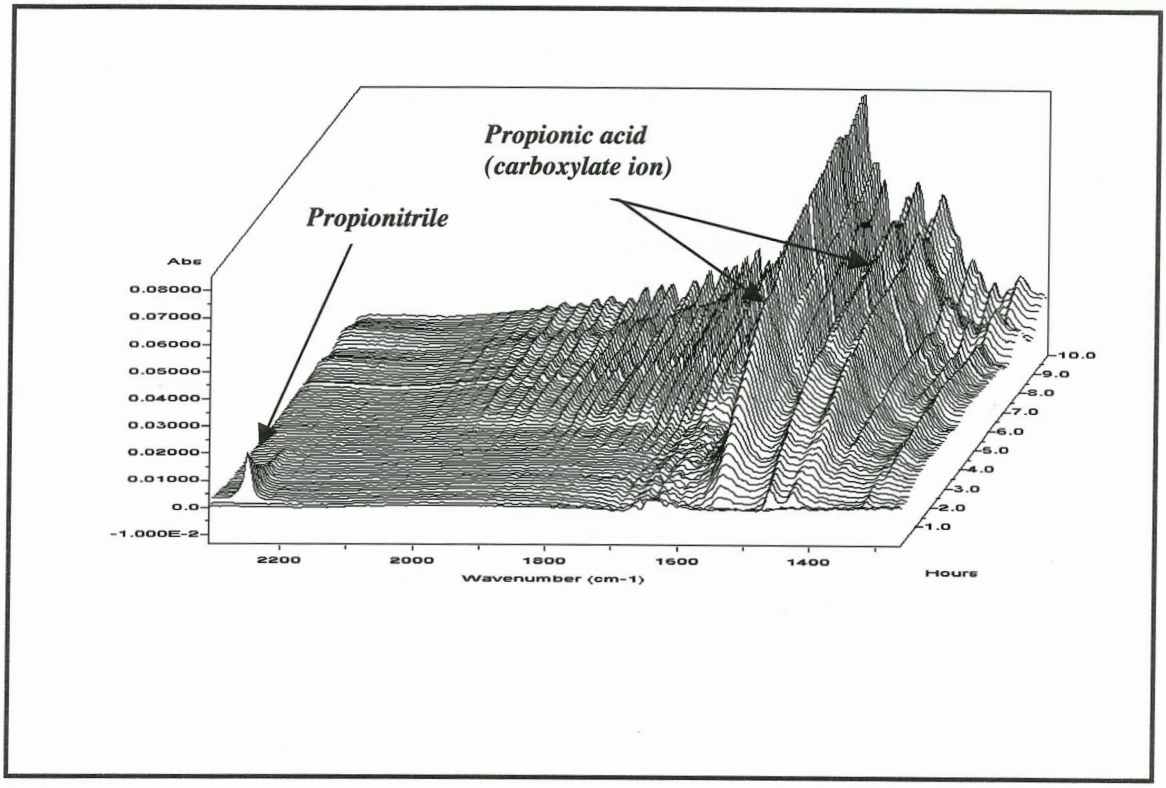


Figure 4.11: Real-time 3D spectrum of propionitrile (250 mM) by suspensions of bacteria (OD_{600} 0.4-0.8 concentrated 10 fold) that had been grown on propionitrile (10mM). The nitrile is seen to be rapidly hydrolysed directly to propionic acid by the nitrilase enzyme.

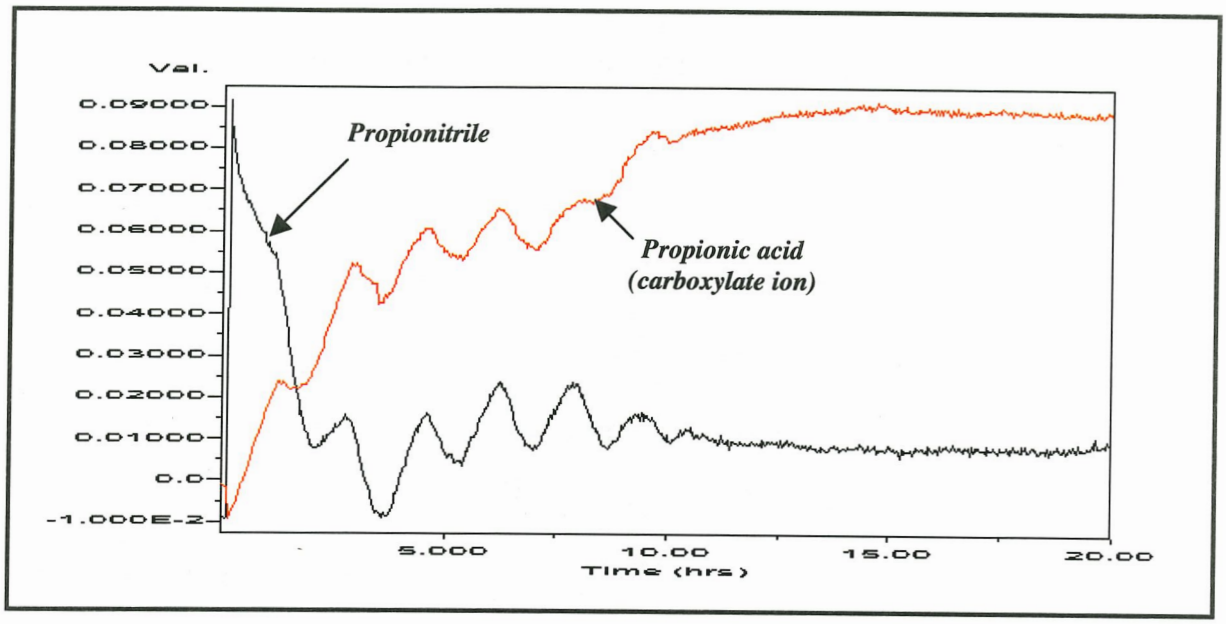


Figure 4.12: Reaction profile of the biotransformation of propionitrile (250 mM) by bacteria grown on propionitrile (10mM). The nitrile is converted directly to the corresponding carboxylic acid with no evidence of any intermediates suggesting conversion by a nitrilase enzyme.

4.6 Induction of the enzymatic pathways

Table 4.1 summarises the results obtained from the previous experiments.

4.6.1 Acetonitrile grown bacteria

Bacteria were grown on acetonitrile (10mM). The resulting spectra showed the two-step nitrile hydratase/amidase pathway was induced in all cases (biotransformation of benzonitrile not determined) as seen in table 4.1.

4.6.2 Benzonitrile grown bacteria

Bacteria grown on benzonitrile induce the one step nitrilase pathway. Table 4.1 suggests that during all biotransformations the nitrile was converted directly to the corresponding carboxylic acid even when aliphatic mononitriles (acetonitrile and propionitrile) are used as substrates. However, it is also possible that amide was formed but its further conversion was rapid, meaning that it was only present in undetectably low concentrations.

4.6.3 Propionitrile grown bacteria

Propionitrile induces both the one and two-step nitrile degrading pathways, therefore, either the nitrilase or nitrile hydratase could convert the nitrile starting substrate. Acetonitrile (250mM) was biotransformed via the two-step pathway whereas propionitrile seemed to be hydrolysed by the one step (benzonitrile (250mM) was not determined). This led to the suggestion that either the nitrile hydratase enzyme could only biotransform the simplest nitrile (acetonitrile) and was more active than the nitrilase or the amide intermediate was formed but was not detected.

Table 4.1: Summary of the biotransformation of acetonitrile, benzonitrile and propionitrile (250mM) monitored using React IR MP and a silicon probe. Bacteria were grown on acetonitrile, benzonitrile or propionitrile (10mM) to induce either the one step or two-step nitrile-degrading pathways.

	Growth substrate (10mM)		
	Acetonitrile	**Benzonitrile**	**Propionitrile**
Acetonitrile	Nitrile/Amide/Acid	Not determined (Nitrile/acid?)	Nitrile/amide/acid
Benzonitrile	Not determined	Nitrile/acid	Nitrile/acid
Propionitrile	Nitrile/amide/acid	Not determined	Nitrile/acid

(250mM)

4.7 Chiral application for React IR

An envisaged application for this technique was to monitor kinetic resolutions of chiral substrate conversions by the bacterium *R. rhodochrous* LL100-21. Firstly solubility profiles were carried out by monitoring a range of chiral nitrile concentrations using React IR MP with a silicon probe. The optimum concentration for detection for these chiral substrates was found to be 100mM (data not shown).

Initial studies on the biotransformation of α-methylbenzyl cyanide (2-phenylpropanenitrile) (100 mM) (figure 4.13) and 2-methylhexanitrile (100 mM) (figure 4.14) were then carried out.

Bacteria were grown on propionitrile (10mM) and benzonitrile (10mM) and the biotransformations were carried out by suspensions of bacteria in phosphate buffer (pH 7.3, 30°C) over a 24-hour time period. The reaction was again monitored by React IR MP using a silicon probe.

These substrates were used as a previous study had suggested that another closely related *Rhodococcus* spp. *Rhodococcus rhodochrous* NCIMB 11216, was enantioselective towards the S-(+) enantiomers of these substrates (Gradley and Knowles (1994a)).

A profile generated from the data using 2-methylhexanitrile showed a 50 % biotransformation of the substrate, then a lag period followed by a slower biotransformation of the remaining substrate. These data suggested that the bacteria could be enantioselective towards either the S-(+) or the R-(-) enantiomer of the racemate but further studies are need to unequivocally prove this.

Data from the α-methyl benzyl cyanide experiments were not determined as the spectrum was contaminated with water vapour.

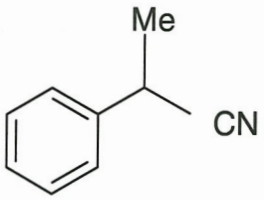


Figure 4.13: Representation of the chiral nitrile α-methyl benzyl cyanide (2-phenylpropanenitrile)

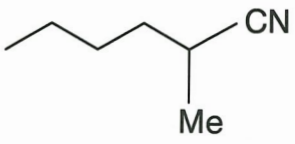


Figure 4.14: Representation of the chiral nitrile 2-methylhexanitrile

4.8 Discussion and conclusions

4.8.1 Introduction

The only published applications in biotechnology of on-line FTIR were as given by (Doak and Phillips (1999), and by Fairbrother *et al.* (1991). Doak and Phillips (1999) utilised mid infrared spectroscopy to follow fermentation of *E.coli in situ* using a diamond composition ATR probe. They studied the application for *in situ* large scale monitoring of metabolites produced from glucose within a complex medium during a fermentation process. Their aims were to improve the fermentation process to achieve maximum efficiency. Alternatively, Fairbrother *et al.* (1991) monitored the production of lactose and lactic acid in whey fermentation using FTIR spectroscopy.

In the present study the technique was utilised on a smaller scale and revealed the potential for wider application of the technique for the study of biocatalysis.

4.8.2 Diamond probe studies

Initial studies monitored the biotransformation of acetonitrile (250mM) by cells grown on acetonitrile (10mM). This induced the two-step nitrile hydratase/amidase pathway. The study then used React IR MP fitted with a diamond probe (ASI technology) to follow the reaction. The intermediates of the reaction were detected but the disappearance of the nitrile was impossible to follow as the diamond probe has a natural absorbency in the 1900 cm^{-1}-2200 cm^{-1} region. Therefore, all further studies used a silicon probe, which enabled nitrile degradation to be followed in real-time.

4.8.3 Biotransformation of acetonitrile (250mM)

Acetonitrile (250mM) was biotransformed using suspensions of bacteria grown on acetonitrile, benzonitrile and propionitrile (10mM) in phosphate buffer.

The spectra of the biotransformation of acetonitrile (250 mM) grown on acetonitrile (10 mM) showed clearly the disappearance of the nitrile and that the reaction proceeded through acetamide to acetic acid. These data illustrated the two-step nitrile hydratase/amidase pathway and showed for the first time the reaction in real-time.

Again, when bacteria were grown on propionitrile (10 mM) the spectral data revealed that acetonitrile was biotransformed via the two-step nitrile hydratase/amidase pathway. Both these spectra and profiles gave a good real-time indication of formation and breakdown of products/intermediates, along with the number of species present within the reaction mixture at any given stage of the reaction process.

No data are obtained for benzonitrile grown bacteria as the probe became contaminated with water vapour, which made determination of the spectra impossible. Future studies should address this problem using a sealed and dried nitrogen source, as attempts to use silica gel were unsuccessful.

4.8.4 Biotransformation of benzonitrile (250 mM)

Benzonitrile (250mM) was biotransformed using bacteria grown on acetonitrile (10 mM), benzonitrile and propionitrile (10mM). The reactions were monitored using mid infrared spectroscopy with a silicon probe.

Spectra for the benzonitrile reaction showed stoichiometric conversion of benzonitrile to benzoic acid suggesting the action of a nitrilase enzyme. Furthermore, benzamide production was not detected by this technique. Its production during the reaction cannot be discounted as it may be removed rapidly via an amidase such that it is only present in a very low concentration. However, earlier work showed that bacteria grown on benzonitrile exhibit little or no amidase activity (Linton and Knowles (1986); Dadd, unpublished). A real-time profile of this reaction confirmed these data by indicating only two species present in the reaction (benzonitrile and benzoic acid).

Propionitrile is known to induce both the one and two step nitrile degrading pathways, (Dadd, unpublished; Thompson (1988)). The resulting spectra showed benzonitrile again being converted directly to benzoic acid with no evidence of any intermediates. During the reaction an initial lag period was observed and was attributed to the insolubility of the benzonitrile as the generated reaction profile showed production of benzoic acid from time zero. As with benzonitrile grown cells, no evidence of an amide intermediate was seen. This could be due to rapid and total removal of the amide or conversion within the cell. However, a more likely explanation was direct conversion of benzonitrile to benzoic acid by a nitrilase enzyme.

The reaction spectra for bacteria grown on acetonitrile were impossible to interpret due to water contamination within the probe.

4.8.5 Biotransformation of propionitrile (250 mM)

Finally, the biotransformation of propionitrile (250mM) was carried out using bacteria grown on acetonitrile, benzonitrile and propionitrile (10mM). Again the reaction was monitored by React IR MP using a silicon probe.

The spectra obtained for acetonitrile grown cells showed rapid conversion of propionitrile via the two-step nitrile hydratase/amidase pathway.

Conversely, bacteria grown on propionitrile converted the propionitrile directly to propionic acid. This result is interesting as it suggests that the nitrile hydratase enzyme could be specific only to simple substrates e.g. acetonitrile therefore enabling the reaction to proceed via the two-step pathway. This observation could explain why such low yields were extracted from all biotransformations using (cyanomethyl) benzonitrile compounds (chapter 3). More complex substrates, for example, propionitrile and 2-(cyanomethyl) benzonitrile seem to proceed via the one-step nitrilase pathway (Dadd, unpublished).

As in previous experiments, the amide intermediate could be present but was not detected. Growth of the bacteria on the nitriles (acetonitrile, benzonitrile and propionitrile) and subsequent biotransformation of the corresponding amides could test for putative propionamidase activity. Alternatively, studies using the amidase inhibitors chloroacetone or urea (Graham, 1999) could be used in conjunction with React IR MP to monitor any accumulation of amide within the reaction supernatant.

Spectra obtained for bacteria grown on benzonitrile were impossible to follow due to water vapour obscuring the 3D image, therefore future experiments could address this problem to eliminate this contamination.

Further to these observations, *Rhodococcus rhodochrous* LL100-21 grown on benzonitrile (10 mM) were originally thought to preferentially biotransform aromatic nitriles. However, it was demonstrated that this is not necessarily always the case as an inducible aliphatic nitrilase was found in *Rhodococcus rhodochrous* K22 by Kobayashi *et al.* (1991).

In this study, bacteria grown on benzonitrile seemed to convert the aliphatic mononitriles, acetonitrile and propionitrile directly to the corresponding carboxylic acids and ammonia This suggested that the benzonitrile-induced nitrilase can biotransform aliphatic as well as aromatic substrates. Future studies could investigate the possibility of different nitrile hydrolysing enzymes produced under different growth conditions. This has been carried out using the bacterium *Rhodococcus rhodochrous* NCIMB 11216 by Hoyle *et al.* (1998). Furthermore, investigation at the genetic level using PCR, RT-PCR or other available techniques could unequivocally elucidate the nature and presence of these enzymes under different induction conditions.

4.8.6 Chiral applications for mid infrared spectroscopy

Another application envisaged for this technique was to monitor kinetic resolutions of a racemate by *R. rhodochrous* LL100-21. Previous studies were performed by Gradley *et al.* (1994) who used the bacterium *Rhodococcus rhodochrous* NCIMB 11216 to produce a kinetic resolution of the substrate R-(-), S-(+)-2-methylbutyronitrile. They found that the S-(+) enantiomer was biotransformed more rapidly than the R-(-) enantiomer using whole cell suspensions and cell free extracts previously grown on propionitrile and benzonitrile.

Chiral biotransformations of cyclic nitrile compounds: chapter 4

In this study, bacteria were grown on propionitrile and benzonitrile (10mM) and biotransformation of the chiral substrates α-methyl benzyl cyanide and 2-methylhexanitrile (100mM) were investigated. Reactions were monitored using React IR MP and a silicon probe.

Unfortunately, only the spectrum for 2-methylhexanitrile could be determined as water contamination obscured peaks when α-methyl benzyl cyanide was used. A putative lag period in the reaction profile was seen suggesting the bacteria was enantioselective towards either the R-(-) or S-(+) enantiomer of the racemate.

With further analysis and experimentation, reactions such as these could be terminated at 50 % substrate disappearance to give an enantiomeric excess of 100 % of the product, thus giving maximum stereo-control to these types of reaction. These results need to be supported using other techniques, such as CCE chiral capillary electrophoresis or chiral HPLC.

The use of React IR MP for biotransformations is in its infancy and some drawbacks were encountered which should be overcome in due course. These include problems with the water solubility of the nitriles; the more insoluble the nitrile the less it was detected by the probe. Therefore, all nitriles used in this study were water-soluble or partially water-soluble. For substrates with low water solubility the use of low water systems e.g. water/water-miscible solvent mixtures could perhaps overcome their insolubility problems.

In this study relatively high concentrations of nitriles 250 mM (g/litre scale) were utilised and therefore the limits of detection need to be improved. Once sensitivity has been improved the technology could be utilised to detect pharmaceutically interesting secondary metabolites, both *in situ* and in real-time, and utilised within other aspects of biocatalysis.

Ultimately, this study has shown that this is a novel technique to monitor biocatalysis in real-time, capable of giving both quantitative and qualitative data.

Applications that are envisaged include elucidation of single and multiple-step enzyme kinetics, detection of regioselective biotransformations, and its use in the monitoring of reaction kinetics as a means of optimising enantioselective biotransformations.

Chapter 5: Biotransformation of prochiral substrates

5.1 General Introduction

A range of biotransformations have been carried out using prochiral symmetrical nitriles. Utilising the bacterium *Rhodococcus rhodochrous* LL100-21, it was envisaged that substrates could be desymmetrised and then subjected to further chemical rearrangements. An example of this is a Hoffman rearrangement (amide to primary amine) to form novel intermediates of neurokinin inhibitors for pharmaceutical usage as inhibitors of neurokinin receptors, which are G protein-coupled receptors involved in pain transmission and neurogenic inflammation.

5.2 Biotransformation of 3,5 piperidinedicarbonitrile

5.2.1 Introduction

The biotransformation of 3,5 piperidinedicarbonitrile (a symmetrical dinitrile) (figure 5.1) was undertaken. If regiospecificity was shown by the bacteria towards 3,5 piperidinedicarbonitrile a chiral centre could be formed during the biotransformation. Previous work (Chapter 3) suggested the cyano group at position 2 may be hydrolysed in preference to the cyano group in position 6 due to steric hindrance caused by the phenyl group.

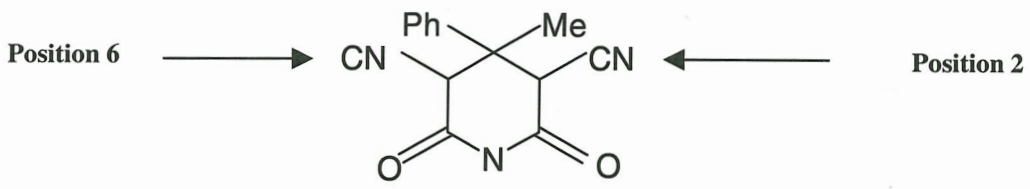


Figure 5.1: Structural representation of the imide 3,5 piperidinedicarbonitrile (10 mM) to be tested for biotransformation by *Rhodococcus rhodochrous* LL100-21 that had been grown on the mononitriles propionitrile, benzonitrile and acetonitrile (10 mM).

5.2.2 Propionitrile grown bacteria

Rhodococcus rhodochrous LL100-21 was grown on propionitrile (10mM) to induce both the one step and two step nitrile hydrolysing pathways (Linton and Knowles, 1986). Cells were harvested in the mid-exponential phase (OD_{600} 0.4-0.8) and concentrated 10-fold in phosphate buffer (pH 7.3). The substrate, 3,5 piperidinedicarbonitrile (10 mM), was added from a methanol stock solution and the reaction was followed by the production of ammonia into the supernatant.

Propionitrile-grown bacteria produced approximately 7 mM of ammonia after 96 hours which suggested that one cyano group had been hydrolysed (figure 5.2). Therefore, it was possible that the bacterial biotransformation was regioselective, resulting in formation of a chiral centre but no definite conclusion could be made. Moreover, subsequent analysis by conventional HPLC revealed more than one product had formed during the time course of the reaction.

5.2.3 Acetonitrile grown bacteria

Bacteria were grown on acetonitrile to induce the two step nitrile hydratase /amidase enzymatic pathway (Collins and Knowles, 1983). Bacteria were harvested in mid exponential phase (OD_{600} 0.4-0.8) and concentrated 10-fold in phosphate buffer (pH 7.3). The substrate 3,5 piperidinedicarbonitrile (10 mM) was added (from a methanol stock) and the reaction was followed by the production of ammonia in the supernatant (figure 5.2). After 96 hours only 2 mM ammonia was produced in the supernatant. This indicated a lowly active biotransformation to the corresponding carboxylic acid had occurred. Subsequent HPLC spectra of the reaction showed more than one product being formed therefore the exact nature of the reaction would have to be determined using alternative analytical techniques.

5.2.4 Benzonitrile grown bacteria

Bacteria were grown on benzonitrile (10 mM) to induce the one step nitrilase pathway (Collins and Knowles, 1983). Bacteria were harvested in mid exponential phase (OD_{600} 0.4-0.8) and concentrated 10-fold in phosphate buffer (pH 7.3). The substrate 3,5 piperidinedicarbonitrile (10 mM) was added and the reaction was followed by the production of ammonia in the supernatant. After 96 hours approximately 1.5 mM was produced in the supernatant (figure 5.2). HPLC spectra again suggested production of more than one product during the time course of the reaction.

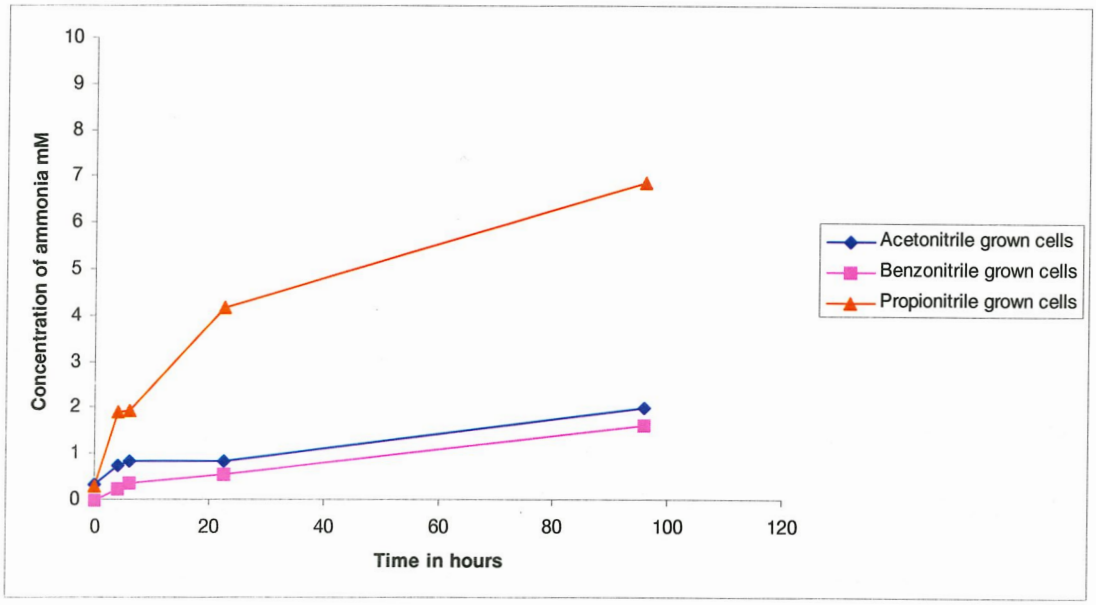


Figure 5.2: Biotransformation of the imide 3,5 piperidinedicarbonitrile (10mM) by *Rhodococcus rhodochrous* LL100-21 that had been grown on propionitrile, benzonitrile and acetonitrile (10mM). Propionitrile grown cells gave the highest activity with approximately 7mM of ammonia being produced. Standard errors were calculated from duplicate samples and found to be negligible, and were therefore omitted from the graph.

5.3 Biotransformation of 3-phenyl-1, 3, 5, pentanetricarbonitrile

5.3.1 Introduction

The biotransformation of this substrate was of interest as it is a prochiral substrate and hydrolysis by the bacterium could produce a homochiral quaternary centre making it of interest as a pharmaceutical intermediate. Figure 5.3 gives the structure of the substrate to be biotransformed.

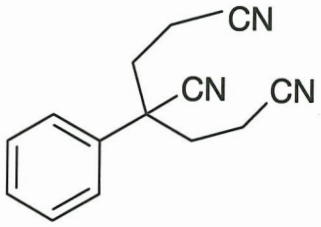


Figure 5.3: Structural representation of 3-phenyl-1, 3, 5, pentanetricarbonitrile.

5.3.2 Results

Rhodococcus rhodochrous LL100-21 was grown on acetonitrile benzonitrile and propionitrile (10 mM). Cells were harvested in mid exponential phase (OD_{600} 0.4-0.8) and concentrated 10-fold in phosphate buffer (pH 7.3). The substrate 3-phenyl-1, 3, 5, pentanetricarbonitrile (10 mM) was added and the reaction was followed for 1 h by the production of ammonia in the supernatant. Ammonia production was observed in all cases (figure 5.4) but at a very low yield. Therefore, no conclusions could be drawn as to the number of cyano groups hydrolysed.

The experiment was repeated using bacteria grown on propionitrile (10 mM) but the reaction was carried out over 200 h (figure 5.5).

The total concentration of ammonia produced was approximately 12 mM, which suggested one cyano group had been hydrolysed. HPLC of the reaction revealed that in all cases just one major product was formed during the biotransformation (figure 5.6).

Larger scale biotransformations were carried out using bacteria grown on propionitrile (10 mM). Chemical extraction using diethyl ether was also performed but no product was recovered. The biotransformation was repeated and an alternative extraction procedure was carried out using chloroform instead of diethyl ether. A small amount of a white solid was recovered but subsequent ^{1}HNMR revealed only the starting substrate 3-phenyl-1, 3, 5, pentanetricarbonitrile. These results were also confirmed using Thin Layer Chromatography (TLC) analysis.

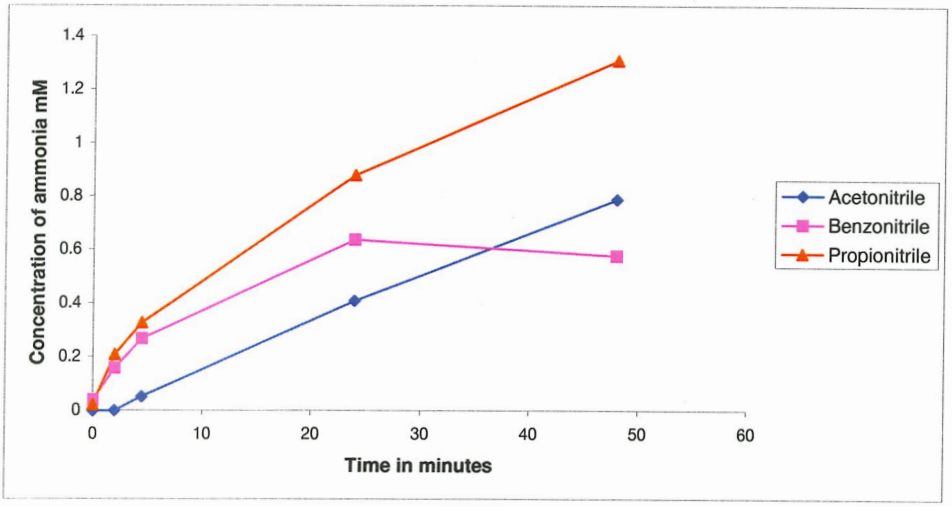


Figure 5.4: Biotransformation of the trinitrile 3-phenyl-1, 3, 5, pentanetricarbonitrile (10 mM), using *Rhodococcus rhodochrous* LL100-21 that had been grown on acetonitrile, benzonitrile and propionitrile (10mM). Error bars (range of duplicate values) are within the symbols plotted.

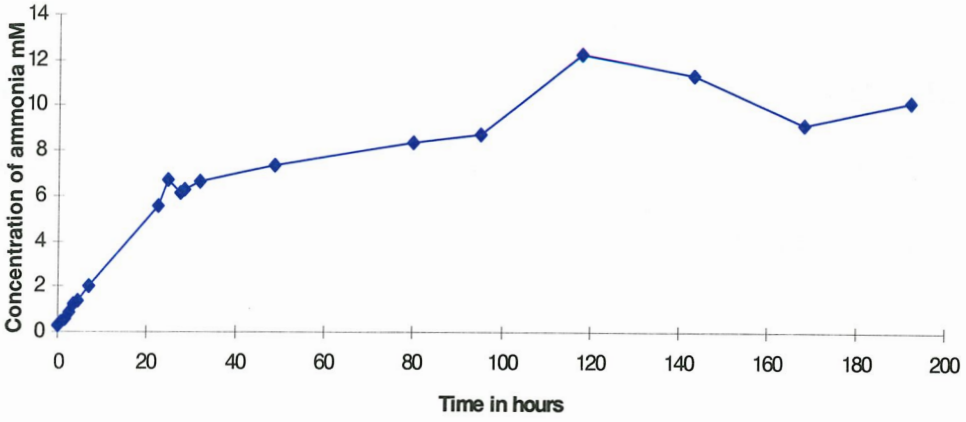


Figure 5.5: Concentration of ammonia produced during the larger scale biotransformation of the trinitrile 3-phenyl-1,3,5,pentanetricarbonitrile (10 mM) using bacteria that had been grown on propionitrile (10 mM). Standard error was calculated from duplicate samples but errors were found to be negligible. Therefore, error bars were omitted from the graph.

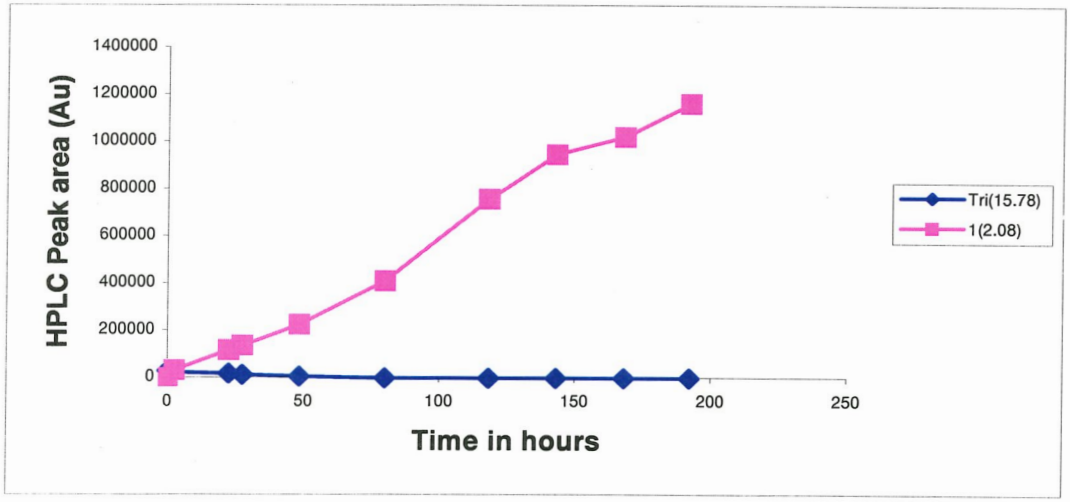


Figure 5.6: Formation of the sole major product formed during the biotransformation of the trinitrile 3-phenyl-1, 3, 5, pentanetricarbonitrile. No other products were observed over the time course of the reaction. The trinitrile was seen at very low concentrations due to its insolubility in the aqueous phase.

Tri(15.78) = Trinitrile retention time 15.78 min

1(2.08) = Major product retention time 2.08 min

5.4 Biotransformation of 2-(1-cyanoethyl) benzonitrile

5.4.1 Introduction

2-(1-cyanoethyl) benzonitrile is a chiral derivative of 2-(cyanomethyl) benzonitrile and was synthesised as a racemate. As a conventional HPLC technique was not available to separate the two enantiomers, Chiral Capillary Electrophoresis (CCE) was used (figure 5.8). A structural representation of the compound is given in figure 5.7.

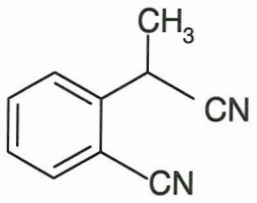


Figure 5.7: Diagrammatic representation of the chiral substrate 2-(1-cyanoethyl) benzonitrile.

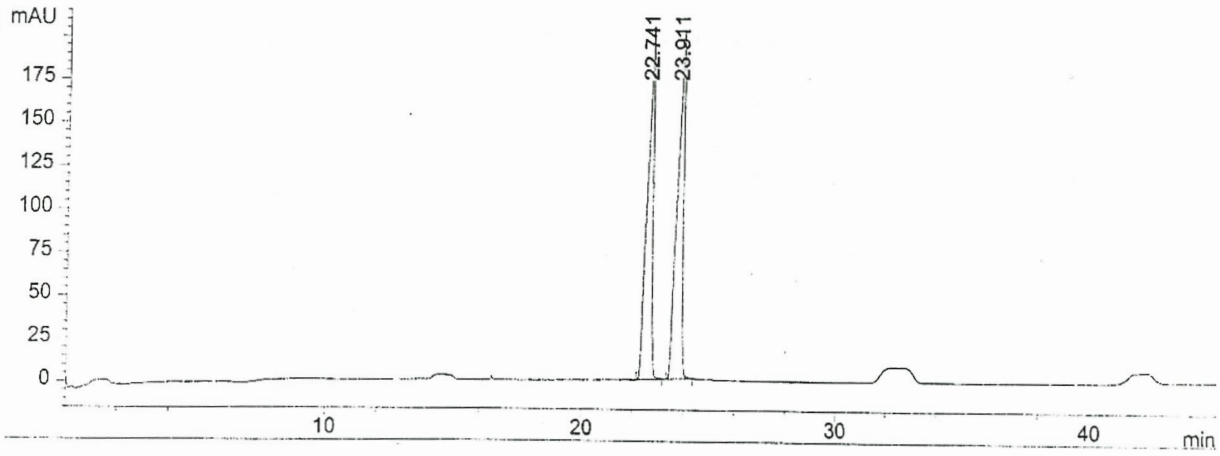


Figure 5.8: Separation of the two enantiomers of the substrate 2-(1-cyanoethyl) benzonitrile by Chiral Capillary Electrophoresis (CCE).

5.4.2 Propionitrile, benzonitrile and acetonitrile grown bacteria

A total of 10mM ammonia was detected in the supernatant from the biotransformation of 2-(1-cyanoethyl) benzonitrile (10mM) of propionitrile grown cells, suggesting that either the aromatic cyano group, or both the (+/-) aliphatic side chains of the enantiomers had been hydrolysed. From previous results using 2-(cyanomethyl) benzonitrile, it is tentatively supposed that the aliphatic side chain cyano group of both enantiomers had been hydrolysed, as the aromatic group would have been inaccessible to the enzyme due to steric hindrance. This result suggested that the bacteria have no preference for either of the stereoisomers of (+/-) 2-(1-cyanoethyl) benzonitrile.

Ammonia release from benzonitrile- and acetonitrile-grown cells was minimal, therefore, the nature of the enzymology of the biotransformation are hard to interpret using these data. (figure 5.9).

HPLC analysis was carried out throughout the time course of the reaction. Propionitrile- and benzonitrile-grown bacteria yielded two products and acetonitrile-grown bacteria three. An example spectrum is shown in figure 5.10.

Following larger scale biotransformations and diethyl ether extraction a yellow oil was seen within the cell biomass. No products were recovered from the supernatant. Subsequent attempts to recover the oil were unsuccessful.

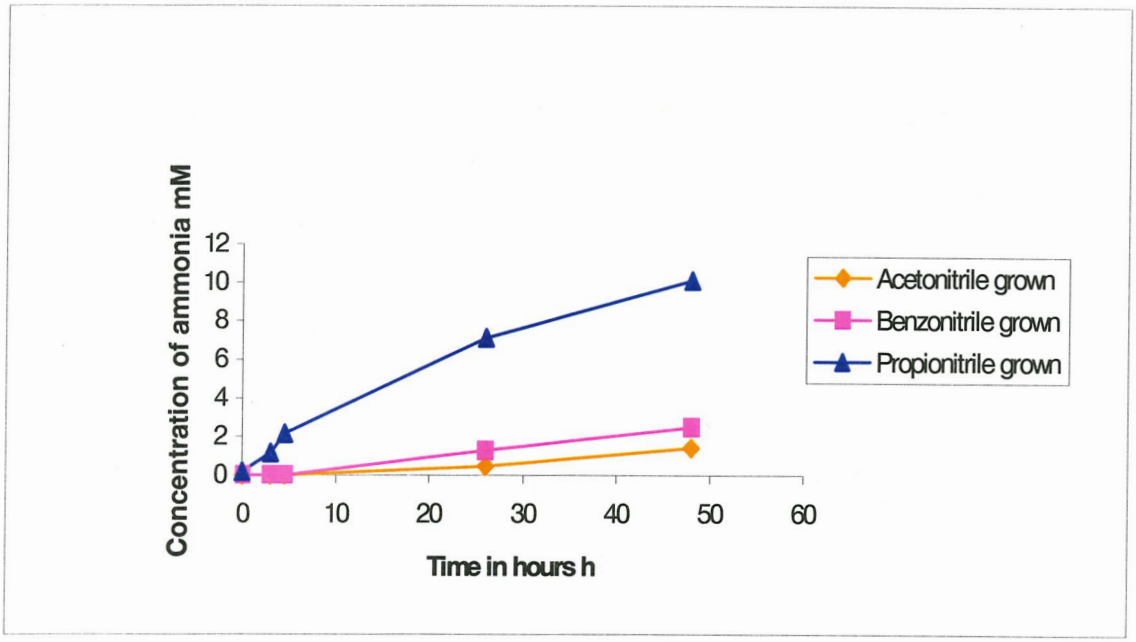


Figure 5.9: Ammonia production during the biotransformation of the chiral substrate 2-(1-cyanoethyl) benzonitrile (10mM) using cells previously grown on propionitrile, benzonitrile or acetonitrile. Standard errors were negligible and therefore were omitted.

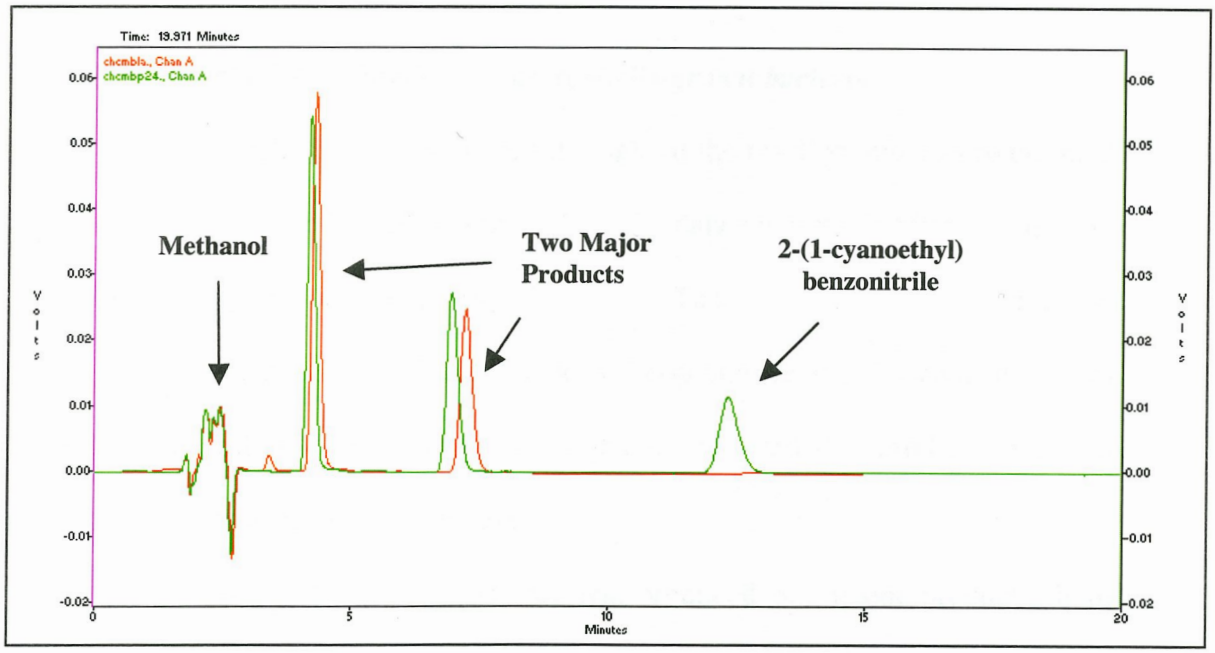


Figure 5.10: Example HPLC spectrum of the two products formed during the biotransformation of 2-(1-cyanoethyl) benzonitrile (10 mM) using cells that had been grown on propionitrile (10 mM). The substrate was fully biotransformed after 48 hours (the spectra indicate two time points 24 hours and 48 hours).

5.5 Biotransformation of 2-phenyl 1,3 propanedicarbonitrile

5.1 Introduction

2-Phenyl 1,3 propanedicarbonitrile (figure 5.11) is a prochiral dinitrile. *Rhodococcus rhodochrous* LL100-21 that had been grown on the mononitriles propionitrile, benzonitrile and acetonitrile was used to hydrolyse the prochiral dinitrile in an attempt to generate a homochiral quaternary centre.

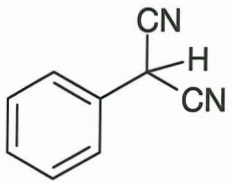


Figure 5.11: Diagrammatic representation of the prochiral substrate 2-phenyl 1,3 propanedicarbonitrile

5.5.2 Propionitrile- benzonitrile- and acetonitrile-grown bacteria

Ammonia release was monitored throughout the reaction and was found in all cases to be very low (< 1.4mM) (figure 5.12). The data suggested a biotransformation had taken place, but only at a very low level. Therefore HPLC was carried out throughout the time course of the reactions (propionitrile and benzonitrile grown bacteria). Spectral analyses were carried out and indicated that products had been formed but it was unclear as to how many.

In contrast, acetonitrile-grown bacteria produced one major product (figure 5.13). A larger scale biotransformation was carried out (50 mM substrate) and upon chemical extraction a yellow oil was recovered.

Further analysis by H^1 NMR and 2D NMR suggested the major product was benzoic acid.

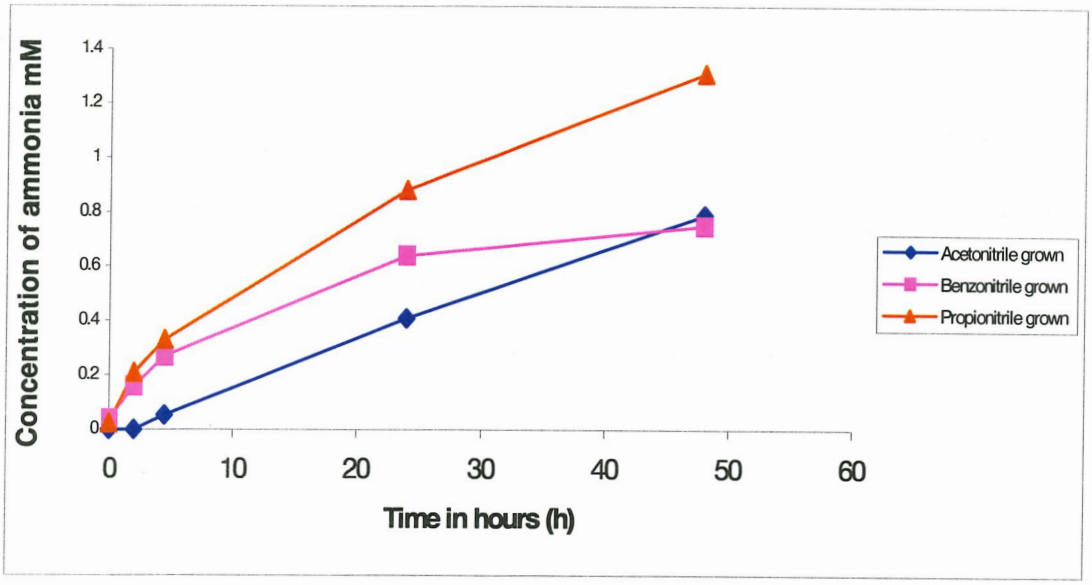


Figure 5.12: Ammonia release during the biotransformation of 2-phenyl 1,3 propanedicarbonitrile (10mM). Standard errors of duplicate samples were calculated and found to be negligible therefore, were omitted.

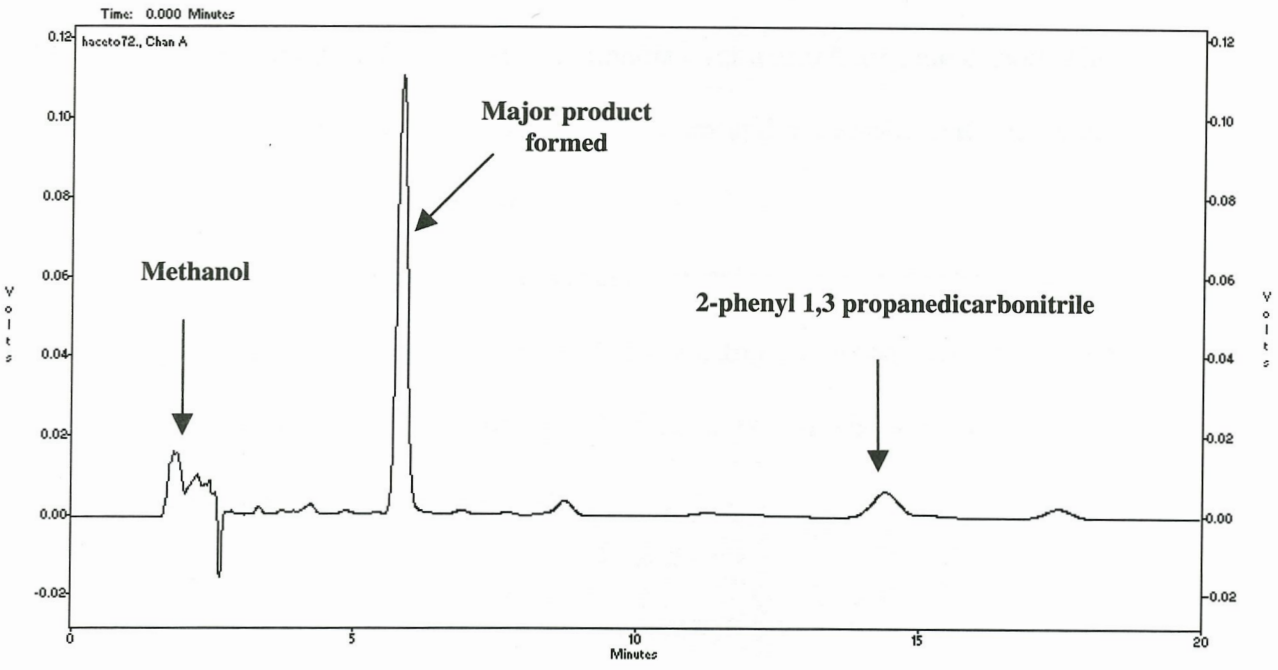


Figure 5.13: HPLC of the biotransformation of 2-phenyl 1,3 propanedicarbonitrile (10 mM) using bacterial cells that had been grown on acetonitrile (10 mM). After 72 hours one major product was formed in the supernatant.

5.6 Discussion and conclusion

5.6.1 Biotransformation of 3,5 piperidinedicarbonitrile

Rhodococcus rhodochrous LL100-21 was shown to be capable of biotransforming this novel prochiral substrate. Propionitrile grown bacteria exhibited the greatest activity towards the substrate with 7 mM of ammonia being produced from 10 mM substrate. At this stage there is no evidence for which position was hydrolysed. It is possible that it was the cyano group in position 2 (figure 5.1) due to potential steric hindrance preventing the other cyano group being hydrolysed (cf. Chapter 3). Acetonitrile and benzonitrile grown bacteria produced only negligible amounts of ammonia in the supernatant, therefore no conclusions could be suggested for the biotransformations.

5.6.2 Biotransformation of 3-phenyl-1, 3, 5, pentanetricarbonitrile

Biotransformation of the trinitrile 3-phenyl-1, 3, 5, pentanetricarbonitrile (10 mM) yielded approximately 10-12 mM of ammonia over a 200 hour time period. This was equivalent to 33-40 % of the potential maximum and suggested that one cyano group was hydrolysed during the reaction.

HPLC analysis of the resultant supernatant revealed one major product, which was possibly a chiral intermediate of the starting substrate. Attempts to recover the product yielded only a small quantity of a white solid which upon analysis by ^{1}H NMR revealed only the starting substrate.

5.6.3 Biotransformation of 2-(1-cyanoethyl) benzonitrile

Rhodococcus rhodochrous LL100-21 is capable of biotransforming the substrate 2-(1-cyanoethyl) benzonitrile. Growth of the bacteria on propionitrile yielded 10 mM of ammonia and suggested either the aromatic cyano group or both the (+/-) aliphatic side chains were hydrolysed. Previous work (cf. Chapter 3) suggested that the (+/-) aliphatic side chain was hydrolysed due to steric hindrance. In this study conventional HPLC was used, therefore no separation of enantiomers was detected.

Chiral Capillary Electrophoresis (CCE) was found to be a useful technique to separate the two enantiomers of the racemate and could be utilised in further studies on the putative chiral products.

5.6.4 Biotransformation of 2-phenyl 1,3-propanedicarbonitrile

2-Phenyl 1,3-propanedicarbonitrile was in part biotransformed by the bacterium *Rhodococcus rhodochrous* LL100-21 but with only small amounts of ammonia release. Using HPLC analysis the time course of the reactions were followed. Propionitrile and benzonitrile grown bacteria formed 2 to 3 products but most interestingly acetonitrile grown cells formed only one single product. These products need to be analysed by Chiral Capillary Electrophoresis and/or LC-MS. This further suggested the reaction products were unstable and other spurious products were formed, possibly by double decarboxylation reactions. Recovery of products from this biotransformation is unlikely due to their instability, and hence no future work was planned.

5.6.5 Conclusions

The study of novel prochiral nitriles using the bacterium *Rhodococcus rhodochrous* LL100-21 following growth and induction on the mononitriles (acetonitrile, benzonitrile or propionitrile) has illustrated that this bacterium is capable of their biotransformation to novel products. HPLC analysis indicated intermediates/products formed during the reaction but as these compounds were not commercially available identification was difficult. Also, only small amounts of the compounds were available, which limited the experiments that could be carried out. Furthermore, analytical techniques such as Chiral Capillary Electrophoresis (CCE) or chiral HPLC (for the separation of the enantiomers) need to be developed further to enable the identification of all intermediates and products of these types of biotransformation.

Chapter 6: Biotransformation of benzonitrile in the presence of hydroxylamine

6.0 Introduction

As hydroxylamine (NH_2OH) is more nucleophilic than water, the following experiments were designed to investigate if its addition to the biotransformation medium would compete with the normal nitrilase hydrolysis reaction to form novel products. Benzonitrile was chosen as it was easily detected by HPLC and it is known to induce the one step nitrilase enzymatic pathway, which converts the benzonitrile to benzoic acid (Collins and Knowles, 1983). Benzohydroxamic acid (figure 6.1), and benzamide oxime (figure 6.2) are precursors of many pharmaceutical-interesting products.

Hydroxamic acids (R-CONHOH) form stable chelates with metal ions and are constituents of several interesting compounds, for example growth factors, food additives, antibiotics, tumour inhibitors, anti-fungal agents, cell division factors and enzyme inhibitors (Kurzak *et al.*, 1992 and Holmes, 1996). Several studies have concentrated on the use of the amidase enzyme in the presence of hydroxylamine from bacteria (Vaysse *et al.*, 1997; and Hirrlinger and Stolz, 1997 (figure 6.3)). The synthesis of either of these compounds has not been reported by whole cell or cell free extracts using a nitrilase enzyme.

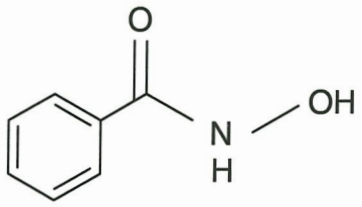


Figure 6.1: Structure of benzohydroxamic acid

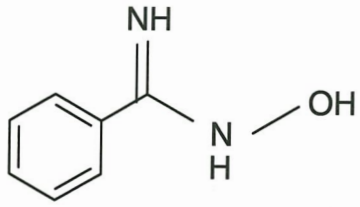


Figure 6.2: Structure of benzamide oxime

6.1 Biotic experiment

R. rhodochrous LL100-21 was grown on benzonitrile (10 mM), harvested at the mid exponential phase of growth (OD_{600} 0.4-0.8), and concentrated 10-fold in 100 mM phosphate buffer (pH 7.3). In initial experiments a suspension of cells in phosphate buffer (pH 7.3) was added to 50 mM benzonitrile in a solution of 100 mM hydroxylamine, (total volume 25 ml). The reaction was carried out for 24 hours with shaking (200 rpm) at 30 °C.

Samples were taken at intervals and analysed by HPLC, using relevant reference compounds. Benzonitrile was converted to benzoic acid and another unidentified product. In HPLC spectra taken after a reaction time of 45 min, the benzonitrile, benzoic acid and the unknown compound had retention times of 6.22, 3.70 and 1.59 min respectively (figure 6.4). Subsequent chemical extraction and analysis yielded only benzoic acid. Larger-scale biotransformations were carried out to investigate if the unknown product could be chemically extracted using diethyl ether extraction (section 2.1.5). To initiate the reaction 50 mM benzonitrile was added to hydroxylamine (375 mM) and a phosphate buffer/cell suspension (harvested at an OD_{600} of 0.4-0.8, concentrated 10-fold, total volume 50 ml) and incubated for 48 hours at 30 °C and 200 rpm.

The supernatant was chemically extracted using diethyl ether as described in section 2.1.5. Upon rotary evaporation a yellow solid was recovered at approximately 3% yield.

^{1}H NMR was carried out and analysis of the spectrum concluded that the product was benzohydroxamic acid with trace amounts of benzoic acid (figure 6.5).

From the GC-MS spectra obtained the biotransformation product had a molecular ion of 137, confirming that the unknown product was benzohydroxamic acid (figure 6.1).

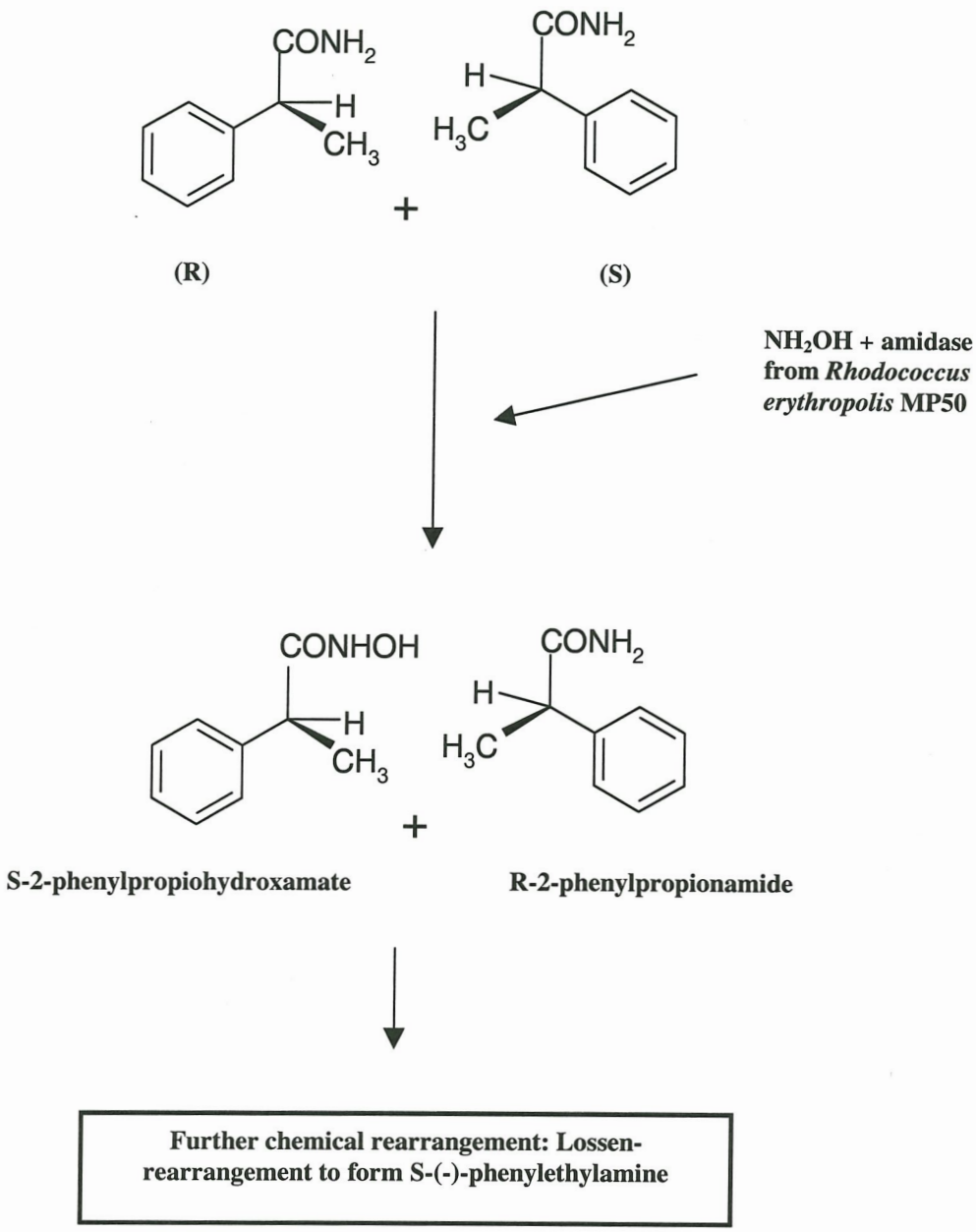


Figure 6.3: Reaction scheme for the enantioselective formation of S-2-phenylhydroxamate by the amidase from *R. erythropolis* MP50 and subsequent Lossen rearrangement (Hirrlinger and Stolz, 1997).

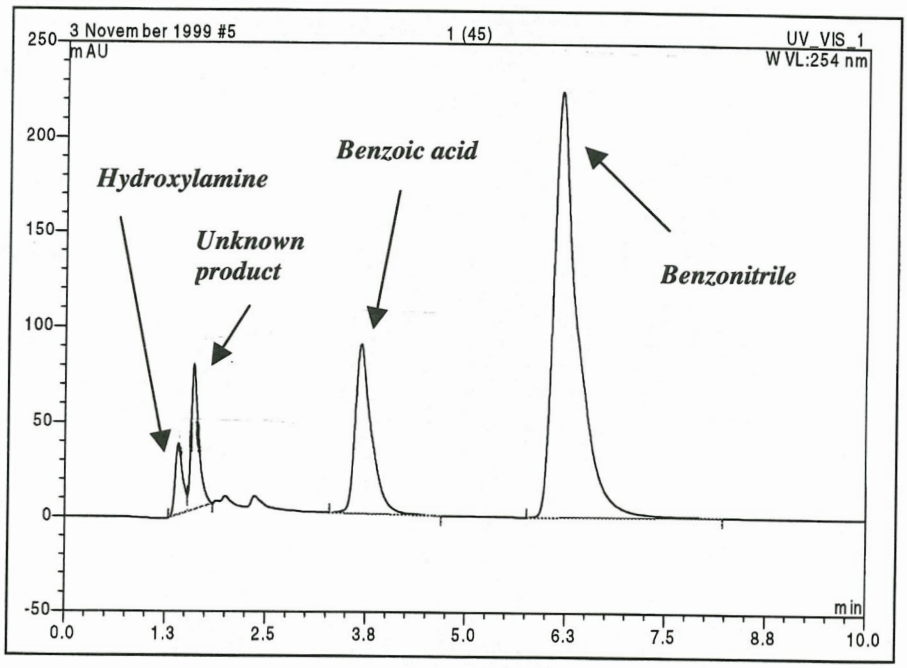
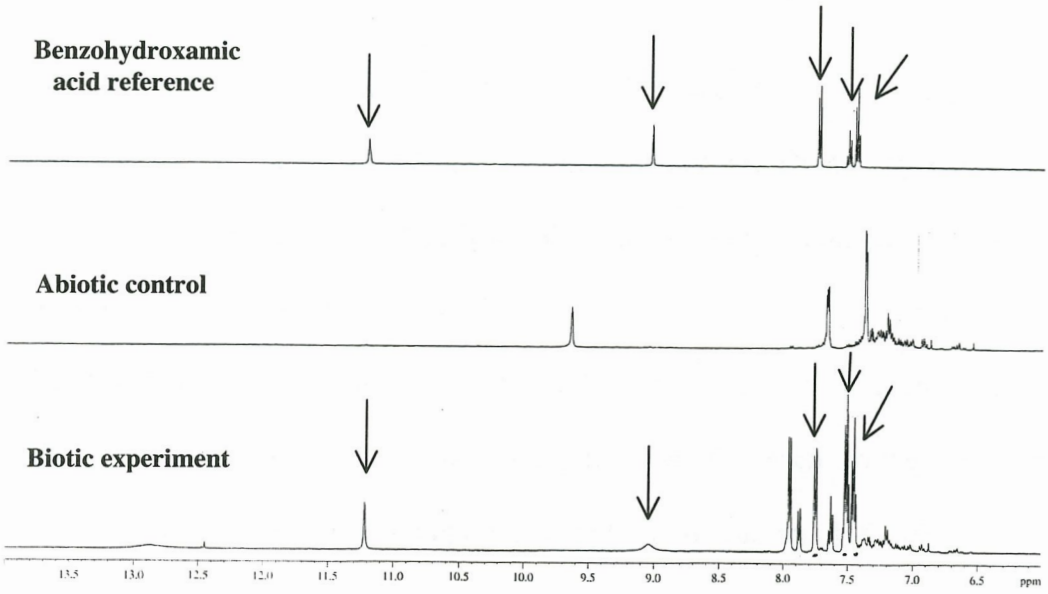
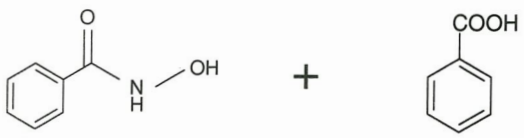


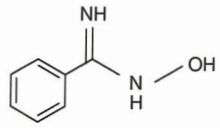
Figure 6.4: HPLC spectrum of the reaction products from the biotransformation of benzonitrile (50 mM) by *Rhodococcus rhodochrous* LL100-21in the presence of hydroxylamine (375 mM). After a 45 min incubation a single unknown product was formed in the biotransformation supernatant. A C18 HPLC column was used.





(a) (b)

Benzohydroxamic acid (a) and benzoic acid (b): The products from the biotic reaction of hydroxylamine, benzonitrile and *Rhodococcus rhodochrous* LL100-21. Arrows on the spectrum line up the signals from the benzohydroxamic acid in the reference and biotic experiments.



(c)

Benzamide oxime (c): The product from the abiotic control reaction.

Figure 6.5: Biotransformation of benzonitrile in the presence of hydroxylamine. The biotic reaction yielded the products benzohydroxamic acid (a) and small amounts of benzoic acid (b). Conversely, the abiotic reaction yielded synthetic benzamide oxime (c) (a Sigma rare chemical).

6.2 Abiotic control

An abiotic control also was undertaken, which consisted of all components described above except the bacterial cells. The supernatant was chemically extracted with diethyl ether, and the resultant yellow solid was recovered, although at a very low yield compared to the starting substrate.

^{1}H NMR was carried out, and the unknown product was identified as benzamide oxime (figure 6.2). The benzamide oxime had also been synthesised in a cleaner form than the reference compound supplied by Sigma, UK which was contaminated with the starting substrate used to synthesise the product.

From GC-MS spectra the biotransformation product had a molecular ion of 136 thus confirming the unknown product to be benzamide oxime (figure 6.2).

6.3 Discussions and conclusions

This is the first documented occurrence of this type of synthesis using whole bacterial cell cultures or cell free extracts. The results from the biotransformation were unusual as a novel product, benzohydroxamic acid, was produced. Expected was the usual nitrilase reaction that converts the nitrile to the corresponding carboxylic acid and ammonia. This suggested that the more nucleophilic hydroxylamine had competed with water in the reaction of the nitrilase enzyme (figure 6.6).

As the final pH of the supernatant was 8.5 it is possible that lysis of the bacterial cells had occurred. Therefore, future work could investigate the viability of the cells under these conditions and the reaction could be repeated using cell free extracts.

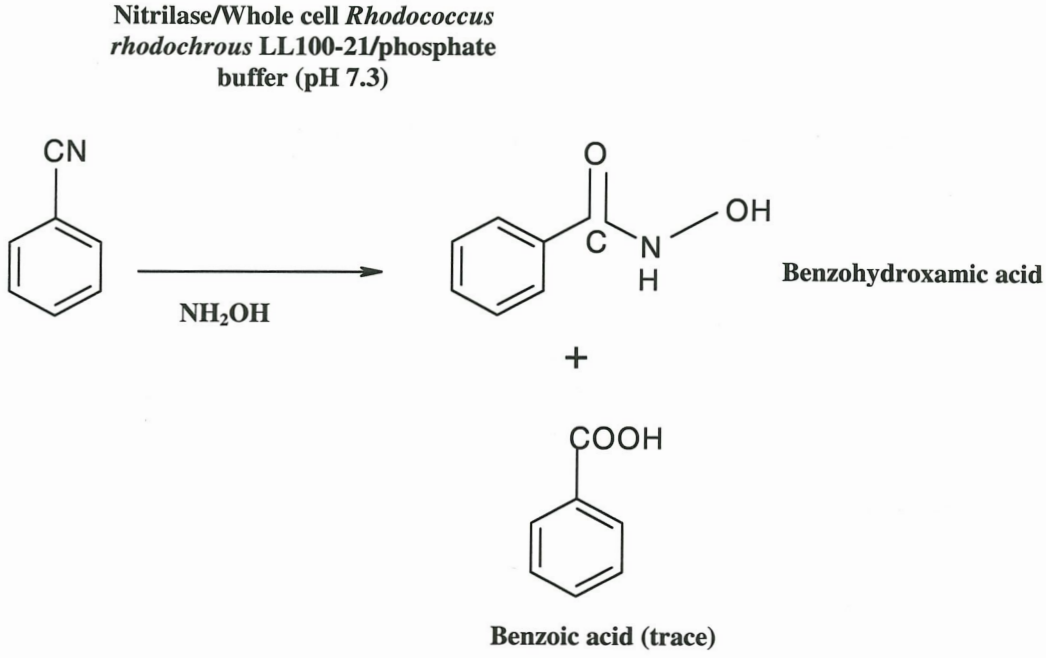


Figure 6.6: Reaction scheme for the production of benzohydroxamic acid using whole cells of *Rhodococcus rhodochrous* LL100-21 (grown on benzonitrile (10 mM) to induce the nitrilase enzyme) in the presence of hydroxylamine (375 mM).

More interestingly, the abiotic control yielded the Sigma rare chemical benzamide oxime which upon analysis by ^{1}H NMR was found to be of a higher quality than Sigma UK could supply, although this was extracted at only a very low yield (>1 %) compared to the starting substrate.

This study set out to investigate if this type of chemical synthesis could be carried out using whole cell suspensions of the bacterium *Rhodococcus rhodochrous* LL100-21 in the presence of hydroxylamine. Using nitrile biotransformations as a model system, with a suitable range of nitrile as substrates, the results have suggested that high value commodity chemicals could be produced in this way although a more detailed investigation is needed to optimise the procedures.

Chapter 7: Molecular biology techniques

7.1 Growth of Rhodococcus sp. *DSM 11397 and Pseudomonas fluorescens DSM 11387*

7.1.2 Introduction

The isolation, characterisation and growth of *Pseudomonas fluorescens* DSM 11387 (a bacterium that contains nitrilase but not nitrile hydratase) and *Rhodococcus* sp. DSM 11397 (a bacterium that contains nitrile hydratase but not nitrilase) has been previously been described (Layh *et al.*, 1997).

In this study the growth of these bacteria on the mononitriles propionitrile, acetonitrile and benzonitrile (carbon and/or nitrogen source) was investigated as a prelude to investigating the presence or absence of these enzymes using molecular techniques. These molecular experiments were preliminary and were conducted later in the project after examining the nitrile and nitrile hydratase pathways in more detail. This approach was the next step in looking at the genetic pathways of *R. rhodochrous* and other bacteria to resolve unanswered questions concerning the induction of the nitrile hydrolysing enzymes.

7.2.2 Results of the growth experiments

P. fluorescens DSM 11387 and *Rhodococcus* sp. DSM 11397 were grown in minimal medium consisting of M-9 salts (minus ammonium salts) (Miller, 1972), 1 ml trace metals 1^{-1} (Bauchop and Elsden, 1960) and the following carbon and/or nitrogen sources: benzonitrile (10 mM), propionitrile (10 mM) or acetonitrile (10 mM). Cultures of 50 ml were grown in 250 ml flasks at 30 °C with shaking (200 rpm) for 24 to 72 hours.

Table 7.1 illustrates growth observed when nitriles were used as a source of carbon and nitrogen. Table 7.2 shows growth when nitriles were the nitrogen source and glucose (30 mM) the source of carbon.

The results show that both *P. fluorescens* DSM 11387 and *Rhodococcus* sp. DSM 11397 can grow on either acetonitrile or propionitrile as the sole carbon and/or nitrogen source but not on benzonitrile.

As a consequence of this, the presence of the nitrilase and nitrile hydratase enzymes in these bacteria was investigated using molecular techniques (time constraints prevented a detailed study of biotransformations of the mononitriles).

Table 7.1: Growth of the bacteria *Rhodococcus* sp. DSM 11397 and *Pseudomonas fluorescens* DSM 11387 on the mononitriles acetonitrile, benzonitrile and propionitrile as a sole carbon and nitrogen source.
Growth = OD_{600} (1.0 +)
No growth = OD_{600} (0)

Carbon and Nitrogen source	***Rhodococcus* sp. DSM 11397**	***Pseudomonas fluorescens* DSM 11387**
Acetonitrile	Growth	Growth
Benzonitrile	No growth	No growth
Propionitrile	Growth	Growth

Table 7.2: Growth of the bacteria *Rhodococcus* sp. DSM 11397 and *Pseudomonas fluorescens* DSM 11387 on the mononitriles acetonitrile, benzonitrile and propionitrile as a sole nitrogen source.

Nitrogen source only	*Rhodococcus* sp. DSM 11397	*Pseudomonas fluorescens* DSM 11387
Acetonitrile	Growth	Growth
Benzonitrile	No growth	No growth
Propionitrile	Growth	Growth

7.2 Southern hybridisation

Southern hybridisation is used to detect specific DNA sequences from (in this case) genomic DNA extracted from bacterial isolates. In this study specific primers were designed to investigate the presence or absence of the nitrilase and nitrile hydratase genes from *Rhodococcus* sp. DSM 11397 and *P. fluorescens* DSM 11387

7.2.1 Acquisition of primers for Southern hybridisation analysis

Nitrile hydratase and nitrilase primers were designed using the following method. Firstly, the Entrez nucleotide database was searched, (http://www.ncbi.nlm.nih.gov/entrez) with the search terms "nitrile and hydratase" or "nitrilase". The search was refined by selecting organisms closely related to *Rhodococcus* (X54074, X14668, Z48769, E12518, AB016078, M60264, D14454) and *Pseudomonas* species (U89363).

The same procedure was used for the design of the nitrilase gene primers. The sequences were then aligned and analysed using the suite of programs in GCG Wisconsin Package Version 10.1, (Genetics Computer Group (GCG), Madison, Wisc). Table 7.3 details the design of the primers.

Table 7.3: Sequence acquisition for Southern hybridisation. The nitrile hydratase was designed for both organisms as the sequences were conserved. The nitrilase primers were designed specifically for *Rhodococcus rhodochrous* LL100-21 and *Pseudomonas fluorescens.*

Enzyme	Sequence	Length (bp)	Mwt (Da)
Nitrile-hydratase (Both organisms)	5'-CGC GTC TAC GAC ACC ACC GCC GAA ACT CGC TAC ATG G -3'	37	11861.4
***Pseudomonas*-nitrilase**	5'- CCG GAA TAA ATG CCT CCG GGA ACG -3'	24	7110.4
***Rhodococcus rhodochrous*-nitrilase**	5'-GTA CCC GTA CCA CAT CTG G -3'	19	6054.8

7.2.2 Extraction of genomic DNA

Genomic DNA from *Rhodococcus* sp. DSM 11397 was extracted using the modified CTAB procedure detailed in section 2.3.1. The genomic DNA of *P. fluorescens* DSM 11387 was extracted using the chromosomal DNA extraction procedure given in section 2.3.2. In both cases the resultant DNA was electrophoresed using a 0.7 % agarose gel run at 80 volts for 45 min. The high molecular weight DNA extracted from *Rhodococcus* sp. DSM 11397 can be seen in figure 7.1. A similar result was obtained with *P. fluorescens* DSM 11387.

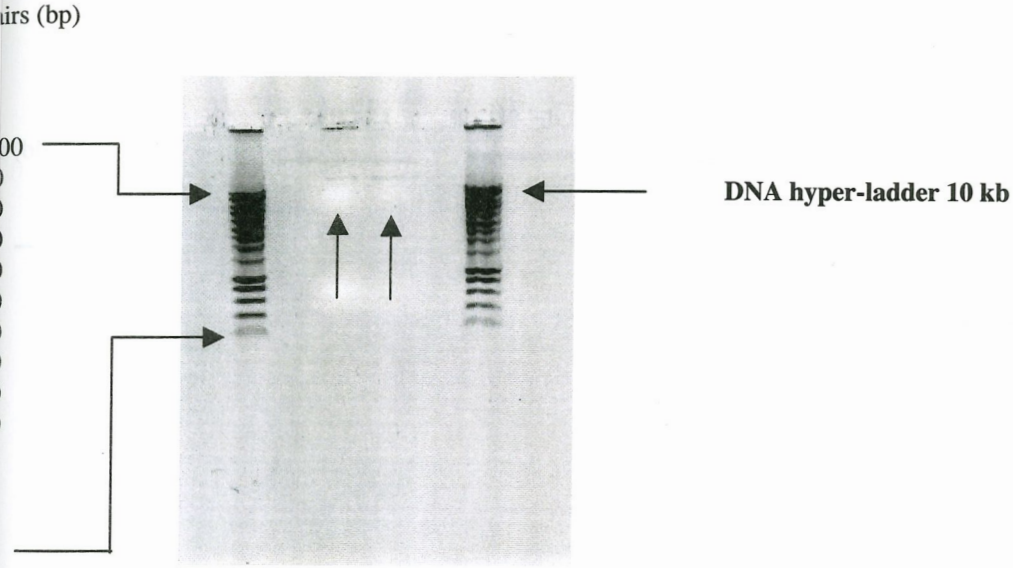


Figure 7.1: An example of high molecular DNA (indicated by arrows) extracted (using a modified CTAB procedure) from *Rhodococcus* sp. DSM 11397

7.2.3 Genomic DNA quantification and endonuclease restriction digests

Before Southern blotting could take place genomic DNA was quantified using standard spectrophotometric methods (Sambrook *et al.*, 1989) and digested. Individual restriction endonuclease digests were carried out using the restriction enzymes *Pst*I and *Sty*I (20 U of each). Reactions were made up to a total volume of 20 µl with ddH_2O and digests were performed at 37°C for 2 to 24 hours. DNA fragments were observed using gel electrophoresis (section 2.3.2) and smears resulting from successful digestion of the genomic DNA (for all three bacteria) are shown in figure 7.2.

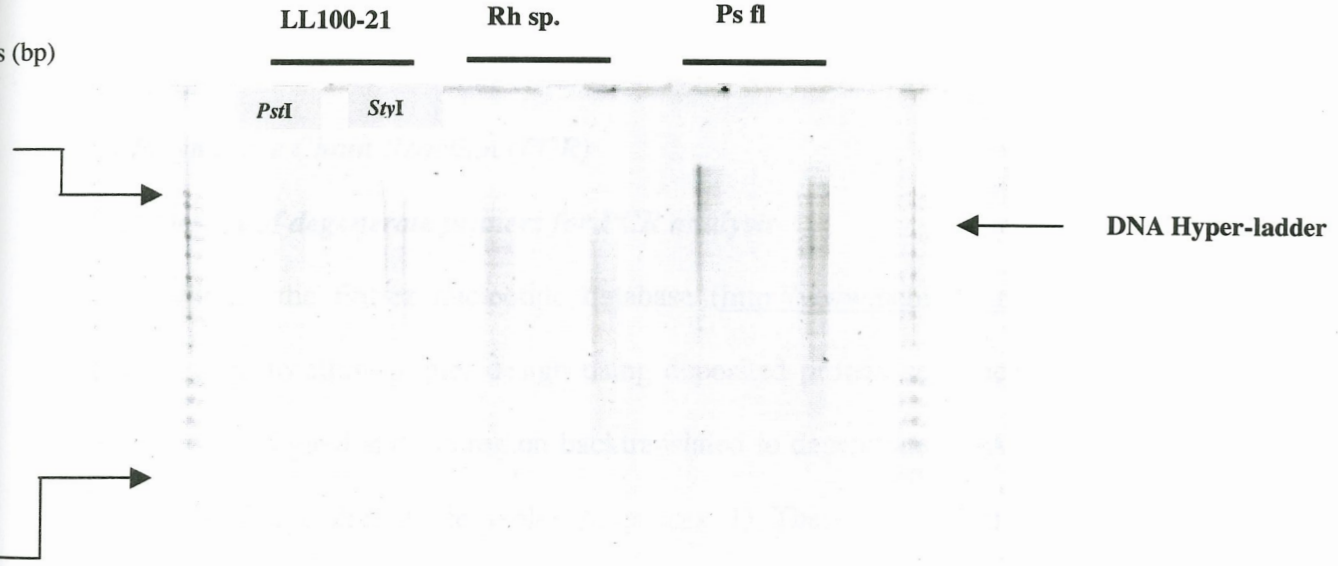


Figure 7.2: Restriction digest using genomic DNA from the bacteria *Rhodococcus rhodochrous* LL100-21 (LL100-21), *Rhodococcus* sp. DSM 11397 (Rh sp.) and *Pseudomonas fluorescens* DSM 11387 (Ps fl). The restriction enzymes used were *Pst*I and *Sty* I. The resultant digested DNA was used for Southern hybridisation. Each 'smear' indicates the genomic DNA was successfully digested.

7.2.4 Blotting and probing for the nitrile hydratase and nitrilase DNA sequences

For Southern analysis (Southern, 1975) digested DNA was blotted directly from agarose gels onto nitro-cellulose membranes. Subsequent detection of fragments corresponding to either nitrile hydratase or nitrilase genes was carried out by hybridisation to the DNA of gene specific probes. The probes used were either 5' digoxigenin-11-d UTP (DIG) labelled primers or fluorescien labelled primers as described in table 7.3.

When DIG and fluorescien labelled primers were used no DNA fragments were detected probably due to the relative insensitivity of the detection kits used or probe design. Future work would refine the parameters of the technique which are discussed in the conclusions (section 6.3.5).

7.3 Polymerase Chain Reaction (PCR)

7.3.1 Design of degenerate primers for PCR analysis

Firstly the Entrez nucleotide database (http://www.ncbi.nlm.nih.gov/entrez) was searched to allow primer design using deposited protein sequences. Conserved regions were aligned and the region backtranslated to degenerate DNA codons using the appropriate codon usage tables (appendix 1) These were then converted to degenerate primers again using codon usage tables. The degeneracy of each primer was calculated (appendix 2 and 3) and primers with less than 30 % degeneracy were synthesised. Primers designed are shown in table 7.4.

Table 7.4: Degenerate primers designed from aligned protein sequences of bacteria.

F= Forward primer
R=Reverse primer
FASR= Forward primer designed as a reverse primer.
Rhod = *Rhodococcus* sp.
Pseu = *Pseudomonas* sp.

Primer	Region/name	Forward / Reverse	Sequence	Length (bp)	Mwt (μg/μmole)
1	Rhod region 1 (F) Nit	F	5'- KTN AAR GTN GCN RCN GTN SMN GC -3'	23	7358.9
2	Pseu region 1 (F) Nit	F	5' -TTY AAR GCN GCN GTN CAR GC -3'	20	6452.9
3	Rhod region 2 (F) Nit	F	5'- TTY CCN GAR GTN TKB ATY CCN GGN TAY CC -'	29	9302.3
4	Pseu region 2 (F) Nit	F	5' -TTY CCN GAR GCN TTY ATY CCN GGN TAY CC -3'	29	9264.0

Table 7.4 continued.

5	Rhod region 3 (R) Nit	R	5' -GCY TCN ACR TGN GTN GGY TTN ARY TTY C -3'	28	9020.8
6	Pseu region 3 (R) Nit	R	5' -CGY TCN RCN ACR CTN ACY CTC ATY TTY C -3'	28	8850.0
7	Rhod region 4 (R) Nit	R	5'- GGC CAN GAN GCN ACR TGN AYY TGY TC -3'	26	8396.9
8	Pseu region 4 (R) Nit	R	5'- GGC CAN GCN GCN ACR TGN ACY TGY TC -3'	26	8365.4
9	Rhod region 1 (F) N/H	F	5'- ATG TCN GTN CAN ATH GAY CAY CAN CAN G -3'	28	9009.6
10	Pseu region 1 (F)	F	5'- ATG GGN CAR TCN CAY CAN CAY GAY CAY CAY C -3'	31	9924.1
11	Rhod region 2 (F) N/H	F	5'- CAR GSN CCN GTN TCN GAY MGN AG -3'	23	7336.1

Table 7.4 continued.

12	Pseu region 2 (F) N/H	F	5'- CAR GCN CCN CCN GAR TCN ATY AG -3'	23	7360.3
13	Rhod region 3 (R) N/H	R	5'- TAY TGN GCN CAN GCN GCN GTN CCR TC -3'	26	8348.5
14	Pseu region 3 (R) N/H	R	5'- GCN GTN GCR TCN GCN AGN AGN CGN GC -3'	26	8450.8
15	Rhod region 4 (R) N/H	R	5'- CGY TGN GGN CCR CTD ATN CGR TAY TG -3'	26	8409.9
16	Pseu region 4 (R) N/H	R	5'- CCA NAC NCG DAT YTC YTT RTT NGC NGG RAT -3'	30	9652.5
17	Rhod region 2 (FASR) N/H	FASR	5'- CTN CKR TCN GAN CAN GGN SCY TG -3'	23	7302.6
18	Pseu region 2 (FASR) N/H	FASR	5'- CTR ATN GAY TCN GGN GGN GCY TG -3'	23	7477.8

7.4.2 PCR reactions

Genomic DNA was extracted from the bacteria *Rhodococcus* sp. DSM 11397, *P. fluorescens* DSM 11387, *Rhodococcus rhodochrous* LL100-21 (positive control), and the yeast *Saccharomyces cerevisiae* (negative control). PCR reactions were carried out as described in section 2.3.6 using the forward and reverse primers detailed in table 7.5. These primer sets were designed from table 7.4 as indicated.

Table 7.5: Primers used and expected product size for the PCR reactions carried out. Forward and reverse primers were taken from table 7.4 and numbers correspond to the specific primers used (as detailed in table 7.4).

Bacterial primer	Forward	Reverse	DNA fragment (bp)
Rhodococcus nitrilase	1	5	393
Pseudomonas nitrilase	2	6	393
Rhodococcus nitrile hydratase	9	13	249
Pseudomonas nitrile hydratase	10	14	249

The subsequent PCR products were run on a 0.7% agarose gel stained with ethidium bromide (0.2 μg/ml). As many non-specific products were visualised no conclusions could be made without further optimisation of the parameters.

7.4 Discussion and conclusions

7.4.1 Southern hybridisation

This technique did not highlight any DNA fragments corresponding to nitrile hydratase and nitrilase in any of the test bacterial genomes. However, both genes are known to be present in *Rhodococcus rhodochrous* LL100-21 (Collins and Knowles, (1983); Linton and Knowles (1986)), suggesting the techniques used need to be refined. Possible parameters that could be investigated include hybridisation temperature, concentrations of primer/template DNA, probe length and the probe design.

The detection step of DIG and fluorescien labelled primers is extremely insensitive therefore a chemiluminescent or radioactive approach could be used in the future for greater sensitivity.

7.4.2 PCR reactions

Due to time constraints restricting experimental design the probes described yielded many non-specific products during the PCR reactions. Therefore, as with the Southern hybridisation technique, conditions need to be refined to yield PCR products of the correct size. For example combinations of the primers synthesised could be used as could increased annealing temperatures, and varying concentrations of primer and template DNA. Once identified a nested PCR (to increase specificity of the product) followed by sequencing and cloning could be carried out.

Chapter 8: Project conclusions

The main aims of the project were linked to the utilisation of the bacterium *Rhodococcus rhodochrous* LL100-21 or any other isolated microorganism to biotransform a range of novel multifunctional nitriles. These nitriles ultimately could be of commercial interest, for example in the pharmaceutical industry.

As a prelude to the study of these more diverse nitriles, the biotransformations of (cyanomethyl) benzonitriles were studied. These compounds are dinitriles with an aliphatic side chain of $-CH_2CN$ plus an aromatic nitrile group directly attached to the benzene ring.

Analysis of these compounds revealed that regioselectivity can be achieved by this bacterium. Bacteria that had been grown on either benzonitrile or propionitrile converted 2-(cyanomethyl) benzonitrile to 2-(cyanophenyl) acetic acid. The lack of transformation of the aromatic nitrile group was possibly due to steric hindrance encountered by the nitrilase enzyme.

Biotransformation of 3- and 4-(cyanomethyl) benzonitrile confirmed this supposition as in both cases it was the aromatic rather than the aliphatic cyano group that was preferentially hydrolysed. This suggested that the nitrilase enzyme would only hydrolyse the aliphatic cyano group of 2-(cyanomethyl) benzonitrile when another substituent is present in the *ortho* position.

Further studies were carried out to investigate the initial rates of hydrolysis when different functional groups were present in the *ortho* position.

Interestingly, when a cyano or bromo group was present in the *ortho* position, the initial rate was much faster (up to 7 times that for cyano) if there was either substitution by a methyl group or no ring substitution in the *ortho* position. This suggested that these groups could play an important role in the hydrolysis reaction, although further experiments using a wide range of *ortho* substituents are needed to confirm these data.

In contrast bacteria grown on (and induced by) acetonitrile converted the (cyanomethyl) benzonitrile compounds to different products. One example is 2-(cyanomethyl) benzonitrile which was converted to a mixture of the amide intermediates, although in low yields. This suggested the nitrile hydratase enzyme of this bacterium does not exhibit any regioselectivity towards these compounds. Furthermore, the results indicated the amidase enzyme was unable to further convert the products.

These results were exciting as they showed that *R. rhodochrous* could exhibit regioselectivity depending on the inducer/growth substrate and the position of substitution within the aromatic ring. These studies helped to predict the putative intermediates/products formed during the biotransformation of more diverse nitriles utilised within this project.

A small but diverse range of prochiral substrates were then studied to investigate the production of a chiral centre which, with further chemical rearrangement, might afford products that would be of interest to the pharmaceutical or fine chemicals industries. Biotransformations using these prochiral substrates suggested that novel intermediates and products could be formed using *R rhodochrous* LL100-21.

Unfortunately, so far the biotransformation data has not been fully completed on the substrates tested, as only a small amount of the substrates were available. Also time constraints within the project prevented analytical and experimental parameters to be fully optimised.

Studies were carried out on the nitrilase enzyme of *R. rhodochrous* with 2-(cyanomethyl) benzonitrile as the substrate, as previous data (cf. Chapter 3) had suggested that the bacterium was unable to grow on (but could transform) the substrate. The data obtained suggested that the nitrilase reaction was diffusion limited as the enzyme is located inside the cell.

The exact nature of the enzyme could not be determined, and more extensive studies (which were out of the scope of this project) would need to be conducted using the nitrile hydratase, amidase and nitrilase enzymes with different multifunctional nitriles as the substrates. This, coupled with a genetic approach, will give a clearer picture of the enzyme structure, kinetics, regulation and induction.

Genetic studies would conclusively show the presence or absence of these genes in bacterial isolates and confirm if under different induction conditions different nitrile hydrolysing enzymes are produced. Furthermore, probes could be designed to isolate novel nitrile hydrolysing microorganisms from the environment. Therefore, as a prelude to such genetic studies, this project investigated the presence/absence of nitrile hydrolysing genes in different bacteria (*Rhodococcus* and *Pseudomonas*) and yeast (*Saccharomyces cerevisiae*) using Southern hybridisation and PCR approaches.

Specific primers were designed for *Rhodococcus* and *Pseudomonas* nitrile hydratase and nitrilase enzymes. As both techniques have yielded no specific data on the presence or absence of the nitrile hydrolysing genes, more time is needed to optimise the experimental procedures.

Addition of solvents to biotransformation media are known to change the way in which enzymes act on substrates (Klibanov, 1989). Using a known biotransformation (benzonitrile to benzoic acid) activity of the nitrilase enzyme from *R. rhodochrous* was studied in the presence of hydroxylamine. Hydroxylamine is more nucleophillic than water so it was envisaged that it might compete with the water to form a novel compound.

Using suspensions of bacteria, the biotransformation yielded a novel product (benzohydroxamic acid) which is a potential precursor to many pharmaceutically interesting compounds. More interestingly the abiotic control yielded the compound benzamide oxime (a Sigma rare chemical) which, although was only produced in small quantities, was cleaner than the standard synthesised by Sigma UK. Ultimately, this part of the project has shown for the first time a new and novel way to synthesise these compounds. More extensive studies are needed to optimise the procedures and test a wide range of substrates for the biotransformation.

During the project it was discovered that mid infrared spectroscopy was a useful technique to monitor biotransformations in real-time. Nitrile biotransformations were used as a model system using React IR MP with either a diamond or silicon probe. Initial problems including solubility of the nitriles and sensitivity of the probe were overcome and the technique proved to be very powerful in monitoring the enzymatic action of the bacteria in real-time.

This study also investigated the ability of *R. rhodochrous* to resolve racemates and monitor them in real-time. This appears to be possible, paving the way to a technique in which these types of reaction could be studied in real-time and terminated at the appropriate stage, to give 100 % enantiomeric excess (e.e) of the product and hence maximum stereo-control.

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Appendices

Appendix 1: The universal genetic code

First Position (5' end)	Second position				Third position (3' end)
	U	**C**	**A**	**G**	
U	Phe	Ser	Tyr	Cys	**U**
	Phe	Ser	Tyr	Cys	**C**
	Leu	Ser	**STOP**	**STOP**	**A**
	Leu	Ser	**STOP**	Trp	**G**
C	Leu	Pro	His	Arg	**U**
	Leu	Pro	His	Arg	**C**
	Leu	Pro	Gln	Arg	**A**
	Leu	Pro	Gln	Arg	**G**
A	Ile	Thr	Asn	Ser	**U**
	Ile	Thr	Asn	Ser	**C**
	Ile	Thr	Lys	Arg	**A**
	Met	Thr	Lys	Arg	**G**
G	Val	Ala	Asp	Gly	**U**
	Val	Ala	Asp	Gly	**C**
	Val	Ala	Glu	Gly	**A**
	Val	Ala	Glu	Gly	**G**

Appendix 2: Degenerate codon table

DNA symbol	Meaning	A C G T
A	Adenine	-
G	Guanine	-
C	Cytosine	-
T	Thiamine	-
U	Uracil	-
Y	pYrimidine	C or T
R	puRine	A or G
W	"Weak"	A or T
S	"Strong"	C or G
K	"Keto"	T or G
M	"aMino"	C or A
B	not A	A or G or T
D	not C	A or C or T
H	not G	A or C or T
V	not T	A or C or G
X, N, ?	Unknown	A or C or G or T
O	Deletion	-
-	Deletion	-

Appendix 3: Degeneracy in Rhodococcus rhodochrous

Rhodococcus rhodochrous [gbbct]: 36 CDS's (10752 codons)

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fields: [triplet] [frequency: per thousand] ([number])

UUU 2.0 (22) UCU 3.4(37) UAU 4.4(47) UGU 1.9(20)
UUC 30.2 (325) UCC 16.6(179) UAC 25.2(271) UGC 6.8(73)
UUA 0.5 (5) UCA 4.7(50) UAA 0.5(5) UGA 2.3(25)
UUG 12.6 (135) UCG 17.1(184) UAG 0.6(6) UGG 18.4(198)

CUU 4.7(50) CCU 6.0(65) CAU 6.0(64) CGU 9.7(104)
CUC 37.4(402) CCC 18.5(199) CAC 22.2(239) CGC 29.6(318)
CUA 1.9(20) CCA 3.2(34) CAA 5.2(56) CGA 11.4(123)
CUG 32.8(353) CCG 25.8(277) CAG 22.9(246) CGG 16.5(177)

AUU 4.8(52) ACU 5.3(57) AAU 4.2(45) AGU 3.8(41)
AUC 38.4(413) ACC 34.9(375) AAC 21.0(226) AGC 13.0(140)
AUA 1.4(15) ACA 6.5(70) AAA 5.0(54) AGA 1.2(13)
AUG 22.4(241) ACG 15.0(161) AAG 20.3(218) AGG 2.1(23)

GUU 6.0(65) GCU 9.3(100) GAU 16.5(177) GGU 16.3(175)
GUC 41.5(446) GCC 50.5(543) GAC 44.4(477) GGC 42.0(452)
GUA 6.6(71) GCA 16.7(180) GAA 22.9(246) GGA 14.5(156)
GUG 24.9(268) GCG 33.1(356) GAG 41.3(444) GGG 13.3(143)

Coding GC 63.81% 1st letter GC 65.35% 2nd letter GC 46.95% 3rd letter GC 79.13%

Appendix 4: Media and buffers

1: M9 minimal media

Phosphate buffer (X10)

Na_2HPO_4	60 g/l
KH_2PO_4	30 g/l
NaCl	5 g/l
NH_4Cl	10 g/l (nitrogen source)

Magnesium sulphate/Calcium chloride solution (X100)

$CaCl_2.2H_2O$	0.736 g in 500 ml dd H_2O
$MgSO_4$	12.3 g in 500 ml dd H_2O

Trace Metal Solution (X 1000)

MgO	5.375 g/l
$CaCO_3$	1.0 g/l
$FeSO_4.7H_2O$	2.25 g/l
$CuSO_4.5H_2O$	0.125 g/l
H_3BO_3	0.03 g/l
$ZnSO_4.4H_2O$	0.72 g/l
$MnSO_4.4H_2O$	0.56 g/l
HCl (conc)	22.65 g/l

For 50 ml add 5 ml phosphate buffer, 0.5 ml Magnesium sulphate/Calcium chloride solution and 50 µl trace metal solution.

Note: Autoclave Magnesium sulphate/Calcium chloride solution and trace metal solution separately and add to the phosphate buffer after it is autoclaved.

2: Luria-Bertani broth (LB)

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agar (if required)	12 g/l

3:Lysis buffer (1) 1mg/ml of proteinase K and protease P 5147 (Sigma, UK) (5u/tube / 200 µl of 5 mg/ml) were added to 0.5 % (w/v) sodium dodecylsulphate (SDS).

4: Lysis buffer (2): Protease P5147 (Sigma, UK) (5u/tube, 200 µl of 5 mg/ml) and proteinase K (1 mg/ml) were added to 10 % (w/v) sodium dodecylsulphate (SDS).