NITROARYL REDUCTASE: PURIFICATION FROM *SACCHAROMYCES CEREVISIAE* AND USE IN BIOTRANSFORMATIONS

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This thesis is submitted in part fulfillment of the requirements for the degree of Doctor of Philosophy at the university of Edinburgh. Unless otherwise stated, the work described is original and has not been previously submitted, in whole or in part for any degree at this or any other university.

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ABSTRACT

It is well known that baker's yeast (*Saccharomyces cerevisiae)* can be used as a reagent for the reduction of a variety of functional groups, especially the enantioselective reduction of carbonyl groups to the corresponding optically active alcohols.[1] In the context of this thesis it has been shown that aromatic nitro compounds undergo reduction with *S. cerevisiae* to the corresponding anilines under conditions of neutral pH, room temperature and in an aqueous medium.[2] Mechanistic studies employing putative intermediates, suggest that nitroso and hydroxylamino species are involved on the reduction pathway. Further insights into the mechanism have been gained by investigating the reduction of a series of dicyanonitroaromatic substrates. In all cases, in addition to reduction of the nitro group, the nitrile group *ortho* to the nitro group undergoes conversion to the amide, *via* a proposed heterocyclic intermediate.[3]

The broad range of nitroaromatic substrates reduced by the baker's yeast prompted us to commence purification of the enzyme(s) responsible for the biotransformations. Ten litre fermentations were used in order to provide a sufficient quantity of cells for the subsequent purification steps. The use of size exclusion, anion exchange, hydrophobic interaction and affinity chromatography led to partial purification of the nitroaryl reductase enzymes. The reduction of 1,4-dinitrobenzene to 4-nitroaniline provided an ideal enzyme assay for the production of kinetic data, the enzyme demonstrating Michaelis-Menten kinetics. Subsequent to the successful optimisation of the purification protocol, Marina Alexeeva (University of Edinburgh) has completed the purification of three nitroaryl reducatases with molecular weights of approximately 50 KDa, 45 KDa and 32 KDa as judged by SDS-PAGE.

REFERENCES:

1. R. Csuk and Glänzer, *Chem. Rev.*, 1991, **91**, 49.

2. C. L. Davey, L. W. Powell, N. J. Turner and A.S. Wells, *Tetrahedron Lett.*, 1994, **35**, 7867.

3. J. A. Blackie, N. J. Turner and A. S. Wells, *Tetrahedron Lett.*, 1997, **38**, 3043.

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CONTENTS

ABBREVIATIONS

ADP	adenosine dinucleotide phosphate
AEx	anion exchange
BSA	bovine serum albumin
BTP	bis-tris propane
DTT	dithiothreitol
FF	fast flow
FPLC	fast protein liquid chromatography
GF	gel filtration
h	hours
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
KDa	kilodalton
M	molar concentration
min	minutes
mol	moles
mp	melting point
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nmr	nuclear magnetic resonance
nOe	nuclear Overhauser effect
PMSF	phenylmethylsulphonyl fluoride
rpm	revolutions per minute
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
sec	seconds
TK	transketolase
tlc	thin layer chromatography
TPP	thiamine pyrophosphate
U	enzyme units
UV	ultra-violet
YPD	yeast peptone dextrose
° C	degrees centigrade

RESEARCH AIMS

The aims of this research project were several fold; firstly the optimisation of a biotransformation protocol involving the baker's yeast reduction of nitroaromatic compounds. Following this optimisation, a number of nitroaromatic compounds were to be tested as substrates for baker's yeast aiming to obtain further insight into the substrate specificity of the yeast.

Secondly, to gain further information on the mechanism of the reductive biotransformation and to compare the findings with previously published work.

Thirdly, to extend the use of dicyanonitroarenes which, when reduced with baker's yeast, would produce novel trifunctionalised aromatic structures.

Finally, a major part of the project was to attempt to identify the enzyme(s) responsible for the reduction by protein purification techniques. The isolated proteins may demonstrate differing selectivity to the whole cells from which they are purified, if this is favourable it may be possible in the long term to overexpress the individual nitroreductases in suitable host systems.

1. INTRODUCTION

1.1 GENERAL

The use of biocatalysts dates back to the early nineteenth century with the fermentation of 'beer mash' to produce ethanol. Since these pioneering discoveries were made, a number of progressive findings has led the diverse field of biotransformations (**Table 1.1**).[1]

Year	Founder	Discovery
1830	Coffey	Fermentation of beer mash to ethanol
1833	Payen & Persoz	'a soluble ferment' (amylases)
1838	Berzelium	"catalysis - a new power to produce chemical activity belonging to both inorganic and organic nature'
1858	Pasteur	Kinetic resolution of DL-ammonium tartrate by *Penicillium glaucum*
1864	Pasteur	Oxidation of ethanol to acetic acid by *Acetobacter aceti*
1886	Brown	Oxidation of mannitol to fructose by *Bacterium aceti* and *Acetobacter xylinum.*
1874	Dumas	Reduction of sulphur to hydrogen sulphide by *Saccharomyces cerevisiae*
1921	Neuberg	Chiral acetoin condensation by Yeast
1934	Reichstein	Oxidation of D-sorbitol to L-sorbose by *Acetobacter xylinum*

Table 1.1

With an increasing number of enzymes becoming available for use as 'reagents' in synthetic organic chemistry, the existing limitations encountered for increasing the commercial application of biotransformations needs addressing. Although there is not a widespread use of biotransformations in industry, there are many examples of large scale biocatalytic processes.[1] With the chemical industry facing increasing pressure to perform 'cleaner chemistry' and the Food and Drug Administration (FDA) regulating the marketing of single enantiomers as opposed to racemates in new drug admissions, biocatalysts are set to become an invaluable tool in the industry.

The advantages of using biocatalysts (either whole cell or isolated enzyme) are well established and address both the previously mentioned concerns within the chemical industry. The obvious requirement for safety, including environmental pollution, for processes being performed on a large scale is resulting in the restriction of the use of some organic solvents and extreme conditions such as heat, pH and pressure. These issues can be predominantly averted by using a biologically catalysed reaction which would ordinarily use water as reaction medium and would utilize mild conditions of room temperature, atmospheric pressure and neutral pH.

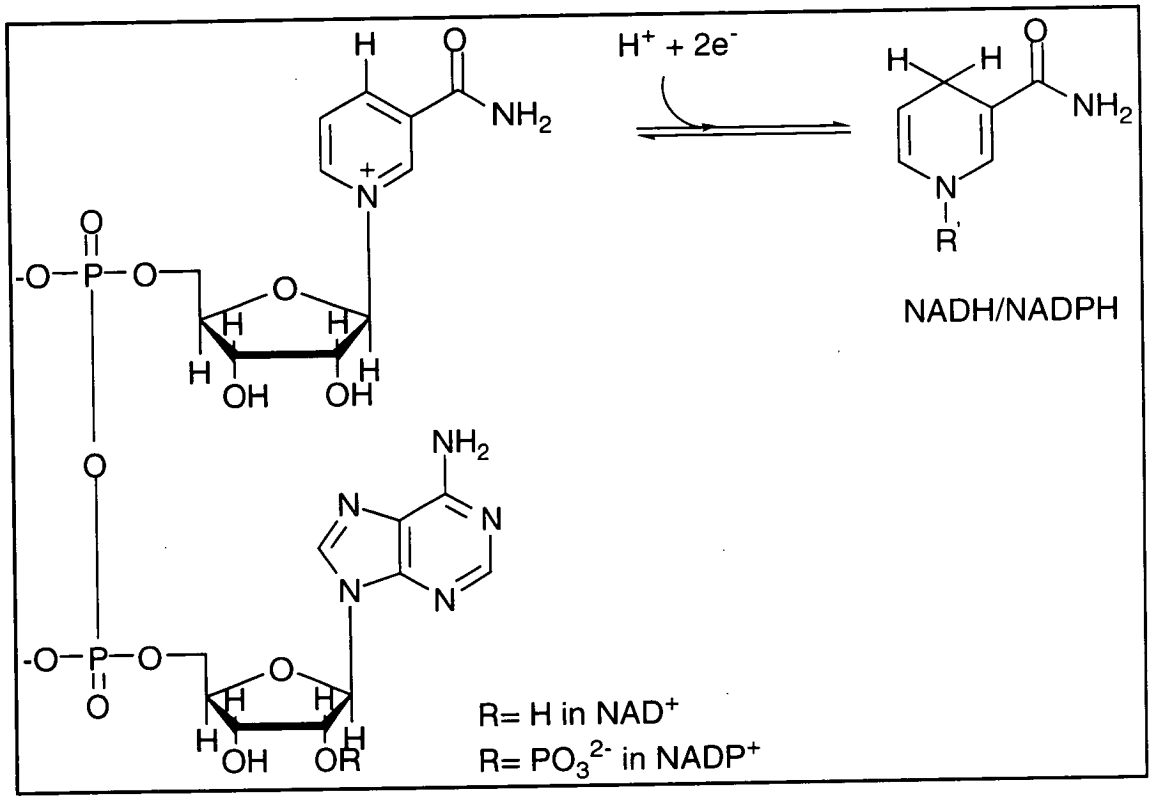
Enantioselectivity in organic synthesis has become a key issue in the pharmaceutical industry for three reasons:[2] Firstly a single enantiomer product can be administered at half the racemic dose (or less if there is more than one asymmetric centre). Thus the second reason becomes apparent, if a chiral centre can be introduced at an early stage in the synthetic process, the yield should be doubled. The third reason for promoting single enantiomer products became very apparent with the thalidomides (one of many examples). Thalidomide was given to pregnant women for reducing blood pressure and early morning sickness, the *R*-isomer was responsible for these sedative effects, the *S*-isomer was the cause of the irreparable defects in many of the births.

Chemo-, regio- and stereoselective reactions may be difficult to perform using conventional synthetic organic chemistry. Biocatalysts can promote these reactions under mild conditions and often with a broad range of substrates and with high turnover numbers.

With the field of biocatalysis making great advances and the benefits becoming apparent, the disadvantages that presently limit the use of enzyme catalysed reactions are being confronted. The instability of enzymes or whole cell systems can often be overcome by immobilisation techniques.[3] Immobilisation enables the continuous use of the biocatalyst, thus making it more cost effective. One method of immobilisation is the adhesion or adsorption of the biocatalyst onto a water-insoluble support such as a commercial resin.[3] Immobilisation of this kind is a mild method that may require the washing out of any new grown cells during operation. Enzymes can also be immobilised by cross-linking them to solid supports using the functional groups found on the surface of the enzyme to covalently couple to the support. Alternatively, cross-linked enzyme crystals (CLECS) can be prepared in which enzymes are directly cross-linked with bifunctional chemical reagents can be achieved. Entrapment or encapsulation of enzymes or cells in gels and other polymers are further methods of immobilisation. The biocatalyst, either enzyme or whole cell, does not itself bind to the matrix or the membrane. Numerous matrices have been employed for this method:

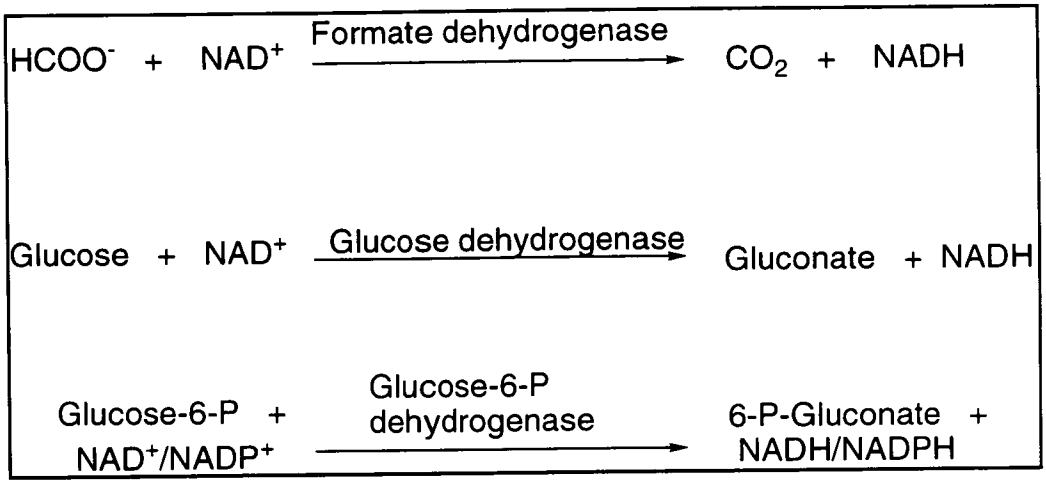
agar, agarose, alginate, carrageenan, collagen, glucan, cellulose acetate, polyacrylamide and polyurethanes. Calcium alginate has been most extensively used. Gel entrapment can be performed under sterile conditions and once the biocatalyst is immobilised it is protected from contamination. κ-Carrageenan, an anionic polysaccharide extracted from seaweed, and polyacrylamide are also commonly used for immobilisation.

A second disadvantage in the use of biocatalysis is the requirement by certain enzymes for cofactors, some of which (NADH and NADPH, **Scheme 1.1**) are too expensive to be used in stoichiometric amounts.



Scheme 1.1

The expense of cofactors can be avoided by the use of a whole cell system which has an in-built cofactor recycling system, or alternatively a recycling system can be employed. There are three well established recycling systems that can be used for the regeneration of nicotinamide cofactors (**Schemes 1.2-1.4**).



Scheme 1.2-1.4

The issue of poor substrate solubility in the aqueous reaction medium of a biotransformation has received a lot of attention. Water-immiscible solvents are normally less toxic to the biocatalyst than water-miscible ones, though presently the most common method of adding a hydrophobic substrate to an aqueous solution is by the addition of a minimum amount of water-miscible solvent to the system. As well as substrate solubility the use of organic media has further benefits:[4]

i. the ability to shift the reaction equilibrium,
ii. regulation of the concentrations of substrates / products in contact with the catalyst and thus prevention of substrate / product inhibition,
iii. reduction in the amount of foaming in bioreactors,
iv. simplification of product removal,
v. reduction of microbial contamination,
vi. enantioselective control over the reactions using different solvents.

As a rule enzymes require a monolayer of water to retain their structure, and therefore activity, and do not function under totally anhydrous conditions. The amount of water required can be very small and is dependent on the enzyme in question and the solvent.

1.2 BIOCATALYTIC REACTION TYPES

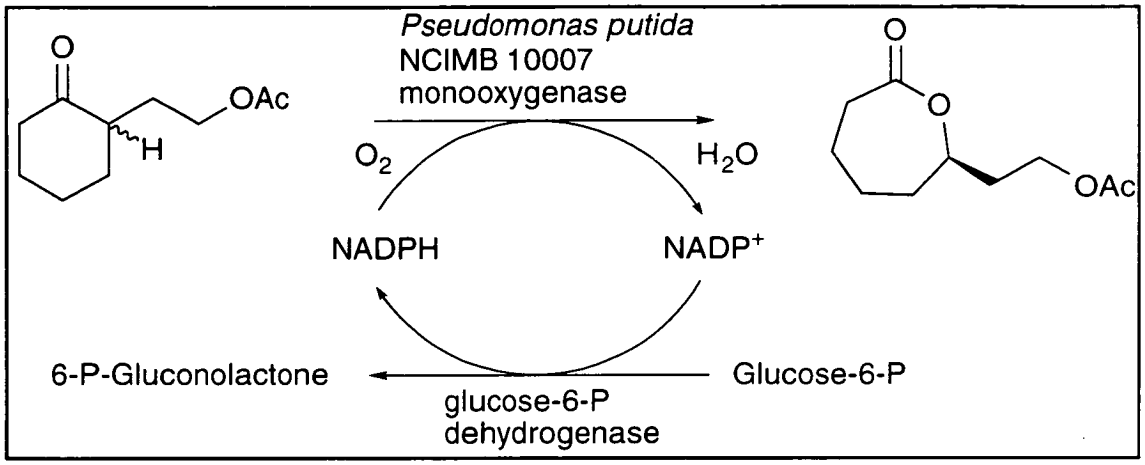
Biotransformations can be performed either using a whole cell system or an isolated enzyme derived from a whole cell. There are advantages and disadvantages to both methods (**Table 1.2**). Enzymes are classified by the Enzyme Commission (EC) according to their function and fall into one of six classes; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

Biotransformation System	Advantages	Disadvantages
Whole Cells	Inexpensive Enzyme cofactors present	Large glassware required Messy work-up Side reaction interference
Isolated Enzymes	Simple apparatus Simple work-up Specific for the selected reaction Co-solvents better tolerated	Expensive Addition of enzyme cofactors required or cofactor recycling required

Table 1.2

1.2.1 OXIDOREDUCTASES

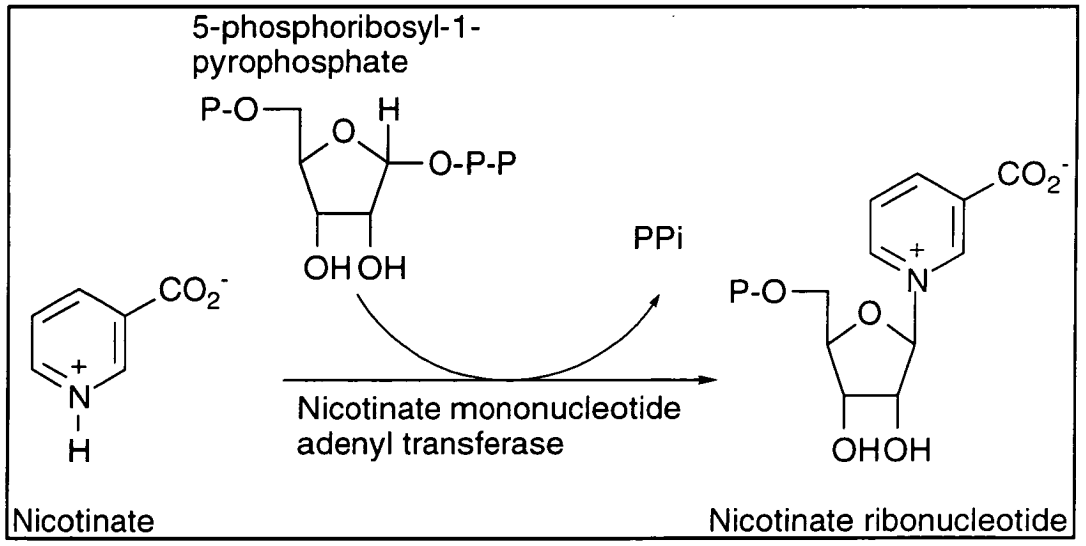
The choice between the use of a whole cell system or isolated enzymes is most relevant within the group of enzymes that catalyse oxidation and reduction reactions since there is a requirement for cofactors when using enzymes for these reaction types. Oxidoreductases make up approximately 26 % of identified enzymes and 26 % of commercially available enzymes.[5] The reduction of ketones and carbon-carbon double bonds using whole cells is dominated by baker's yeast as the biocatalyst. Dehydrogenases, from micro-organisms or animal sources, are capable of the reduction of ketones to alcohols and the reverse process under appropriate conditions with the addition of co-factors. Enzyme catalysed oxidation of alcohols to ketones is not as common as the reverse reaction and generally has no advantage over the use of chemical methods. Baeyer-Villiger oxidations, performed chemically using a peracid, can be achieved with chemo, and regioselectivity, when using a mono-oxygenase (**Scheme 1.5**).[6, 7]



Scheme 1.5

1.2.2 TRANSFERASES

Transferases are the largest group of identified enzymes, comprising 29 % of isolated enzymes and 26 % of the total number that are commercially available (the same proportion as the oxidoreductases).[5] These enzymes, as their name suggests, catalyse the transfer of a group such as an acyl, phosphoryl, sugar or amino acid from one substrate to another. The biosynthesis of the nicotinamide cofactors, for example, utilizes a transferase in the early stages (**Scheme 1.6**).



Scheme 1.6

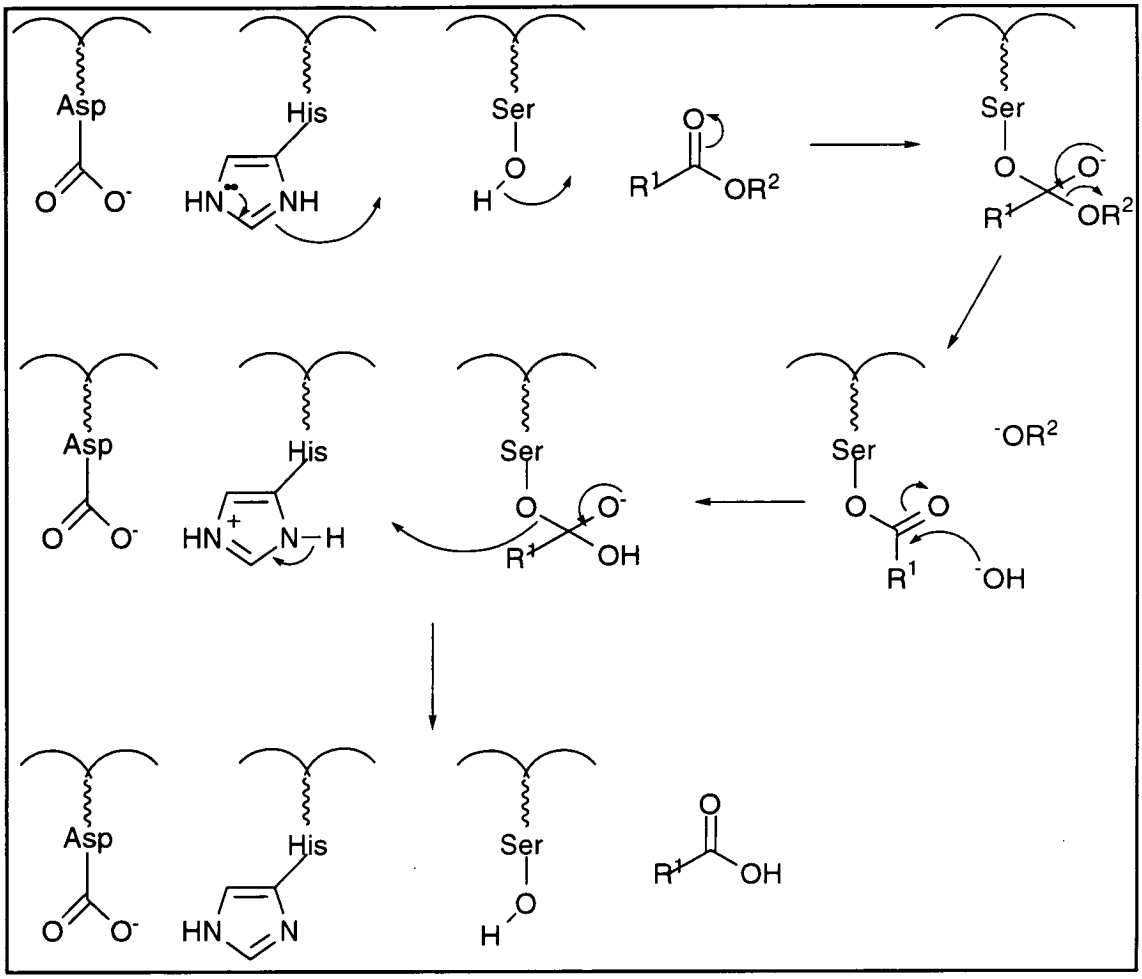
1.2.3 HYDROLASES

Hydrolytic enzymes are the largest group of commercially available enzymes, making up 36 % of the total and though they are not the largest group of identified enzymes (transferases), they are still a significant proportion at 26 %. Hydrolytic reactions encompass the hydrolysis of esters, amides, nitriles and epoxides without the requirement of a cofactor. The enzymes show high enantioselectivity and perform predominantly kinetic resolutions. The mechanism of the catalytic reaction by chymotrypsin, for example, involves the nucleophilic attack by a serine residue to the carbonyl group with formation of the tetrahedral intermediate which subsequently collapses to liberate the acyl enzyme intermediate (**Scheme 1.7**). The nucleophile may be the hydroxyl group of serine (serine proteases), the carboxylate of aspartic acid (pepsin) or the thiol of cysteine (papain). α-Chymotrypsin is a widely used hydrolytic enzyme with activity linked to three amino acids in the catalytic site; serine-195, histidine-57, and aspartate-102. This 'catalytic triad' is typical of hydrolytic reactions. Hydrolytic enzymes can often be used in organic solvents such as methanol, dimethylsulfoxide and DMF provided there is some water present. Under these hydrophobic conditions the hydrolytic enzymes can work in reverse, coupling the acid to the alcohol to give the ester, and often do so enantioselectively.

1.2.4 LYASES

Lyases are responsible for the catalytic addition of a nucleophile to double bonds such as C=O (aldolases and mandelonitrile lyase) and C=C (aspartase and fumerase). The lyases are a smaller group of enzymes than those previously mentioned, both commercially (10 %) and in total number identified (10 %).

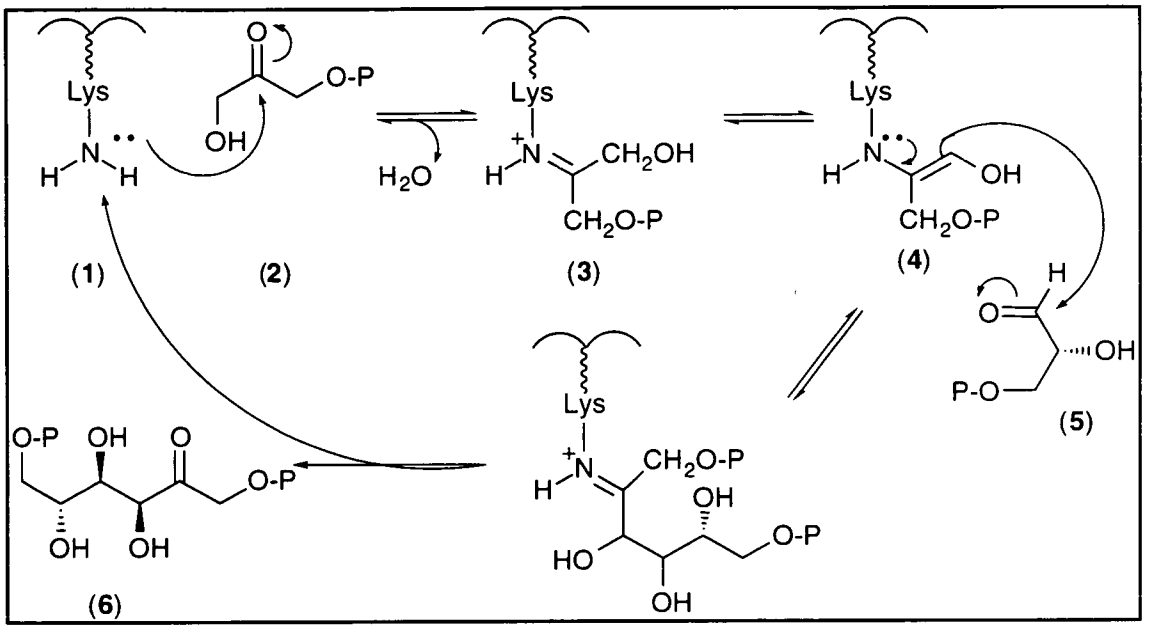
C-C bond forming enzymes, found naturally in the glycolytic pathway, catalyse an important class of biotransformations. Biological aldol condensations, catalysed by aldolases, are the result of coupling an aldehyde (variable in structure) to a carbonyl containing compound, usually either pyruvic acid or dihydroxyacetone phosphate (DHAP), often forming new chiral centres. For example, rabbit muscle aldolase (RAMA) catalyses the condensation of D-glyceraldehyde-3-phosphate (**5**) with DHAP (**2**) to yield fructose-1,6-bisphosphate (**6**).



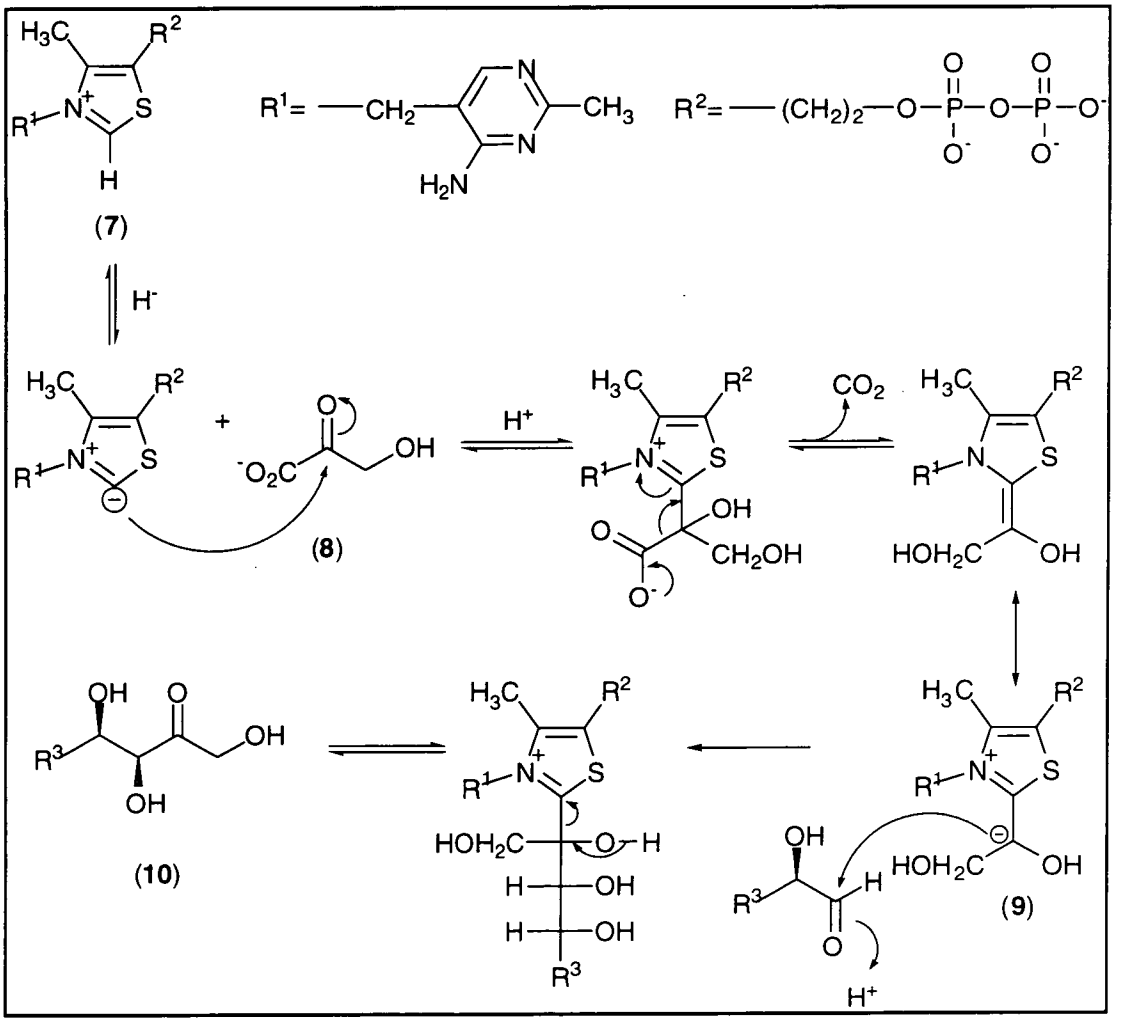
Scheme 1.7

Initially, nucleophilic attack of the amino group of a specific lysine residue in the active site of the enzyme (**1**) with DHAP (**2**) forms a protonated Schiff's base (**3**). The enamine tautomer of the Schiff's base (**4**) attacks the adlehyde (**5**) to form, after hydrolysis, the aldol product (**6**) and the enzyme is regenerated (**Scheme 1.8**). RAMA is capable of coupling up to 75 different electrophilic aldehydes with all products having the same configuration.[8]

Transketolase, which has been overexpressed in *E. coli*, gives rise to similar products to the aldolases and requires magnesium ions and catalytic thiamine pyrophosphate (**7**, TPP) as cofactors. TPP attacks the ketol donor (highly specific for hydroxypyruvate, **8**) to form a carbanion (**9**) which in turn attacks the aldehyde (wide ranging specificity) to from the product (**10**). The evolution of carbon dioxide as a by-product with hydroxypyruvate ensures the reaction goes to completion (**Scheme 1.9**).



Scheme 1.8

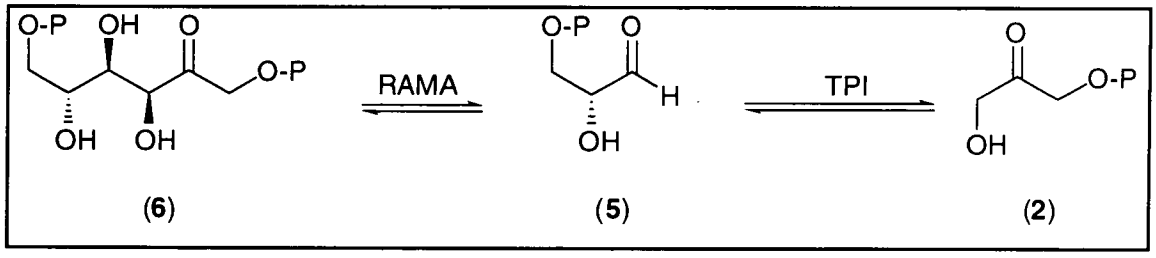


Scheme 1.9

Other examples of lyases include mandelonitrile lyase which catalyses the synthesis of *R*-cyanohydrins from the corresponding aldehyde, or ketone, and HCN. Carbon-carbon bond formation is also catalysed by enzymes present in baker's yeast, (*Saccharomyces cerevisiae*) resulting in the acyloin condensation. A number of lyases and haloperoxidases[9] are available for the synthesis of C-X bonds (where X= O, N, S and halogen).

1.2.5 ISOMERASES

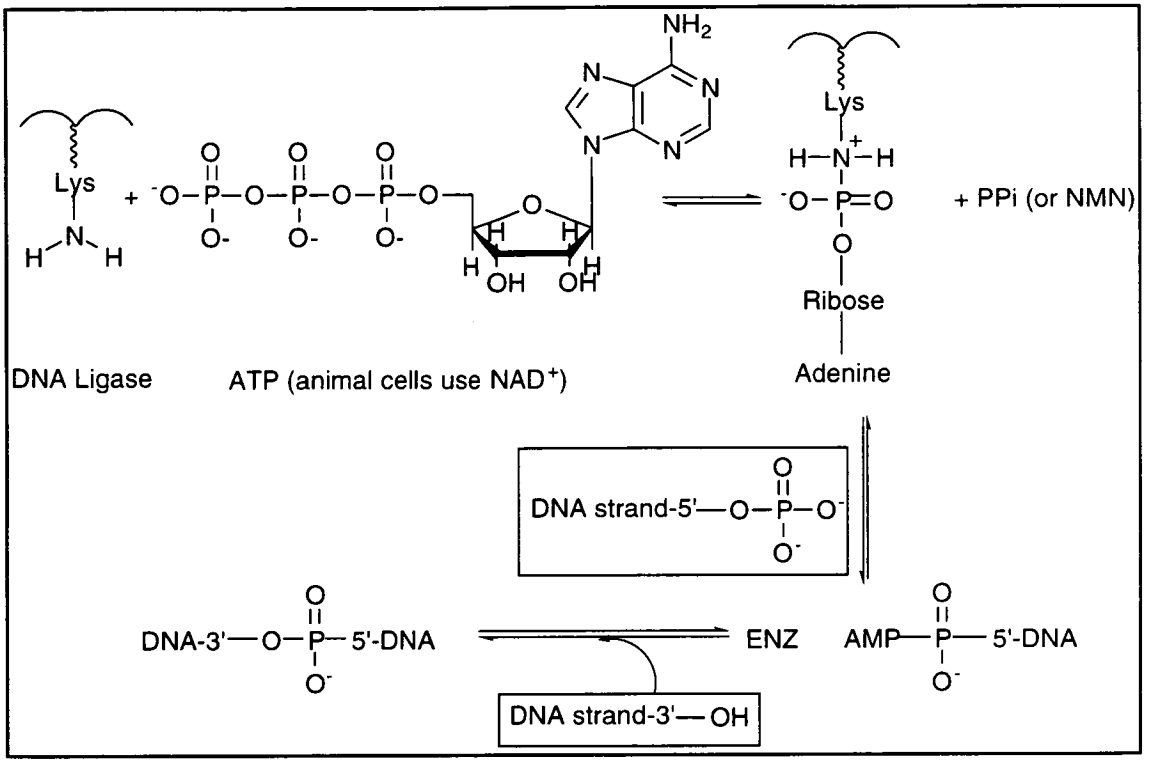
Isomerases are responsible for epimerizations, racemizations and other isomerizations, they make up 5 % of the total number of identified enzymes but only 2 % of those that are commercially available. Previously an example was provided showing the production of fructose-1,6-bisphosphate (**6**) from DHAP (**2**) and glyceraldehyde-3-phosphate (G-3-P, **5**) catalysed by RAMA. This example will be used again here to demonstrate a catalytic isomerase reaction. Triose phosphate isomerase (TPI) is found in the glycolytic pathway where it interconverts DHAP (**2**) and G-3-P (**5**) (**Scheme 1.10**), the glyceraldehyde-3-phosphate then going on further in glycolysis. At equilibrium 96 % of the triose phosphate is DHAP.



Scheme 1.10

1.2.6 LIGASES

Ligases catalyse the formation of C-O, C-S, C-N and phosphoryl bonds and are very important in molecular biology. DNA ligase, for example, is involved in the replication of DNA. This enzyme catalyses the formation of a phosphodiester bond between the 3'-OH group at one end of a DNA chain and the 5'-phosphate group at the other end of a DNA chain (**Scheme 1.11**). The DNA strands must be part of a double helical molecule.



Scheme 1.11

1.3 BAKER'S YEAST (*SACCHAROMYCES CEREVISIAE*) MEDIATED BIOTRANSFORMATIONS

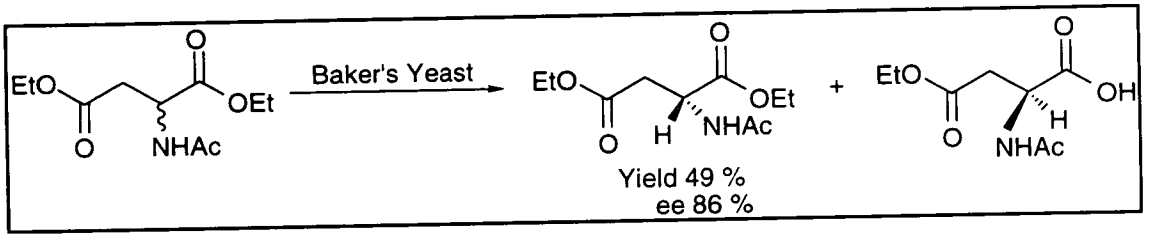
Saccharomyces cerevisiae is a readily available microorganism (world output 600,000 ton/year[10]). Economically it has great potential as a biocatalyst as it is inexpensive and easy to use.

Among the types of biotransformations performed by whole cells or enzymes, baker's yeast, as a whole cell shows oxidoreductase, hydrolase and lyase activities. Baker's yeast biotransformations, however, are dominated by reductions, predominantly carbonyl reductions, in which the resulting product possesses an asymmetric carbon. There are a large number of baker's yeast (whole cell and isolated enzymes) catalysed biotransformations known, the majority of which have been summarised in a number of reviews.[10-12] Below are given a few examples to demonstrate the range of biotransformations that are possible.

1.3.1 HYDROLYSIS OF ESTERS

The hydrolytic ability of baker's yeast was discovered inadvertently as an undesirable side reaction. Three active enzymes have since been recognised;[11] sterol

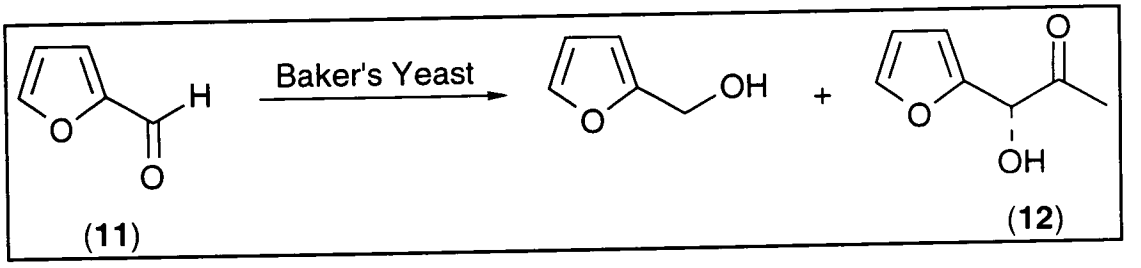
ester hydrolase, triacylglycerol hydrolase (lipase) and carboxylic ester hydrolase (esterase). Although baker's yeast has shown hydrolytic activity in a number of cases, the commercially available hydrolytic enzymes from other sources are far superior. Baker's yeast has however found application in the resolution of various α-amino acids by hydrolysis of the corresponding N-acetyl derivatives (**Scheme 1.12**).[10] Also, baker's yeast shows regioselectivity in the hydrolysis of the ester moiety α to the centre of chirality. However, β-amino acids and cyclic amino acids are not substrates for hydrolysis by baker's yeast.



Scheme 1.12

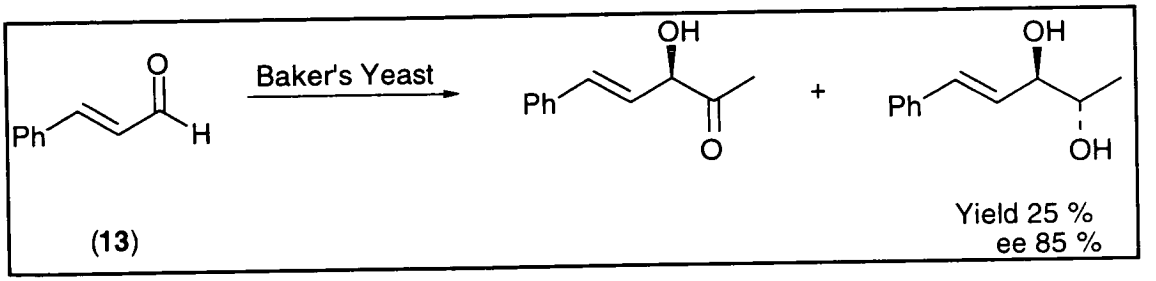
1.3.2 FORMATION OF C-C BONDS

First reported by von Liebig in 1913, the acyloin condensation was observed during yeast fermentation of furfural (**11**) in which furylic alcohol (**12**), the condensation product (identified by Neuberg), was found in addition to the reduction product (**Scheme 1.13**).[11]



Scheme 1.13

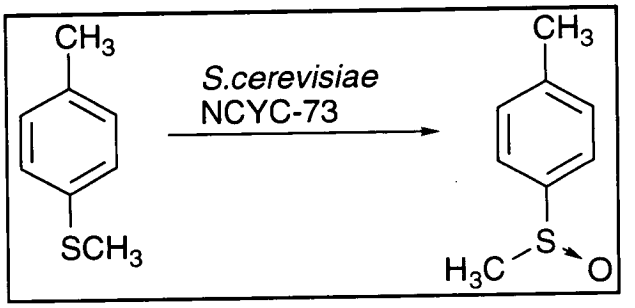
The acyloin condensation is similar to that catalysed by transketolase (see earlier). The source of the acetate unit is pyruvate. α,β-Unsaturated aldehydes, for example cinnamaldehyde (**13**), give optically active diols (**Scheme 1.14**).[11]



Scheme 1.14

1.3.3 OXIDATIONS

There are few examples of baker's yeast catalysed oxidations in the literature, however a limited number of isolated examples of oxidations involving the formation of sulphoxides from corresponding sulphides do exist (**Scheme 1.15**).[13] In this instance a strain of *S. cerevisiae* obtained from the National Collection of Yeast Cultures (NCYC) was used. Benzyl, furfuryl or haloaromatic functionality result in lower yields and stereoselectivity, bulkier side chains also lead to a decrease in rate and selectivity.



Scheme 1.15

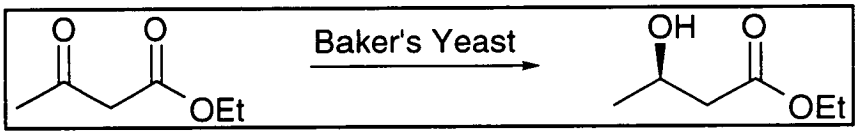
1.3.4 REDUCTIONS

As mentioned above, reduction reactions constitute the largest class of baker's yeast catalysed transformations. Typical substrates include:[10]

- Monocarbonyl compounds - including cyclic and aliphatic alkenes, sulphur containing molecules, α-heterocyclic substituted ketones and nitrocarbonyl compounds.
- Dicarbonyl compounds - including cyclic and acyclic diketones.
- α-Keto esters.
- β-Keto esters - including β-Keto esters with keto group as part of a ring, and aliphatic keto esters.

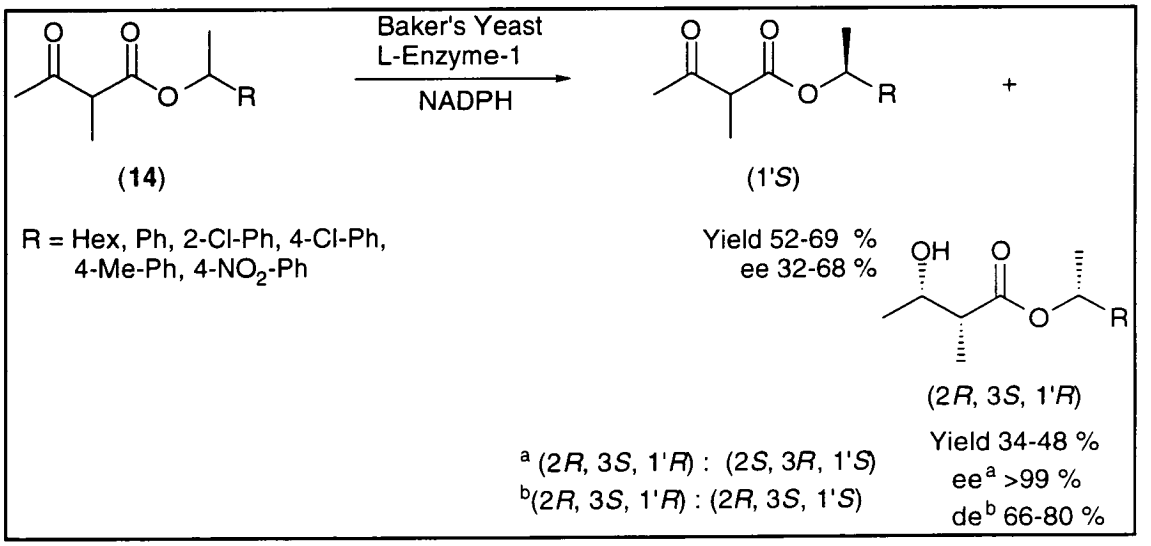
- γ– and δ–Keto acids and esters.
- C=C Double bonds.
- Organometallic compounds.
- Fluorine containing ketones and ketoesters.

The simplest example of the reductive process, the reduction of a ketone to a secondary alcohol, can be achieved by baker's yeast to produce optically active chiral alcohols. Among the many types of carbonyl containing compounds that can be reduced by baker's yeast, the most studied is the enantioselective reduction of β-keto esters,[10-12] a simple example of which is shown in **Scheme 1.16**.



Scheme 1.16

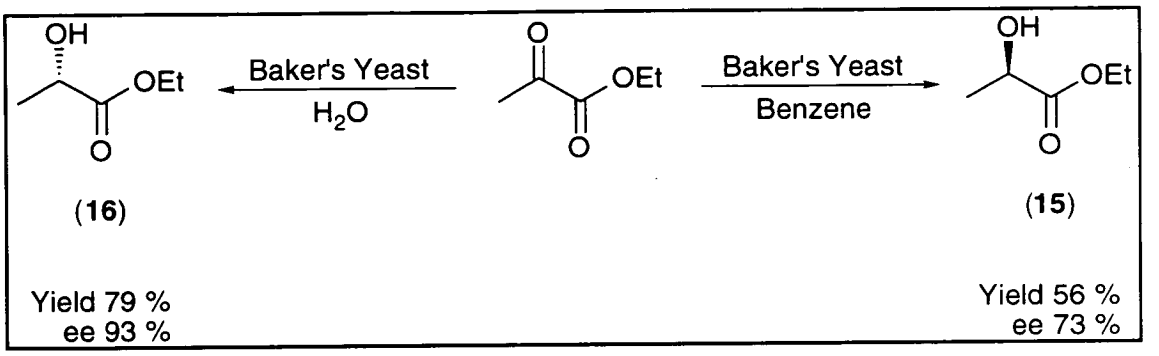
The introduction of plural asymmetric centres in one step is of great value for the production of chiral building blocks. For example, a β-keto ester reductase (known as L-Enzyme-1) from baker's yeast catalyses asymmetric reduction accompanied by simultaneous kinetic resolution of 1'-arylethyl-2-methyl-3-oxobutanoates (**14**) (**Scheme 1.17**).[14]



Scheme 1.17

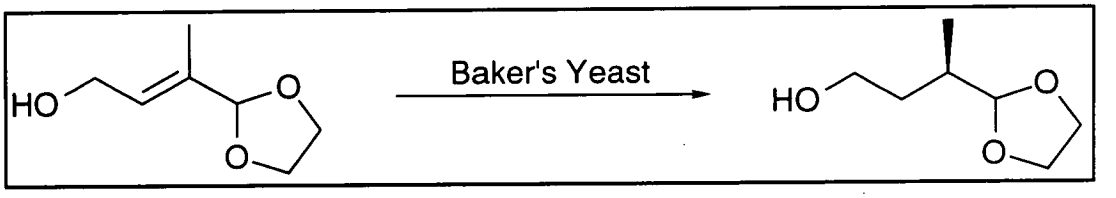
There are reports of baker's yeast biotransformations occurring in the presence of organic solvents.[15] A recent report describes the effect of using an organic solvent

on the stereochemistry of reduction of α-keto esters (**Scheme 1.18**).[16] Non-polar solvents favour formation of the *R*-stereoisomer (**15**), whereas an aqueous medium favours the formation of the *S*-isomer (**16**).



Scheme 1.18

The baker's yeast reduction of C-C double bonds has predominantly involved the preparation of the chiral isoprenoid unit found in natural products. One of the early transformations leading to a chiral isoprenoid unit of this type is shown below (**Scheme 1.19**).[11]



Scheme 1.19

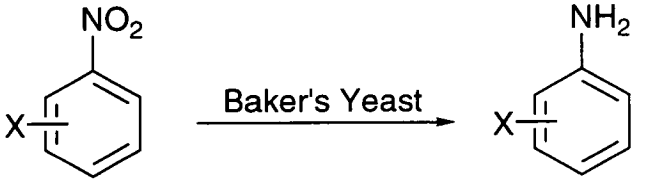
1.4 BAKER'S YEAST MEDIATED REDUCTIONS OF N-O AND N=N CONTAINING COMPOUNDS

The reduction of nitroarenes using baker's yeast was first reported by Neuburg in 1914.[17] Since then the baker's yeast reduction of N-containing substrates has become a field of increasing interest, encompassing the reductions of nitroarenes, nitrosoarenes, nitroalkanes, hydroxylamines, azides, N-oxides, isoxazoles and oximes.

1.4.1 BAKER'S YEAST REDUCTION OF NITROARENES

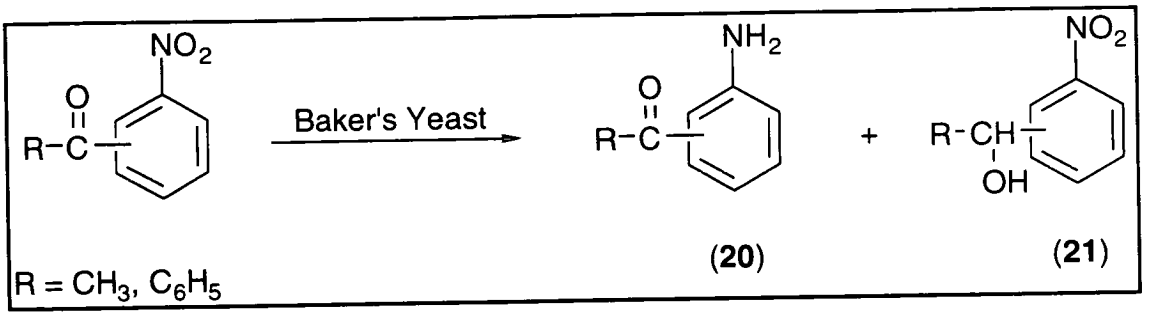
Although Neuberg reported the conversion of nitrobenzene to aniline using fermenting baker's yeast in 1914[17], little had been reported on the influence of the

substituents on the reduction of nitroarenes. Takeshita *et al.*, studied the reduction of a number of substituted nitroarenes with baker's yeast (**Table 1.3**).[18] The results indicated that reduction proceeds smoothly, and with a good yield of the aniline, when electron withdrawing groups are present on the ring and conversely reduction is sluggish or fails to occur at all when electron donating groups are present. Some differences of reactivity between *ortho*- and *para*-nitro compounds and the *meta*-isomers were observed. This paper reports that when a ketone group is attached to the aromatic ring, selective reduction took place to give either amino ketones or nitroalcohols without any amino alcohol being obtained. The *o*- and *p*-aminoketones (**20**) were preferentially obtained at temperatures of 32-33 °C (72-108 h), while the *m*-isomer gave a slight excess of the nitroalcohol (**21**). Under milder conditions (24 °C, 24 h) the nitroalcohol (**21**) was formed preferentially (**Scheme 1.20**).



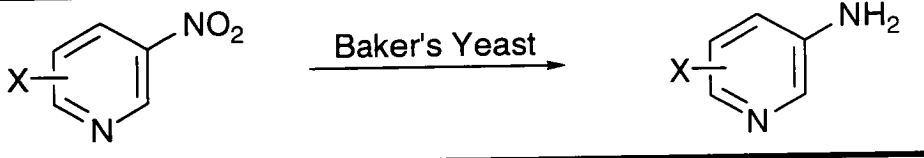
Entry No	Substituent	Yield (%)	Entry No	Substituent	Yield (%)
1	*o*-NH_2	0	16	*o*-NO_2	87
2	*m*-NH_2	0	17	*m*-NO_2	66
3	*p*-NH_2	0	18	*p*-NO_2	87
4	*o*-OH	0	19	*o*-CN	30
5	*m*-OH	18	20	*m*-CN	88
6	*p*-OH	0	21	*p*-CN	80
7	*o*-CH_3	0	22	*o*-CF_3	66
8	*m*-CH_3	17	23	*m*-CF_3	55
9	*p*-CH_3	24	24	*p*-CF_3	48
10	*o*-CH_3O	22	25	*o*-$COOC_2H_5$	28
11	*m*-CH_3O	25	26	*m*-$COOC_2H_5$	28
12	*p*-CH_3O	0	27	*p*-$COOC_2H_5$	61
13	*o*-Br	62	28	*o*-$NHCOCH_3$	25
14	*m*-Br	42	29	*m*-$NHCOCH_3$	59
15	*p*-Br	37	30	*p*-$NHCOCH_3$	4

Table 1.3



Scheme 1.20

Takeshita went on to study the reduction of heteroaromatic nitro compounds with baker's yeast.[19] Nitropyridine derivatives with electron rich substituents present were shown to be reduced in low yields or not at all. However, when electron withdrawing groups were present, reduction proceeded smoothly and in good yields at 33 °C over 41-115 h (**Table 1.4**), consistent with the earlier work.[18] Also reported was the selective reduction of the nitro group of 1-methyl-3-nitro- and 1-methyl-5-nitro-2(1H)-pyridones over a carbonyl group at 33 °C over 77-95 h.[19]



Entry No	X	Yield (%)	Entry No	X	Yield (%)
1	6-OCH_3	26	5	6-Cl	88
2	6-NH_2	0	6	2-Cl	78
3	6-OH	0	7	6-Cl, 4-CH_3	77
4	2-OH	40	8	2-Cl, 6-Cl	41

Table 1.4

Four years after Takeshita *et al.*, released their findings on the baker's yeast reduction of nitroarenes, Davey *et al.*, reported the regioselective reduction of dinitro-arenes using baker's yeast.[20] Initially the baker's yeast reduction of mononitroarenes were studied confirming that reduction was enhanced when electron withdrawing groups were present on the ring. An unexpected result arose from the reduction of 2-nitrobenzonitrile (**22**) to 2-aminobenzamide (**23**) in 56 % yield, the expected product being 2-cyanoaniline (**24**). **Scheme 1.21** shows a mechanism that was proposed for the reduction.

The report also discusses the regioselective reduction of a series of dinitroarenes. In most cases one regioisomer of the two possible aniline products

predominated (**Table 1.5**). The results led to the proposal of a model for predicting the regiospecificity of the reduction (**Figure 1.1**).

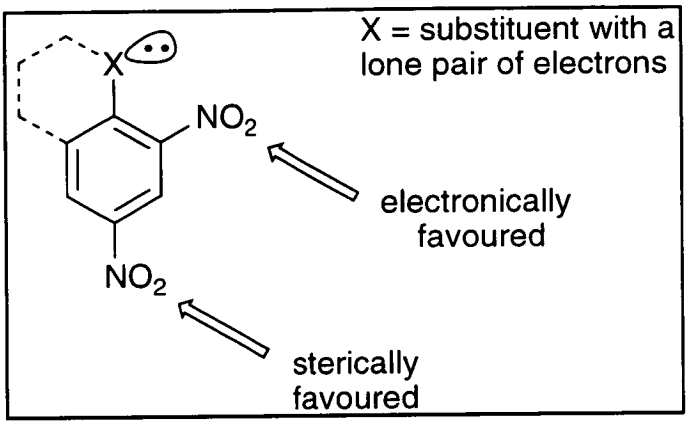
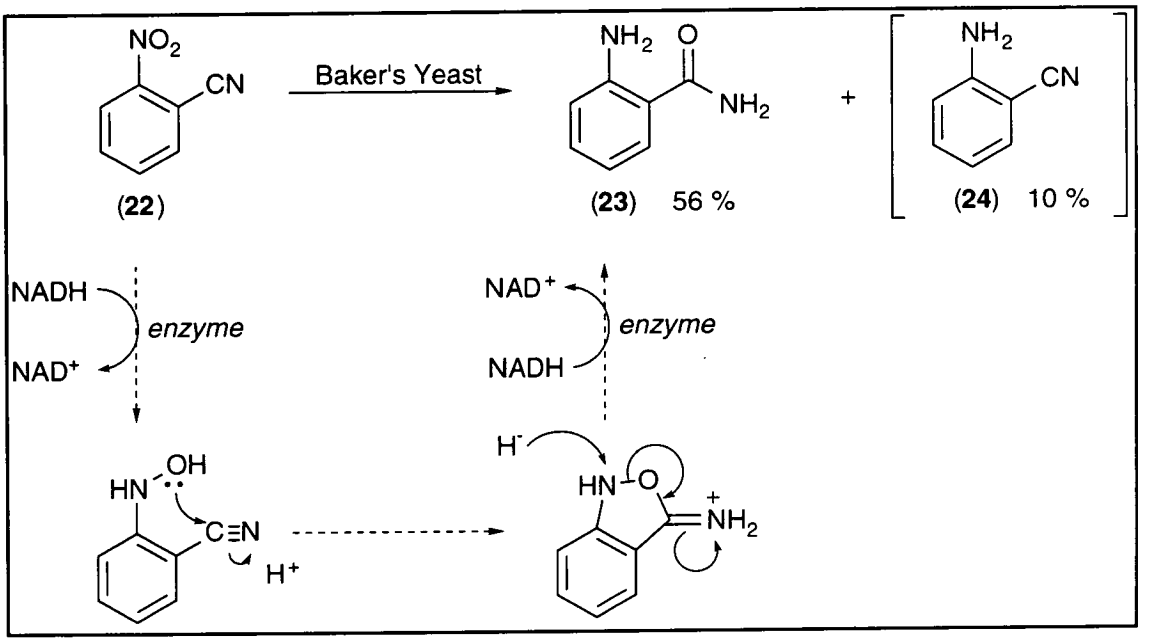
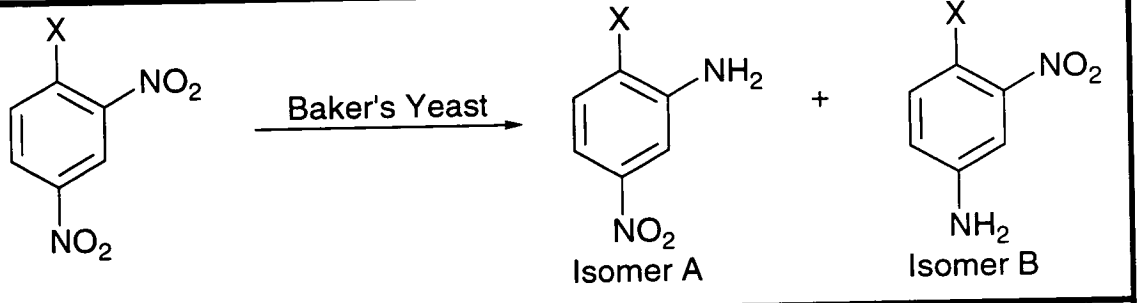


Figure 1.1



Scheme 1.21

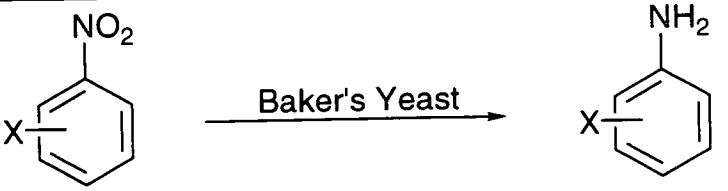


Entry No	X	Time (d)	Yield of A	Yield of B	Ratio A:B
1	CH_3	4	22	22	1:1
2	C_2H_5	4	11	26	1:3
3	OCH_3	4	30	6	5:1
4	OC_2H_5	4	20	7	3:1
5	SCH_3	4	13	27	1:2
6	$S(O)CH_3$	$^1/_4$	7	7	1:1

Table 1.5

All the biotransformations performed in this report were carried out in water at neutral pH and 32 °C. In the same year as the report by Davey *et al.*, was published, Baik *et al.*, released their findings on the selective reduction of aromatic nitro compounds to aromatic amines by baker's yeast in basic solution.[21] It is worth noting that the conditions employed by Baik and co-workers in their research (typically water/ethanol or water/methanol medium, sodium hydroxide to produce pH >12 and high temperature, 70-80 °C) differ from the mild conditions used by Davey *et al.*, and other research groups when using baker's yeast. The report describes baker's yeast as an efficient and selective reagent in the reduction of aromatic nitro compounds, with higher yields and shorter reaction times than those previously reported (**Table 1.6**).

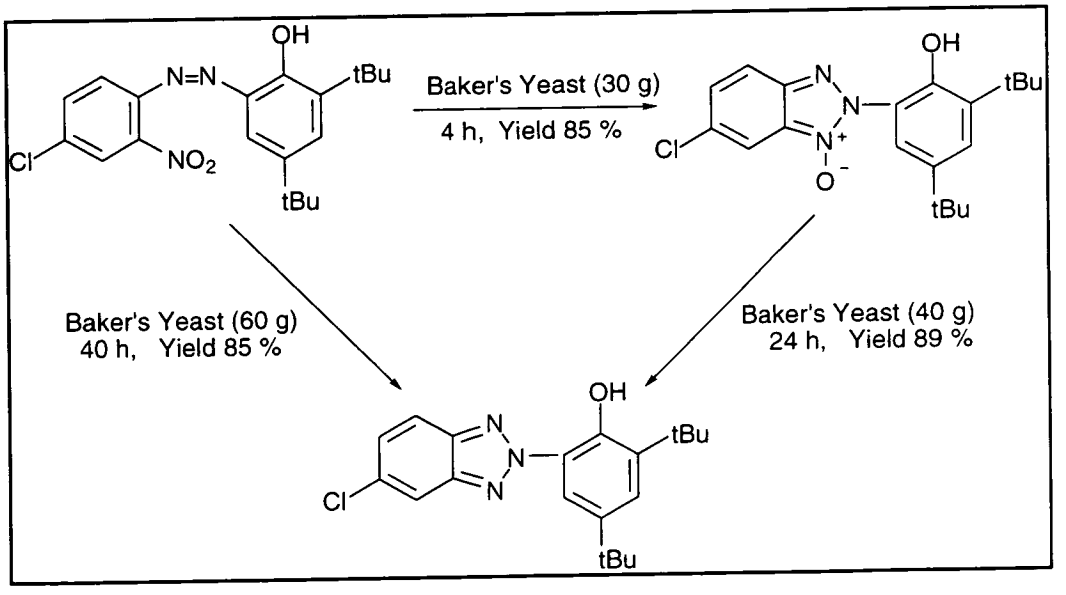
A year later in 1995, Baik and co-workers released a similar report on the reductive cyclisation of nitrophenylazo dyes using baker's yeast in sodium hydroxide as a method for synthesising benzotriazoles-1-oxide. It was shown that by using twice the amount of yeast and a longer reaction time, the benzotriazoles were obtained (**Scheme 1.22**). As before the reaction conditions employed involved high pH and high temperature.[22]



Entry No	X	Reaction Time (h)	Yield (%)
1	H	20	55
2	2-$COCH_3$	2	91*
3	4-$COCH_3$	2	91*
4	4-COC_6H_5	5	85
5	4-CHO	5	84*
6	3-I	5	91
7	3-Br	4	90*
8	2-Cl	5	90*
9	3-Cl, 6-NH_2	3	85
10	3-NO_2	2	95*
11	2-$OCH_2CH{=}CH_2$	24	70*
12	4-$OCH_2CH{=}CH_2$	24	69
13	4-OCH_3	24	25*
14	2-OH	24	19
15	4-OH	24	18
16	1-Nitronaphthalene	7	85

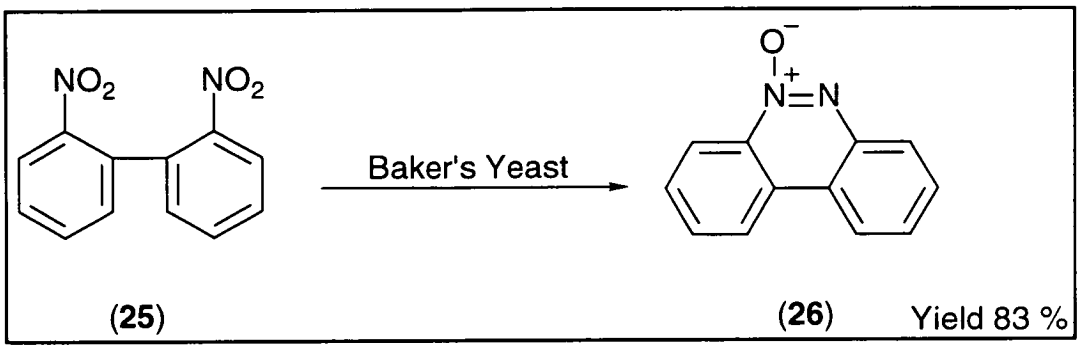
* Yields determined by GLC

Table 1.6



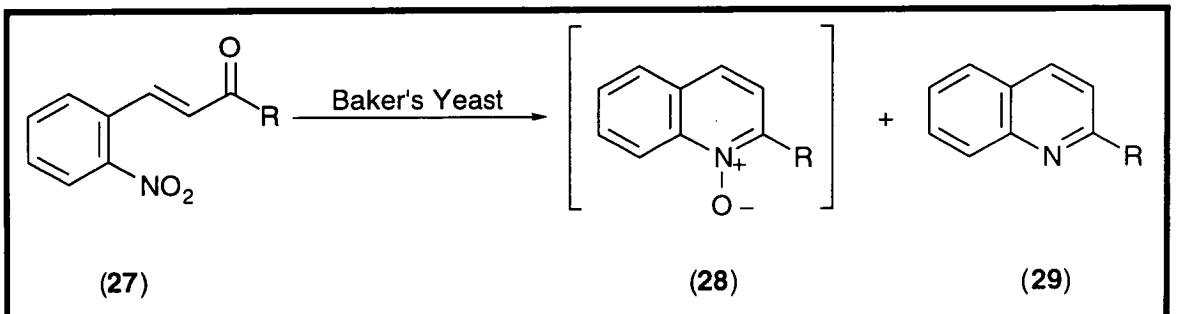
Scheme 1.22

Under the same conditions 2,2'-dinitrobiphenyl (**25**) was reduced to the corresponding N-oxide (**26**) in 83 % yield (**Scheme 1.23**).



Scheme 1.23

More recently Baik *et al.*, have reported the use of baker's yeast in basic media to produce quinolines (**29**) from nitrocinnamaldehydes (**27**) via the N-oxides (**28**, **Table 1.7**).[23] As before electron withdrawing groups were found to enhance the rate of reaction and electron donating groups to impede the rate of reaction.

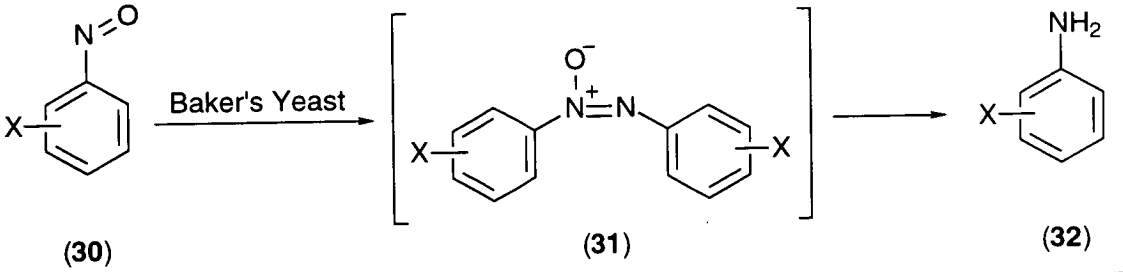


Reaction Conditions	Time (h)	Yield of (**28**) (%)	Yield of (**29**) (%)
30 g baker's yeast, 0.5 g **27**, aq EtOH	24	0	0
2 g NaOH, 0.5 g **27**, 30 g baker's yeast, aq EtOH	1	91	0
4 g NaOH, 0.5 g **27**, 30 g baker's yeast, aq EtOH	24	trace	88

Table 1.7

1.4.2 BAKER'S YEAST REDUCTION OF NITROSOARENES

To date there is only one report published on the reduction of nitrosoarenes by baker's yeast, again by Baik *et al.*[24] As with their previous work on the reduction of nitroarenes, Baik and co-workers performed their transformations at high temperatures (80 °C). However in the reduction of nitrosoarenes, the conversion is performed in aqueous media without the addition of sodium hydroxide. The report describes the selective reduction of aromatic nitroso compounds (**30**) to the corresponding anilines (**32**) via azoxybenzenes (**31**) using baker's yeast (**Table 1.8**). Not surprisingly, by stopping the reaction at an earlier stage (by using less yeast or allowing a shorter reaction time) azoxybenzene was formed in a higher yield than if allowed to proceed for longer. Under the conditions employed for these transformations there was no evidence to favour reduction of an electron deficient system over an electron rich one. The selectivity described is the reduction of the nitroso group over a ketone substituent (**Table 1.8** entries 11 and 12).



(**30**) (**31**) (**32**)

Entry No	X	Time (h)	Yield of (**31**) (%)	Yield of (**32**) (%)
1	H	0.5	77	22
2	H	1.0	26	73
3	H	8.0	0	95
4	H	8.0[a]	71	21
5	2-Cl	0.5	0	95
6	3-Br	1.0	0	99
7	3-I	1.0	0	94
8	4-CH_3	0.5	0	95
9	4-OCH_3	1.0	0	92
10	4-OH	8.0	10	90
11	4-$C(O)CH_3$	0.5	36	37
12	4-$C(O)CH_3$	8.0	25	69

Table 1.8 [a] 5 g of baker's yeast was used instead of 15 g.

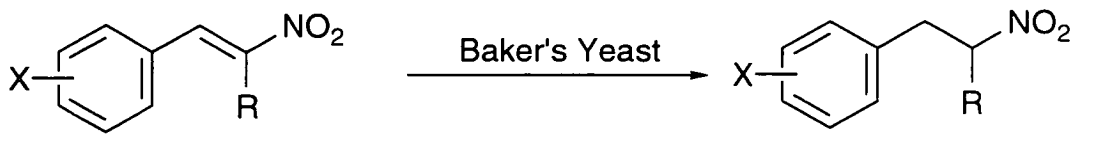
1.4.3 BAKER'S YEAST REDUCTION OF NITROALKENES

The reported literature on the reduction of nitroalkenes with baker's yeast can be subdivided into two groups; (i) the chemoselective reduction of the double bond, leaving the nitro group untouched, and (ii) the reduction of the nitro group in preference to the carbon-carbon double bond.

(i) Reduction of C=C in preference to the nitro group

The chemoselective reduction of nitroalkenes with baker's yeast was reported by Takeshita *et al.*[25] Initial studies on the reduction of *E*-β-nitrostyrenes (**33**) to the corresponding nitroalkanes (**34**) were made (**Table 1.9**), and the effect of substituents on the aromatic ring examined (entries 1-15). The conditions used for the reactions, like those used previously by Takeshita, include fermentation of the substrate with baker's yeast in water at 31-33 °C over a period of 72-74 h. Little difference was observed between the effects of electron withdrawing and electron donating groups in the ring. Slightly higher yields resulted when the substrates were methyl substituted *E*-β-nitrostyrenes (entries 16-29).

When the aromatic ring was substituted with a nitro group the methyl substituted substrates behaved very differently. Lower yields of the nitroalkane (**36**) were observed with reduction of the nitro substituent on the ring being favoured (**Table 1.10**). Similar trends were observed with the reduction of heteroaromatic nitroalkenes.

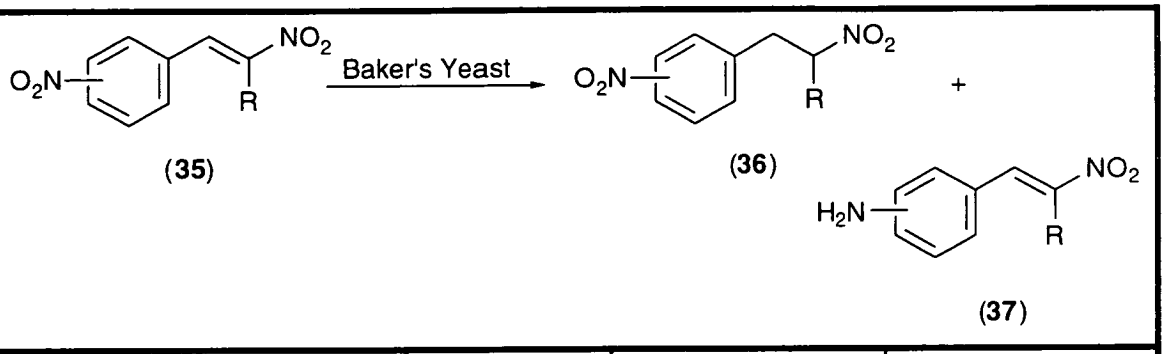


(33) (34)

Entry No	X	R	Yield of (34)(%)	Entry No	X	R	Yield of (34)(%)
1	H	H	43	16	H	CH_3	79
2	*o*-CH_3O	H	57	17	*m*-CH_3O	CH_3	60
3	*m*-CH_3O	H	81	18	*p*-CH_3O	CH_3	42
4	*p*-CH_3O	H	75	19	*o*-CH_3	CH_3	69
5	*o*-CH_3	H	73	20	*m*-CH_3	CH_3	59
6	*m*-CH_3	H	41	21	*p*-CH_3	CH_3	61
7	*p*-CH_3	H	67	22	*p*-OH	CH_3	69
8	*p*-OH	H	37	23	*m*-CN	CH_3	52
9	*m*-CN	H	69	24	*p*-CN	CH_3	48
10	*p*-CN	H	28	25	*o*-Cl	CH_3	48
11	*o*-Br	H	61	26	*m*-Cl	CH_3	42
12	*m*-Br	H	52	27	*p*-Cl	CH_3	73
13	*p*-Br	H	46	28	*o*-Br	CH_3	44
14	*o*-Cl	H	73	29	*m*-Br	CH_3	52
15	*p*-Cl	H	67				

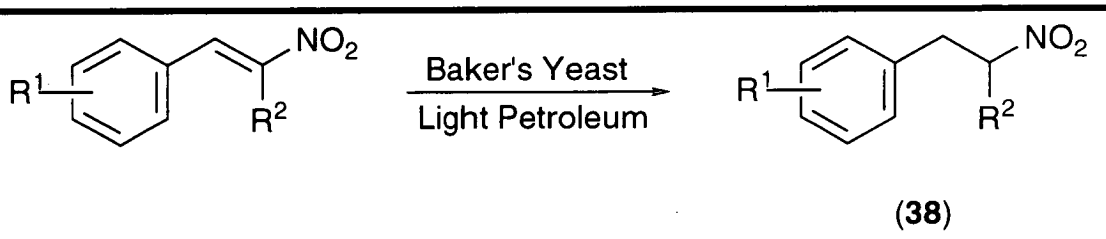
Table 1.9

The yeast mediated reduction of nitrostyrenes in organic solvent was reported by Bak *et al.*[26] Optimisation of the protocol was performed using β-nitrostyrene in order to ascertain the amount of water required to be present in the organic media. Light petroleum was found to be the best solvent with water present at 0.8 ml/g of yeast. The report discusses whether a similar trend is present in an organic solvent system to that of an aqueous system. Nitrostyrenes with different substituents attached to the aromatic ring were tested with baker's yeast (**Table 1.11**). Those with electron donating groups attached to the aromatic ring gave higher yields than those with electron withdrawing groups. This differs from Takeshita's report where little or no difference was observed, and is contrary to the observations found with nitro- and nitrosoarenes.



Entry No	NO_2 Position	R	Yield of (**36**) (%)	Yield of (**37**) (%)
1	4	H	44	5
2	3	H	42	0
3	2	H	65	0
4	4	CH_3	24	61
5	3	CH_3	10	28
6	2	CH_3	24	16
Temp = 33 °C, Time =52-72 h				

Table 1.10

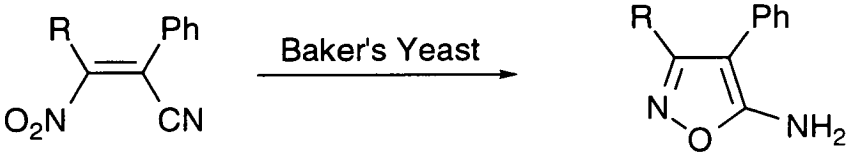


Entry No	R^1	R^2	Yield of (**38**) (%)
1	H	H	74
2	CH_3	H	76
3	OCH_3	H	82
4	CN	H	12
5	NO_2	H	8
6	H	CH_3	80
7	CH_3	CH_3	80
8	OCH_3	CH_3	43
9	CN	CH_3	52
10	NO_2	CH_3	30
Reactions performed at room temperature			

Table 1.11

(ii) Reduction of the nitro roup in preference to C=C

The preparation of isoxazoles (**40**) from nitroalkenes (**39**) was reported by Navarro-Ocaña *et al.*[27] The reaction is thought to proceed through initial reduction of the vinylic nitro group followed by formation of the aminoisoxazole (**Table 1.12**).



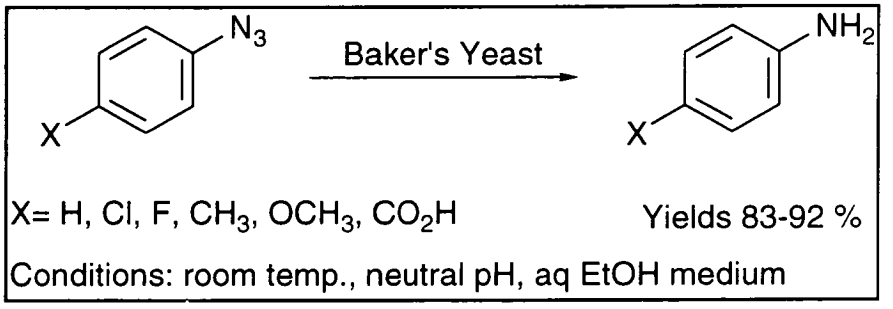
(**39**) (**40**)

Entry No	R	Yield of (**40**) (%)
1	Ph	75
2	4-Chlorophenyl	82
3	4-Methoxyphenyl	70
4	4-Nitrophenyl	74
5	2-Thienyl	52
6	2-Furyl	50
7	Methyl	80
8	H	75
Reactions performed at 30 °C, neutral pH over 2-6 h		

Table 1.12

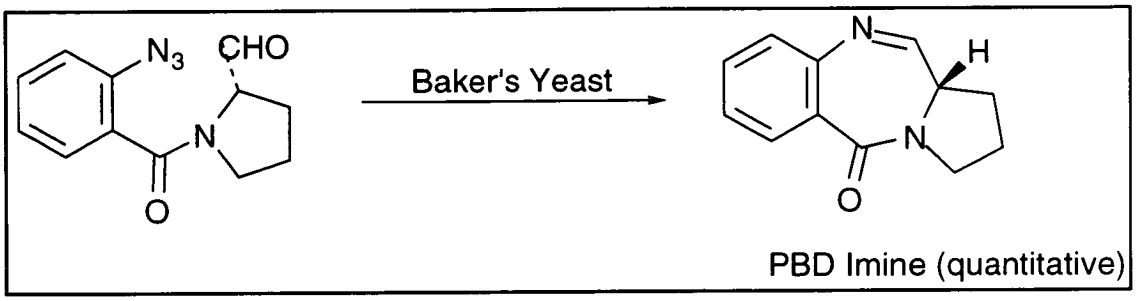
1.4.4 BAKER'S YEAST REDUCTION OF AZIDES

Two reports by Kamal *et al.*, in 1997 discuss the reduction of azides by baker's yeast.[28, 29] The first report[28] starts with some representative aryl azide reductions (**Scheme 1.24**).



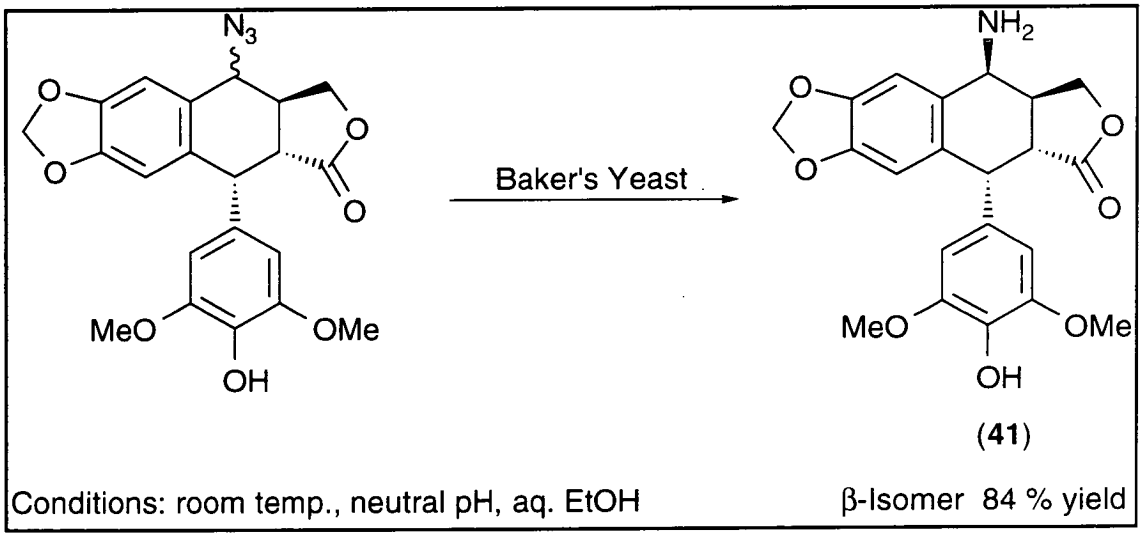
Scheme 1.24

The authors have incorporated this biocatalytic reduction into the synthesis of pyrrolo-benzodiazepine (PBD) analogues. It is noteworthy that chemoselective reduction of the azido group is achieved in the presence of an aldehyde (**Scheme 1.25**).



Scheme 1.25

The second report by Kamal *et al.*, uses baker's yeast as a step in the synthesis of 4-β-aminopodophyllotoxins (**41**), derivatives of naturally occurring podophyllotoxin. These compounds are known to be cytotoxic by blocking the activity of DNA topoisomerase II leading to DNA damage (**Scheme 1.26**).



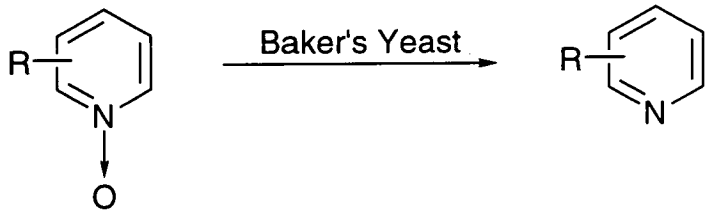
Scheme 1.26

1.4.5 BAKER'S YEAST REDUCTION OF N-OXIDES

Of the two papers published on the reduction of N-oxides by baker's yeast, the earlier paper by Takeshita *et al.*, uses the mild conditions of room temperature and neutral pH as employed in all their previous work.[30] The second paper by Baik *et al.*,

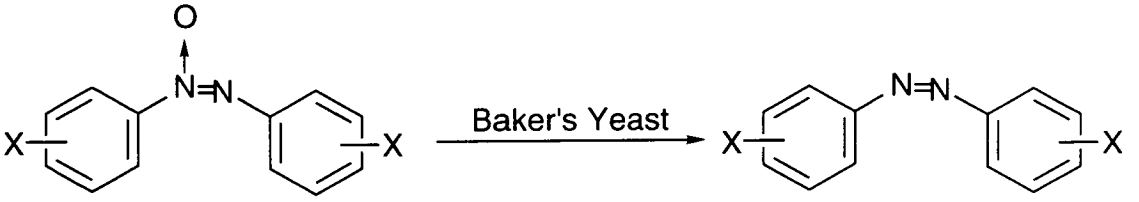
uses the more extreme conditions of high pH (>12) and refluxing temperature typical of their other research.[31]

Takeshita reported on the chemoselective reduction of pyridine N-oxides with baker's yeast (**Table 1.13**). Under the extreme conditions employed by Baik *et al.*, higher yields were obtained and sodium hydroxide was shown to be essential for reduction. A study of the reduction of substituted azoxybenzenes to the corresponding azobenzenes was made (**Table 1.14**). Though enhancement of reduction is shown when X is an electron withdrawing group, high yields are still observed when electron donating groups are present on the ring, contrary to the observations made by Takeshita and co-workers.



Entry No	R	Yield (%)	Entry No	R	Yield (%)
1	2-CH_3	22	6	4-Ph	35
2	3-CH_3	29	7	4-NO_2	0
3	4-CH_3	22	8	4-CN	0
4	3-OH	31	9	2-Br	7
5	4-OCH_3	44			

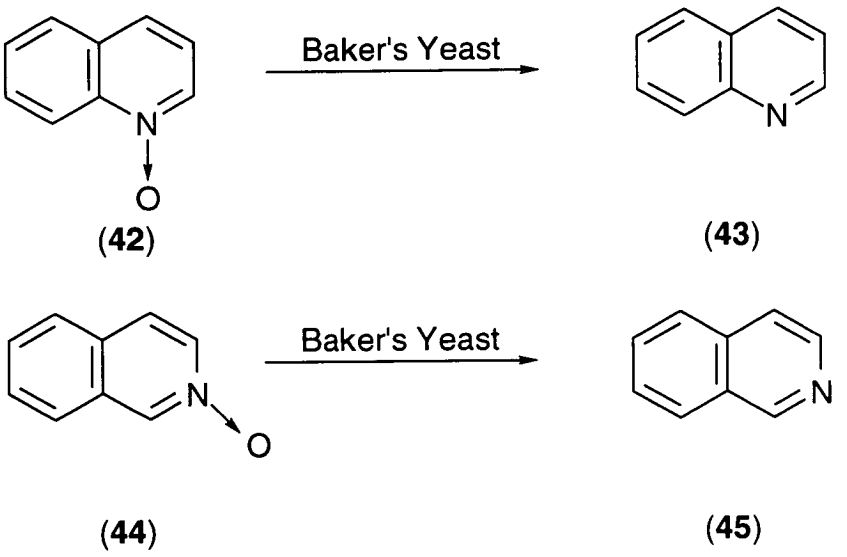
Table 1.13



Entry No	X	Yield of azobenzenes (%)
1	H	96
2	2,2'-Cl	85
3	3,3'-Br	93
4	3,3'-I	95
5	4,4'-OCH_3	71
6	4,4'-CH_3	72

Table 1.14

A better comparison can be made with quinoline N-oxide (**42**) and isoquinoline N-oxide (**43**) (**Table 1.15**). Under the extreme conditions employed by Baik *et al.*, there appeared to be little difference in yield when electron rich and electron deficient substituents were present on the quinoline N-oxides, or the position of the substituent.



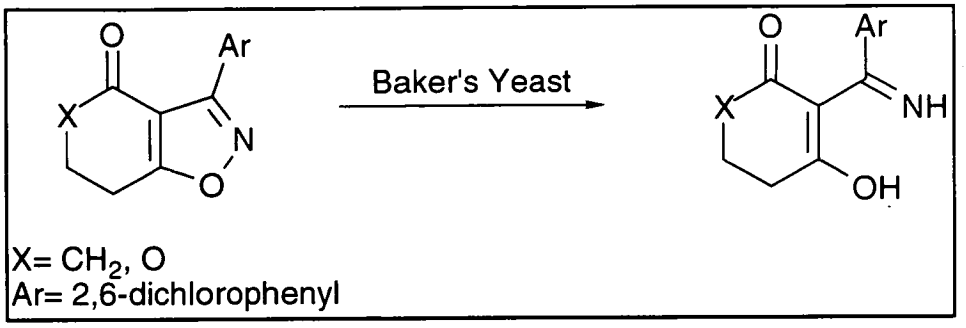
(**42**) (**43**) (**44**) (**45**)

Author	Conditions	Yield of (**43**) (%)	Yield of (**45**) (%)
Takeshita et al	Neutral pH, 35 °C, H_2O, 166 h	8	-
Baik et al	High pH, reflux, 70 % EtOH, 8 h	91	-
Takeshita et al	Neutral pH, 33 °C, H_2O, 168 h	-	44
Baik et al	High pH, reflux, 70 % EtOH, 6 h	-	90

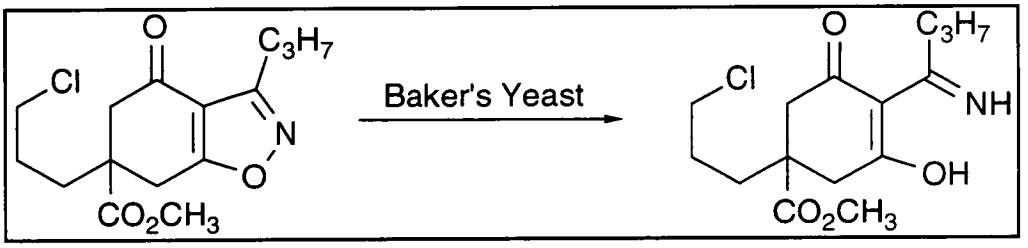
Table 1.15

1.4.6 BAKER'S YEAST REDUCTIVE CLEAVAGE OF ISOXAZOLES

Reported by Easton *et al.*, in 1994,[32] the baker's yeast catalysed reductive cleavage of the N-O bond of isoxazoles under mild conditions was examined but reported to be low yielding (**Schemes 1.27 and 1.28**).



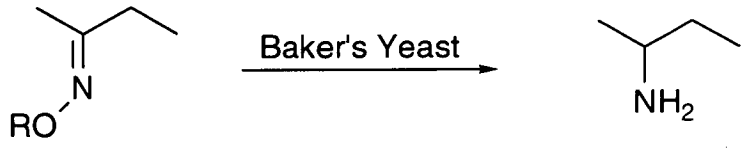
Scheme 1.27



Scheme 1.28

1.4.7 BAKER'S YEAST REDUCTION OF OXIMES

Gibbs and Barnes reported the asymmetric synthesis of amines by the action of baker's yeast on oximes.[33] The low yields were attributed to the products being strongly adsorbed by the yeast.



	Substrate		Product		
Entry No	R	Excess of E-isomer	ee (%)	Configuration	Yield (%)
1	H	44	58	R	20
2	CH_3	40	24	R	15
3	$COCH_3$	44	Racemic		4

Table 1.16

1.4.8 CONCLUSION

In reviewing the literature of baker's yeast mediated reductions of N-O and N=N containing compounds the importance of this area of research is clear, especially with regard to the chemo-, regio- and stereoselective activities of the baker's yeast. It is also clear that two distinct sets of conditions have been employed in this field of study : the mild conditions of neutral/near neutral pH, room temperature - 35 °C and aqueous medium employed by most research groups, and the more extreme conditions employed by Baik and co-workers of high pH (>12), high temperature and aqueous ethanol/methanol medium. This dichotomy will be examined further later in this thesis.

1.5 REDUCTION OF NITROAROMATIC COMPOUNDS BY MICROORGANISMS OTHER THAN BAKER'S YEAST

Nitroaromatic compounds are used in pesticides as well as in explosives and precursors to dyes. As a result they are found in abundance in the environment, and in most cases they are highly toxic or mutagenic. The conversion of these compounds in natural systems is of special interest with the bioremediation of contaminated sites becoming an important field of research.

Aside from *S. cerevisiae*, a number of organisms have been identified as capable of metabolising aromatic nitro compounds including the antibiotic chloramphenicol[34-36], nitrobenzoic acids[37, 38], 2,4,6-trinitrotoluene (TNT)[39] and 2,4-dinitrotoluene (DNT)[40], 4-chloronitrobenzene[41], picric acid[42] and nitrobenzene.[43] The microorganisms utilising nitroaromatic compounds as growth substrates derive carbon, nitrogen and energy from the substrates. A variety of strategies are employed for the biotransformations:

i. Monooxygenase catalysed initial reaction.
ii. Dioxygenase catalysed initial reaction.
iii. Initial reduction yielding an amine which may be further metabolised.
iv. Complete reductive removal of the nitro group from nitroaromatic compounds.
v. Metabolism of nitroaromatic compounds via partial reduction and replacement reactions.
vi. Reduction of nitroaromatic compounds to arylamines.

1.5.1 MONOOXYGENASE CATALYSED INITIAL REACTION

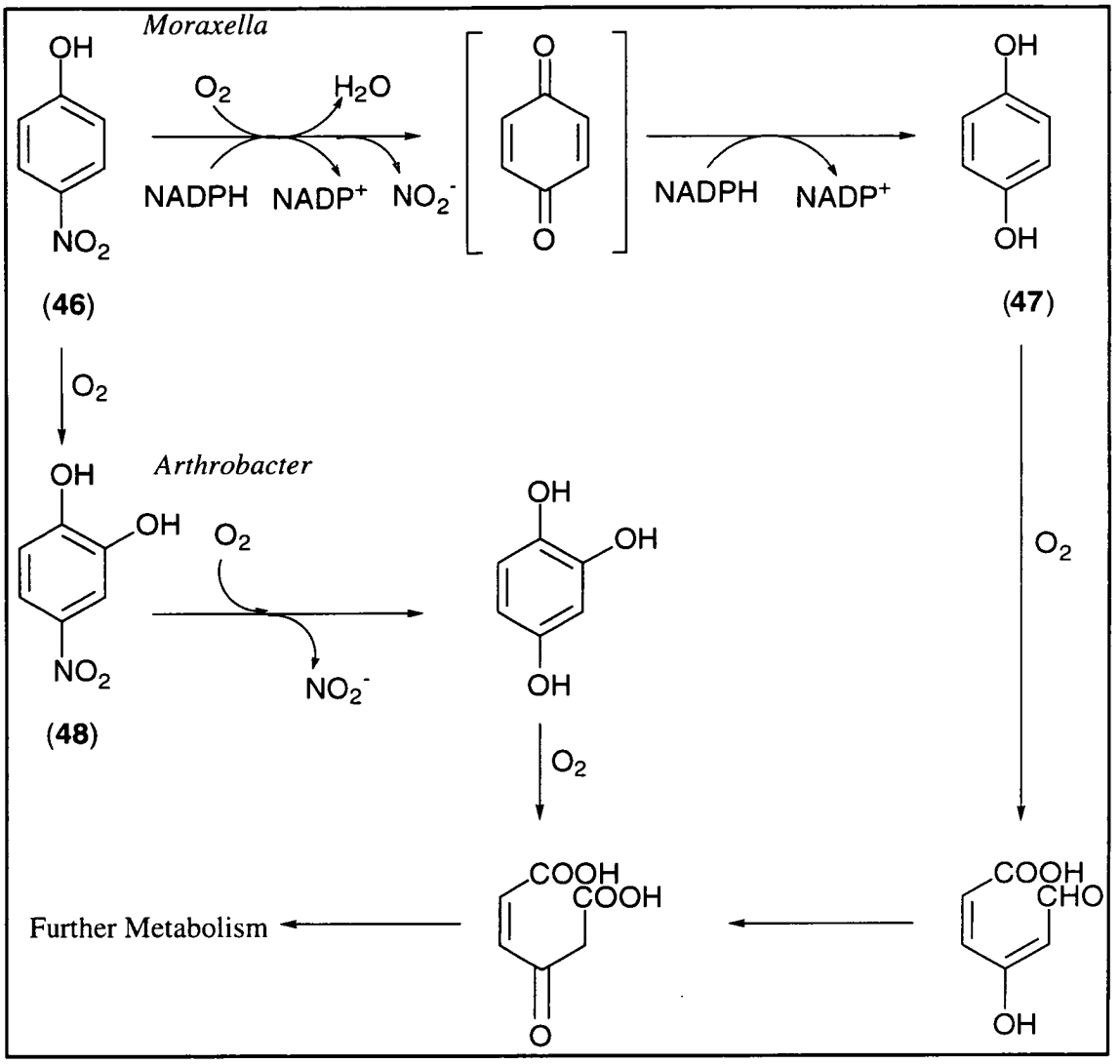
A partially purified enzyme from a strain of *Moraxella* species has been shown to degrade 4-nitrophenol (**46**) by an initial oxygenase catalysed reaction releasing nitrite.[44] The accumulated hydroquinone (**47**) is further degraded. In contrast to the *Moraxella* species, a species of *Arthrobacter* degrades 4-nitrophenol via 4-nitrocatechol (**48**, **Scheme 1.29**).

1.5.2 DIOXYGENASE CATALYSED INITIAL REACTION

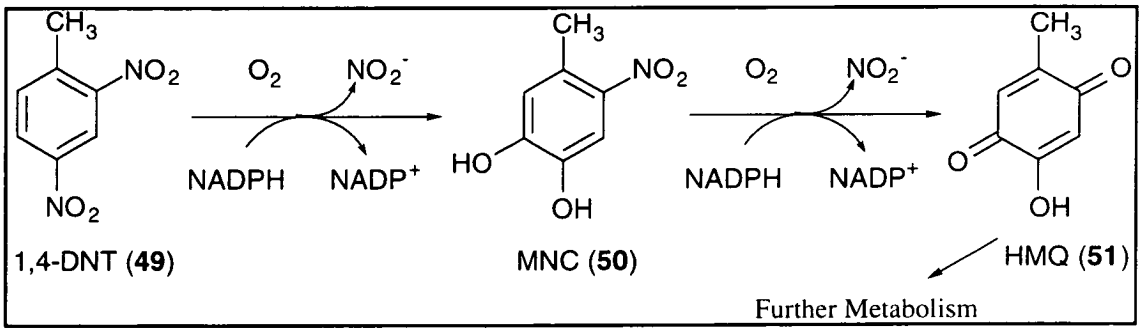
2,4-Dinitrotoluene (2,4-DNT, **49**), a by-product in the manufacture of TNT, is degraded by a species of *Pseudomonas*.[44] A dioxygenase enzyme catalyses the initial reaction to produce 4-methyl-5-nitrocatechol (MNC, **50**) and nitrite is eliminated. The product of the dioxygenase reaction, MNC, is subsequently converted to 2-hydroxy-5-methylquinone (HMQ, **51**) by a monooxygenase enzyme (**Scheme 1.30**).

1.5.3 INITIAL REDUCTION TO AROMATIC AMINES WHICH MAY BE METABOLISED FURTHER

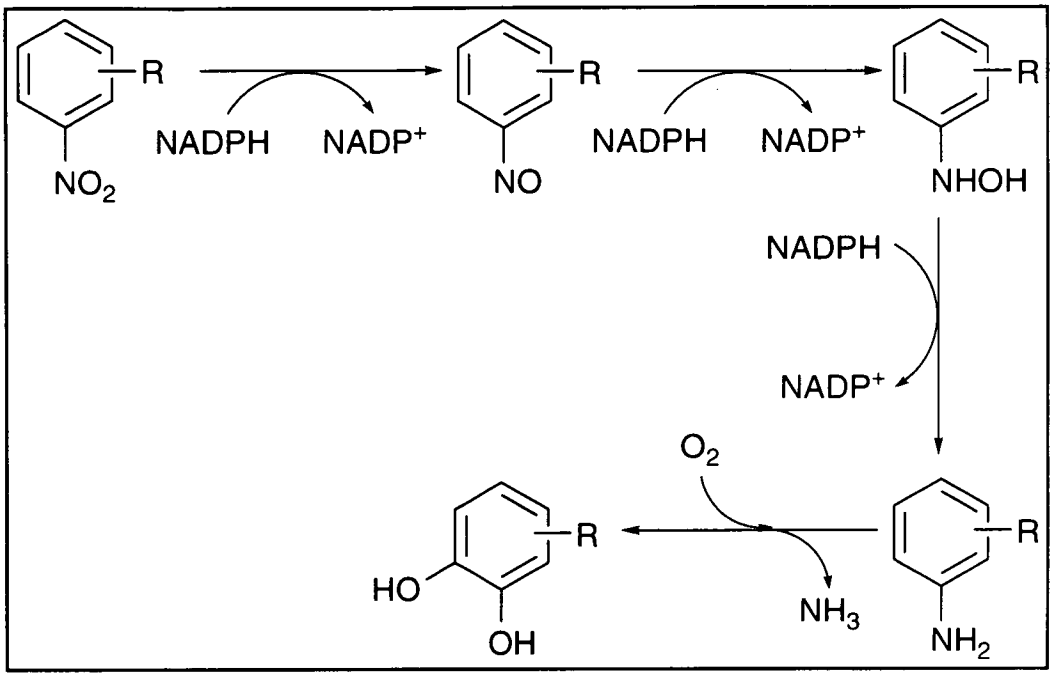
Several microorganisms reduce nitroaromatic compounds via nitroso and hydroxylamine intermediates, to the corresponding amine. The amine product can be further metabolised by aniline oxygenases to ammonia and catachol (**Scheme 1.31**).[37]



Scheme 1.29



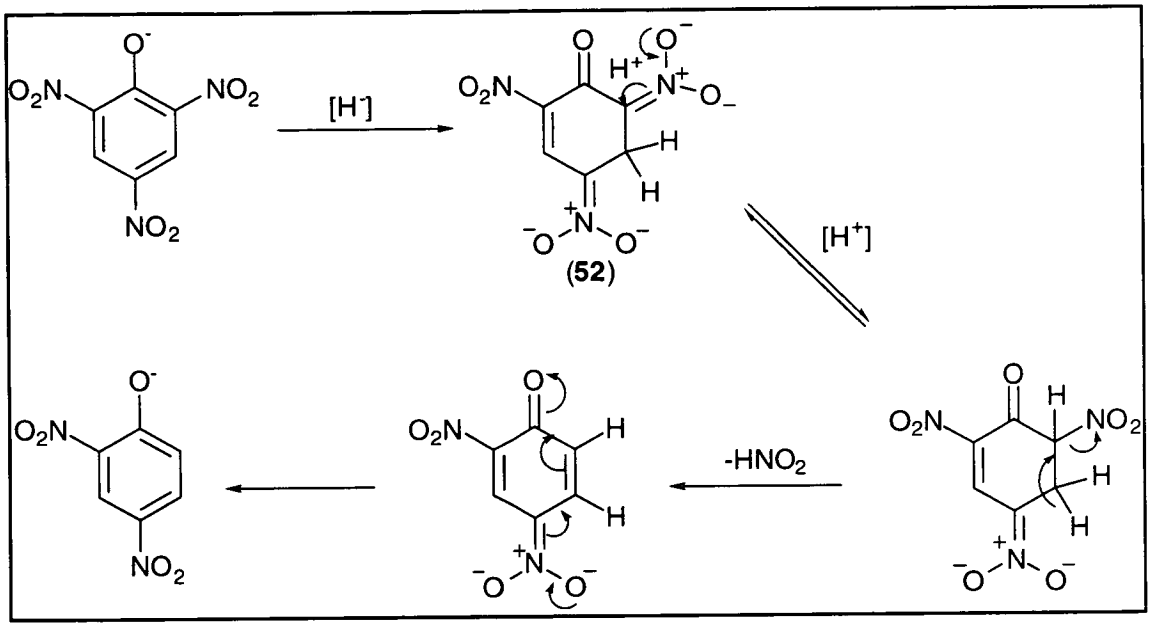
Scheme 1.30



Scheme 1.31

1.5.4 COMPLETE REDUCTIVE REMOVAL OF THE NITRO GROUP FROM NITROAROMATIC COMPOUNDS

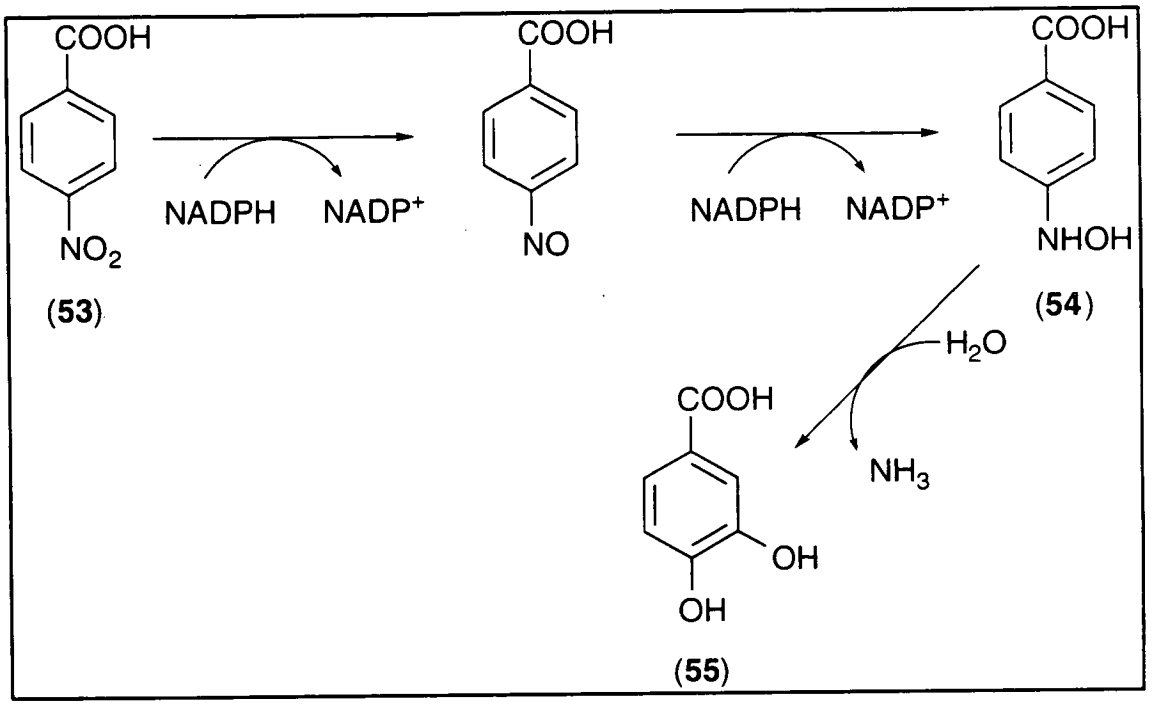
Rhodococcus erythropolis has been shown to completely remove the nitro group of nitroaromatic compounds as nitrite. The removal is initiated by nucleophilic attack on the aromatic ring. A mutant of *R. erythropolisI,* known as the HL PM-1 variant, can utilise picric acid as a nitrogen source. During growth the organism accumulated an orange-red metabolite which was identified as the hydride-Meisenheimer complex (**52**) of picric acid. The complex is metabolised further to liberate nitrite (**Scheme 1.32**).



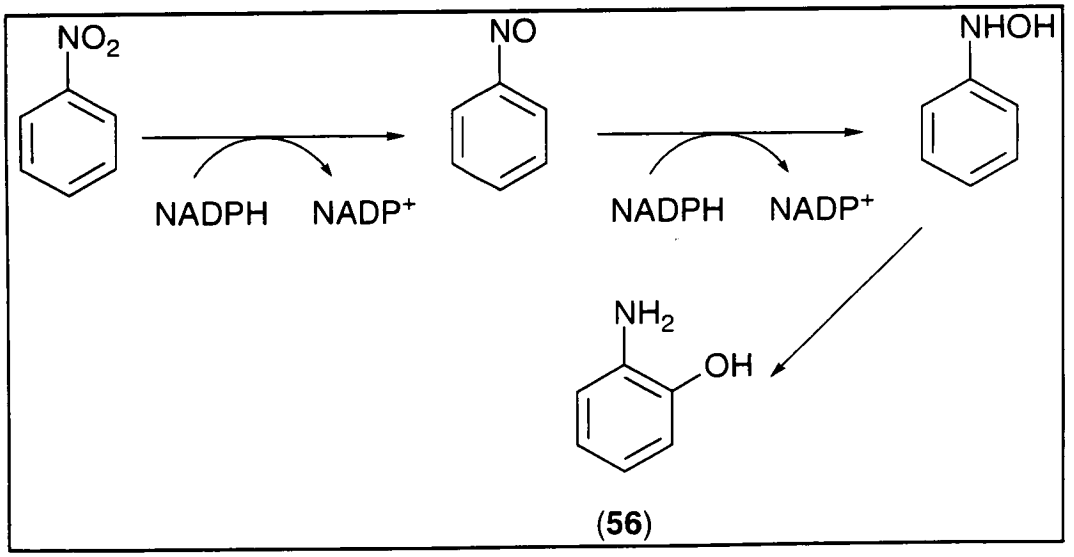
Scheme 1.32

1.5.5 METABOLISM OF NITROAROMATIC COMPOUNDS VIA PARTIAL REDUCTION AND REPLACEMENT REACTIONS

A nitroreductase from *Comamonas acidivorans* reduces 4-nitrobenzoate (4-NBA, **53**) to 4-hydroxylaminobenzoate (4-HABA, **54**) but does not catalyse further reduction to 4-aminobenzoate (4-ABA).[38] A second enzyme converts 4-HABA to protocatechuate (**55**, **Scheme 1.33**). A different enzyme isolated from a *Pseudomonas pseudoalcaligenes* species, hydroxylaminobenzene mutase, performed a comparable conversion from nitrobenzene to hydroxylaminobenzene. However, in this case the hydroxylaminobenzene was not converted to protocatechuate, but to 2-aminophenol (**56**).[43] This enzyme-mediated reaction parallels the Bamberger rearrangement (**Scheme 1.34**).



Scheme 1.33



Scheme 1.34

1.5.6 REDUCTION OF NITROAROMATIC COMPOUNDS TO ARYLAMINES

A number of microorganisms capable of reducing the nitro group of nitroaromatics to the corresponding amines have been identified though the products of the reduction are not converted further. The nitroreductase enzymes responsible for the conversions generally reduce a broad spectrum of analogous substrates. Although there are many

examples of the reduction of nitroaromatics to arylamines, only a few nitroreductases have been purified.

The nitroreductase enzymes that have been isolated have been purified from a variety of sources. Early work by Saz and Slie described the use of cell free extracts of *Escherichia coli* for the reduction of the nitro groups of chloramphenicol and *para* nitrobenzoic acids to the corresponding aryl amines.[45] Further studies were made using other aromatic nitro compounds as substrates for reduction, the best substrates were those with an electron withdrawing group *para* to the nitro substituent.

The nitroreductase activity of *Aspergillus niger* was reported in 1961 by Higgins.[46] *meta*-Dinitrobenzene, a potent growth inhibitor of *A. niger*, was detoxified by reduction when added to established cultures. A three fold purification lead to the retention of 30 % of the nitro reducing activity of the original culture. This partially pure enzyme extract displayed maximum activity at pH 7.8. The specificity of the nitroreductase was tested with other substrates with *meta*-dinitrobenzene proving to be the most effective (**Table 1.17**).

Entry No	R	Aryl amine formed (mμmoles)
1	*m*-NO_2	148.5
2	*p*-SO_2NH_2	53.5
3	*p*-CO_2H	52.5
4	*m*-CO_2H	34.9
5	*o*-CO_2H	8.0
6	Chloramphenicol	0.0

Table 1.17

The isolation of a nitroreductase in highly purified form from a species of *Nocardia* was reported by Villanueva in 1964.[47] Acetone powdered forms of the microorganism were prepared and the nitroreductase activity was assayed using the reduction of *para*-dinitrobenzene by the measurement of *para*-nitroaniline formed at 37 °C. Purification was achieved by isolating the precipitated protein from a 45-65 % ammonium sulphate cut and applying this fraction to a DEAE-cellulose column at a pH greater than 7.0. The active component was shown to be a monomer with a molecular weight of 24 KDa.

In 1983 Kinouchi and Ohnishi purified four nitroreductases from *Bacteroides fragilis* GAI0624.[48] The reduction of 1-nitropyrene was used as an assay to follow the purification which started with ultrasonication to produce a crude extract. This extract was fractionated with ammonium sulphate, retaining the 40-60 % fraction and desalting by dialysis. Ion exchange chromatography using DEAE cellulose at pH 6.0 gave two active fractions, the first failing to adsorb, the second eluted with a salt concentration of 0.125-0.15 M. The adsorbed fraction was applied to a gel filtration column (sephadex G-200) at pH 6.8 and eluted to produce two peaks, one with high nitroreductase activity (NRase I) and the other with low activity (NRase III) with estimated molecular weights of 52 KDa and 180 KDa respectively. The unadsorbed fraction from the ion-exchange stage was passed through two gel filtration columns (sepharose 2B and sepharose 6B). Two active peaks were found, one with high activity (NRase II) and the other with lower activity (NRase IV) with estimated molecular weights of 320 KDa and 680 KDa respectively. These nitroreductases were tested for substrate specificity at this stage but all four nitroreductases were further purified to a near pure state for characterisation. The substrate specificity of the enzymes was determined by the effect of mutagenic nitro compounds on mutagenic activity (**Table 1.18**). NRase I markedly reduced the mutagenicity of 1-nitropyrene (Entry 1) but showed little effect on the dinitropyrenes (Entries 2,3 and 4). NRase III on the other hand decreased the mutagenicity of the dinitropyrenes (Entries 2,3 and 4). NRases I, II and III enhanced the mutagenicity of nitronaphthalene (Entry 8). NRase IV was specific for 4-nitroquinoline 1-oxide (Entry 9). Nitroreductase activity required a coenzyme; NRases II, II and IV were NADPH linked, NRase I was NADH linked.

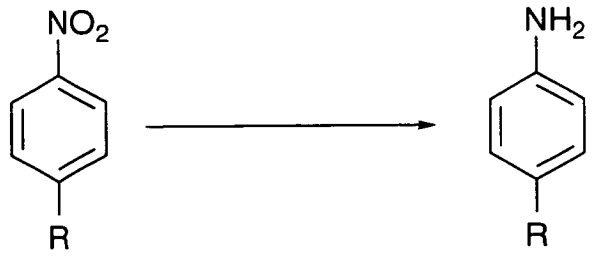
The purification and characterisation of an oxygen insensitive NAD(P)H nitroreductase from *Enterobacter cloacae* 96-3 was reported by Bryant and DeLuca in 1991.[49] Cell lysis was achieved by sonication and acetone precipitation was used to fractionate the crude extract. Active fractions were run on an anion-exchange column followed by a propyl hydrophobic interaction column, a second anion-exchange column (MonoQ) and finally an HPLC size exclusion column to give homogeneous nitroreductase.

TNT was used as the assay substrate, monitoring the conversion of NADH to NAD^+ at 340 nm. Analysis of the physical properties of the nitroreductase indicated that the enzyme is a monomer with a molecular weight of 27 KDa with flavin mononucleotide (FMN) required as a cofactor. The purified nitroreductase was able to utilise NADH or NADPH for reduction. The rate of nitro reduction for a number of substrates was tested (**Table 1.19**).

Chemical	% Mutagenicity (revertants on plate)[a]				
	No Enz.	NRase I	NRase II	NRase III	NRase IV
1-Nitropyrene	100	18	31	71	63
1,3-Dinitropyrene	100	61	35	29	51
1,6-Dinitropyrene	100	98	51	36	99
1,8-Dinitropyrene	100	90	49	22	92
1,3,6-Trinitropyrene	100	83	78	52	91
1,3,6,8-Tetranitropyrene	100	78	81	81	92
2-Nitrofluorene	100	73	64	64	78
2-Nitronaphthalene	100	144	118	107	97
4-Nitroquinoline-1-oxide	100	71	49	97	9
Nitrofurantoin	100	97	99	103	103
Benzo(a)pyrene	100	99	98	97	100
1-Aminopyrene	100	92	90	99	101

Table 1.18 [a]Results are accurate to ± 0-7.8 %

A pure protein with a molecular weight of 24 KDa was isolated from *E. coli* B by Anlezark *et al.*, in 1992.[50] The enzyme was shown to be an FMN containing flavoprotein which requires either NADH or NADPH as a cofactor. Purification of the nitroreductase enzyme from *E. coli* B was achieved by obtaining a crude extract by sonication. This extract was applied to a hydrophobic interaction column (phenyl sepharose CL-6B) at pH 7, 0.3 M ammonium sulphate. The protein was eluted at pH 7.6. Active fractions were desalted by dialysis and then applied to an anion exchange column (Q-sepharose), the nitroreductase was eluted at 0.1-0.12 M potassium chloride. Desalting the active proteins into bis-tris propane, pH 7, was achieved using a gel filtration column (sephadex G-25). Anion-exchange with Q-sepharose was repeated using the new buffer, the active protein was eluted at 0.07-0.09 M potassium chloride.



Entry	R	Velocity (μmol/min/mg)
1	$CH_2SO_3^-$	32.75
2	$COCH_3$	18.51
3	CHO	13.52
4	COOH	2.37
5	H	1.56
6	CH_3	1.01
7	OH	0.71
8	NH_2	0.71
9	menadione (**58**)	118.9

Table 1.19

The nitroreductase from *E. coli* B was shown to reduce the antitumour agent CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] (**Figure 1.2, 57**) as well as menadione (**58**) and nitrofurazone (**59**). The gene coding for the *E. coli* B nitroreductase enzyme was cloned and expressed in *E. coli* K12[51] and crystallographic data has since been established for this enzyme.[52]

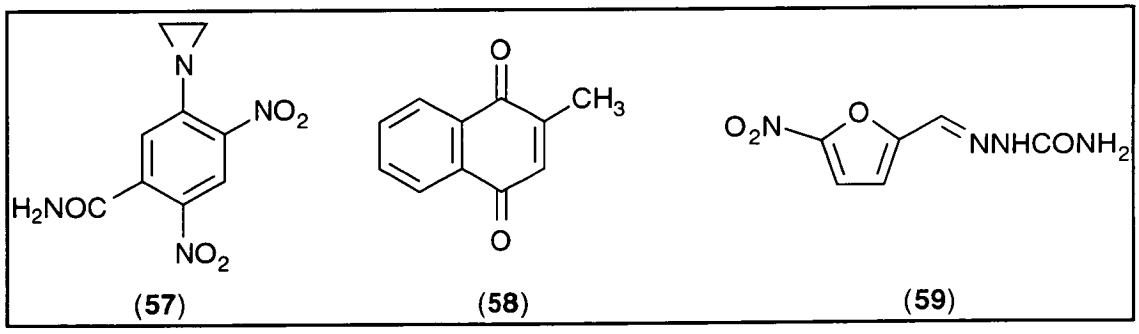


Figure 1.2

In 1993 Blasco and Castillo reported the characterisation of a nitrophenol reductase from *Rhodobacter capsulatus* E1F1.[53] This enzyme is responsible for the photoreduction of 2,4-dinitrophenol (DNP) to 2-amino-4-nitrophenol and was purified to electrophoretic homogeneity. The organism was grown in the light, under anaerobic

conditions in the presence of 2,4-DNP. A crude extract was obtained and fractionated with ammonium sulphate, retaining the 35-70% cut. This fraction was passed through a gel filtration column (sephadex G-75), the resulting fractions with activity were pooled and applied to an anion-exchange column (DEAE sephacel), and eluted with sodium chloride. Activity was found at a salt concentration of 0.15 M. An HPLC assay was used to follow the reduction of 2,4-DNP.

Nitrophenol reductase from *R. capsulatus* E1F1 used several nitrophenols as substrates (**Table 1.20**). It is interesting to note that no conversion was seen with dinitrobenzene, 2,4-dinitrotoluene and 2,4-dinitroaniline (Entries 8,9 and 10), the phenolic group being a prerequisite for enzyme activity.

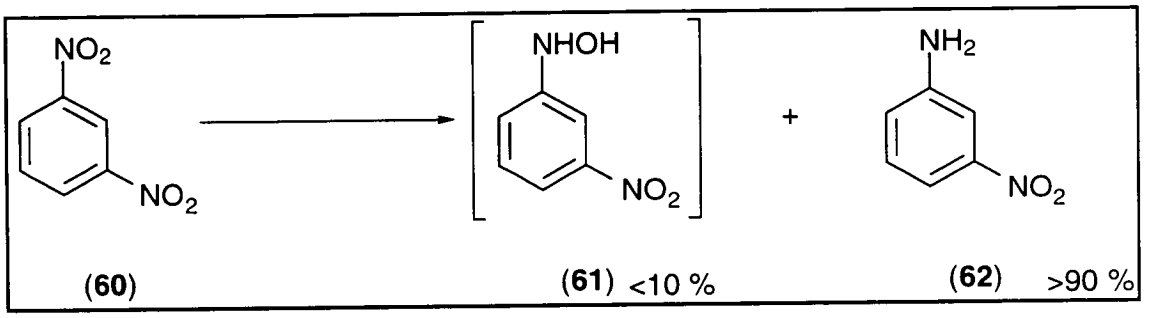
Entry No	Substrate	NRase activity (mU/mg protein)
1	2-Nitrophenol	16
2	3-Nitrophenol	0
3	4-Nitrophenol	0
4	2,4-Dinitrophenol	42
5	2,5-Dinitrophenol	121
6	3,4-Dinitrophenol	30
7	2,4,6-Trinitrophenol	37
8	Dinitrobenzene	no conversion
9	Dinitrotoluene	no conversion
10	Dinitroaniline	no conversion

Table 1.20

This enzyme exhibits different substrate specificity to those mentioned previously. Phenol substituted nitroaromatics are usually unfavourable substrates for nitroreductases. Dinitrobenzene typically proves to be an excellent substrate, the reverse is seen to be the case for nitrophenol reductase from *R. capsulatus* E1F1. It seems likely that a different type of enzyme is responsible for these reactions. The molecular weight of the enzyme is 54 KDa (two subunits of 27 KDa each), similar to that observed from *B. fragilis*. Nitrophenol nitroreductase was shown to require NADH or NADPH as cofactor and had a pH optimum of 6.

A membrane associated aromatic nitroreductase was identified in *Phanerochaete chrysosporium* by Rieble *et al.*, in 1994.[54] Although the enzyme has not yet been purified, a number of nitroaromatic substrates were tested with the cell free extract of the fungus. The extract required the addition of NADPH and had an optimum temperature of 50 °C and pH of 6.5. No enzyme activity was observed in the presence

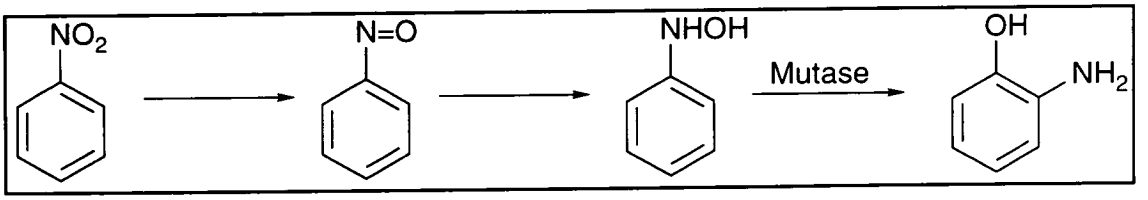
of molecular oxygen. Nitroreductase assays were performed on 1,3-dinitrobenzene (**60**) identifying 1-hydroxylamino-3-nitrobenzene (**61**) and 1-amino-3-nitrobenzene (**62**) as products by HPLC and GC-MS (**Scheme 1.35**).



Scheme 1.35

Reduction of 2,4-dinitrotoluene yielded hydroxylamino-, amino- and diamino-toluene isomers with the amino-nitrotoluene isomer being the most abundant. 2,4,6-Trinitrotoluene was reduced to the hydroxylamino- and amino-dinitrotoluene. 2,4-Dichloro-1-nitrobenzene and 1-chloro-2,4-dinitrobenzene also served as substrates but only their respective amino derivatives were observed.

In 1995 Somerville *et al.*, reported the purification and characterisation of nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45.[55] In this instance the microorganism is reported to reduce nitrobenzene to nitrosobenzene then further to N-phenylhydroxylamine as usual, but no aniline is formed by these whole cells. Instead, N-phenylhydroxylamine is converted by a mutase enzyme to 2-aminophenol (**Scheme 1.36**).



Scheme 1.36

Purification of the nitroreductase enzyme began with cell lysis using a French Press. The lysate was treated with ammonium sulphate retaining the 45-60 % cut. This fraction, once desalted, was loaded onto an anion-exchange column (Q-sepharose FF) and the protein eluted with a potassium chloride gradient. The active fractions were concentrated and resolved through a gel filtration column (sephacryl S-200). The assay used to follow the purification was the oxidation of NADPH in a reaction mixture

containing nitrobenzene. The purified enzyme had maximum activity at pH 8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) indicated the protein had a molecular weight of 33 KDa , confirmed as a monomer by gel filtration results.

1.6 ENZYME KINETICS

Once an enzyme has been isolated, the kinetic parameters relating to the enzyme can be examined. The Michaelis-Menten equation can be used to calculate the kinetic parameters of many enzymes. When an enzyme obeys Michaelis-Menten kinetics the rate of the reaction, v, varies with substrate concentration, [S], as shown in **Figure 1.3.** The substrate concentration at which the reaction rate is half its maximumm is known as the Michaelis constant, Km. The maximal velocity, Vmax, is achieved when the enzyme is saturated with substrate.

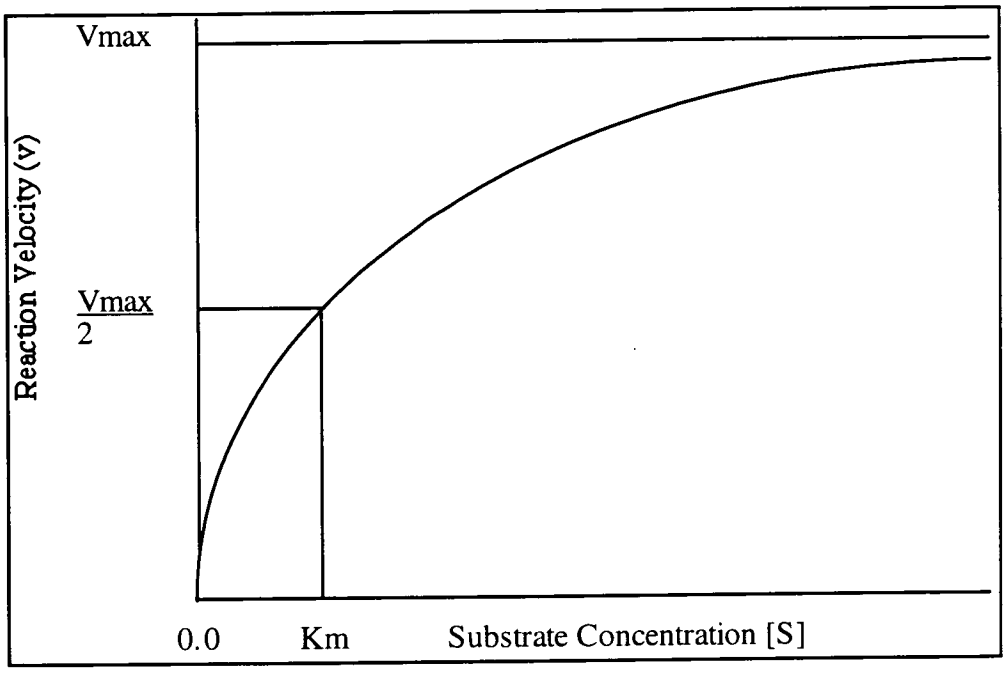


Figure 1.3 A normal hyperbolic relationship between v and [S].

The rate v is defined as the number of moles of product formed per second. When a low substrate concentration is used, v is almost linearly proportional to [S]; when [S] is high, v is nearly independant of [S]. The model proposed by Michaelis and Menten to account for the kinetic characteristics of enzymes is shown in **Scheme 1.37**.

$$\text{Enzyme + Substrate} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Enz-Sub} \xrightarrow{k_3} \text{Enzyme + Product}$$

Scheme 1.37

Kcat, often referred to as the turnover number, is expressed as moles of substrate consumed per unit time per mole of enzyme. The velocity of an enzyme catalysed reaction, v, is expressed as a function of the substrate and enzyme concentration by the Michaelis-Menten equation:

$$v = \frac{[S] \,.\, Vmax}{Km + [S]}$$

The Michaelis constant, Km, and the maximal rate, Vmax, can be derived from rates of catalysis measured at different substrate concentrations. Graphically, a double reciprocal of the graph shown in Figure 1.3 provides a straight line plot, the Linweaver-Burke plot. The Y-intercept is 1/Vmax, the X-intercept is -1/Km and the slope is Km/Vmax (**Figure 1.4**).

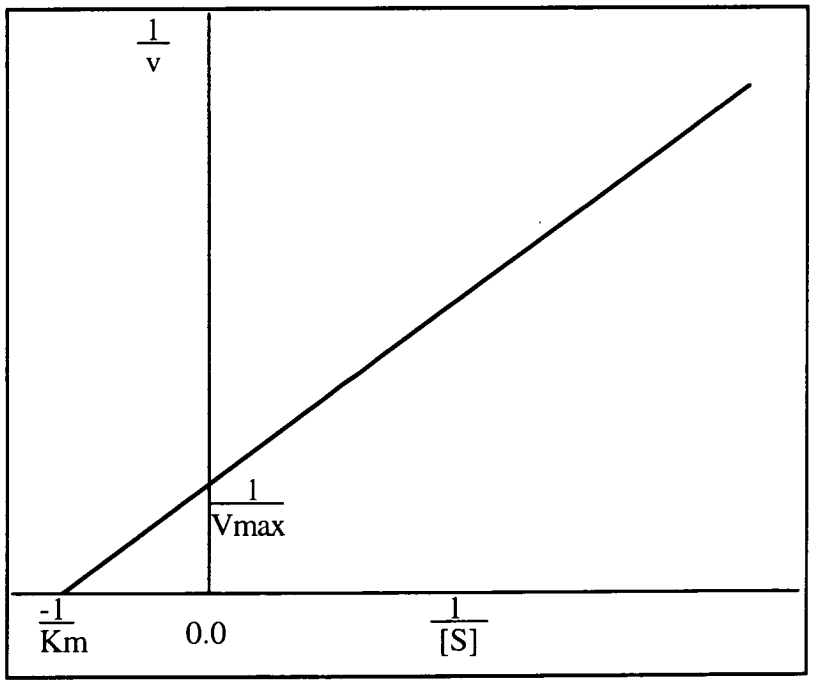


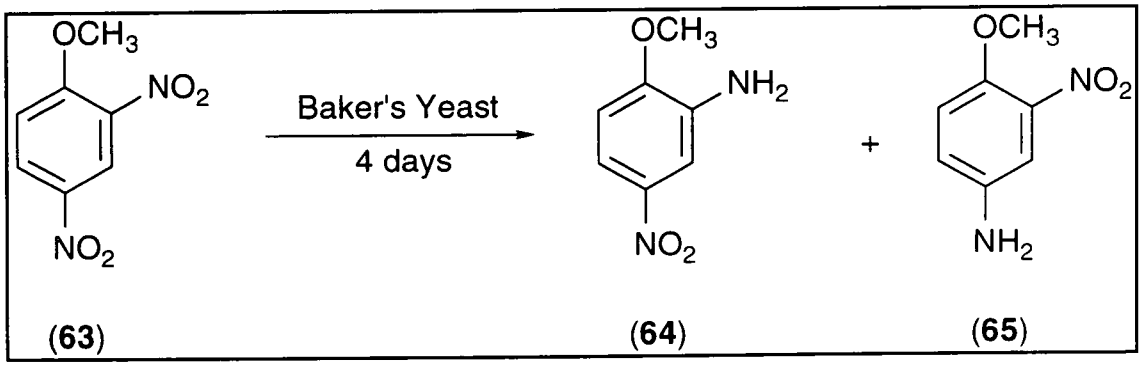
Figure 1.4 A double reciprocal Lineweaver-Burke plot

2. RESULTS AND DISCUSSION: BIOTRANSFORMATIONS

The baker's yeast reduction of aromatic nitro compounds could become a useful tool for synthetic organic chemists. Currently, such reductions are performed by the chemical industry using classical approaches, generating large amounts of waste by-products. An alternative procedure that could operate under milder conditions, with the additional benefits of chemo- and regioselectivity, could prove to be pivotal in the synthesis of functionalised aromatic derivatives. Baker's yeast has shown to be an efficient biocatalyst for these reductions[18, 20, 21] and initial work concentrated on providing an improved and reliable protocol for the bioreductions.

2.1 OPTIMISATION STUDIES

Previous work within our research group on the regioselective reduction of dinitroarenes using baker's yeast by Davey *et al.*,[20] indicated that these reactions were relatively low yielding processes. Prior to the commencement of optimisation studies, the baker's yeast reduction of 2,4-dinitroanisole (**63**, **Scheme 2.0**) was performed in order to gain experience with the protocol used at the time and to verify that the work already performed within the group could be repeated.



Scheme 2.0

The reduction of 2,4-dinitroanisole (**63**) to 2-amino-4-nitroanisole (**64**) and 4-amino-2-nitroanisole (**65**) had been reported in the literature to give yields of 30 % and 6 % respectively.[20] When repeated the reduction was shown to be reproducible and was adopted as the system on which the optimisation studies would be performed.

Initial methodology involved the addition of the substrate to the yeast suspension and the biotransformation was monitored by thin-layer chromatography (TLC). Upon completion of the reduction the yeast/product suspension was filtered through a celite bed and the products were extracted from the filtrate with ethyl acetate. Purification by flash chromatography provided the isolated products.

This method had several weaknesses, predominantly it was a time consuming messy process that would be problematic on a large scale. TLC proved to be an inadequate monitoring system due to the fact that it appeared to indicate complete conversion but upon work-up significant quantities of starting material were recovered. The extraction process also proved to be inefficient. It became apparent that the aniline products remained on the celite bed and in the aqueous phase of the system (clearly visible by the distinct yellow coloration of the anilines).

In order to achieve improved yields gas-liquid chromatography (GLC) was used to monitor the biotransformation and on work-up the suspension was filtered through celite as before. However, this time the pH of the filtrate was raised and crude product obtained after continuous extraction overnight with chloroform. This improved the yields of (**64**) and (**65**) to 59 % and 7 % respectively upon isolation after flash chromatography.

The reaction was repeated with slight modification. When the reaction was shown to be complete by GLC, the yeast/product suspension was taken to pH 8 and saturated with salt then extracted overnight with chloroform. The isolated yields of (**64**) and (**65**) using this methodology were shown to be 80 % and 15 % respectively. This work-up method was employed for all further biotransformations, a summary of the optimisation can be seen in **Figure 2.0**.

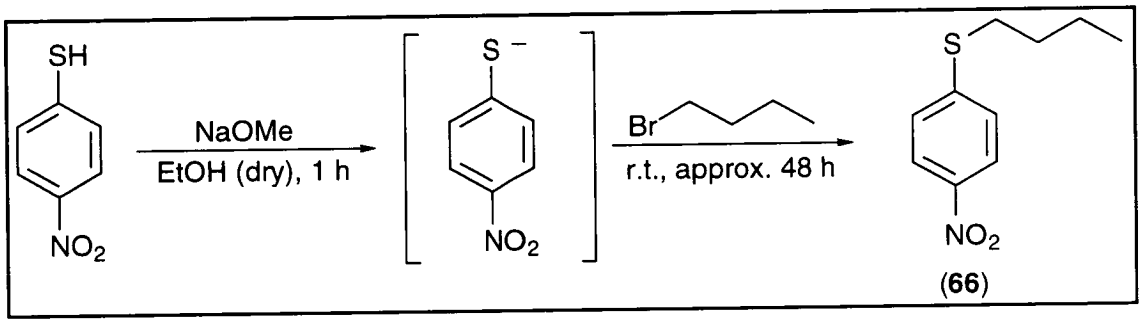
Published Method[20]	*Improved Method*	*Optimised Method*
Yeast/Product Suspension TLC Monitoring	Yeast/Product Suspension GLC Monitoring	Yeast/Product Suspension GLC Monitoring
↓	↓	↓
Filter through celite bed	Filter through celite bed	Continuous extraction through yeast/product suspension
↓	↓	↓
Extract pH 6 and dry organic phase	Adjust aqueous phase to pH 8 and continuously extract, dry organic phase	Dry organic phase
↓	↓	↓
Flash chromatography	Flash chromatography	Flash chromatography
Yield: (**64**) 30 % (**65**) 6 %	Yield: (**64**) 59 % (**65**) 7 %	Yield:(**64**) 80 % (**65**) 15 %
Recovery: 36 %	Recovery: 66 %	Recovery: 95 %

Figure 2.0

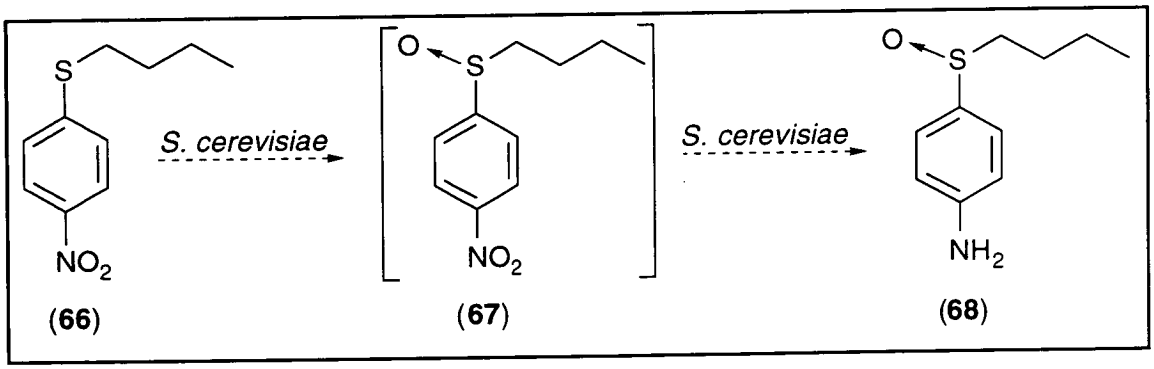
2.2 SUBSTRATE SPECIFICITY

Using the optimised methodology, a number of compounds were treated with baker's yeast in order to determine the breadth of substrates that could be reduced, particularly with respect to chemo- and regioselectivity.

It had been reported that a strain of baker's yeast (*Saccharomyces cerevisiae* NCYC-73) was capable of oxidising aryl sulphides to the corresponding sulphoxides.[56] With this in mind butyl-4-nitrophenylsulphide (**66**) was prepared as demonstrated in **Scheme 2.1** with the view to perform a tandem oxidation-reduction of the sulphide to sulphoxide and nitro to amine respectively (**Scheme 2.2**). It was believed, on the basis of previous work, that the nitro group would not be reduced without initial oxidation of the sulphide.



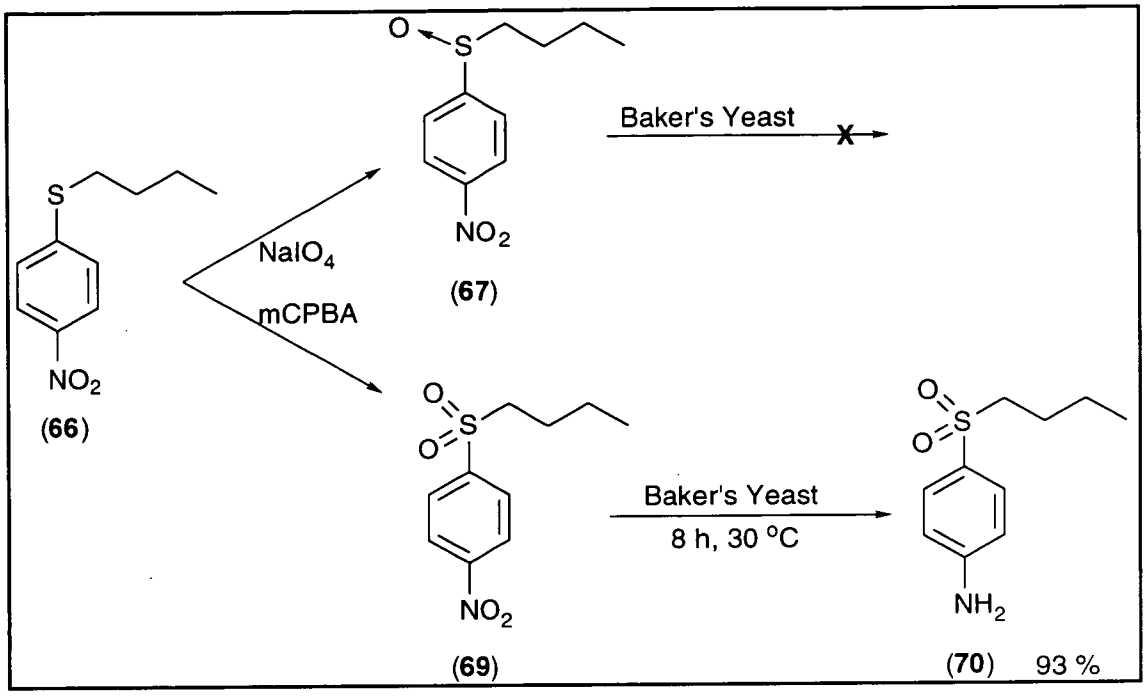
Scheme 2.1



Scheme 2.2

Unfortunately no conversion occurred with either the dried baker's yeast (*S. cerevisiae*) used for all biotransformations in this project, or the NCYC-73 strain which had been reported to oxidise sulphides. The conditions required by the oxidising enzyme are likely to be different to those required by the reducing enzyme in yeast and this could account for the lack of reaction.

Racemic butyl-4-nitrophenylsulphoxide (**67**) was synthesised from the sulphide (**66**) and tried as a substrate with baker's yeast in order to ascertain whether any enantioselective reduction occurred. However, any products formed rapidly decomposed.



Scheme 2.3

The bioreduction of the sulphone (**69**) was considerably faster than most biocatalysed reductions attempted (8 hours compared with 4 days) forming a very stable product in excellent yield (**Scheme 2.2**). This reduction was monitored in detail by GLC using 4-nitrothioanisole as an internal standard (**Figure 2.1**). It is clear that with the 4-butylphenylsulphide, sulphoxide and sulphone examples, reaction rate is enhanced by increasing the electron-withdrawing capacity of the substituent.

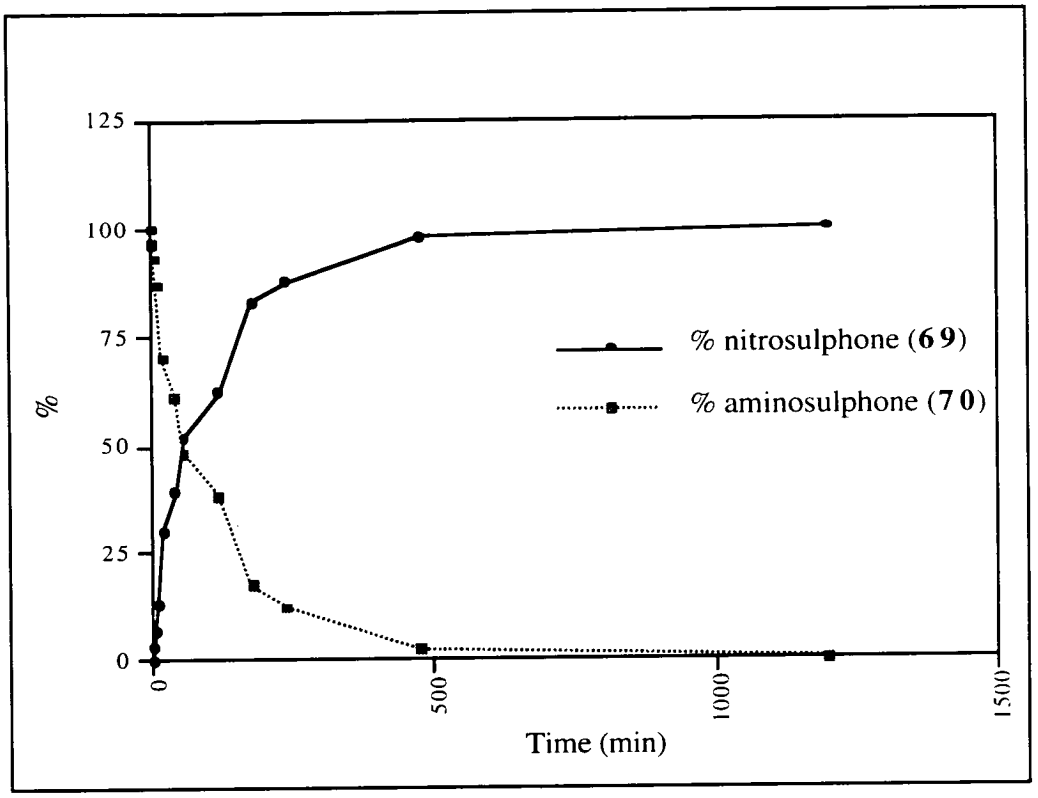


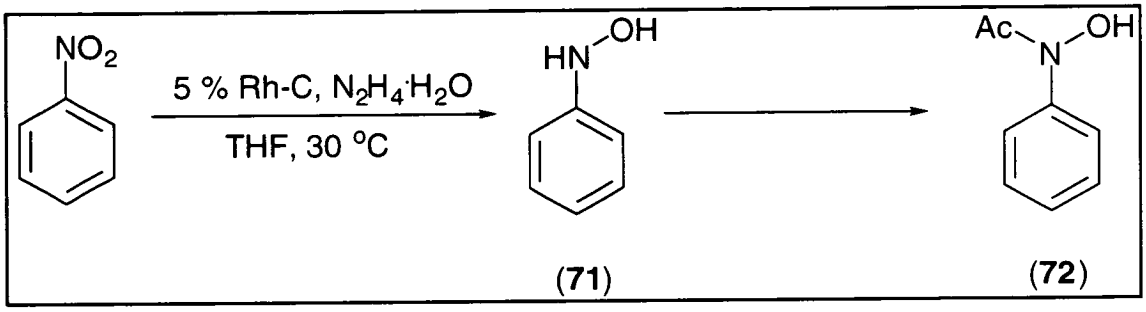
Figure 2.1 The Reduction of 4-Nitrophenylsulphone (**69**)by Baker's Yeast.

2.3 MECHANISM OF REDUCTION

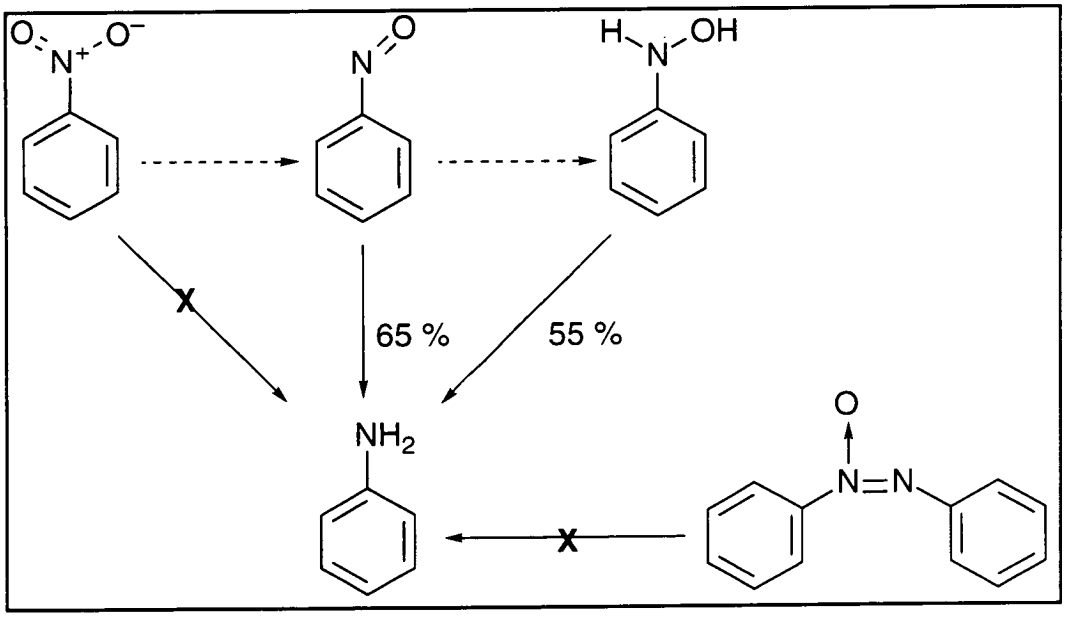
It became apparent when reviewing the literature (section 1.5) that two distinct sets of conditions had been employed in the reduction of nitroarenes by baker's yeast. The first set of conditions, covering most of the work[18, 20] and employed by ourselves, used baker's yeast in aqueous media at approximately 30 °C (thereafter called type I conditions). The second set of conditions, employed by Baik *et al* [21, 24], used aqueous ethanol or methanol as the reaction medium with a high pH (>12) and temperatures of 70-80 °C and even reflux (type II conditions). These two sets of conditions impart different selectivity on the reduction of nitroarenes. Type I conditions require the presence of an electron-withdrawing substituent on the aromatic ring in order to achieve successful reduction, yet under type II conditions electron-donating substituents can be tolerated. In this section the mechanisms of reduction under each set of conditions will be discussed and compared. The reduction of nitrobenzene performed under type I and type II conditions is used for comparison.

In our research, type I conditions were used throughout (unless otherwise stated) using high performance liquid chromatography (HPLC) to monitor the reactions. Under type I conditions nitrobenzene was not reduced by baker's yeast. However nitrosobenzene and N-phenylhydroxylamine (**71**), the latter prepared by the

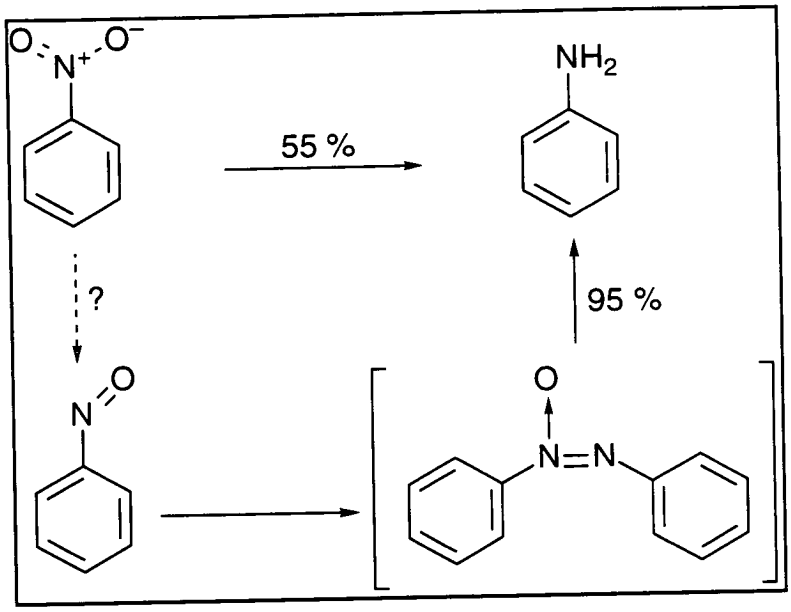
partial reduction of nitrobenzene using rhodium on carbon and hydrazine hydrate[57] (**Scheme 2.4**), were found to be rapidly reduced to aniline in moderate yields (65 % and 55 % respectively). The N-phenylhydroxylamine was further characterised as the N-acetylphenylhydroxylamine (**72**) by treatment with acetylchloride. Azoxybenzene, prepared by refluxing nitrobenzene with glucose in aqueous medium at high pH[58], was not reduced by baker's yeast under type I conditions. Under type II conditions nitrobenzene is reported to be successfully reduced to aniline in a 55 % yield[21], and nitrosobenzene (under slightly modified type I conditions of neutral pH) is reported to be reduced to aniline via azoxybenzene intermediate.[24] The reaction pathways summarised in **Schemes 2.5** and **2.6** clearly show the differences between the two sets of conditions.



Scheme 2.4



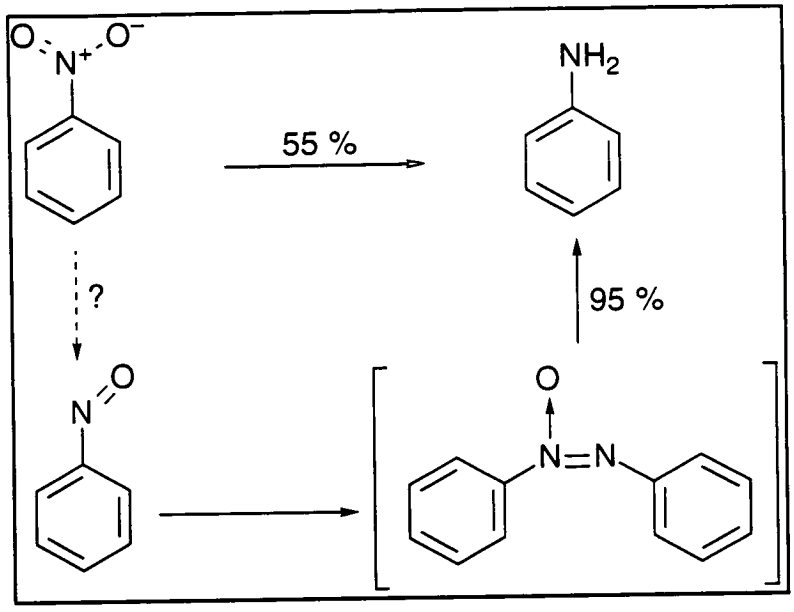
Scheme 2.5 Type I Reaction Conditions.



Scheme 2.6 Type II Reaction Conditions.

Further evidence that reduction occurred by different mechanisms under the two sets of conditions was provided by investigating the reduction of 2,4-dinitroanisole. As described earlier, under type I conditions a ratio of 5.3:1 of 2-amino-4-nitroanisole to 4-amino-2-nitroanisole was obtained in a combined yield of 95 %. When the same substrate was tested under type II conditions, the reaction was less clean and only afforded 20 % of the 2-amino-4-nitroanisole isomer. Under the harsh type II conditions employed by Baik *et al.*, it is highly unlikely that the enzymes in the yeast cell would remain active. When we repeated the type II conditions we found that the yeast cells rapidly aggregated, typically a sign of loss of activity.

It is well known that the chemical reduction of a nitro group to the corresponding aniline proceeds via a series of two electron additions to form firstly the nitroso derivative followed by the hydroxylamine finally resulting in the aniline.[59] Azoxybenzene (**73**) can be prepared by the condensation of nitrosobenzene and N-phenylhydroxylamine. This well established synthesis[58] occurs by heating nitrobenzene with glucose in aqueous sodium hydroxide for one hour, followed by refluxing for two hours, after which azoxybenzene can be isolated (**Scheme 2.7**). Galbraith *et al.*, tested the applicability of the glucose-alkali reducing mixture with a variety of substituted aromatic nitro compounds to form the corresponding azoxy products.[60] The reaction conditions employed used 30 % sodium hydroxide heated at 80 °C together with the substrate and glucose. Many simple aromatic nitro compounds were reduced under these conditions. Newbold and LeBlanc studied the reduction of a series of monochloro-, bromo- and iodonitrobenzenes to the corresponding



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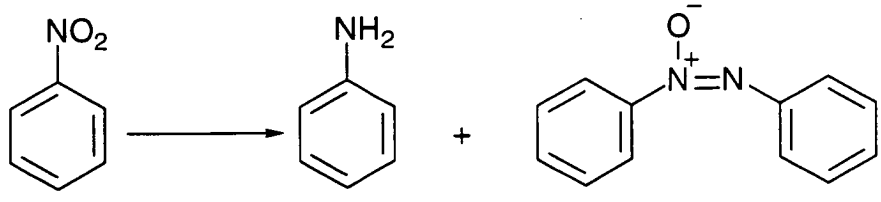
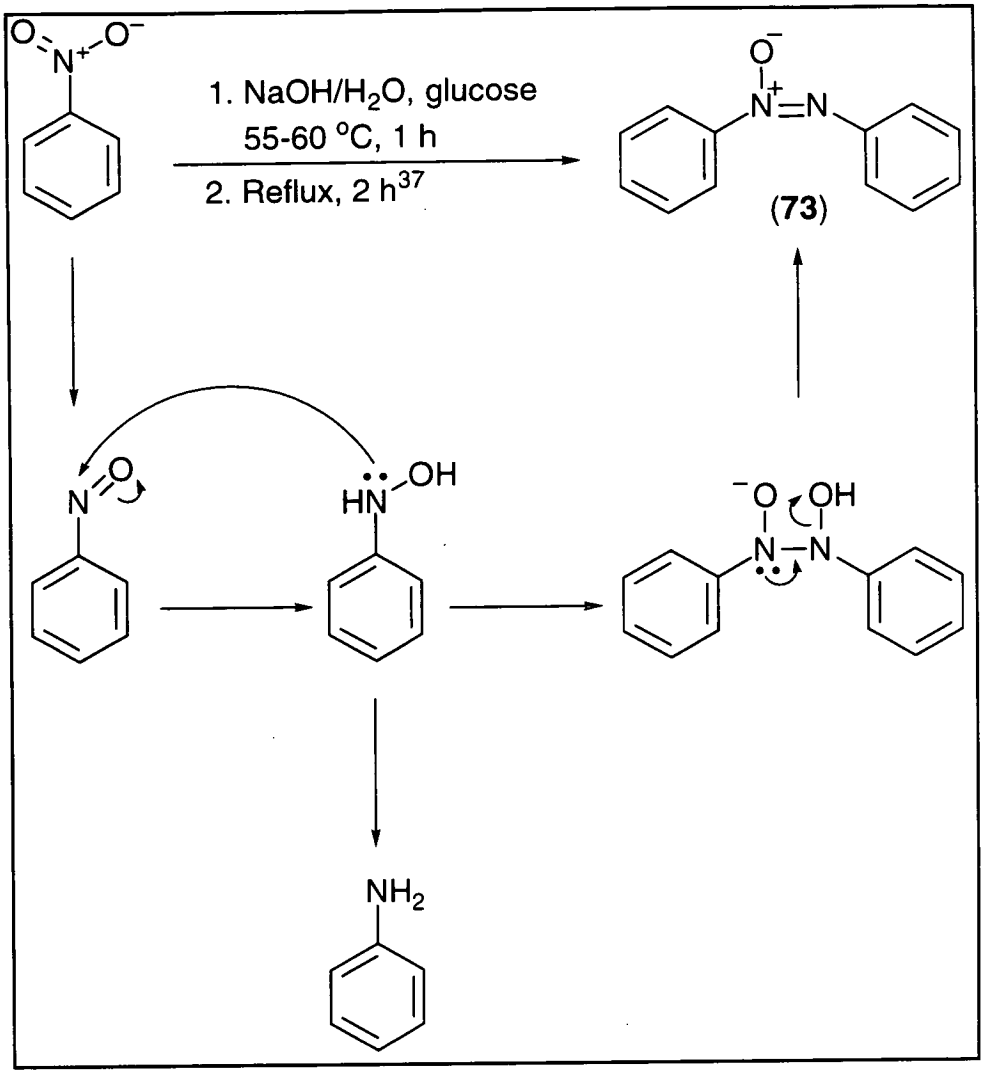
azoxybenzenes using reducing monosaccharides and disaccharides in alkaline medium.[61] Initial studies on nitrobenzene using galactose, fructose, lactose and maltose indicated that azoxybenzene was obtained in fair yield from galactose and fructose, 66.1 % and 67.3 % respectively and no aniline was produced. With the disaccharides, lactose and maltose, much lower yields were obtained, 19.5 % each, and again no aniline. With the chloro-, bromo- and iodonitrobenzenes similar results were obtained, with the monosaccharides proving to be the better reducing agents. The reaction conditions employed were similar to those previously mentioned, with high pH and high temperature. Thus it occured to us that the type II conditions reported by Baik *et al.*, could simply be a variation of these classical chemical conditions. It is unlikely that the yeast will be catalysing the reduction reactions under such harsh conditions, more likely, the yeast is acting as a carbohydrate source for the reduction. To test this notion a series of experiments involving the reduction of nitrobenzene under a variety of conditions were performed by Dr Andrew Wells at SmithKline Beecham, Tonbridge (**Table 2.0**). The results of these tests demonstrate the necessity of the basic reaction media, without which no reaction occurs. Production of aniline is favoured by increasing the amount of carbohydrate made available and the effect of adding baker's yeast to the reaction medium is comparable to adding glucose, providing evidence that baker's yeast is indeed acting as a source of carbohydrate.



(74) **(73)**

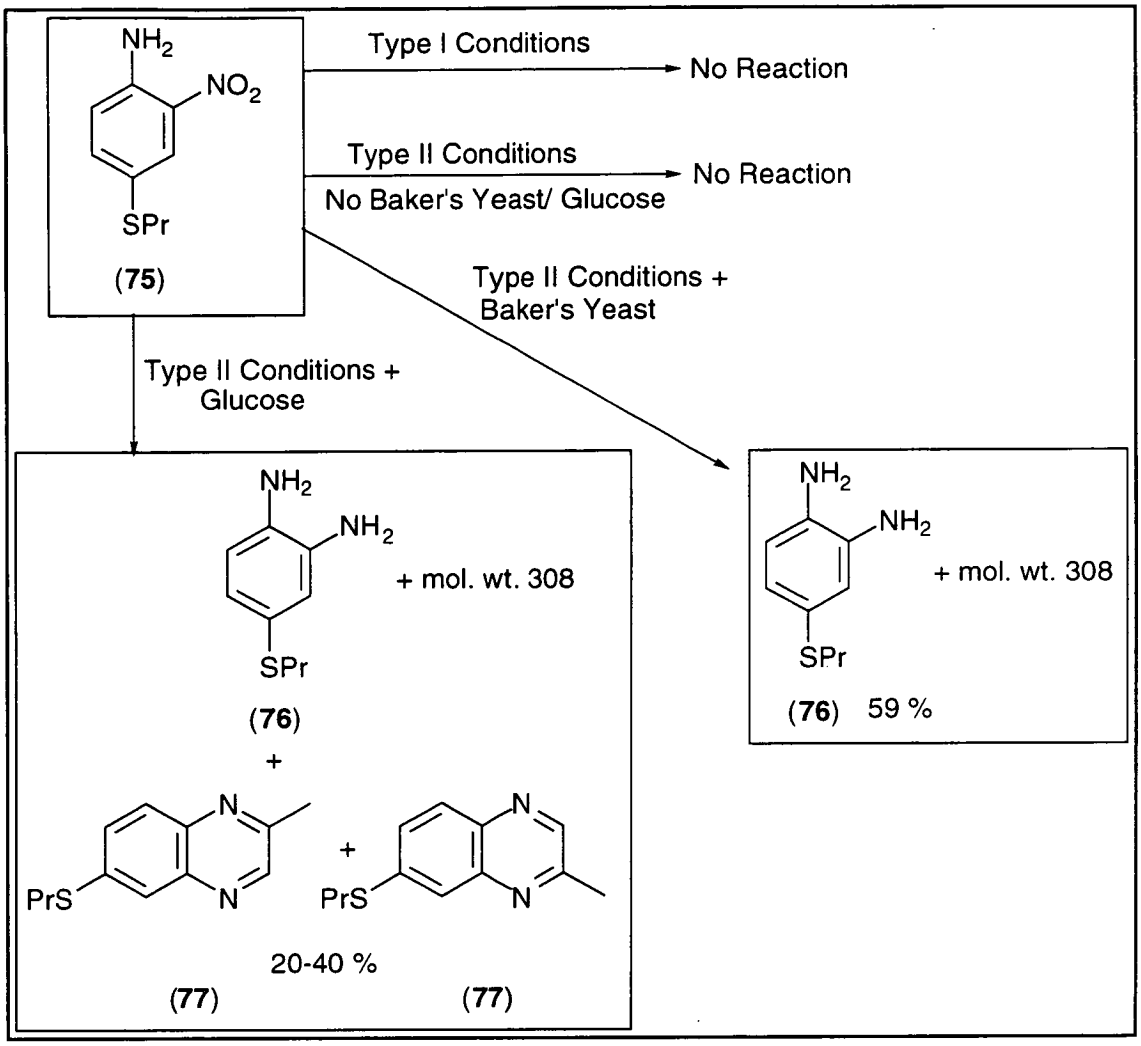
Entry No	Conditions	Yield of (**74**) %	Yield of (**73**) %
1	No NaOH	0	0
2	NaOH 5 g, glucose 1 g	11	60
3	NaOH 5 g, glucose 5 g	30	0
4	NaOH 5 g, baker's yeast 5 g	30	0

Table 2.0

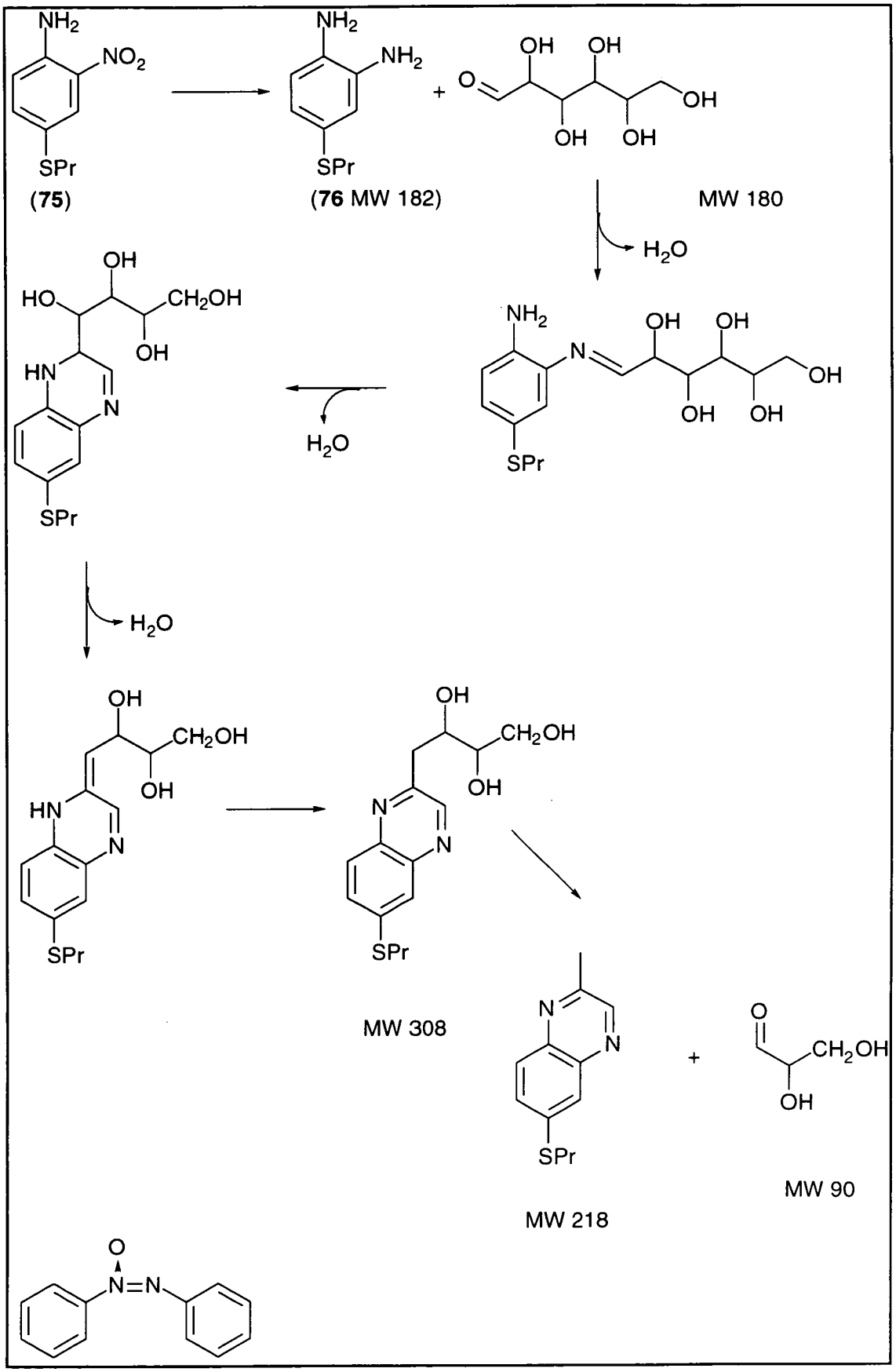


Scheme 2.7

4-Thiopropyl-2-nitroaniline (**75**) is an example of another compound that fails to be reduced under type I conditions but is successfully reduced under type II conditions (**Scheme 2.8**). A control experiment in absence of baker's yeast and glucose failed to give any reduction. Using HPLC-MS to monitor the reactions the experiment was repeated with the addition of baker's yeast giving 4-thiopropyl-2-aminoaniline (**76**) in 59 % yield, along with a product/intermediate with a molecular weight of 308. When the experiment was repeated using glucose or fructose in place of the baker's yeast, 3-methyl-7-thiopropylquinoxaline (**77**) and 2-methyl-7-thiopropylquinoxaline (**78**) were obtained alongside (**76**) and the compound with MW 308. This led to the proposed mechanism shown in **Scheme 2.9**. The differences in yields could be attributed to the slow release of carbohydrate from the yeast cells.


NH2
NO2
SPr
(75)
Type I Conditions
No Reaction
Type II Conditions
No Baker's Yeast/ Glucose
No Reaction
Type II Conditions +
Baker's Yeast
Type II Conditions +
Glucose
NH2
NH2
+ mol. wt. 308
SPr
(76)
+
N
N
PrS
+
N
N
PrS
20-40 %
(77)
(77)
NH2
NH2
+ mol. wt. 308
SPr
(76) 59 %

Scheme 2.8


NH2
NO2
SPr
(75)
NH2
NH2
SPr
(76 MW 182)
OH
OH
O
OH
OH
OH
MW 180
H2O
NH2
OH
OH
N
OH
OH
OH
SPr
H2O
OH
HO
CH2OH
OH
HN
N
SPr
H2O
OH
CH2OH
OH
HN
N
SPr
OH
CH2OH
OH
N
N
SPr
MW 308
N
N
SPr
MW 218
+
O
CH2OH
OH
MW 90
O
N=N

Scheme 2.9

A summary of the differences observed between type I and type II conditions can be seen in **Table 2.1**.

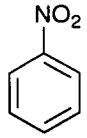
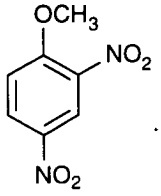
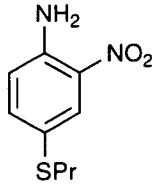
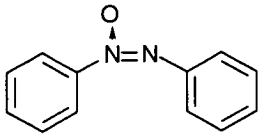
Substrate	Products	
	Type I Conditions	Type II Conditions
	No conversion	55 % Aniline
	80 % 2-amino-4 nitro anisole, 15 % 4-amino-2-nitroanisole	20 % 2-amino-4-nitro anisole
	No conversion	4-thiopropyl-2-amino aniline + **77** & **78**
	No conversion	Aniline (neutral pH) Azobenzene (high pH)

Table 2.1

Other substrates that failed to be successfully reduced by baker's yeast under type I conditions include nitroarenes **79-85** (**Figure 2.2**). The biphenyl compounds were initially tested to see if the effect of substituents on the aromatic ring could be transmitted through a biphenyl ring system. The failure of reaction of biphenyls could be due to the structures being too large to fit into the active site of the enzyme responsible for the reduction.

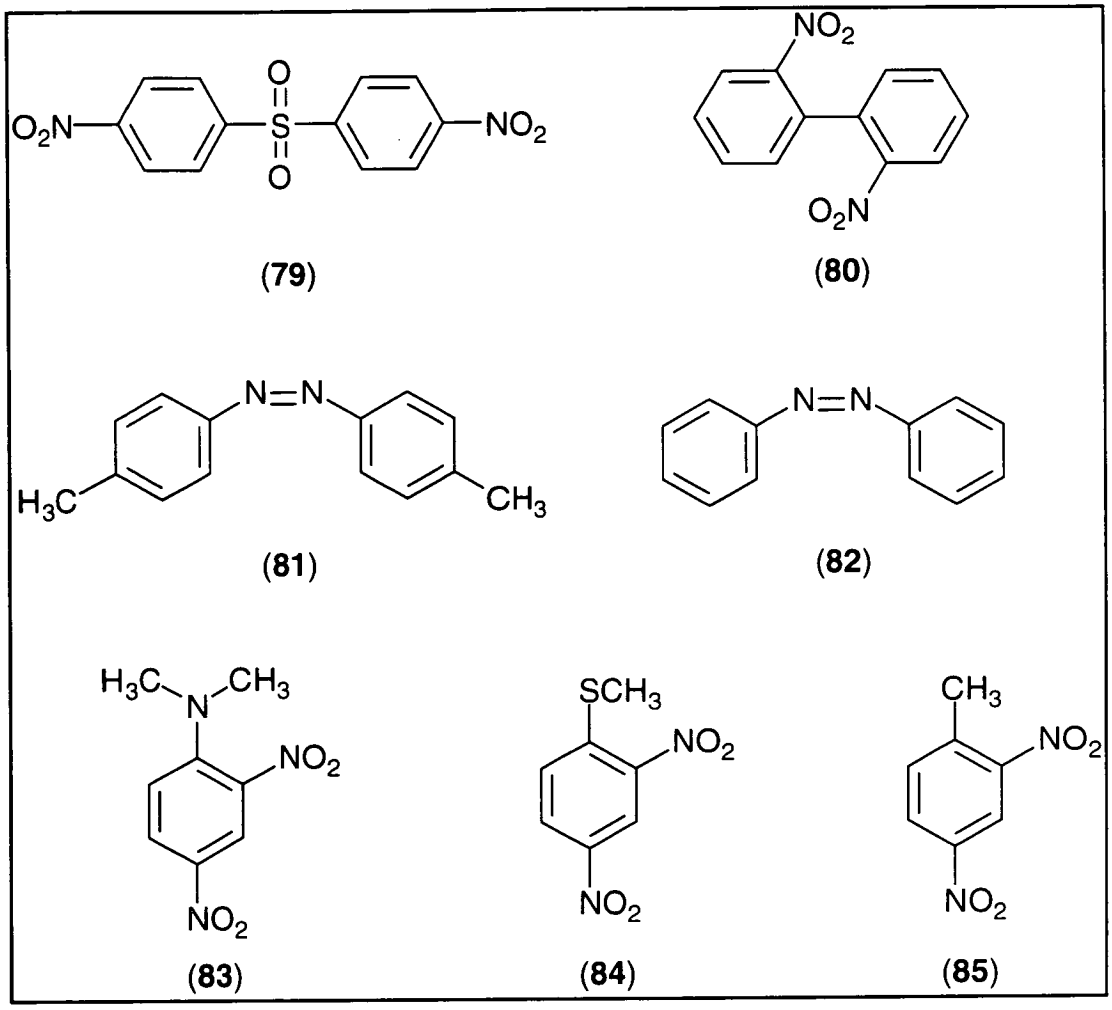


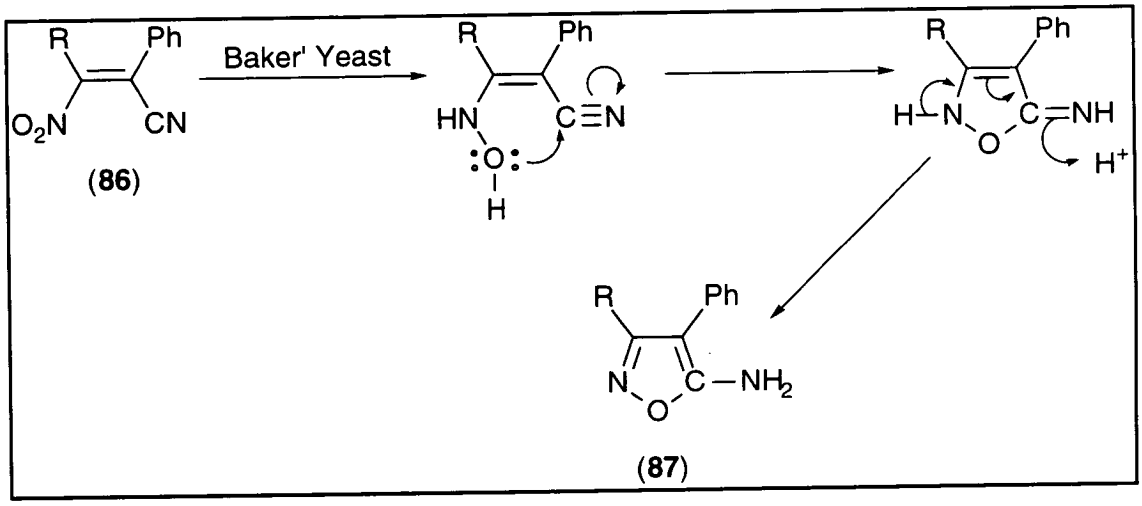
Figure 2.2

2.3.1 CONCLUSION

Under the type II conditions employed by Baik *et al.*, a variety of substrates are reduced irrespective of the presence of an electron withdrawing group. As previously suggested, it could be advantageous to provide the necessary carbohydrate in the form of baker's yeast instead of glucose but this could result in a more difficult work-up. Under type I conditions an electron withdrawing group present on the aromatic ring of a substrate facilitates reduction. The baker's yeast demonstrates a more limited substrate range under type I conditions but those that are successfully reduced can show selectivity to a significantly greater degree than that observed under type II conditions.

2.4 REDUCTION OF NITROBENZONITRILES

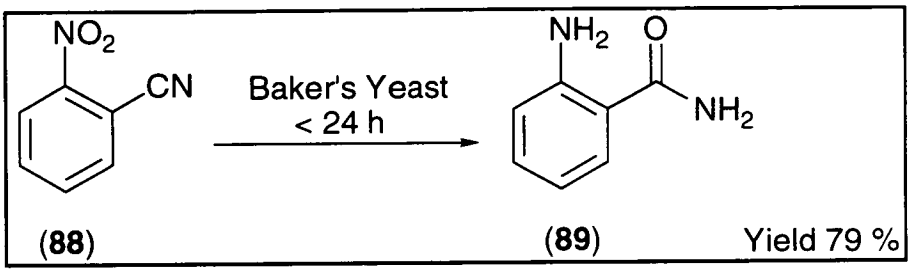
Previous work undertaken in our research group by Davey *et al.*, led to a proposed mechanism for the reduction of 2-nitrobenzonitrile (section 1.4.1). It was proposed that formation of 2-aminobenzamide, instead of the expected 2-aminobenzonitrile, could be via intramolecular attack of the hydroxyl group of the hydroxylamine intermediate onto the nitrile to give benzo-isoxazolidine. This mechanism is supported by Navarro-Ocãna *et al.*, (section 1.4.1) who reported the formation of isoxazoles (**87**) from (Z)-3-alkyl-3-nitro-2-phenylpropenonitriles (**86**, **Scheme 2.10**). With this mechanism in mind it was decided to see whether it was possible to exploit the conversion of nitrile to amide through a cyclic intermediate as a strategy in the regioselective transformation of dinitriles.



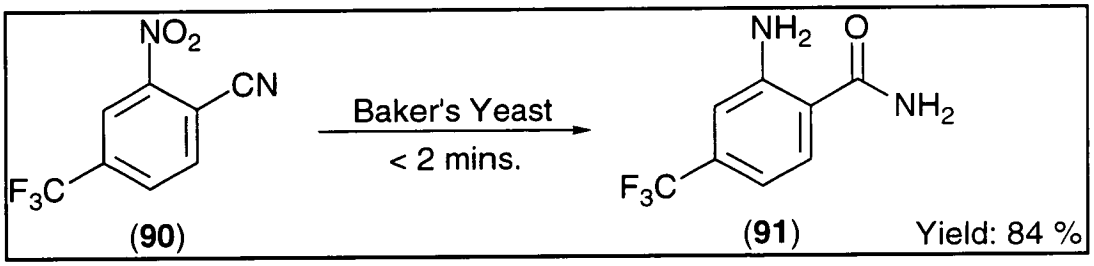
Scheme 2.10

The biotransformation reported by Davey *et al.*, on 2-nitrobenzonitrile was repeated but purification of the products was difficult due to contaminants extracted from the aqueous fraction of the biotransformation. The contaminants were attributed to a new batch of dried baker's yeast obtained. The problem was overcome by pre-washing the dried yeast in acetone at -20 °C, this resulted in the extraction of the contaminants from the yeast prior to use whilst maintaining activity. This pre-washed yeast was used for all the nitrobenzonitriles tested. The reduction of 2-nitrobenzonitrile (**88**) was repeated with the "clean" yeast using the optimised work-up conditions. The yield was improved by 23 %, producing a 79 % isolated yield of 2-aminobenzamide (**89**, **Scheme 2.11**). This reaction took less than 24 h, significantly quicker than many other biotransformations. To see whether an electron withdrawing substituent

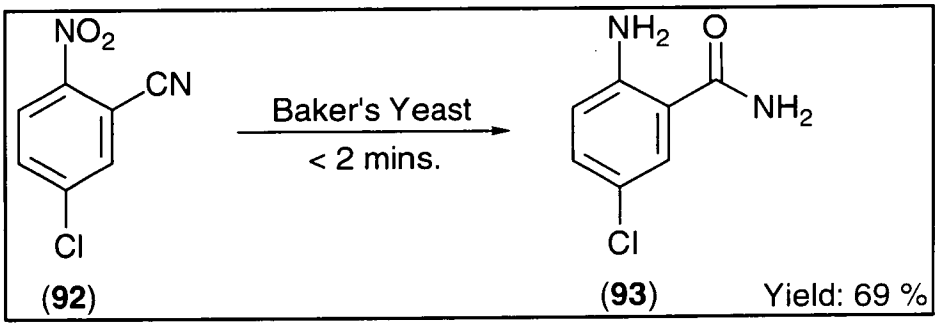
would enhance this reaction, 2-nitro-4-trifluoromethylbenzonitrile (**90**) and 2-nitro-5-chlorobenzonitrile (**92**) were tried as substrates with baker's yeast, giving the corresponding amides (**91** and **93**) in 84 % and 69 % yields respectively (**Scheme 2.12** and **2.13**).



Scheme 2.11



Scheme 2.12



Scheme 2.13

As well as a significant enhancement in yield when electron withdrawing groups were present on the ring, the reaction times were considerably reduced to less than two minutes, *i.e.* as soon as the substrate was added a sample was taken and run on HPLC which indicated conversion of the starting material. Nitrobenzonitriles that failed to be reduced included 2-nitro-4,5-dimethoxybenzonitrile (**94**), and 2,6-dinitrobenzonitrile (**95**, **Figure 2.3**).

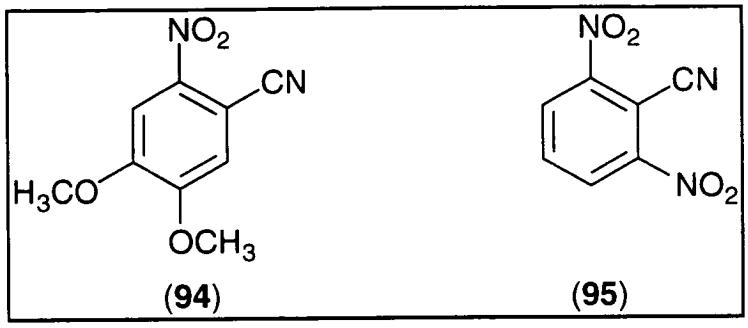
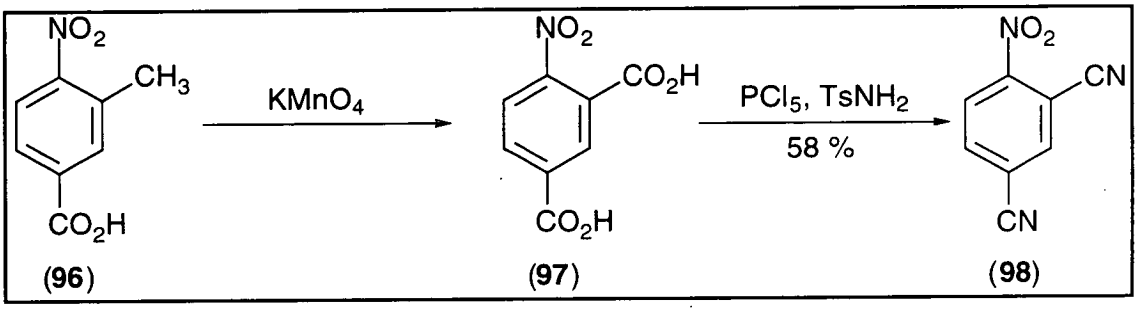


Figure 2.3

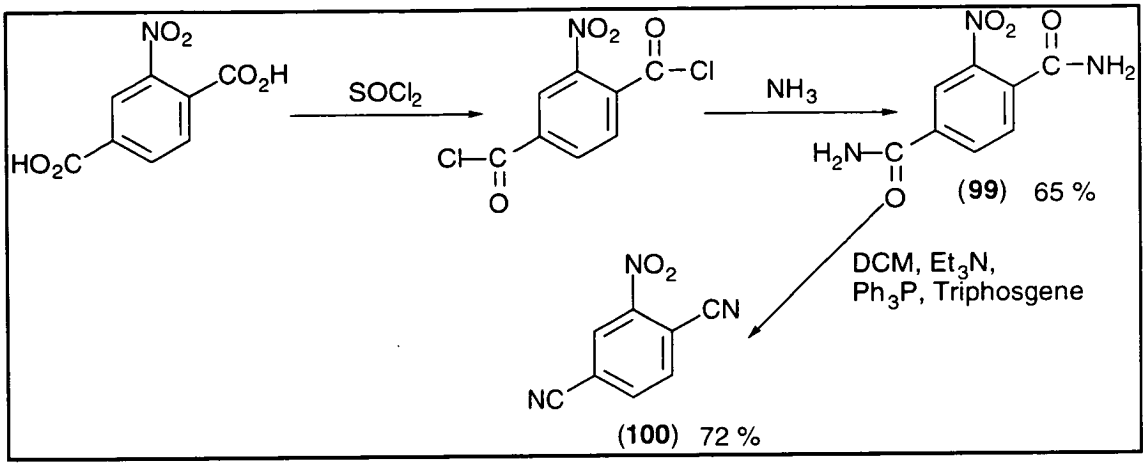
An alternative explanation for the production of amides from nitrile containing substrates could be due to the presence of amidase activity in baker's yeast. To examine this theory a series of dicyanonitroarenes was investigated.

4-nitroisophthalonitrile (**98**) was prepared from 3-methyl-4-nitro-benzoic acid (**96**) via nitroisophthalic acid (**97**) as shown in **Scheme 2.14**.



Scheme 2.14

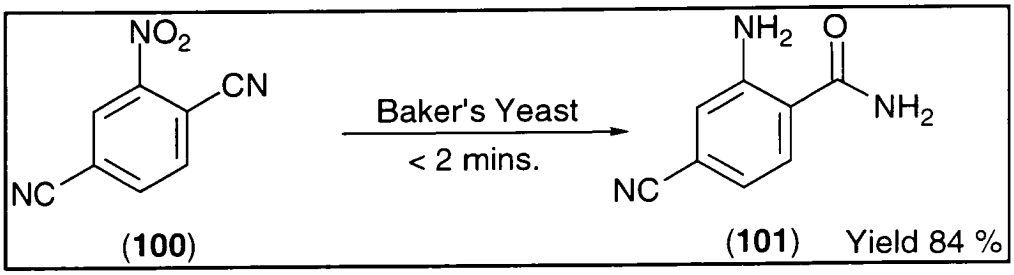
The same method was used in the attempted preparation of 3-nitroterephthalonitrile (**100**) from 3-nitroterephthalic acid, however this proved unsuccessful. The dinitrile (**100**) was prepared instead by forming 3-nitroterephthalamide (**99**) from the acid and then treating the diamide with triphosgene and triphenylphosphine as shown in **Scheme 2.15**.[62] Other dicyanonitroarenes tested as substrates for baker's yeast were commercially available.



Scheme 2.15

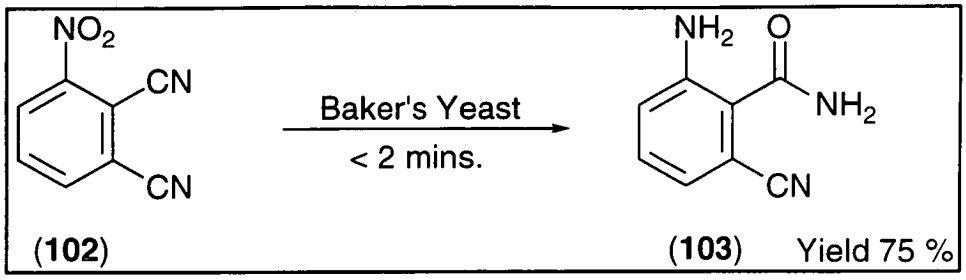
The reduction of 4-nitroisophthalonitrile (**98**) by baker's was monitored by HPLC and the work-up performed in the usual manner. Compounds extracted by this method and purified by flash chromatography failed to resemble any of the expected products. Extraction with ethyl acetate after filtering the cells through a celite bed also failed to produce any identifiable products. Ion-exchange chromatography also failed. It is thought that the presence of the *para*-nitrile group activated the nitro group towards displacement. There have been reports in the literature that a nitro group *ortho* to a nitrile and *para* to an electron withdrawing group is fairly easily displaced.[63-65] It may be that in this instance the nitro moiety is being displaced by a nucleophilic group (e.g. amino acid of a protein) present in the reaction medium. To test this theory 4-nitroisophthalonitrile (**98**) was stirred in water separately with cysteine and lysine both in the absence of baker's yeast. No reaction occurred in the presence of cysteine but HPLC clearly demonstrated rapid reaction between the substrate and lysine.

3-nitroterephthalonitrile (**100**) proved to be an excellent substrate for baker's yeast with selective conversion of the *ortho*-nitrile to produce 4-amido-3-aminobenzonitrile in 84 % yield (**101**, **Scheme 2.16**).



Scheme 2.16

The reduction of 3-nitrophthalonitrile (**102**) to 2-amido-3-aminobenzonitrile (**103**) also proved to be successful, again showing selectivity for the ortho-nitrile as would be expected if reduction occurred via the isoxazolidine intermediate (**Scheme 2.17**) This result further confirms the theory that under type I conditions the reduction follows the pathway shown in **Scheme 1.21**. The selectivity observed, confirmed by nOe studies (**Figure 2.4**), shows that the conversion of the nitrile group to the amide is not the result of amidase activity in the yeast as this would be expected to convert both nitrile groups.



Scheme 2.17

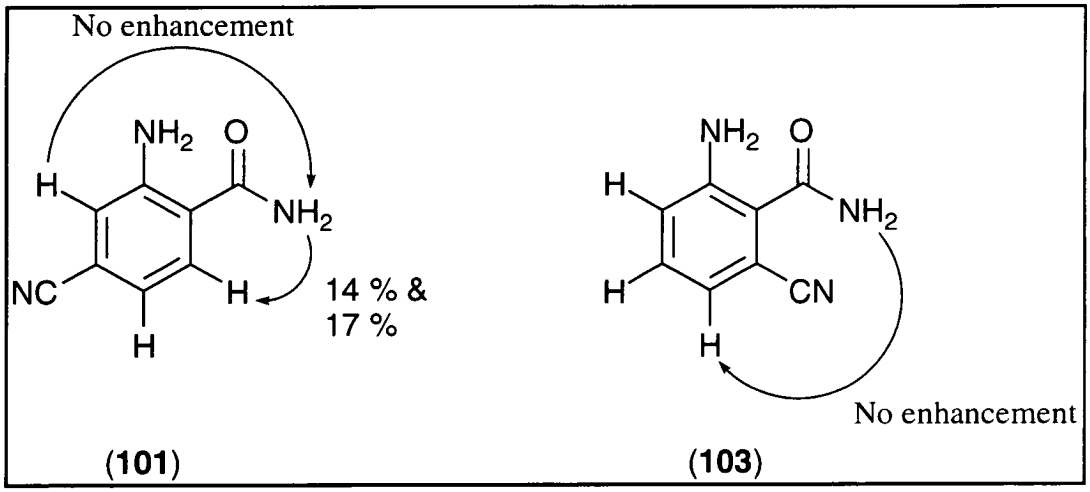
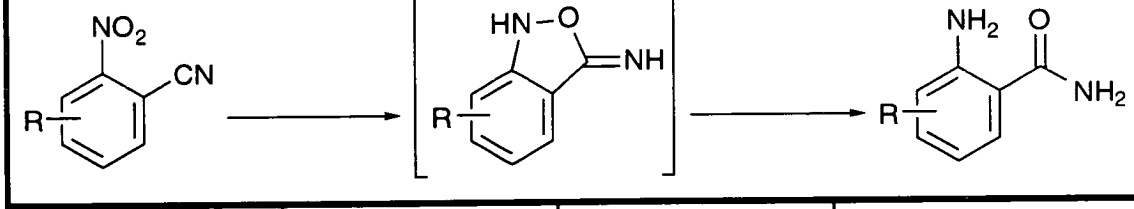


Figure 2.4 Further evidence of the structures of 4-amido-3-aminobenzonitrile (**101**) and 2-amido-3-aminobenzonitrile (**103**) was provided by comparison of the expected frequency positions of carbon atoms of one regioisomer in the $^{\delta}C$ nmr spectrum with the alternative regioisomer.[‡]

A summary of the results of baker's yeast reductions of substituted 2-nitrobenzonitriles is given in **Table 2.2**.

[‡] Calculation of expected frequencies taken from:
Williams, D.H. and Flemming, I. Spectroscopy Methods in Organic Chemistry (5th ed.). McGraw-Hill UK



Entry No	R	Reaction Time	Yield of Product (%)
1	H	<24 h	79
2	5-Cl	<2 min	69
3	4-CF_3	<2 min	84
4	4,5-CH_3O	–	–
5	6-NO_2	–	–
6	6-CN	<2 min	75
7	5-CN	–	–
8	4-CN	<2 min	84

Table 2.2

Due to the speed of the bioreductions we were unable to isolate any intermediates to confirm the formation of the isoxazolidine intermediate. Close monitoring by HPLC however, clearly shows the formation of a number of intermediates during the reaction. The reaction of 3-nitroterephthalonitrile (**100**) to 4-amido-3-aminobenzonitrile (**101**) is shown in **Figure 2.5** (i-vi).

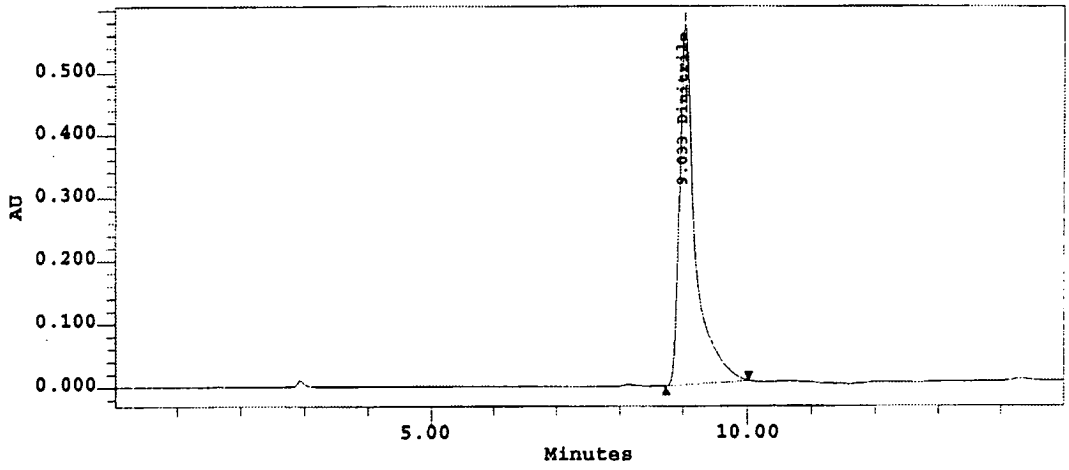


Figure 2.5 i Reaction time = 0 min

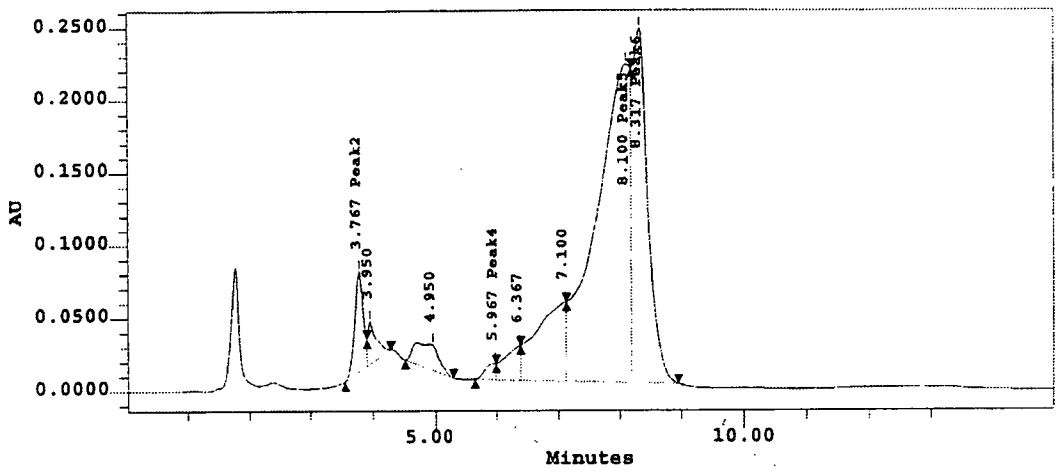


Figure 2.5 ii Reaction time = 2 min

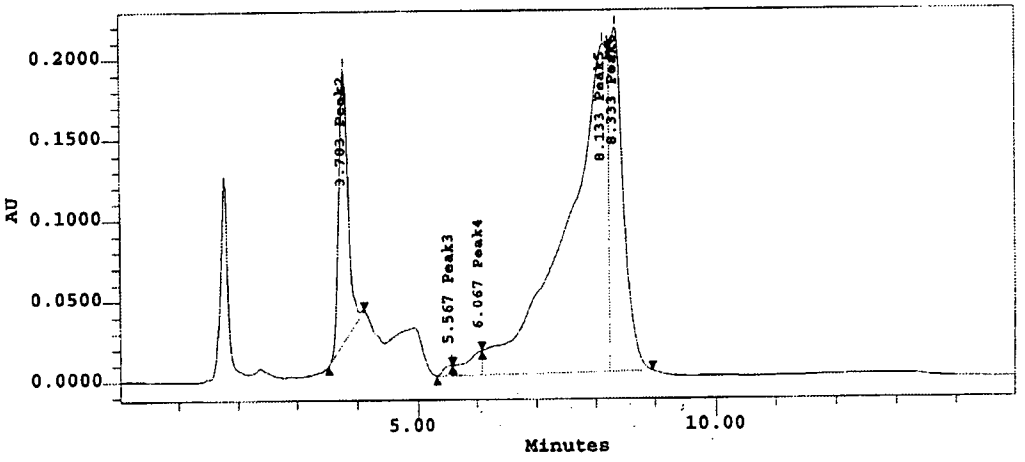


Figure 2.5 iii Reaction time = 5 min

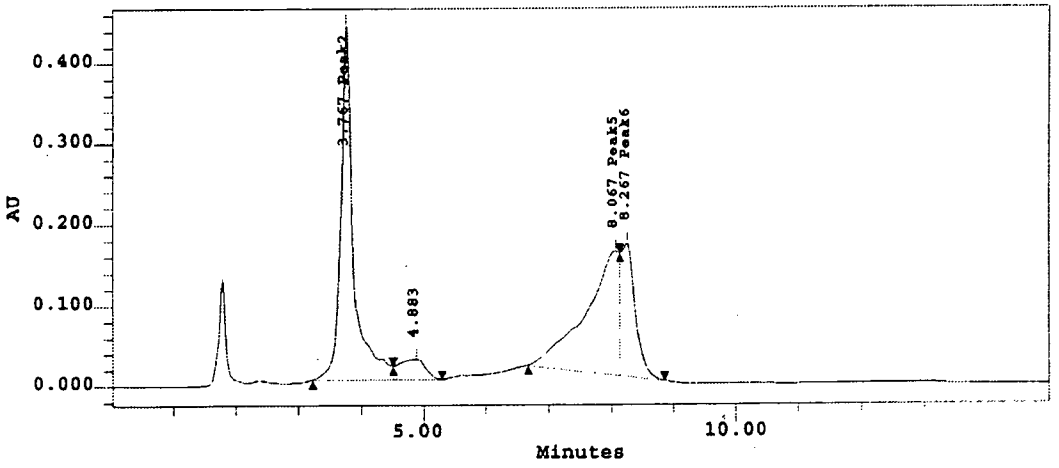


Figure 2.5 iv Reaction time = 10 min

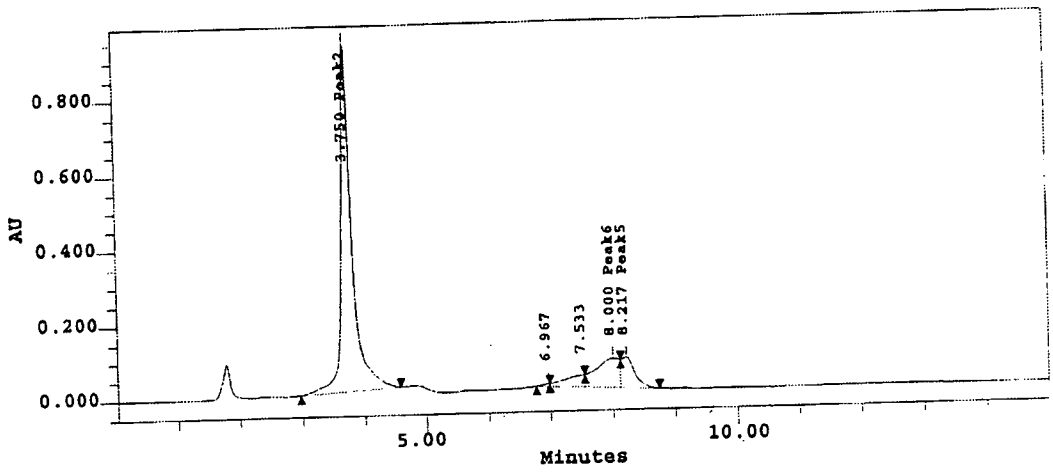


Figure 2.5 v Reaction time = 20 min

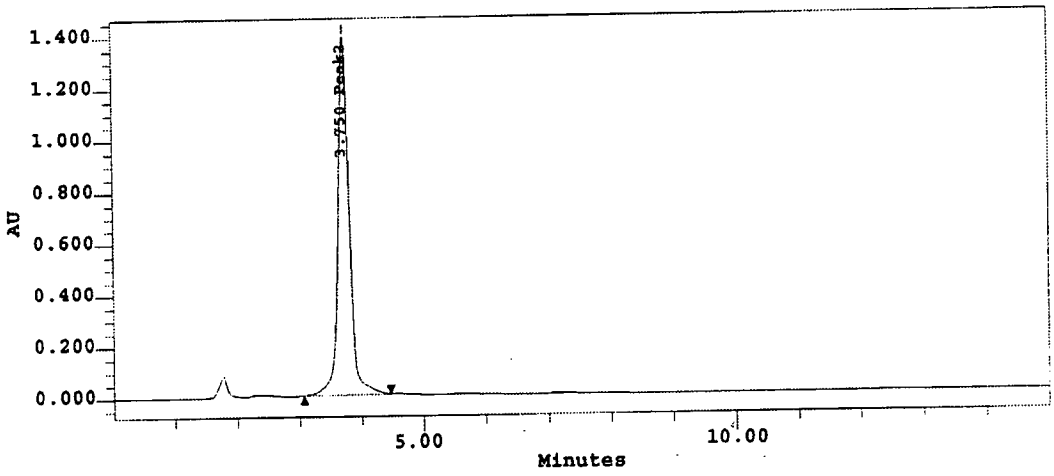


Figure 2.5 vi Reaction time = 40 min

2.5 CONCLUSION

Using the optimised reaction conditions, a work-up protocol that greatly enhances the yield of products formed by biotransformations has been produced. Two different pathways have been proposed for the bioreductions according to the reaction conditions employed. Under type I conditions, the reduction of nitroarenes procedes via the nitroso and hydroxylamine intermediates, finally forming the corresponding aniline. Evidence for this mechanism is provided by the bakers yeast reduction of nitrobenzonitriles. These substrates are reduced via an isoxazolidine intermediate that would not be formed unless reduction first proceeded to form the hydroxylamine intermediate.

Under type II conditions, a variety of substrates are reduced which fail to be substrates under type I conditions. Alongside the unfavourable conditions for enzyme activity, it is proposed that type II conditions are merely an adaptation of a classical chemical reaction.

Baker's yeast conversions performed under type I conditions provides a method for selectively manipulating the reduction/hydrolysis of a number of 2-nitrobenzonitriles leading to synthetically useful and novel multifunctionalised aromatic structures.

3. EXPERIMENTAL: BIOTRANSFORMATIONS

3.1 GENERAL

IR absorption spectra were recorded on a Perkin-Elmer 881, Perkin-Elmer 781 or a Biorad FTS-7 spectrophotometer using standard techniques. Melting points were determined on an Electrothermal instrument and are uncorrected. Low and high resolution mass spectra were recorded on a Kratos Profile HV3, Finnigan 4500, Kratos MS50TC, VG TRIO-2-quadropole or a VG 70-250 SEQ intrument and recorded under electron-ionisation (EI) conditions.

^{1}H and ^{13}C were recorded on a Bruker AM300, Bruker AM400, Bruker AC250, Varian Gemini 2000, Jeol GX 270 or a Jeol GSX 400 spectrometer. Chemical shifts are reported in ppm (δ_H and δ_C) using TMS as an internal standard. Coupling constants (J) are stated in Hertz (Hz).

GLC chromatograms were recorded on a Pye-Unicam PU4500 or a Shimadzu 14A instrument using an HT8 capilliary column (supplied by SGE UK). HPLC chromatograms were obtained using a Beckman Gold HPLC system or a Waters system with UV detection.

A New Brunswick G25 orbital shaker was used for incubations.

Petroleum ether, referred to as petrol (BPt 40/60 °C), and ethyl acetate were distilled prior to use, ethanol was dried over 3Å sieves. All other reagents were obtained from commercial suppliers. TLC was performed on pre-coated silica gel glass backed plates (Merck 60 F_{254}, 0.25 mm). The plates were visualised using UV light (254 nm) or ninhydrin dip. Column chromatography was carried out with silica gel 60H (0.04-0.063 mm) (Merck 9385) or Fisons matrix silica 60 particle size 35-70 μ as absorbant.

GLC Methods:

	Column (°C)	Detector (°C)	Injector (°C)	He Pressure (psi)
1.	190	250	250	11
2.	215	250	250	14
3.	230	250	250	15
4.	165	250	250	14

HPLC Methods:

1. Column: Highcrom S5ODS2 125 x 4.6 mm
   Gradient 25 % MeCN, 75 % H_2O (0.1 % TFA) to
   80 % MeCN, 20 % H_2O (0.1 % TFA) over 30 min.
2. Column: Phenomonex Prodigy 5µ C8 150 x 4.6 mm
   Gradient 25 % MeCN, 75 % H_2O (0.1 % TFA) to
   80 % MeCN, 20 % H_2O (0.1 % TFA) over 30 min.
3. Column: HPLC Technology SperisorbODS2 250 x 4.6 mm
   Isocratic 30 % MeCN, 70 % H_2O (0.1 % TEA)

3.2 GENERAL PROCEDURE A FOR BAKER'S YEAST REDUCTIONS

Baker's yeast (Sigma Type II, YSC-2) (25 g) was suspended in distilled water (100 ml) in a conical flask (500 ml) and incubated in an orbital shaker at 30 °C and 200 rpm. After approximately 1h, the substrate (250 mg) dissolved in the minimum amount of DMSO was added to the suspension. The conversion of the substrate to the reduced products was monitored by TLC and/or GLC. For analysis of the product/yeast suspension by TLC or GLC, 250 µl of the suspension was withdrawn and added to 100 µl of ethyl acetate (containing internal standard where necessary) in an Eppendorf tube. After microcentrifugation the organic layer containing the product was then separated. Upon reaction completion, the yeast suspension was continuously extracted with chloroform overnight. The organic extract was filtered and dried over $MgSO_4$, then the solvent was removed under reduced pressure. Purification was achieved by flash column chromatography.

3.3 GENERAL PROCEDURE B FOR BAKER'S YEAST REDUCTIONS

Baker's yeast (Sigma Type II, YSC-2) was added to pre-cooled (-20 °C) acetone and the suspension was stirred for 15 mins. Once the yeast had settled out, the acetone was removed and the procedure repeated with fresh pre-cooled acetone. The yeast was then filtered and dried in a vacuum oven at room temperature. The acetone treated yeast (20 g) was suspended in distilled water (100 ml) in a 250 ml conical flask and incubated in an orbital shaker at 30 °C and 200 rpm. After 1 h, the substrate (100 mg) dissolved in a minimum amount of DMSO was added to the suspension. The conversion of the substrates to the products was monitored by TLC,GLC and/or HPLC. To get the product/yeast suspension into a state by which TLC or GLC could be used, 250 µl of the suspension was withdrawn and added to 100 µl of ethyl acetate

in an Eppendorf tube. After microcentrifugation the organic layer containing the product was then separated. To follow the reaction by HPLC, 1 ml of the suspension was added to 2 ml of acetonitrile and shaken, filtration through syringe filters produced a sample fit for injection. Upon reaction completion the product suspension was taken up to pH 8 using NaOH (1 M) and saturated with salt, then subjected to continuous extraction with chloroform overnight. The organic extract was filtered and dried over $MgSO_4$ and the solvent removed under reduced pressure. Purification was achieved by flash column chromatography.

3.4 CHEMICAL SYNTHESES AND BIOTRANSFORMATIONS

BAKER'S YEAST REDUCTION OF 2,4-DINITROANISOLE (**63**).

Following the general proceedure A, 2,4-dinitroanisole (**63**) (0.25 g, 1.26 mmol) was added to the yeast suspension. After 3-4 days GLC indicated complete reduction of the substrate. Purification of the crude product on SiO_2 (eluent 7:3, DCM:Petrol) yielded two products:

2-AMINO-4-NITROANISOLE (**64**) (0.17 g, 80.3%) Orange/yellow coloured powder; R_f 0.71 (DCM); M.Pt 116 °C (from EtOH)(lit.[66] 118 °C); υ_{max} (KBr)/cm^{-1} 3471 and 3374s (N-H), 1662 and 1584s (CH arom.), 1510 and 1332s (N-O); δ_H (300 MHz; $CDCl_3$) 3.96 (3H, s, CH_3); 4.06 (2H, br s, NH_2), 6.80 (1H, d, $^3J_{6.5}$ 8.8, H-6), 7.56 (1H, d, $^4J_{3.5}$ 2.7, H-3), 7.68 (1H, dd, $^4J_{5.3}$ 2.7 and $3J_{5.6}$ 8.8, H-5); nOe experiment: Irradiation of NH_2 (at 4.06 ppm) resulted in a 6% enhancement of the signal corresponding to H-3 (at 7.56 ppm); δ_C (75 MHz; $CDCl_3$) 42.4 (CH_3) 116.6 (CH) 124.2 (CH), 127.7 (CH); *m/z* (EI) 168 [M^+], 153 [M^+-CH_3], 138 [M^+-OCH_3+H], 122 [M^+-NO_2], 122 [M^+-OCH_3+H-NH_2]; HRMS: $C_7H_8N_2O_3$ requires 168.0539, found 168.0533 (1.1 ppm dev.); GLC (Method 1) RT 7.30-7.70 min.

4-AMINO-2-NITROANISOLE (**65**) (0.03 g, 14.9%) Orange/red coloured gum; R_f 0.2 (DCM); υ_{max} (KBr)/cm^{-1} 3479 and 3388s (N-H), 1624 and 1572m (CH arom.), 1524 and 1348s (N-O); δ_H (300 MHz; $CDCl_3$) 3.64 (2H, br s, NH_2), 3.88 (3H, s, CH_3), 6.86 (1H, dd, $^4J_{5.3}$ 2.8 and $^3J_{5.6}$ 8.8, H-5), 6.93 (1H, d, $^3J_{6.5}$ 8.8, H-6), 7.18 (1H, d, $^4J_{3.5}$ 2.8, H-3); nOe experiment: Irradiation of NH_2 (at 3.64 ppm) resulted in a 12% enhancement of the signal corresponding to H-3 (at 7.18 ppm) and a 6% enhancement of the signal corresponding to H-5 (at 6.86 ppm); δ_C (75 MHz; $CDCl_3$) 57.2 (CH_3), 111.5 (CH), 115.7 (CH), 124.7 (CH); *m/z* (EI) 168 [M^+], 92 [M^+-NO_2-

OCH_3+H]; HRMS: $C_7H_8N_2O_3$ requires 168.0539, found 168.0535 (0.2 ppm dev.); GLC (Method 1) RT 6.40-6.75 min.

SYNTHESIS OF BUTYL-4-NITROPHENYLSULPHIDE (66)

To a stirred solution of p-nitrothiophenol (0.50 g, 4.06 mmol) in dry ethanol (50 ml) at room temperature under an inert atmosphere was added sodium methoxide (0.50 g, 2 eq.). After 30 mins. bromobutane (1.28 g, 2.3 eq.) was added and the reaction mixture was stirred for a further 48 hrs. The reaction was quenched by the careful addition of water and the mixture extracted with ethyl acetate (3 x 50 ml). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated under pressure and the crude material was purified on SiO_2 (eluent 6:4, petrol:DCM) to yield butyl-4-nitrophenylsulphide (**66**) as a yellow oil (0.59 g, 68%); R_f 0.83 (DCM); υ_{max} ($CDCl_3$)/cm^{-1} 2964 and 2873s (C-H), 1580s and 1509 (CH arom.), 1339s (N-O), 1092s (C-S); δ_H (300 MHz; $CDCl_3$) 0.94 (3H, t, $^3J_{4',3'}$ 7.9, H-4'), 1.49 (2H, m, H-3'), 1.69 (2H, m, H-2'), 3.03 (2H, t $^3J_{1',2'}$ 7.9, H-1'), 7.16 (2H, AB, H-2 and H-6), 8.11 (2H, AB, H-3 and H-5); δ_C (75 MHz; $CDCl_3$) 13.5 (CH_3), 22.0 (CH_2), 30.5 (CH_2), 31.7 (CH_2), 123.9 (CH), 126.0 (CH), 144.9 (C), 148.2 (C); *m/z* (EI) 211 [M^+], 155 [M^+-$(CH_2)_3CH_3$]; HRMS: $C_{10}H_{13}NO_2S$ requires 211.0667, found 211.0663 (1.9 ppm dev.); GLC (Method 2) RT 4.25 mins.

SYNTHESIS OF BUTYL-4-NITROPHENYLSULPHOXIDE (67)

Butyl-4-nitrophenylsulphide (**66**) (0.36 g, 1.71 mmol) was partially dissolved in 1:1 methanol/water (50 ml) and cooled to 0 °C with stirring. To this was added precooled sodium meta periodate (0.54 g, 1.5 eq) dissolved in the minimum amount of 1:1 methanol/water. The reaction was stirred overnight at 0 °C. The reaction mixture was extracted into ethyl acetate (3 x 50 ml) and the combined extracts dried over Na_2SO_4. The crude product was purified on SiO_2 (eluent 6:4, ethyl acetate:petrol) to yield butyl-4-nitrophenylsulphoxide (**67**) as a yellow/orange coloured oil (0.220 g, 56.8%); R_f 0.59 (6:4, ethyl acetate :petrol); υ_{max} ($CHCl_3$)/ cm^{-1} 2966 and 2935s (C-H stretch), 1603 and 1527s (CH arom.), 1347s (N-O), 1042s (S-O); δ_H (200 MHz; $CDCl_3$) 0.90 (3H, t, $^3J_{4',3'}$ 7.0, H-4'), 1.44 (2H, m, H-3'), 1.75 (2H, m, H-2'), 2.80 (2H, m, H-1'), 7.79 (2H, AB, H-3 and H-5), 8.35 (2H, AB, H-2 and H-6); δ_C (200 MHz; $CDCl_3$) 150.31 and 144.13 (C), 123.80 and 122.97 (CH), 55.37, 22.41 and 20.37 (CH_2), 12.14 (CH_3); *m/z* (EI) 227 (M^+), 171 (M^+-C_4H_9), 155 (M^+-C_4H_9-O), 125 (M^+-

C_4H_9-NO_2), 76 (M^+-NO_2-SO-C_4H_9); HRMS $C_{10}H_{13}NO_3S$ requires 227.0616, found 227.0610 (2.7 ppm dev.)

SYNTHESIS OF BUTYL-4-NITROPHENYLSULPHONE (**69**)

To a solution of butyl-4-nitrophenylsulphide (**66**) (2.54 g, 12 mmol) at -78 °C in CH_2Cl_2, m-CPBA (4.45 g, 1.5 eq) in CH_2Cl_2 was added dropwise. The mixture was stirred at -78 °C for one hour then washed with 10% sodium hydroxide (2M) followed by water. The crude product was purified on SiO_2 (eluent DCM) to yield butyl-4-nitrophenylsulphone (**69**) as a white crystalline solid (1.38 g, 47.2%); R_f 0.36 (DCM); M.Pt 43 °C (from DCM/Hexane); υ_{max} ($CHCl_3$)/cm^{-1} 2968-2877m (C-H), 1531s (CH arom.), 1350s (N-O), 1148s (S=O), 1329m (S=O), 1087s (C-S); δ_H (300 MHz; $CDCl_3$) 0.89 (3H, t, $^3J_{4',3'}$ 7.9, H-4'), 1.41 (2H, m, H-3'), 1.68 (2H, m, H-2'), 3.13 (2H, m, H-1'), 8.10 (2H, AB, H-3 and H-5), 8.40 (2H, AB, H-2 and H-6); δ_C (75 MHz; $CDCl_3$) 150.9 and 144.9 (C), 129.6 (CH), 55.9 (CH_2), 24.5 (CH_2), 21.5 (CH_2), 13.4 (CH_3); *m/z* (EI) 243 [M^+], 227 [M^+-O], 213 [M^+-2O], 188 [M^+-2O-C_2H_3], 156 [M^+-2O-C_4H_9], 93 [M^+-2O-C_4H_9-SO_2]; HRMS: $C_{10}H_{13}NO_4S$ requires 243.0565, found 243.0573 (3.0 ppm dev.); GLC (Method 3) RT 5.38 mins.

BAKER'S YEAST REDUCTION OF BUTYL-4-NITROPHENYLSULPHONE (**69**)

Following the general procedure the sulphone substrate (**69**) (0.25 g, 1.05 mmol) was added to the yeast suspension. After approx. 8 h GLC indicated complete reduction of the substrate. Purfication of the crude product on SiO_2 (eluent ethyl acetate) yielded butyl-4-aminophenylsulphone (**70**) as cream colured crystals (0.21 g, 93%); M.Pt 108 °C (from EtOAc/petrol)(lit. 107-109 °C); υ_{max} ($CHCl_3$)/cm^{-1} 3510 and 3416m (N-H), 2967-2876m (C-H), 1620 and 1594s (CH arom.) 1299 and 1139s (S=O), 1089m (S-C); δ_H (300 MHz; $CDCl_3$) 0.88 (3H, t, $^3J_{4',3'}$ 7.9, H-4'), 1.37 (2H, m, H-3'), 1.67 (2H, m, H-2'), 3.03 (2H, m, H-1'), 4.17 (2H, br s, NH_2), 6.70 (2H, AB, H-3 and H-5), 7.63 (2H, AB, H-2 and H-6); δ_C (75 M Hz; $CDCl_3$) 13.5 (CH_3) 21.5 (CH), 24.9 (CH), 56.4 (CH), 130.1 and 114.1 (C); *m/z* (EI) 213 [M^+], 184 [M^+-CH_2CH_3], 156 [M^+-C_4H_9], 93 [M^+-C_4H_9-SO_2]; HRMS: $C_{10}H_{15}NO_2S$ requires 213.0823, found 213.0813 (4.7 ppm dev.); GLC (Method 3) RT 7.7 mins.

SYNTHESIS OF *N*-PHENYLHYDROXYLAMINE (**71**)[57]

Nitrobenzene (4.1 g, 33.3 mmol) and THF (20 ml) were placed in a flask fitted with a condenser, thermometer and a pressure equilising dropping funnel. Rhodium on carbon (0.1 g) was added to the solution which was cooled, with stirring, to 15 °C. Hydrazine hydrate (1.7 g) was slowly added via the dropping funnel to the stirred reaction mixture, whilst maintaining the temperature at 25-30 °C. The evolution of H_2 gas and the necessity of an ice bath to keep the temperature low indicated that the exothermic reaction was proceeding and the reaction mixture was stirred for 90 mins. The reaction mixture was filtered and the THF removed under vacuum. Crystallisation from hexane gave *N*-phenylhydroxylamine (**71**) as a cream coloured solid (46 %); R_f 0.46 (eluent 7:3 Petrol:EtOAc); M.Pt 79 °C(lit.[57] 81 °C); (Found: C, 65.8; H, 6.5; N, 12.7. C_6H_7NO requires C, 66.0; H, 6.5; N, 12.8 %); υ_{max} 3250m (N-H), 3000-2800br,s (O-H); δ_H (270 MHz; $CDCl_3$) 6.5 (2H, br, NHOH), 6.95 (3H, m, H-2, H-4, H-6), 7.25 (2H, m, H-3 and H-5); δ_C (100 MHz; d_6-DMSO) 113.0 (CH), 119.0 (CH), 128.5 (CH), 152.0 (C); HPLC (Method 2) RT 3.6 mins.

SYNTHESIS OF *N*-ACETYLPHENYLHYDROXYLAMINE (**72**)[57]

To a flask containing N-phenylhydroxylamine (**71**, 1.25 g, 11.5 mmol) in THF (10 ml) was added sodium hydrogen carbonate (1.45 g, 17.3 mmol) in water (1.5 ml). This solution was cooled, with stirring, to 4 °C. Acetylchloride (0.9 g, 11.5 mmol) was introduced slowly to the reaction mixture whilst maintaining the temperature below 0 °C. Stirring was continued for a further 30 mins. Sodium hydroxide (0.7 g) in water (7 ml) was added to the stirred solution and the temperature kept below 10 °C. The reaction mixture was stirred for a further 10 min. before separation of the aqueous phase. The organic phase was diluted with hexane (10 ml) and the aqueous phase again separated. The organic phase was extracted with sodium hydroxide (2 M, 2 x 5 ml) and the aqueous phases were combined and cooled to 5 °C and neutralised with conc. HCl. The aqueous phase was extracted with DCM (3 x 30 ml) and the fractions combined, dried over $MgSO_4$, and reduced under vacuum, giving*N*-acetylphenylhydroxylamine (**72**) as a white coloured solid in 72 % yield; R_f 0.56 (EtOAc); M.Pt 64-65 °C (from EtOAC/petrol)(lit.[57] 66-67 °C); υ_{max} (Nujol/cm^{-1}) 3300-3100br (O-H), 2900-2800br (N-O), 1630s (C=O), 1500s (CH_3); δ_H (270 MHz; d_6-DMSO at 40 °C) 2.19 (3H, s, CH_3), 7.14 (1H, m, C-4), 7.37 (2H, m, C-3 and C-5), 7.61 (2H, m, C-2 and C-6); δ_C (100 MHz; d_6-DMSO at 30 °C) 22.2 (CH_3), 128.0 (CH), 142.2 (C), 169.3 (C=O); *m/z* (EI) 151 [M^+], 135 [M^+-CH_4], 109 [M^+-CH_2CO],

93 [Ph-NH_2^+]; HRMS (EI) $C_8H_9NO_2$ requires 151.0633, found 151.0627 (4.1 ppm dev.)

SYNTHESIS OF AZOXYBENZENE (**73**)[58]

Nitrobenzene (5.1 g, 41.5 mmol) was added to a flask containing NaOH (7.5 g) in water (25 ml). The solution was warmed to 55-60 °C with stirring. Glucose (5.25 g) was added in portions to the stirred mixture and the temperature maintained at 55-60 °C for 1 h. After this time the mixture was refluxed for a further 2 h and was then cooled. Distillation removed any aniline and/or nitrobenzene and the remaining material was poured into an ice-cold beaker. A solid precipitated out and was filtered off. Recrystallisation from methanol gave azoxybenzene (**73**) as a brown coloured solid (32 %); M.Pt 36 °C (from MeOH)(lit.[58] 36 °C); R_f 0.80 (1:1, EtOAc:petrol); υ_{max} (Nujol)/cm^{-1} 1570m (N=N0); δ_H (270 MHz; $CDCl_3$) 7.49 (6H, m, H-3, H-4, H-5 and H-3', H-4', H-5'), 8.17 (2H, dd, $^3J_{6',5'}$ 7.5 and $^3J_{2',3'}$ 7.5, H-6' and H-2'), 8.31 (2H, dd, $^3J_{6,5}$ 7.5 and $^3J_{2,3}$ 7.5, H-6 and H-2); δ_C (63 MHz; $CDCl_3$) 122.23 (CH), 125.41 (CH), 128.58 (CH), 128.68 (CH), 129.49 (CH), 131.47 (CH), 143.88 (C),148.22 (C); *m/z* (E.I.) 198 [M^+], 91 [M^+ -$C_7H_7^+$]; HRMS: $C_{12}H_{10}N_2O$ requires 198.0793, found 198.0784 (4.8 ppm dev.).

SYNTHESIS OF N,N-DIMETHYL-2,4-DINITROANILINE (**83**)

2,4-Dinitrochlorobenzene (9.92 g, 46.27 mmol) was dissolved in acetone (25 ml) and poured over crushed ice (approx. 330 g) with constant stirring. Dimethylammonium chloride (9.95 g, 0.12 mol, 2.6 eq.) was added to the stirring solution and the temperature was kept under 20 °C. Potassium carbonate (approx. 25 g) dissolved in water (100 ml) was added and the mixture was warmed to 45 °C for 30 mins. Filtration of the reaction mixture produced an oil, the filtrate was extracted into chloroform and dried over Na_2SO_4. The solvent was removed under reduced pressure and purification of the crude product on SiO_2 (eluent 1:1, DCM:petrol) produced a white crystalline product, *N,N*-dimethyl-2,4-dinitroaniline (**83**) (4.78 g, 48.9%); R_f 0.26 (1:1, DCM:petrol); M.Pt 84 °C (from DCM/petrol)(lit.[67] 87 °C); υ_{max} (KBr)/cm^{-1} 2921w (N-CH_3), 1608 and 1507s (CH arom.), 1332s (N-O); δ_H (300 MHz; $CDCl_3$) 3.06 (6H, s, CH_3), 7.01 (1H, d, $^3J_{6,5}$ 9.4, H-6), 8.22 (1H, dd, $^4J_{5,3}$ 2.7 and $^3J_{5,6}$ 9.4, H-5), 8.71 (1H, d, $^4J_{3,5}$ 2.7, H-3); δ_C (75 MHz; $CDCl_3$) 42.4 (CH_3), 116.6 (CH), 124.2 (CH), 127.7 (CH), 149.1 (CH); *m/z* (EI) 211 [M^+], 181 [M^+-$(CH_3)_2$], 166 [M^+-$N(CH_3)_2$],

119 [M^+-2(NO_2)]; HRMS: $C_8H_9N_3O_4$ requires 211.0593, found 211.0596 (1.3 ppm dev.).

When N,N-dimethyl-2,4-dinitroaniline was used as a substrate for Baker's yeast following the general procedure, no reduction occurred.

SYNTHESIS OF 2,4-DINITROTHIOANISOLE (**84**)

To a solution of 2,4-dinitrochlorobenzene (5.06 g, 25.36 mmol), in THF (25 ml) was added a mixture of sodium methylsulphide (2.06 g, 1 eq) in THF (25 ml, partially soluble) and sodium hydroxide (1M) (1 g, 1 eq). The mixture was refluxed for approx. 10 mins. and filtered hot through a hot sinter. The solvent was removed under reduced pressure and the crude product purified on SiO_2 (eluent 8:2, petrol:ethyl acetate) to yield 2,4-dinitrothioanisole (**84**) as a yellow coloured powder (2.28 g, 42%); R_f 0.53 (1:1, ethyl acetate:petrol); M.Pt 122 °C (from EtOAc/petrol)(lit.[68] 123-126 °C); υ_{max} ($CHCl_3$)/ cm^{-1} 1594s and 1523 (CH arom.), 1343s (N-O); δ_H (300 MHz; $CDCl_3$) 2.61 (3H, s, CH_3), 7.56 (1H, d, $^3J_{6,5}$ 12.0, H-6), 8.40 (1H, dd, $^4J_{5,3}$ 3.95 and $^3J_{5,6}$ 12.0, H-5), 9.07 (1H, d, $^4J_{3,5}$ 3.95, H-3); δ_C (75 MHz; $CDCl_3$) 16.3 (CH_3), 121.7 (CH), 126.3 (CH), 127.2 (CH); *m/z* (EI) 214 [M^+], 199 [M^+-CH_3], 168 [M^+-SCH_3-H], 121 [M^+-SCH_3-NO_2]; HRMS: $C_7H_6N_2O_4S$ requires 214.0048, found 214.0053 (2.4 ppm dev.)

REDUCTION OF 2,4-DINITROTHIOANISOLE (**84**)

Following the general proceedure A the substrate, 2,4-dinitrothioanisole (0.20 g, 0.92 mmol), was added to a yeast suspension. After 3-4 days the extracted organic material was purified on SiO_2 (eluent 1:1, ethyl acetate:petrol) to yield:

2-AMINO-4-NITROTHIOANISOLE (60.5 mg, 36%); Orange/brown coloured gum; R_f 0.69 (1:1, ethyl acetate:petrol); υ_{max} ($CHCl_3$)/cm^{-1} 3483 and 3384m (N-H), 2929 (CH_3), 1618s (CH arom.), 1590w (N-H), 1514m (CH arom.), 1344s (N-O); δ_H (400 MHz; $CDCl_3$) 2.48 (3H, s, CH_3), 4.38 (2H, br s, NH_2), 7.27 (1H, d, $^3J_{6,5}$ 8.8, H-6), 7.51 (1H, d, $^4J_{3,5}$ 2.4, H-3), 7.56 (1H, dd, $^4J_{5,3}$ 2.4 and $^3J_{5,6}$ 8.8, H-5); nOe experiment: Irradiation of NH_2 (at 4.38 ppm) resulted in a 1.5% enhancement of the signal corresponding to H-3 (at 7.51 ppm); δ_C (100 MHz; $CDCl_3$) 16.07 (CH_3), 108.48 (CH), 113.38 (CH), 128.82 (CH), 129.98 (C), 145.51 (C), 146.97 (C); *m/z* (EI) 184 [100%, M^+], 169 [9, M^+-CH_3], 138 [16, M^+-SCH_3+H]; HRMS: $C_7H_8N_2O_2S$ requires 184.0307, found 184.0311 (2.5 ppm dev.)

BAKER'S YEAST REDUCTION OF 2,4-DINITROTOLUENE (**85**)

Following the general proceedure A the substrate, 2,4-dinitrotoluene (0.24 g, 1.31 mmol) , was added to a yeast suspension. After 3-4 days GLC indicated no further reduction. Upon purification of the extracted organics (eluent DCM) the products were identified as:

2-AMINO-4-NITROTOLUENE (0.03 g, 13%); A red/brown coloured solid; R_f 0.41 (DCM); M.Pt 107 °C (from DCM)(lit.[69] 107 °C); υ_{max} ($CHCl_3$)/cm^{-1} 3017s (CH arom.), 2929s (CH_3), 1525m (N-H), 1350w (N-O); δ_H (400 MHz; $CDCl_3$) 2.23 (3H, s, CH_3), 3.91 (2H, br s, NH_2), 7.15 (1H, d, $^3J_{6,5}$ 8.2, H-6), 7.50 (1H, d, $^4J_{3,5}$ 2.28, H-3), 7.53 (1H, dd, $^4J_{5,3}$ 2.28 and $^3J_{5,6}$ 8.2, H-5); nOe experiment: Irradiation of NH_2 (at 3.91 ppm) resulted in a 4.1% enhancement of signal corresponding to H-3 (at 7.49 ppm); δ_C (100 MHz; $CDCl_3$) 17.6 (CH_3), 108.8 (CH), 113.3 (CH), 129.451 (C), 130.7 (CH), 145.3 (C), 147.4 (C); *m/z* (EI) 152 [M^+], 106 [M^+-NO_2]; HRMS: $C_7H_8N_2O_2$ requires 152.0586, found 152.0588 (1.5 ppm dev.); GLC (Method 4) RT 7.00-7.05 mins.

4-AMINO-2-NITROTOLUENE (0.03 g, 16.4%); Yellow coloured needles; R_f 0.33 (DCM); M.Pt 76 °C (from H_2O)(lit.[66] 78 °C); υ_{max} ($CHCl_3$)/cm^{-1} 3017s (CH arom.), 2929s (CH_3), 1525w (N-H), 1350w (N-O); δ_H (400 MHz; $CDCl_3$) 2.46 (3H, s, CH_3), 3.84 (2H, br s, NH_2), 6.80 (1H, dd, $^4J_{5,3}$ 2.5 and $^3J_{5,6}$ 8.2, H-5), 7.08 (1H, d, $^3J_{6,5}$ 8.2, H-6), 7.28 (1H, d, $^4J_{3,5}$ 2.5, H-3); nOe experiment: Irradiation of NH_2 (at 3.84 ppm) resulted in a 2.7% enhancement of the signal corresponding to H-3 (at 7.28 ppm) and a 2.4% enhancement of the signal corresponding to H-5 (at 6.80 ppm); δ_C (100 MHz; $CDCl_3$) 19.5 (CH_3), 110.3 (CH), 119.8 (CH), 122.9 (C), 133.4 (CH), 145.2 (C), 149.6 (C); *m/z* (EI) 152[M^+], 137 [M^+-CH_3], 106 [M^+-NO_2]; HRMS: $C_7H_8N_2O_2$ requires 152.0586, found 152.0584 (1.3 ppm dev.); GLC (Method 4) RT 6.00-6.10 mins.

SYNTHESIS OF 4-NITROISOPHTHALIC ACID (**97**)

Into a three-necked flask fitted with a high speed air stirrer and a water condenser, was added 3-methyl-4-nitrobenzoic acid (**96**) (20 g, 0.1 mol) in water (400 ml). Potassium permanganate (40.2 g, 2.5 eq) was added to the solution which was vigorously stirred. The temperature was raised to 85 °C and the reaction cooled in an ice-bath (an exothermic reaction occured) to maintain a temperature of about 80 °C. Once the

reaction appeared complete the solution was refluxed for 2 h at approx. 90 °C then allowed to cool to 75 °C where it was filtered through a bed of celite. After further cooling the solution was acidified to pH 3 by the addition of conc. HCl which resulted in the product, 4-nitroisophthalic acid (**97**), crystallising as a white crystaline solid (3.71 g, 16 %); M.Pt 246 °C(lit.[70] 245 °C); υ_{max} (Nujol)/cm^{-1} 2666 and 2548br (CO_2H), 1700s (C-O), 1558s (N-O assym. stretch), 1351s (N-O sym. stretch); δ_H (270 MHz; d_6-DMSO) 8.07 (1H, d, $^3J_{6.5}$ 7.8, H-6), 8.27 (1H, d, $^3J_{5.6}$ 7.8, H-5), 8.35 (1H, s, H-3), 13.5 (2H, br, CO_2H); δ_C (63 MHz; d_6-DMSO) 124.41 (CH), 126.92 (CH), 131.04 (C), 133.62 (CH), 134.58 (C), 151.19 (C), 165.00 (CO_2H),165.29 (CO_2H); *m/z* (-ve ion spray) 210 $[M-H]^-$, 166 [210-CO_2]; HRMS: $C_8H_5NO_6$ requires 211.0117, found 211.0108 (4.4 ppm dev.).

SYNTHESIS OF 4-NITROISOPHTHALONITRILE (**98**)

Into a three-necked flask fitted with an air condenser,was placed 4-nitroisophthalic acid (**97**) (2 g, 9.5 mmol), p-toluenesulphonamide (3.5 g, 20.7 mmol) and phosphorous pentachloride (8.6 g, 41.3 mmol). The mixture was stirred, at 200 °C for 30 mins. then cooled to ambient temperature and the air condenser replaced with a water condenser. $CHCl_3$ (20 ml) was added and the solutionrefluxed for 30 mins. The reaction was basified to pH 8 with 10 % $NaHCO_3$, the aqueous phase removed and the organic phase dried over $MgSO_4$. Solvent was removed under reduced pressure and crude product purified by flash chromatography on silica gel with $CHCl_3$:EtOAc (9:1) as eluent to give 4-nitroisophthalonitrile (**98**) as a yellow/brown coloured powder (93 mg, 58 %); R_f 0.70 (1:1, EtOAc:Petrol); M.Pt 125 °C (from EtOAc/petrol)(lit.[70] 125 °C); (Found: C,55.4; H, 1.6; N, 24.1. $C_8H_3N_3O_2$ requires C, 55.5; H, 1.7; N, 24.3%); υ_{max} ($CHCl_3$)/cm^{-1} 2990m (CH arom), 2210w (C≡N), 1525s (N-O), 1360s (N-O); δ_H (270 MHz; $CDCl_3$) 8.12 (1H, d, $^3J_{5.6}$ 9.36, H-5), 8.24 (1H, s, H-3), 8.49 (1H, d, $^3J_{6.5}$ 9.36, H-6); δ_C (63 MHz; $CDCl_3$) 109.54 (C), 112.76 (CN), 114.70 (CN), 118.69 (C), 126.39 (CH), 136.96 (CH), 138.59 (CH),150.12 (C); *m/z* (EI) 173 [M^+], 143 [173-NO], 127 [173-NO_2]; HRMS: $C_8H_3N_3O_2$ requires 173.022526, found 173.02275 (1.4 ppm dev.).

SYNTHESIS OF 3-NITROTEREPHTHALOMIDE (**99**)

Thionyl chloride (10 ml, 137 mmol, 10 eq.) was added to a flask containing nitroterephthalic acid (3 g, 14.2 mmol). The solution was refluxed under an inert atmosphere for 30 mins, the reaction was monitored by TLC (after adding a drop of the

reaction mixture to methanol to make the ester). On completion of the reaction distillation removed any residual thionyl chloride. The acid chloride was allowed to cool, ammonia (0.88, 35%, 30 ml) was added dropwise until gas evolution ceased. The yellow solution was left to stir overnight, filtered and washed with water. Recrystalisation from hot water gave nitroterephthalamide (**99**) as a cream coloured solid in 64.5 % yield; R_f 0.1 (EtOAc); M.Pt 260 °C (from H_2O) (Lit[71]262 °C); (Found: C, 46.0; H, 3.7; N, 19.4. $C_8H_7N_3O_4$ requires C, 45.9; H, 3.4; N, 20.1%); υ_{max} (Nujol)/cm^{-1} 2900-2800s (N-H), 1662m (C=O), 1615m (N-H), 1533m (N-O), 1464m (N-H), 1378m (N-H), 1349m (N-O); δ_H (200 MHz; d_6DMSO) 7.68 (1H, d, $^3J_{3,4}$ 8.0, H-3), 7.73 (1H, br, NH), 7.77 (1H, br, NH), 8.18 (1H, d, $^3J_{4,3}$ 8.0, H-4), 8.23 (1H, br, NH), 8.34 (1H, br, NH), 8.41 (1H, s, H-6); δ_C (50 MHz; d_6-DMSO) 122.25 (CH), 128.48 (CH), 131.55 (CH), 134.13 (C), 135.33 (C), 146.18 (C), 164.85 (C=O), 166.32 (C=O); *m/z* (EI) 209 [M^+], 193 [M^+-NH_2], 119 [M^+-$CONH_2$-NO_2]; HRMS: $C_8H_7N_3O_4$ requires 209.0436 found 209.0426 (4.9 ppm dev.); HPLC (Method 3) RT 3.7 mins

SYNTHESIS OF NITROTEREPHTHALONITRILE (**100**)

Nitroterephthalamide (**99**) (0.76 g, 3.63 mmol), TEA (1.9 g, 18.81 mmol) and triphenylphosphine (3 g, 11.44 mmol) were added to a flask containing DCM (40 ml) and heated to reflux under an inert atmosphere. A solution of triphosgene (1.14 g, 3.84 mmol) in DCM (16 ml) was then added slowly to the mixture and left to reflux overnight. The reaction mixture was left to cool, filtered and washed with DCM. The filtrate was reduced under vacuum. Chromatography of the crude product on SiO_2 (eluent DCM) gave nitroterephthalonitrile (**100**) as a yellow coloured solid in 71.5% yield; R_f 0.5 (eluent DCM); M.Pt 123 °C(lit.[72]; (Found: C, 55.4; H, 2.0; N, 23.6. $C_8H_3N_3O_2$ requires C, 55.5; H, 1.75; N, 24.3 %); δ_H (200 MHz; d_6-DMSO) 8.39 (1H, d, $^3J_{3,4}$ 8.0, H-3), 8.47 (1H, dd, $^4J_{4,6}$ 1.5 and $^3J_{4,3}$ 8.0, H-4), 8.95 (1H, d, $^4J_{6,4}$ 1.5, H-6); δ_C (50 MHz; d_6-DMSO) 110.43 (C), 111.75 (C), 115.36 (C), 115.88 (C), 128.76 (CH), 135.88 (CH), 137.54 (CH),147.98 (C); *m/z* (EI) 173 [M^+], 127 [M^+-NO_2], 100 [M^+-NO_2-CN]; HRMS: $C_8H_3N_3O_2$ requires 173.0225 found 173.0220 (3.0 ppm dev.); HPLC (Method 3) RT 9.0 mins

BAKER'S YEAST REDUCTION OF NITROTEREPHTHALONITRILE (**100**)

4-AMIDO-3-AMINOBENZONITRILE (**101**) Yield 83%; Yellow coloured solid; M.Pt 204 °C (Lit[73] 203-204 °C); δ_H (200 MHz; d_6-DMSO) 3.35 (2H, br, NH_2), 6.83

(1H, dd, $^4J_{4,6}$ 1.5 and $^3J_{4,3}$ 8.5, H-4), 7.04 (1H, d, $^4J_{6,4}$ 1.5, H-6), 7.37 (1H, br, N-H), 7.63 (1H, d, $^3J_{3,4}$ 8.5, H-3), 7.94 (1H, br, N-H); δ_C (63 MHz; d_6-DMSO) 114.02 (C), 116.84 (CH), 117.51 (C), 118.91 (C), 119.44 (CH), 129.98 (CH), 150.15 (C),170.11 (C=O); *m/z* (EI) 161 [M^+], 117 [M^+-$CONH_2$]; HRMS: $C_8H_7N_3O$ requires 161.0589 found 161.0588 (0.51 ppm dev.)

All the following biotransformations followed the general procedure B previously given.

BAKER'S YEAST REDUCTION OF 2-NITROBENZONITRILE (**88**)

2-AMINOBENZAMIDE (**89**) (79 %); Cream/brown coloured powder; R_f 0.20 (1:1, EtOAC:petrol); M.Pt 100 °C (from EtOAc /petrol)(lit.[74] 103-106 °C); υ_{max} (Nujol)/cm^{-1} 3409, 3321 and 3192m (N-H), 1627, 1608, and 1585m (N-H); δ_H (270 MHz; $CDCl_3$) 5.67 (2H, br, NH_2), 5.89 (2H, br, $CONH_2$), 6.65 (2H, m, H-3 and H-5), 7.22 (1H, dd, $^3J_{4,3}$ 7.5 and $^3J_{4,5}$ 7.5, H-4),7.36 (1H, d, $^3J_{6,5}$ 7.5, H-6); δ_C (63 MHz; $CDCl_3$) 113.88 (C), 116.27 (CH), 117.29 (CH), 127.88 (CH), 132.85 (CH), 149.28 (C),171.61 (C=O); *m/z* (E.I.) 136 [M^+], 119 [M^+-NH_2], 92 [M^+-NH_3CO]; HRMS: $C_7H_8N_2O$ requires 136.0637, found 136.0626 (7.7 ppm dev.); HPLC (Method 1) RT 6.3 mins.

BAKER'S YEAST REDUCTION OF 2-NITRO-4-TRIFLUOROMETHYLBENZONITRILE (**90**)

2-AMINO-4-TRIFLUORMETHYLBENZAMIDE (**91**) (84 %); Cream/brown coloured powder; R_f 0.30 (1:1, EtOAc:petrol); M.Pt 148 °C (from EtOAc/petrol)(lit.[75] 151-152 °C); υ_{max} (Nujol)/cm^{-1} 3516-3182m (N-H), 1659-1557m (N-H); δ_H (270 MHz; $CDCl_3$) 5.84 (4H, br, NH_2), 6.88 (1H, d, $^3J_{5,6}$ 8.9, H-5), 6.92 (1H, s, H-3), 7.32 (1H, d, $^3J_{6,5}$ 8.9, H-6); δ_C (63 MHz; $CDCl_3$) 112.3 (CH), 113.9 (CH), 116.13 (C), 128.54 (CH), 134.72 (C), 149.27 (C),170.34 (C=O); *m/z* (EI) 204 [M^+], 187 [M^+-NH_3], 160 [M^+-$CONH_2$]; HRMS: $C_8H_7F_3N_2O$ requires 204.0510, found 204.0497 (6.5 ppm dev.); HPLC (Method 2) RT 5.0 mins.

BAKER'S YEAST REDUCTION OF 5-CHLORO-2-NITROBENZONITRILE (**92**)

2-AMINO-5-CHLOROBENZAMIDE (**93**) (69 %); Cream coloured solid: R_f 0.30 (1:1, EtOAc:petrol); M.Pt 168 °C (from EtOAC/petrol)(lit.[76] 169-171 °C): υ_{max}

(Nujol)/cm^{-1} 3397, 3354, 3296, and 3167w (N-H), 1680, 1618, 1585, and 1551m (N-H); δ_H (270 MHz; $CDCl_3$) 6.62 (4H, br, NH_2), 6.7 (1H, d, $^3J_{3,4}$ 9.4, H-3), 7.15 (1H, dd, $^3J_{4,3}$ 9.4 and $^4J_{4,6}$ 1.9, H-4), 7.58 (1H, d, $^4J_{6,4}$ 1.9, H-6); δ_C (63 MHz; d_6-DMSO) 114.58 (C), 117.61 (C), 118.20 (CH), 128.07 (CH), 131.77 (CH), 149.21 (C), 170.17 (C=O); *m/z* (EI) 170 [M^+], 153 [M^+-NH_3], 126 [M^+-$CONH_2$], 125 [M^+-Cl]; HRMS: $C_7H_7ClN_2O$ requires 170.0247, found 170.0251 (2.7 ppm dev.); HPLC (Method 2) RT 2.8 mins.

BAKER'S YEAST REDUCTION OF 3-NITROPHTHALONITRILE (**102**)

2-AMIDO-3-AMINOBENZONITRILE (**103**) (74.5 %); Yellow coloured solid; R_f 0.14 (1:1, EtOAc:petrol); M.Pt 204 °C; υ_{max} 3504-3146w (N-H), 2234s (C≡N), 1702-1555m (N-H); δ_H (300 MHz; $CDCl_3$) 5.61 [2H, br, NH_2], 6.94 (1H, d, $^3J_{3,4}$ 8.3, H-3), 6.98 (1H, d, $^3J_{5,4}$ 8.3, H-5), 7.22 (1H, dd, $^3J_{4,3}$ 8.3 and $^3J_{4,5}$ 8.3, H-4), 7.7 (1H, br, N-H),7.95 (1H, br, N-H); δ_C (63 MHz; d_6-DMSO) 110.1 (C≡N), 118.5 (C), 120.1 (CH), 120.5 (CH), 122.4 (C), 130.4 (CH), 147.1 (C), 167.8 (C=O); *m/z* (EI) 161 [M^+], 144 [M^+-NH_3], 117 [M^+-$CONH_2$]; HRMS: $C_8H_7N_3O$ requires 161.0589, found 161.0583 (3.5 ppm dev.); HPLC (Method 3) RT 3.2 mins.

4. RESULTS AND DISCUSSION: PROTEIN PURIFICATION

4.1 GENERAL

PROTEIN PURIFICATION- In order to obtain any information concerning the mechanism of action of an enzyme it is important to be able to isolate the protein in pure form. Once pure, the amino acid sequence of the protein can be established, leading to the primary structure of the protein.

The purification of a protein will typically involve the use of ammonium sulphate in a technique known as 'salting-out', followed by chromatographic techniques such as ion exchange, gel filtration, hydrophobic interaction and affinity chromatography.

SALTING-OUT- This process is dependent on the hydrophobic regions found on the surface of the protein which create an ordered aqueous environment within the vicinity of these regions. When salt ions, usually ammonium sulphate, are added to the system the water is stripped from the hydrophobic regions in order to solvate the salt ions. These exposed hydrophobic regions on one protein can interact with those on another resulting in aggregation. Thus protein with larger or more hydrophobic regions on the surface will aggregate faster. This technique is often used at an early stage in the purification of a protein to fractionate the crude extract prior to chromatography.

ION-EXCHANGE CHROMATOGRAPHY- Ion-exchange is probably the most frequently used chromatographic technique for the separation and purification of proteins. This technique separates proteins on the basis of their charge. Once the ion-exchange resin has been equilibrated in terms of pH and ionic strength, the sample can be applied. Proteins with the appropriate charge bind reversibly to the matrix and those that remain unbound can be washed free from the column. By changing the elution conditions, the bound protein can be removed from the column, typically using an increasing ionic gradient. Proteins are released from the column in the order of their binding strength, the most weakly bound is eluted first. A pH gradient can be employed instead of an ionic gradient, which would alter the charges on the protein, thus leading to altered binding efficiency.

The insoluble matrix that is used to pack the columns has covalently bound charged groups on its surface; either positively charged (anion-exchangers), or

negatively charged (cation-exchangers). Typical ion-exchange resins and the functional groups can be seen in **Table 4.1**. Sepharose (cross-linked agarose), sephadex (cross-linked dextran) and sephacel (cross-linked cellulose) are common matrices for ion-exchangers.

Anion-Exchange	Functional Group
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$
Quaternary aminoethyl (QAE)	$-O-CH_2-CH_2-N^+(C_2H_5)_2-CH_2-CHOH-CH_3$
Quaternary ammonium (Q)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$
Cation-Exchange	**Functional Group**
Carboxymethyl (CM)	$-O-CH_2-COO^-$
Sulphopropyl (SP)	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$
Methyl Sulphonate (S)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^{--}$

Table 4.1 Typical ion-exchange matrices.

GEL FILTRATION CHROMATOGRAPHY (GF)- This technique separates proteins according to their size as they pass through a column packed with a gel. The gel contains pores and is available in a range of accurately controlled pore size. Similar to the matrices in ion-exchange, cross-linked polymers like sephadex are used to prepare the gels. The column is packed with the gel through which the liquid phase is eluted. Having loaded the sample on to the column, small proteins can diffuse into the gel pores from the solution. The proteins that are larger in size than the pores in the gel are washed from the column. Continuous elution brings the larger proteins through the column first with the smallest being the most delayed. By running proteins of a known molecular weight, it is possible to standardise the column and thus ascertain the approximate size of the native protein in question. Gel filtration chromatography is also an efficient way to de-salt a sample.

Sephacryl, superdex, superose, sephadex and sepharose are typical matrices for use in gel filtration chromatography.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)- The utilisation of an immobilised ligand attached to a polymer matrix forms the basis of hydrophobic interaction chromatography. Protein samples are applied in an eluent containing a high salt concentration, usually ammonium sulphate. By the same mechanism that is used to describe the salting-out process, the ordered water molecules surrounding the matrix bound ligand and the hydrophobic regions on the protein is stripped away leaving the

two to interact. Elution of the protein is achieved by decreasing the concentration of the salt in the eluent.

In general, straight chain alkyl ligands and aryl ligands are employed in the technique, for example butyl, octyl and phenyl ligands. Phenyl sepharose, butyl sepharose and phenyl superose are typical hydrophobic interaction column packings.

AFFINITY CHROMATOGRAPHY- Affinity chromatography enables the purification of almost any protein on the basis of its biological function. The protein to be purified is adsorbed specifically and reversibly to a complimentary ligand immobilised on a matrix, unwanted proteins can be washed free from the column. Bound proteins can be eluted by changing the experimental conditions to those which favour desorption.

HiTrap columns are commonly used for general purpose affinity chromatography. HiTrap Blue for example, specifically binds nucleotide cofactor requiring proteins. There are a large number of group specific adsorbents available but information about the protein to be purified is necessary in order to decide which adsorbent to use, alternatively a trial and error procedure would be required.

Ion-exchange, gel filtration, hydrophobic interaction and affinity chromatography are all commonly used purification procedures. Reverse phase HPLC is also often used but generally leads to inactivation of the protein.

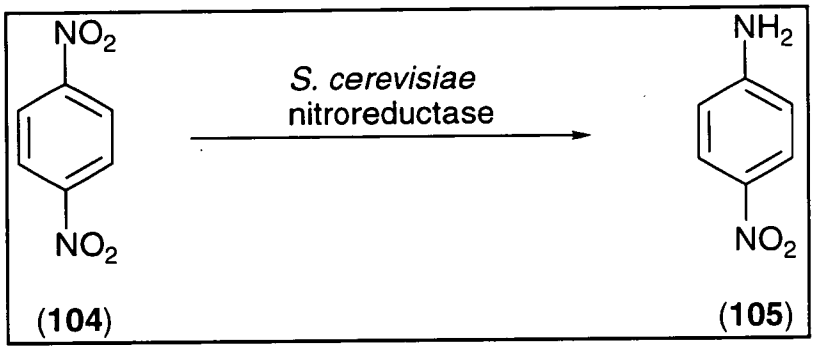
4.2 EXPERIMENTAL PROCEDURES

REAGENTS- All chemicals purchased were of biological or HPLC grade unless otherwise specified. YPD broth, potassium phosphate, phenylmethylsulphonyl fluoride (PMSF), dithiothreitol (DTT), ammonium sulphate, Tris base, Tris-HCl, bovine serum albumin (BSA), SDS-PAGE low molecular weight standards, and dialysis tubing (12,000 MW exclusion limit) were purchased from Sigma Chemical Co. (Dorset, UK). NADPH was purchased from Fluka (Dorset, UK). 1,4-Dinitrobenzene was purchased from Aldrich Chemical Co. (Dorset, UK). EasyGel was purchased from Scotlab.

CHROMATOGRAPHIC MATERIAL- All chromatography was performed at 4 °C using the Äkta Explorer-10 (Pharmacia Biotech, St. Albans, UK) unless otherwise stated. Elution of protein was measured by the absorbance at 280 nm When the Äkta was not being used for a purification step the CECIL 1000 Series UV/Vis spectrophotometer was used to measure absorbance. Mono Q® HR5/5 (1 ml), Phenyl

superose® (1 ml), Resource™ Q™ (1 ml), Superose® 12, Sephacryl® S-200 HR, Q-Sepharose® Fast Flow, XK 26/20 and XK 16/70 columns were all purchased from Pharmacia Biotech (St. Albans, UK).

ENZYME AND PROTEIN ASSAYS- The reduction of 1, 4-dinitrobenzene (**104**) to 4-nitroaniline (**105**) was used as a standard assay throughout the purification of the nitroreductases (**Scheme 4.1**).



Scheme 4.1

The assay mixture contained 50 mM 1,4-dinitrobenzene (in 5 µl DMSO), 1 mM NADPH in a final volume of 800 µl of a buffer composed of 100 mM potassium phosphate (pH 6 or 7). 200 µl of the protein solution to be assayed was added to the buffer mixture. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmole of 4-nitroaniline per minute at 30 °C. 4-Nitroaniline has an extinction coefficient (ε) of 15,090 and λmax of 370 nm. The assay was monitored at 410 nm as the absorbance of the product overlaps with the absorbance of the cofactor NADPH. The formation of the product was monitored using a Hewlett Packard 8453 UV/Vis spectrophotometer. In order to ascertain the total amount of protein present in each solution the Bradfords Assay[77] was employed. This assay is based on the fact that a shift in A_{max} of an acidic solution of coomassie blue G-250 from 465 nm to 595 nm occurs when the dye binds to proteins present. Protein concentrations were determined using bovine serum albumin as a protein standard.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) was carried out as described by Laemelli[78] using a 4 % stacking gel and either a 12, 15 or 18 % separating gel. Proteins were visualised by staining with Coomassie blue R-250.

PREPARATION OF A SUSTAINABLE COLONY OF *S. CEREVISIAE*- Lyophilised *S. cerevisiae* (Sigma) was hydrated in deionised water for 1 h, then plated out onto YPD agar (Sigma) and allowed to grow at 30 °C for 24 h. A single colony of the *S. cerevisiae* was picked off the plate and used to inoculate 100 ml of sterile YPD broth in a shake flask. The inoculum was grown at 30 °C and 200 rpm for 24 h. A sample of this inoculum was grown on a second agar plate as before and a single colony was again picked off the plate and used to produce a stock culture in the form of YPD agar slopes.

CELL GROWTH- 500 ml of sterile YPD media was inoculated with *S. cerevisiae* from a stock culture and allowed to grow at 30 °C and 200 rpm for 24 h. This 500 ml culture was used to inoculate 7-10 L of sterile media in a 10 L BioFlow 1000 fermenter (New Brunswick Scientific, Hatfield, UK). Mixing was achieved at 200 rpm by two six-bladed turbines. The contents of the vessel were maintained at 30 °C by circulation of cold water through a metal probe. Ingoing air at 2.5-3.0 L/min was sterilised by passage through a filter. After 24 h cells were harvested by centrifugation (Sorvall RC 5C) at 8,000 rpm for 15 min at 4 °C. 10 L of cells grown in this fashion typically yielded 160 g of wet cell paste. The growth curve of a 7 L inoculation is shown in **Figure 4.1**.

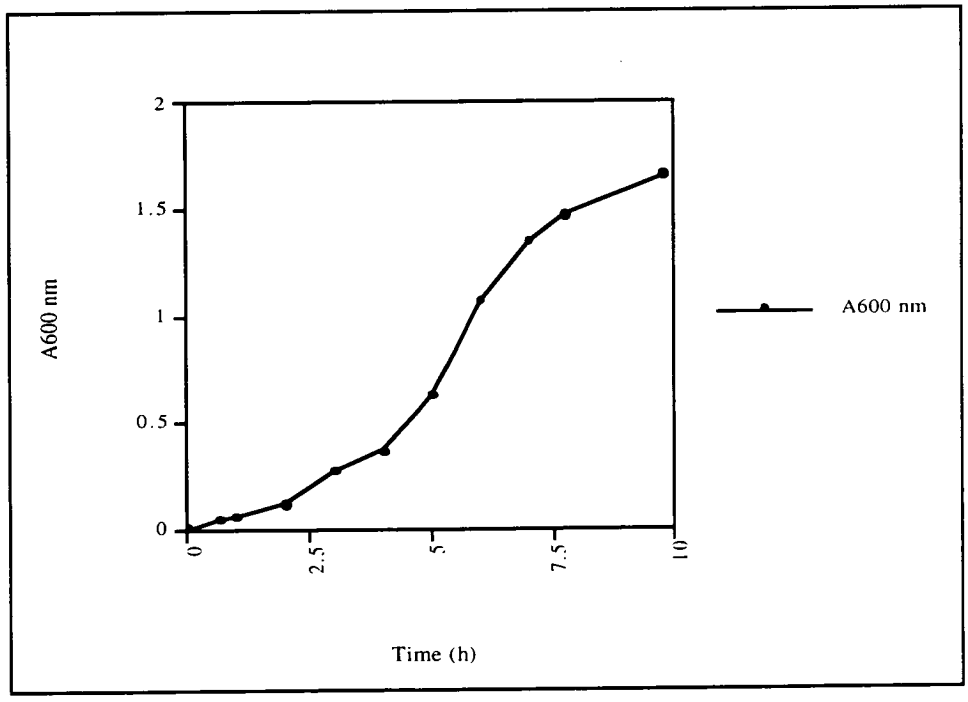


Figure 4.1 A growth curve for a 7 L fermentation.

CELL LYSIS (LYTICASE)- The harvested cells from a 200 ml growth were washed in ice-cold phosphate buffered saline and collected by centrifugation at 3000 rpm for 5 min at 0 °C. The cell paste was resuspended in a volume of stabilising buffer A [1M sorbitol, 10 mM $MgCl_2$, 50 mM potassium phosphate (pH 7.8), 100 µg/ml PMSF and 2 mM DTT] equal to that of the original culture and incubated at 30 °C for 10 min, the cell pellet was collected by centrifugation at 3000 rpm for 10 min at 0 °C. The supernatant was discarded and the pellet resuspended in a volume of stabilising buffer B [1M sorbitol, 10 mM $MgCl_2$, 25 mM sodium succinate (pH 5.5), 25 mM potassium phosphate (pH 7.8), 100 µg/ml PMSF and 2 mM DTT] equal to that of the original culture. This suspension was incubated at 30 °C for 2 min, Lyticase (12.5 U/ml suspension) was added to the suspension which was incubated at 30 °C for 45-60 min. The resulting protoplasts were harvested by centrifugation at 500 g for 30 min and resuspended in lysis buffer (0.01x original culture volume). The suspension was stored at 0 °C for 30 min and then harvested by centrifugation at 3000 rpm for 30 min at 0 °C, the supernatant was retained as the cell free extract.

Nitroreductase activity was apparent until the protoplasts were suspended in the lysis buffer. Thereafter the enzyme activity reduced dramatically.

CELL LYSIS (NEBULISATION)- The harvested cells were resuspended in approximately 400 ml of 100 mM potassium phosphate buffer, pH 6 or 7, containing 100 µg/ml PMSF and 2 mM DTT. The cells were disrupted by three passages through a BioNeb™ cell disruption system (Glas-Col, UK) using helium at a pressure of 200 psi and a flowrate of 15-20 L/min. The cell debris was removed by centrifugation at 10,000 rpm for 20 min at 4 °C. The supernatant was decanted and retained as the cell free extract and the pellet discarded.

4.3 PROTEIN PURIFICATION

4.3.1 METHOD 1

(Step 1) Protamine Sulphate Precipitation- Nucleic acids were removed from the crude extract of a 7 L fermentation by the addition of a 1% protamine sulphate solution used at a concentration of 1 part in 10 of cell extract, stirred at 4 °C for 1 h. The precipitate was removed by centrifugation at 18,000 rpm for 20 min at 4 °C.

(Step 2) Ammonium Sulphate Precipitation- The extract was taken to 80 % saturation with ammonium sulphate with stirring at 4 °C for 1 h and the precipitated protein collected by centrifugation at 18,000 rpm for 20 min at 4 °C. The pellet was resuspended in the minimum amount of 100 mM potassium phosphate buffer, pH 7, containing 100 µg/ml PMSF and 2 mM DTT and dialysed against 1 L of the same buffer in order to remove the ammonium sulphate salts.

(Step 3) Q-Sepharose FF Anion Exchange Chromatography - The protein extract was applied to a 50 ml (XK 26/20) column of Q-Sepharose FF anion exchange resin which had been previously equilibrated with 100 mM phosphate buffer pH 7.0 containing PMSF and DTT. Protein was eluted from the column using a gradient of 0-600 mM KCl over 8.5 column volumes (**Figure 4.2**) 5 ml fractions were collected and assayed for nitroaryl reductase activity (**Figure 4.3**) and the active fractions were pooled and concentrated in Centriprep-10 tubes (Amicon) to 1.5 ml.

(Step 4) Superose 12 Gel Filtration Chromatography - 200 µl samples of the concentrated protein from the previous anion exchange step were applied to a Superose 12 gel filtration column that had been equilibrated with 100 mM potassium phosphate buffer, pH 7. The protein was eluted with the same buffer at a rate of 1ml/min (**Figure 4.4**) and the fractions containing protein were assayed for enzyme activity (**Figure 4.5**).

After all of the protein mixture had been passed through the gel filtration column (7 runs) the active fractions were pooled and again concentrated in Centriprep-10 tubes. This step didn't prove to be very beneficial for purifying the protein.

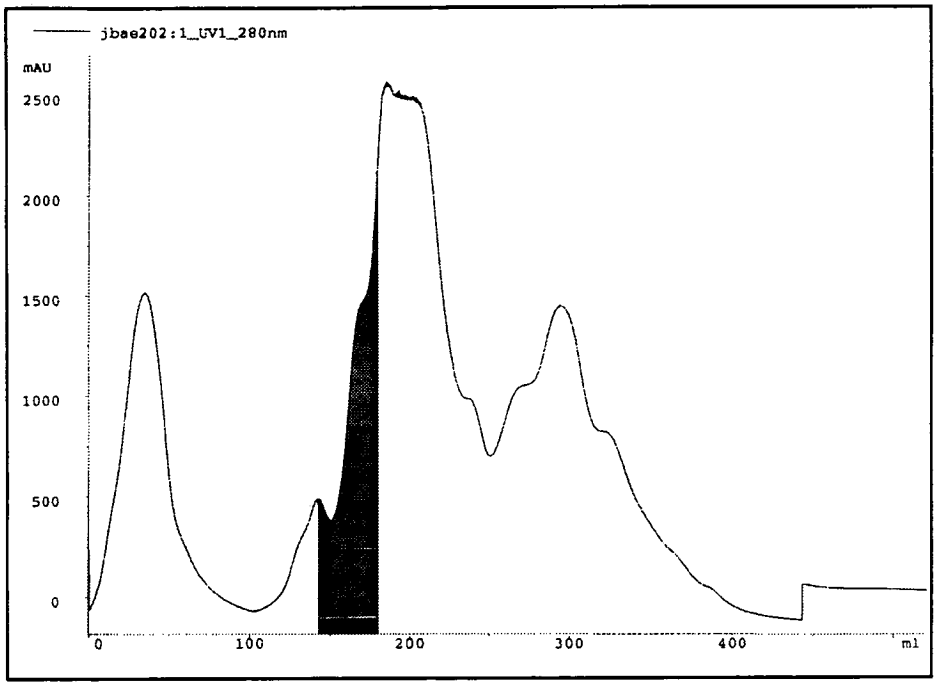


Figure 4.2 Q-Sepharose FF anion exchange chromatography. Shaded area indicates activity.

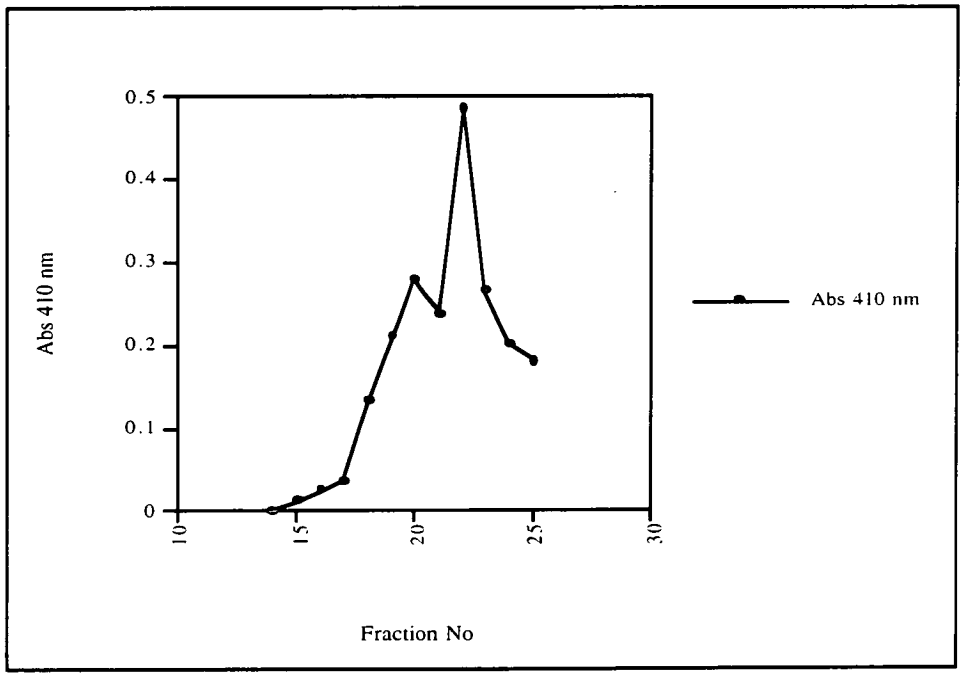


Figure 4.3 Enzyme activity after Q-Sepharose FF (AEx) column.

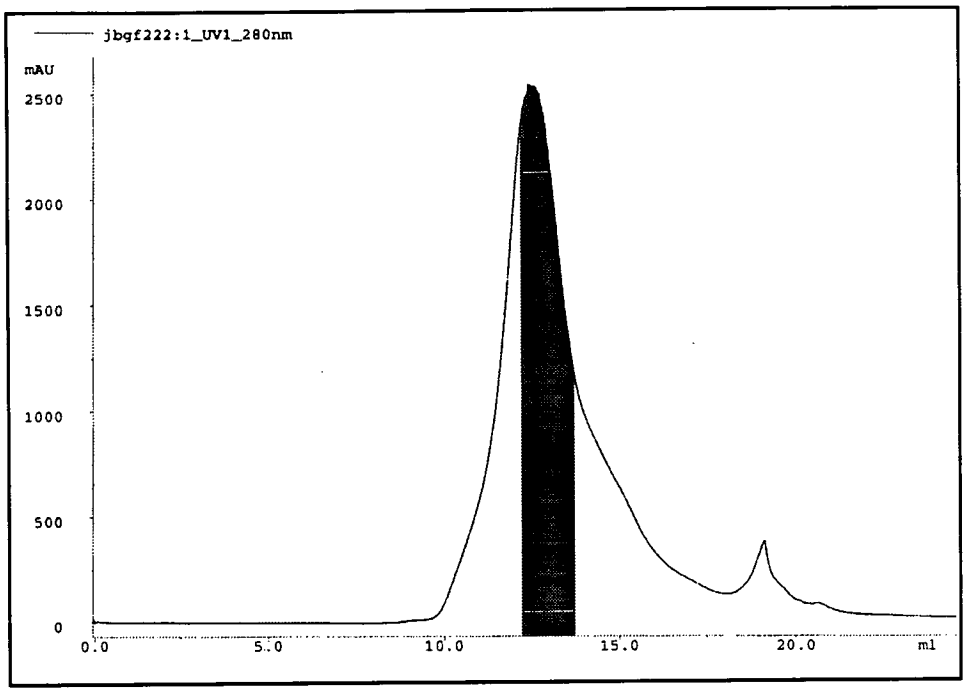


Figure 4.4 Superose 12 gel filtration chromatography. Shaded area indicates activity.

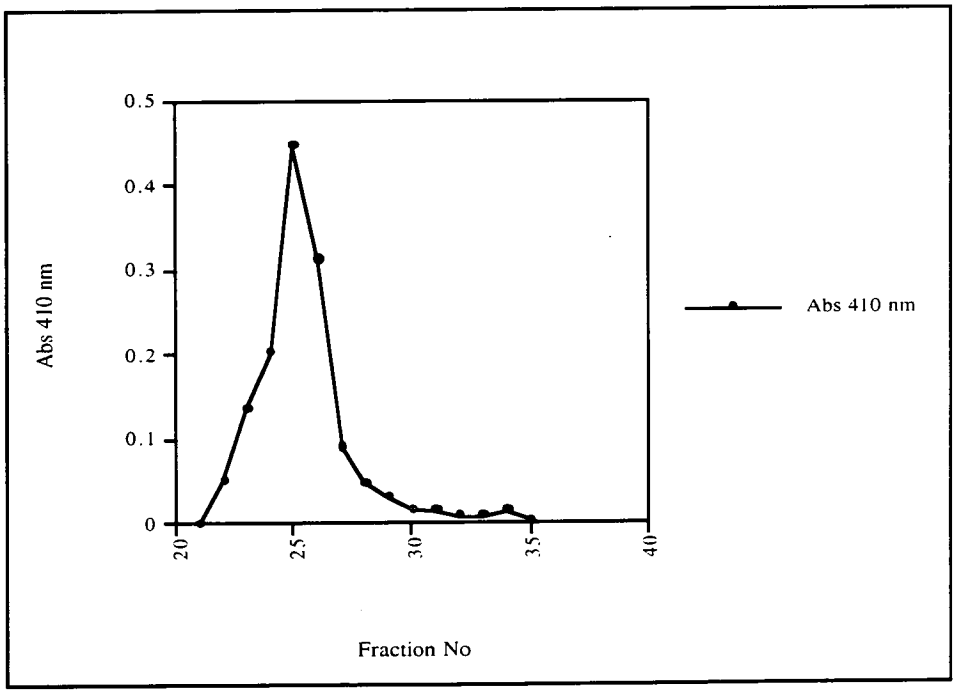


Figure 4.5 Enzyme activity after Superose 12 (GF) column.

(Step 6) Phenyl Superose Hydrophobic Interaction Chromatography - The concentrated protein from the gel filtration step was taken to a concentration of 2 M ammonium sulphate and applied to a Phenyl Superose hydrophobic interaction column. The column had been previously equilibrated with 100 mM potassium phosphate

buffer containing 2 M ammonium sulphate. Unbound protein was washed from the column and bound protein was eluted over 20 column volumes (**Figure 4.6**), 2 ml fractions were collected and assayed for activity (**Figure 4.7**).

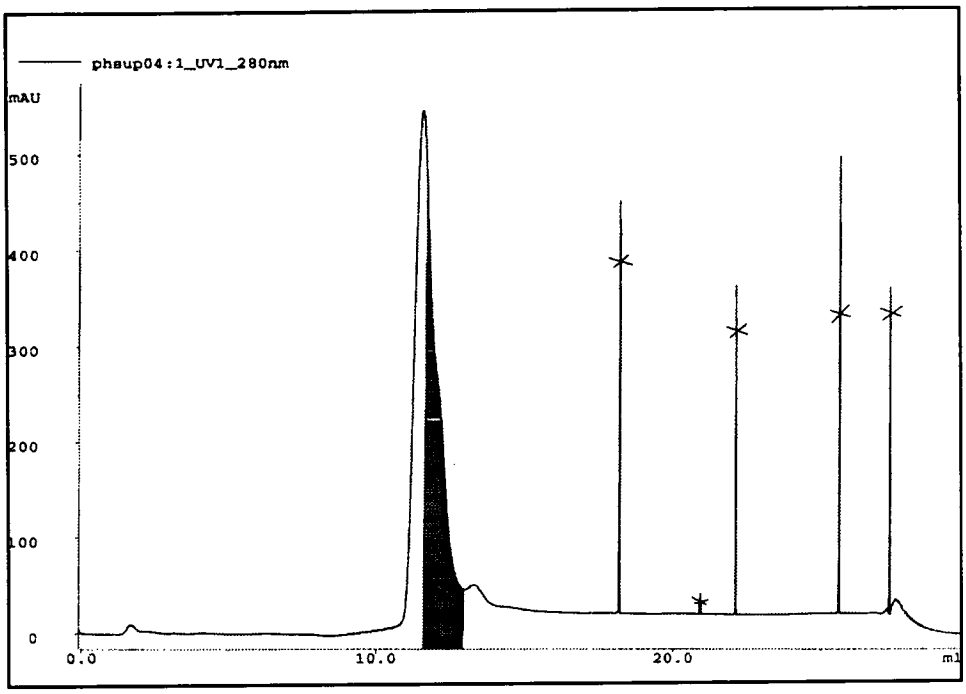


Figure 4.6 Phenyl Superose hydrophobic interaction chromatography. Shaded area indicates activity. X indicate peaks produced by air in the system.

Again this step didn't result in isolation of a single protein so the active fractions were pooled and concentrated.

(Step 7) HiTrap Blue Affinity Chromatography - The concentrated protein from the previous step was applied to a pre-equilibrated HiTrap blue affinity column but failed to bind. The unbound protein was concentrated and taken onto the next step.

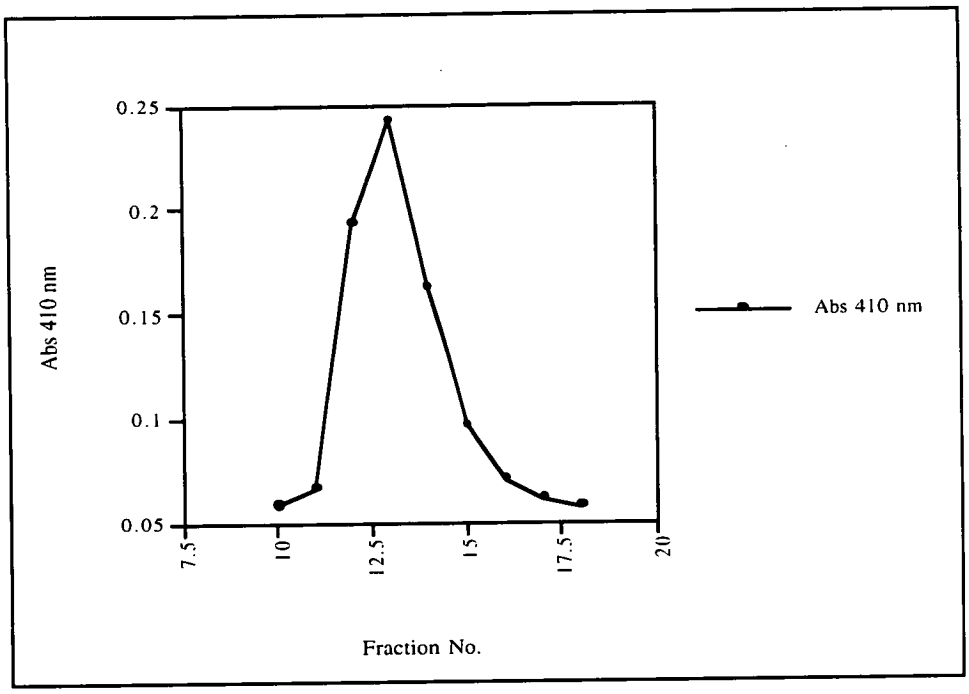


Figure 4.7 Enzyme activity after Phenyl Superose (HIC) column.

(Step 8) Resource Q Anion Exchange Chromatography - The concentrated protein that failed to bind to the affinity column was applied to a Resource Q anion exchange column that had been equilibrated with 100 mM potassium phosphate buffer, pH 7, any unbound protein being washed off the column. Bound protein was eluted with an increasing potassium chloride gradient over 20 column volumes (**Figure 4.8**) and 2 ml fractions were collected. The fractions were assayed for enzyme activity and an 18 % gel run on the active fractions, a comparison could then be made at each stage of the purification (**Figure 4.9**).

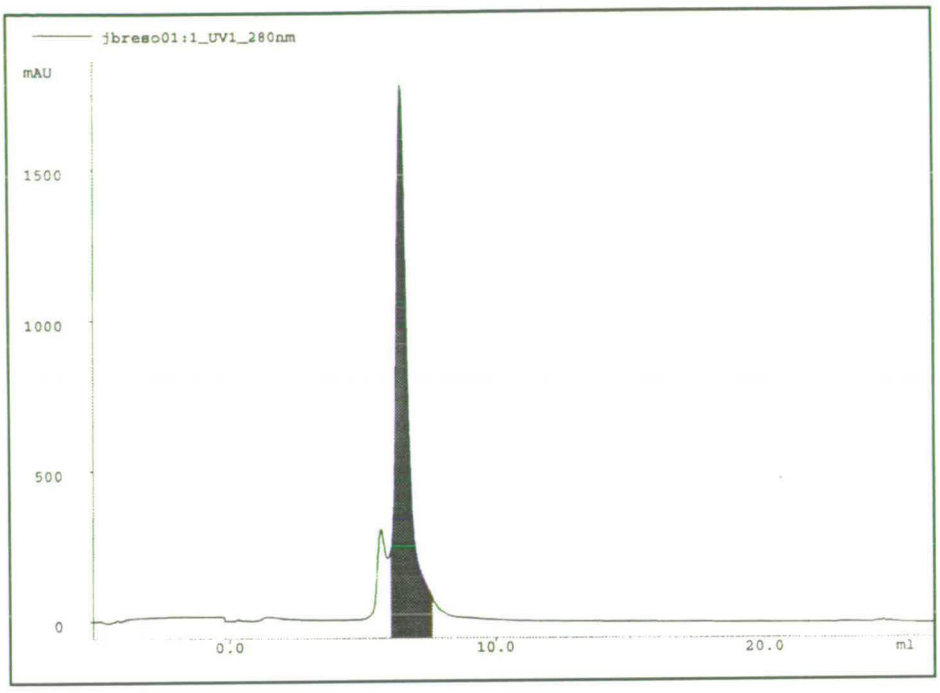


Figure 4.8 Resource Q anion exchange chromatography. Shaded area indicates activity.

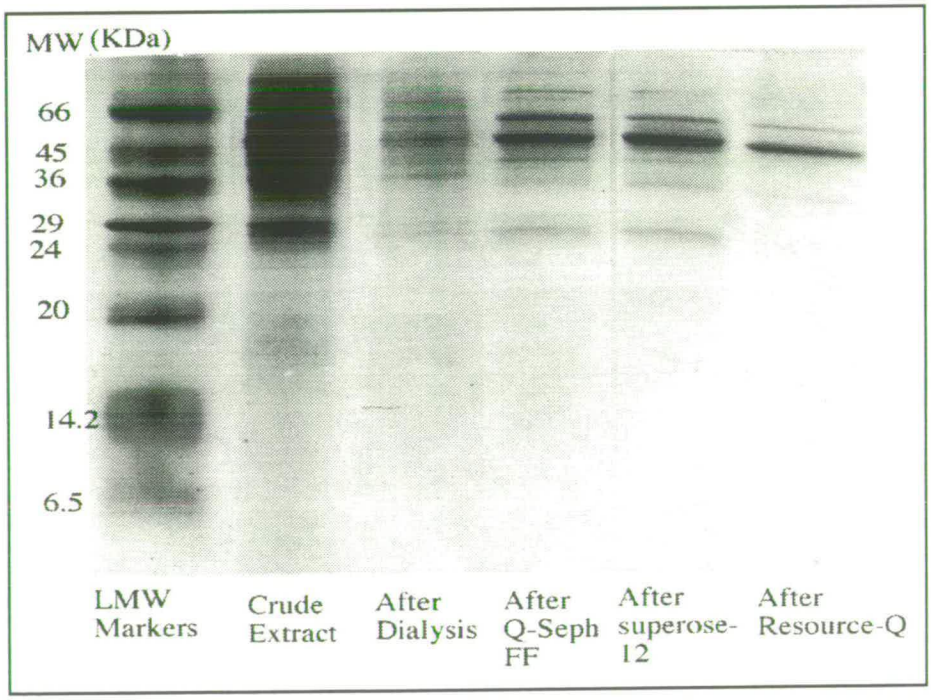


Figure 4.9 SDS-PAGE (18 %) analysis of the purification of nitroaryl reductase for *S. cerevisiae*. Four major proteins are present at approximately 66, 55, 45 and 36 KDa.

ASSAY RESULTS

Kinetics results from the crude extract:

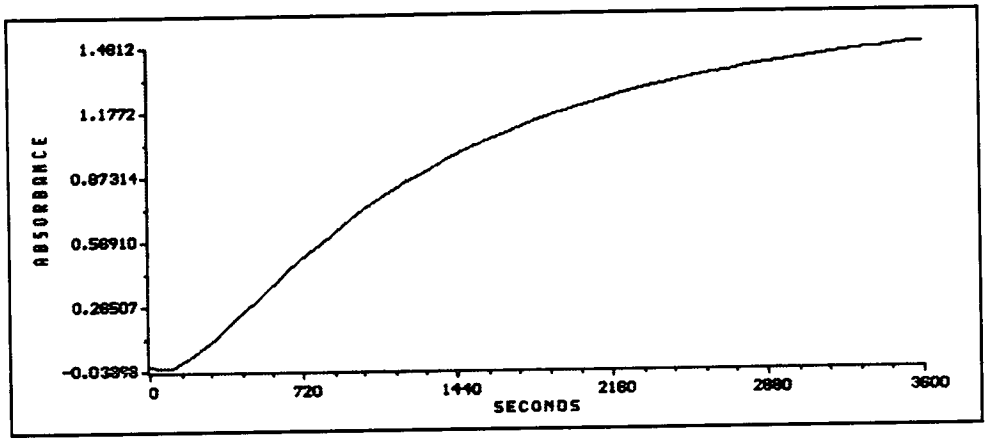


Figure 4.10 Timed based assay of the crude extract indicating the production of 4-nitroaniline at $A_{410\ nm}$

Substrate concentration = 298 µM

Rate (v) : $\Delta A = 0.152 / 15{,}090 = 1.01 \times 10^{-8}$ mol/ml

1.01×10^{-8} mol/ml / 192 s = 5.25×10^{-11} mol/s

$= 5.25 \times 10^{-5}$ µmol/s

$= 3.15 \times 10^{-3}$ µmol/min (in 200 µl sample)

$= 15.75 \times 10^{-3}$ U/ml (in 1 ml)

$1 / v = 1.91 \times 10^{11}$ s/mol

Kinetics results from Q-Sepharose FF anion exchange column:

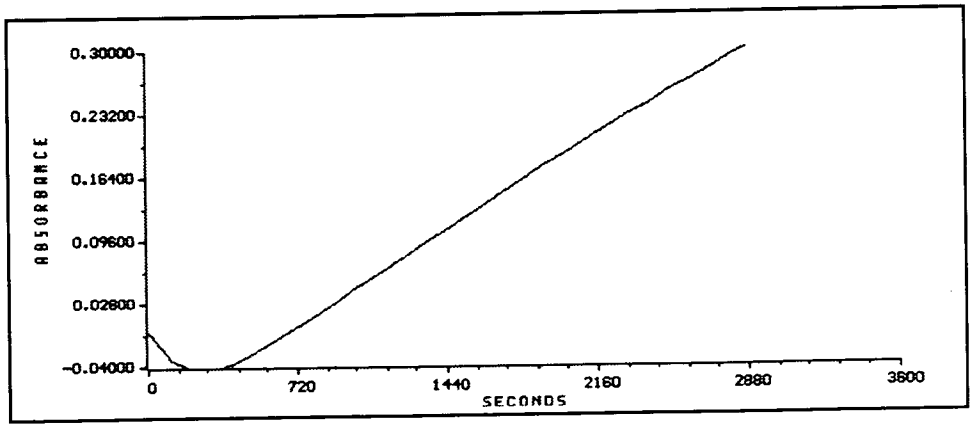


Figure 4.11 Timed based assay after Q-sepharose FF anion exchange column indicating the production of 4-nitroaniline at $A_{410\,nm}$

Substrate concentration = 298 µM

Rate (v) : $\Delta A = 0.034 / 15{,}090 = 2.25 \times 10^{-9}$ mol^{-1}

2.25×10^{-9} mol/ml / 252 s = 8.94×10^{-12} mol/s

= 8.94×10^{-6} µmol/s

= 5.36×10^{-4} µmol/min (in 200 µl sample)

= 2.68×10^{-3} U/ml (in 1 ml)

$1 / v = 1.12 \times 10^{12}$ s/mol

Kinetics results from Superose 12 gel filtration column:

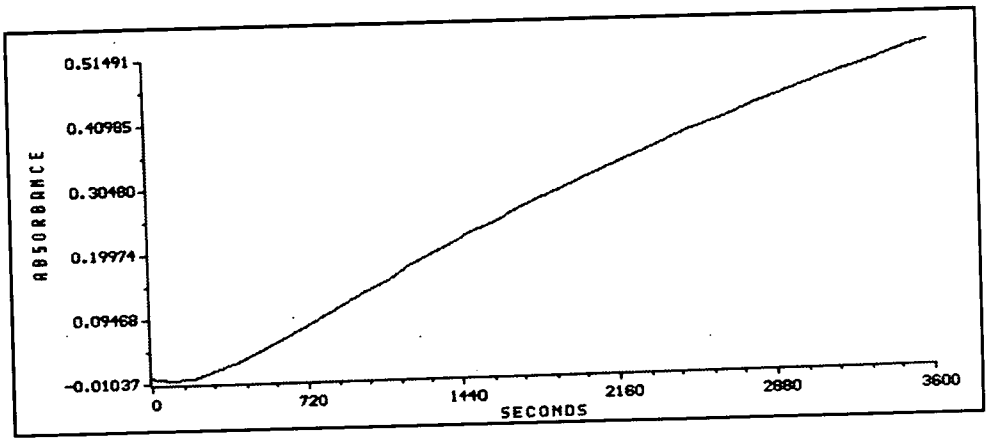


Figure 4.12 Timed based assay after Superose 12 gel filtration column indicating the production of 4-nitroaniline at $A_{410\,nm}$

Substrate concentration = 298 µM

Rate (v) : $\Delta A = 0.105 / 15{,}090 = 6.96 \times 10^{-9}$ mol/ml

6.96×10^{-9} mol/ml / 552 s = 1.26×10^{-11} mol/s

= 1.26×10^{-5} µmol/s

= 7.56×10^{-4} µmol/min (in 200 µl sample)

= 3.78×10^{-3} U/ml (in 1 ml)

$1 / v = 7.93 \times 10^{10}$ s/mol

Bradfords Assay Results:

Protein solutions to be investigated are compared with a known standard, bovine serum albumin (**Figure 4.13**). The same protein solution is assayed at three different volumes in order that an average can be taken.

Crude Extract: Average $A_{595\ nm}$ per µl = 0.171
Amount of protein *c.f.* BSA standard = 29.6 µg/µl

After Q-Sepharose FF: Average $A_{595\ nm}$ per µl = 0.019
Amount of protein *c.f.* BSA standard = 3.3 µg/µl

After Superose 12: Average $A_{595\ nm}$ per µl = 0.028
Amount of protein *c.f.* BSA standard = 5.02 µg/µl

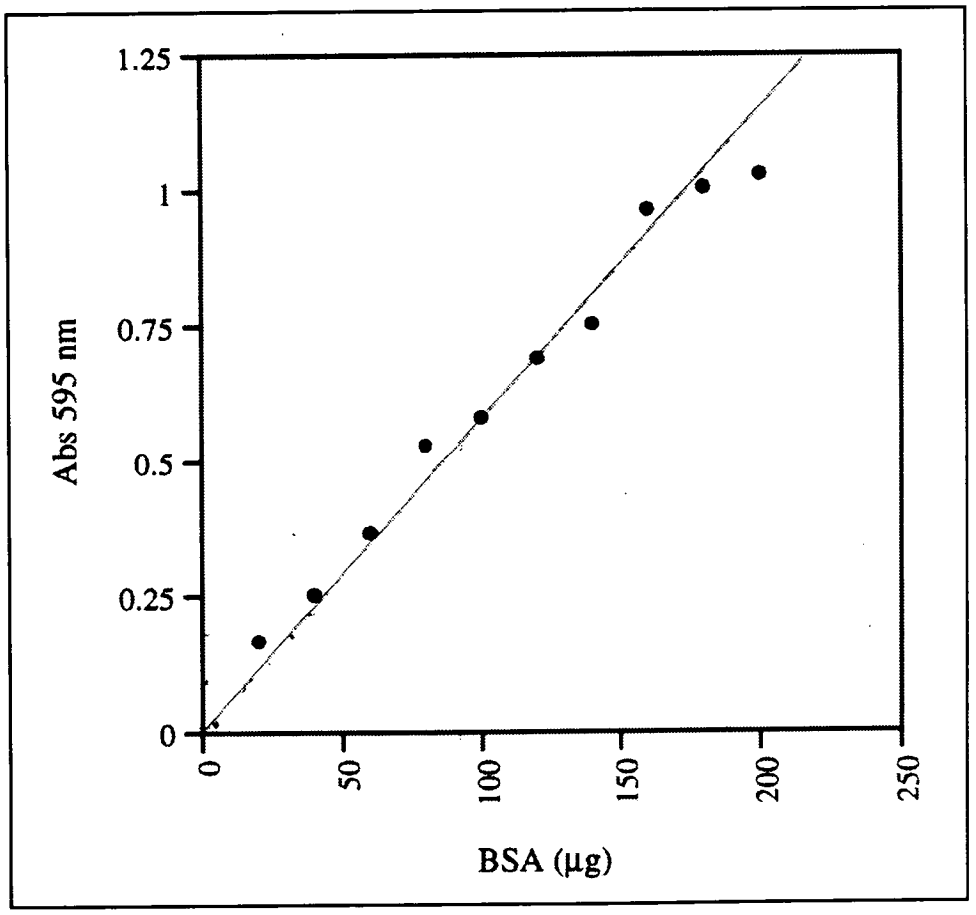


Figure 4.13 Standard BSA plot for reference with Bradfords Assay

Specific Activities:
Crude Extract = 15.75×10^{-3} U/ml / 29.6 µg/µl
= 0.53 mU/mg
After Q-Sepharose FF = 2.68×10^{-3} U/ml / 3.3 µg/µl
= 0.81 mU/mg

After Superose 12 = 3.78×10^{-3} U/ml / 5.02 µg/µl

= 0.75 mU/mg

4.3.2 METHOD 2

Despite the first attempt at purification being relatively successful it was decided that possible improvement could be made by increasing the number of ammonium sulphate fractionation steps. It was also decided to avoid dialysis by performing a gel filtration step after the ammonium sulphate precipitation.

(Step 1) Protamine Sulphate Precipitation - Nucleic acids were removed from the cell free extract of a 10 L culture of *S. cerevisiae* as in Method 1.

(Step 2) Ammonium Sulphate Precipitation - The resulting extract from the protamine sulphate precipitation was then taken to 20 % saturation with ammonium sulphate with stirring at 4 °C for 1 h and the precipitated protein collected by centrifugation at 18,000 rpm for 20 min at 4 °C. The pellet was resuspended in the 3 ml of 100 mM potassium phosphate buffer, pH 7, containing 100 µg/ml PMSF and 2 mM DTT, this procedure was repeat with 30 %, 50 %, 65 % and 80 % ammonium sulphate saturation of the supernatent at each stage. When assayed for the production of 4-nitroaniline only the 65 % and 80 % cuts demonstrated signinficant activity. A 15 % SDS-PAGE gel was run on all the precipitated fractions (**Figure 4.14**). The 80 % fraction was used for further purification as the specific activity was significantly higher.

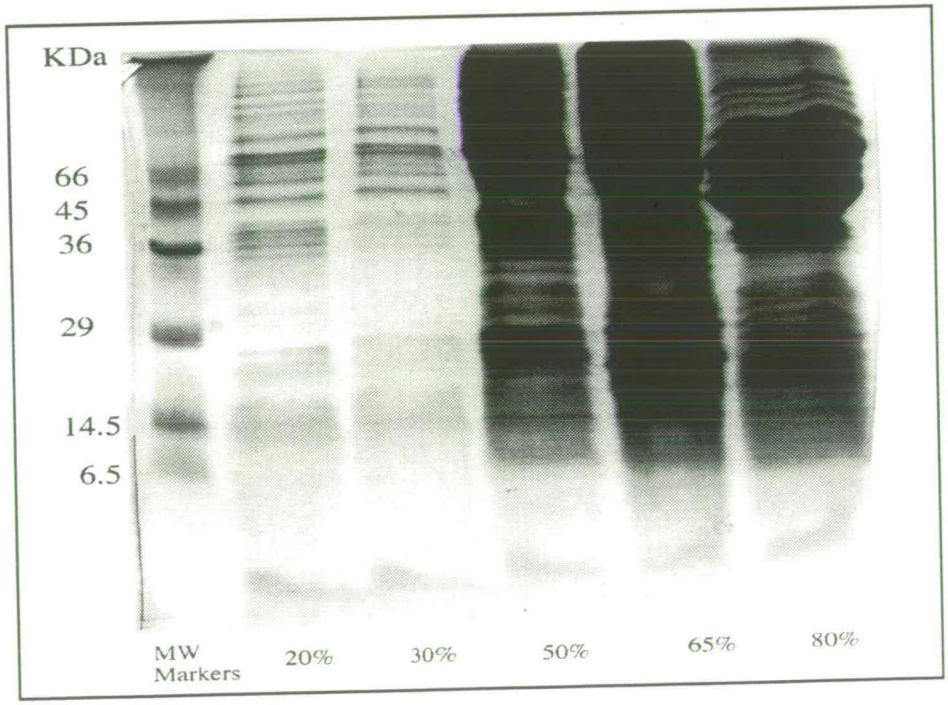


Figure 4.14 SDS-PAGE of the ammonium sulphate precipitated proteins.

(Step 3) Sephacryl S-200 Gel Filtration Chromatography - An XK16/70 column was packed with Sephacryl S-200 gel filtration resin to produce a bed volume of approximaetly 120 ml. The column was pre-equilibrated with 100 mM potassium phosphate buffer (pH 7.0) and the 80 % ammonium sulphate cut, dissolved in a minimum amount of buffer, was applied to the column. Proteins were eluted at a rate of 1ml/min using a peristaltic P-1 pump (Pharmacia Biotech) and collected in 8 ml fractions (**Figure 4.15**). All fractions were assayed for nitroreductase activity which was shown to be present in fraction 5.

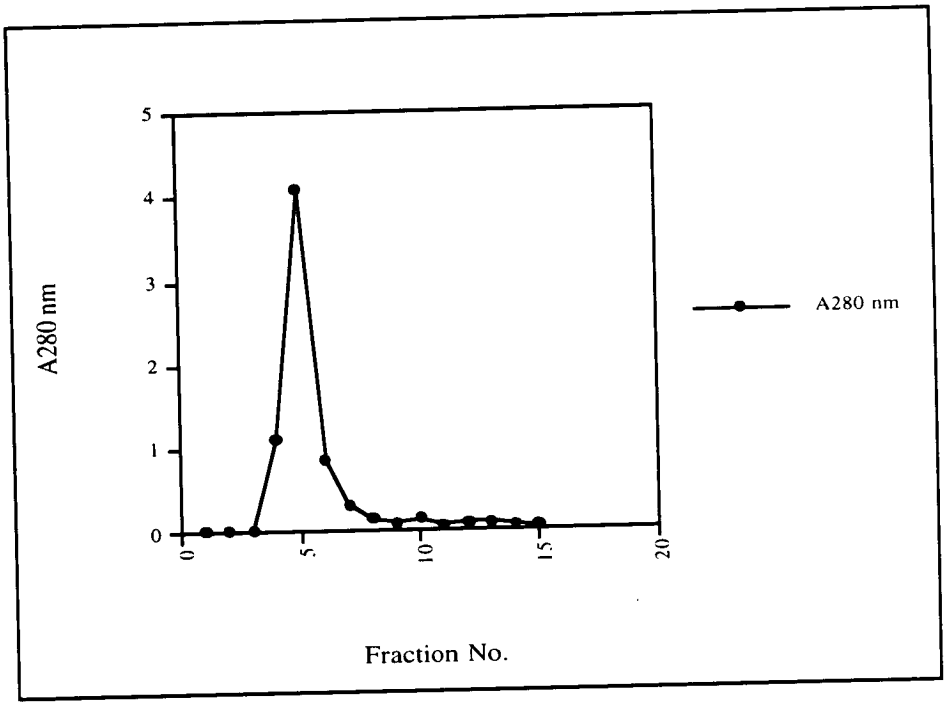


Figure 4.15 Sephacryl S-200 gel filtration chromatography.

Attempts to separate the proteins in fraction 5 (after Sephacryl S-200) using Resource Q, phenyl superose and Q-sepharose FF failed due to either the protein binding too strongly to the columns resulting in a significant loss in protein concentration, or the proteins lack of binding to the columns. It is believed that the problems encountered duting Method 2 are due to technical problems experienced with the Äkta.

ASSAY RESULTS

Kinetics results after ammonium sulphate fractionation

65 % Ammonium sulphate cut:

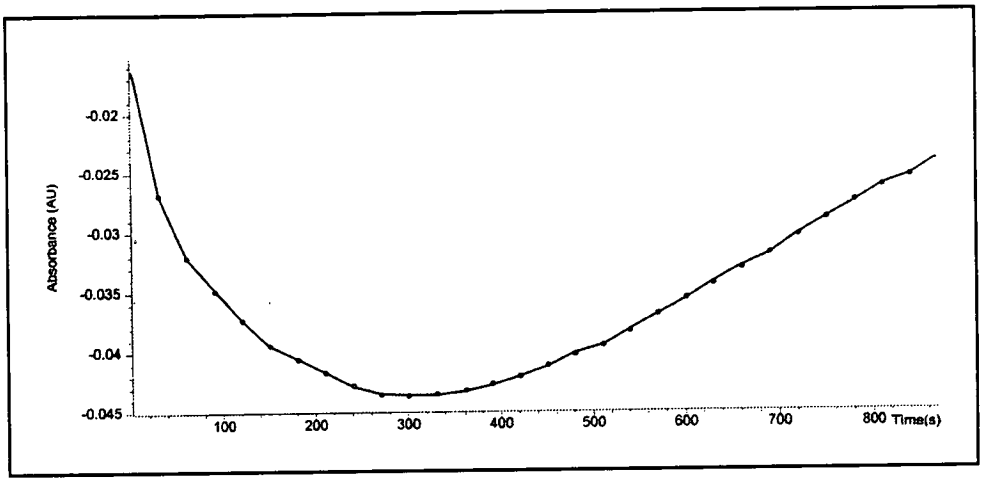


Figure 4.16 Time based assay for the 65 % ammonium sulphate precipitate

Substrate concentration = 298 μM

Rate (v) : $\Delta A = 4.55 \times 10^{-3} / 15{,}090 = 3.02 \times 10^{-10}$ mol/ml

3.02×10^{-10} mol/ml / 120 s = 2.5×10^{-12} mol/s

$= 2.5 \times 10^{-6}$ μmol/s

$= 1.5 \times 10^{-4}$ μmol/min (in 50 μl sample)

$= 3 \times 10^{-3}$ U/ml

Bradfords Assay indicated a total protein concentration of 46 mg/ml

Specific Activity of 65 % cut = 3×10^{-3} U/ml / 46 mg/ml

= 0.065 mU/mg

80 % Ammonium sulphate cut:

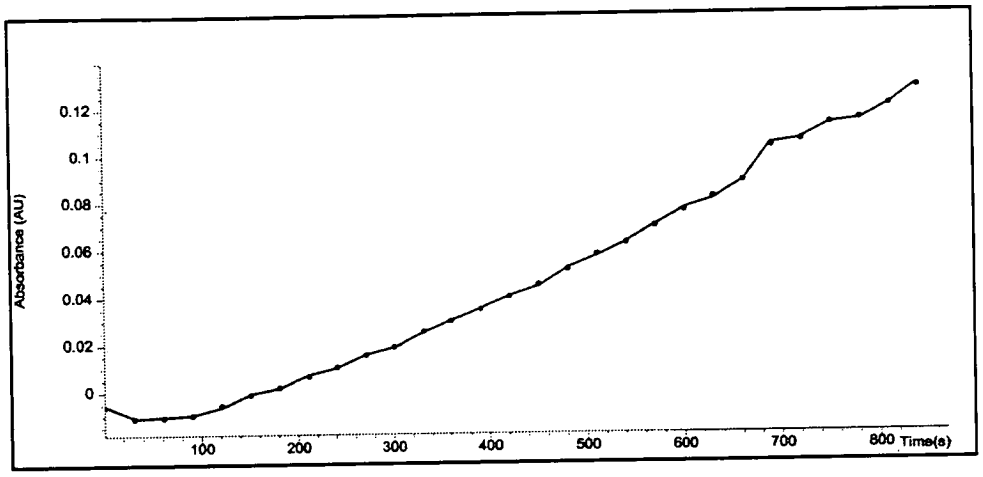


Figure 4.17 Time based assay for the 80 % ammonium sulphate precipitate

Substrate concentration = 298 μM

Rate (v) : $\Delta A = 0.066 / 15{,}090 = 4.37 \times 10^{-9}$ mol/ml

4.37×10^{-9} mol/ml / 300 s = 1.46×10^{-11} mol/s

$= 1.46 \times 10^{-5}$ μmol/s

$= 8.76 \times 10^{-4}$ μmol/min (in 50 μl sample)

$= 17.5 \times 10^{-3}$ U/ml

Bradfords Assay indicated a total protein concentration of 50 mg/ml

Specific Activity of 80 % cut = 17.5×10^{-3} U/ml / 50 mg/ml

= 0.35 mU/mg

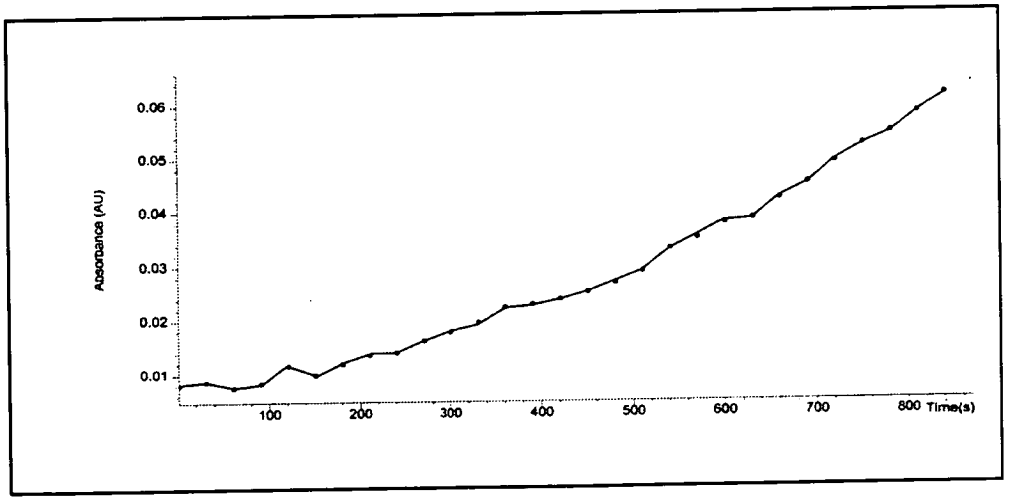


Figure 4.18 Time based assay for fraction 5 from Sephacryl S-200 gel filtration column. Specific activity of this fraction = 1.31×10^{-3} U/ml

4.3.3 METHOD 3

A repeat of the unsuccessful Method 2 was performed with less ammonium sulphate fractionation in order to determine if the gel filtration step after fractionation was causing the problem.

(Step 1) Protamine Sulphate Precipitation - Nucleic acids were removed from the cell free extract of a 9 L culture of *S. cerevisiae* as in Method 1.

(Step 2) Ammonium Sulphate Precipitation - The resulting extract from the protamine sulphate precipitation was then taken to 60 % saturation with ammonium sulphate with stirring at 4 °C for 1 h and the precipitated protein collected by centrifugation as before. This procedure was repeat with 80 % ammonium sulphate saturation. The 80 % cut was concentrated through centriprep-10 tubes to remove the salt.

(Step 3) Sephacryl S-200 Gel Filtration Chromatography - The concentration protein from the 80 % ammonium sulphate cut was applied to a Sephacryl S-200 gel filtration column and run in the same way as in Method 2. The elution profile is shown in **Figure 4.19.** Fractions 3 to 16 were assayed for reductase activity (**Figure 4.20**) and a gel run on the relevant fractions (**Figure 4.21**).

(Step 4) Q-Sepharose Fast Flow Anion Exchange Chromatography - Fractions 6 and 7 were combined and applied to a Q-Sepharose FF anion exchange column that had previously been equilibrated with 100 mM potassium phosphate buffer (pH 6). Unbound material was washed from the column and bound protein was eluted at a rate of 5 ml/min with a gradient of 0 to 1M sodium chloride over 10 column volumes. All fractions containing protein were assayed for reductase activity, the assay needed to be left overnight before any absorption at 410 nm could be identified (**Figure 4.22**).

Active fractions were run on a 15 % SDS-PAGE gel (**Figure 4.23**). Fractions 20 to 24 were combined and run on Phenyl Superose hydrophobic interaction column, unfortunately there was not enough protein eluted to produce adequate assay results. The protein band at approximately 55 KDa (**Figure 4.23**) was thought to be the nitroaryl reductase, unfortunately there was not enough protein to perform an N-terminal analysis to confirm these suspicions. Further attempts during this method to obtain enough protein to enable N-terminal analysis to be performed failed.

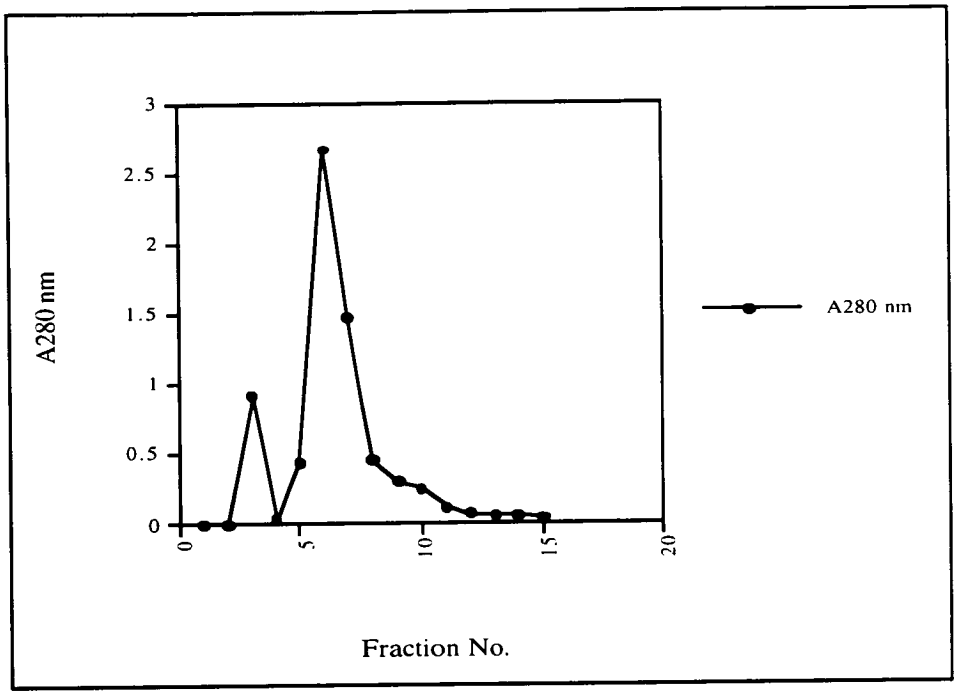


Figure 4.19 Elution profile for Sephacryl S-200 gel filtration chromatography.

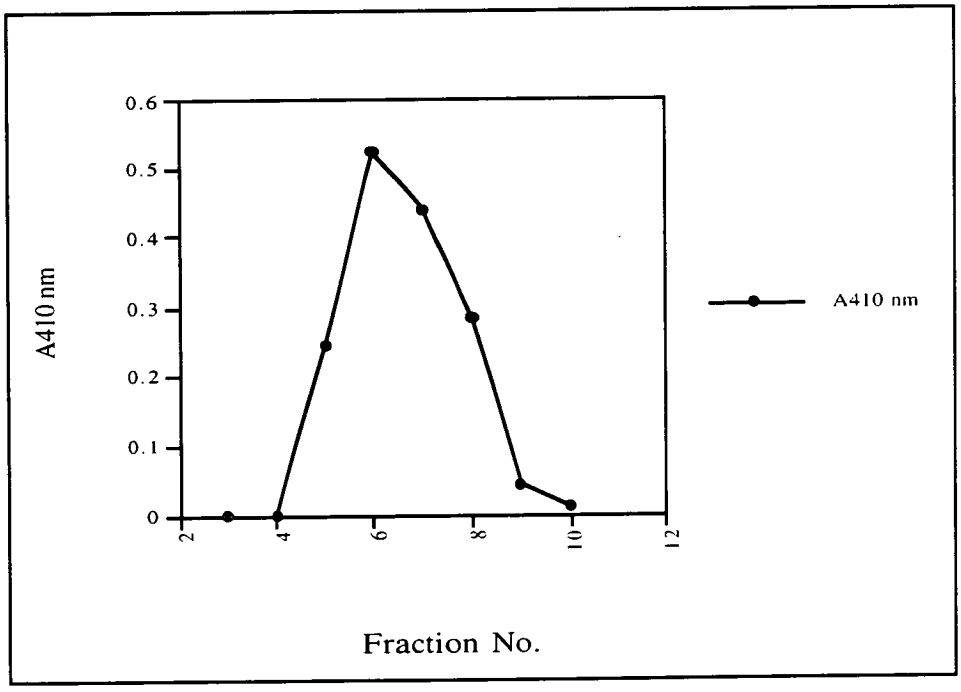


Figure 4.20 Enzyme activity after Sephacryl S-200 gel filtration chromatography.

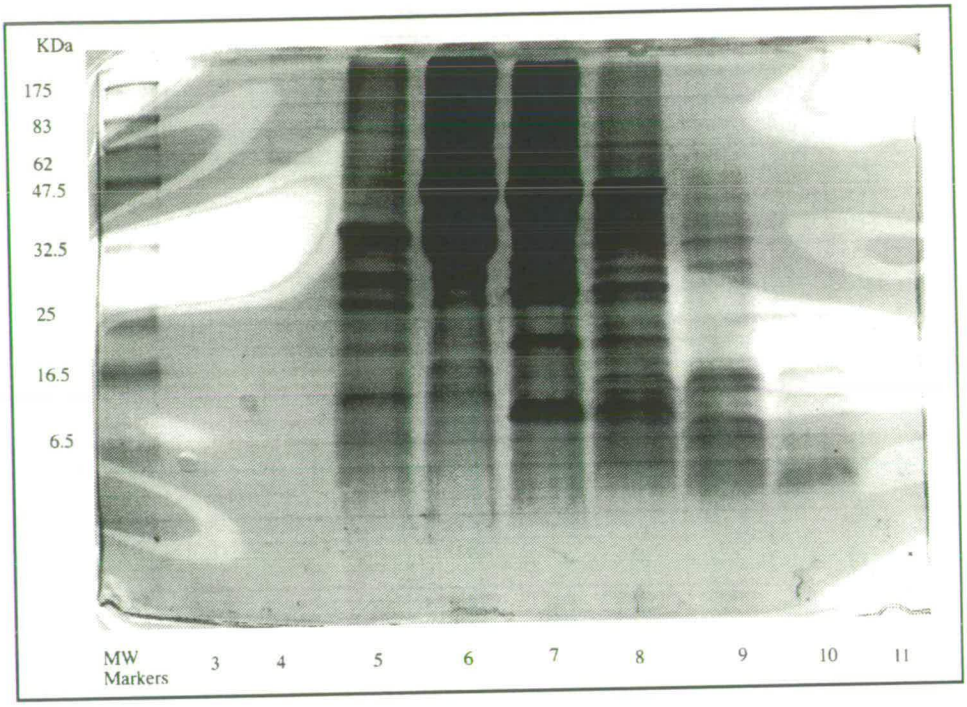


Figure 4.21 SDS-PAGE (15 %) gel of fractions 3-11 from Sephacryl S-200 gel filtration column.

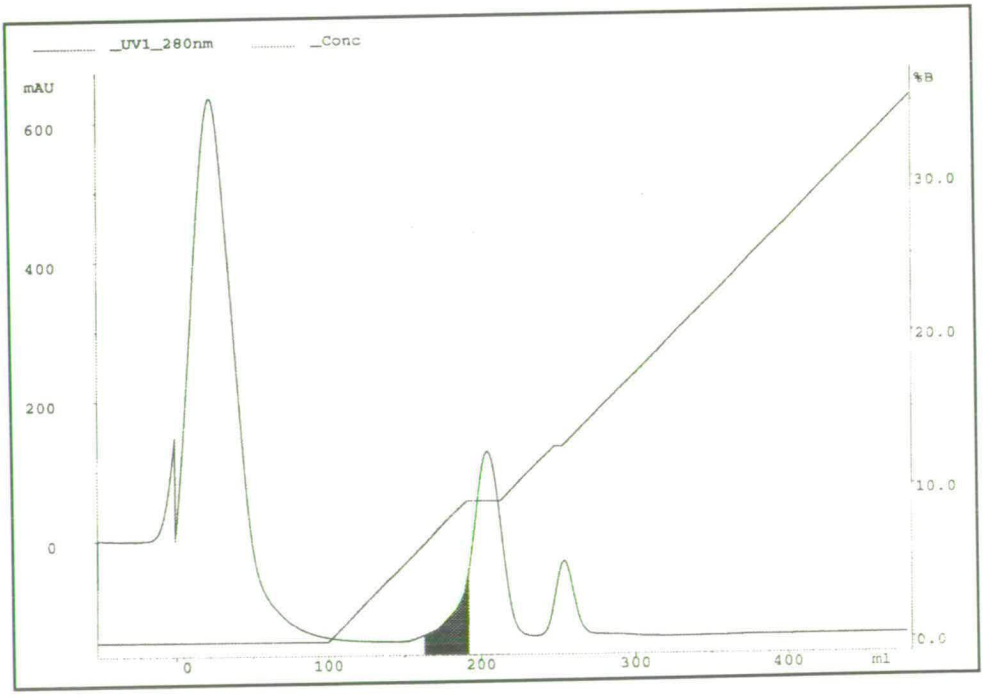


Figure 4.22 Elution profile from Q-Sepharose FF anion exchange column. Shaded area indicates activity.

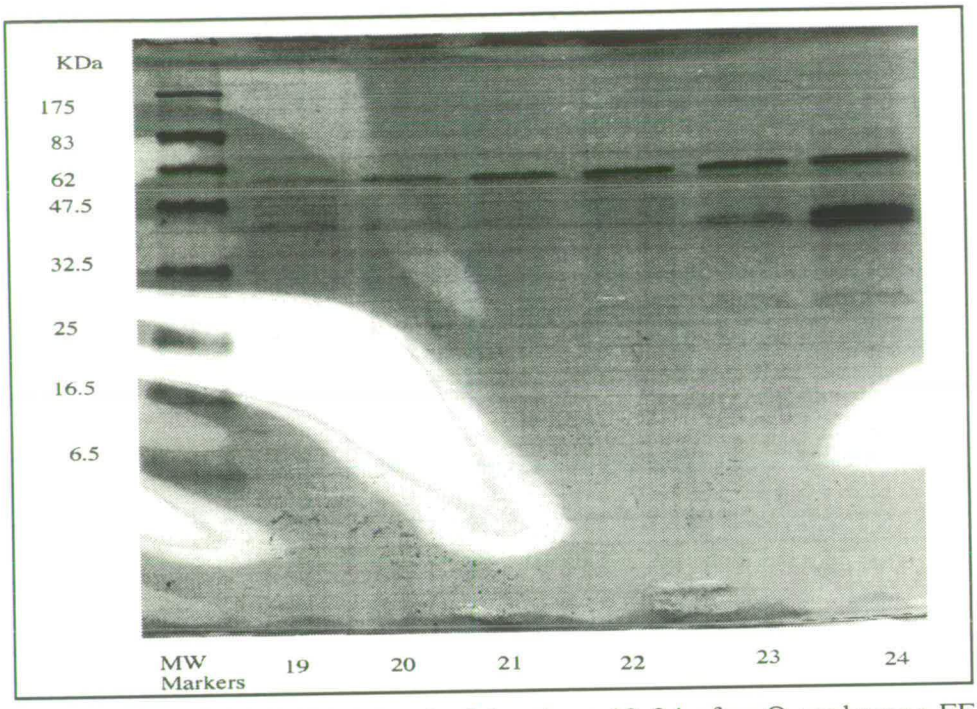


Figure 4.23 SDS-PAGE (15 %) gel of fractions 19-24 after Q-sepharose FF anion exchange column.

4.3.4 METHOD 4

It was shown in Method 3 that using a gel filtration step in place of dialysis proved to be successful. In Method 4 a further attempt was made to improve the technique by performing additional ammonium sulphate fractionations with the view of removing a larger amount of unwanted protein at an early stage in the purification.

(Step 1) Protamine Sulphate Precipitation- Nucleic acids were removed from the cell free extract of a 20 L (2x10L) culture of *S. cerevisaie* as in Method 1.

(Step 2) Ammonium Sulphate Precipitation- The crude extract was taken to 50 % ammonium sulphate saturation and was left stirring for 1 h at 4 °C, the precipitated protein was collected by centrifugation at 18,000 rpm for 20 min. at 4 °C. The pellet was retained and the procedure repeated with the supernatant at 60, 70 and 80 % ammonium sulphate saturation. The four pellets were resuspended in 50 mM phosphate buffer pH 6.0 (5-10 ml) containing PMSF (100 µg/ml) and DTT (2mM) and assayed for nitroreductase activity and protein concentration. As expected the 60 and 70 % fractions were highly active, however, the 50 % fraction also demonstrated high activity. The 50 % cut was refractionated with 25, 30, 33, 35, 40, 45 and 55 % ammonium sulphate and each fraction assayed for activity. The 30 % fraction, in particular, indicated activity.

(Step 3) Sephacryl S-200 Gel Filtration Chromatography- The 60 and 70 % ammonium sulphate fractions were combined and loaded onto a 1.5L column of Sephacryl S-200 gel filtration resin that had been pre-equilibrated with 50 mM phosphate buffer, pH 6.0. Proteins were eluted at a rate of 2 ml/min against gravity and collected in 20 ml fractions. All fractions were assayed for protein concentration and nitroreductase activity (**Figure 4.24**). Fractions with activity were pooled (8x20 ml fractions).

(Step 4) Q-Sepharose FF Anion Exchange Chromatography- The active protein from Step 3 was applied to a 50 ml (XK 26/20) column of Q-Sepharose FF anion exchange resin which had been pre-equilibrated with 50 mM phosphate buffer, pH 6.0. Unbound protein was washed from the column using two column volumes of start buffer. Bound proteins were eluted using a 0-1 M sodium chloride gradient over 15 column volumes (**Figure 4.25**). All fractions were assayed for nitroreductase activity

(**Figure 4.26**) and a 15 % SDS-PAGE gel was run on significant fractions (**Figure 4.27**).

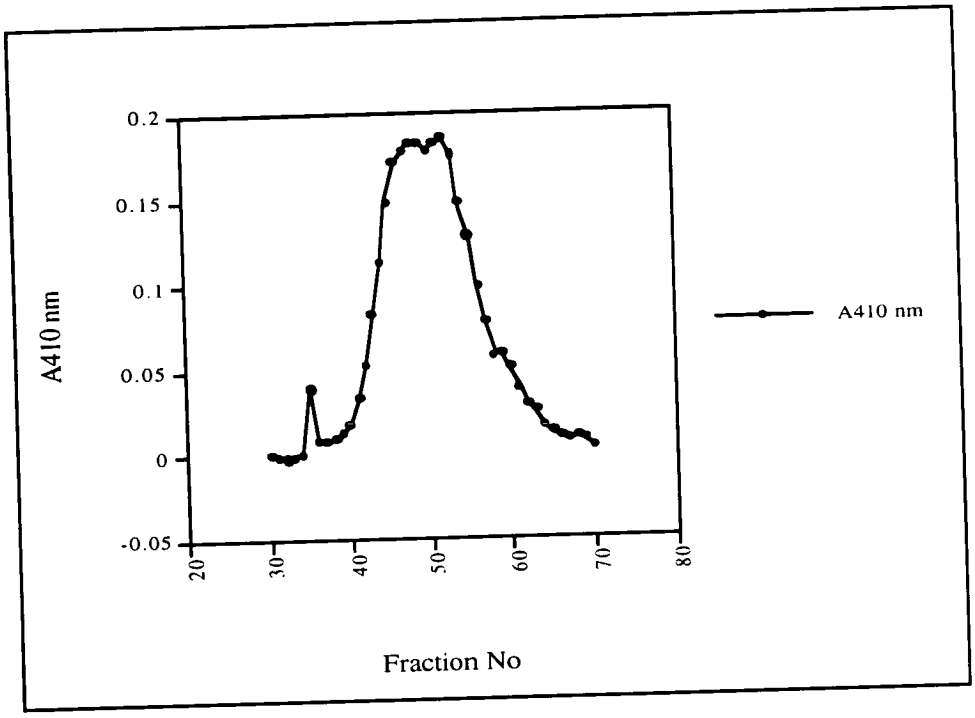


Figure 4.24 Enzyme activity after Sephacryl S-200 gel filtration chromatography.

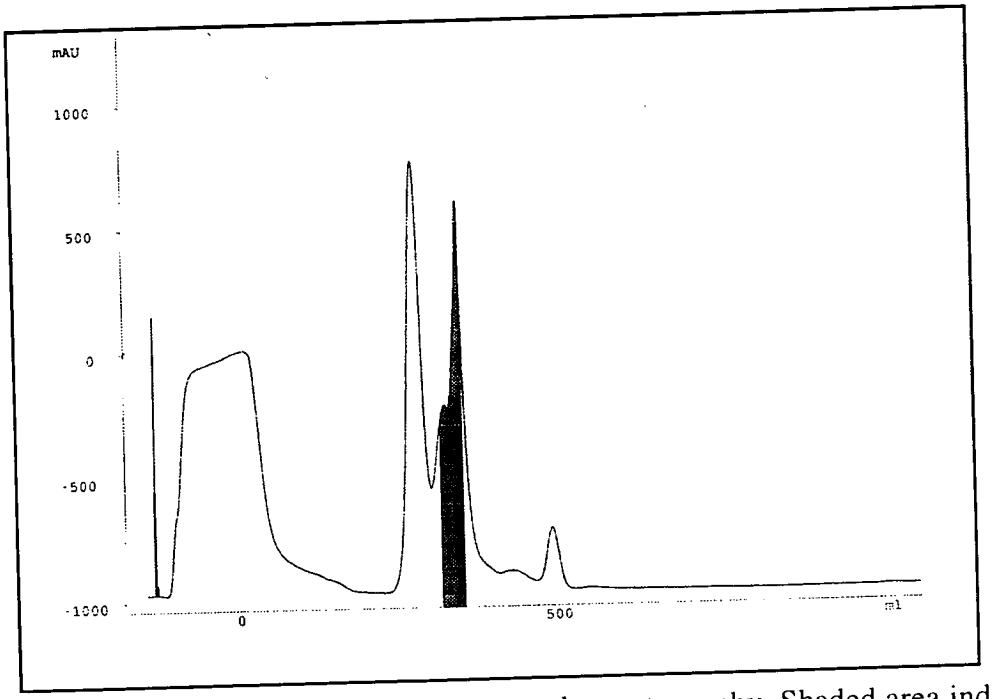


Figure 4.25 Q-Sepharose FF anion exchange chromatography. Shaded area indicates activity.

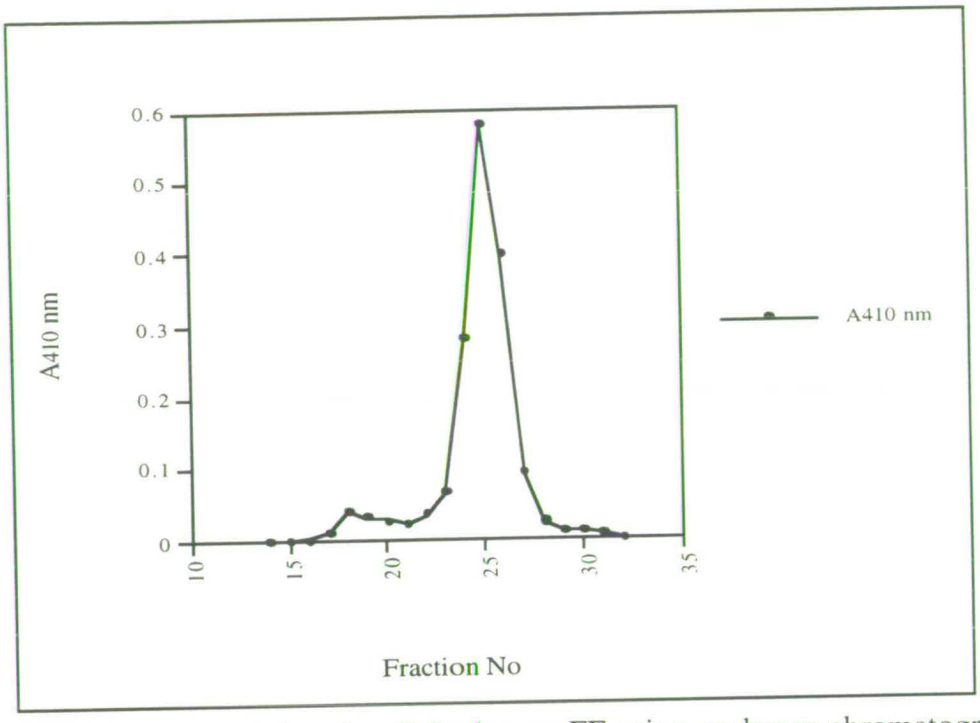


Figure 4.26 Enzyme activity after Q-Sepharose FF anion exchange chromatography

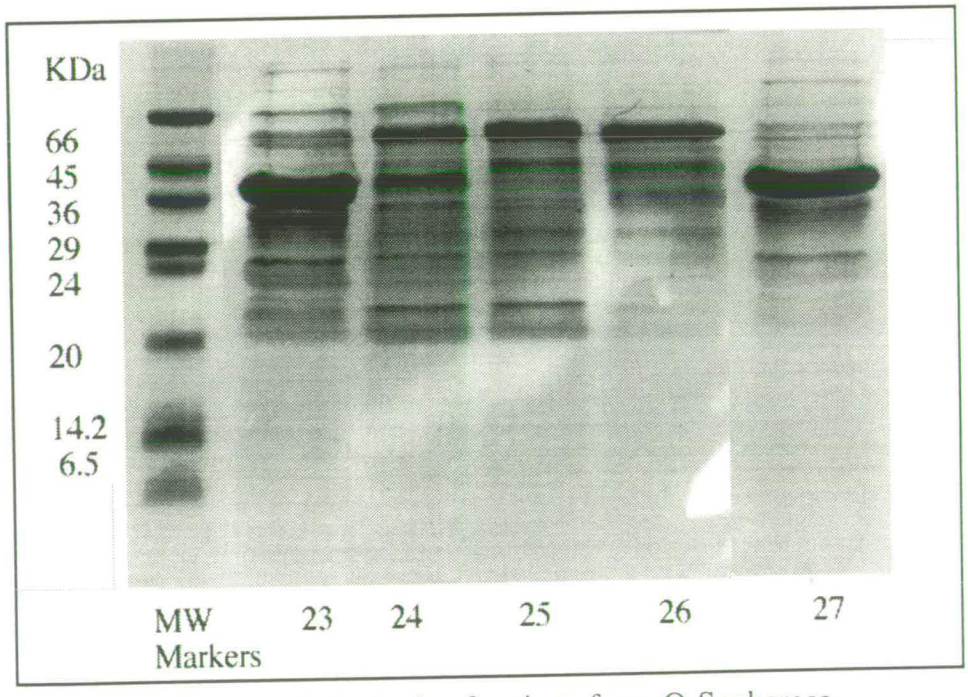


Figure 4.27 SDS-PAGE (15 %) Active fractions from Q-Sepharose .

(Step 5) Superose 12 Gel Filtration Chromatography- The column was standardised with four proteins of known molecular weight (**Figure 4.28**): Bovine Serum Albumin (66 KDa), Chicken Egg Ovalbumin (45 KDa), Carbonic Anhydrase (29 KDa) and Cytochrome c (12.4 KDa). A 200 µl sample from the active 30 % ammonium sulphate precipitate was run on the Superose 12 column (**Figure 4.29**) and all

fractions assayed for activity (**Figure 4.30**). By comparing the region of activity observed with the unknown protein with the standards, the active enzyme is shown to have a molecular weight of < 12 KDa.

A 200 µl sample from the active fraction No 25 after step 4 was applied to the gel filtration column. The protein eluted was not very concentrated but activity was observred in two fractions corresponding to a molecular weight of approximately 50 KDa (**Figure 4.31**), unfortunately there was insufficient protein to observe on a gel.

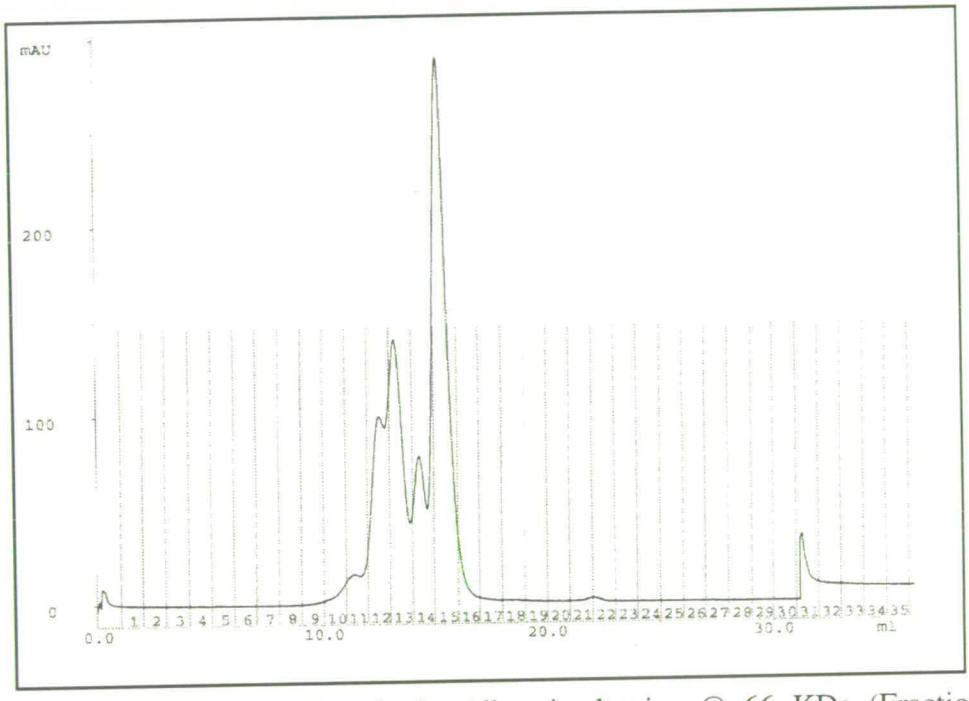


Figure 4.28 Superose-12 Standards: Albumin, bovine @ 66 KDa (Fraction 12), Albumin, Chicken Egg @ 45 KDa (Fraction 13), Carbonic Anhydrase @ 29,000 KDa (Fraction 14) and Cytochrome C @ 12,384 KDa (Fraction 15).

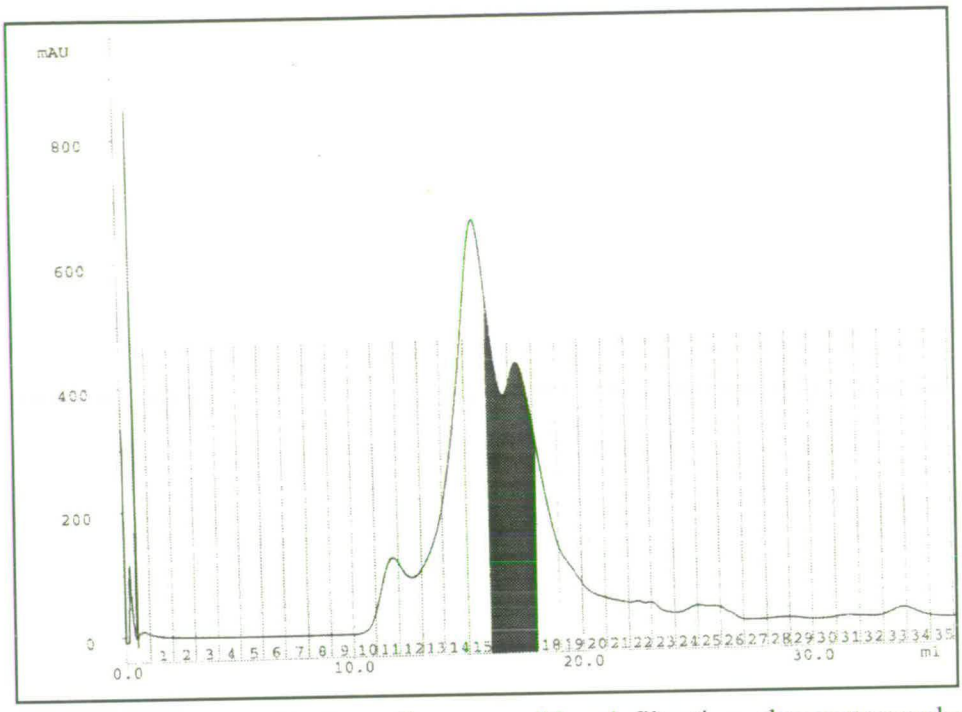


Figure 4.29 Elution profile from Superose 12 gel filtration chromatography (30% standards comparison). Shaded area indicates activity.

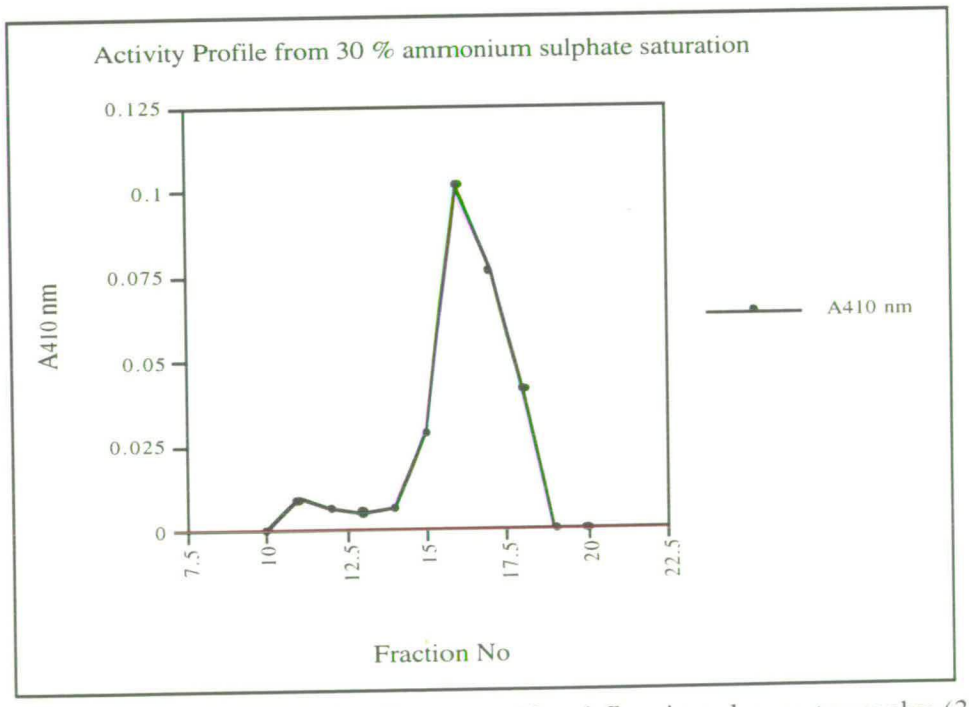


Figure 4.30 Enzyme activity after Superose 12 gel fltration chromatography (30 %).

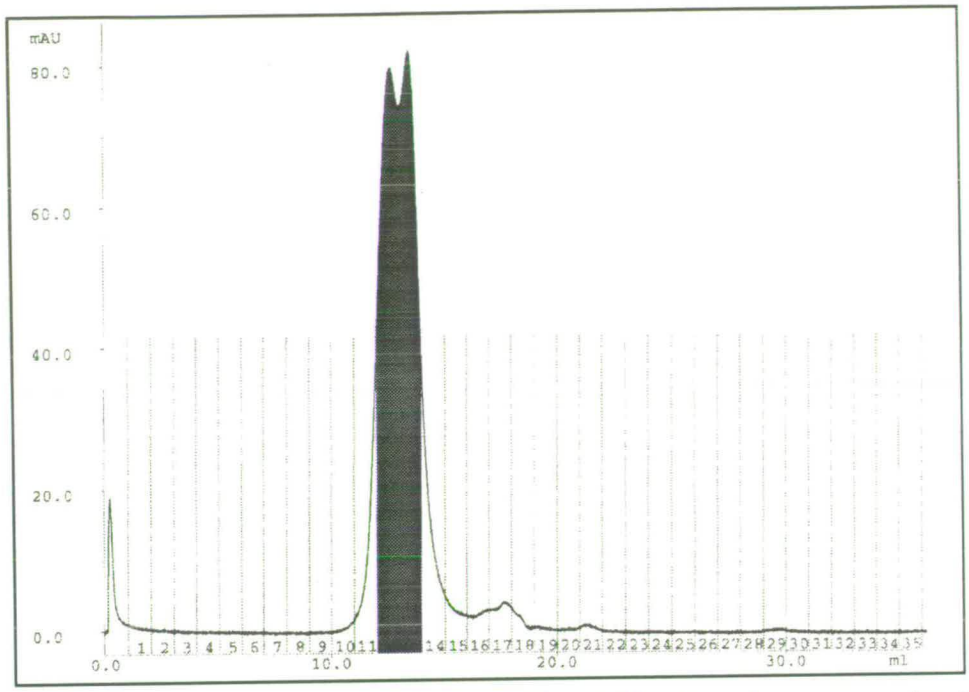


Figure 4.31 Elution profile from Superose 12 gel filtration chromatography (after Q Sepharose). Shaded area indicates activity.

(Step 6) Phenyl Superose Hydrophobic Interaction Chromatography- Protein from the active fractions 25, 26 and 27 after Q-Sepharose FF anion exchange chromatography (Step 4) was combined and adjusted to a concentration of 1.5 M ammonium sulphate. The protein was applied to a Phenyl Superose column that had been pre-equilibrated with 50 mM phosphate buffer, pH 6.0 containing 1.5 M ammonium sulphate. Unbound protein was washed from the column and bound protein was eluted over 30 column volumes with a decreasing salt gradient (**Figure 4.32**). All fractions were assayed for nitroreductase activity (**Figure 4.33**) and the active ractions were run on a 15 % SDS-PAGE gel (**Figure 4.34**). The most active fractions (13-22) were combined and dialysed to remove the ammonium sulphate.

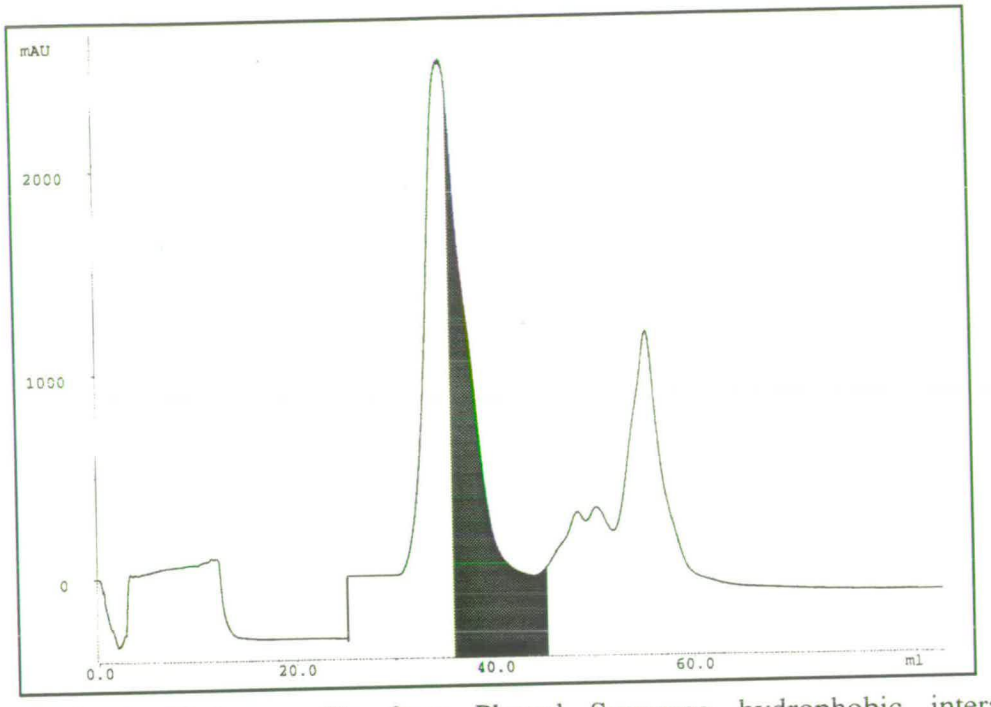


Figure 4.32 Elution profile from Phenyl Superose hydrophobic interaction chromatography. Shaded area indicates activity.

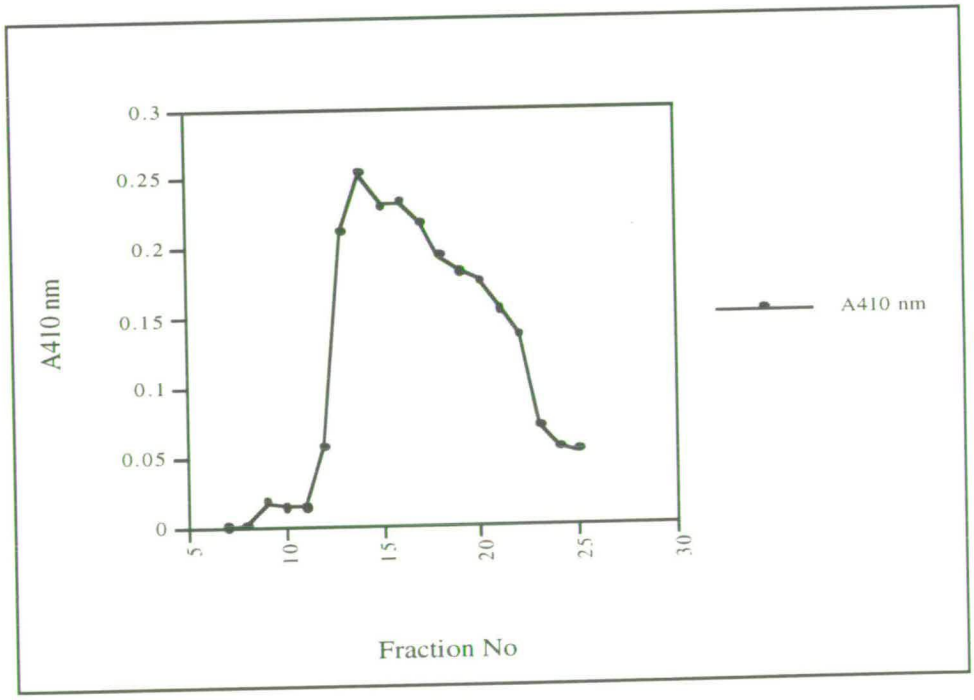


Figure 4.33 Enzyme activity from Phenyl Superose hydrophobic interaction chromatography.

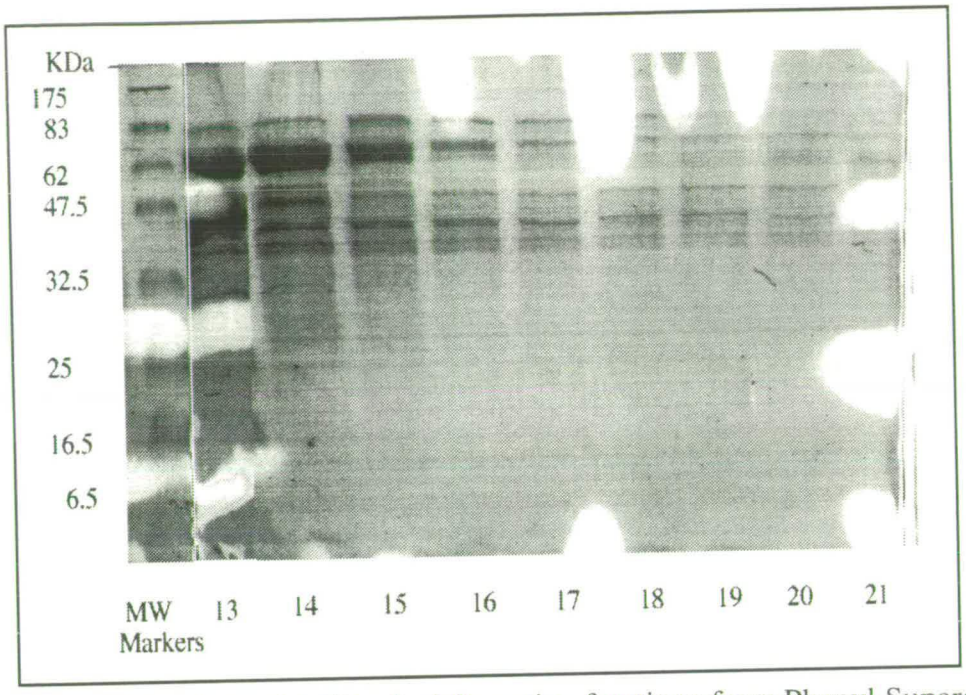


Figure 4.34 SDS-PAGE (15 %) gel of the active fractions from Phenyl Superose.

(Step 7) ADP Sepharose Affinity Chromatography- The combined protein from Step 6 was applied to a pre-eqiuilibrated ADP Sepharose column. Protein was eluted with a stepwise sodium chloride gradient (**Figure 4.35**). All fractions were assayed for activity and a 15 % SDS-PAGE gel was run on the active fractions (**Figure 4.36**). Fraction 8 appeared to be almost pure, a western blot was performed on the sample which was then submitted for N-terminal sequencing.

Results from the N-terminal sequence analysis indicated the presence of a contaminating protein from *E.coli*. When comparing with other gels it appears that the contamination occured as a result of using the ADP Sepharose column. It was therefore not possible to pursue this purification further.

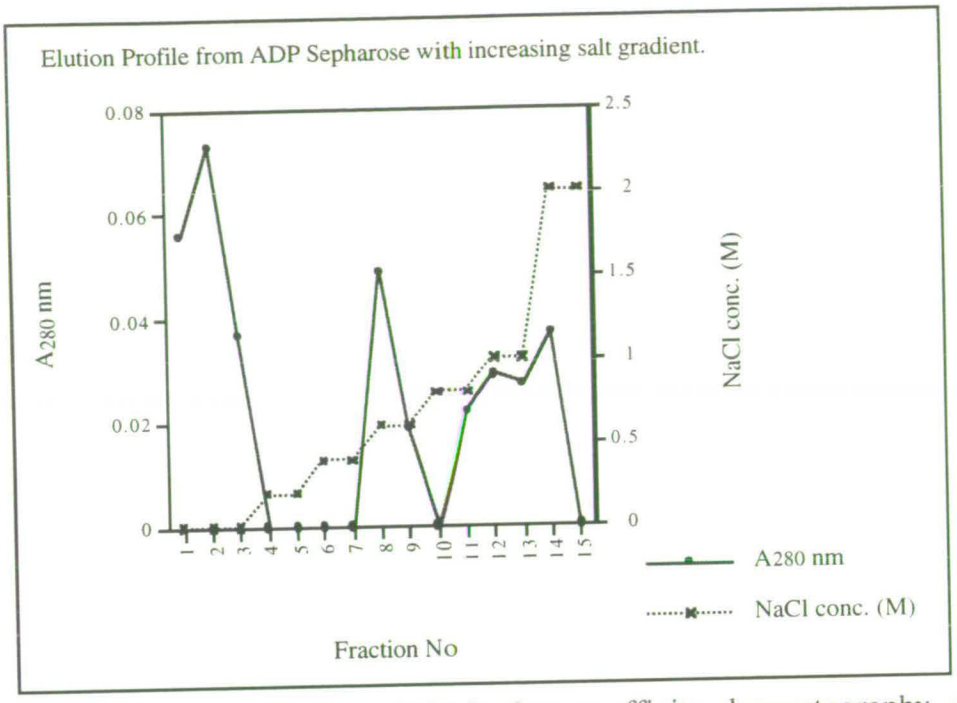


Figure 4.35 Elution profile from ADP Sepharose affinity chromatography. Activity was observed after 0.5 M NaCl.

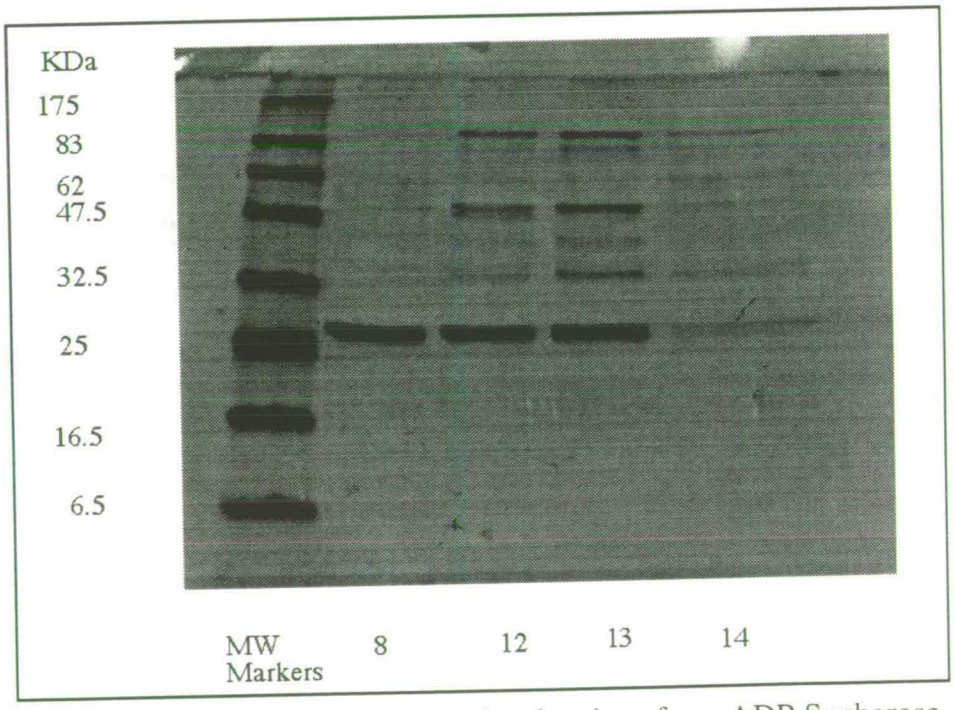


Figure 4.36 SDS-PAGE (15 %) gel of active fractions from ADP Sepharose.

ASSAY RESULTS:

50 % : 2.52 x 10^{-2} U/ml, in 10 ml = 0.252 U
60 % : 2.72 x 10^{-2} U/ml, in 5 ml = 0.136 U
70 % : 6.23 x 10^{-2} U/ml, in 5 ml = 0.312 U
80 % : 1.60 x 10^{-2} U/ml, in 5 ml = 0.08 U

Bradfords Assay:

The four ammonium sulphate cuts were diluted by taking 10 µl of protein extract and adding 990 µl of buffer.

	A595 nm	From standard curve
50 %	0.675	1.02 mg/ml
60 %	0.575	0.88 mg/ml
70 %	0.580	0.86 mg/ml
80 %	0.568	0.85 mg/ml

Specific Activity:

50 % : 2.52 x10^{-2}/100.2 = 2.5 U/mg
60 % : 2.72 x10^{-2}/88 = 2.4 U/mg
70 % : 6.23 x10^{-2}/86 = 5.36 U/mg
80 % : 1.60 x10^{-2}/85 = 1.36 U/mg

The 50 % ammonium sulphate pellet was active, so was dissolved in 80 ml 50 mM phosphate buffer pH6 and the fraction was re-cut:

25 % : 0.12 U/ml	In 1.5 ml = 0.18 U
30 % : 4.77 x 10^{-2}U/ml	In 3 ml = 0.14 U
33 % : 1.33 x 10^{-2}U/ml	In 3 ml = 0.04 U
35 % : 5.30 x 10^{-2}U/ml	In 1.5 ml = 7.95 x 10^{-3} U
40 % : 5.30 x 10^{-2}U/ml	In ml = 0.016 U
45 % : 8.60 x 10^{-2}U/ml	In 4 ml = 0.034 U
55 % : 7.30 x 10^{-2}U/ml	In 2 ml = 0.014 U

% CUT	A595 nm	From calibration plot	Specific Activity
20	0.081	0.125	
25	0.194	0.29	4.14 x 10^{-3} U/mg

30	0.232	0.34	1.4×10^{-3} U/mg
33	0.173	0.25	5.32×10^{-4} U/mg
35	0.120	0.18	2.9×10^{-4} U/mg
40	0.410	0.625	8.48×10^{-5} U/mg
45	0.661	0.98	8.78×10^{-5} U/mg
55	0.360	0.52	1.4×10^{-4} U/mg

As diluted 10 µl with 990 µl then x100 dilution.

4.3.5 METHOD 5 - OPTIMISATION

Although the majority of the techniques used were relatively successful, it was often observed that there was a rapid reduction in protein concentration, and therefore activity. In the light of this observation an optimisation experiment was performed where the aim was to determine the best conditions for maintaining enzyme activity.

(Step 1) Protamine Sulphate Precipitation- Nucleic acids were removed from the cell free extract of a 10 L culture of *S. cerevisiae* as in Method 1.

(Step 2) Ammonium Sulphate Precipitation- The protein extract was taken to 50 % saturation with ammonium sulphate with stirring at 4 °C for 1 h. The precipitated proteins were collected by centrifugation at 18,000 rpm for 20 min at 4 °C. The pellet was retained and the procedure repeated with the supernatent at 70 % ammonium sulphate saturation. The proteins collected between 50-70 % were divided approximately equally into seven, each fraction was dissolved in a different buffer in order to ascertain the buffer for retention of activity (**Table 4.2**).

Entry No	Buffer (50 mM)	Activity Under 1 week	Activity Under 2 weeks
1	Bis-Tris Propane, pH 6.0	+	-
2	Bis-Tris Propane, pH 6.5	+	-
3	Bis-Tris Propane, pH 7.0	+	+
4	Bis-Tris Propane, pH 7.5	+	+
5	Bis-Tris Propane, pH 8.0	+	+
6	Phosphate, pH 6.0	-[a]	-
+: Active, -: Inactive, [a] Precipitation started in 2 days.			

Table 4.2 Optimisation of buffer conditions for maintaining maximum activity.

The 50 % ammonium sulphate precipitate was re-fractionated with 35 % ammonium sulphate which indicated activity. The 70 % cut was approximately 10x more active than the 35 % cut so all efforts were concentrated on the more active fraction.

(Step 3) Superose 12 Gel Filtration Chromatography- A 200 µl sample of the protein from each of the seven buffers was applied to a Superose 12 gel filtration column. The proteins were eluted in the same buffer by gravity. The best separation was achieved with 50 mM Bis-Tris Propane (BTP), pH 8.0 (**Figure 4.37**). All fractions were

assayed for activity and the active fractions from each run were pooled whilst keeping the seven buffer types separate. The active protein from the 35 % ammonium sulphate cut was also applied to the column in BTP pH 7.0 (**Figure 4.38**).

(Step 4) Resource Q Anion Exchange Chromatography- Samples of protein from pHs 6.5, 7.0, 7.5, and 8.0 were applied to a Resource Q anion exchange column that had been pre-equilibrated with buffer of the appropriate pH. The elution profile of BTP pH 7.0 can be seen in **Figure 4.39** with activity seen in fractions 7 and 8. The corresponding 15 % SDS-PAGE gel can be seen in **Figure 4.40**. At pH 8.0 separation proved more succesful (**Figure 4.41**) with activity seen in fractions 11, 12, 13 and 14, the corresponding 15 % SDS-PAGE gel can be seen in **Figure 4.42**.

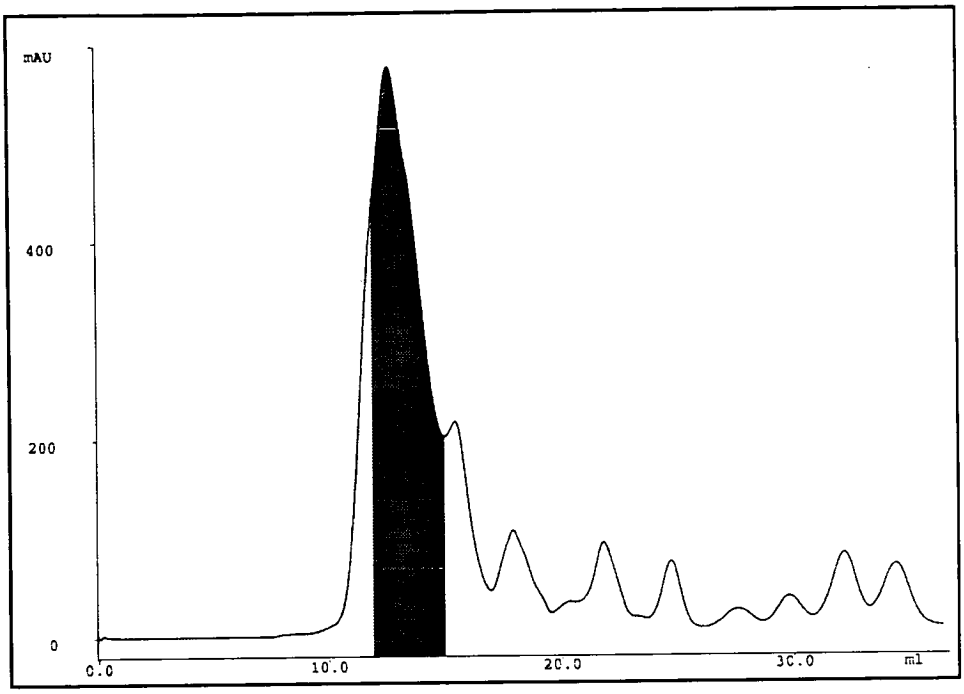


Figure 4.37 Elution profile from Superose 12 gel filtration chromatography (70 % ammonium sulphate fraction). Shaded area indicates activity. Start conditions: 50 mM BTP pH 8.0.

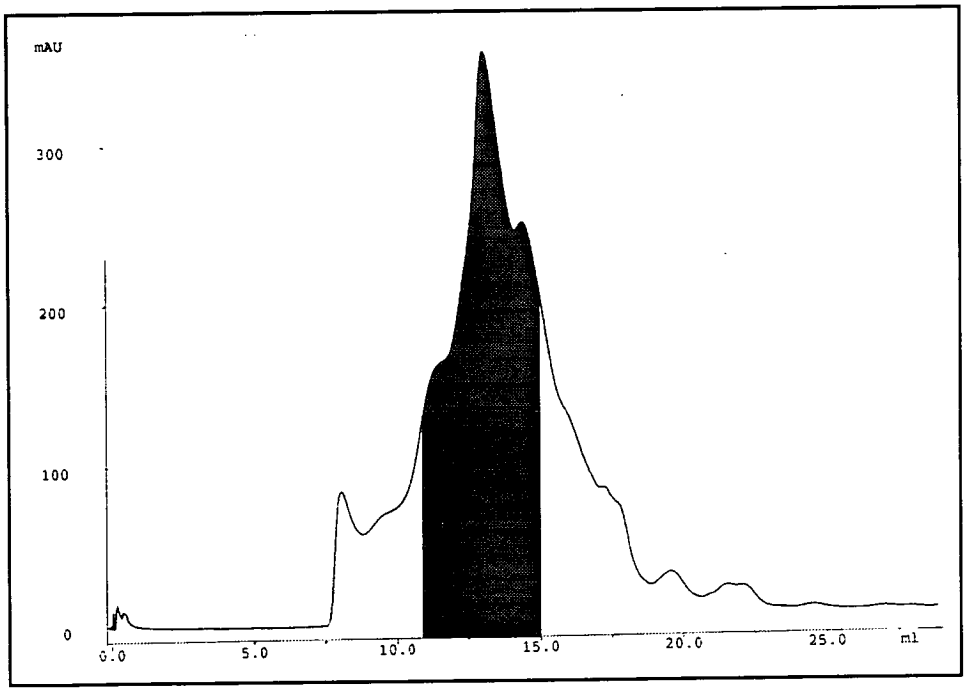


Figure 4.38 Elution profile from Superose 12 gel filtration chromatography (35 % ammonium sulphate fraction). Shaded area indicates activity. Start conditions: 50 mM BTP pH 7.0.

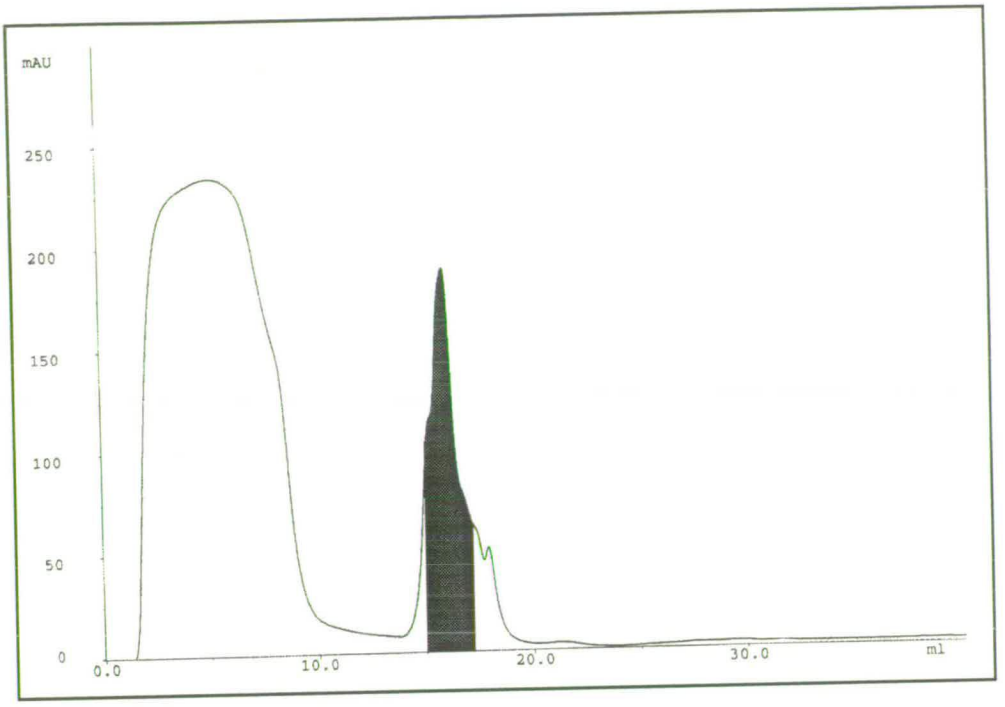


Figure 4.39 Elution profile from Resource Q anion exchange chromatography. Shaded area indicates activity. Start condition: 50 mM BTP pH 7.0.

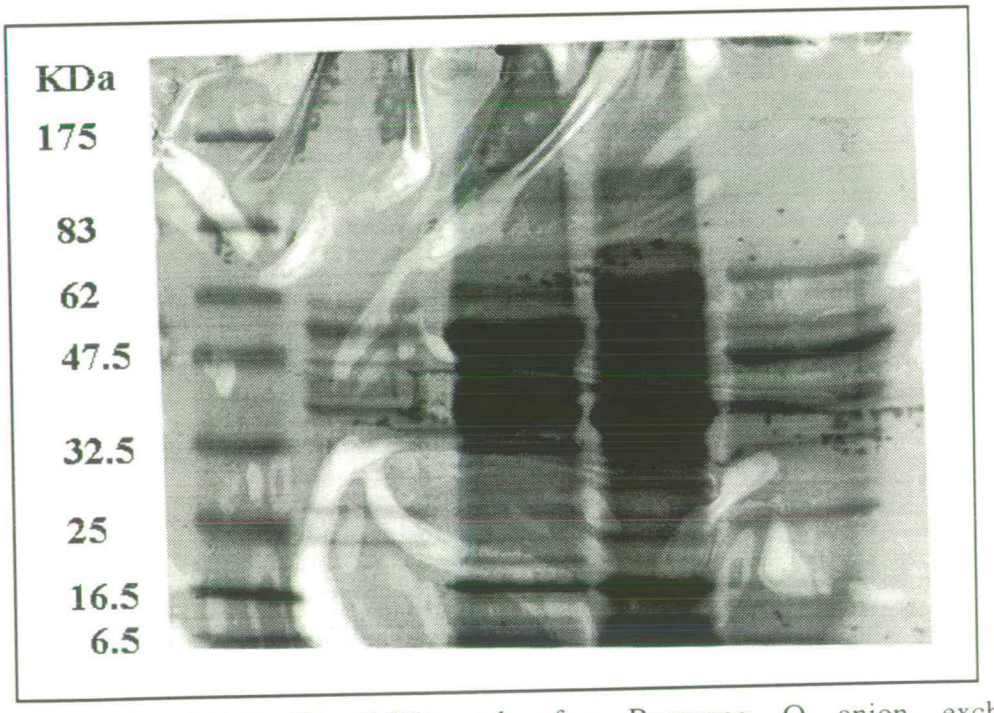


Figure 4.40 SDS-PAGE (15%) gel after Resource Q anion exchange chromatography at pH 7.0. Fractions 6,7,8 and 9 shown.

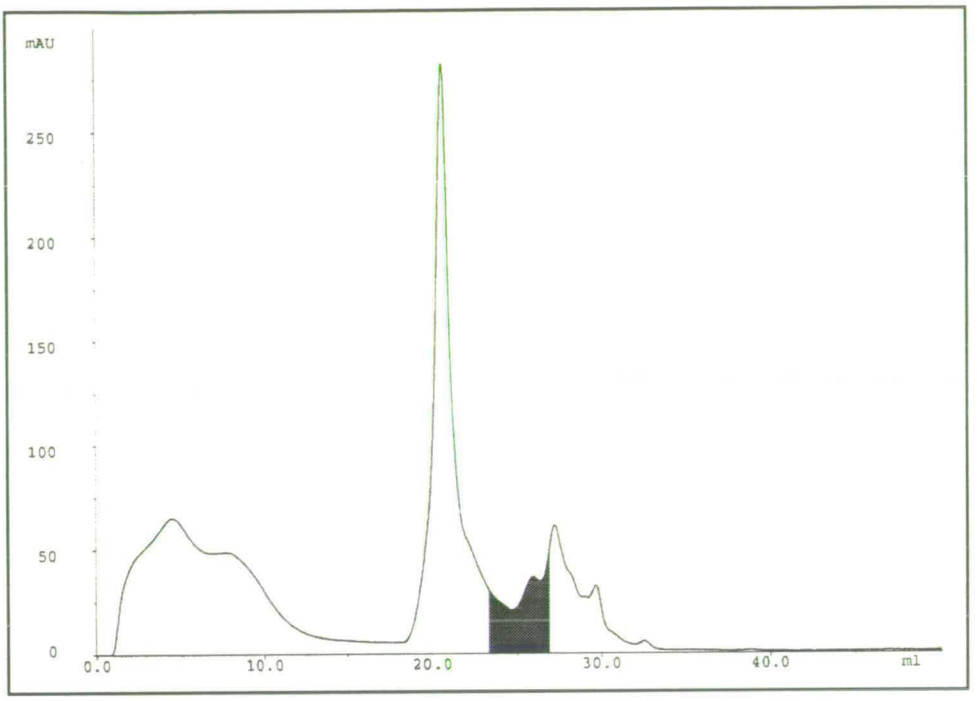


Figure 4.41 Elution profile from Resource Q anion exchange chromatography. Shaded area indicates activity. Start condtions: 50 mM BTP pH 8.0.

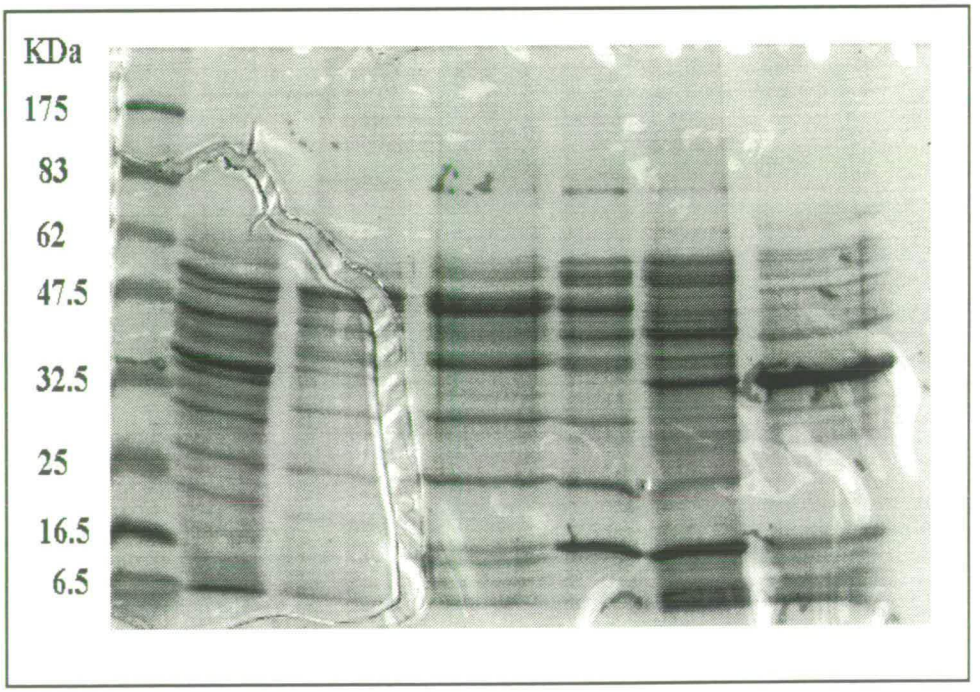


Figure 4.42 SDS-PAGE (15%) gel after Resource Q anion exchange chromatography at pH 8.0. Fractions 10-15 inclusive are shown.

(Step 5)- At this point in the optimisation it was necessary to combine all the protein from the 70 % ammonium sulphate cut in the different buffers. Any precipitated protein

was removed by centrifugation and the supernatant was dialysed against 50 mM BTP, pH 8.0

(Step 6) Q-Sepharose FF Anion Exchange Chromatography- 10 ml of this crude protein at pH 8.0 was applied to a Q-Sepharose FF anion exchange column that had been pre-equilibrated with 50 mM BTP, pH 7.2. Protein was eluted from the column with 50 mM BTP pH 7.2 using an increasing gradient of 0-300 mM sodium chloride over 12 column volumes (**Figure 4.43**). 5 ml fractions were collected and assayed for activity. The active fractions were pooled (70 ml) and concentrated using Centriprep-30 to 7 ml and dialysed against BTP pH 6.0. To see if better separation could be achieved at pH 6.0, the protein was reloaded onto the anion exchange column which had been pre-equilbrated with 50 mM BTP, pH 8.0. Unfortunately most of the protein failed to bind and was washed from the column. The protein that did bind, however, bound too strongly for elution under normal conditions and required 2 M sodium chloride for removal. The unbound protein was dialysed against 50 mM BTP, pH 6.0.

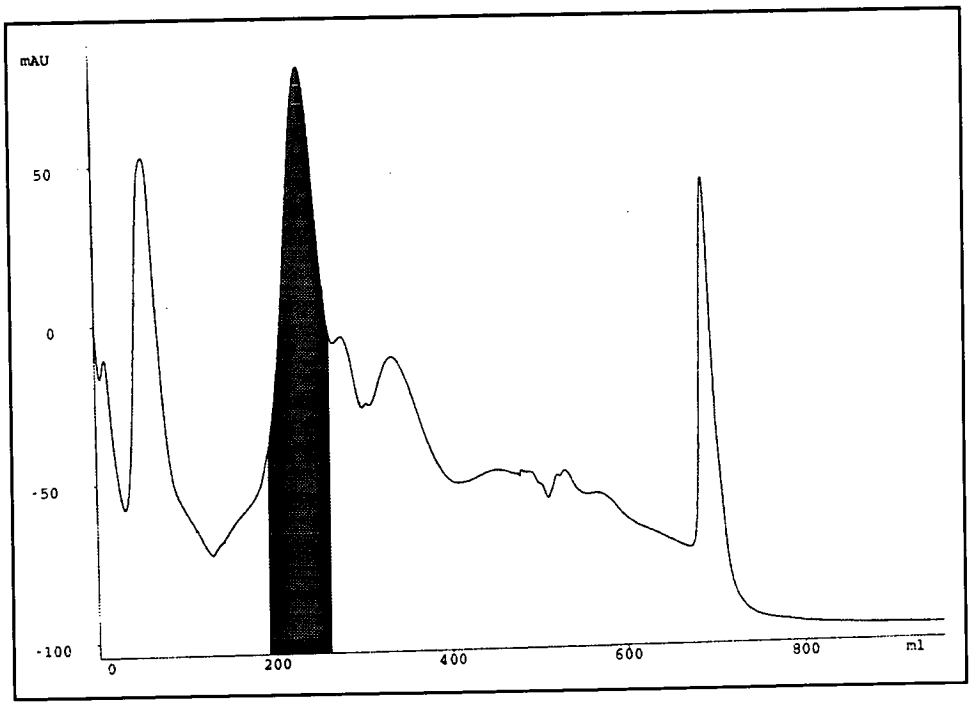


Figure 4.43 Elution profile from Q Sepharose FF anion exchange chromatography. Shaded area indicates activity. Start conditions: 50 mM BTP pH 7.2.

(Step 7) HiTrap Blue Affinity Chromatography- 4x1 ml samples of the dialysed protein at pH 6.0 were applied to a HiTrap Blue affinity column that had been pre-equilibrated with 50 mM BTP, pH 6.0. Any unbound protein was washed from the column and

collected in 1 ml fractions. Bound protein was eluted using 1 M and 1.5 M sodium chloride, 1 ml fractions were collected. All fractions were assayed, activity was observed in two of the unbound fractions and in the 1 M salt fractions (**Figure 4.44**). The active fractions were run on a 15 % SDS-PAGE gel (**Figure 4.45**). This procedure was repeated using a very gradually increasing salt gradient again binding and eluting with 50 mM BTP, pH 6.0. Although protein was eluted using this method, none of the fractions were active. After the column had been washed several times with 2 M sodium chloride with no success, the pH was increased to 8.0. It appeared that very little protein was eluting from the column at this stage, in spite of this, however, one fraction (42) produced activity (**Figure 4.46**). A 15 % SDS-PAGE gel was run on these results (**Figure 4.47**).

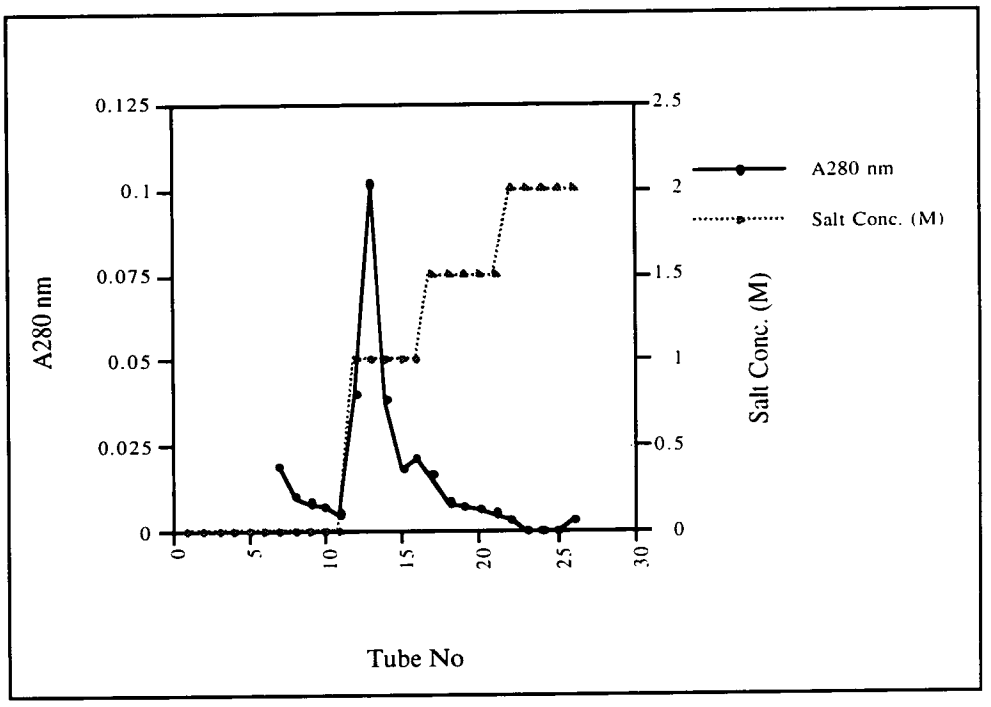


Figure 4.44 Elution profile from HiTrap Blue affinity chromatography. Activity was observed at in the unbound and1 M NaCl fractions. Start conditions: 50 mM BTP pH 6.0.

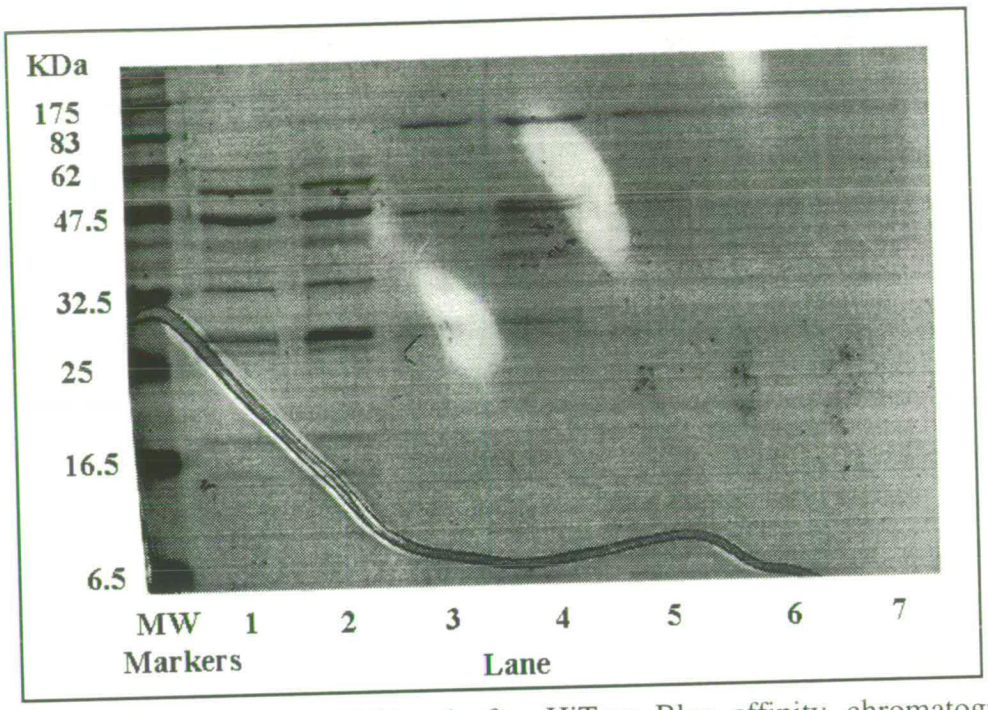


Figure 4.45 SDS-PAGE (15%) gel after HiTrap Blue affinity chromatography. Lanes 1 and 2 correspond to activity observed in the unbound fraction and lanes 3-7 correspond to the active fractions eluted at 1 M NaCl.

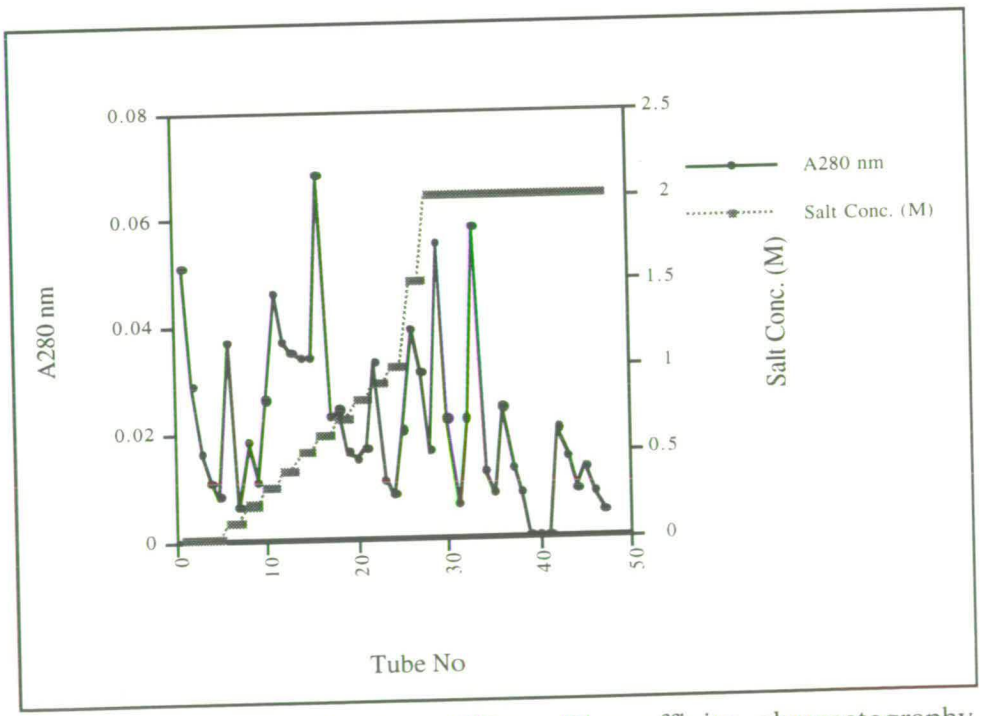


Figure 4.46 Elution profile from HiTrap Blue affinity chromatography. Start conditions: 50 mM BTP pH 6.0 increasing to 8.0 at tube number 37. Activity was observed after the pH was increased.

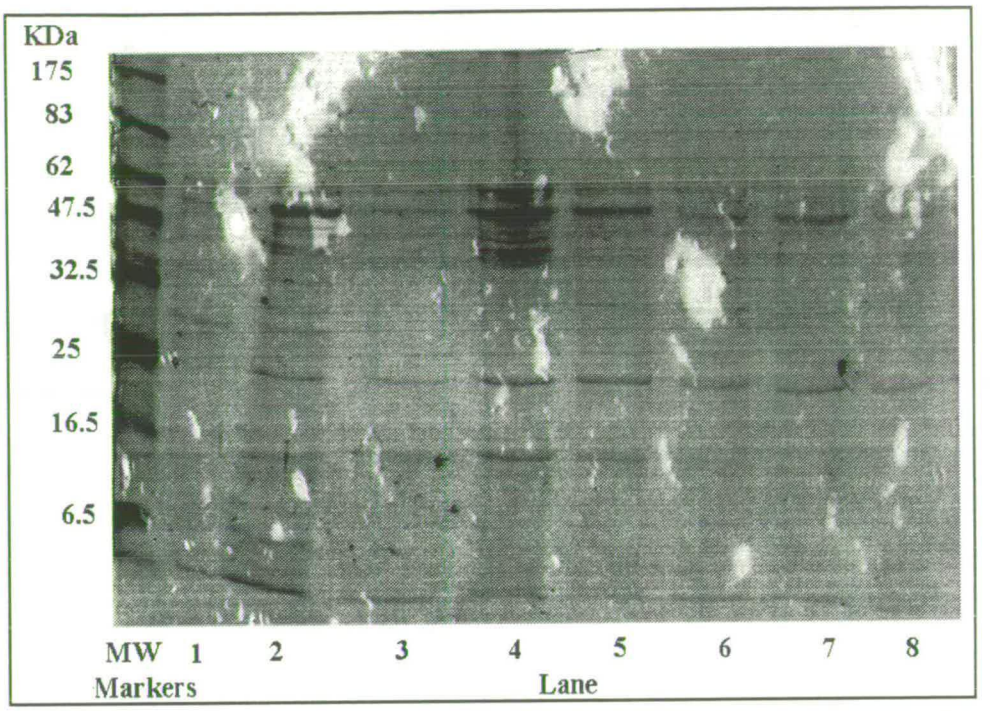


Figure 4.47 SDS-PAGE (15%) gel after HiTrap Blue affinity chromatography at pH 8.0. Lanes 1-8 correspond to fractions 40-47 with fraction 42 (lane 3) being the active fraction.

(Step 8) Phenyl Superose Hydrophobic Interaction Chromatography- Using the crude protein combined at Step 5, different pH's were tested for optimisation of the hydrophobic interaction stage. Initially 500 µl of the crude protein was diluted to 5 ml and taken to a concentration of 1.5 M ammonium sulphate, in 50 mM BTP pH 5.5. This was applied to the column and protein eluted with a decreasing salt concentration (**Figure 4.48**). The actual pH, however, was shown to be 6.4 (BTP fails to hold pH steady below pH 6.0). All fractions were assayed for activity and a corresponding 15 % SDS-PAGE gel was run on the active fractions (**Figure 4.49**).

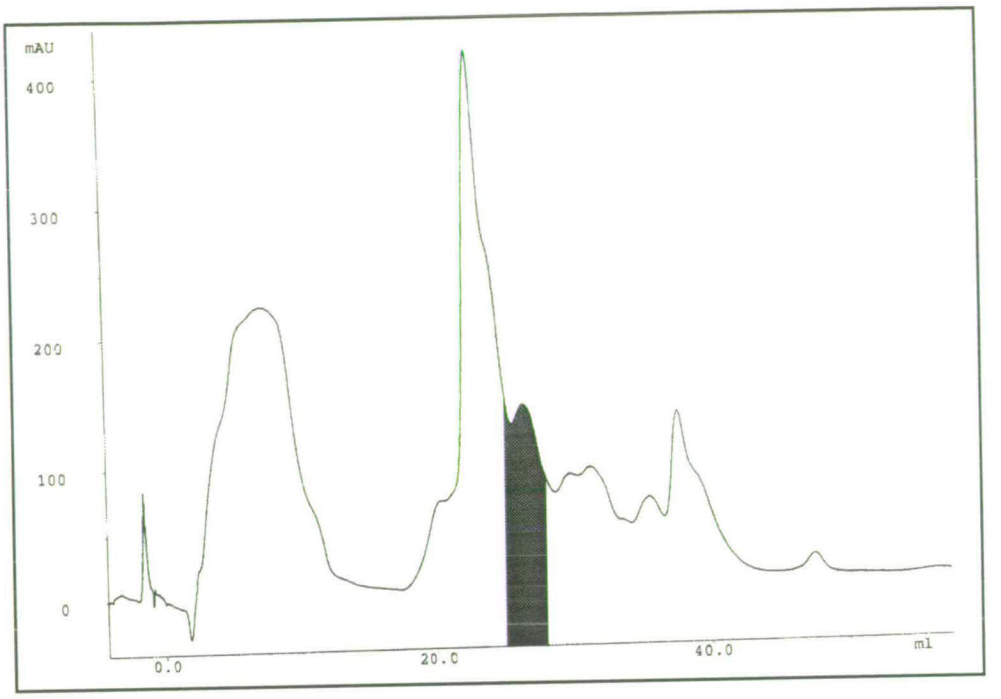


Figure 4.48 Elution profile from Phenyl Superose hydrophobic interaction chromatography. Shaded area indicates activity. Start condtions: 50 mM BTP pH 6.4 at 1.5 M ammonium sulphate.

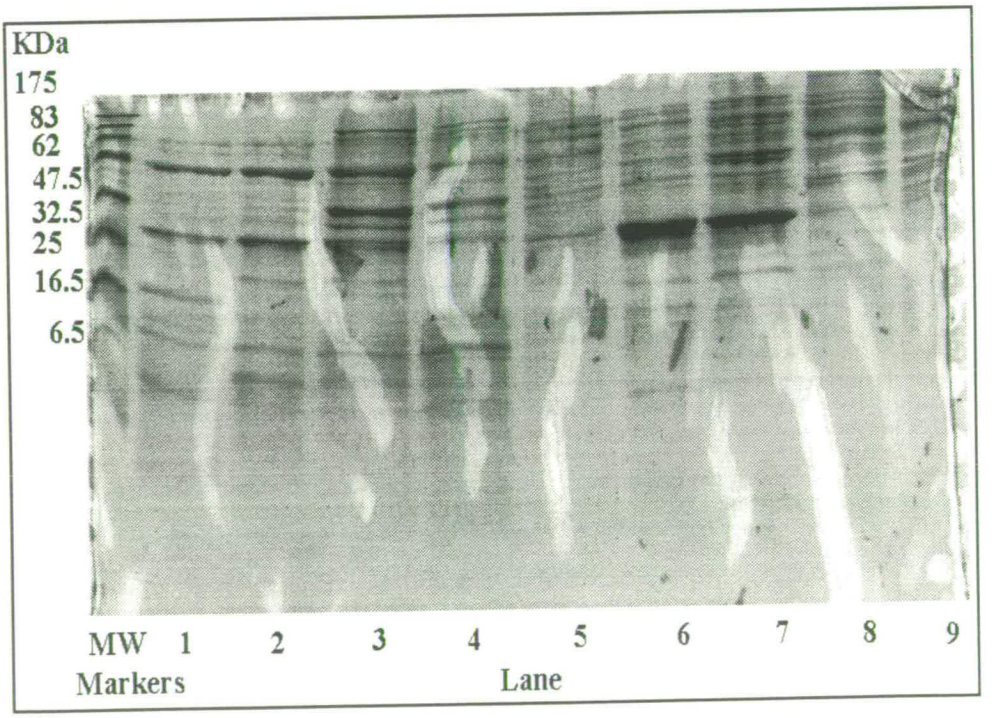


Figure 4.49 SDS-PAGE (15%) gel after Phenyl Superose hydrophobic interaction chromatography at pH 6.4. Lanes 1-9 correspond to fractions 13-21 with lanes 4, 5 and 6 being the most active.

The hydrophobic interaction step was repeated using 50 mM phosphate buffer, pH 5.5, again separation was good (**Figure 4.50**) and the active fractions were run on a 15 % SDS-PAGE gel (**Figure 4.51**).

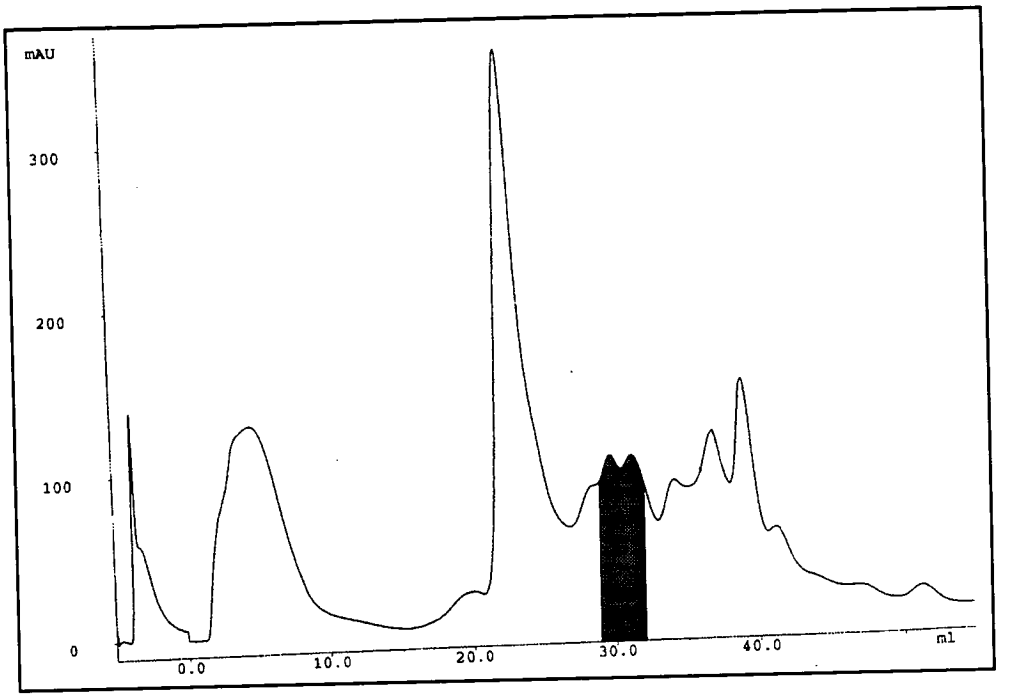


Figure 4.50 Elution profile from Phenyl Superose hydrophobic interaction chromatography. Shaded area indicates activity. Start conditions: 50 mM phosphate buffer pH 5.5 at 1.5 M ammonium suphate.

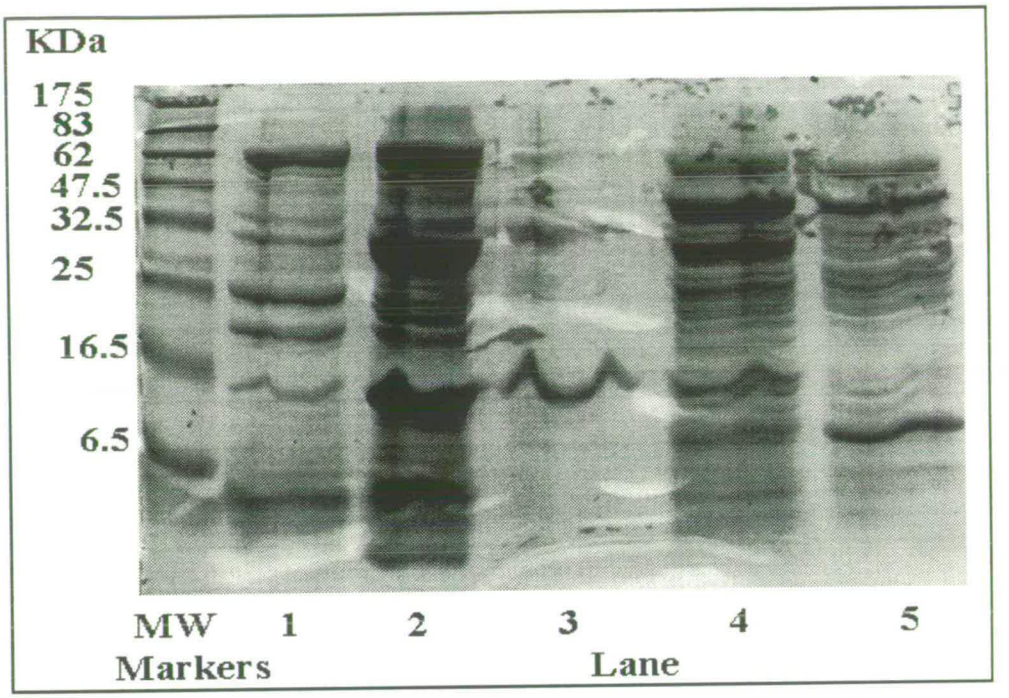
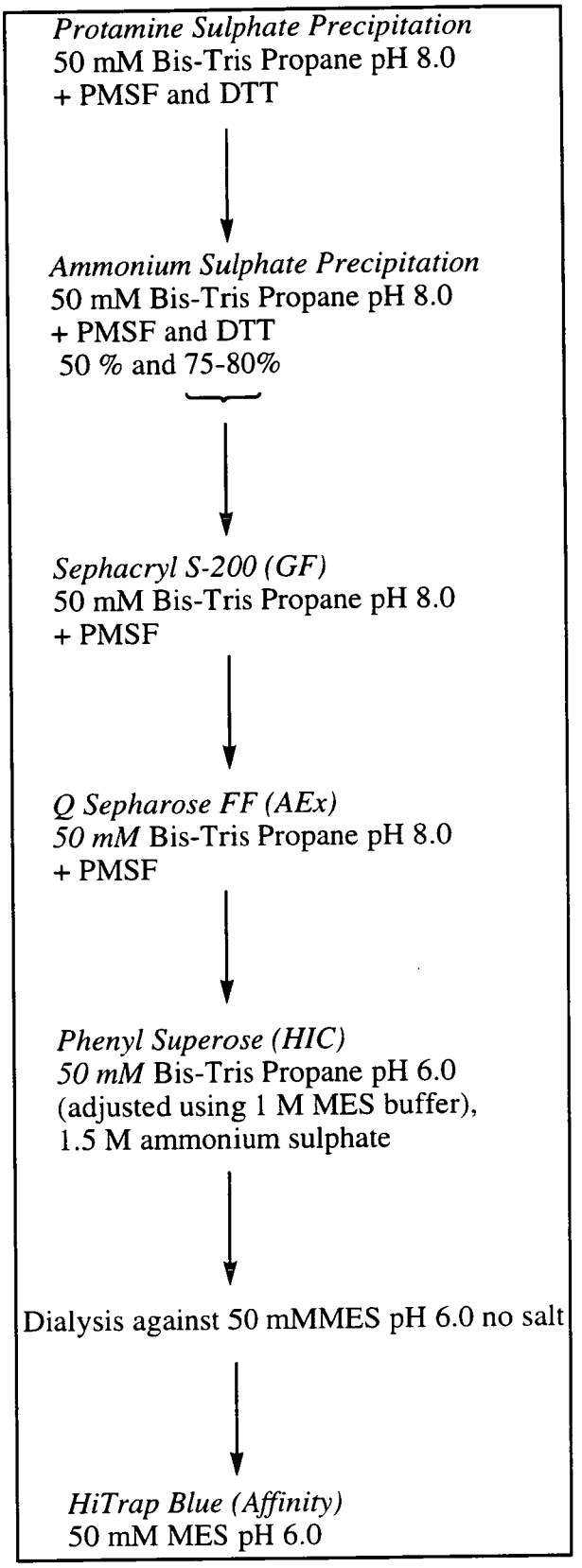


Figure 4.51 SDS-PAGE (15%) gel after Phenyl Superose hydrophobic interaction chromatography at pH 5.5. Lanes 1-5 correspond to fractions 9-23, lane 3 being the most active.

Although no kinetic experiments were performed during this optimisation due to time constraints, we have been able to produce an optimised protocol for use in further experiments (**Scheme 4.2**).



Scheme 4.2 Optimised purification protocol.

4.3.6 METHOD 6

Subsequent to the work carried out by the author and using the optimised protocol worked out in Method 5, another purification was attempted by Marina Alexeeva using FPLC as well as the Äkta. The use of nebulisation for breaking open the cells was proving inefficient so was used in conjunction with sonication.

(Step 1) Protamine Sulphate Precipitation- Nucleic acids were removed from the cell free extract (25 mM BTP pH 8.0 + PMSF and DTT) of a 10 L culture of *S. cerevisiae* as in Method 1.

(Step 2) Ammonium Sulphate Precipitation- The protein extract was taken to 50 % ammonium sulphate saturation with stirring at 4 °C for 1 h. The precipitated protein was collected by centrifugation and the pellet discarded. The buffer was taken to 75 % saturation with ammonium sulphate, the proteins precipitated between 50-75 % were retained.

(Step 3) Sephacryl S-200 Gel Filtration Chromatography- The proteins from the 50-75 % ammonium sulphate saturation were applied to a 1.5 L Sephacryl S-200 gel filtration column that had been previously equilibrated with 25 mM BTP pH 8.0 containing PMSF and DTT. Proteins were eluted at a rate of 2 ml/min. 15 ml fractions were collected and assayed for activity. The most active fractions (33-41) were pooled.

(Step 4) Q-Sepharose FF Anion Exchange Chromatography- The pooled fractions from Step 3 were applied to a 50 ml Q-Sepharose FF anion exchange column which had been previously equilibrated with 25 mM BTP pH 8.0 containing PMSF and DTT. Proteins were eluted with an increasing salt gradient upto 0.7 M NaCl over 16 column volumes (**Figure 4.52**). Two areas of activity were observed, fractions 44-53 and fractions 71-80.

(Step 5) Phenyl Sepharose Hydrophobic Interaction Chromatography- The combined fractions of 44-53 from Step 4 were adjusted to pH 6.0 by the addition of 1 M MES. The protein was applied to a 25 ml Phenyl Sepharose hydrophobic interaction column which had been previously equilibrated with 25 mM MES pH 6.8 at 1.5 M ammonium sulphate. Elution was achieved with a decreasing ammonium sulphate gradient at a rate of 4 ml/min. 8 ml fractions were collected and one area of activity was observed. The procedure was repeated with fractions 71-80 from Step 4. In this instance two areas of

activity were observed (**Figure 4.53**), one significantly more active than the other. At this stage the most active fraction was taken further and the less active fraction discarded.

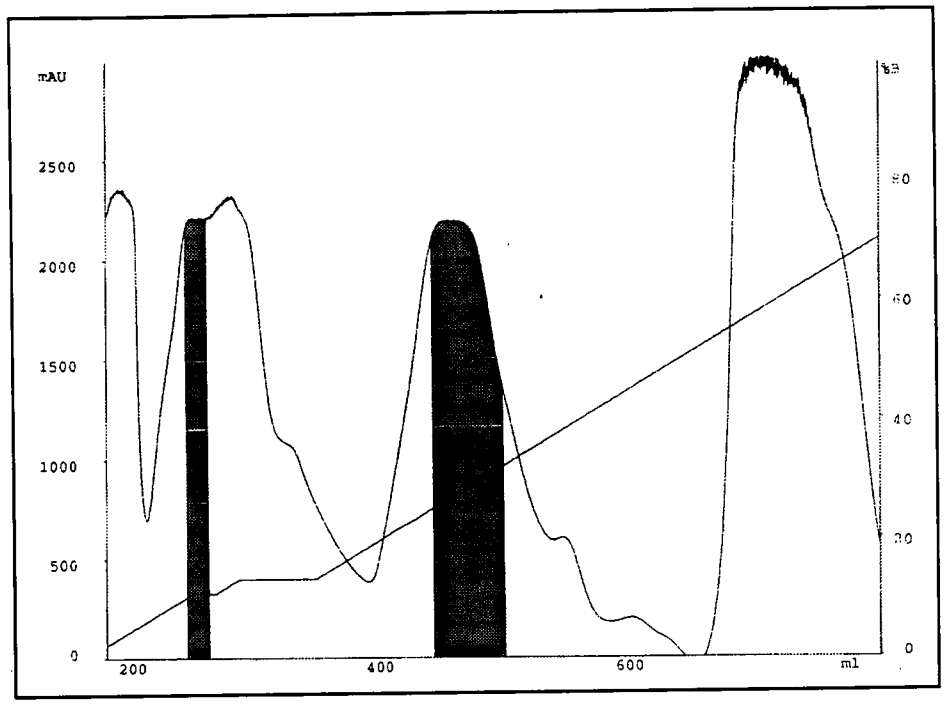


Figure 4.52 Elution profile from Q-Sepharose FF anion exchange chromatography. Shaded areas indicate activity in fractions 44-53 and 71-80. Start conditions: 25 mM BTP pH 8.0 containing PMSF and DTT.

(Step 6) HiTrap Blue Affinity Chromatography- The two active fractions from Step 5 (from 44-53 and 71-80 after Step 4) were kept separate and both dialysed against 25 mM MES pH 6.0 to remove the ammonium sulphate. The first fraction (44-53) was applied to a 1 ml HiTrap Blue affinity column that had been previously equilibrated with 25 mM MES pH 6.0. Proteins were eluted using a stepwise salt gradient (0.5, 0.8, 1.0 and 2.0 M NaCl), this was repeated at pH 6.5, 7.0, 7.5 and 8.0. Activity was observed in pH's 6, 6.5 and 7.0 at 2 M salt. At pH 6.0 six bands were observed on a gel, four of which were strong. At pH 6.5 one of the major bands was still strong and at pH 7.0 only one band was apparent. This protein had a molecular weight of approximately 45 KDa. The procedure was repeated on the second fraction (71-80) where a protein of approximately 32 KDa was observed.

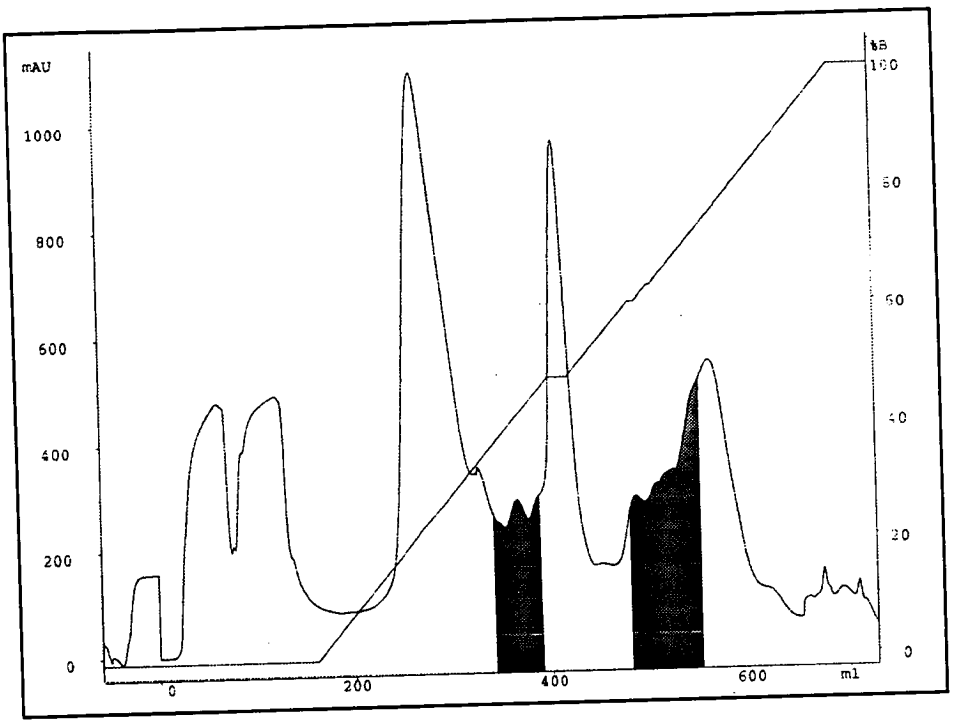


Figure 4.53 Elution profile from Phenyl Sepharose hydrophobic interaction chromatography. Shaded areas indicate activity. Start conditions: 25 mM MES pH 6.0 at 1.5 M ammonium sulphate, protein sample from fractions 71-80 after Step 4.

(Step 7) N-Terminal Analysis- The N-terminus of the 32 KDa protein was blocked. The ten N-terminal residues of the 45 KDa protein demonstrated 100 % homology to an enzyme known as Old Yellow Enzyme, also known as NADPH dehydrogenase 2 (EC 1.6.99.1).[79-83]

ASSAY RESULTS

45 KDa Protein (Old Yellow Enzyme):

Purification Step	Specific Activity	Fold	Total Protein	Total Activity
Crude Extract	8.28×10^{-4} U/mg	1	1750 mg	1.5 U
Gel Filtration	3.4×10^{-3} U/mg	4	120 mg	0.09 U
Anion Exchange	6.6×10^{-3} U/mg	8	15 mg	0.1 U
Hydrophobic Interaction	9.4×10^{-3} U/mg	11	3.5 mg	0.03 U
Affinity*	0.38 U/mg	450	0.05 mg	0.02 U

Table 4.3 Assay results for the purification of a nitroreductase with molecular weight 45 KDa. *Most active fraction.

Purification Step	Specific Activity	Fold	Total Protein	Total Activity
Crude Extract	$8.28x10^{-4}$ U/mg	1	1750 mg	1.5 U
Gel Filtration	$3.4x10^{-3}$ U/mg	4	170 mg	0.09 U
Anion Exchange	$3.6x10^{-2}$ U/mg	43	24 mg	0.85 U
Hydrophobic Interaction	$4.2x10^{-1}$ U/mg	507	1 mg	0.40 U
Affinity*	1.8 U/mg	2170	0.035 mg	0.06 U

Table 4.4 Assay results for the purification of a nitroreductase with molecular weight 32 KDa. *Most active fraction.

A summary of Method 6 can be seen in **Scheme 4.3**. Using a bead mill to break open the cells has further enhanced the method. The area of less activity seen at the Phenyl Sepharose stage has since been shown to be a protein of approximately 50 KDa and has been submitted for N-terminal analysis. The three purified proteins can be seen in **Figure 4.54**.

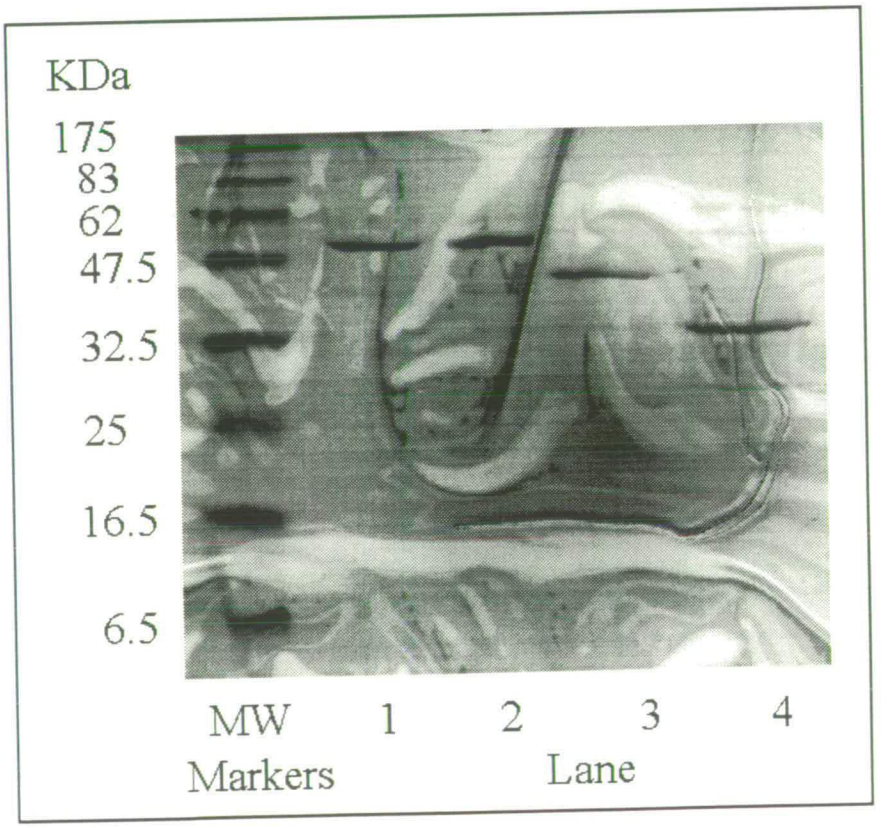
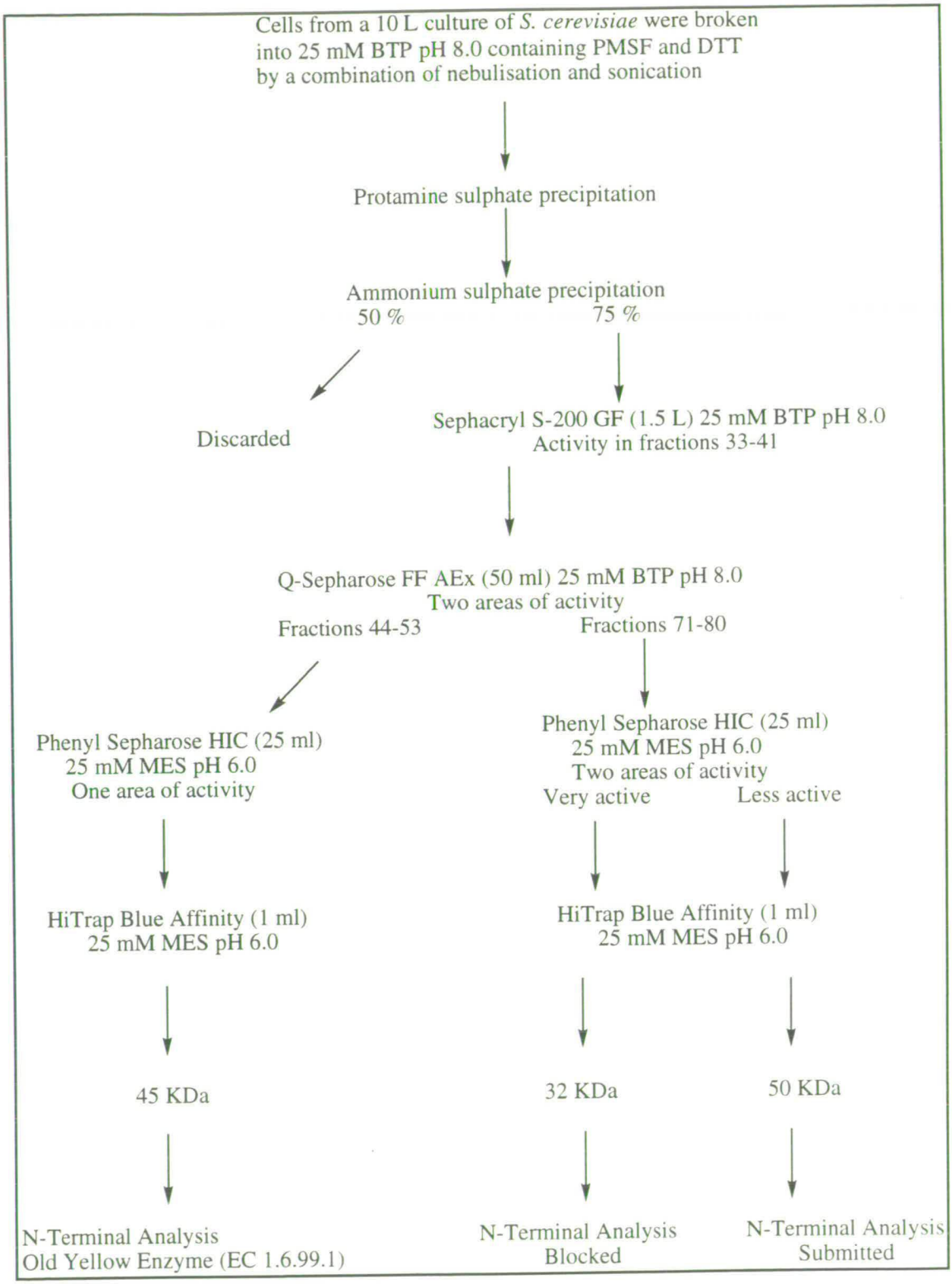


Figure 4.54 SDS-PAGE (15 %) gel of purified proteins at 50 KDa (Lanes 1 and 2), 45 KDa (Old Yellow Enzyme, Lane 3) and 32 KDa (Lane 4).



Scheme 4.3 Summary of Method 6.

4.4 CONCLUSION

Few comparisons can be made between the three nitroaryl reductase enzymes purified from *S. cerevisiae* and those already purified from the other sources mentioned in the introduction.

A 52 KDa nitroreductase from *B. fragilis*[48] is comparable in size to the 50 KDa nitroaryl reductase from *S. cerevisiae* but there is little other information to compare the two enzymes. The 24 KDa nitroreductase from *E. cloacae* was shown to be capable of reducing a variety of diverse substrates (**Table 1.19**).[49] Despite there being no examples of substrate specificity with the nitroaryl reductases from *S. cerevisiae*, a number of substrates were tested with the whole cells of the yeast. Nitrobenzene is successfully reduced by enzyme for *E. cloacae* but not by the yeast whole cells, it can therefore be assumed that the enzymes from the two different sources are displaying differing activities.

The 52 KDa nitrophenol reductase from *R. capsulatus* E1F1[53] and the aromatic nitroreductase from *P. chrysosporium*[54] are not comparable with the nitroaryl reductases from *S. cerevisiae*. Whole cells of *S. cerevisiae* fail to reduce nitrophenols to any significant degree and the enzyme purified from *P. chrysosporium* is oxygen sensitive and fails to exhibit any activity in the presence of molecular oxygen.

A 33 KDa nitroreductase purified from *P. pseudoalcaligenes* JS45[55] is comparable in size to the 30 KDa nitroaryl reductase from *S. cerevisiae* but demonstrates a different mechanism of reduction, reducing nitrobenzene to 2-aminophenol instead of the expected aniline.

Of the nitroreductases purified from the microorganisms previously mentioned, none discuss an enzyme of approximately 45 KDa corresponding to the Old Yellow enzyme from *S. cerevisiae*. The 50 KDa and 30 KDa nitroaryl reductase from *S. cerevisiae* can be compared in molecular weight to those from *B. fragilis, R. capsulatus and P. pseudoalcaligenes* JS45, however no other comparisons can be made with these enzymes.

Although there is no significant homology between the nitroaryl reductase enzymes from *S. cerevisiae* and those from other sources at this stage, it is believed that there are other nitroaryl reductases that have yet to be purified from the yeast.

4.5 FUTURE WORK

Concerning the baker's yeast whole cell biotransformations, the mechanism of reduction of the dicyanonitroarene substrates could be achieved by identifying the intermediate compounds observed by HPLC.

Regarding the isolated enzymes, any further nitroreductase enzymes should be purified and those already isolated need to be identified. To enable any significant research on the isolated proteins, it would be advantageous to overexpress the individual nitroreductase in suitable host systems in order to simplify the purification protocol and produce a greater quantity of enzyme(s).

The kinetic parameters of the individual enzymes could be ascertained if they are unknown and a comparison of the selectivity could be made between the isolated enzymes and the whole cell system.

To gain insight into the specificity of the isolated enzymes it would be necessary to optimise the reaction conditions of each enzyme individually.

X-ray crystallography studies of the enzyme would be a useful method of gaining further information into the mechanism of reduction, particularly if crystal structures of the enzyme-substrate, enzyme-intermediate and enzyme-product could be obtained.

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Concerning the Baker's Yeast (*Saccharomyces cerevisiae*) Mediated Reduction of Nitroarenes and Other N-O Containing Functional Groups

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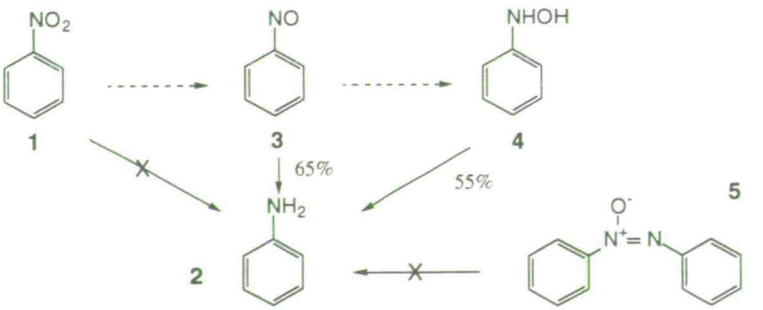
Abstract: Nitro- and nitrosoarenes can be reduced using baker's yeast (*Saccharomyces cerevisiae*) under two distinct sets of conditions, one of which is in fact a well established non-enzymic process. In order to clarify reports in the literature a comparison of the two methods has been made. © 1997 Elsevier Science Ltd.

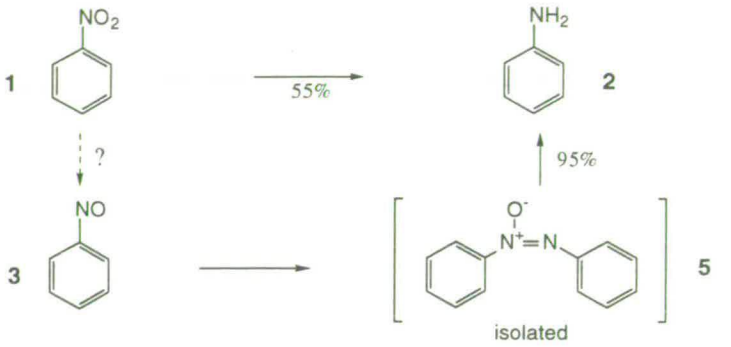
Recently there have been a number of reports concerning the use of baker's yeast (*Saccharomyces cerevisiae*) for the reductive cleavage of N-O bonds in a variety of functional groups including nitro arenes[1], nitroalkenes[2], nitrosoarenes[3], isoxazoles[4] and *N*-oxides[5]. In general the reactions proceed under mild conditions and may present synthetic advantages in terms of chemo- and regioselectivity. Typically yeast catalysed reactions are carried out at neutral pH in aqueous media with a substrate concentration of 1-2 mg ml^{-1}. However, inspection of the literature relating to yeast catalysed N-O reductions revealed that two distinct sets of reaction conditions could be employed for this biotransformation. Whereas most of the papers [1a-d, 2b, 4, 5a] describe the use of *S. cerevisiae* at pH 5.5-6.0 and 30 °C with fermenting or non-fermenting yeast (thereafter called type I conditions), the group of Baik *et al.*,[1e-f, 2a, 3, 5b] report quite different reaction conditions, namely reaction temperatures of 70-80 °C and even reflux (*sic*)[5b], high pH (> 12) and the inclusion of methanol/ethanol in the reaction medium (type II conditions). Even with respect to the reduction of nitroarenes (*vide infra*), the two different reaction conditions result in different selectivity, *e.g.* under type I conditions electron-withdrawing groups are required for successful reduction [1a,b] whereas with type II conditions electron-donating groups can be tolerated.[1e] This discrepancy suggested to us a difference in reaction mechanism, especially in view of the fact that the yeast is unlikely to be stable at high pH and temperature. In this letter we report on our investigations into the likely mechanisms of these two reductions.



Scheme 1: Reduction of nitrobenzene and derivatives using *S. cerevisiae* under type I conditions.
typical conditions: *S. cerevisiae* (20g), substrate (100 mg), water (100 ml), 30 °C, pH 5.5-6.0.

An indication of the difference between the two sets of conditions can be gained by comparing the reduction of nitrobenzene **1** (**Schemes 1** and **2**). Under type I conditions, no reduction of nitrobenzene

can be detected whereas with type II conditions a 55% yield of aniline **2** is obtained. Use of nitrosobenzene **3** with type I conditions gives a clean conversion to aniline (65%) whereas under type II conditions, modified by exclusion of the NaOH, the azoxybenzene **5** is detected as an intermediate *en route* to aniline. By comparison, azoxybenzene is not reduced under type I conditions. We have also shown that phenylhydroxylamine **4**[6] can be reduced under type I conditions to give aniline in 55% yield leading us to propose the sequence of events shown in **Scheme 1**.

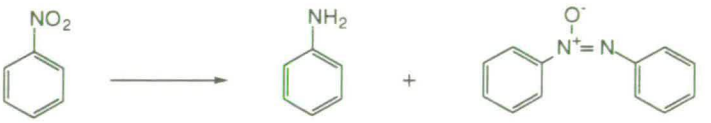


Scheme 2: Reduction of nitrobenzene and derivatives using *S. cerevisiae* under type II conditions.
typical conditions: *S. cerevisiae* (30g), substrate (500 mg), NaOH (4g) (except for nitroso reduction), water (900 ml), methanol (40 ml), 70-80 °C, pH >12.

In order to gain further insight into the difference between the two sets of conditions we subjected 2,4-dinitroanisole to the reduction. Using our optimised protocol for carrying out these yeast catalysed reductions[7] we obtained a 5.3:1 ratio of 2-amino-4-nitroanisole : 2-nitro-4-aminoanisole in a combined yield of 95%. However, under type II conditions the reaction was found to be less clean and resulted only in isolation of 2-amino-4-nitroanisole in a yield of 20%.

In addition to the evidence provided above, the following points should be noted. Firstly, under type II conditions, it is highly unlikely that the yeast cells remain active during the reaction. Indeed, our experience has been that the cells appear to coagulate very rapidly presumably followed by cell death and subsequent inactivation of the enzyme activity under the high pH and high temperature of the medium. Secondly, it is well known that nitroarenes can be reduced to the corresponding anilines and azoxybenzenes under strongly basic conditions in solutions that contain simple alcohols and/or glucose or fructose.[8] It thus occurred to us that perhaps the *S. cerevisiae* type II conditions were in fact simply a variation of these classical conditions and that the yeast was merely acting as an alternative source of carbohydrate.

In order to test this notion we carried out a series of comparative experiments involving the reduction of nitrobenzene under a variety of conditions as shown in **Scheme 3**. The principal observation from these experiments is that the effect of adding glucose to the reaction at the appropriate concentration is essentially the same as that of adding *S. cerevisiae*, leading to the conclusion that the function of the yeast is to act as a source of carbohydrate.

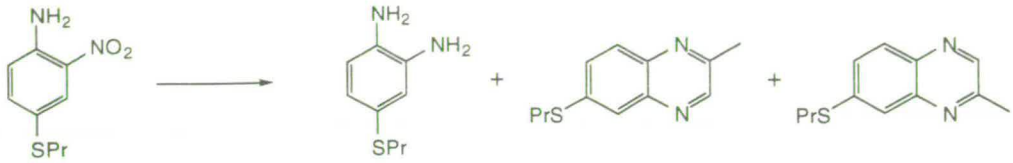


conditions		yield/%
no NaOH, glucose	0	0
NaOH (5g), glucose (1g)	11	60
NaOH (5g), glucose (5g)	30	0
NaOH (5g), *S. cerevisiae*	30	0

Scheme 3: Reduction of nitrobenzene.
conditions: nitrobenzene (1g), water (80 ml), MeOH (40 ml).

We considered the possibility that the use of *S. cerevisiae* under type II conditions may offer some advantage over simple addition of glucose in that the carbohydrate may be provided in a slow release form

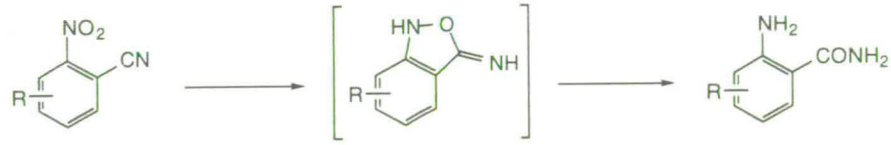
as the yeast cells degrade. Some support for this proposal was obtained from the reduction of the nitroarene **6** (**Scheme 4**). The use of *S. cerevisiae* resulted in a cleaner reaction (59% of the aniline **7**) compared to the use of glucose/fructose which gave a lower yield of the aniline and a 30% yield of the quinoxalines **8** and **9**. It is noteworthy that nitroaniline **6** was totally inactive under type I conditions providing further evidence for the difference between type I and II conditions.



conditions	yield/%		
S. cerevisiae (30g)	59	0	
glucose/fructose (4g)	trace	30	

Scheme 4: Reduction of nitroaniline **6**.
conditions: substrate (1g), water (80 ml), MeOH (40 ml), NaOH (5g).

The presence of arylhydroxylamines as intermediates in the reduction pathway under type I conditions has been demonstrated above (**Scheme 1**) and inferred from previous experiments[1a] in which it was found to be possible to reduce 1,2-nitrocyanoarenes to the corresponding 1,2-aminobenzamides, presumably *via* the corresponding isoxazoline intermediates (**Scheme 5**). We have extended this approach by investigating the reduction of some dicyanonitroarenes and have found that in all cases the nitrile group *ortho*- to the cyano group undergoes selective transformation to the benzamide resulting in a means for selectively manipulating one nitrile group in the presence of another.



R	time	yield/%
H		79
3-CN		75
4-CN		--
5-CN		84
4-Cl		69
5-CF_3		84

Scheme 5: Conversion of 1,2-nitrocyanoarenes to 1,2-aminobenzamides.

In conclusion it seems clear that the baker's yeast mediated reduction of nitroarenes and related N-O containing substrates is best carried out under conditions that maintain the integrity of the yeast thereby exploiting the inherent enzymic catalytic activity. In our hands, the reactions using type I conditions are simpler and cleaner and result in higher yields. Regarding the type II conditions it seems that there is no significant advantage in using baker's yeast over glucose/NaOH/MeOH for simple nitro reductions. Moreover, any claims that there are advantages in terms of chemoselectivity (*i.e.* reduction of nitro groups in the presence of ketones[5b]) are almost certainly due to the destruction of all enzyme activity under the reaction conditions used leading to non-enzymic processes only. It is noteworthy that previous claims of baker's yeast mediated reactions have subsequently been revised by others in the light of more carefully executed experiments with the appropriate controls.[9]

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6. Phenylhydroxylamine is relatively unstable but can be prepared and conveniently stored as its *N,O-bis*-acetyl derivative; *Org. Syn.*, 1989, **67**, 187.
7. *Typical conditions for the baker's yeast reduction of nitroarenes*: The baker's yeast purchased from Sigma (Sigma type II) was initially purified using the following procedure (*N.B.* this purification of commercially available baker's yeast has been found to result in cleaner product isolation). To a solution of acetone (1l) at -20 °C was added baker's yeast (200-300g) and the suspension stirred gently for 20 minutes after which the acetone was removed by decanting and the procedure repeated with a further quantity of acetone (1l). After the second washing the yeast was collected and dried. Acetone-washed baker's yeast (10g) was suspended in tap water (40 ml) and incubated at ~32 °C for 1h, after which the substrate (100 mg) dissolved in DMSO or hot ethanol (~5 ml) was added. The reaction was shaken at 32 °C, in an orbital shaker, and the conversion monitored by t.l.c. Upon completion of the reaction, the aqueous medium was saturated with NaCl and the pH adjusted to 8. The entire mixture was then continuously extracted overnight with chloroform after which the chloroform layer was washed, dried and evaporated to yield the crude product. Standard chromatographic procedures lead to the isolation of the aniline products.
8. Vogel's *Textbook of Practical Organic Chemistry*, 4th Ed., p. 724.; Prato, M.; Quintily, U.; Scaplo, L.; Scorrano, G. *Bull. Chim. Soc. France*, 1987, N1, 99.
9. For a discussion of the purported baker's yeast catalysed 1,3-dipolar cycloaddition reactions of nitrile oxides see Easton, C.J.; Hughes, M.M.; Tiekink, E.R.T.; Savage, G.P.; Simpson, G.W. *Tetrahedron Lett.*, 1995, **36**, 629.

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